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MORPHOLOGICAL STUDIES ON *CULEX MOLESTUS* OF THE *CULEX PIPIENS* COMPLEX (DIPTERA: CULICIDAE) IN UNDERGROUND PARKING LOTS IN WUHAN, CENTRAL CHINA

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ABSTRACT

Mosquito surveillance was conducted in 42 underground parking lots in the densely populated metropolitan area of Wuhan, Hubei Province, central China from Aug to Oct 2012. Autogenous *Culex p. molestus* mosquitoes, which inhabit underground sites elsewhere in China, were identified and recorded for the first time in Hubei Province. Also *Culex quinquefasciatus* and intermediates (presumably with *Cx. p. pallens*) were identified from the samples, but *Cx. p. pallens*, which is known to hybridize with *Cx. quinquefasciatus*, was not found during the study. We attempted to determine how to differentiate between the subspecies of the *Culex pipiens* complex in Wuhan. In the female wings of *Cx. p molestus,* the subcosta intersected the costa beyond or at the furcation of R_{2+3}; however in *Cx. quinquefasciatus* and intermediates, the subcosta intersected the costa before the furcation of R_{2+3}. In female abdomens of *Cx. p. molestus,* lateral patches on tergum II reached the posterior margins; however in *Cx. quinquefasciatus* and intermediates, the lateral spots on tergum II did not reach the posterior margins. In the male genitalia of *Cx. p. molestus,* the DV/D ratios were -0.125 to 0.104 with a mean of -0.016 (n = 102), and DV/D ratios ≤ 0 were frequently found (68%). The dorsal arm of the phallosome of *Cx. quinquefasciatus* and intermediates usually was slightly divergent outwardly, and DV/D ratios were 0.316 to 1.571 with a mean of 0.582 (n = 118). Nineteen percent were intermediates with DV/D ratios between 0.2 and 0.4. Eighty-one percent were *Cx. quinquefasciatus* in which DV/D ratios between 0.4 and 0.6 were found frequently (53%). *Culex p. molestus* could be distinguished from *Cx. quinquefasciatus* and intermediates through characteristics of the female wing, the abdominal terga, the male scutum and the phallosomes.

Key Words: autogenous, *Culex quinquefasciatus*, DV/D ratio, intermediates, morphology, phallosome, scutum, tergum, wing venation

RESUMEN

Se realizó un sondeo de mosquitos en 42 estacionamientos subterráneos en el área metropolitana densamente poblada de Wuhan, Provincia de Hubei, centro de China desde agosto hasta octubre del 2012. Se identificaron y registraron por primera vez en la provincia de Hubei mosquitos *Culex p. molestus* autógena que habitan los sitios subterráneos en otras partes de China. También se identificaron de las muestras *Culex quinquefasciatus* y sus intermedios, pero *Culex p. pallens,* que se puede hibridar con *Cx. quinquefasciatus*, no fue encontrado durante el estudio. Tratamos de determinar cómo diferenciar entre las subespecies del complejo *Culex p. pipiens* en Wuhan. Las alas de las hembras de *Cx. p. molestus* tiene el interceptó de la subcosta con la costa más allá o en la furca de la R_{2+3}; sin embargo, en *Cx. quinquefasciatus* y los intermedios, presento el interceptó de la subcosta con la costa antes de la furca de R_{2+3}. En el abdomen de la hembra de *Cx. p. molestus*, los parches laterales en tergo II llegaron hasta el margen posterior; pero en *Cx. quinquefasciatus* y los intermedios, los puntos laterales en tergo II no llegaron hasta el margen posterior. La genitalia del macho de *Cx. p. molestus* tiene la proporción DV/D de -0.125 a 0.104, con un promedio de -0.016 (n = 102) y se encontraron con frecuencia (68 %) proporciones DV/D de ≤ 0. El brazo dorsal de la falosoma de *Cx. quinquefasciatus* y los intermedios generalmente fue ligeramente divergentes hacia el exterior y la proporción de DV/D fue 0.316 a 1.571, con un promedio de 0.582 (n = 118). Diecinueve por ciento de ellos fueron intermediarios con una proporción de DV/D entre

0.2 y 0.4. Ochenta y uno por ciento de ellos fueron *Cx. quinquefasciatus* en el que se encuentran con frecuencia una proporción de DV/D entre 0.4 y 0.6 (53%). Se pudo distinguir *Culex p. molestus* de *Cx. quinquefasciatus* y los intermedios a través de las características del ala de la hembra, los tergitos abdominales y el escudo y la falosoma del macho.

Palabras Clave: autógenas, *Culex quinquefasciatus*, proporción de DV/D, intermedios, morfología, falosoma, escudo, tergitos, venación alar

Culex molestus Forskal is one of the vectors of filariasis (Southgate 1979) and a competent laboratory vector of Japanese encephalitis virus (Weng et al. 2000). This species is known to frequent underground sites (Byrne & Nichols 1999; King et al. 1960). Indeed, *Cx. molestus* was first recorded in China in the Beijing underground water system in 1992 (Zhao & Lu 1993), and subsequently in an underground parking lot in Shanghai in 2009 (Ji et al. 2010). Until this report, *Cx. molestus* had not been found in Hubei Province, central China.

Recently, traffic jams in the densely populated metropolitan area of Wuhan, Central China have become a serious problem, and the construction of underground parking lots has progressed rapidly. Concrete water storage cisterns and ditches serve as the primary habitats for *Cx. pipiens* complex immatures. *Culex quinquefasciatus* Say was the most abundant species in urban Wuhan (Bao et al. 2005; Tian et al. 2010). Also, *Cx. quinquefasciatus* and *Culex pallens* Coquillett of the *Culex pipiens* complex subspecies were reported in Hubei Province (Lei & Zhou 1998).

The objective of the present study in Wuhan, Central China was to determine (i) whether *Cx. molestus* exists in underground parking lots, and (ii) which morphological traits of *Cx. molestus* differ from those of other subspecies of the *Culex pipiens* complex in Wuhan.

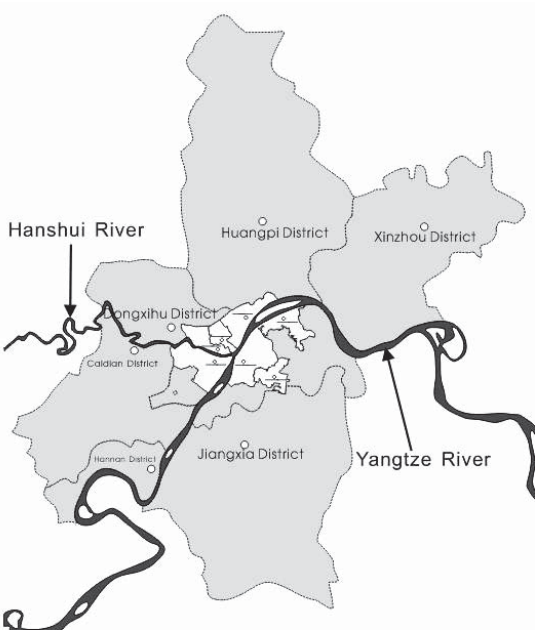

Fig. 1. Map of Wuhan. White areas are the 7 central districts. Hankou lies to the northwest of Yangtze River, and to the northeast of Hanshui River, including (from west to east) Qiaokou, Jianghan and Jiang'an District.

Materials and Methods

We conducted mosquito surveillance in 42 underground parking lots of the densely populated metropolitan area of Jianghan and Jiang'an District of Wuhan (Fig. 1, at N 30° 36' 34" E 114° 15' 34"; 27 m asl) from Aug to Oct 2012. Mosquito habitats were sampled using a hand held dipper (500 mL), hand net, or pipette. Twenty to 400 immature mosquitoes were sampled and placed into a 3.2 L plastic box one from each underground parking lot, labeled, and sent to the Vector Laboratory of Wuhan Centers for Disease Prevention & Control. Larvae were reared on a diet of brewers' yeast (Gerberg et al. 1994). Late stage *Culex* larvae and pupae from each parking lot were transferred to a separate mosquito cage and reared to adults. Adult mosquitoes were identified morphologically to species (Belkin 1962; Lu & Wu 2003). Specimens of male *Culex pipiens* complex with narrow and pointed apex dorsal arms (D) and the measurement of the dorsal and ventral arms (DV) in male genitalia (DV/D ratios) greater than 0.4 were considered to be *Cx. quinquefasciatus*, and similar specimens with DV/D ratios between 0.2-0.4 were considered intermediates (Barr 1957) of *Cx. quinquefasciatus* and *Cx. pallens*. Since the intermediates were indistinguishable morphologically from *Cx. quinquefasciatus,* other than by the DV/D ratio, the 2 forms may have come from the same egg raft, these specimens were considered *Cx. quinquefasciatus* and intermediates with *Cx. pallens*.

Adult mosquitoes were retained for several days and provided rearing water in the cages for the observation of autogenous oviposition without blood feeding. *Cx. molestus* was identified when specimens were morphologically identical to *Cx. molestus* as described by Harbach et al. (1984). To test stenogamy (Tate & Vincent 1936), autogenous eggs from 5 underground parking lots were

isolated and reared separately. Pupae from each underground parking lot were placed in a Plexiglass box (W 3 cm × L 7 cm × H 18.2 cm) with approximately 3 cm^3 of rearing water.

Four cages of *Culex* mosquitoes from 4 separate underground parking lots, each with mosquitoes (> 100), were identified as *Cx. quinquefasciatus* and intermediates without other sub-species of *Culex pipiens*, and were selected for the detailed morphological study.

To learn if *Cx. pallens* was mixed with *Cx. molestus*, 4 other cages with *Cx. molestus* were selected, in which 2 cages had autogenous egg rafts.

The total of 8 cages of mosquitoes were provided with albino mice for blood meals. The subsequent egg-rafts were recorded, morphological characters were recorded, and reared individually. Morphological characteristics of several 4th stage larvae from each egg-raft were studied. One to 3 pupae from an egg-raft were allowed to emerge in a glass container. The rest of the late stage larvae and pupae were placed into a cage, and reared for several days with rearing water in the cage to observe autogenous oviposition. Morphological characters of adult males and females from each egg-raft were recorded.

Egg-rafts, 4th stage larvae, and adult males and females from autogenous eggs were obtained for detailed study and comparison with *Cx. quinquefasciatus* and intermediates.

Siphon indices of 4th stage larvae were calculated using the width measured at mid length of the siphon. Pectens, 1-S setae and the shape of siphons were compared. Morphology of the scutum, wings, abdomen and male phallosomes of the 2 subspecies were compared. To observe female wings clearly, each female was killed with chloroform, one of the wings was removed and placed on a microscope slide, and the scales were brushed off by a small brush dipped into a small amount of solution contained laundry detergent. A microscope with an eyepiece reticle was used. The coordinate origin was adjusted at intersecting point of subcosta and costa. The reticle was rotated until one of the coordinate lines was rotated to the wing margin. The furcation points of R_{2+3} were observed in the reticle.

The length of the stem of the upper fork cell and the cell of female wing (Dobrotworsky 1965), the distance between the tips of the dorsal and ventral arms and that between the 2 dorsal arms of the male phallosome were measured with an ocular micrometer.

Results

Culex quinquefasciatus and intermediates were found in 37 of the 42 underground parking lots sampled (Fig. 2). *Culex* mosquitoes collected from underground parking lots laid autogenous egg rafts in 11 cages, and the adults were identi-

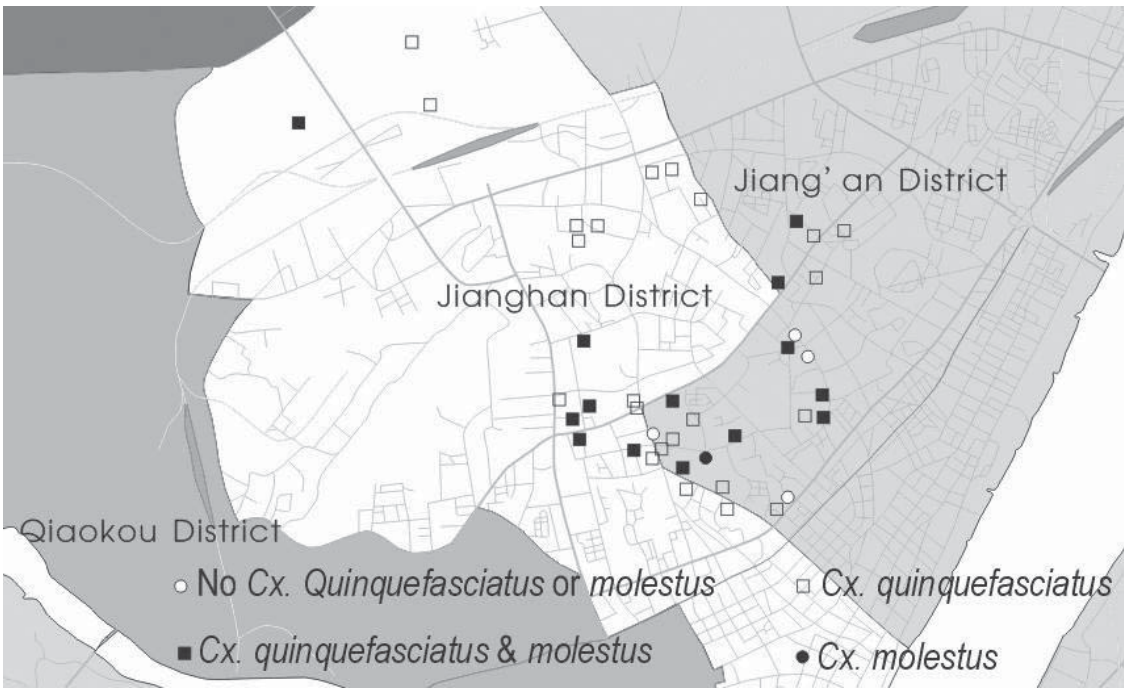

Fig. 2. Locations and occurrence of mosquito species of the 42 underground parking lots (UPLs) in Jiang'an and Jianghan District of Wuhan.

fied as *Cx. molestus*. In stenogamy tests, autogenous egg rafts were observed in each of the 5 small boxes after several days, and they hatched subsequently. The autogenous mosquitoes showed stenogamy in the laboratory. Males were identified as *Cx. molestus* due to their possession of narrow ventral arms of the genitalia in 4 cages in which autogenous eggs were not observed from the same underground parking lot.

Four cages were selected with *Cx. molestus* that produced egg-rafts after a blood meal and 35 of these egg-rafts were studied. Adult mosquitoes from 33 egg-rafts were identified as *Cx. quinquefasciatus* and intermediates and *Cx. molestus*. Adult mosquitoes were similar morphologically to *Cx. pallens* from 2 egg-rafts from one underground parking lot in Jianghan District. These adults did not lay autogenous eggs, but deformed mosquitoes were found in the next generation. The dorsal arms of the male genitalia of the next generation were similar to those of the *Cx. pallens*, or they were morphological intermediates of *Cx. pallens* and *Cx. quinquefasciatus* (see Feng & Liu 1954). The 2 egg-rafts may have consisted of hybrids of *Cx. molestus* and *Cx. quinquefasciatus*. Typical *Cx. pallens* were not found in this study.

Egg-Rafts

Culex molestus. The shapes of the autogenous eggs were rounded, rhombus-shaped, triangular, oblong, or indefinite and a number of eggs were arranged obliquely. Fifty-five egg-rafts from unfed females each contained 15-111 eggs with an average of 66 eggs. Most egg-rafts from females fed with albino mouse blood were symmetrical and boat-shaped, and formed of numerous eggs arranged in straight rows. Several egg-rafts were irregular. Thirty blood-meal fed egg-rafts contained 64-216 eggs with an average of 147.

Culex quinquefasciatus and Intermediates. Thirty egg-rafts were mostly boat shaped and contained 92-249 eggs with an average of 151. Egg-rafts of *Cx. quinquefasciatus* and intermediates were indistinguishable from the egg-rafts laid by blood-fed *Cx. molestus* mosquitoes.

Fourth Stage Larva

Culex molestus. The siphon was usually more slender with the ventral side in lateral view slightly sinuate in comparison to that of *Cx. quinquefasciatus* and intermediates. Their siphon indexes were 3.84-5.24 with a mean of 4.44 (Table 1). The pecten had 11-17 teeth including 0-2 basal abortive teeth, each with several (usually 3-5) ventral denticles. In 1-S 4-5 (usually 4) pairs of setae each 2-6 branched were found and the first seta was usually located beyond the pectin, but occasionally within it.

Culex quinquefasciatus and Intermediates. They were similar to *Cx. molestus*. The basal half of the siphon was slightly stout and the siphon was tapered distally. The mean siphon index was 3.94 (Table 1), which was significantly less than the index of *Cx. molestus* (t =10.76, df = 77, P = 0.0001). The pectens had 8-15 teeth including 0-2 basal abortive teeth, each with several (usually 3-5) ventral denticles. On 1-S 4 pairs of setae each had 2-7 branches. The first seta was located beyond the pecten.

Culex molestus, Females

Thorax. Scutal scales were golden brown with a reddish tint, while scutal setae were dark reddish brown. Integument and scales between supraalar and posterior dorsocentral setae were often darker, forming a pair of conspicuous ovoid spots (Harbach 1984). Sometimes the ovoid spots were indistinct.

Wing. Subcosta intersected the costa beyond furcation of vein R_{2+3} in about half of the females while others had a furcation of vein R_{2+3}, cell R_2/R_{2+3} 3.41-5.27, mean 4.05 (Table. 2).

Abdomen. Sterna were usually entirely yellowish or with only inconspicuous patches of dark scales present medially on sterna III-VI. Basal bands of abdominal terga were yellowish, usually the same color as sterna and basolateral spots. Sometimes basolateral spots were white and slightly different from the color of basal bands of abdominal terga and sterna. Tergum II had a basomedian spot of yellowish scales. Basal bands were slightly convex on terga II-V, often straight on terga VI-VII, and occasionally basal bands were slightly concave on terga VI-VII. Usually basal bands on terga III and IV did not reach spots and on terga V-VII were contiguous with spots. The lateral patches of yellowish or white scales on tergum II reached posterior margins and sometimes there were several pieces of dark scales near the lateral margin. Basolateral spots on terga III-VI of some females did not narrow

TABLE 1. SIPHON INDICES OF FOURTH STAGE LARVAE OF *CULEX MOLESTUS* AND *CX. QUINQUEFASCIATUS* AND INTERMEDIATES IN UNDERGROUND PARKING LOTS IN WUHAN, CHINA.

Subspecies	Number	Range	Mean
Culex molestus	60	3.84-5.24	4.44
Cx. quinquefasciatus and intemediates	60	3.53-4.21	3.94

TABLE 2. CELL R2 /R2+3 IN FEMALES OF *CULEX MOLESTUS* AND *CX. QUINQUEFASCIATUS* AND INTERMEDIATES IN UNDERGROUND PARKING LOTS IN WUHAN, CHINA.

Subspecies	Number	Range	Mean
Culex molestus	60	3.41-5.27	4.05
Cx. quinquefasciatus and intemediates	60	2.61-4.33	3.30

near lateral margins (Fig. 3A). Basolateral spots on terga III-VI of some females narrowed near lateral margins, similar to those of *Cx. quinquefasciatus* and intermediates, and the scale colors were more likely to be white (Fig. 3B). Other females had lateral patches of yellowish scales on terga II-VI often with small basal patches of white scales near lateral margins on terga II-VI (Fig. 3C).

Culex quinquefasciatus and Intermediates, Females

Thorax. Scutal scales were buff-colored and scutal setae were dark brown. No ovoid spots existed between supraalar and posterior dorsocentral setae. Wing: The subcosta intersected the costa before the furcation of vein R_{2+3}, cell R_2/R_{2+3} 2.61-4.33 (Table. 2), which was significantly less when compared to *Cx. molestus* ($t = 9.72$, df = 118, $P = 0.0001$). Yet there was considerable overlap in the range of ratios.

Abdomen (Fig. 3D). Sterna were mainly pale scaled, usually with dark scales or spots of dark scales present medially and lateroapically and occasionally median line entirely dark except II. Basal bands of abdominal terga were nearly white, usually the same color as sterna and slightly darker than basolateral white spots. Tergum II had median basal triangular patch of scales, while terga III-VII had convex basal bands. Bands usually did not touch spots on terga III-VI, while bands often were continuous with spots on tergum VII. White basolateral spots on tergum II did not reach posterior margins. Basolateral spots on terga II-VI narrowed near lateral margins.

Culex molestus, Males

Head: The combined length of the first 4 segments of palpi did not exceed the length of proboscis. Thorax: Similar to female except scales between supraalar and posterior dorsocentral se-

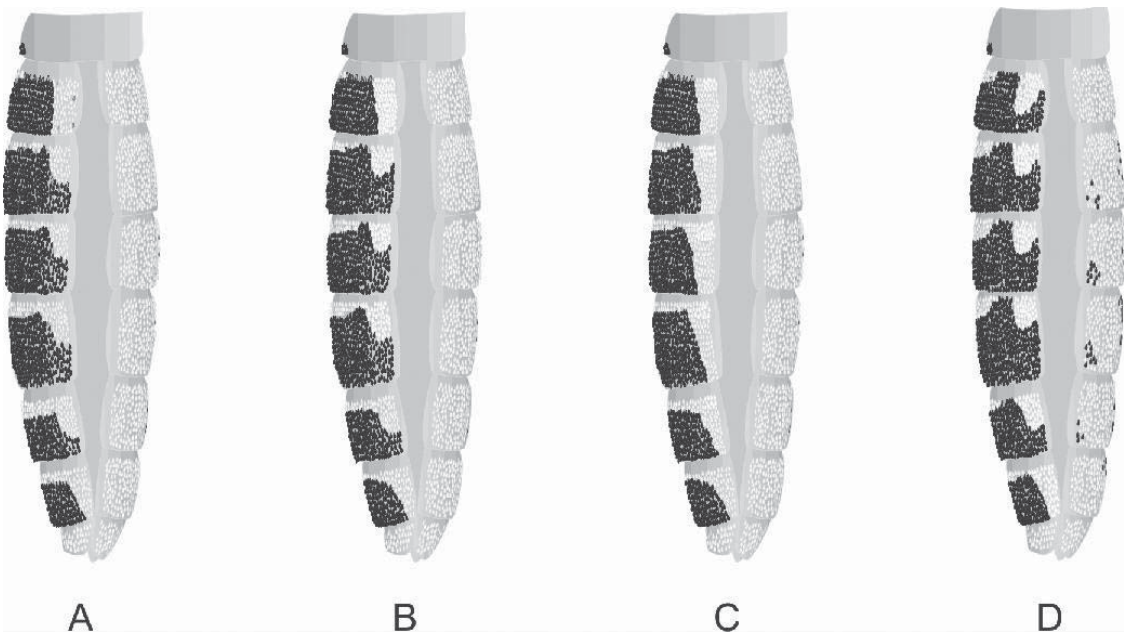

Fig 3. Female abdomen in lateral view. (A) *Cx. molestus* with yellowish basolateral spots not narrowed near lateral margins on terga III-VI. (B) *Cx. molestus* with white basolateral spots narrowed near lateral margins on terga III-VI. (C) *Cx. molestus* with lateral patches of yellowish scales on terga II-VII. (D) *Cx. quinquefasciatus* and intermediates with white basolateral spots narrowed near lateral margins on terga II-VI.

tae were noticeably darker and ovoid spots were distinct.

Wing. Subcosta intersecting costa before or occasionally beyond furcation of R_{2+3}.

Abdomen. Terga II had basomedian yellowish spot. Terga III-VII had basal yellowish bands (usually straight, or nearly so) produced posteriorly along lateral scale-free areas. There were particularly produced on terga V-VII, which gave them a concave appearance (Harbach et al. 1984). Sterna II-VII were yellowish (same color as basal bands of terga) and more frequently with median dark scales in comparison with the female (occasionally with lateroapical dark scales).

Male genitalia. Dorsal arms were broad, even width, broad subtruncate apex, and strongly divergent apically. The distance between the 2 ventral arms was often shorter than that between the tips of the 2 dorsal arms (Fig. 4A) and sometimes longer than that between the tips of the 2 dorsal arms (Fig. 4B). Lateral arms were broad in lateral view. Posterior margin more or less trilobed, and the ventral lobe more prominent than the others, bending ventrolaterally. The ventral lobe of the lateral arm in some specimens was larger in the dorsal view (Fig. 4B); however it was not necessarily larger in the lateral view. The lateral arm had a small, apically rounded subbasal knob (Tanaka et al. 1979), namely a lateral basal process (Sirivanakarn 1976), which was distinct, and smaller in comparison with *Cx. quinquefasciatus* and intermediates in dorsal view. After dissection of the outer division of the 2 subspecies, the subbasal knob of *Cx. molestus* was almost the same size as that in *Cx. quinquefasciatus* and intermediates. The ventral arm was usually narrower and more heavily sclerotized than in *Cx. quinquefasciatus* and intermediates. The DV/D ratio -0.125 to 0.104 had a mean -0.016 (n = 102). DV/D ratios ≤ 0 were found frequently (68%) Fig. 5).

Cx. quinquefasciatus and Intermediates, Males

Head: Palpi and proboscis were like those of male *Cx. molestus*.

Thorax. Scutum was similar to the female.

Wing: Subcosta intersecting costa before furcation of R_{2+3}.

Abdomen: Tergum II had basomedian spot. Terga III-VII had nearly white basal bands, usually straight, or nearly so, produced posteriorly along lateral scale-free areas, particularly on terga V-VII. Sterna II-VII had pale scales (same color as basal bands of terga) more frequently with median and lateroapical dark scales than in the female.

Male genitalia (Fig. 4C). The dorsal arm was narrower than that of *Cx. molestus*, straight, slightly divergent outwardly, and sometimes parallel with its counterpart of the opposite side. The apex was pointed and often slightly slanted inwardly in the dorsal view. The lateral arm was not trilobed in the lateral view as described by Sirivanakarn & White (1978). The subbasal knob seemed larger in comparison with *Cx. molestus* in the dorsal view. The ventral arm was larger, broader and more weakly sclerotized than in *Cx. molestus*. The DV/D ratios were 0.316 to 1.571 and had a mean of 0.582 (n = 118). Nineteen percent were intermediates with DV/D ratios between 0.2 and 0.4. Eighty one percent were the *Cx. quinquefasciatus* in which the DV/D ratios were between 0.4 and 0.6, and they were found frequently (53%) Fig. 5).

Discussion

We do not know the origin of the infestation of *Cx. molestus* in Wuhan due to a lack of surveys from underground sites. A few underground parking lots were built in Wuhan 10 yr ago. However, during World War II air-raid shelters were excavated in Wuhan, and possibly they were colonized by *Cx. molestus*. Also, the species may have been

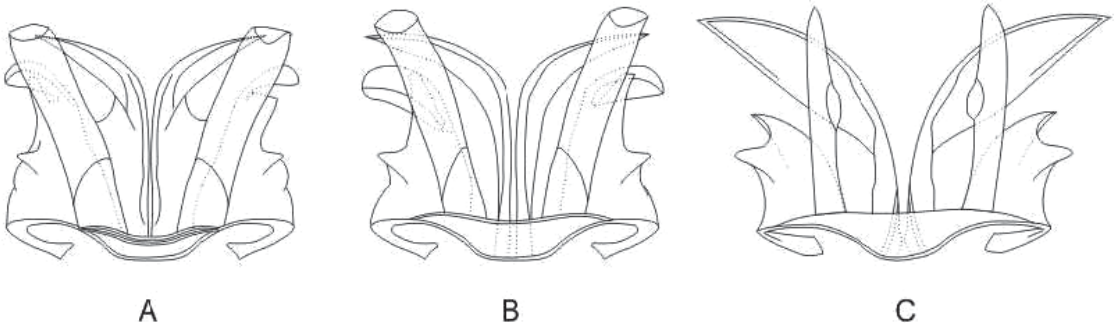

Fig. 4. Male genitalia in dorsal view. (A) *Cx. molestus,* the distance between the two ventral arms is shorter than that between the tips of the two dorsal arms, ventral lobe of lateral arm smaller. (B) *Cx. molestus ,* the distance between the two ventral arms is longer than that between the tips of the two dorsal arms, ventral lobe of lateral arm larger. (C) *Cx. quinquefasciatus* and intermediates.

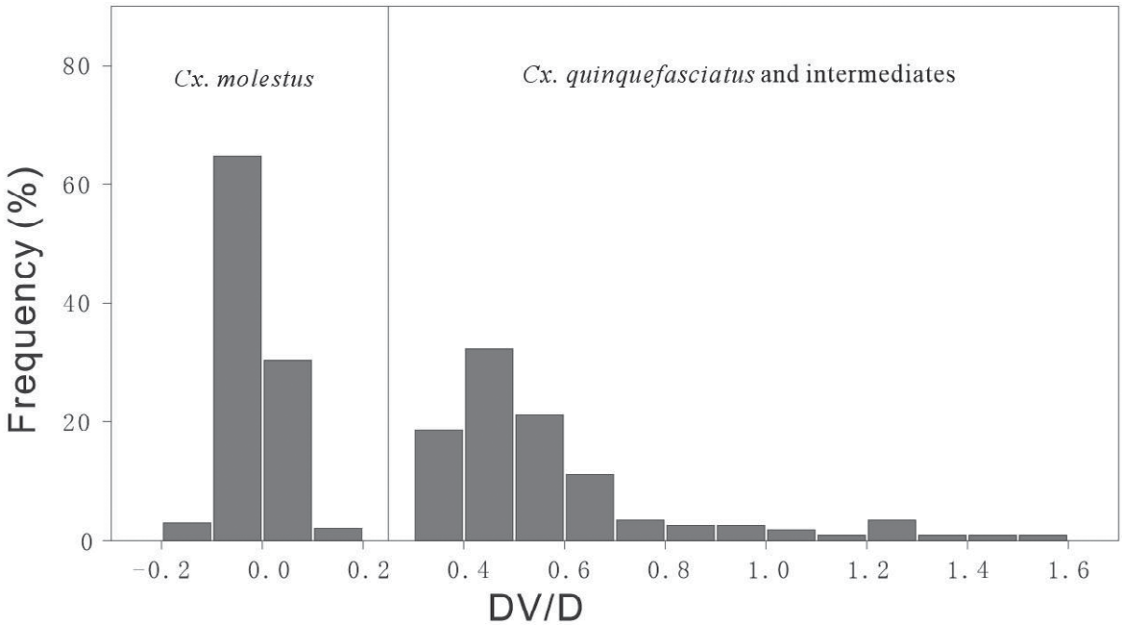

Fig. 5. The frequency distribution of the DV/D ratio of *Cx. Molestus* (left side) and the frequency distribution of the DV/D ratio of *Cx. quinquefasciatus* and intermediates (right side).

misidentified as *Cx. pallens,* because autogenous oviposition was not observed. Maximum DV/D ratios of male genitalia of *Cx. molestus* populations of Beijing (Zhao & Lu 1993) and Shanghai (Ji et al. 2010) were -0.0167 and -0.019, respectively, but the ratios of some *Cx. molestus* of Wuhan were greater.

Culex quinquefasciatus and *Cx. pallens* are widely distributed in China, the former in southern China and the latter in northern China, generally with the boundary line at the Yangtze River. The intermediate forms are thought to occur in the range of 28°-32° N (Chen & Lu 1983), which includes Wuhan. The DV/D ratios of *Culex pipiens* complex showed a negative correlation with latitude (Zhao & Lu 1994). Qiu et al (1986) reported that a composition of 28.1% *Cx. pallens,* 43.8% *Cx. quinquefasciatus*, and 18.1% intermediates was found in 1984 in Wuhan. However, we did not find *Cx. pallens* in this study.

Male genitalia (DV/D ratio) identifications revealed numerous discrepancies to accurately determine the identity of *Culex pipiens* complex members in the hybrid zone (McAbee et al. 2008). Intermediates (Jacob & Francy 1984) of *Cx. quinquefasciatus* and *Cx. pallens* in this study were slightly different from intermediate forms of *Cx. quinquefasciatus* and *Cx. pallens* as described by Feng & Liu (1954). The former intermediates were morphologically more similar to typical *Cx. quinquefasciatus,* except in their DV/D ratio, while the latter were morphologically intermediate between *Cx. quinquefasciatus* and *Cx. Pallens.*

There was some difficulty in distinguishing late stage larvae of *Cx. molestus* from those of *Cx. quinquefasciatus* and intermediates. The siphon index of *Cx. molestus* was often larger than those of *Cx. quinquefasciatus* and intermediates, but there was a range of overlap.

Females of the 2 subspecies can be distinguished by where the subcosta intersects the costa in the wing, the shape of basal bands in terga III-VII of the abdomen, and basolateral spots on tergum II. Males can be distinguished by dark ovoid spots between supraalar and posterior dorsocentral setae on the thorax and characteristics of male genitalia. Scales of mosquitoes should be carefully preserved. Characteristics of mosquitoes taken from cages might not be observed owing to the scales having fallen off.

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