SYSTEMIC ORGANIZATION OF *Tetraponera rufonigra* (Jerdon, 1851) (HYMENOPTERA: FORMICIDAE): HISTOLOGICAL OBSERVATION

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ABSTRACT

Tetraponera rufonigra Jerdon, 1851, is one of the most dangerous ants, whose apex of gaster region contains a sting structure with a complex venom gland. Here, we describe the systemic organ system of *T. rufonigra* using histological techniques. The venom gland of *T. rufonigra* was located above the mesentero-proctodeal region, which could be histologically classified into two parts; the venom gland reservoir and the venom gland tubes. The venom gland tubes were lined by a simple cuboidal epithelium, whereas the venom gland reservoir was enclosed with simple squamous epithelium. The pygidial gland was localized near the cuticle as clusters, and each of the oval shape pygidial gland cells contained a spherical nucleus surrounded by acidophilic cytoplasm. High magnification observation showed that the integumentary system consists of three layers; epicuticle, exocuticle, and endocuticle. The digestive system was a single narrow tube consisting of the foregut, midgut, and hindgut. Five to six rectal papillae were found in the hindgut. The urinary system was made up of Malpighian tubules, which lined with simple cuboidal epithelium and tracheal embedded in the adipose tissue. The respiratory system was composed of spiracles and trachea adjacent to the integument, all of which were surrounded by a simple squamous epithelium. In the nervous system, several ganglia were found in this study. There were two layers in each ganglion: outer cortex and inner medullar layers. Several cell types were observed in the cortex layer including the neurosecretory cells, the neuroglia, and neurons. The outcomes of this study could provide a useful basis for future research on the family Formicidae.

Keywords: Arboreal bicolor ant, carnivorous ant, histology, Thailand, venom gland.

ABSTRAK

Tetraponera rufonigra Jerdon, 1851, ialah salah satu spesies semut yang paling berbahaya, yang mana hujung gaster mengandungi struktur bersengat dengan kelenjar racun kompleks. Dalam kajian ini, sistem organ *T. rufonigra* diperihalkan menggunakan teknik histologi. Kelenjar racun *T. rufonigra* terletak di atas bahagian mesentero-proktodeal dan boleh dikelaskan secara histologi kepada dua bahagian; takungan kelenjar racun dan tiub kelenjar racun. Tiub kelenjar racun dipenuhi oleh epitelium kubus ringkas, manakala takungan kelenjar racun dicirikan dengan epitelium skuamos ringkas. Kelenjar pigidial terletak pada kutikula dalam bentuk kelompok dan setiap sel kelenjar pigidial berbentuk oval mengandungi nukleus sfera yang di kelilingi oleh sitoplasma asidofili. Pemerhatian pembesaran tinggi menunjukkan bahawa sistem integumentari terdiri daripada tiga lapisan; epikutikel, eksokutikel, dan endokutikel. Sistem penghadaman terdiri daripada satu tiub sempit yang terdiri daripada usus depan, usus tengah, dan usus belakang. Lima hingga enam papila rektum dijumpai pada usus belakang. Sistem perkumuhan terdiri daripada tubul Malpighi, yang dipenuhi dengan epitelium kubus dan trakea yang terdapat dalam tisu adipos. Sistem pernafasan terdiri daripada spirakel dan trakea pada integumen, semuanya di kelilingi oleh epitelium skuamos ringkas. Bagi sistem saraf, beberapa ganglion didapati dalam kajian ini. Terdapat dua lapisan dalam setiap ganglion: korteks luar dan lapisan medula dalaman. Beberapa jenis sel diperhatikan dalam lapisan korteks termasuk sel-sel neurosekretori, neuroglia dan neuron. Hasil kajian ini dapat memberikan asas yang berguna untuk kajian lanjut Formicidae pada masa akan datang.

Kata Kunci: Semut dwiwarna arboreal, semut karnivor, histologi, Thailand, kelanjar racun.

INTRODUCTION

Tetraponera rufonigra Jerdon, 1851, is an arboreal ant that belongs to the order Hymenoptera and family Formicidae. This ant is found widely in the tropical zone, typically in natural forests but also in human habitations. The prominent morphological characters of *T. rufonigra* includes the bicolored body of black and yellowish-brown colors, dispersed hairs, long thorax, small flat oval pronotum, protrude oval mesonotum and two nodes on the petiole segment. Pedicels are 3-5 mm, and the average body length of the ant is approximately 10.5-13.0 mm (Bodlah et al. 2017; Potiwat & Sitcharungsi 2015). This ant also has a wide head and large mandible with five or six teeth, as well as dorsally positioned eyes, indicating the carnivorous habit (Bodlah et al. 2017; Chong & Lee 2010).

Currently, *T. rufonigra* is considered to be one of the most dangerous ants because its apex of gaster region contains a sting structure with a complex venom gland, which produces a substance toxic to human (Potiwat & Sitcharungsi 2015). A previous observation showed that the venom causes adverse symptoms such as constriction, breathing problems and vomit when injected into human. Furthermore, *T. rufonigra*-induced anaphylaxis has been reported in Thailand. In one case, the patient displayed two episodes of urticarial, angioedema, dyspnea and even unconsciousness (Ratnatilaka et al. 2011; Wanotayan et al. 2005). Biochemical identification of the venom is an enthralling topic, which is necessary to treat the allergic inflammation of human. Additionally, morphological characterization of the venom gland will also contribute to our understanding of how the venom is injected to human, which may lead to the development of novel defense systems. In this study, we describe the systematic organization of *T. rufonigra* for the first time using histological techniques with a particular attention to its venom gland.

MATERIALS AND METHODS

Twenty-five individuals of *T. rufonigra* (Figure 1) with the total length of 10-12 mm were collected from agricultural and human residence areas at three sites in Phitsanulok, Nakhon Pathom and Nan provinces, Thailand, during January to June in 2011-2014. All samples were euthanized by rapid cooling shock and fixed as a whole in Davidson's fixative for 36-48 hours at room temperature. For histological examination, fixed samples were processed according to the standard histological procedures (Presnell & Schreibman 1997; Suvarna et al. 2013). The paraffin blocks were cut at 4 and 5 µm thicknesses and stained with hematoxylin and eosin (H&E). Longitudinal histological sections were observed for the systematic organs and photographed with a Leica DM750 light microscope. In addition, diagram of ant anatomy was re-drawn based on Hölldobler et al. (1982) and Solis et al (2013).

Figure 1. *Tetraponera rufonigra* in natural habitat.

RESULTS

Venom Gland

The venom gland of *T. rufonigra* was situated above the mesentero-proctodeal region. Two distinct parts were identified in the venom gland: the venom gland reservoir and venom gland tubes (Figures 2A-2C). The venom gland reservoir contained the eosinophilic substance, which was lined by the simple squamous epithelium (Figure 2C). The venom gland tubes were also observed in longitudinal histological sections (Figures 2F-2G), which are also represented in the schematic diagram (Figure 2H). This gland was mostly lined with simple cuboidal epithelium (Figures 2D-2E). The eosinophilic substance is likely to be released into the sting from the venom gland (Figures 2F-2G).

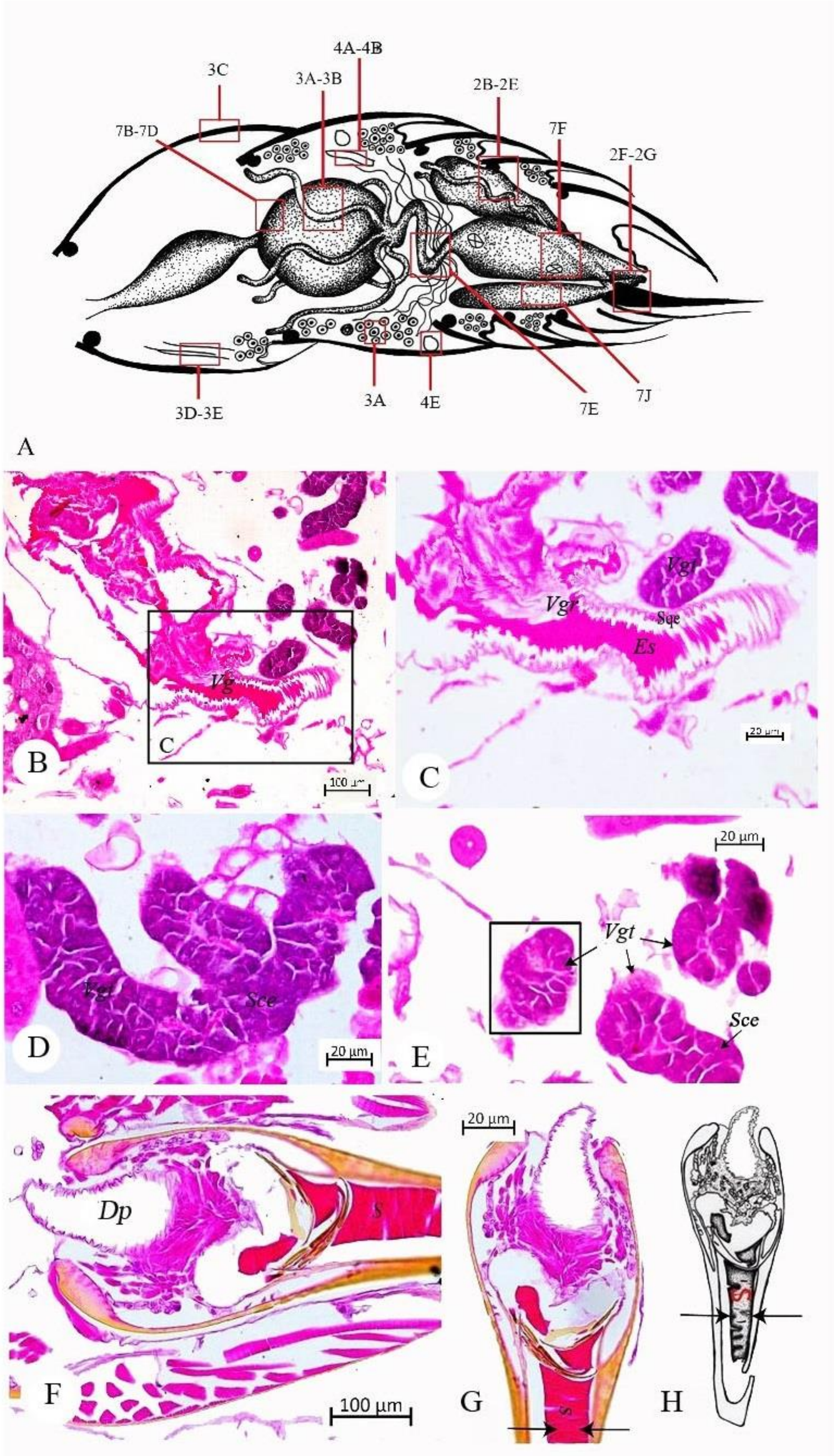

Figure 2. Schematic diagrams of the abdomen (A) and light microscopic images (B-E) of the venom gland (Vg) of *Tetraponera rufonigra*. B-C: The venom gland (Vg) was composed of the venom gland reservoir (Vgr) and venom gland tubes (Vgt). Vgr was covered by a layer of simple squamous epithelium (Sqe)

and contained the eosinophilia (Es; acidophilic substances stained in red). D-E: Under high magnification, Vgt was lined with simple cuboidal epithelium (Sce). F-H: Structure and schematic diagram of the sting (S). The sting was jointed to the duct of the venom gland (Dp). The acidophilic structures (arrows) indicate toxic components.

(Illustration of ant abdomen (2A) redrawn from Solis et al. (2013)).

Pygidial Gland

The pygidial gland was composed of several clusters that were clearly observed near the integumentary part. Each cluster contained approximately 2-6 cells (Figure 3A). The pygidial gland cells were classified into active and inactive cells. Active pygidial cells were large cells containing a spherical nucleus surrounded by the acidophilic cytoplasm (Figure 3A). On the other hand, inactive pygidial cell had a small size and the nucleic structure was rarely observed (Figure 3B).

Integumentary and Muscular Systems

The integumentary system is essential to support the body of *T. rufonigra*. It had a three cuticle layers including epicuticle, exocuticle, and endocuticle (Figure 3C). The epicuticle was the outermost layer covered with a waxy brownish-black layer throughout the ant body. Exocuticle was the light-colored middle layer and relatively thicker than the epicuticle (Figure 3C). The endocuticle was the thinnest layer that was easily identified with a dark pink color (Figure 3C).

Skeletal muscle fibers of *T. rufonigra* were clearly observed using the light microscopy. There were compact muscle bundles that dispersed along the long axis structure in longitudinal section of the ant body. Figures 3D shows the muscle bundles attached to the cuticle. The muscle fibers attached to the cuticular layer by tonofibrillae. It was a striated muscle containing skeletal muscle fibers (Figure 3E).

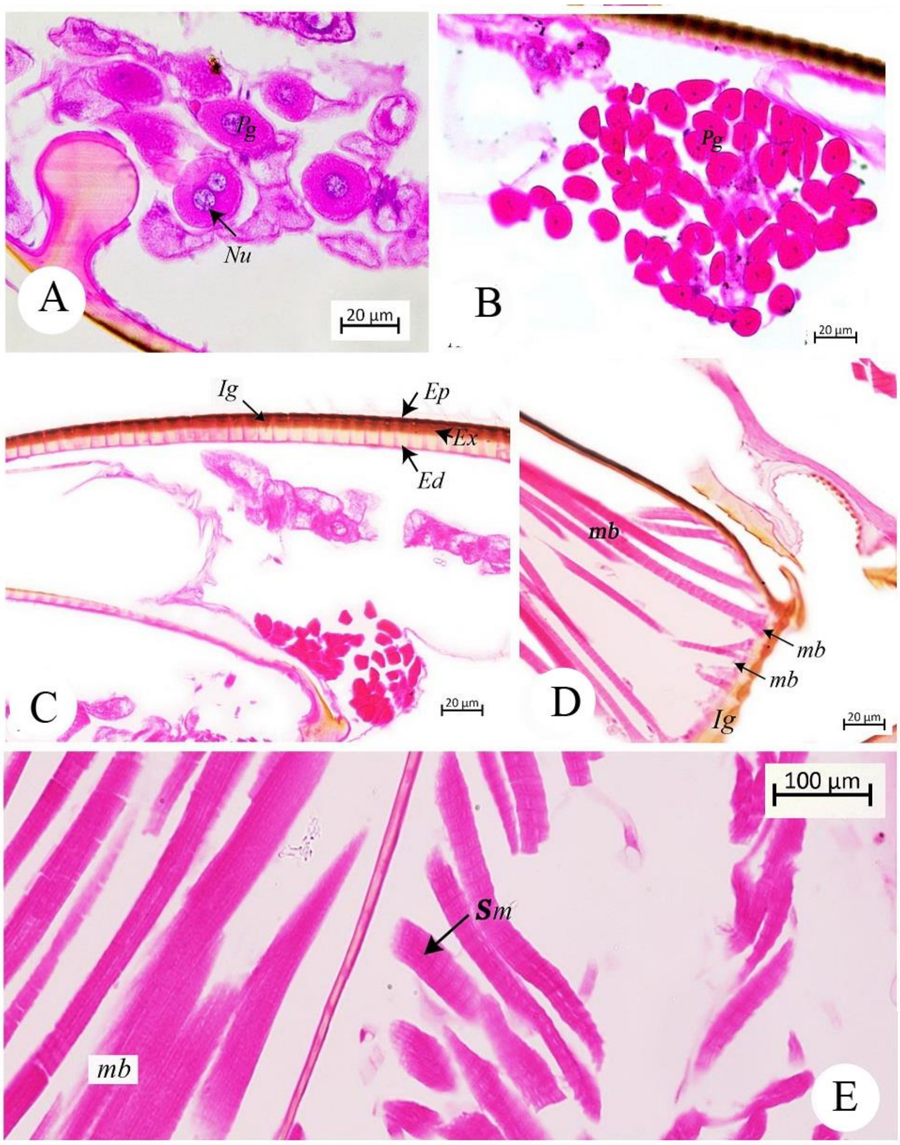

Figure 3. Light microscopic images of several structures of *Tetraponera rufonigra*. A: Pygidial gland (Pg) contained active cells characterized by the large size and the presence of nucleus (Nu). B: Pygidial gland also contained small inactive cells without clearly distinguishable nuclei. C-D: The integumentary system (Ig) was composed of three layers, epicuticle (Ep), exocuticle (Ex) and endocuticle (Ed), and was linked to muscular bundles (mb). E: Striated skeletal muscle (Sm) was clearly observed near the muscular bundles linked to the integumentary system.

Excretory System

The Malpighian tubule is the main excretory system of insects. It was commonly located between midgut and hindgut of the digestive system in ants (Solis et al. 2013), as also in the case of *T. rufonigra* (data not shown). This tubule was composed of a single layer of cuboidal epithelium cells (Figure 4A). In general, this structure is enclosed by a thin layer of peritoneal membrane, which is associated with muscle structure. However, under the light microscope it was difficult to clearly observe the peritoneal membrane (Figure 4B).

Respiratory System

Two structures including spiracles and trachea were observed in the respiratory system. The spiracles of *T. rufonigra* were scattered laterally along the thorax and abdomen. Histological structure showed that the trachea is an elongated tube (Figures 4C-4E).

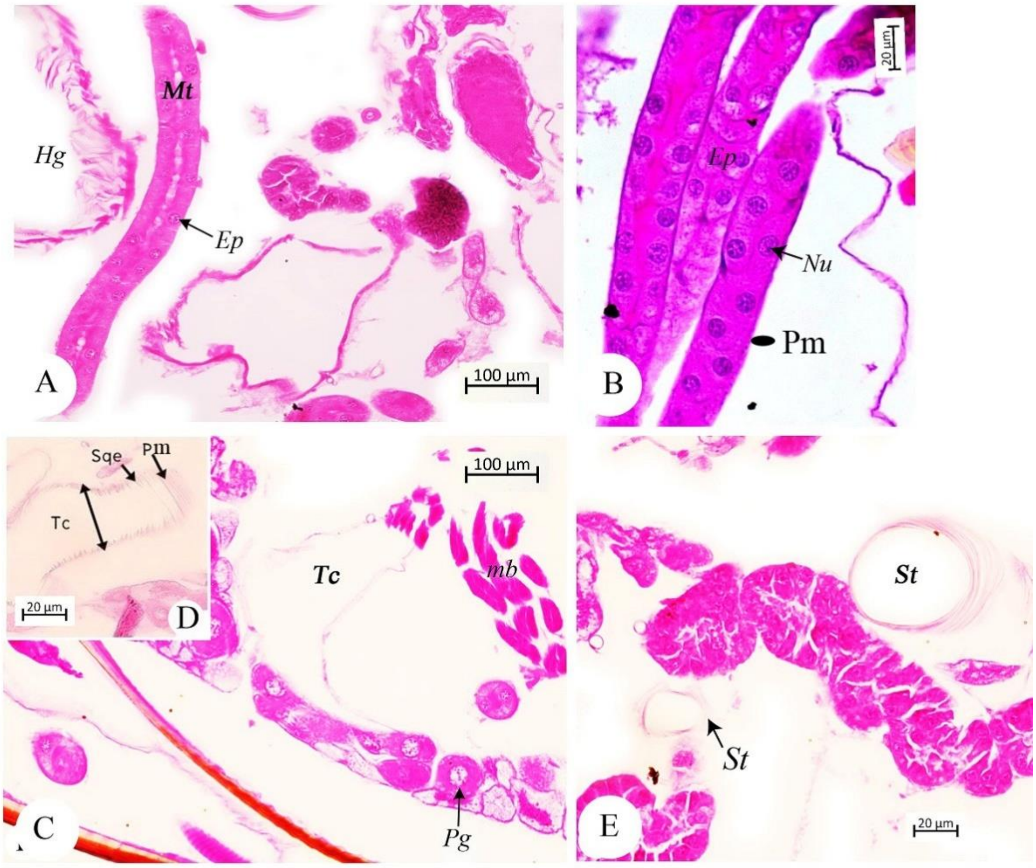

Figure 4. Light microscopic images of the excretory and respiratory systems of *Tetraponera rufonigra*. A-B: Structure of the excretory system. The Malpighian tubule (Mt) was enclosed by a layer of epithelium cells (Ep). Observations at high magnification showed that the oval nucleus was centrally located in the epithelium. C-E: Structure of the respiratory system. D: Trachea (Tc) was lined by simple squamous epithelium (Sqe) on the peritoneal membrane (Pm). E: The tracheal tubules (Tt) were observed along with the trachea. Abbreviations: Hg = hind gut, Mb = muscular bundle, Pg = pygidial glands.

Nervous System

We observed the ganglion in two parts of the *T. rufonigra* body; the brain and ventral nerve cord. The brain was embedded in the head capsule (Figure 5A), which was connected with compound eyes. The brain structure was divided into two parts; (i) the inner medulla composed of a layer of nerve fiber without neuronal cells and (ii) the outer cortex containing various types of cells including neurosecretory cells, neuroglia and neuronal cells (Figures 5B-5C). Although the locations of these ganglia were different, they were remarkably similar in basic components (Figures 5D-5F and Figure 6). Each ganglion had an outer cortex and an inner medulla (Figures 5D-5F and Figure 6). The second and fourth ganglia were bigger than the first thoracic ganglion possibly because there are formed by the fusion of ganglia during insect evolution (see Discussion).

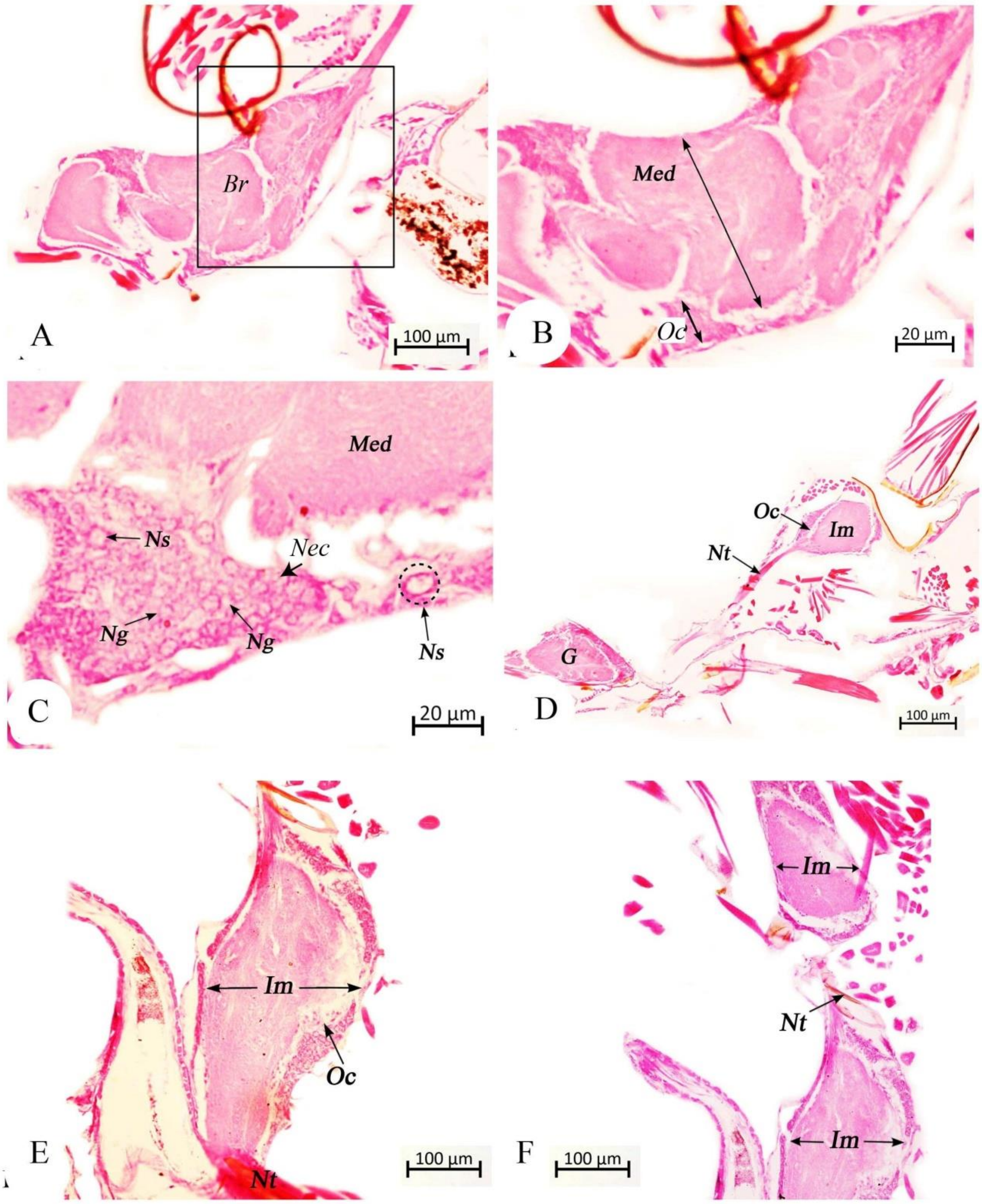

Figure 5. Light microscopic images of the nervous system of *Tetraponera rufonigra*. A: Localization of the brain (Br) in the ant head. B: Higher magnification image showed that the brain is composed of the medullar region (Med) and outer cortex (Oc). C: Three cell types were observed in the outer cortex: neuroglia (Ng), neuronal cell (Nec) and neurosecretory cells (Ns). D: the nerve tract (Nt) was linked to ganglia (G). E-F: Subesophageal ganglion (Sg) was associated with another ganglion by nerve tract (Nt). All ganglia were structurally similar being consisted of the inner medulla (Im) and outer cortex (Oc). Fg = fore gut.

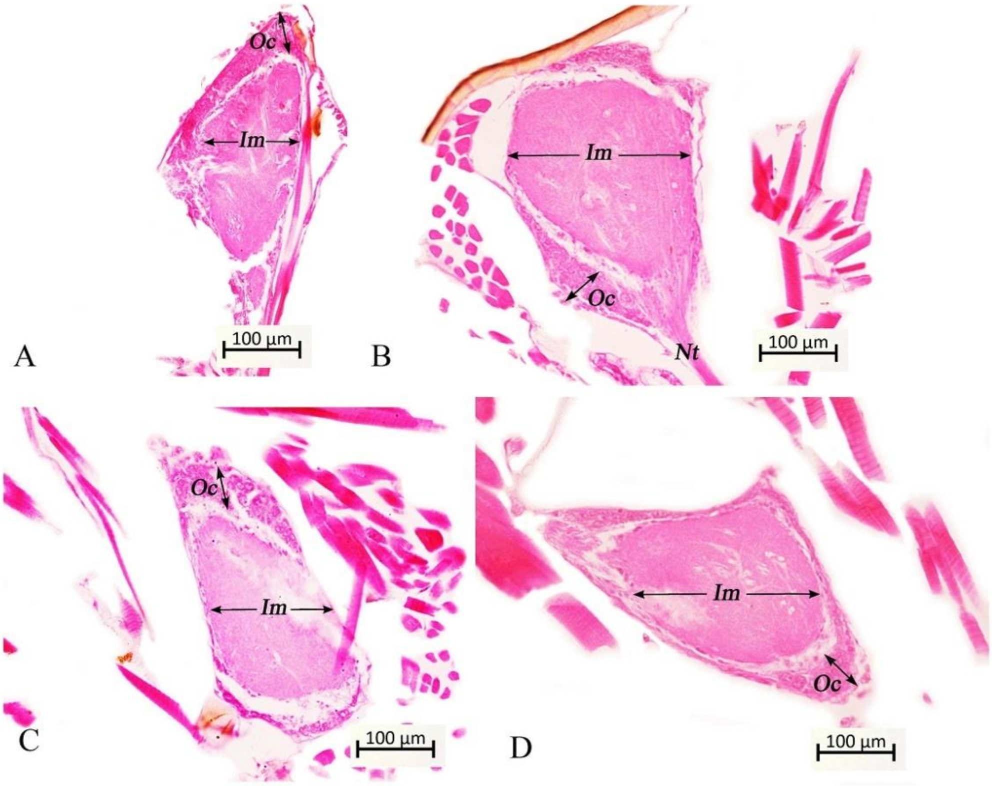

Figure 6. Light microscopic images of the ganglia of *Tetraponera rufonigra***.** A: ganglion 1 B: ganglion 2. C-D: ganglion 3. All ganglia were structurally similar being consisted of inner medulla (Im) and outer cortex (Oc). Abbreviation: Nt = nerve tract.

Digestive System

The digestive system of *T. rufonigra* was divided into three parts: foregut, midgut and hindgut (Figures 2A and 7). At the light microscopic level, the mucosal foregut was histologically lined by simple squamous epithelium (Figure 7A). In the longitudinal section, the midgut was clearly observed in the abdomen and contained the food component (Figure 7B). The mucosal layer of the midgut was composed of two sub-layers: epithelium and muscular sub-layers (Figure 7C). At higher magnification, the epithelial sub-layer was well-covered with low simple cuboidal epithelium with microvilli (Figure 7D). The hindgut contained rectal papillae, and in longitudinal sections the hindgut was composed of two layers; epithelium and muscularis (Figure 7F). The ileum and rectum were hardly distinguishable. Longitudinal fold of the muscular structural unite was obviously observed (Figure 7F). At higher magnification, the structure of rectal papilla was likely to be the bud structural type (Figures 7G-7H). Five to six rectal papillae can be found in the mucosa layer of the hindgut; in which each papilla was clearly distinguished with simple squamous epithelium (Figures 7F-7H).

Dufour's Gland

The Dufour's gland is an abdominal gland of insects, which is the source of a trail pheromone. The wall of this structure was folded, lined by simple cuboidal epithelium (Figure 7I) and surround with a thin layer of connective tissue. A thin layer of muscular layer was identified (Figure 7I).

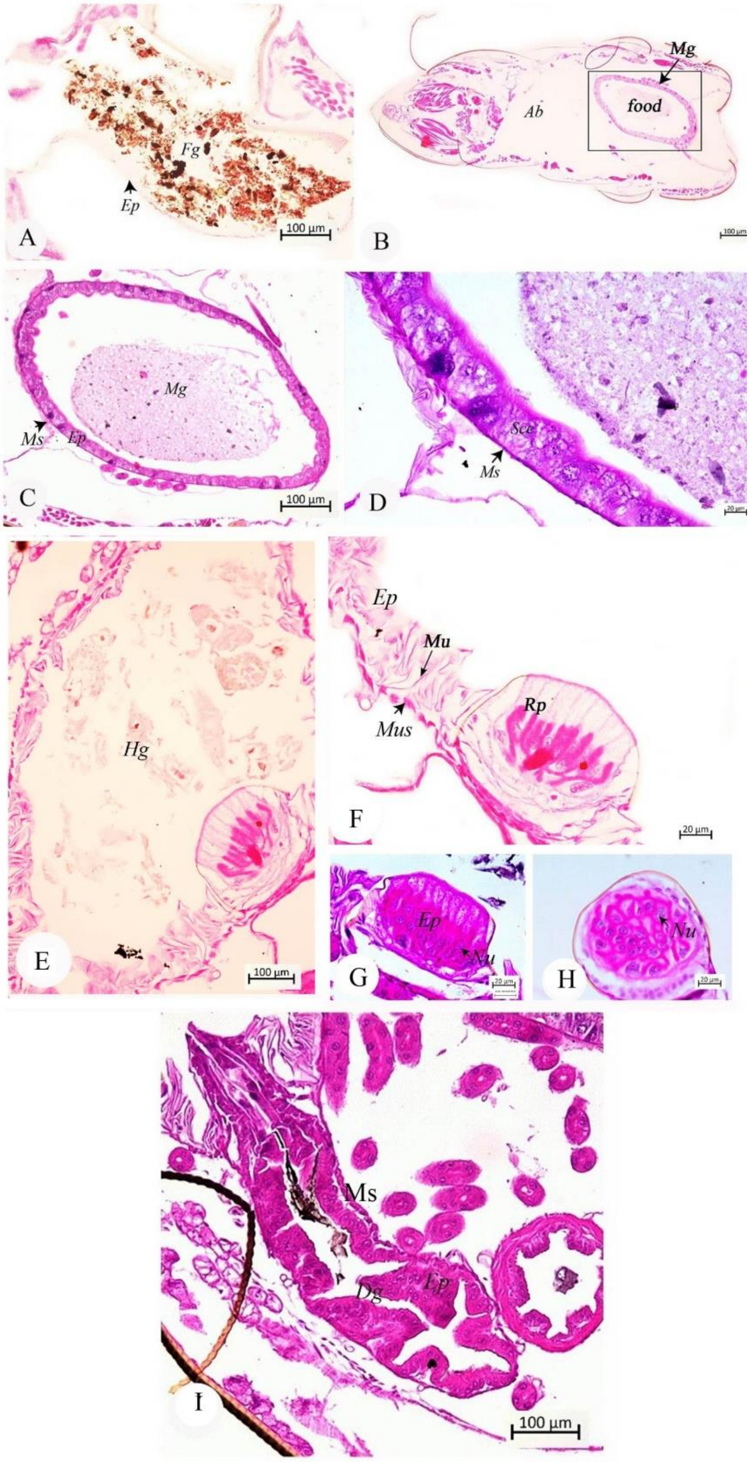

Figure 7. Light microscopic images of the digestive system of *Tetraponera rufonigra*. A: Foregut (Fg). B: Midgut (Mg). C-D: High magnification observation of the midgut (Mg) lined by epithelium [Ep; simple cuboidal epithelium (Sce)]. E: Hindgut (Hg). F-H: Several rectal papillae (Rp) were observed in the mucosa (Mu). I: structure of Dufour's gland. Abbreviations: Ab = abdominal region, Ep = epithelium, Ms = muscular layer, Mus = muscularis, Mt = Malphigian tubules, Nu = nucleus.

DISCUSSION

In this study, we showed the systematic organization of *T. rufonigra*. The venom gland (also called poison gland) of *T. rufonigra* consisted of two parts [venom gland tubes and venom gland reservoir (poison sac)], which is similar to that of *Camponotus pennsylvanicus* reported previously (Schoeters & Billen 1998). The venom gland tubes were lined with simple cuboidal epithelium, while the venom gland reservoir was covered with simple squamous epithelium. These finding are consistent with reports on *S. invicta* (Callahan et al. 1959) and *C. pennsylvanicus* (Hermann et al. 1975). These histological features support the known function of venom gland tubes to transfer the venom to the sting, and that of the venom gland reservoir to synthesize and store the venom.

The presence of the pygidial gland has been reported in other ant species (Hölldobler & Traniello 1980). The function of pygidial gland is to produce and release chemicals, and the function is diverse depending on the species. For example, the pygidial gland releases communicating pheromones in *Pachycondyla laevigata* (Hölldobler & Traniello 1980). In *Pheidole biconstricta* (subfamily Myrmicinae), this gland is involved in the production of the defense pheromone and alarm pheromone (Kugler 1979).

The integument system of *T. rufonigra* consisted of three distinct layers (epicuticle, exocuticle and procuticle). Both epicuticle and exocuticle were obviously stained in pink/orange by eosin, indicating the presence of protein component in these two layers. Endocuticle, the thinnest layer with chitin component, was stained light pink as reported previously (Locke 1969). However, epidermis and basement membrane were not clearly observed under the light microscope in contrast to a previous report (Triplehorn & Johnson 2005). Future transmission electron microscopy (TEM) will allow us to further observe detailed structures.

The insect respiratory system consists of the tracheal system and spiracles that disperse throughout the body. The functions of this system are to deliver oxygen from the air to the tissues as well as to evacuate carbon dioxide resulting from cellular respiration to the air (Hetz & Bradley 2005; Weibel 1984). Similar structures have been visualized in *Oligotoma saundersii* (Poolprasert & Senarat 2014) and *Catopsilia pomona* (Senarat et al. 2015).

The digestive system of insects is normally divided into three main parts; foregut (stomodeum), midgut (mesenteron or ventriculus) and hindgut (proctodeum). In this study, we identified all three main parts of the digestive system in *T. rufonigra.* However, only mucosa and connective layer were observed in digestive tract wall although the midgut and hindgut of Striped blister beetle, *Epicauta waterhousei* (Coleoptera: Meloidae) contained four layers including mucosa, submucosa, muscularis and serosa (Senarat et al. 2014), which are similarly seen in other insects such as stick insect, *Pylaemenes mitratus* (Phasmid: Basillidae) (Harris et al. 2019) and firefly, *Pteroptyx tener* (Coleoptera: Lampyridae) (Othman et al. 2018). It is possible that each layer of digestive tract wall of *T. rufonigra* is extremely thin; thus, the anatomical study under the light microscope could not identify these layers. Meanwhile, in *T. rufonigra,* midgut mucosa and hindgut mucosa were lined with simple columnar epithelium and simple squamous epithelium, respectively. This difference suggests that midgut functions mainly in digestion, whereas hindgut functions mainly in absorption, which is echoed by the previous study (Borror et al. 1989; Triplehorn & Johnson

2005). Moreover, Malpighian tubules were lined with simple cuboidal epithelium and mainly found between midgut and hindgut. The important roles of these tubules could relate to the excretion of metabolic wastes, especially nitrogenous wastes (uric acid), from the hemolymph, along with the excretion of undigested foods via the anus (Berridge & Oschman 1969).

The insect central nervous system (CNS) includes three parts: (1) brain or supraesophageal ganglion, (2) subesophageal ganglion, and (3) ventral nerve cord (Adams & Selander 1979; Nation 2007). In this study, five CNS compartments were observed, including the brain and four abdominal ganglia. The histological examination revealed that each ganglion consists of two parts: (1) inner medulla, which consists of nerve fibers and neuroglia (supporting cell) and (2) outer cortex, which consists of neurosecretory cells (NS cells), neurons (NR), and neuroglia (NG). The identification of NS cells may need further verification since NS cells look darker in some previous studies (Bliss & Welsh 1952; Preciado et al. 1994). In this study, the location and morphological characters are similar to some NS cells (Poolprasert & Senarat 2014; Soomcham et al. 2017), which led us to conclude that these are likely to be NS cells. Thus, further experiments such as TEM will help us to strengthen our conclusion. The result from this study is consistent with those from previous studies on other insects, e.g., blister beetle, *Epicauta waterhousei* (Langkawong et al. 2013), common emigrant, *Catopsilia pomona* (Soomcham et al. 2017) and webspinner, *Oligotoma saundersii* (Poolprasert & Senarat 2014). Normally, ant nervous system consists of brain, subesophageal ganglion located posterior to the brain, and two thoracic ganglia (Gillott 2005). The number of ganglia indicates the evolutionary level of insects. Insects in primitive orders normally have more ganglia than those in advanced orders. For example, three thoracic ganglia and eight abdominal ganglia are found in Coleoptera, whereas three thoracic ganglia and seven abdominal ganglia are found in Embioptera; three thoracic ganglia and only six abdominal ganglia can be found in Blattodea. Interestingly, only one thoracic ganglion can be found in Diptera with lack of abdominal ganglion (Lacombe 1971; Triplehorn & Johnson 2005). Two thoracic ganglia and three to four abdominal ganglia are commonly found in Hymenoptera, which includes ants, bees, wasps and sawflies. Ball & Vinson (1984) reported that *Solenopsis invicta* has two thoracic ganglia and four abdominal ganglia, which is in agreement with the number of ant abdominal ganglia from this study as well as the number in Lepidoptera (Heywood 1965; Kim et al. 1998). The 2nd and 4th ganglia identified in this study have a large size, suggesting that these are formed by fusion of other ganglia.

CONCLUSION

This histological study revealed essential systems in *T. rufonigra* obtained from the field. The information can provide an almost complete view on the systematic characteristics of this ant, which will be useful for future taxonomical and physiological research.

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