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Tongue Structure of *Rhinolophus pusillus* and *Miniopterus schreibersii*

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ABSTRAK

Kelelawar memiliki berbagai peran penting baik secara ekologis maupun ekonomis yaitu dalam pengendalian hama, siklus nutrien, pollinator, dan penghasil guano. Namun, kelelawar mengalami ancaman kepunahan yang disebabkan karena kerusakan lingkungan maupun perburuan liar, sehingga upaya konservasi sangatlah penting. Upaya ini dapat dilakukan melalui pendekatan studi struktur histologis lidah untuk memahami kesukaan pakan binatang tersebut. Di Yogyakarta, Indonesia, terdapat dua spesies kelelawar, *Rhinolophus pusillus* dan *Miniopterus schreibersii* yang hidup di habitat sama. Penelitian ini bertujuan untuk mengkaji struktur histologis lidah *R. pusillus* dan *M. schreibersii*. Tiga ekor kelelawar pada masing-masing spesies ditangkap menggunakan *sweep net* dan *mist net*, selanjutnya kelelawar dianastesi menggunakan kloroform dan dikorbankan. Lidah dikoleksi dan difiksasi dalam *neutral buffered formalin* 10%. Preparasi histologis dilakukan menggunakan metode parafin dengan pewarnaan Hematoxylin Eosin dan tebal irisan 6 µm. Data histologis dianalisis secara deskriptif komparatif. Hasil menunjukkan bahwa struktur histologis lidah *R. pusillus* dan *M. schreibersii* serupa. Secara histologis, lidah kelelawar tersebut tersusun atas 3 lapis yaitu tunika mukosa, tunika submukosa, dan tunika muskularis. Pada bagian lapisan epitel pipih berlapis berkeratin terdapat tonjolan papila filiformis, fungiformis, dan sirkumvalata. Tunika muskularis tersusun dalam 3 orientasi. Pada lidah kedua spesies tersebut juga terdapat kelenjar ludah dengan sel-sel penghasil kelenjar serosa dan mukosa. Kelenjar ludah *R. pusillus* terdapat di bagian sublingual dan anterior lidah, sedangkan kelenjar ludah *M. schreibersii* terdapat di bagian antero-inferior dan posterior lidah. Berdasarkan hasil tersebut dapat disimpulkan bahwa kedua spesies kelelawar memiliki struktur lidah yang serupa sehingga dapat mengembangkan pola adaptasi yang serupa terhadap pakan.
Kata kunci: *Rhinolophus pusillus, Miniopterus schreibersii*, lidah, struktur histologis, papila, kelenjar ludah

ABSTRACT

Bat plays many important roles environmentally and economically as pest control, nutrient cycling, pollinator as well as guano's producer. The threat of extinction arises due to environment damage or illegal hunting, thus, conservation effort is crucial that can be approached through studying the histological structure of tongue to understand its food preferences. In Yogyakarta, Indonesia, there are two bats, *Rhinolophus pusillus* and *Miniopterus schreibersii* which live in the same habitat. This study evaluated the histological structure of tongue of *R. pusillus* and *M. schreibersii*. Three individuals each species was caughted using sweep net and mist net, further anesthetized using chloroform and sacrificed. The tongues were collected and fixed in 10% neutral buffered formalin. Histological preparation was done based on paraffin method with Hematoxylin Eosin staining and sectioned with 6 µm thickness. The histological data were compared descriptively. The results showed that the histological structure of tongue of *R. pusillus* and *M. schreibersii* are similar. There are 3 layers; mucous, submucous, and muscular layers. The keratinized stratified squamous epithelial layer protrudes as filiform, fungiform, and circumvallate papillae. The muscular layer arranged in 3 orientations. We found serous and mucous producing cells in salivary gland's of both bats. The salivary glands of *R. pusillus* are found in the sublingual and anterior part of the tongue, whereas, the salivary gland of *M. schreibersii* are found in the antero-inferior part and posterior part of the tongue. Based on these results, we suggested that both species have similar tongue structure thus may develop similar food adaptation.
Keywords: *Rhinolophus pusillus, Miniopterus schreibersii*, tongue, histological structure, papillae, salivary gland

INTRODUCTION

Bats play many important roles in ecosystem as pollinators, seed dispersals, arthropod suppressors, and nutrient turnover agents (Castillo-Figueroa, 2020). Therefore, bat is important as ecological indicator of habitat quality (Hassi, 2018). In addition, bats produce guano which has economic value (Kasso & Balakrishnan, 2013; Sakoui *et al.*, 2020). Meanwhile, the ecosystem disturbances caused by deforestation, land conversion, and illegal hunting are serious threat to the biodiversity in South East Asia, including bats (Hughes, 2017; Tanalgo & Hughes, 2019). The bat meat trading was also contributing to this massive extinction, especially in Indonesia (Struebig *et al.*, 2007; Sheherazade & Tsang, 2015).

Conservation is crucial to prevent bats extinction. Understanding what are the diets of a species is one of the basic efforts to prevent extinction. Tongue is an organ that plays a role in the initial process of digestion. Each species has different tongue characteristics depending on the type of diet (Selim *et al.*, 2008; Abayomi *et al.*, 2009; Adeniyi *et al.*, 2010; Taki-El-Deen *et al.*, 2013). Adeniyi *et al.*, (2010) studied that structural differences of tongue were particularly evident in the epithelium layer and salivary glands.

There are limited studies related to the histological structure of bat's tongue in South Asia. Therefore, this study aimed to evaluate the tongue structure of *Miniopterus schreibersii* and *Rhinolophus pusillus*, which further can be used as data based to compile the conservation strategy of bats based on their diets.

MATERIALS AND METHODS

Bats and Sampling Area

Rhinolophus pusillus (Figure 1A.) and *Miniopterus schreibersii* (Figure 1B.), three individu each were capture from Goa Jepang aisle number 23, Bukit Plawangan, Taman Nasional Gunung Merapi, Yogyakarta, Indonesia (Figure 2. and 3.) using sweep net and mist net. Bat sampling is based on license no: S. 120/TINGM-1.4/2012. The captured specimens were then placed in separated cages based on species.

Histological Preparation

Bats were euthanized and dissected to collect the tongue samples. The samples were fixed in 10% neutral buffered formaline for 1–2 days. The samples were then dehydrated using graded ethanol (70, 80, 90, 96, and 100%) and subsequently cleared with xylene overnight. Afterwards, the samples were infiltrated with series of paraffin wax and then embedded with freshly melted paraffin wax. The samples were sectioned longitudinally with 6 μm of thickness using microtome and placed into clean glass slides. The sections were then proceed with Hematoxylin-Eosin staining procedure (Bancroft & Cook, 1988) and observed under a light microscope. The histological images were taken using microscope mounted DSLR camera.

Data Analysis

Data were analyzed descriptively by comparing the histological structure of the tongue of *R. pusillus* and *M. schreibersii*.

RESULTS AND DISCUSSION

General Tongue Structure of Rhinolophus pusillus and Miniopterus schreibersii

R. pusillus' tongue is morphologically shorter and wider, whereas *M. schreibersii* is longer and slender. As the general structure of other bats' tongue which composed of three layers: mucous layer, submucous layer, and muscular layer (Taki-El-Deen *et al.*, 2013), the tongue of these two species are also composed by three layers. The mucous layer of tongue on both *R. pusillus* and *M. schreibersii* are arranged by keratinized stratified squamous epithelium which protruded into tongue papillae. Abayomi *et al.*, (2009) studied that pangolins (*Manis tricuspis*), which are insectivores (ants and termites eater), have a keratinized structure on the epithelial tissue of the tongue to prevents abrasion and tissue damage when eating termites or ants. In addition, this keratinization also acts as an adhesive to keep foods stay to the surface of the tongue. Taki-El-Deen *et al.*, (2013) reported that stratified squamous epithelial layer of insectivorous bat is thicker compared to that of frugivorous bat. Furthermore, Abayomi *et al.*, (2009) suggested that the keratinization differences on mammalian tongue are not only about functional adaptation but are also related to phylogenetic adaptation.

There are two layers underneath the mucous layer, which are submucous layer and muscular layer. The submucous layer consists of connective tissue enriched with blood capillaries. The tongue's muscular layer of *R. pusillus* and *M. schreibersii* composed of striated muscle arranged in 3 orientations, which are longitudinal, transversal, and vertical. The longitudinal muscles can be found in two locations, namely under the lamina propia mucosae (the internal longitudinal muscles) and at the base of the tongue (the external longitudinal muscles). The vertical and transverse

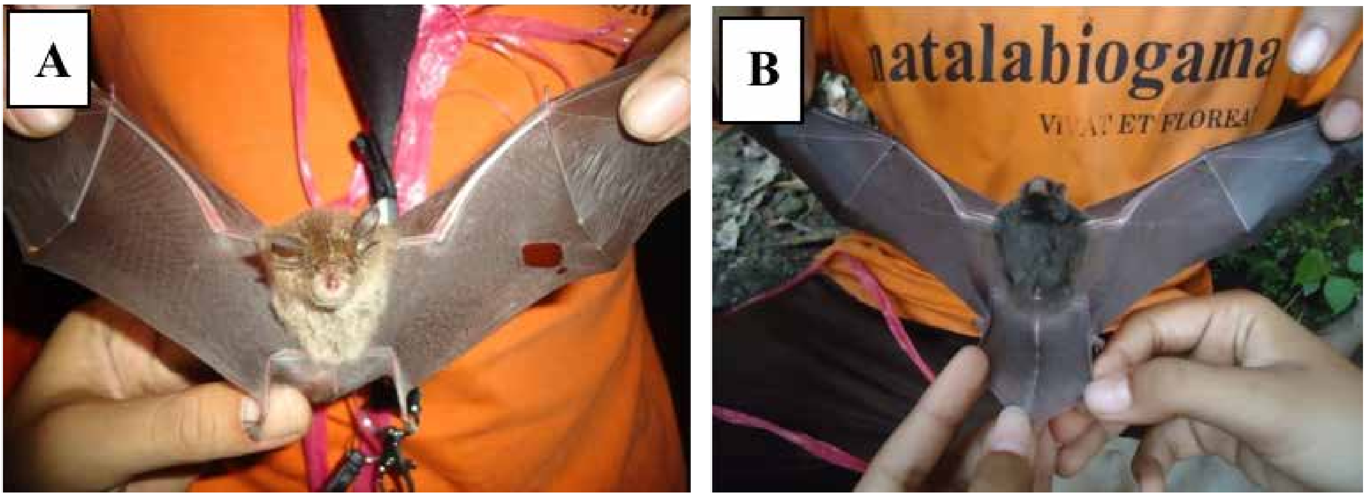

Figure 1. *Rhinolophus pusillus* (A) and *Miniopterus schreibersii* (B) (Doc. Hermawan, 2012)

Figure 2. Map of Special Region of Yogyakarta

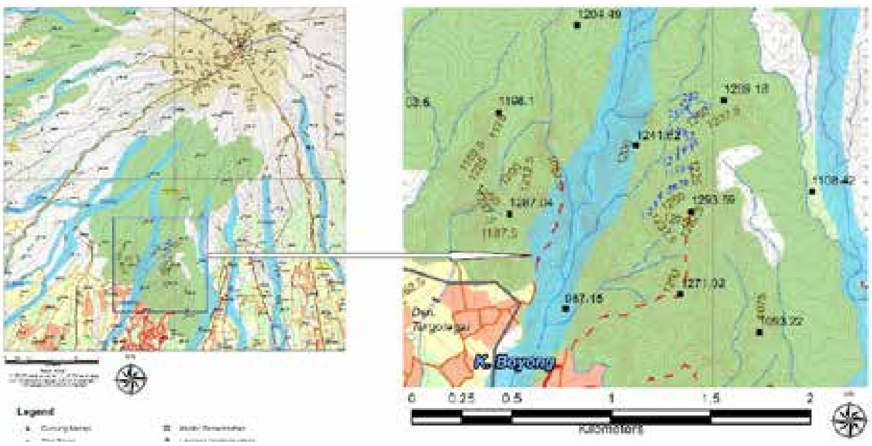

Figure 3. Map of Goa Jepang, Kaliurang, Pakem, Sleman, Special Region of Yogyakarta

* The aisle number of Goa Jepang shown in blue.

muscles are arranged between the internal and external longitudinal muscles (Figure 4.). The muscle bundles are surrounded with connective tissues containing many blood vessels and nerves. The tongue muscles of *R. pusillus* and *M. schreibersii* are complex and dense due to their role in mechanically digesting the food.

In this study, we did histological measurement of these 3 layers of tongue. We found that epithelial layer thickness of *R. pusillus* is similar with that of *M. schreibersii*. This might be related to the similar type of insect consumed by both *R. pusillus* and *M. schreibersii* since they live in the same habitat. The both bats tongue epithelium are quite thick to protect the tongue tissue from the exoskeleton of the insects while chewing. Moreover, the similar thickness were also shown by the muscular layer of *R. pusillus* and *M. schreibersii.* The ratio of the longitudinal muscle's width to the muscular layer's thickness between *R. pusillus* and *M. schreibersii* were similar (Table 1.). Furthermore, the ratio of the vertical and transverse muscle's thickness to the muscular layer's thickness were also similar between these two bats (Table 1.).

Based on the histological measurement of muscular layer, we found that longitudinal muscles have a smaller proportion compared to transverse and vertical muscles (Table 1.). The longitudinal muscles include the superior longitudinal muscles and the inferior longitudinal muscles. The superior and inferior longitudinal muscles play a role in the narrowing and widening of the tongue, lifting or lowering the tip of the tongue. The transverse muscles play a role in the tongue narrowing while the vertical muscles play a role in the tongue widening. However, these two types of muscle work together to lengthen the tongue. Therefore, *R. pusillus* and *M. schreibersii* are tend to have a similar tongue elasticity in processing food due to greater proportion of the longitudinal muscles. Mammals such as bat, hedhog, and rat have similar muscle orientation which important to hold the structural integrity of tongue (Adeniyi *et al*., 2010). Harper *et al*., (2013) reported that in nectar-feeding bat, *Glossophaga soricina* the muscle layer of the tongue function as muscular hydrostat to maximize the gathering of nectar.

The Tongue Pappilae of Rhinolophus pusillus and Miniopterus schreibersii

The mamalian tongue generally has 3 - 4 types of papillae, which are filiform, fungiformis, foliate (found in certain mammals), and circumvallate papillae. Our results showed that the surface of the tongue of *R. pusillus* and *M. schreibersii* contains three types of papillae, those are filiform papillae, fungiform papillae, and circumvallate paillae (Figure 5.). These results were similar with the previous study in frugivorous bat *Rousettus aegyptiacus*, insectivorous bat *Rhinopoma hardwicke* (Taki-El-Deen *et al*., 2013), and *Pipistrillus kuhli* (Selim *et al*., 2008; Mutlak *et al*., 2015).

The filiform papillae dominantly occupied almost the entire epithelial layer of R. *pusillus* and M. *Schreibersii tongue*. These papillae are clearly visible, tapered, long, curved posteriorly, and has no taste buds. Both bat species in this study have cornified filiform papillae which gave rough texture (Figure 6.). Previous studies reported some morphological variations of filiform papillae. *Eidolon heluum* has crown-like filiforom papillae (Abayomi *et al*., 2009), Taki-El-Deen *et al*., (2013) reported that filiform papillae of *Rousettus aegyptiacus* have curve form. These papillae were abundant and scaterred on the dorsal surface of tongue. Whereas, filiform papillae of *Rhinopoma hardwicke* have conical, slender, and sharp shape. Mutlak et al., (2015) found that filiform papillae of *Pipistrillus kuhli,* the Egyptian bat, has sharp and slender cone-like protrusion which numerous and distributed on the dorsal anterior of the tongue. In addition, the filiform papillae on the anterior surface of the tongue plays a role in binding food (Okon, 1974). Furthermore, the filiform papillae become thinner in the median part to increase the friction between the tongue and the food substances ensuring the food movement in the oral cavity. Therefore, this structure effectively retain foods in the mouth as long as bats fly and hold the foods until ingested (Okon, 1974; Pastor *et al*., 1993). In addition, Taki-El-Deen *et al*., (2013) studied that filiform papillae of Egyptian fruit bat have mechanical function which help to gather semi liquid food.

The fungiform papillae of *R. pusillus* and *M. schreibersii* are scaterred among filiform papillae in the anterior and median part of the tongue, which similar with that of *Eidolon heluum* (Abayomi *et al*., 2009). Fungiform papillae are mushroom-like and less abundant than filiform papillae. Taki-El-Deen *et al*., (2013) studied that fungiform papillae *Rousettus aegyptiacus* were like a dome and scattered between filiform pappilae. *Rhinopoma hardwicke* also has fungus-like fungiform papillae. Mutlak *et al*. (2015) reported that *Pipistrillus kuhli,* the Egyptian bat has fungiform papillae are mushroom-like and can be found in the presence of taste buds. Fungiform papillae have taste buds, that consist of taste sensor (Hwang & Lee, 2007). Chung & Kwun (1977) reported that the number of fungiform papillae depends on the type of food consumed by the species.

Two circumvallate papillae were found in the

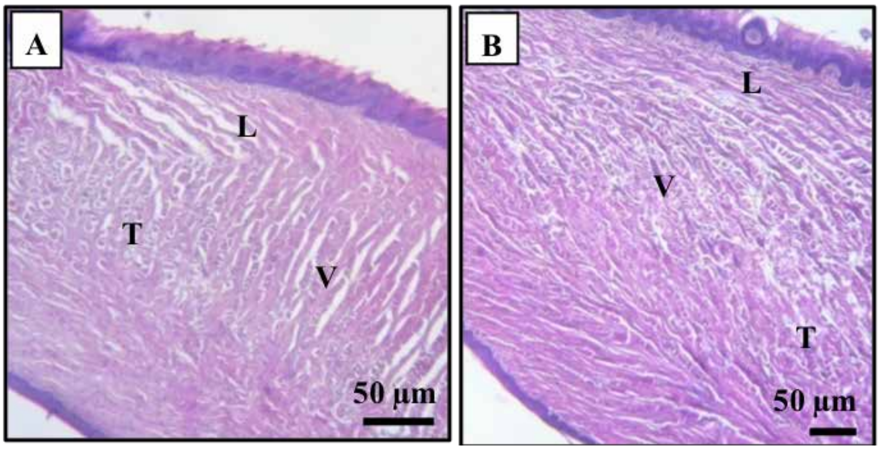

Figure 4. Histological structure of the tongue muscles of *Rhinolophus pusillus* (A) and *Miniopterus schreibersii* (B). HE staining. L: longitudinal muscle, T: transverse muscle, and V: vertical muscle. Scale bar: 50 µm.

Table 1. Tongue's histological measurement of *Rhinolophus pusillus* and *Miniopterus schreibersii*

	R. pusillus **(µm)** (Mean ± SD)	***M. schreibersii*** **(µm)** (Mean ± SD)
Epithelial layer's thickness (middle centre of tongue)	62.33 ± 2.52	55.67 ± 5.13
Longitudinal muscular layer's thickness	305.00 ± 13.23	314.67 ± 4.51
Vertical and transverse muscular layer's thickness	335.00 ± 5.57	373.33 ± 15.28
Muscular layer's thickness	691.67 ± 7.64	756.33 ± 30.55
Ratio of the longitudinal muscular layer's thickness : the muscular layer's thickness	1 : 2.3	1 : 2.3
Ratio of the vertical and transverse muscular layer's thickness : the muscular layer's thickness	1 : 2	1: 2

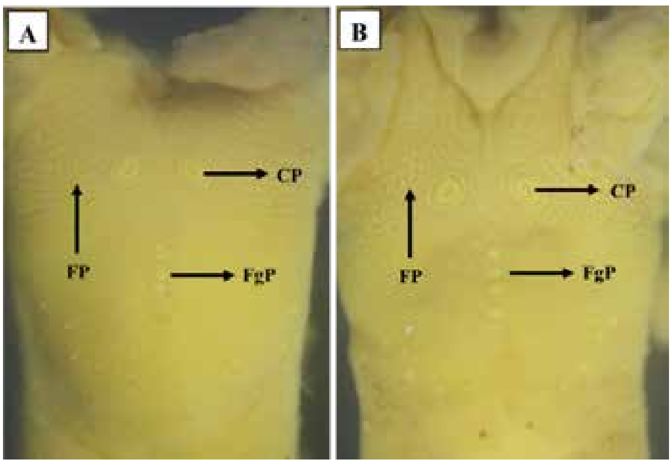

Figure 5. Anatomical structure of the upper surface of the tongue *Rhinolophus pusillus* (A) and *Miniopterus schreibersii* (B). FP: filiform papilla; FgP: fungiform papilla; CP: circumvallate papilla.

posterior portion of the tongue in both species (Figure 5.). The number of circumvalate papillae depends on the different types of food consumed by the bat species. Frugivorous bats typically have three circumvalate papillae while insectivorous bats have two circumvalate papillae (Pastor *et al*., 1993; Son *et al*., 2000; Emura *et al*., 2001; Gregorin, 2003; Hwang & Lee, 2007). Abayomi *et al*., (2009) studied that *Eidolon heluum* has circumvallate papillae found in the distal part of the tongue and there are taste buds. Taki-El-Deen *et al.,* (2013) reported that the circumvallate papillae of *Rousettus aegyptiacus* are wide-flattened, protruding on the surface of the tongue surrounded by a deep invagination of survace epithelium. Moreover, *Rhinopoma hardwicke* also has flattened circumvallate papillae. Mutlak *et al*., (2015) observed that *Pipistrillus kuhli,* the Egyptian bat, has circular circumvallate papillae and can be found in the presence of taste buds.

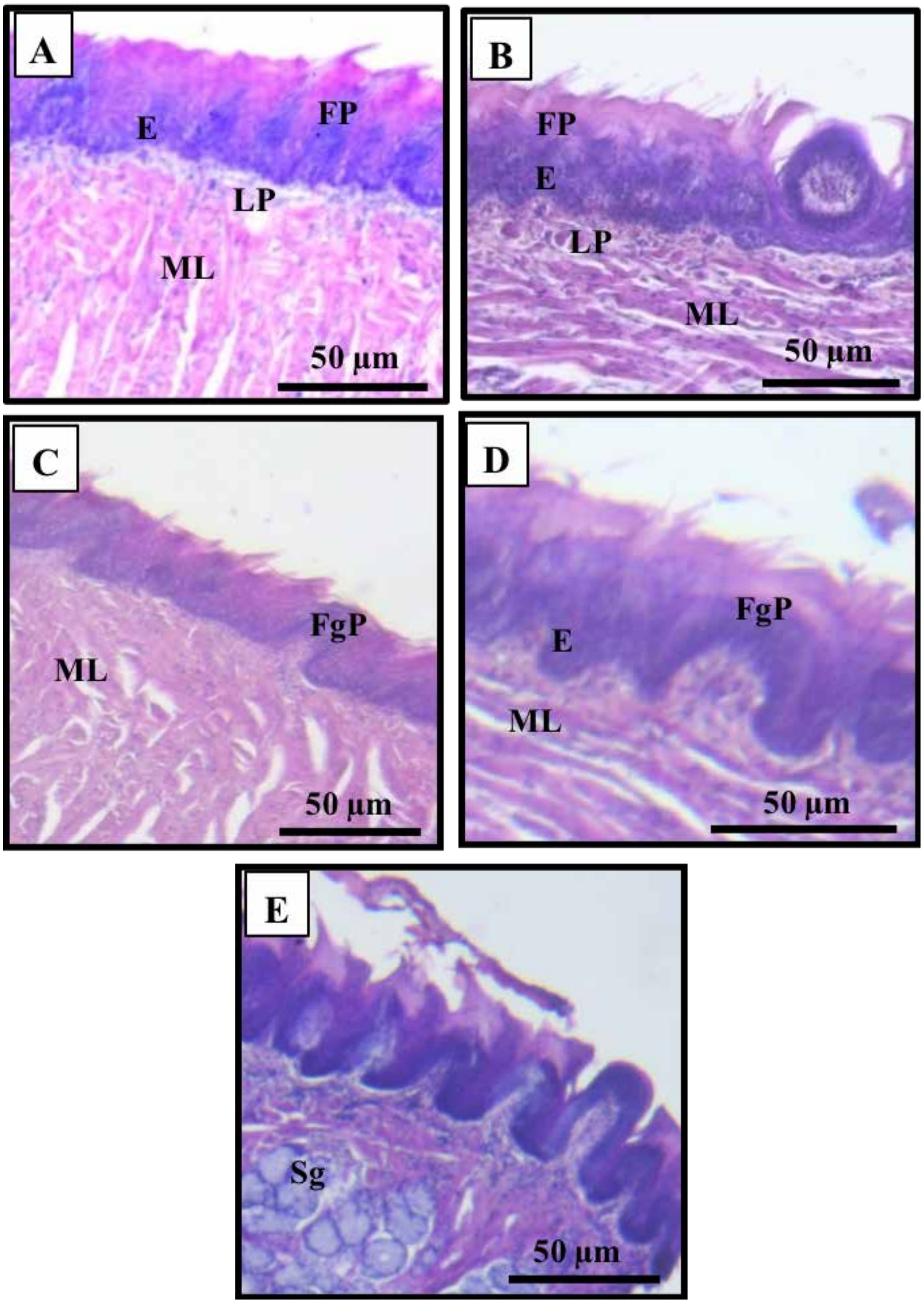

Figure 6. Histological structure of tongue papillae on *Rhinolophus pusillus* (A & C) and *Miniopterus schreibersii* (B, D, & E). HE staining. FP: filiform papillae, FgP: fungiform papillae, E: stratified squamous epithelium with cornification, LP: lamina propia, ML: muscle layer, and Sg: salivary glands. Scale bar: 50 µm.

Besides of its keratinization, the bat tongue papillae in epithelial tissue were known adapted to the insects that are usually eaten (Kobayashi & Shimamura, 1982). The tongues of the bats *Pipistrellus pipistrellus* (Pastor *et al*., 1993) and *Myotis macrodactylus* (Hwang & Lee, 2007) are also highly adapted to the insects they eat. This study evaluated the distribution of papillae which are considered to have a relationship with the eating habits of a species. Harper *et al*., (2013) studied that the tongue of nectar-feeding bat, *Glossophaga soricina* is provided with long filamentaous papillae which function dynamically to take up the nectar. Moreover, the type of diet determines the shape and allocation of lingual papillae (Mutlak *et al*., 2015).

Histological Structure of the Salivary Glands of Rhinolophus pusillus and Miniopterus schreibersii

In Mammals, the chemical digestion on the tongue vere provided by the precence of salivary glands. The salivary glands of R. pusillus are found in the sublingual and the anterior part of the tongue. In the other hand, the salivary gland of *M. schreibersii* are found in the antero-inferior part and the posterior part of the tongue (Figure 7).

The anterior part of R. *Pusillus'* salivary glands are located between the muscular layer. This salivary glands are composed of serous and mucous acini. The serous acini is darker and the cell nucleus is in the middle. Whereas the mucosal acini is brighter and the epithelial cell nucleus located periphery. There are also interlobular septa around the acini salivary glands separating each glandular lobules (Figure 8A.). The sublingual part of *R. pusillus'* salivary glands are found under the tongue. The shape of sublingual gland is resembling almond seeds. This sublingual glands have a mixture of acini, namely serous acini and mucosal acini. The structure of the sublingual part of salivary glands is the same as in the anterior side. Serous acini is dark stained and is usually present in the form of serous demilunes and mucosal acini is stained lighter. The intercalary excretory duct and the interlobular excretory duct are present within sublingual gland (Figure 8B.).

The salivary glands in *M. schreibersii* are located in the antero-inferior part of the tongue between the muscle bundles and in the posterior part of the tongue. The antero-inferior salivary gland has the same structure as the anterior salivary gland in *R. pusillus*. The glands are composed of darker stained serous acini and lighter stained mucosal acini. In addition, intercalary ducts can be observed (Figure 8C.). The posterior salivary gland usually associated with the circumvalate papilla (Hand *et al*., 1999). These glands are under the circumvalate papilla. These glands have only serous acini (Figure 8D.). These glands secrete serous liquid through a small opening around the circumvalate papilla.

Glandular secretions from the acini asini will be excreted through several intercalar ducts which will fuse to form the interlobularis duct. The minor salivary glands have serous acini which produce aqueous secretions rich in amylase and lysozim enzymes and mucosal acini which secrete a viscous fluid rich in mucopolysaccharides which functions as protection and mucus secretion for lubricants (Health *et al*., 1993 in Abayomi *et al*., 2009). Some studies proposed that the salivary gland composition in mammals may varies depend on their food (Estecondo *et al*., 2005; Mutlak *et al*., 2015; Guerrero-Hernández & Moreno-Mendoza, 2016; Goździewska-Harłajczuk *et al*., 2018). Adeniyi *et al*., (2010) studied that in mammals such as rat which consumes dry food has prominent serous salivary gland. The serous fluid may help lubrication and swallowing mechanism.

Weterings *et al*., (2015) evaluated that different species of insectivorous bats may have different diets preferences especially when they inhabit at different location. Previously, Clare *et al*., (2011) stated that

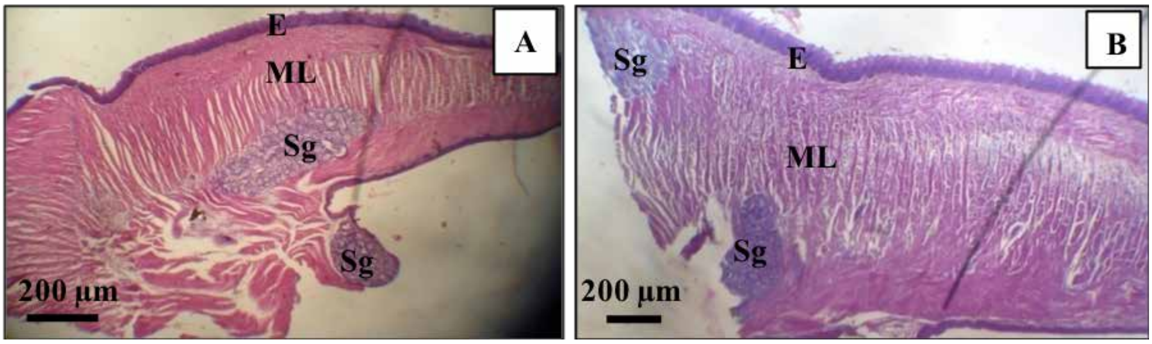

Figure 7. Histological structure of tongue of (A) *Rhinolophus pusillus* and (B) *Miniopterus schreibersii*. HE staining. E: epithelium, ML: muscle layer, Sg: salivary glands. Scale bar: 200 µm.

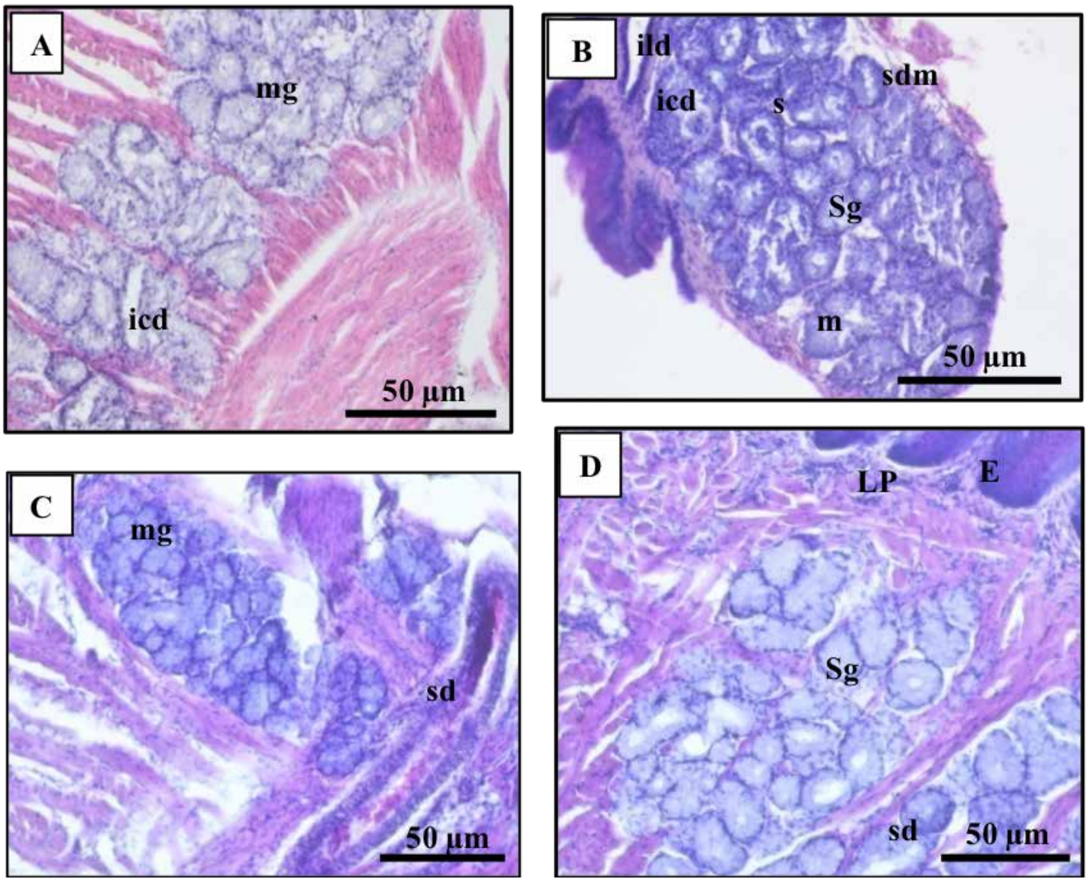

Figure 8. Histological structure of the anterior part of salivary glands on the tongue of *Rhinolophus pusillus* (A), the sublingualis part of salivary gland of *Rhinolophus pusillus* (B), the antero-inferior part of salivary gland of *Miniopterus schreibersii* (C), and the posterior part of salivary gland of *Miniopterus schreibersii* (D). HE staining. E: epithelium, LP: lamina propia, mg: mucosal gland, Sg: salivary gland, m: mucosal secretion unit, s: serous secretory unit, sdm: serous demilunes; icd: intercalar duct; ild: interlobular duct, and sd: secretory duct. Scale bar: 50 µm.

the same insectivorous bat, *Myotis lucifugus,* inhabit at different location had different diets preferences as well. In this study, we proposed that there is no difference in histological structure of tongue between *R. pusillus* and *M. schreibersii* since both of them might share similar diets preferences. Hence, they might also share similar digestive mechanism based on their insect food preferences.

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