

Structure of reticulated python (*Malayopython reticulatus*) tongue using scanning electron microscopy and light microscopy

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Abstract. Marina M, Saragih GR, Kustiati U, Budipitojo T, Wijayanto H, Pangestinarsih TW, Ariana, Wendo WD, Budiariati V, Kusindarta DL. 2024. Structure of reticulated python (*Malayopython reticulatus*) tongue using scanning electron microscopy and light microscopy. *Biodiversitas* 25: 2121-2129. Reticulated python (*Malayopython reticulatus*) is the longest snake in the world. It belongs to order Squamata, suborder Serpentes, family Pythonidae, and genus *Malayopython*. The size of this snake can reach up to 6-8 m, which makes it the largest member of Pythonidae family. The tongue of *Malayopython reticulatus* (Schneider, 1801) is unique in that it serves the chemosensory process by capturing and carrying airborne chemical particles to Jacobson's or vomeronasal organs, so it would be very interesting to know the morphology of the lingual papillae of the tongue of *M. reticulatus*. This research aimed to determine the morphological and histological structures of *M. reticulatus* tongue via Scanning Electron Microscopy (SEM) and Hematoxylin-Eosin (HE) staining. Macroscopically, the tongue was divided into the apex, corpus, and radix. This organ is elongated with bifurcation. The dorsal anterior part tends to be blackish, whereas the posterior part tends to be lighter to reddish in color. SEM observation showed that the reticulated python tongue has microfacets and micropores on the dorsal side but lack papillae and taste buds. Histological results (HE staining) revealed that the tongue is divided into a mucosal layer and a muscular layer. The mucosal layer consists of lamina epithelial mucosa and lamina propria mucosa. Melanin exists in the lamina propria mucosa. The muscular layer comprises longitudinal, transverse, and vertical muscles and also lack papillae and taste buds.

Keywords: Histology, *Malayopython reticulatus*, morphology, tongue

INTRODUCTION

Snakes are legless reptiles with elongated bodies covered in scales (Lillywhite 2014). These animals are classified under order Squamata, which includes 96% of all reptile species. To date, more than 3400 species of snakes in the world have been divided into 18-27 families, one of which is Pythonidae (Mattison 2014). Pythonidae family consists of nonvenomous snakes that are generally large to gigantic in size (Vitt and Caldwell 2014). Reticulated python (*M. reticulatus*) is a member of Pythonidae family and can be found in Indonesia. This snake is distributed throughout Southeast Asia and several other Asian countries, such as Bangladesh and India (Kalki et al. 2018). This animal, also known as the python, has three subspecies based on habitat diversity, morphology, and genetics: *M. r. reticulatus*, *M. r. saputrai*, and *M. r. jampeanus* (Hanifa et al. 2016).

As a carnivorous animal, the Reticulated python (*M. reticulatus*) obtains food by hunting other animals. This hunting process requires the help of various sensory organs, one of which is the tongue. The tongue is a muscular organ located in the oral cavity and extends to the oropharynx. In mammals, the tongue functions in the digestion process, namely, promoting the process of prehensile and food swallowing. Taste, temperature, and pain receptors can also be found on the tongues of mammals

(König and Liebich 2020). Snakes have a different tongue structure and function compared with mammals. They have slender, forked tongues that are not glandular, highly keratinized, and lack taste buds (Girling and Raiti 2019; Vitt and Caldwell 2014). Snake tongue does not participate in the prehensile or tasting process but rather assists in the chemosensory process by capturing and carrying airborne chemical particles to Jacobson's or vomeronasal organs (Girling and Raiti 2019; Lillywhite 2014).

Previous studies on the structure of snake tongue have been conducted on rat snake (*Elaphe climacophora*) (Iwasaki et al. 1996), Chinese cobra (*Naja naja atra*), Many-banded krait (*Bungarus multicinctus*), bamboo viper snake (*Trimeresurus stejnegeri* subsp. *stejnegeri*) (Mao et al. 1991), rat snake (*Elaphe obsoleta*) (Morgans and Heidt 1978), and plains garter snake (*Thamnophis radix*) (Ridlon 1985), with the results observed in the form of micropores (Mp), microfacets (MFs), and microvilli on the tongue surface. The presence of Mp, MFs, and microvilli make an interesting exploration to the morphology of the snake tongue related to the chemosensory function of the snake tongue. Histologically, researchers have studied the tongues of water snake (*Enhydryis chinensis*), water snake (*Xenochrophis piscator*) (Yin et al. 1996), and Hissing sand snake (*Psammophis sibilans*) (El-Mansi et al. 2020). However, to date, no research has discussed the

morphological and histological structures of reticulated python (*M. reticulatus*) tongue.

MATERIALS AND METHODS

Animal sample

Six reticulated python (*Malayopython reticulatus*) were captured by setting traps around the Special Region of Yogyakarta. The animals were identified at the Animal Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia to determine the exact species. The six sample were prepared by opening the cavum oris and separating the maxilla and mandible.

Conservation status

Malayopython reticulatus used in this study is not on any conservation list in Indonesia. According to the International Union for Conservation of Nature Red List of Threatened Species, *M. reticulatus* is categorized as a Least Concern species (Stuart et al. 2018)

Ethical approval

The experimental procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia (approval number: 0109/EC-FKH/Int./2021).

Gross macroscopy analysis

The tongue was extracted from the sheath and cut at the base (at the musculus hyoideus) to separate it from the mandible. After separation, the samples were cleaned by brushing with a fine toothbrush and rinsed with working Phosphate-Buffered Saline (PBS) (Nacalai Tesque, Kyoto, Japan). Then, the samples were washed through immersion in 0.9% physiological NaCl solution (Nacalai Tesque, Kyoto, Japan). Three tongue samples were kept in Hematoxylin-Eosin (HE) fixative solution (4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan)), and another three were kept in Scanning Electron Microscopy (SEM) fixative solution (glutaraldehyde 0.5% (ChemCruz), 1.5% paraformaldehyde (Nacalai Tesque), HEPES (ChemCruz), and working PBS (Nacalai Tesque) for at least 6-8 h. Macroscopic observation was performed on all samples using a Canon EOS 7000D camera. After macroscopic observation, the samples were prepared for SEM and light microscopy.

SEM

Tongue samples that had been fixed for 24 h were washed five times using 0.9% physiological NaCl (Nacalai Tesque) for 5 min. The samples were then cut into three parts: the apex, corpus, and radix. After cutting, sample dehydration was carried out by immersing the samples in graded ethanol (KgaA) solutions ranging from 70, 80, 90, and 95% for 3 min each in stages at room temperature, followed by immersion in absolute ethanol for 5 min twice to remove the remaining liquid from the samples. The samples were then fixed on a conductive metal plate with carbon tape. Subsequently, the samples were dried using a

vacuum system (BUEHLER, Castable, Vacuum System) for 60 min, and the surface was coated with conductive material using a coater (JEOL autofine coater JEC-3000FC) that applied platinum for 120 s. This process ensured that the samples were conductive to emitted electrons. Finally, an SEM sample was inserted into an electron microscope (JEOL JSM-6510LA) and observed at a voltage of 15 kV and magnifications of 30x, 100x, 1000x, 3000x, 5000x, and 10000x.

Hematoxyline eosin staining

Tongues that had been stored in 4% paraformaldehyde fixative solution (Nacalai Tesque) for 24 h were trimmed using a rotary microtome (Yamato RV 240) in the apex, corpus, and radix regions. The samples were then placed in a gauze and washed under running water for 30 min. Subsequently, the samples were dehydrated by immersing them in graded ethanol solutions (KgaA) starting from 70, 80, and 90% followed by immersion in absolute ethanols I, II, and III for 60 min each at room temperature. After the dehydration process, the clearing process was performed in xylol I, II, and III (KgaA) solutions for 45 min each at room temperature. Sample processing was followed by paraffin infiltration for 45 min in a 60°C incubator. Samples on paraffin blocks were cut at a thickness of 5 µm using a rotary microtome. The cut samples were placed in a water bath and then transferred to the surface of the coated slide. The slides were then placed in a slide warmer for 24 h at 40°C. The sample slides were then separately subjected to HE staining (Leica Biosystem, Illinois, USA). Staining results of the samples were observed using a light microscope connected to Optilab software to capture images.

Immunohistochemistry for PGP 9.5

Immunohistochemistry staining procedure based on the Biocare IHC staining manual (Biocare, California, USA), staining begins with a deparaffinization process with xylene, followed by rehydration with absolute alcohol and graded alcohol. After rehydration, slides were washed with PBS, and then peroxidase blocking was performed with 3% H2O2 in methanol and washed again with PBS. They were blocking serum with a sniper background for 35 minutes. Slides were washed again, and anti-PGP 9.5 primary antibodies (Invitrogen, Massachusetts, USA) (dilution 1:5000 with PBS) were dripped on slides as much as 30 µL for each preparation and incubated at room temperature for 3 hours. After incubation of the primary antibody, followed by the secondary antibody with Trakie Universal Link for 25 minutes, the slides were washed with PBS. The staining stage was continued by staining with Trakie Avidin HRP for 20 minutes. Wash the slides with PBS and homogenize them using Diaminobenzidine (DAB) in the substrate for 20 minutes. To stop the reaction, the slide was dipped in distilled water. The next stage is dehydration with graded alcohol and alcohol absolute, then clearing with xylene, and the last stage is mounting using balsam Canada (Merck, New Jersey, USA).

RESULTS AND DISCUSSION

Gross morphology of the tongue

Macroscopic observation showed that reticulated python tongue is elongated with a forked anterior part. This branching is referred to as bifurcation, and thus, the reticulated python tongue is bifurcated. In the in-situ cavum oris, the tongue extended to the base of the mandible and ventral to the glottis (Figure 1.A). The organ can extend into and out of the cavum oris. A sheath can be found on the posterior part of the tongue (precisely at the ventral glottis), and it envelops and stores the tongue when it is in the cavum oris. Once primarized, the tongue can be viewed in its entirety from anterior to posterior. On the dorsal side, the anterior part of the tongue tends to be blackish, whereas the posterior part is lighter to reddish (Figure 1.B). The color difference can also be observed on the ventral side but not as distinct as that on the dorsal part, which tends to be grayish on the anterior and light on the posterior side (Figure 1.C). In close observations, the median sulcus (MS) can be found in the medial part on the dorsal and ventral sides of the tongue. The sulcus on the ventral side is referred to as the ventral sulcus, whereas that on the dorsal side is the dorsal sulcus. On the ventral side, the sulcus extends from anterior to posterior, but on the dorsal side, the more posterior sulcus begins to disappear.

In general, the tongue is divided into three parts from anterior to posterior, namely, the apex, corpus, and radix. The apex is the most anterior part of the tongue, and it is bifurcated (bifurcation) and blackish in color. This part is slender and thin with a pointed anterior end. The tongue apex has a grayish black color, which becomes lighter at the further anterior end of the tongue. The center of the tongue is called the corpus. This part is blackish in color and becomes fainter on its posterior side. The corpus is unbranched but remains outside the tongue sheath. On the ventral side of the corpus, the sulcus is still clearly visible. Meanwhile, the sulcus starts to disappear on the dorsal side. The apex and corpus are outside the cavum oris when

the tongue is extended. Radix is the most posterior part and the longest part of the tongue. It is located ventral to the glottis and covered by a sheath. When the tongue is extended, the radix remains in the cavum oris of the snake. The dorsal and ventral sides of the tongue radix are light in color. On the ventral side, the sulcus is still clearly visible, whereas on the dorsal side, the sulcus is not visible. The most posterior part of the radix is in direct contact with the bifurcated external hyoglossal musculus.

SEM

Apex

SEM observation of the *M. reticulatus* tongue was conducted on the dorsal side, at the apex, corpus, and radix. Overall, the tongue of *M. reticulatus* has a typical structure, i.e., a flat surface without protrusions, such as taste buds or papillae. At high magnifications, irregular polygonal thickening of epithelial cell borders can be observed. With the increase in magnification, protrusions with round to irregular shapes called MFs can be observed. The MFs are surrounded by small holes called Mp. Both formations are modifications of the snake tongue epithelium. Specifically, two types of MFs exist: large MFs and small MFs (Mf). Large MFs are large, and Mf have exactly the same size as Mp.

At 30x and 100x magnifications, SEM of the apex of the *M. reticulatus* tongue showed a flat dorsal surface with a MS along the medial surface (Figures 2.A and 2.B). No notable and special formations were observed under these magnifications. When the magnification was increased to 1000x, polygonal thickening of the epithelial cell borders was observed (Figure 2.C). Irregular shape and distribution of this thickening were detected along the tongue surface. At 5,000x and 10,000x magnifications, epithelial cell modifications in the form of large MF, Mf, and Mp were observed (Figures 2.D and 2.E). Large MFs are larger than Mp. Meanwhile, Mf are relatively of the same size as Mp. The distribution and size of each formation were evenly distributed across the tongue surface.

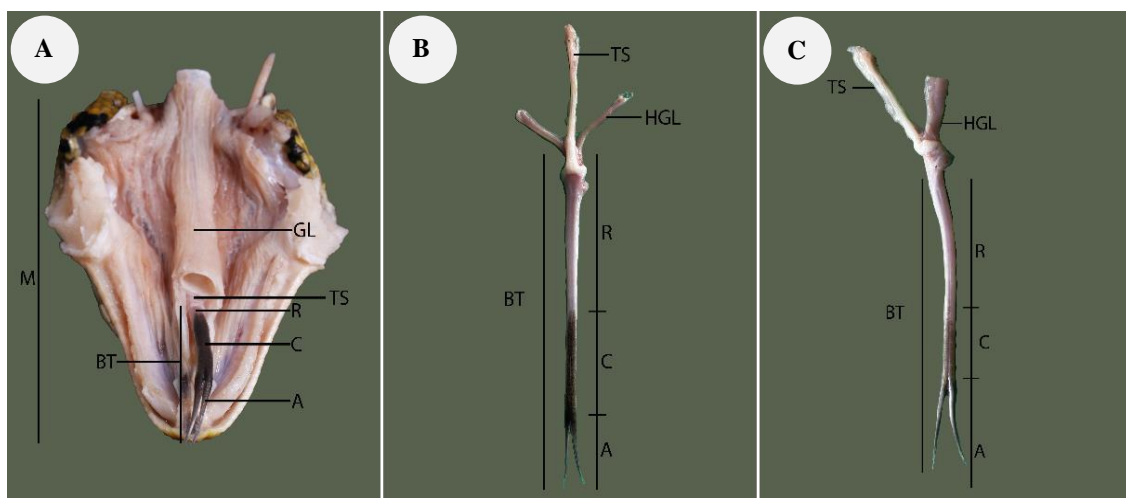


Figure 1. Macroscopic image of the *Malayopython reticulatus* tongue along with the division of tongue regions. A. In situ tongue on the mandible. B. Dorsal view of the tongue separated from the mandible. C. Ventral view of the tongue separated from the mandible (A: apex, BT: bifurcated tongue, C: corpus, GL: glottis, HGL: hyoglossus muscle, M: mandible, R: radix, and TS: tongue sheath)

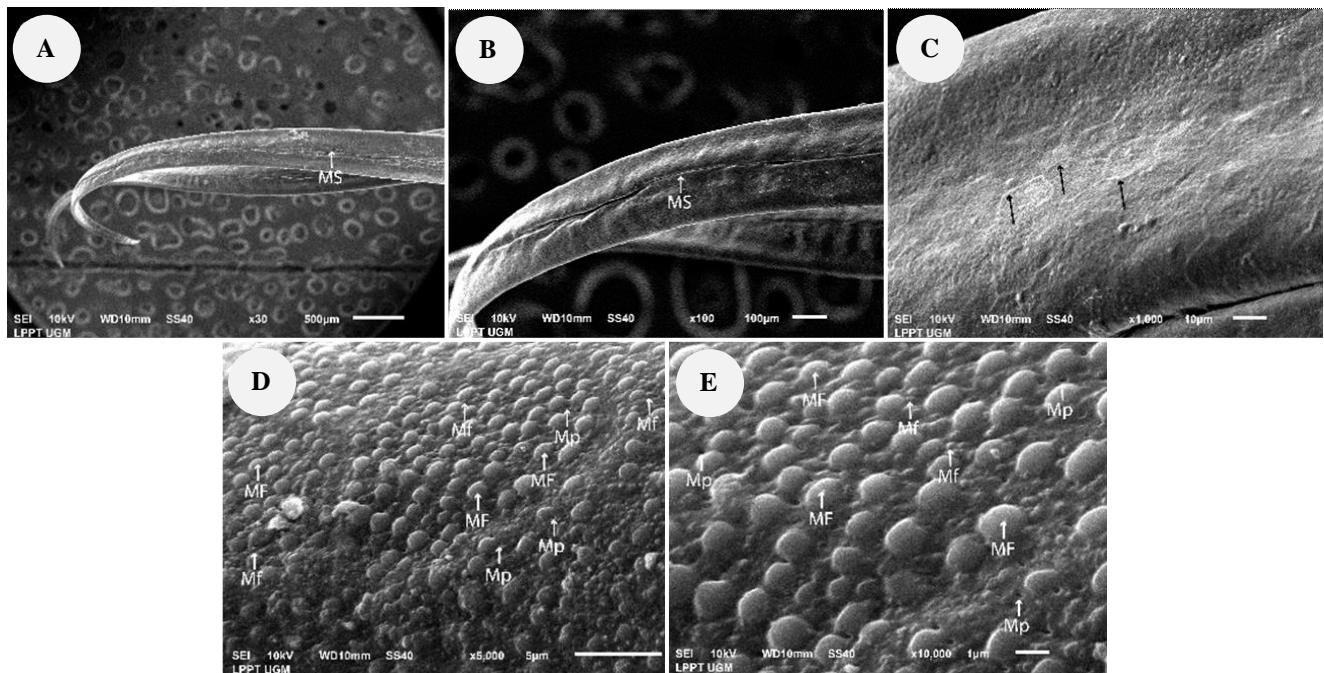


Figure 2. SEM image of *Malayopython reticulatus* tongue apex. A. Apex anterior part at 30x with magnification showing a visible flat surface and MS; B. Apex at 100x magnification revealing a visible flat surface and MS; C. Apex at 1000x magnification displaying the visible thickening of epithelial cell boundaries in polygonal form (arrow); D. Apex at 5000x magnification presenting visibly large MFs, MF, and Mp; E. Visibly large MFs, Mf, and Mp at 10000x magnification

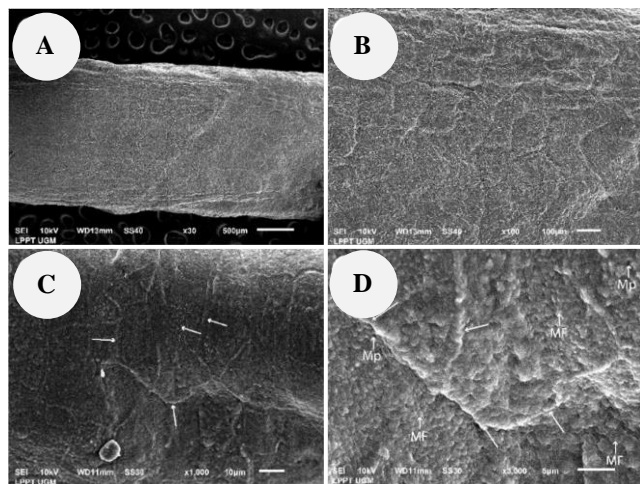


Figure 3. SEM image of *Malayopython reticulatus* tongue corpus. A. Corpus at 30x magnification showing a flat surface; B. Corpus at 100x magnification displaying a flat surface; C. Corpus at 1000x magnification revealing thickened polygonal epithelial cell boundaries (arrows); D. Corpus at 3000x magnification showing thickened polygonal epithelial cell boundaries (arrows), large MFs, and Mp

Corpus

The results of SEM observations at 30x and 100x magnifications of the dorsal surface of the corpus of the *M. reticulatus* tongue showed a flat surface without notable formations (Figures 3.A and 3.B). No sulcus median was found on the corpus surface. At 1000x magnification, the formation comprised thickened epithelial cell borders (arrows) with irregular shape and distribution (Figure 3.C). At this magnification, large but vaguely visible MFs appeared. At a high magnification of 3000x, the formation

of large MFs and Mp was visible (Figure 3.D). Large MFs in the corpus part of the *M. reticulatus* tongue showed a less rounded shape with uneven distribution and size. Large MFs at the corpus were smaller than those found at the apex, but their size remained larger than that of Mp. Mp were also less frequent at the corpus on the tongue surface of the snake.

Radix

The dorsal surface of the most posterior part of the tongue, that is, the radix, showed a smooth surface with a fold-shaped structure at 30x and 100x magnification (Figures 4.A and 4.B, respectively). At a high magnification (1000x), the formation of polygonal epithelial cell boundary thickening (arrow) can be observed (Figure 4.C). This thickening exhibited an irregular shape and distribution along the tongue surface. At a magnification of 3000x, epithelial cell border thickening can still be observed, along with large MFs that were less round in shape and Mp (Figure 4.D). The large MFs remained larger than the Mp, although the size increasingly became smaller compared with the apex and corpus. Mp were also less common on the dorsal surface of the tongue apex.

HE staining

Observation of the tongue of *M. reticulatus* using apex, corpus, and radix region preparations with HE-stained cross-sections was performed to observe and identify the histological structure of the tongue in the three parts. In general, the results of histological observation of the tongue structure of *M. reticulatus* showed a similar structural arrangement. The microscopic layer of the tongue comprises the tunica mucosa and tunica muscularis. In the muscularis tunica, different intrinsic muscles can be found in each part. Neither the apex, corpus, nor radix had taste

buds or papillae lingualis.

Apex

The histological structure of the cross-section of the *M. reticulatus* tongue apex from dorsal to ventral consists of the tunica mucosa and tunica muscularis. Tunica mucosa itself is divided into two laminae, namely, Lamina Epithelialis Mucosa (LEM) and Lamina Propria Mucosa (LPM) (Figure 5.C). The LEM comprises layered flat cells, which are referred to as complex squamous epithelium, whereas the LPM consists of a dense connective tissue. In the tunica muscularis, several intrinsic musculatures and rami of the hypoglossal nerve (N) can be found (Figures 5.A and 5.B). The intrinsic musculus of the tongue on the most dorsal side is the Dorsal Longitudinal Muscle (DLM). On the more ventral part, a pair of Hyoglossus Muscle (HGL) bundles can be found. The HGL and N are surrounded by a Circular Muscle (CM), which is composed of Transverse Muscle (TM) (Figures 5.A and 5.B). In the LPM, melanin (ME) showed an even distribution (Figure 5.C). This pigment renders the tongue with a blackish color at the apex. The MS can be observed on the dorsal and ventral sides of the tongue apex. The ventral MS is deeper than the dorsal one. The histological image of the tongue apex of *Malayopython reticulatus* showed no keratinization, salivary glands, goblet cells, nor papilla lingualis.

Corpus

The cross-section of the corpus has a histological structure similar to that of the apex. The structural layers in the corpus are the tunica mucosa and tunica muscularis when viewed from dorsal to ventral conditions. Tunica mucosa comprises two laminae, namely, the LEM and LPM (Figure 6.C). The LEM comprises complex squamous epithelial cells, whereas the LPM is composed of dense connective tissue. ME is still found, which gives the tongue a blackish color, in the LPM of the corpus part of the tongue. The tunica muscularis is composed of = DLM on the dorsal part and a pair of HGL bundles on the ventral part (Figures 6.A and 6.B). The tunica muscularis also has a pair of N. Both of these structures in the corpus of the tongue are surrounded by the CM. The most striking difference with the apex is that the CM on the corpus side is composed of a TM and a Vertical Muscle (VM). The MS on the dorsal and ventral

sides is still found but not as deep as the apex. No keratinization salivary glands, goblet cells, or papilla lingualis were found at the tongue corpus.

Radix

At the most posterior part of the tongue, that is, the radix, the transverse histological structure appeared slightly different. The structural layer from dorsal to ventral was still composed of the tunica mucosa and tunica muscularis. One difference compared with the apex and corpus is that the tunica mucosa consisted only of the LEM (Figures 7.B and 7.C). Neither LPM nor ME was found in the radix. The intrinsic muscular arrangement of the tunica muscularis also varied. No DLM was observed on the dorsal side (Figures 7.A and 7.B). The tunica muscularis contained a pair of HGL bundles and a pair of N. As in the corpus, these two structures are surrounded by a CM composed of a TM and a VM. The similarity between the apex and corpus is the absence of keratinization, salivary glands, goblet cells, or papilla lingualis.

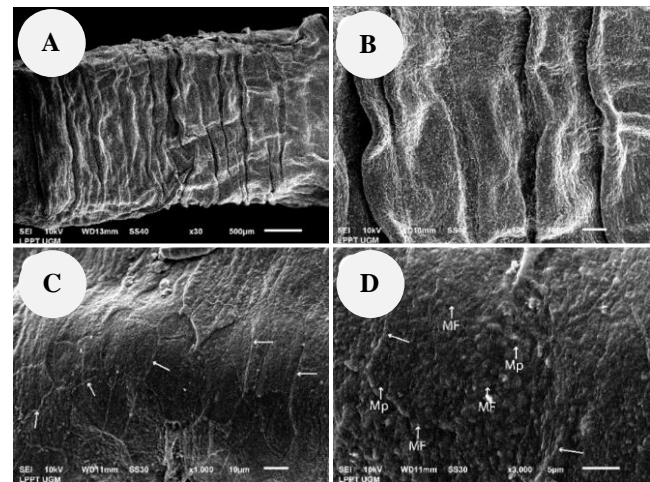


Figure 4. SEM image of the radix of *Malayopython reticulatus*. A. Radix at a 30x magnification showing visible folds; B. Radix at 100x magnification revealing visible folds; C. Radix at 1000x magnification presenting the visible thickening of polygonal epithelial cell boundaries (arrows); D. Corpus at a magnification of 3000x displaying visible thickening of polygonal epithelial cell boundaries (arrows), large MFs, and Mp

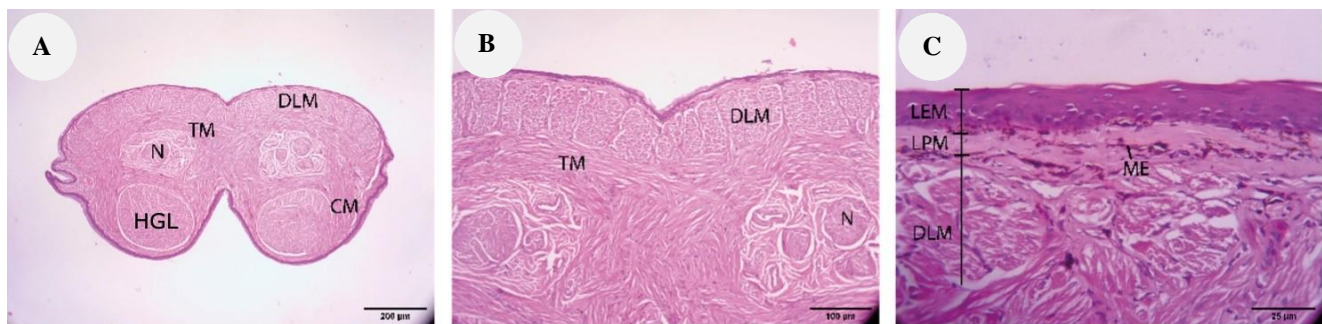


Figure 5. Histological image of the tongue apex of *Malayopython reticulatus* with HE staining. A) Magnification of 10 x 4 at a scale bar of 200 µm showing the intrinsic musculature of the tongue and nerve (CM: circular muscle, DLM: dorsal longitudinal muscle, HGL: hyoglossus muscle, N: rami of hypoglossal nerve, TM: transverse muscle); B. Magnification of 10 x 10 at scale bar of 100 µm; C. Magnification of 10 x 40 at a scale bar of 25 µm

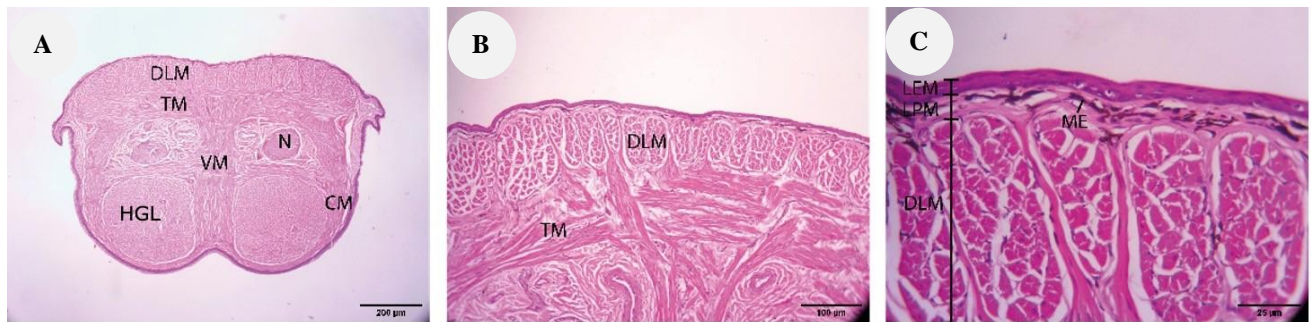


Figure 6. Histological images of the tongue corpus of *Malayopython reticulatus* with HE staining. A. Magnification at 10 x 4 at a scale bar of 200 µm showing the intrinsic musculus of the tongue and nerve (CM: circular muscle, DLM: dorsal longitudinal muscle, HGL: hyoglossus muscle, N: rami of hypoglossal nerve, TM: transverse muscle, and VM: vertical muscle). B. Magnification 10 x 10 at a scale bar of 100 µm. C. Magnification of 10 x 40 at a scale bar of 25 µm showing the visible division of mucosal lamina (LEM: lamina epithelialis mucosa, LPM: lamina propria mucosa, ME: melanin)

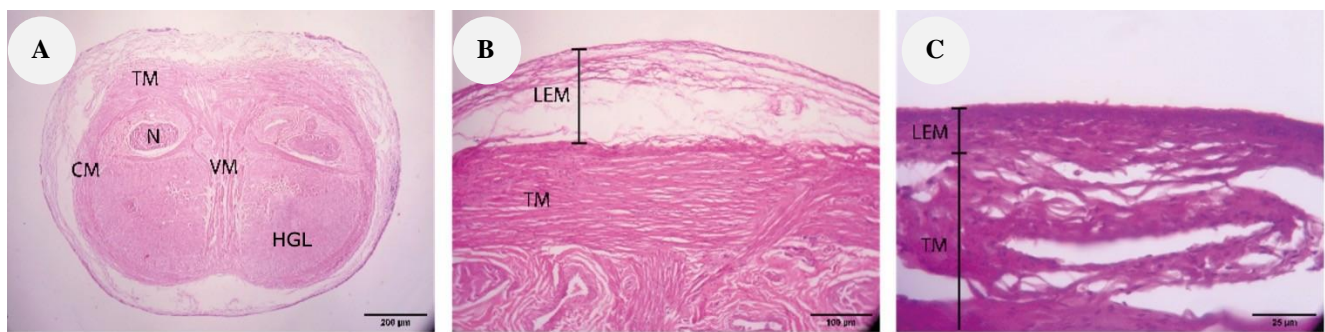


Figure 7. Histological image of the tongue radix of *Malayopython reticulatus* with HE staining. A. Magnification of 10 x 4 at a scale bar of 200 µm, showing the intrinsic musculus of the tongue and nerve (CM: circular muscle, HGL: hyoglossus muscle, N: rami of hypoglossal nerve, TM: transverse muscle, and VM: vertical muscle). B. Magnification of 10 x 10 at a scale bar of 100 µm. C. Magnification of 10 x 40 at a scale bar of 25 µm showing visible LEM: lamina epithelialis mucosa)

Discussion

The macroscopic observation of the tongue of (*Malayopython reticulatus*) showed that it has a smooth surface, is slender and elongated, and has a deep branching/forked on its anterior part, similar to the tongues of other snakes, such as *Elaphe climacophora* (Iwasaki et al. 1996) and diamondback water snake (*Natrix rhombifera*) (Morgans and Heidt 1978). The same tongue shape was found in *Varanus niloticus* (Sheren et al. 2018). However, differences in tongue shape were observed compared with other animals in order Squamata. In lizards (except *Varanus*), such as those belonging to *Xantusia*, *Abronia*, *Coleonyx*, *Dasia*, *Cordylus*, and *Crotaphytus*, the tongue shape tends to widen and fill the cavum oris. Bifurcation can still be observed in these genera, but it is very short (Filoramo and Schwenk 2009). In general, the tongue is divided into three parts: the apex, corpus, and radix. This is in accordance with the works of De Groot et al. (2004) and El-Mansi et al. (2020), who also divided the tongue into three parts: the apex (branching part), corpus (the part that comes out when the tongue is extended), and radix (the most posterior part that remains in cavum oris when the tongue is extended). *Malayopython reticulatus* has a tongue that is black on the dorsal side and a grayish on the ventral side, similar to *Xenochrophis piscator* (Yin et al. 1996). SEM morphological observations of the

Malayopython reticulatus tongue showed the absence of papillae or taste buds on the dorsal surface; thus, the tongue surface appeared smooth at 10x and 30x magnifications. The tongues of *Elaphe climacophora* (Iwasaki et al. 1996), *Elaphe quadrivirgata* (Iwasaki and Kumakura 1994), *Xenochrophis piscator*, *Enhydris chinensis* (Yin et al. 1996), and *Thamnophis radix* (Ridlon 1985) have similar surfaces. This feature is different from that of other squamate species, namely, *Scincus scincus* and *Uromastix ornata*, which have taste buds and several types of papillae, namely, multiangular, rhomboid, and comb-like papillae at the apex, triangular and leaf-like papillae at the corpus, and scaly or bird wing-shaped papillae at the radix, on the dorsal surface of their tongue (Salem et al. 2019). The formation of MS on the dorsal surface of the apex (fork) can also be found in *Malayopython reticulatus* and *Trimeresurus mucrosquamatus* (Mao et al. 1991). At 1000x magnification of the tongue surface, epithelial cell modification in the form of irregular polygonal thickening of epithelial cell boundaries can be observed. This polygonal thickening can also be found in other snakes, such as *Elaphe climacophora* (Iwasaki et al. 1996), *Trimeresurus mucrosquamatus*, *Naja naja atra*, *Bungarus multicinctus*, *Trimeresurus mucrosquamatus*, *Trimeresurus stejnegeri* subsp. *stejnegeri* (Mao et al. 1991), and *Thamnophis radix* (Ridlon 1985).

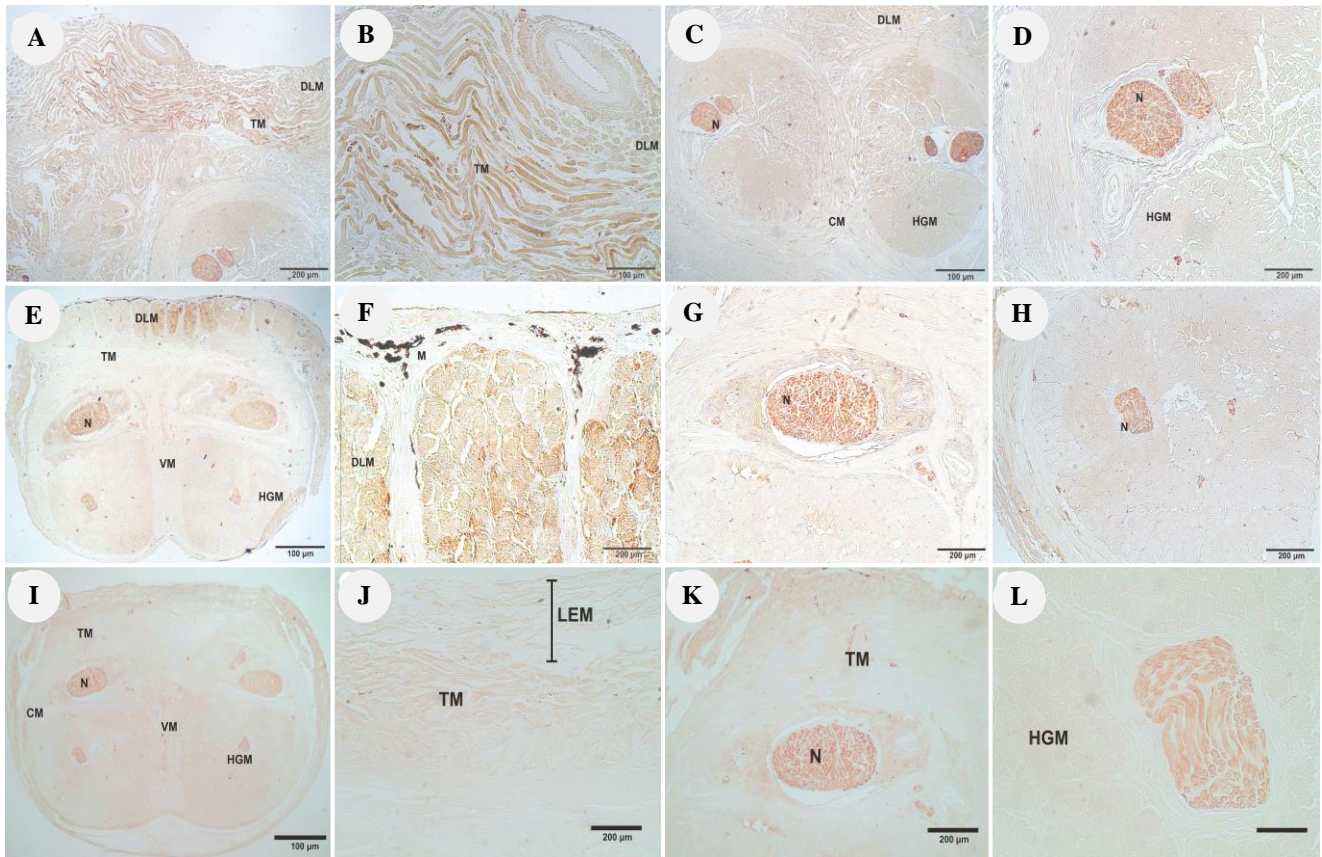


Figure 8. Immunohistochemical reactivity of PGP 9.5 of *Malayopython reticulatus* (A-D) Apex; (E-H) Corpus; (I-L) radix. (A, C, E) Magnification of 10 x 4 at a scale bar of 200 µm; (B, D, F-H) Magnification of 10 x 4 at a scale bar of 100 µm, showing the intrinsic musculus of the tongue and nerve (CM: circular muscle, HGL: hyoglossus muscle, N: rami of hypoglossal nerve, TM: transverse muscle, DLM= dorsal longitudinal muscle, and VM: vertical muscle)

SEM observation at magnifications of 3000x, 5000x, and 10000x revealed the presence of special formations called MFs, which are rounded protrusions scattered on the dorsal surface of *Malayopython reticulatus* tongue. The term MFs is derived from their shape, which resembles the facets of insect eyes (Morgans and Heidt 1978). At the apex, the shape, size, and distribution of microfacets are very regular. MFs at the apex are similar to those found in *Trimeresurus mucrosquamatus*, *Naja naja atra*, *Bungarus multicinctus*, *Trimeresurus mucrosquamatus*, and *Trimeresurus stejnegeri* subsp. *stejnegeri* (Mao et al. 1991). The more posterior the tongue, the more irregular the shape, size, and distribution of the MFs of the *Malayopython reticulatus*. The corpus and radix are less rounded, less prominent, and of different sizes. The shape of the MFs on the corpus and radix of *Malayopython reticulatus* is similar to that of water snakes *Enhydris chinensis* and *Xenochrophis piscator* (Yin et al. 1996). Another similarity is the presence of large MFs and Mf at the apex. The difference in the structure of these MFs may be due to the semiaquatic behavior of *Malayopython reticulatus*. Mp are also present on the tongue of *Malayopython reticulatus*, Mp are small holes that surround MFs. These formations can be found in many snakes, such as *Thamnophis radix* (Ridlon 1985), *Natrix*

rhombifera, and *Elaphe obsoleta* (Morgans and Heidt 1978). No microridges or microvilli can be found on the dorsal surface of the tongue of *Elaphe climacophora* (Iwasaki et al. 1996), *Trimeresurus mucrosquamatus*, *Naja naja atra*, *Bungarus multicinctus*, *Trimeresurus mucrosquamatus*, *Trimeresurus stejnegeri* subsp. *stejnegeri* (Mao et al. 1991), *Enhydris chinensis*, and *Xenochrophis piscator* (Yin et al. 1996).

Histological observation of the tongue via HE staining showed two tongue layers, namely, the tunica mucosa and tunica muscularis. The tunica mucosa at the apex and corpus consists of the LEM, which comprises stratified squamous epithelium, and LPM, which consists of dense connective tissue. ME can be found in the LPM, and it gives the snake tongue a blackish color that is visible to the naked eye. The radix part of tunica mucosa is composed of the LEM and contains no ME. This structure is similar to that of *Psammophis sibilans* (El-Mansi et al. 2020) and *Natrix rhombifera*, *Elaphe obsoleta* (Morgans and Heidt 1978), and *Naja naja atra* (Mao et al. 1991). The difference is that ME in *Natrix rhombifera* is located in the epithelium, whereas that in *Elaphe obsoleta* is concentrated in the basal part of cells (Morgans and Heidt 1978). No keratinization was observed on the entire tongue surface. According to Girling and Raiti (2019), the tongue is

generally highly keratinized. This keratinized structure blocks excessive water from entering the tongue (Yin et al. 1996). The loss of this keratinized layer is likely due to its periodic shedding (Mao et al. 1991). Papillae, taste buds, or salivary glands were not found at the apex, corpus, or radix of the tongue. This finding is in accordance with that of Girling and Raiti (2019), who stated that snake tongues lack glands (aglandular). Papillae and taste buds can be found at the base of cavum oris (El-Mansi et al. 2020). Mucus-producing glandules can be found on the tongue sheath, and they lubricate the snake tongue when it is extended from the mouth (Morgans and Heidt 1978).

The muscularis tunica of the tongue consists of intrinsic muscle fibers arranged longitudinally, vertically and transversally and a pair of rami of hypoglossal nerve. The most dorsal intrinsic musculus is the dorsal longitudinal muscle, which is found only at the apex and corpus of the tongue. A pair of hyoglossus muscle bundles exists on the more ventral part. The hyoglossus muscle and rami of hyoglossus nerve are surrounded by circular muscle. The circular muscle from the apex is composed of transverse muscle, whereas the corpus and radix consist of transverse muscle and vertical muscle. This arrangement can also be found in the tongue of *Python regius* (Smith and Mackay 1990). The difference in the intrinsic musculature of each tongue part is a result of the different functions of each muscle. The tongue musculature operates in a muscular-hydrostatic manner. The extrinsic part of the tongue contains the v-shaped hyoglossus muscle, which is important in the mechanism of tongue movement. This musculus originates from the ceratobranchial cartilage. Activation of this extrinsic hyoglossus muscle results in retraction movements of the entire tongue. Meanwhile, activation of the intrinsic hyoglossus muscle shortens the organ. Contractions of the circular muscle and transverse muscle cause the decrease in tongue diameter and an increase in tongue length that extends the tongue out of the mouth. When the dorsal longitudinal muscle contracts together with the hyoglossus muscle, the tongue bends upward. Contraction of the dorsal longitudinal muscle and hyoglossus muscle in turn triggers an important tongue-flicking mechanism in snakes (Smith and Mackay 1990; De Groot et al. 2004).

As shown by macroscopic, SEM, and histological observations via HE and immunohistochemistry staining, the tongue of *Malayopython reticulatus* is elongated, has a smooth surface, and is forked on its anterior part. In addition, this organ has Mp and MFs and lacks papillae or taste buds. This morphology is a form of adaptation to the manner of foraging and lifestyle of reticulated python. The snake's tongue has no swallowing function, but it aids in chemosensory mechanisms. The mandibles and maxillae, teeth, and gravity become the main factors for snakes when swallowing food. Thus, tongue assistance is no longer needed. The snake's tongue has a forked anterior end that functions in tropotaxis, which is the detection of chemical stimuli from two different points simultaneously (Yang and Wang 2016). This function improves the snake's ability to detect important chemical stimuli, such as pheromone trails and prey. As active foragers and ambush predators, these

animals require maximum prey detection chemically, visually, and mechanically. The tongue flicking mechanism enables snakes to obtain chemical particles from the environment. Adaptations of the shape of tongue epithelium in the form of MFs and Mp play a role in this process. The collected chemical molecules are then passed on to vomeronasal/Jacobson's chemosensory organ through two holes referred to as vomeronasal fenestrae. Thus, the tongue is not an actual chemosensory organ but rather a supporting organ for the vomeronasal chemosensory mechanism (Department of Wildlife and National Parks Peninsular Malaysia 2021; McDowell 1972; Moon et al. 2019; Schwenk 1994).

In conclusion, the structure adaptation of the *Malayopython reticulatus* tongue is affected by the snake's habitat and function or ecological constraints. Overall, the tongue in *Malayopython reticulatus* is a remarkable sensory tool that allows them to gather crucial information about their surroundings, aiding in hunting and defense.

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