COI Nucleotide Sequence Confirms the Species of Common Posy Butterfly, Drupadia ravindra Horsfield, 1829 and Histological Feature Reveals Its Microanatomical Structure of Nervous System and Sense Organs

Pisit Poolprasert1*, Anongnat Chitchamnong1, Piyakorn Boonyoung2, Sinlapachai Senarat3, Paradorn Dokchan4, Ezra Mongkolchaichana5, Wirot Likittrakulwong6 and Grant Berry7

1 Biology Program, Faculty of Science and Technology, Pibulsongkram Rajabhat University, Phitsanulok, 65000, Thailand
2 Department of Anatomy, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand
3 Department of Marine Science, Faculty of Science, Chulalongkorn University, Pathum Wan, Bangkok, 10330 Thailand
4 Environmental Entomology Research and Development Center, Kamphaengsaen Research and Development Institute at Kamphaengsaen, Kasetsart University, Kamphaengsaen Campus, Nakhon Pathom 73140, Thailand
5 Department of General Science, Faculty of Science and Health Technology, Navamindrahiraj University, Bangkok 10300, Thailand
6 Animal Science program, Faculty of Food and Agricultural Technology, Pibulsongkram Rajabhat University, Phitsanulok, 65000, Thailand
7 New Cambridge International School, Phitsanulok, 65000, Thailand

*Corresponding author: poolprasert_p@psru.ac.th

KHON KAEN AGR. J. 48 SUPPL. 1: (2020).

บทคัดย่อ: ดีเอ็นบาร์โค้ดเป็นเครื่องมือที่มีประสิทธิภาพ รวดเร็ว และเชื่อถือได้สำหรับการระบุสิ่งมีชีวิตชนิดต่าง ๆ ทั้งในระดับสกุลและชนิด ในการศึกษาครั้งนี้มีการใช้ลําดับลําดับเบส 631 คู่เบส จากยีน COI ในไมโทคอนเดรียของผีเสื้อแต้มแสดธรรมดา Drupadia ravindra Horsfield, 1829 หลังจากเทียบลําดับเบสที่ได้กับฐานข้อมูล GenBank โดยใช้โปรแกรม BLAST พบว่า มีความคล้ายคลึงมากกว่า 99 เปอร์เซ็นต์ จึงยืนยันได้ว่าเป็นผีเสื้อแต้มแสดธรรมดา (Drupadia ravindra) ประเภทนี้ได้ทำการวิเคราะห์แผนภูมิต้นไม้ทางวิวัฒนาการด้วยวิธี Neighbor Joining (NJ) ร่วมกับตัวอย่างที่มีการฝากไว้ก่อนหน้า พบว่า ผีเสื้อแต้มแสดธรรมดาในประเทศไทยเป็นแบบวิวัฒนาการเชิงเดี่ยว นอกจากนี้ ยังมีการศึกษาเพื่อให้เข้าใจโครงสร้างประสาทส่วนหน้าและตาของผีเสื้อแต้มแสดธรรมดา ด้วยเทคนิคทางด้านเนื้อเยื่อวิทยา ที่สามารถใช้สำหรับการศึกษาการสร้างหนักผักโดยไบโอเทคนิคและสรีรวิทยาการสืบพันธุ์ พบว่า มุขวิทยาของระบบประสาทของผีเสื้อชนิดนี้ ประกอบด้วยสมองและปมประสาท ที่มีฟันเจาะขึ้นสู่ส่วนกลาง ประกอบด้วยชั้นส่วนนอกและส่วนใน โดยที่เซลล์ส่วนนอกได้แก่ เซลล์นิวโรซีคลีทอรี เซลล์ประสาท และเซลล์ประสาทที่มีลักษณะเฉพาะในส่วนนี้ ขณะที่เซลล์ส่วนใน เทียบกับสมองหน้าของผีเสื้อชนิดนี้ ซึ่งมีเซลล์สามชนิด เซลล์นิวโรเกลีย เส้นใยประสาท และเซลล์ประสาทส่วนที่มีรูปแบบของออมมาติเดีย ประกอบเป็น 3 ส่วน คือ โปรโตเซรีบรัมส่วนหน้า โปรโตเซรีบรัมส่วนกลาง และไตรโทเซรีบรัมส่วนล่าง ซึ่งจะคาดเดาได้จากแผนภูมิวิวัฒนาการในสายพันธุ์ พบว่า ผีเสื้อแต้มแสดธรรมดาในประเทศไทยมีการวางลายปิลาร์ส่วนหน้า แบบดีเอ็นบาร์โค้ดที่มีความคล้ายคลึงกันกับตัวอย่างที่มีการฝากไว้ก่อนหน้า

คำสำคัญ: ดีเอ็นบาร์โค้ด ผีเสื้อแต้มแสดธรรมดา Drupadia ravindra ชนิดนิวโร "เซลล์นิวโร" รังผึ่ง

1 Biological Program, Faculty of Science and Technology, Pibulsongkram Rajabhat University, Phitsanulok, 65000, Thailand
2 Department of Anatomy, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand
3 Department of Marine Science, Faculty of Science, Chulalongkorn University, Pathum Wan, Bangkok, 10330 Thailand
4 Environmental Entomology Research and Development Center, Kamphaengsaen Research and Development Institute at Kamphaengsaen, Kasetsart University, Kamphaengsaen Campus, Nakhon Pathom 73140, Thailand
5 Department of General Science, Faculty of Science and Health Technology, Navamindrahiraj University, Bangkok 10300, Thailand
6 Animal Science program, Faculty of Food and Agricultural Technology, Pibulsongkram Rajabhat University, Phitsanulok, 65000, Thailand
7 New Cambridge International School, Phitsanulok, 65000, Thailand
8 Corresponding author: poolprasert_p@psru.ac.th
INTRODUCTION

At present, the molecular method based on the DNA sequence can be applied to the taxonomic identification of known and unknown specimens. Even though several different DNA based techniques are potentially available for quarantine and forensic examinations. DNA barcoding, a modern technique for the rapid identification of any species, has also been widely used as a method to efficiently describe biodiversity (Yang et al., 2018). Also, this technique uses DNA fragments obtained from specific genes, normally from mitochondrial DNA such as the cytochrome c oxidase I (COI) gene, a fragment of about 500-800 base pairs, has been extensively used in several animal species (Hebert et al., 2003a, b). In general, Allio et al. (2017) postulated that mitochondrial DNA (mtDNA) evolves at a faster rate than nuclear DNA (nuDNA) in animals. This has contributed to the popularity of mtDNA as a molecular marker in evolutionary studies. In this regard, the COI is more conserved and it is very suitable for species identification since its sequence has a low variability (less than 1-2%). Even for the closely-related species its value is less than 1%. In addition, the COI gene is the most common gene used to analyze the relationship among closely-related species in several insect groups (butterflies, beetles, and flies), as individual gene or its combination with other genes (Hebert et al., 2003a, b; 2004; Wilson, et al., 2013).

The insect nervous system generally comprises the central nervous system (CNS) and the peripheral nervous system. The former consists of the brain (frontal ganglion), ventral nerve cord and ventral ganglia. In the same manner, the CNS is primarily located ventrally along the length of the body, so insects are said to have a ventral nerve cord (Gullan and Cranston, 2004; Triplehorn and Johnson, 2005). The latter includes a stomatogastric...
ganglion, sensory and motor nerves. In addition, the insect nervous system includes pars intercerebralis-corpus cardiacum-corpus allatum and neuroendocrine system that consists of store and release organs (Klowden, 2002). In particular the brain structure comprises three pairs of lobes: the protocerebrum, the deutocerebrum, and the tritocerebrum. These lobes are fused ganglia, clusters of neurons that process sensory information. Each lobe controls different activities and functions. Neurons vary in number among insect brains. The common fruit fly has at least 100,000 neurons, while a honeybee has one million neurons (Gullan and Cranston, 2004; Eichler et al., 2017; Howard et al., 2018). Studies on the brain in the insects have been carried out by several investigators like Hughes (1980), Ayali and Ziberstein (2003) and Zilberstein et al. (2006). It is well-known that all regions concern to have a role the production of neurotransmitters and neuromodulators. These two chemical peptides are speculated to play a key role for controlling reproduction and food uptake functions (Hughes, 1980; Ayali and Ziberstein, 2003; Zilberstein et al., 2006). Evidence of these functions was reported in the gut activity of two locust species (Schistocerca gregaria and Locusta migratoria) using the brain ganglionectomy technique. (Hingham et al., 1966; Bignell, 1974). It was revealed that the presence of feeding activity and food uptake of these species were immediately reduced. According to important function of brains mentioned above, most researchers seem to agree that the existence of the histological and histochemical investigations of insect nervous system have been exclusively identified before understanding the neuroendocrinological mechanism for further study. Several techniques regarding histological description in many insect groups have been observed i.e. honeybees, Apis mellifera (Calabria et al., 2010), migratory locusts, Locusta migratoria (Kumas and Karakiṣt, 2012), blister beetles, Epicauta waterhousei (Lungkawong et al., 2013), webspinners, Oligotoma saundersii and Eosemia auripecta (Poolprasert and Senarat, 2014; 2015). For histology among lepidopterans, only one preliminary observation regarding the common emigrant, Catopsillia pomana frontal ganglion, thoracic and abdominal ganglia has been viewed (Soomcham et al., 2014).

As mentioned earlier, studies concerning molecular and histological traits of the common posy Drupadia ravindra, a small butterfly and major common butterfly species in Thailand, are still scarce in Thailand. Therefore, the authors attempted to use molecular taxonomic identification on D. ravindra. The authors also histologically identified the nervous system with emphasis of brain or frontal ganglion and eye structure in D. ravindra. These findings could be useful for further management regarding agriculture or forestry. Besides, the basic knowledge of this histological description could contribute not only to understand the structure and cell types but also to be used in further research in the function of the neurotransmitter and neuromodulator of this butterfly and other butterfly species.

MATERIALS AND METHODS

Butterfly Sampling

A total of five common posies, Drupadia ravindra with wingspan of about 3.13±0.65 cm (Figure 1) were collected from a mix-deciduous forest, Sakaerat District, Nakhon Ratchasima Province, Thailand. All butterflies were mercy-killed by pinching their thoraces (middle body segment) between thumb and forefinger following the guidance of Triplehorn and Johnson (2005). All specimens were initially identified based on external morphology following the guildbook as described by Ek-Amnuay (2012). Afterwards, a single butterfly specimen was chosen to confirm the species using DNA barcoding. Remaining specimens they were fixed immediately in Davidson’s fixative for 24-36 hours prior to histological study.
With regard to molecular identification, the total genomic DNA was extracted from a single leg of *D. ravindra* using DNeasy Blood & Tissues kit (Qiagen, Germantown, MD, US, catalog #69504). The protein-coding mitochondrial COI gene for molecular analysis was used in this study. The primers used for the polymerase chain reaction (PCR) amplification were LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al., 1994). Each PCR reaction was performed using a final volume of 20 μl containing 4 μl of 5x PCR Enhancer, 2 μl of 10x HF Reaction Buffer, 0.4 μl 10 mM dNTP Mix, 0.3 of each primer (10 μmol/L), 0.3 μl of Long and High Fidelity DNA Polymerase (0.75 U) (biotechrabbit, Germany), 10.7 μl of nuclear free water and at least 2 ng of genomic DNA template. The cycling program included an initial activation step of 3 min at 94 ºC, followed by 35 cycles of 1 min at 94 ºC (denaturation), annealing temperature at 48 ºC for 1 min, extension at 72 ºC for 1 min and a final extension of 5 min at 72 ºC. The amplification products were visualized under UV light following electrophoresis on an ethidium bromide stained 1% agarose gel in 1x TAE buffer. A single band of PCR products was purified using the GenUP PCR/Gel Cleanup Kit (biotechrabbit, Germany) as described by manufacturer’s instructions and direct sequences by Macrogen, inc (http://www.macrogen.com). Similarity search for a partial mtDNA sequence was verified using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Then a sequence was initially aligned and edited using the MEGA7.0 program (Kumar et al., 2016). The mitochondrial DNA sequence was finally trimmed to 631 base pairs and deposited in GenBank via BankIt (https://www.ncbi.nlm.nih.gov/BankIt/) under accession number MN563578.

**Histological analysis**

To observe the structure and cell types of the nervous system and eyes, whole bodied (except wings) of five common posy specimens were routinely processed using the standard histological technique (Bancroft and Gamble, 2002). The paraffin embed blocks were cut at 6-7 μm thicknesses and stained with Harris’s hematoxylin and eosin (H&E) and Masson’s trichrome (MT) (Bancroft and Gamble, 2002). All histological sections were examined under the light microscope, photographed with a Canon EOS 1100D and photographic plates were generated using Adobe Photoshop CS6.

**Figure 1** Light micrograph of the common posy, *Drupadia ravindra* (A) dorsal and (B) ventral views.
RESULTS AND DISCUSSION

Molecular Identification

The DNA sequence of the mitochondrial 5'COI gene region was analyzed to identify the species based on only a single sample of common posy. The DNA fragment containing 631 base pairs (bp) of COI gene was finally obtained. When conducting a BLAST search, it was found that a single butterfly sample tested could provide 99% of sequence similarity. In this regard, the similarity percentage of different species level should be 97-98% (Wilson et al., 2013). Consequently, it could be identified as Drupadia ravindra. The nucleotide composition of the COI were detected to be A = 32.40%, T = 40.40%, G = 14.10% and C = 13.10%, indicating the mean base composition of the DNA of D. ravindra examined in the current study covered only 15.20% of G-C content whereas A-T content was about 84.4%. However, the overall frequency distributions of nucleotides at the 1st, 2nd and 3rd codon positions were as follows: A = 14.10%, 51.0% and 32.20%; C = 26.20%, 2.40% and 10.70%; G = 16.50%, 0.00% and 10.70% and T = 43.0%, 74.0% and 31.0%, respectively. This finding conforms to the results of the COI gene region of other insect groups exhibiting a bias towards adenine and thymine (Jing and Yingchun, 2006; Karthika et al., 2016; Wongsa et al, 2016).

Like the COI gene of other insects, the base composition analysis for the COI sequence of D. ravindra showed that the average T content was the highest and the average G content was the lowest; the AT content (72.80%) was higher than the GC content (27.2%). In general, the base composition of the COI fragment varied among the species but it was commonly demonstrated with an overall AT bias of 67.27 and GC of 32.73 (Karthika et al., 2016).

With regard to the phylogenetic analysis, the NJ method was used to check the phylogenetic status obtained from a single sequence of partial mtDNA control region using the Kimura 2-parameter model. It was then compared with four published sequences of D. ravindra obtained from Genbank databases (HQ962185; HQ962194 and KT286463). Besides, two species i.e. D. cinesoides and D. theda thesmia retrieved from NCBI served as the out-group. The reliability of the tree topology was assessed by 1,000 bootstrap replications. The numbers at the node indicated the percentage of bootstrap valued for the interior branches. It was revealed that phylogenetic relationship of D. ravindra obtained from a current sequence and other sequences deposited previously formed reciprocally monophyletic clusters (Figure 2). It was noted that 60% of similarity based on NJ bootstrap was observed, indicating that high genetic variation in this D. ravindra was present. It might be suggested that more butterfly samples should be collected to confirm topology of Thai D. ravindra concerning phylogenetic tree position.

Histological analysis

1) Basic histology of the nervous system (NS)

The neuronal structure of Drupadia ravindra could be classified based on localization and histological characterizations into two components; the inner medullae and the outer cortex (Figure 3), as similar to other insects (Kirby et al., 1984; Bolleli et al., 1998). The distinguishing features of the inner medullae were contained in both nerve fibers and the neuroglia. Meanwhile, the outer cortex composed of three cell types, exhibited histological difference. A neurosecretory cell (or neuroendocrine cell) was distinguishable and located in the periphery of the neuronal structure. It was an oval shaped cell and the largest cell was approximately 5-6 µm in diameter. The oval nucleus was also contained in one or two nucleoli, which was surrounded by eosinophilic nucleoplasm. A neuron was oval shape with a diameter of 3-4 µm. The smallest cell was the neuroglian about 2 µm. The presence of this cell was located among the cell types. It had a round nucleus with surrounding the rim of the eosinophilic cytoplasm. In addition to being observed in the neuron, the main function of this cell might be in the protection of neurons (Kumas and Karakist, 2012).
Figure 2 Phylogenetic relationship of *Drupadia ravindra* based on the partial mtDNA-COI sequences. The phylogenetic was a single rooted tree recovered using NJ analysis. Values on the branches represent NJ bootstrap estimates, based on 1,000 replicates. The red MN563578 shows the recent *D. ravindra* sample.

Figure 3 Light micrograph of basic neuronal structure and cellular classification in *Drupadia ravindra* frontal ganglion: 10 µm; IM = inner medulla, Ng = neuroglia, Nc = neurosecretory cell, Ne = neuron, OC = outer cortex.
Figure 4 Light micrograph of frontal ganglion and eye structure of *Drupadia ravindra* with several regions; 200 µm (A), 50 µm (B-D), 20 µm (E-F). Note: Al = antennal lobe, Cb = central body, Cn = cornea, Cp = corpora pedunculata, DC = deutocerebrum, Ey = eye, He = head, Ic = inner of optic chiasma, Ig = inmina gabglionaris, Me = medulla externa, Mi = medulla interna, Oc = outer of optic chiasma, Om = ommatidium, Pb = protocerebral bridge, Pc = protocerebrum, Pi = pars intercerebralis, Rc = retinular cells, Rd = rhabdomere, TC = tritocerebrum.
2) Neuronal classification and structure of NS

According to histological techniques, the nervous system of *Drupadia ravindra* is classified into two components including brain (frontal ganglion) and ventral nerve cord with segmental ganglia. The neuronal structure of *D. ravindra* could be classified based on localization and histological characterizations into two components; the inner medullae and the outer cortex (Figure 3).

The structure of the brain of *D. ravindra* was covered by a thin layer of connective tissue, also called the neuronal capsule. This ganglion was composed of three regions including dorsal protocerebrum, middle deutocerebrum and ventral tritocerebrum (Figure 4 A-D), which was similar to the frontal ganglion in *Catopsilla pomana* (Soomcham et al., 2014) and *Embolyntha batesi* (Lacombe, 1971). Two distinct sub-regions, the paired lateral protocerebrum (pars intercerebralis) and the median protocerebrum (Protocerebral Bridge) were largely and easily observed in the protocerebrum. It is suggested that the structure of this sub-region plays a crucial role in the antennal stimuli, mechanosensory and movements of the antennae (Gullan and Cranston, 2004). In particular, the lateral protocerebrum or corpora pedunculata or mushroom body was present with two prominent lateral lobes to join the eye structure. This region was also separated by the optic nerve (optic stalk). Under high magnification, the optic nerve could be divided into three regions including medulla interna, medulla externa and an inmina gabglionaris, which were connected by the optic chiasma. Investigation of the deutocerebrum was largely composed of the nerve fibers and is also called the paired antennal or intercerebralis lobes. The function of this part is related to the olfactory and the mechanosensory movements of the antennae (Gullan and Cranston, 2004). Additionally, it is believed that the deutocerebrum is shown to play a role in learning olfactory behavior of some insects (Gullan and Cranston, 2004). The tritocerebrum is a small part and not easily seen in the brain (Figure 4A).

The ganglia consist of five ganglia including two thoracic ganglia (pro- and mesothoracic ganglia) and three abdominal ganglia (Soomcham et al., 2017). Typically, the central nervous system of various insects shows the diversity of arrangement of ganglia in the ventral nerve cord. There are three thoracic ganglia...
and eight abdominal ganglia which represent a fused pair, as in webspinners (Embioptera) (Lacombe, 1971; Poolprasert and Senarat, 2015). Meanwhile, highly fused with one thoracic and no abdominal ganglia could occur in flies (Diptera) (Gullan and Cranston, 2004). Based on histological analysis, histological study of all ganglia were shared and could be divided into two regions i.e., inner medular and outer cortex (Figure 3). Several cells in the outer cortex were classified as containing such neurosecretory cell and neuroglia (Figures 5A-5B). It is similarly reported in the lepidopteran nervous system (McLaughlin, 1974; Soomcham et al., 2017).

CONCLUSION

Based on DNA sequencing, it was found that this common posy butterfly could be well classified into species level (*Drupadia ravindra*), suggesting COI mtDNA sequencing is an appropriated technique for rapid species identification and can be applied for other insect groups. Moreover, this examination of the structure of the frontal ganglia and eyes in *D. ravindra* was first report in Thailand. The histological structure of the frontal ganglion was composed of an outer cortex and an inner medulla layers. Three distinct cells i.e. neurosecretory cells, neurons and neuroglia were found in the outer cortex, whereas neuroglia and nerve fibers appeared in the inner medulla. The structure of the frontal ganglion and eyes was detected. The frontal ganglion could be classified into three major regions (dorsal protocerebrum, middle deutocerebrum and ventral tritocerebrum). In this regard, further studies should be made to gain more knowledge about immunoreactivities and gene expression of peptidic hormones. These could be used for hormonal application and physiological pathways.

Acknowledgements

The authors express the gratefulness to aquatic toxicology, Department of Pathobiology, Faculty of Science, Mahidol University, Department of Biology, Faculty of Science and Technology, Pibulsongkram Rajabhat University and Department of Anatomy, Faculty of Science, Prince of Songkla University for their support throughout this study.

REFERENCES


