A method for distinguishing the important malaria vectors *Anopheles dirus* and *An. cracens* (Diptera: Culicidae) based on antennal sensilla of adult females

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Abstract. Some species of the *Anopheles dirus* species complex are considered to be highly competent malaria vectors in Southeast Asia. *Anopheles dirus* is the primary vector of *Plasmodium falciparum* and *P. vivax* while *An. cracens* is the main vector of *P. knowlesi*. However, these two species are difficult to distinguish and identify based on morphological characters. Hence, the aim of this study was to investigate the potential use of antennal sensilla to distinguish them. Large sensilla coeloconica borne on the antennae of adult females were counted under a compound light microscope and the different types of antennal sensilla were examined in a scanning electron microscope. The antennae of both species bear five types of sensilla: ampullacea, basiconica, chaetica, coeloconica and trichodea. Observations revealed that the mean numbers of large sensilla coeloconica on antennal flagellomeres 2, 3, 7, 10 and 12 on both antennae of both species were significantly different. This study is the first to describe the types of antennal sensilla and to discover the usefulness of the large coeloconic sensilla for distinguishing the two species. The discovery provides a simple, reliable and inexpensive method for distinguishing them.

INTRODUCTION

Malaria is a disease caused by plasmodial parasites that are transmitted to humans through the bites of female *Anopheles* mosquitoes. There were approximately 219 million malaria cases in 90 countries and an estimated 435,000 deaths due to the disease in 2017 (WHO, 2018). For example, 1,398 cases of falciparum malaria were reported in 28 provinces of China in 2011, especially in epidemic areas of Yunnan and Hainan provinces (WHO, 2018). China is now aiming to eliminate malaria by 2020. In Thailand, the total number of confirmed malaria cases in 2016 was 37,209 (Bureau of Vector Borne Diseases, Ministry of Public Health, 2017).

Seven taxonomic groups are known to include the main malaria vectors in Southeast Asia, i.e. the Culicifacies, Dirus, Fluviatilis, Leucosphyrus, Minimus and Sundaicus Complexes, and the Maculatus Group (Manguin *et al.*, 2008). According to Sallum *et al.* (2005), the Dirus Complex comprises seven species: *An. baimaii* Sallum & Peyton, *An. cracens* Sallum & Peyton, *An.*

dirus Peyton & Harrison, *An. elegans* James, *An. nemophilous* Peyton & Ramalingam, *An. scanloni* Sallum & Peyton and *An. takasagoensis* Morishita. Takano *et al.* (2010) added an eighth species, informally denoted as "aff. *takasagoensis*". *Anopheles dirus* has a wide distribution in eastern Asia, being recorded in Cambodia, China (Yunnan Province and Hainan Island), Laos, Myanmar, Thailand and Vietnam. *Anopheles cracens* occurs southward from southern Thailand through peninsular Malaysia (Perlis, Terengganu, Kuala Lipis Pahang states) into Sumatra, Indonesia (Sallum *et al.*, 2005; Jiram *et al.*, 2012).

Anopheles dirus and *An. baimaii* have been incriminated as the principle vectors of the malarial protozoa *Plasmodium falciparum* and *P. vivax* in China and Southeast Asia (Manguin *et al.*, 2008; Saeung, 2012; Tainchum *et al.*, 2015), while *An. cracens* has been found to be the main vector of *P. knowlesi* in peninsular Malaysia (Pahang) (Vythilingam *et al.*, 2008; Jiram *et al.*, 2012).

Undoubtedly, correct identification of vector species is important for proper management and control of malaria. However, identification of members of the Dirus Complex is difficult due to overlapping morphological characters (Hii and Rueda, 2013). Various methods for recognition and confirmation of the taxonomic status of the individual species have been investigated, including cytogenetics (Baimai, 1988; Baimai, 1998), enzyme electrophoresis (Green *et al.*, 1992) and molecular genetics (Walton *et al.*, 1999; Sallum *et al.*, 2005; Phunngam *et al.*, 2017). Furthermore, Somboon *et al.* (2009) investigated the structure of the cibarial armature for distinguishing four species of the Dirus Complex but no significant differences were observed. However, the antennal sensilla of members of the complex have not been investigated. In the present study, the various types of sensilla borne on the antennae of females of *An. dirus* and *An. cracens* were examined using scanning electron microscopy, and are described here for the first time.

MATERIALS AND METHODS

Mosquitoes and species identification
Specimens of laboratory strains of *An. dirus* were originally collected in Mae Sod District, Tak Province, Thailand. Specimens of free-mating *An. cracens* were originally from the Armed Forces Research Institute of Medical Sciences laboratory, Bangkok, Thailand. *Anopheles dirus* (Hainan strain, China) was obtained from the Department of Vector Ecology and Environment, Nagasaki University, Japan. The species were identified based on morphology (Rattanarithikul *et al.*, 2006; Somboon and Rattanarithikul, 2013) and the allele-specific polymerase chain reaction (AS-PCR) method of Walton *et al.* (1999). Briefly, genomic DNA was extracted from wings and legs of individual mosquitoes using the PureLink® Genomic DNA Kit (Invitrogen, USA), according to the manufacturer's recommendations. Each PCR reaction was conducted using a 25 µl volume containing 0.5 U of *Taq* DNA polymerase (Invitrogen, USA), 1x *Taq* buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.25 µM of each primer and 1 µl of the extracted DNA. The amplification profile consisted of an initial denaturation at 94°C for 5 min, 35 cycles at 94°C for 30 s, 53°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min. The amplified products were electrophoresed on 1.5% agarose gel and stained with SYBR® Safe DNA gel stain (Invitrogen, USA).

Mosquito rearing
A colony of each of the three strains was established and maintained in an insectary of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, using the procedures described by Choochote and Saeung (2013). The insectary was maintained at 27±2°C, 70–80% relative humidity and illuminated by a combination of natural daylight from a glass window and fluorescent lighting provided for approximately 12 h a day.

Light microscopy
Large sensilla coeloconica (lco) on each antennal flagellum of five-day-old female

mosquitoes were observed using an Olympus BX53 compound microscope. Individual specimens were immersed in 10% potassium hydroxide (KOH) solution in a small bottle and held in an oven at 45°C for 30–45 min. After clearing, they were washed with 80% ethanol, their antennae were removed using an insect needle and the two antennae of each female were mounted together on a microscope slide with Neo-shigaral medium (Tokyo, Japan). The large sensilla coeloconica borne on the left and right flagellum of 30 females of each strain were counted (n = 60 flagella/strain) (Taai *et al.*, 2017).

Scanning electron microscopy
Thirty heads of four- to five-day-old females of each strain were removed under a stereomicroscope and rinsed three times in phosphate buffer (pH 7.4) to remove surface debris. The heads were then dehydrated through an ethanol series of 35, 70, 80 (10 min, two changes) and 95% (15 min, two changes), followed by absolute ethanol (10 min, two changes), and then dried in a critical point dryer. The antennae were carefully dissected from the head capsule under a stereomicroscope, as described by Hempolchom *et al.* (2017). The antennae were mounted on aluminum stubs with double-sided carbon adhesive tape and

sputter-coated with gold. Sensilla were observed and photographed in a JEOL-JSM6610LV scanning electron microscope.

Statistical analysis
The number of large sensilla coeloconica on the antennae of specimens of *An. cracens* and the two strains of *An. dirus* were analyzed using the Kruskal-Wallis test. A post-hoc Dunn's test was used for multiple comparisons of means. All data were analyzed using IBM SPSS statistics, version 24 for Windows (SPSS Inc., Chicago). The level of significance was set at 5% (p-value < 0.05).

RESULTS

Molecular identification
The AS-PCR confirmed the morphological identification of *An. dirus* (Thailand and Hainan strains) and *An. cracens*. PCR species-specific products were 562 bp and 514 bp for *An. dirus* (both strains) and *An. cracens*, respectively (Figure 1).

Morphology of antennal sensilla
In general, the antennae of female mosquitoes consist of two basal segments (scape and pedicel) and an elongate segmented flagellum (Figures 2A–2C). The scape is the
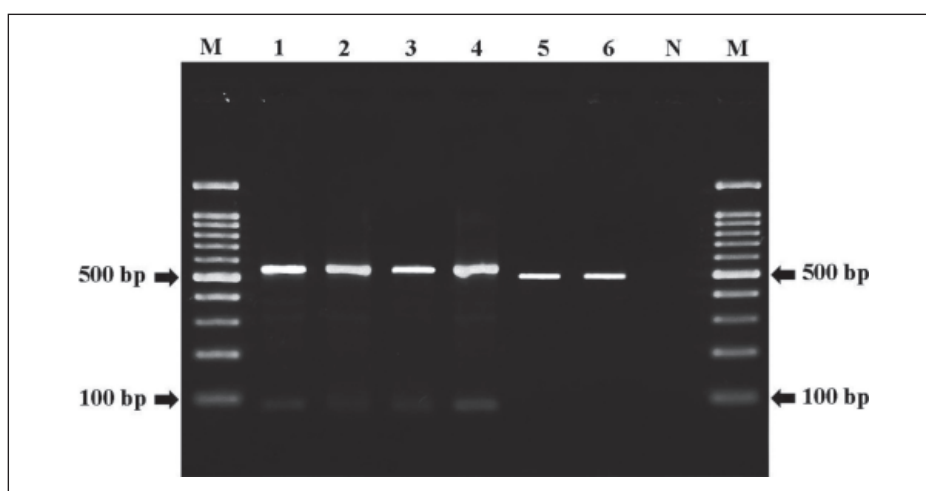

Figure 1. Gel of allele-specific PCR for distinguishing *An. dirus* and *An. cracens*. Lanes 1 and 2: *An. dirus*, Thailand strain (562 bp); lanes 3 and 4: *An. dirus*, Hainan strain (562 bp); lanes 5 and 6: *An. cracens*, Thailand strain (514 bp); lane M: 100 bp ladder.
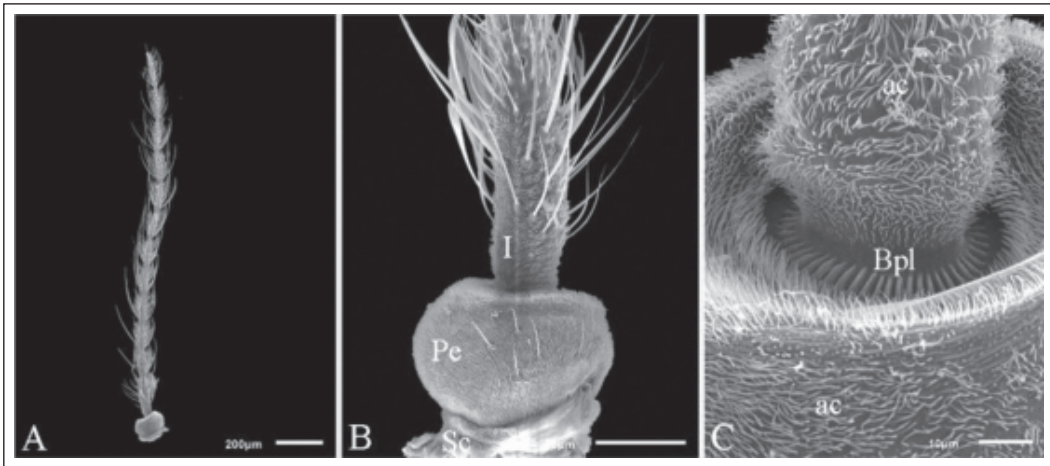
Figure 2. Scanning electron micrographs of the antenna of females of *An. dirus* (virtually identical in *An. cracens*). (A) The flagellum consisting of 13 flagellomeres. (B) The scape (Sc), pedicel (Pe) and first flagellomere (I), densely covered with aculeae (ac). (C) The basal plate (Bpl) of flagellomere I connected with the pedicel.

basal collar-shaped segment (Figure 2B). The pedicel is a large globular segment (Figure 2B) that bears the flagellum (Figure 2C). Each flagellum consists of 13 flagellomeres (Figure 2A). Aculeae (ac, microtrichium-like spicules) densely cover the surface of the scape, pedicel and the first flagellomere (Figures 2B and 2C).

Based on shape and size, five types of sensilla are borne on the antennae of *An. dirus* and *An. cracens*: sensilla ampullacea, sensilla basiconica, sensilla chaetica, sensilla coeloconica and sensilla trichodea (Figure 3). The morphology of each sensillum type is similar in both species.

Sensilla ampullacea are small peg organs in deep pits with a narrow opening. This type of sensillum is abundant on the first antennal flagellomere and decreases in number on flagellomeres 2–5 (Figures 3 and 4A).

Sensilla chaetica are long, thick-walled, sharp-pointed setae that arise from sockets. There are two types: large and small (Figure 3). Six large sensilla chaetica are borne in whorls proximally on flagellomeres 2–13. The small sensilla chaetica usually occur on the distal ends of flagellomeres 2–13. Both types also occur on the ventral surface of the first flagellomere and are often interspersed with aculeae (Figure 3).
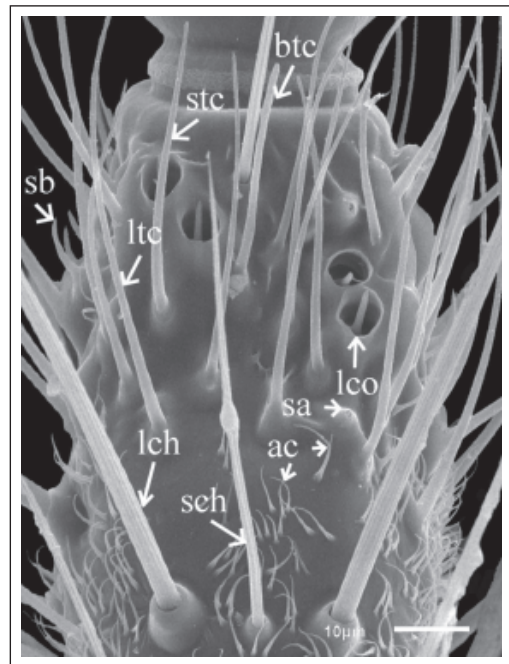

Figure 3. Scanning electron micrograph showing the various types of sensilla borne on the antennae of females of *An. dirus* and *An. cracens*. ac, aculeae; btc, blunt-tipped sensillum trichodeum; lch, large sensillum chaeticum; lco, large sensillum coeloconicum; ltc, long sharp-tipped sensillum trichodeum; sa, sensillum ampullaceum; sb, sensillum basiconicum; sch, small sensillum chaeticum; stc, short sharp-tipped sensillum trichodeum.
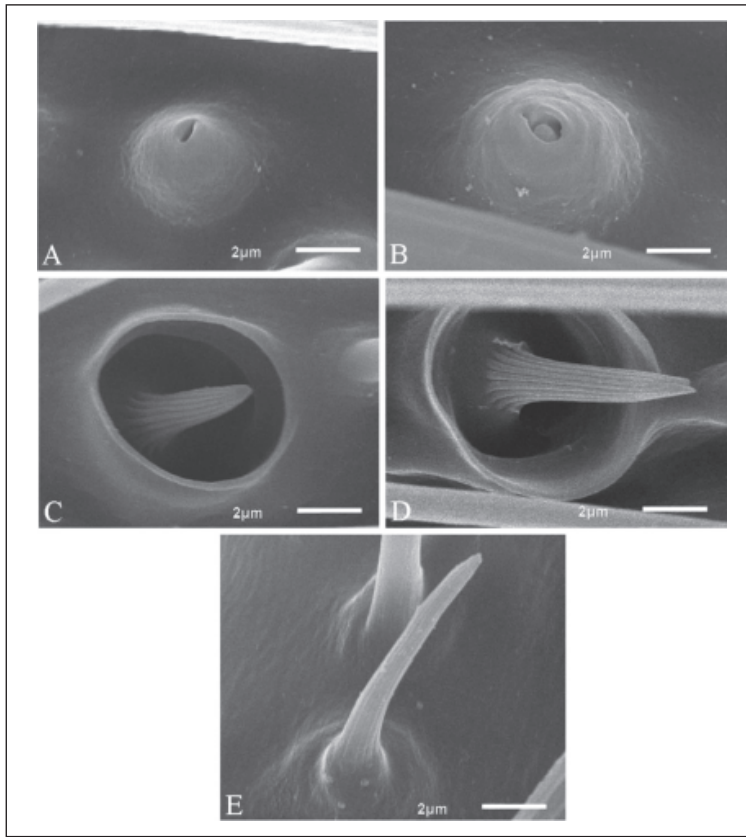
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Figure 4. Scanning electron micrographs of antennal sensilla of females of *An. dirus* and *An. cracens*. (A) Sensillum ampullaceum. (B) Small sensillum coeloconicum. (C) Short form of large sensillum coeloconicum, peg not reaching the orifice of the pit. (D) Long form of large sensillum coeloconicum, peg extending beyond the orifice of the pit. (E) Sensillum basiconicum (grooved peg).

Sensilla trichodea are the most abundant sensilla found on the flagellum of both species. They arise from a small prominent base and have a smooth surface. Three types of sensilla trichodea are present: long sharp-tipped trichodea, short sharp-tipped trichodea and blunt-tipped trichodea. The long sharp-tipped trichodea often bend toward the apex (Figure 3) and their number increases from the proximal to the distal ends of flagellomeres in both species. The short sharp-tipped trichodea of both species are slightly bent and are fewer in number than the former type (Figure 3). Blunt-tipped trichodea are shorter in length, have rounded tips, and have nearly the same diameter from the base to the tip (Figure 3). They also occur in fewer numbers than the sharp-tipped trichodea in both species.

Sensilla coeloconica are thick-walled sensilla. Two types, large and small, can be distinguished based on shape. Large sensilla coeloconica are peg-shaped projections located in deep depressions. They have 10–14 deep longitudinal grooves on their surfaces (Figure 3). The pegs may or may not project from the floor of the depression through the circular openings at the surface of the cuticle (Figures 4C and 4D). Both straight and curved-tipped sensilla coeloconica are found on the antennae of both species. Small sensilla coeloconica arise from the bottom of a shallow pit, but they do not protrude from the opening of the pit. These sensilla have a volcano-like structure with a small opening at the tip and a much smaller cuticular opening at the surface than large coeloconica. This type of
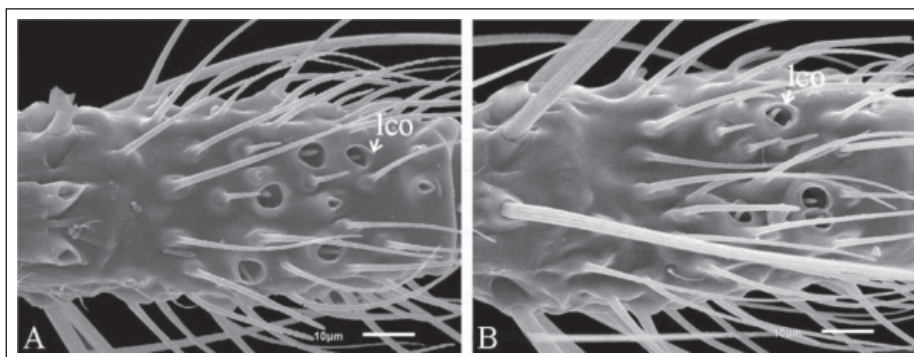
Figure 5. Scanning electron micrographs showing the different numbers of large sensilla coeloconica (lco) observed on flagellomere 2 of antennae of females of (A) *An. dirus* and (B) *An. cracens.*
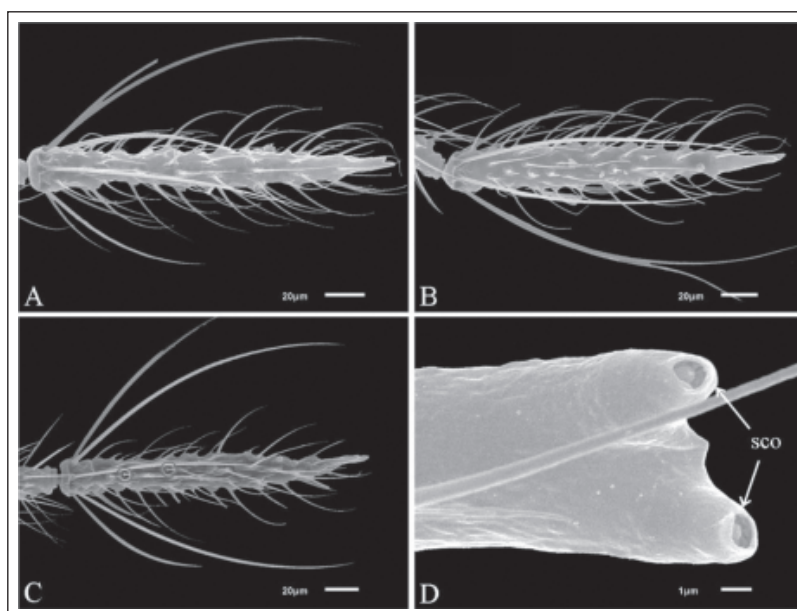

Figure 6. Scanning electron micrographs of flagellomere 13. (A, B) Absence of large sensilla coeloconica in Thai *An. dirus* and Hainan *An. dirus*, respectively. (C) Presence of large sensillum coeloconicum (circled) in *An. cracens*. (D) Higher magnification of sensilla coeloconica (sco) at the tip of flagellomere 13.

sensillum coeloconicum occurs on the first flagellomere (Figures 3 and 4B), and the tip of flagellomere 13 (Figure 6D).

Sensilla basiconica are curved peg-like or horn-shaped sensilla. The surface of sensilla basiconica is grooved lengthwise similar to those of sensilla coeloconica, but the grooves are not deep and are fewer in number (10–12). They arise from small prominences within an ill-defined alveolus (Figures 3 and 4E). Sensilla basiconica are slender, slightly bent, tapered and pointed at the apex, and are scattered on the surfaces of all flagellomeres of both species (Figures 3 and 4E).

Number of large coeloconic sensilla on antennae of females

The number of large sensilla coeloconica per antennal flagellomere ranges from 0–6 in *An. dirus* (both strains) and 0–5 in *An. cracens*. The largest number of large sensilla coeloconica occurs on flagellomere 2 of both strains of *An. dirus* (Table 1 and Figure 5).

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Table 1. Mean numbers of sensilla coeloconica on antennal flagellomeres 1–13 of females of Thai *An. dirus* (DTH), Hainan *An. dirus* (DHN) and *An. cracens* (CR) (30 females/strain, *n* = 60)

Flagellomere	Mosquito species			Kruskal-Wallis Test	Dunn's test
	DTH (range)	DHN (range)	CR (range)		
1	4.13±0.75 (2–6)	3.02±1.17 (1–6)	2.75±0.79 (1–5)	*p* < 0.001*	CR vs DHN (P = 0.288) CR vs DTH (P < 0.001) DHN vs DTH (P < 0.001)
2	4.03±0.86 (2–6)	3.92±1.00 (2–6)	2.95±0.72 (1–4)	*p* < 0.001*	CR vs DHN (P < 0.001) CR vs DTH (P < 0.001) DHN vs DTH (P=1.000)
3	2.80±0.84 (1–4)	2.98±1.05 (1–5)	1.83±0.69 (0–3)	*p* < 0.001*	CR vs DHN (P < 0.001) CR vs DTH (P < 0.001) DHN vs DTH (P = 1.000)
4	2.37±0.74 (1–4)	2.77±0.93 (1–5)	1.93±0.73 (0–3)	*p* < 0.001*	CR vs DHN (P < 0.001) CR vs DTH (P = 0.019) DHN vs DTH (P = 0.037)
5	1.47±0.62 (1–3)	1.93±0.84 (1–4)	1.28±0.56 (0–3)	*p* < 0.001*	CR vs DHN (P < 0.001) CR vs DTH (P = 0.395) DHN vs DTH (P = 0.003)
6	1.55±0.65 (1–3)	1.88±0.78 (1–4)	1.45±0.54 (0–3)	*p* = 0.005*	CR vs DHN (P = 0.007) CR vs DTH (P = 1.000) DHN vs DTH (P = 0.042)
7	1.32±0.47 (1–2)	1.13±0.60 (0–3)	0.73±0.63 (0–2)	*p* < 0.001*	CR vs DHN (P = 0.002) CR vs DTH (P < 0.001) DHN vs DTH (P = 0.236)
8	1.15±0.40 (0–2)	1.28±0.52 (0–2)	1.03±0.45 (0–2)	*p* = 0.014*	CR vs DHN (P = 0.010) CR vs DTH (P = 0.604) DHN vs DTH (P = 0.301)
9	1.00±0.18 (0–2)	0.78±0.45 (0–2)	0.72±0.49 (0–2)	*p* < 0.001*	CR vs DCH (P = 1.000) CR vs DTH (P = 0.000) DHN vs DTH (P = 0.011)
10	1.20±0.40 (1–2)	1.28±0.52 (0–2)	0.98±0.39 (0–2)	*p* < 0.001*	CR vs DHN (P = 0.001) CR vs DTH (P = 0.036) DHN vs DTH (P = 0.819)
11	1.82±0.43 (1–3)	1.53±0.62 (0–3)	1.15±0.58 (0–3)	*p* < 0.001*	CR vs DHN (P = 0.001) CR vs DTH (P < 0.001) DHN vs DTH (P = 0.029)
12	2.15±0.44 (1–3)	2.27±0.71 (0–4)	2.77±0.50 (2–4)	*p* < 0.001*	CR vs DHN (P < 0.001) CR vs DTH (P < 0.001) DHN vs DTH (P = 0.398)
13	0.35±0.58 (0–2)	0	0	*p* < 0.001*	CR vs DHN (P = 1.000) CR vs DTH (P < 0.001) DHN vs DTH (P < 0.001)
Total (range)	25.33±2.70 (19–31)	24.78±3.54 (17–32)	19.58±2.57 (12–26)	*p* < 0.001*	CR vs DHN (P < 0.001) CR vs DTH (P < 0.001) DHN vs DTH (P = 1.000)

The mean number of large sensilla coeloconica borne on each of flagellomeres 2, 3, 7, 10 and 12 in *An. dirus* (both strains) and *An. cracens* is significantly different (Table 1). Likewise, the mean number of large sensilla coeloconica per flagellum of *An. dirus* (Thailand strain, 25.33; Hainan strain, 24.78) and *An. cracens* (19.58) is significantly different (Dunn's test, $p < 0.0001$) (Table 1). Interestingly, large sensilla coeloconica were found on flagellomere 13 of Thai *An. dirus*, with a range of 0–2 (Figure 6C), but were always absent in Hainan *An. dirus* and *An. cracens* (Table 1, Figures 6A and 6B).

DISCUSSION

Based on morphology, the two sibling species, *An. cracens* and *An. dirus*, are difficult to distinguish unambiguously. Molecular methods are now widely used to distinguish and identify them (Walton *et al.*, 1999; Sallum *et al.*, 2005; Sallum *et al.*, 2007; Phunngam *et al.*, 2017). In addition to molecular methods, Cui *et al.* (1992) used gas chromatography of cuticular hydrocarbons to distinguish members of the Dirus Complex in Hainan Province of China, but no significant differences were found between them. However, these advanced methods, need to be performed in the laboratory at high cost. Thus, alternative morphological structures (e.g. antennal sensilla, cibarial armature, wings, etc.) have been successfully used for distinguishing isomorphic or cryptic species (Somboon *et al.*, 2001; Pitts and Zwiebel, 2006; Saeung *et al.*, 2014; Wijit *et al.*, 2016; Wike *et al.*, 2016; Sumruayphol *et al.*, 2016; Hempolchom *et al.*, 2017; Taai *et al.*, 2017). Saeung *et al.* (2014) constructed a robust key for the identification of eight species of the Hyrcanus Group (*An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati* and *An. sinensis*) based on morphometrics and ultrastructure of eggs observed using scanning electron microscopy. Subsequently, Hempolchom *et al.* (2017) constructed a key to reliably distinguish eight species of the Hyrcanus

Group in Thailand based on antennal sensilla of females. More recently, Taai *et al.* (2017) reported an effective method based on antennal sensilla for the identification and separation of females of *An. minimus*, the primary vector of malaria in Thailand, and its sister species *An. harrisoni*. From these studies, it would seem that antennal sensilla are useful structures for distinguishing malaria vectors of species complexes.

Insects rely on olfactory information for locating hosts, mating and locating suitable oviposition sites (Hildebrand and Shepherd, 1997; Qiu *et al.*, 2006). Olfactory receptor neurons in insects are contained in sensilla on antennae and mouthparts (Qiu *et al.*, 2006). In the present study, we describe the ultra-structure of five types of sensilla (ampullacea, basiconica, chaetica, coeloconica and trichodea) on the antennae of females of two important malaria vectors, *An. dirus* (Thailand and Hainan strains) and *An. cracens*. The morphology of these sensilla agrees with the findings of earlier studies on mosquitoes (McIver, 1982; Sutcliffe, 1994; Pitts and Zwiebel, 2006; Hill *et al.*, 2009; Hempolchom *et al.*, 2017). Sensilla trichodea are the most abundant sensilla on the antennae of *An. dirus* and *An. cracens*, which is also true of other insects, such as muscid flies (Diptera: Muscidae) (Wang *et al.*, 2014), yellow dung flies (Diptera: Scathophagidae) (Liu *et al.*, 2016) and other mosquitoes (Pitts and Zwiebel, 2006; Hill *et al.*, 2009; Seenivasagan *et al.*, 2009; Schultze *et al.*, 2014). Onagbola and Fadamiro (2008) noted that sensilla trichodea are putative mechanoreceptors. Schultze *et al.* (2014) confirmed that the blunt-tipped sensilla trichodea of female mosquitoes may respond to oviposition site-related compounds.

Sensilla ampullacea are few in number on the flagellum of both *An. dirus* and *An. cracens*, and this type of sensillum is similar in structure to those reported for other species of *Anopheles* (Boo and McIver, 1975; Taai *et al.*, 2017). This type of sensillum has been classified as hygro- and thermoreceptors. Like sensilla trichodea, sensilla chaetica are putative mechanoreceptors (Hill *et al.*, 2009). Additionally, this sensillum type is presumed to function as a contact chemo-

receptor during oviposition (Seenivasagan *et al.*, 2009). The sensilla basiconica observed on the antennae of *An. dirus* and *An. cracens* females correspond with type I grooved pegs observed in eight species of the Hyrcanus Group (set on small prominences within an ill-defined alveolus) by Hempolchom *et al.* (2017). Several authors have suggested that basiconica are olfactory sensilla (Mclver, 1974), which respond to vapors of ammonia, acetone and water (by excitation), acetic acid and anisole (by inhibition) (Kellogg, 1970), and lactic acid (Davis and Sokolove, 1976).

The fine structure of sensilla coeloconica (large and small) of *An. dirus* and *An. cracens* appears to be similar in other mosquito species (Ismail, 1962; Boo and Mclver, 1976; Pitts and Zwiebel, 2006; Hempolchom *et al.*, 2017). Using light microscopy, the mean number of large sensilla coeloconica on each flagellum of Thai *An. dirus* (25.33) and Hainan *An. dirus* (24.78) is significantly greater than in *An. cracens* (19.58). However, the mean number of these sensilla on the antennae of *An. dirus* and *An. cracens* is less than the mean number on the antennae of *An. harrisoni* (31.98), *An. minimus* (26.25), *An. nigerrimus* (33.10), *An. nitidus* (28.73), *An. paraliae* (37.55), *An. pursati* (27.85), *An. quadriannulatus* (29.00) and *An. sinensis* (36.47) (Gerberg *et al.*, 1994; Wijit *et al.*, 2016; Taai *et al.*, 2017). Although comparison of the ultrastructure of this sensillum type in the two species revealed similar structure, it is important to note that the difference in the number of the large sensilla coeloconica present on their antennae can be used to distinguish them. However, it must be pointed out that this difference may not distinguish these species from other members of the Dirus Complex. A comparative study of antennal sensilla in all members of the complex is needed to answer this question.

CONCLUSIONS

We identified and described, for the first time, the fine structure of five types of sensilla on the antennal flagella of females of *An. dirus* (Thailand and Hainan strains) and *An. cracens*. This study shows that both species bear morphologically similar antennal sensilla, with marked differences in the number of large coeloconic sensilla on each flagellomere and on both flagella. This finding provides an alternative method for distinguishing the two sibling species. The mean total number of large sensilla coeloconica on antennae of *An. cracens* is less than in *An. dirus*. This method is simple, reliable and easy to apply in the field because only light microscopy is required. Thus, it is very useful for entomologists who conduct epidemiological and vector incrimination studies. Further study is needed to determine the usefulness of antennal sensilla in distinguishing all member of the Dirus Complex.

Author Contributions
Conceptualization, AS; Methodology, KT and AS; Resources, PS and PS; Formal Analysis, KT, RP and AS; Investigation, KT, KA, TY, WJ and KP; Writing-Original Draft Preparation, KT and AS; Supervision, WS and AS; Usage of morphological terminology, REH; Writing-Review & Editing, REH, PS and AS; Funding Acquisition, KT and AS.

Conflicts of Interest
The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; nor in the writing of the manuscript and the decision to publish the results.

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