The Hypopharynx of Male and Female Mosquitoes§

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Abstract: In blood-feeder female mosquitoes, the hypopharynx stylet is one part of the fascicle, the structure that pierces into the host skin during blood feeding. As other parts, the hypopharynx is a free stylet. However, since male mosquitoes do not feed blood, their mouthparts are less developed. The hypopharynx fuses with the inner wall of the labium, while maxillae and mandibles are much shorter than the labium. Only the labrum and the labium are well developed and function as food canal and its sheath, respectively. Light microscopy (LM) and scanning electron microscopy (SEM) were done to compare the hypopharynx of males of several mosquito genera and, in addition, females of autogenous mosquitoes.

The hypopharynxs of males of both autogenous and anautogenous mosquitoes fuse with the labium inner wall as long as the labium length, but are distinctly different structures from the labium. Dissociation occurs on the hypopharynx of female autogenous mosquitoes: *Toxorhynchites* spp. have a free hypopharynx as in anautogeny mosquitoes, whereas it fuses with the labium wall in *Malaya genurostris* Leicester, as in male mosquitoes.

INTRODUCTION

As most of female mosquitoes feed on blood, their mouthparts are highly specialized for piercing the host skin and sucking blood. During blood feeding, the fascicle (contains a labrum, a hypopharynx, pairs of mandibles and maxillae) enters the host skin, while the labium-formed sheath remains outside the skin. The maxilla and the mandible function as piercing organs [1,2], but there is no active movement of the hypopharynx. No muscles are either originated from or inserted on the hypopharynx [2]. Thus, practically, there is no active movement of the hypopharynx. Its function is to transfers saliva to the host tissue through salivary canal on its dorsal surface.

Unlike females, male mosquitoes do not feed on blood. Vizzi described the hypopharynx in *Anopheles quadrimaculatus* Say males as a sclerotic plate on the labial gutter [3], while Snodgrass stated that the hypopharynx and the labium of male mosquitoes completely fuse together forming the labio-hypopharynx [4]. Christophers recognized the hypopharynx of *Aedes aegypti* (Linneaus) males as a ridge on the labium [5]. Though the hypopharynx of *Ae. aegypti* males is not free from the labium wall, it is discernible from the labium wall by its texture both under light and scanning electron microscopy (SEM). Fusion of the hypopharynx with the labium is understandable, because the hypopharynx of males does not need to enter into the skin.

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MATERIALS AND METHODS

Mosquitoes

Materials used were the same with those used for examination maxillae and mandibles of male mosquitoes in our previous study [1] listed in Table **1**. The hypopharynx states of males of 44 species of 12 genera, females of 2 partially autogenous and 5 autogenous species were examined. For comparison, the hypopharynx of female Ae. aegypti was examined as a representative of anautogenous species. Specimens were kept in 70% ethanol solution, except for a few specimens kept in 3% glutaraldehide solution for transmission electron microscopy (TEM).

Light Microscopy

Males of all the 44 species were examined under light microscopy. The specimens were stained with Fuschin Acid as desribed in Wahid *et al*. [6]. They then put in methyl cellosolve (Nakarai Chemicals, Kyoto, Japan) on a slide glass and dissected under a dissecting microscope. The head was separated from the thorax. The free hypopharynx of females, together with other free stylets, was easily pried up from the labium gutter by a fine insect pin. On the other hand, it was no easy to separate the hypopharynx of males from labium tissue. The clypeus was pulled upward and forward carefully, to separate the hypopharynx from the inner wall of the labium. The detached hypopharynx was then cover with cover glass and examined under a light microscopy.

Scanning Electron Microscopy (SEM)

SEM was used to confirm the observation made under light microscopy. For this purpose, only males of *Ae. aegypti* were examined, since all male specimens principally have the same attribute. The dissected head, taking the labrum, maxillae and mandibles out of the labial gutter and leaving the hypopharynx undisturbed, was put on a *poly L lysine*-coated glass slide. The specimens were gradually dehydrated

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through 70% to 99% ethanol, and then placed in 99% *t*-butyl alcohol and kept in a freezer (-20°C). They were freeze-dried by using an ID-2 drier (EIKO, Tokyo, Japan) prior to gold coating by an EIKO IB-3 ion coater, then examined by a JSM-5200 LV scanning electron microscope (JEOL, Tokyo, Japan).

Transmission Electron Microscopy (TEM)

Transversal section of the proboscis of males and females of *Ae. aegypti, Malaya genurostris* Leicester*, Topomyia vijayae* Ramalingan and *Toxorhynchites splendes* (Weidemann) were examined by TEM. Fresh or dry specimens were kept in 3% glutaraldehyde solution (dry specimens were first put in 70% ethanol for ± 5 seconds to make them sinkable completely into 3% glutaraldehyde). Heads were then separated from the rest of the body in 3% glutaraldehyde solution buffered to pH 7.4 with phosphate buffer, then fixed for at least 1 hr in phosphate-buffered 1% osmium tetroxide solution. Dehydration was carried out by passing the head through 50%, 75%, 85%, 95%, 100% ethanol, each for 15 min, then, head was kept in n-glycildiethyl solution (QY-1) for 20-40 min, to a 1: 1 mixture of Epon and QY-1 for 1 hr, in a 3: 1 mixture of Epon-QY-1 overnight, and was embeded in Epon 812 for 2 h before heating at 45°C and 60°C, each for 24 h. The block was then cut with an ultra microtome. Sections were mounted on parlodion-carbon coated grids and stained in a saturated uranyl acetate solution followed by lead citrate. Specimens were examined by a transmission electron microscope JEM-1210 (JEOL, Tokyo, Japan).

RESULTS

Hypopharynx of Males

The hypopharynx of male mosquitoes invariably fuses with the inner wall of the labium for all the 44 species (Table **1**). It is a thick longitudinal plate ventrally fused with the inner wall of the labium. Its proximal and distal ends connect to the cibarial ventral wall and the hairy ligula at the proboscis tip, respectively (Fig. **1**). Contrasting to the longitudinally wrinkle surface of inner wall of the labium, the hypopharynx has a smooth surface and a gutter-like salivary canal at the median line (Fig. **2**).

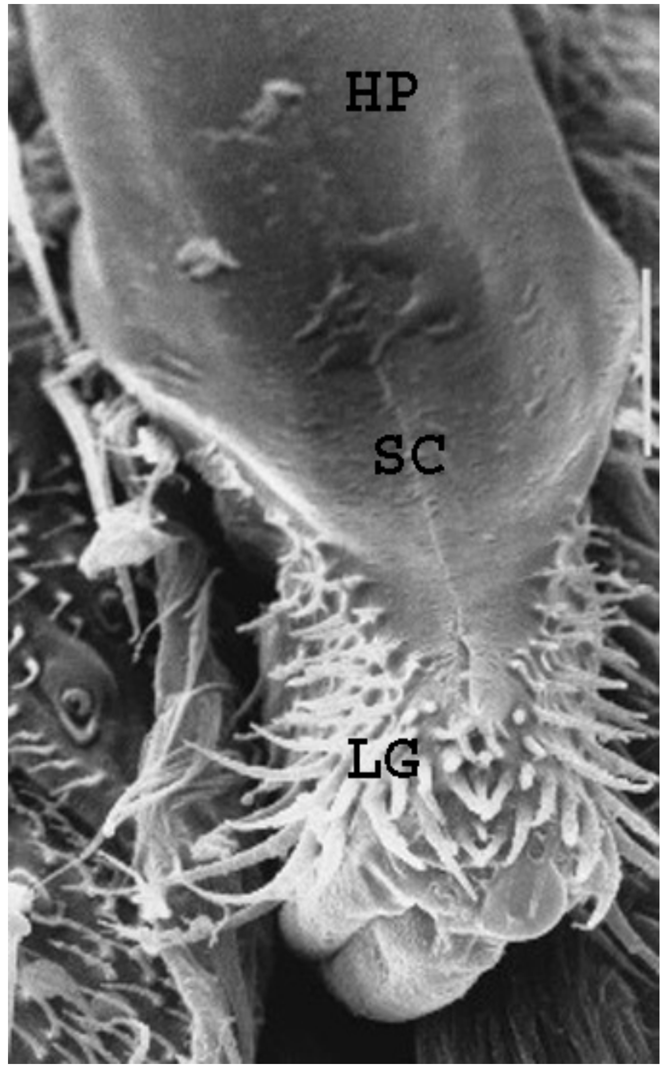

Fig. (1). The hypopharynx tip *Ae. aegypti* males fused with the ligula. HP, Hypopharynx. SC, Salivary canal. LG, Ligula.

By light microscopy, the hypopharynx of males can be recognized as a longitudinal structure in the labial gutter with parallel edges and a midrib representing a salivary canal. A hypopharynx that is detached from the labium has two parallel edges at both sides. The inner edge is the hypopharynx edge and the outer one is the labium wall tissue that is attached to the hypopharynx (Fig. **3**). The transversal section of the proboscis by transmission electron microscopy (TEM) shows different electron density between the hypopharynx tissue and the labium tissue (Fig. **4**).

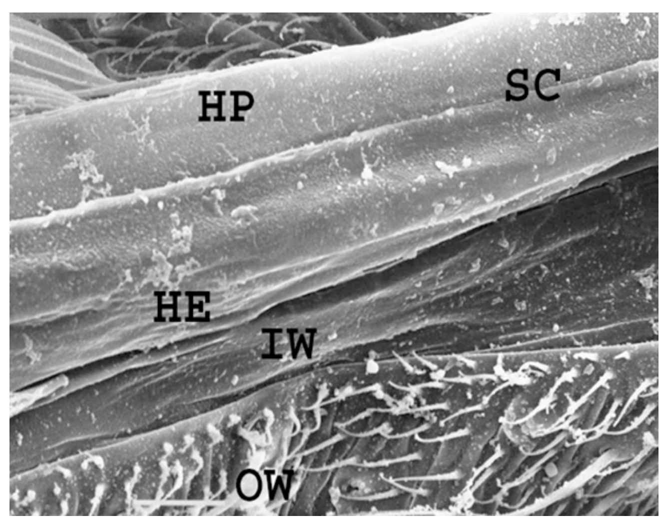

Fig. (2). The hypopharynx trunk of *Ae aegypti* male inside the labium gutter. HP, Hypopharynx. HE, The hypopharynx edge. SC, Salivary canal. IW, Inner wall of labium. OW, Outer wall of labium.

Anatomically, the salivary canal is a dorsally open gutter on the hypopharynx surface. However, its overlapping edges make it functionally a closed tube (Fig. **4A-C**), distally opened at the tip of hypopharynx (Fig. **1**).

Hypopharinx of Females

The hypopharynx of female mosquitoes almost invariably are separated from the labrum as a free stylet. Females of anautogenous mosquitoes, represented by the well known domestic mosquitoes *Ae. aegypti* is a stylet that completely separated from labrum as shown in TEM image (Fig. **5**). Mosquitoes that only autogenous at the first batch of their eggs, but ultimately need blood feeding for the rest of their eggs, as predicted, have hypopharynx attributes similar to those of anautogenous one. The females of *Culex pipiens molestus* Forskal and *Ochlerotatus togoi* (Theobald), are examples of this group, the partially autogenous species. However, despite the distinct non blood-feeding habits of females *Toxorhynchites* spp. and *Topomyia* spp., these lifetime autogenous mosquitoes showing hypopharynx characteristics similar to those of the autogenous females, a free stylet hypopharynx (Fig. **6A**,**B**). The attributes contrasting to those of another lifetime autogenous species, *Malaya genurostris,* which females have a male like-fused hypopharynx (Fig. **7**).

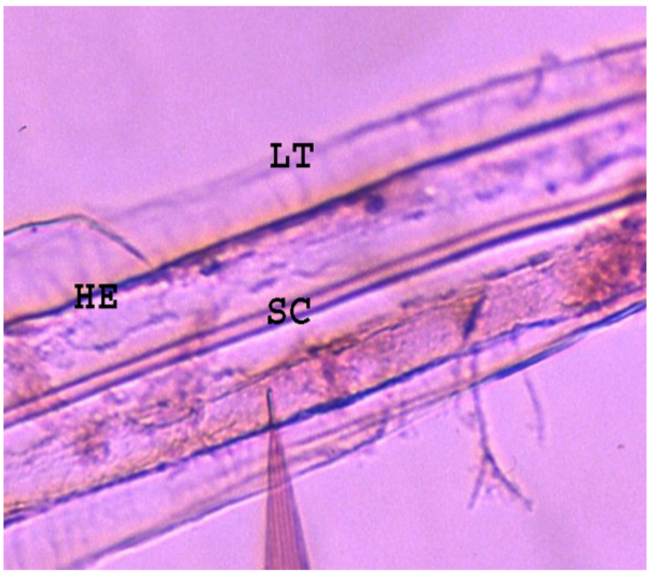

Fig. (3). The detached hypopharynx of a male *Ae. aegypti* from the labium under a light microscope showing a double edges at each side. The inner edge is the hypopharynx edge (HE), while the outer edge is the attached labium tissue (LT). SC, Salivary canal.

Table 1. Status of Hypopharynx of 44 Species Examined

Species	Female	Male
Aedes aegypti (Linneaus)	Free	Fused
Aedes albopictus (Skuse)	Free	Fused
Aedes flavopictus Yamada	Free	Fused
Aedes galloisi Yamada	Free	Fused
Aedes paullusi Stone and Farner	Free	Fused
Aedes pseudoalbolineatus Brug	Free	Fused
Aedes riversi Bohart and Ingram	Free	Fused
Aedes scutellaris (Walker)	Free	Fused
Anopheles kochi Doenitz	Free	Fused
Anopheles lindesayi japonicus Yamada	Free	Fused
Anopheles sinensis weidemann	Free	Fused
Anopheles stephensi Liston	Free	Fused
Anopheles vagus Doenitz	Free	Fused
Armigeres sp. 1	Free	Fused
Armigeres sp. 2	Free	Fused
Armigeres sp. 3	Free	Fused
Armigeres subalbatus (Coquillett)	Free	Fused
Armigeres theobaldi Barraud	Free	Fused
Culex fuscanus weidemann	Free	Fused
Culex halifaxii Theobald	Free	Fused
Culex kyotoensis Yamaguti and La Casse	Free	Fused
Culex pipiens molestus Forskal [a]	Free	Fused
Culex pipiens pallens Coquillett	Free	Fused
Culex quinquefasciatus Say	Free	Fused
Culex tritaeniorhyncus Giles	Free	Fused
Malaya genurostris Leicester [b]	Fused	Fused
Mimomyia chamberlaini metallica (Leicester)	Free	Fused

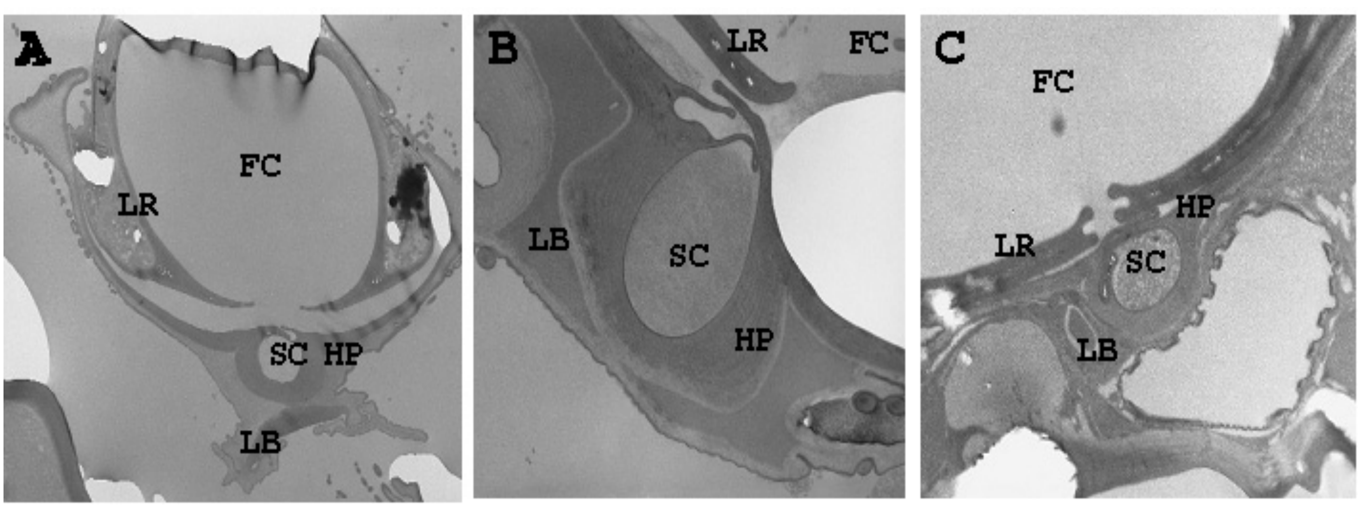

Fig. (4). Transversal sections of the hypopharynx of males of *Ae aegypti* (**A**), *Tx splendens* (**B**), and *To. vijayae* (**C**). FC, Food canal of the labrum. HP, Hypopharynx. LB, Labium. SC, Salivary canal.

(Table 1) contd…..

Species	Female	Male
Ochlerotatus japonicus japonicus (Theobald)	Free	Fused
Ochlerotatus poecilius (Theobald)	Free	Fused
Ochlerotatus togoi (Theobald) [a]	Free	Fused
Orthopodomyia anopheloides (Giles)	Free	Fused
Orthopodomyia sp.	Free	Fused
Topomyia vijayae ramalingan [b]	Free	Fused
Topomyia yanbarensis [b]	Free	Fused
Tripteroides aranoides (Theobbald)	Free	Fused
Tripteroides bambusa bambusa Yamada	Free	Fused
Tripteroides sp. 1	Free	Fused
Tripteroides sp. 2	Free	Fused
Toxorhynchites amboinensis (Doleschall) [b]	Free	Fused
Toxorhynchites manicatus yaeyama Bohart [b]	Free	Fused
Toxorhynchites splendens (weidemann) [b]	Free	Fused
Uranotaenia novobscura novobscura Barraud	Free	Fused
Uranotaenia sp. 1	Free	Fused
Uranotaenia sp. 2	Free	Fused

[a]Partially autogenous species; [b]Lifetime autogenous species.

DISCUSSION

The present study confirmed the previous reports, that hypopharynx of male mosquitoes fuse with the labium, forms the labio-hypopharynx [4]. Although the tissue of the hypopharynx part in the labio-hypopharynx is clearly different from the tissue of the labium part. Males of all the 44 species representing 12 mosquito genera invariably have a hypopharynx fused with inner wall of the labium, regardless feeding habits of their females. Thus, this attribute was established probably at the earliest stage in mosquito evolution, before extensive diversification.

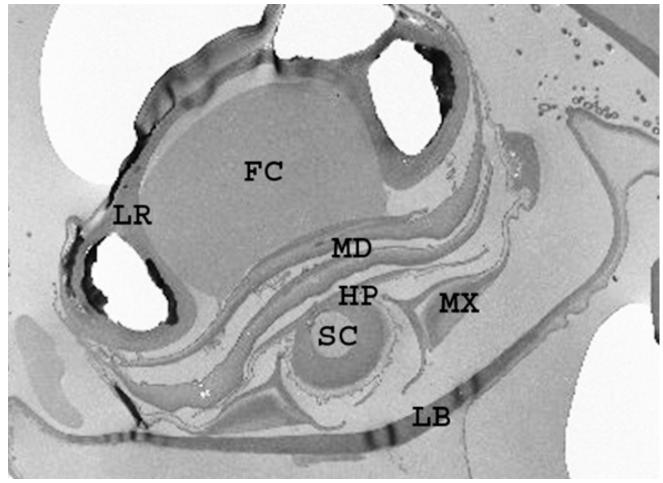

Fig. (5). Transversal section of the proboscis of an *Ae. aegypti* female. The free hypopharynx (HP) is separated from the labium (LB) by a space accommodating the maxillae (MX). FC, Food canal. LR, Labrum. MD, Mandibles. SC, Salivary canal.

It is interesting to note that males of a sand fly, *Lutzomyia migoney* Franca (Psychodidae) has a free hypopharynx [7] and males of black flies of the genus *Simulium* (Simulidae) has a free hypopharynx trunk but its tip fuses with the ligula by a thin transparent membrane (personal observation, unpublished data). Fusion of the hypopharynx and the labium may have taken place after separation of the psychodid line from the lineage including the ancestors of Simulidae and Culicidae.

Despite its fusion with the labium wall, the hypopharynx reaches the tip of the proboscis, with the salivary canal on its

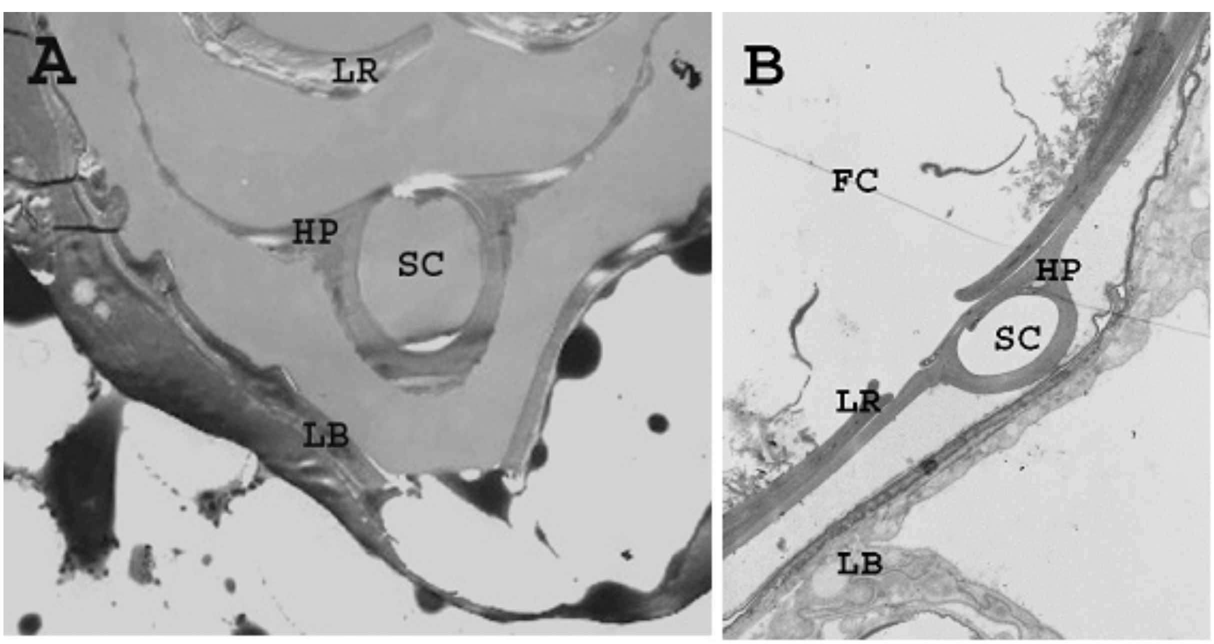

Fig. (6). Transversal sections of the proboscis of autogenous females of *Tx. splendens* (**A**), *To. vijayae* (**B**) showing the free hypopharynx. FC, Food canal. HP, Hypopharynx. LB, Labium. LR, Labrum. SC, Salivary canal.

dorsal surface. Differing from male mandibles and maxillae that probably have no function, the salivary canal of males functions as a closed tube by its dorsal overlapping edges, as in females [1,2,8].

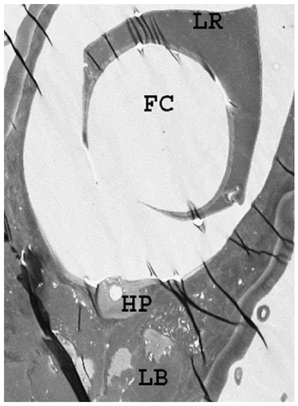

Fig. (7). Transversal section of the proboscis of a *Ml. genurostris* female showing its unique hypopharynx fused with the labium as in males. FC, Food canal. HP, Hypopharynx. LB, Labium. SC, Salivary canal.

Saliva secreted by salivary glands of males contains α-glucosidase, α-amylase and bacteriolytic factors, but lack the polypeptide D7, sialokinins I and II, and apyrase which are secreted from the median lobe and the distal portion of lateral lobes of female's salivary glands [2]. The latter three enzymes are responsible for immunoreaction of hosts [9], endothelium-dependent vasodilatation [10] and anti blood-coagulation [11-13], respectively. Apparently males need saliva for digestion of their main food such as floral and extra-floral nectar and honeydew [14], as well as for protection from pathogenic bacteria in sugar sources [15].

The present study confirmed the previous reports for the free hypopharynx of anautogenous females [4, 5, 8,16-23]. For autogenous females, Hudson reported that an autogenous population of *Wyeomyia smithii* has a free hypopharynx [20].

We described for the first time, the hypopharynx of females of two partially autogenous (*Cx. pipiens molestus* and *Oc. togoi*) and six lifetime autogenous females (3 species of *Toxorhynchites*, 2 *Topomyia* and 1 *Malaya*). As in anautogenous mosquitoes, females of life-time autogenous *Toxorhynchites* and *Topomyia*, as well as partially autogenous *Cx. pipiens molestus* and *Oc. togoi* have a free hypopharynx. However, females of autogenous *Ml. genurostris* have shown a fused hypopharynx with the labium wall as the hypopharynx of males.

The unique case of *Ml. genurostris*, previously known as *Harpagomyia* [24, 25], may correlate with its remarkable feeding habit among mosquitoes that they are acquiring food from a regurgitation fluid by *Crematogaster* ants [25-30] with its peculiar proboscis that swollen at the tip [25]. Jacobson [26,27] in Clements [30] described that adults of *Malaya splendens* feed on a black tree ant, *Crematogaster difformis*. The adult mosquitoes positioned them self on the ant trails, head uppermost, and rocked back and forth, left and right. Ants ascending the tree walked unhindered between the legs of the mosquitoes, but when an ant descending the trunk reached a mosquito, the mosquito palpated the ant's head with its forelegs and antennae. Usually the ant stopped, pressed its thorax to the tree while raising its abdomen and opening its jaws widely. The mosquito immediately started rocking forward and backward while vibrating its wings. When the ant regurgitated a drop of liquid, the mosquito imbibed it, after which the ant continued on its way.

There are two possibilities of the evolution of hypopharynx of genus *Malaya*: Firstly, members of the genus retained an old attribute structure of mosquito ancestor's hypopharynx that might be fused before its separation from labium for the purpose of sucking blood. However, this hypothesis is unlikely since a free hypopharynx, as in anautogenous species, is the most common state in female mosquitoes that suggest that their common ancestor had this attribute [31]. More far, the old genera *Anopheles* that is distributed world wide and placed on the basis of phylogeny tree of mosquitoes based on clasditic analysis [32] has share the free hypopharynx attributes, as well as other autogenous mosquitoes mention above, and hence showing that the hypopharynx of female mosquitoes has a free origin from their common ancestor.

The second hypothesis regarding the attribute of female *Malaya* hypopharynx is that the fused hypopharynx of this genus might recently derived from a free hypopharynx of its ancestor. This hypothesis is supported at least by three facts: (1) the other life-time autogenous genera (*Toxorhynchites* and *Topomyia*) have a free hypopharynx, as well as the partially autogenous species *Cx. pipiens molestus* and *Oc. togoi* showing that even though these mosquitoes do not use their hypopharynx as blood feeding females did, they still retain the clue of their common ancestor's attributes on hypopharynx and other mouthparts such as maxillas and mandibles in some degrees [1,20], so, the fused hypoharynx of *Malaya* should derived from an older attribute of their origin; (2) geographic distribution of genus *Malaya* confined only for Southeast Asia and Papua regions [25,29], showing that this genus might evolved recently in mosquito evolution and hence derived their fused hypopharynx from an older mosquitoes that had a free hypopharynx as other mosquitoes did; (3) phylogeny analyses by Harbach and Kitching, using morphological characters-based cladograms, placed this genus as the most recent group appeared in mosquito evolution [32]. The hypothesis suggests that the genus represents the most advance stage in evolutionary process of the hypopharynx, and that such evolution occurred independently from other autogeneous genera.

Tribes and genera examined by us are still limited in view of the recent classification system of mosquitoes by Harbach and Kitching [32]. Although males of all the species examined have the hypopharynx fused with the labium, there are remain possibilities that different states are discovered for males of the other tribes or genera, especially those put toward the base of the phylogenic tree of mosquitoes based on cladistic analyses of Harbach and Kitching [32]. It is also possible that the hypopharynx fused with the labium is found in life-time autogenous species yet to be examined. We revealed the diverse states of male maxillae and mandibles [1,6], and noted that expansion of the scope to all the mosquito tribes and genera could contribute to phylogenic study of mosquitoes. Though the hypopharynx with functions is much less variable, it could still yield cues to understand the evolution of feeding habits of mosquitoes.

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