Discovery of *Encyclometra bungara* (Digenea: Encyclometridae) in a new host (*Enhydris enhydris*) from Thailand and Cambodia through morphological and molecular identification

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Abstract

The genus *Encyclometra* is one of the two genera in family Encyclometridae, known for parasitising the oesophagus, stomach, and intestine of snakes. Among *Encyclometra*, the species present are: *Encyclometra colubrimurorum*, *Encyclometra japonica*, *Encyclometra asymetrica*, and *Encyclometra bungara*. Species discrimination within *Encyclometra* has predominantly relied on morphological differences, such as the length of the caeca and the position of the testes. Morphological overlaps exist among these species making species discrimination challenging. Additionally, the use of molecular information has been limited for *Encyclometra*. To determine the *Encyclometra* species infecting *Enhydris enhydris* from Thailand and Cambodia, morphological and molecular identification was conducted. Morphological characters and measurements were obtained from 30 *Encyclometra* adults, and they were compared with previous studies of other *Encyclometra* species. Novel sequences of *E. bungara* were generated using the nuclear 18S and 28S ribosomal RNA genes, and the mitochondrial cytochrome c oxidase subunit 1 gene. Our results revealed that the specimens could be morphologically identified as *E. bungara*, with support from molecular information obtained from the phylogenies of the three genetic markers employed. Molecular analysis showed that the *Encyclometra* specimens were distinct from *E. colubrimurorum* and *E. japonica*. Through morphological and molecular identification of the *Encyclometra* specimens found in *Enhydris enhydris* from Thailand and Cambodia, we describe and provide a record of *E. bungara* in a new host and new locality. Additionally, novel molecular sequences were generated, revealing the phylogenetic position of *E. bungara* within the superfamily Gorgoderoidea.

**Keywords:** *Encyclometra bungara; Enhydris enhydris; morphology; molecular identification*
Introduction

*Encyclometra* Baylis & Cannon, 1924, the genus in the Encyclometridae Mehra, 1931, is classified under the superfamily Gorgoderoidea Loose, 1901. *Encyclometra* is one of the two genera in the family Encyclometridae, where they parasitise the oesophagus, stomach, and intestine of snakes (*Tkach, 2008; Saito et al., 2022*). *Encyclometra* is characterised by well-developed oral and ventral suckers, with the oral sucker being smaller or similar in size to the ventral sucker. It is also differentiated from *Encyclobrephus* Sinha, 1949, the other genus in Encyclometridae, by having a tegument without spines (*Tkach, 2008*).

The species of *Encyclometra* include *Encyclometra colubrimurorum* (Rudolphi, 1819), *Encyclometra japonica* Yoshida and Ozaki, 1929, *Encyclometra asymetrica* Wallace, 1936, and *Encyclometra bungara* Srivastava and Ghosh, 1968 (*Rudolphi, 1819; Yoshida and Ozaki, 1929; Wallace, 1936; Yeh, 1958; Srivastava and Gosh, 1968*). The type species *E. colubrimurorum* was first discovered in a grass snake (*Natrix natrix*) in Europe, and it was initially morphologically described by having tandem testes and caeca that are equal in length (*Rudolphi, 1819*). *Encyclometra japonica* was first discovered in a Japanese striped snake (*Elaphe quadrivirgata*) in Japan and was described as a new species due to the testes positioned diagonally relative to each other (*Yoshida and Ozaki, 1929*). The third species, *E. asymetrica*, has been described in various snake species and is characterised by intestinal caeca that are significantly unequal in length (*Wallace, 1936*). Finally, *Encyclometra bungara* was first described in *Bungarus fasciatus* in India (*Srivastava and Ghosh, 1968*) has also been documented in Southeast Asia countries (Thailand and Lao People’s Democratic Republic) in *Xenochrophis piscator* and *Enhydris plumbea* (*Scholz and Ditrich, 1991; Wongsawad et al., 1991*). Species discrimination within *Encyclometra* has primarily relied on morphological differences in the length of the caeca and position of the testes. However, the criteria for species discrimination among the four valid species remains unclear. For example, Yeh (*1958*)
provided the key to distinguish species of *Encyclometra* based on differences in caeca length (equal, subequal, or very unequal), while Gupta and Mehrotra (1977) amended the key and suggested the testes position can be used for species discrimination (Gupta and Mehrotra, 1977).

Molecular methods for species identification are not widely utilized for *Encyclometra*. To date, there are limited sequences in reference databases, with sequences only available for *E. colubrimurom* and *E. japonica*. Here, in order to identify the *Encyclometra* species found in *Enydris enhydris* from Thailand and Cambodia, a comparison of morphological characters among the other valid species of *Encyclometra* was performed. Additionally, molecular identification using both nuclear and mitochondrial genetic markers was used to obtain novel sequences for *E. bungara*.

**Materials and methods**

*Parasite isolation*

A total of 18 rainbow water snakes (*Enydris enhydris*) were obtained from southern Thailand in Nakhon Si Thammarat and adjacent Provinces, north-eastern Thailand in Mahasarakham Province, and Kampong Chhnang Province in Cambodia. The snakes were dissected to examine parasites in the oesophagus. The adult trematodes were isolated, counted, and then preserved in 70% ethanol.

*Morphological analysis*

Thirty adult trematodes were selected, stained in acetic carmine, and mounted in permount for morphological analysis. Morphological characters were observed and measured using an inverted compound microscope equipped with a camera and software (ZEISS primovert). These characters included body length, maximum body width, oral and ventral sucker
dimensions, testes dimensions and position, intestinal caeca symmetry, and egg length and width. The morphological characters and measurements were then compared with those from previous studies on Encyclometra (Yeh, 1958; Gupta and Mehrotra, 1977; Scholz and Ditrich, 1991; Wongsawad et al., 1991; Saito et al., 2022). Morphological drawings of the specimens were conducted under a compound light microscope to illustrate the morphological characters.

**Molecular analysis**

Four trematodes (two from Thailand and two from Cambodia) were selected and individually placed into 1.7 ml microcentrifuge tubes and thoroughly washed with sterile distilled water. Total genomic DNA was isolated using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer’s recommendations. PCR amplification of the partial regions of the nuclear 18S rRNA gene (C for: 5’ – ATGGCTCATTAAATCAGCTAT – 3’, A rev: 5’ – TGCTTTGAGCACTCAAATTTG – 3’), 28S rRNA gene (Digl2: 5’ – AAGCATATCAAGCGG – 3’, 1500R: 5’ – GCTATCCTGAGGGAAACTTCG – 3’), and the mitochondrial COI gene (JB3: 5’ – TTTTTGGGCAATCCTGAGGTTTAT – 3’, JB4.5: 5’ – TAAAGAAAGAACATAATGAAAATG – 3’) were performed in a final PCR volume of 30 µl (Bowles et al., 1992; Curran et al., 2011; Routtu et al., 2014). The lengths of the partial regions of the 18S rRNA, 28S rRNA, and COI genes are 800bp, 1200bp, and 440bp, respectively. Each reaction contained 15 µl of 2X i-Taq™ mastermix (iNtRON Biotechnology, Gyeonggi, South Korea), 0.1 to 0.5 µM of each primer, and 1ng/µl of DNA. PCR was conducted in a T100™ thermocycler (Bio-Rad, California, USA), and the thermocycling conditions followed the respective publications of the primer sequences. PCR amplicons were visualised on 1% agarose gel stained with SYBR™ Safe (Invitrogen, Massachusetts, USA). DNA products were subsequently sent for Barcode Taq sequencing (Celemics, Seoul, South Korea).
Electropherograms of the partial 18S rRNA, 28S rRNA, and COI genes were manually checked using Bioedit 7.0 (Hall, 1999). A dataset containing the concatenated 18S + 28S rRNA genes was also analysed by manually merging the partial 18S and 28S rRNA gene sequences. Multiple sequence alignment with reference sequences obtained from the NCBI database (Supplementary Table S1) was performed using ClustalX 2.1 for each genetic marker (Thompson et al., 2002). The aligned sequences were checked, and a suitable nucleotide substitution model was selected for maximum likelihood (ML) phylogenetic analysis. The substitution model selection and ML phylogenetic analysis was carried out in MEGA X (Kumar et al., 2018). Schistosoma mansoni and Fasciola hepatica were used as outgroups to root the phylogenetic trees. The phylogenetic trees were visualised and labelled using FigTree 1.3.1 (Rambaut, 2009). Pairwise inter- and intra-species genetic distances were calculated using p-distance as the model for each genetic marker in MEGA X (Kumar et al., 2018).

Results

Morphological identification

Out of the 18 Enhydris enhyris examined, 12 were found to be infected with Encyclometra, indicating a prevalence of 66.7%. The infection was in the oesophagus, yielding a total of 103 adult Encyclometra specimens. Morphological identification and measurements confirmed that the specimens obtained from both Thailand and Cambodia are E. bungara. These diagnostic characters include the diagonally placed testes and equal-length intestinal caeca. The morphological measurements (n=30) obtained from these specimens are presented in Table 1 and Supplementary Table S2. Slight differences in the shape of the posterior end were observed between E. bungara specimens from Thailand and Cambodia. The former consistently exhibited a broad posterior end, while the latter consistently displayed a more pointed posterior end. Fig. 1 depicts the differences between E. bungara obtained from the two countries.
The description of *E. bungara* obtained in this study is as follows – whole-body shape flat and lanceolate, posterior end either broad or pointed, cuticle lacking spines. Body length 771–2,996 µm, maximum width 267–926 µm. Oral and ventral suckers well-developed, approximately equal in size. Subterminal oral sucker 120–263 µm by 109–257 µm, ventral sucker 124–316 µm by 122–297 µm, positioned in anterior quarter to a third of the body. Ratio of ventral sucker to oral sucker 1.1:1. Pharynx distinct, oesophagus very short or absent. Intestinal caeca arise near the pharynx and extend to the posterior end, being equal in length. Curved cirrus sac at anterior border of ventral sucker. Ovary oval, near the posterior border of the ventral sucker, next to seminal vesicle. Two oval or slightly lobed testes, always positioned obliquely in the posterior half of body, 56–192 µm x 41–170 µm. Vitelline glands extend along the side of intestinal caeca, starting from the position between the ovary and testes, and extending to the posterior end of the body. Uterus fully filled, with oval-shaped eggs measuring 60–78 µm by 32–44 µm. Excretory bladder Y-shaped.

**Taxonomic summary**

**Host:** Rainbow water snake, *Enhydris enhydris* (Schneider, 1799) (Squamata: Homalopsidae)

**Locality:** Thailand (Southern region: Nakhon Si Thammarat and adjacent provinces; and North-eastern region: Mahasarakham Province) and Cambodia (Kampong Chhnang Province)

**Site of infection:** Oesophagus

**Prevalence:** 66.7% (12 out of 18 host infected)

**Specimens deposited:** Department of Helminthology, Faculty of Tropical Medicine, Mahidol University

**Molecular and phylogenetic analysis**

Three molecular genetic markers — nuclear 18S rRNA gene, 28S rRNA gene, mitochondrial
COI gene, and the concatenated 18S + 28S rRNA genes were used for analysis. Firstly, based on the phylogenies obtained, all three genetic markers supported that the *E. bungara* specimens obtained from *Enhydris enhydris* in this study were genetically different from *E. colubrimurorum* and *E. japonica* (Fig. 2A–D). Inter-species genetic distances also support that *E. bungara* are distinct from *E. colubrimurorm* and *E. japonica*. The genetic distances between *E. colubrimurorum* or *E. japonica* with *E. bungara* ranged from 0.2–0.4%, 1.1–1.2%, and 12.0–14.7% using the 18S, 28S, and COI genes, respectively. Moreover, these genetic distance values obtained were higher than the inter-species genetic distances between *E. colubrimurorum* and *E. japonica*. Table 2 presents the comparison of genetic distances for the three genetic markers.

Secondly, the *E. bungara* specimens from Thailand and Cambodia exhibited genetic similarity, providing evidence that the specimens obtained are the same species. Genetic distances between the specimens using the 18S (0.0–0.13%), 28S (0%), and COI (0.3–6.3%) genes were lower than the inter-species genetic distance observed in *Encyclometra*. Additionally, using the 18S rRNA gene, no sequence variation was observed between *E. colubrimurorum* and *E. japonica*. Contrarily, the use of the 28S rRNA gene and the concatenated 18S + 28S rRNA gene showed slight sequence variations of 0.8% and 0.5%, respectively.

Despite low sequence variation using the nuclear genetic markers (Figs. 2A–C), the phylogeny obtained was well-resolved, with the monophyly of Encyclometridae and its sister group relationship with the family Dicrocoeliidae. Moreover, phylogenetic relationships within the superfamily Gorgoderoidea (comprising eight families) were similar with both the nuclear 18S and 28S rRNA genes, except for Callodistomidae and Orchipedidae.
Discussion

Through morphological and molecular identification of the *Encyclometra* specimens found in *Enhydris enhydris* from Thailand and Cambodia, we provide a record of *E. bungara* in a new host and new locality, along with novel molecular sequences. Secondly, the similarities in morphological characters among the four valid species in *Encyclometra* demonstrate the importance of using molecular information for species identification.

*Encyclometra bungara* was previously found in India, as well as in Southeast Asia, including Lao PDR and Thailand. Although to date, this species is not among the *Encyclometra* species that are listed as valid, the morphological descriptions match with previous records of *E. bungara* found in Southeast Asia. Currently, with the results obtained from this study, *E. bungara* can be found infecting four snake species. These four species are *B. fasciatus*, *X. piscator*, *E. plumbea*, and *E. enhydris*. The first record of *E. bungara* in Southeast Asia was reported by Scholz and Ditrich (1991) in the rice paddy snake (*E. plumbea*) from Vientiane Province in Lao PDR (Scholz and Ditrich, 1991). Subsequently, in Thailand, *E. bungara* was found in the checkered keelback snake *X. piscator* during a survey of trematodes in reptiles and amphibians from Doi Suthep-Pui National Park and suburban areas in Chiang Mai Province (Wongsawad et al., 1991). Aside from host species, another difference between our specimens and previous studies lies in the site of infection. Our specimens were exclusively found in the oesophagus, whereas previous studies found *E. bungara* in the intestine of hosts. However, *Encyclometra* has also been found to infect various organs of hosts, including the oesophagus, stomach, and intestine (Yeh, 1958; Saito et al., 2022; Tkach, 2008). Although no molecular information on *E. bungara* was available for comparison, our specimens can be considered conspecific with *E. bungara* due to similar morphological characters such as the diagonally placed testes and equal-length intestinal caeca. Phylogenetic evidence also supported the differentiation of the *Encyclometra* specimens obtained in this study from *E.*
colubrimurorum and E. japonica, suggesting that the Encyclometra specimens obtained are distinct from other Encyclometra species.

The rainbow water snake, E. enhydris, is endemic in Southeast Asia and has expanded its range to include parts of China, India, and Australia (Murphy et al., 1999; Karns, 2000; Lim and D’Rozario, 2009; Karns et al., 2010a). In the southern and central regions of Thailand, previous surveys demonstrated the dominance of E. enhydris, comprising of more than 80% of the snake species surveyed (Karns et al., 2000; Karns et al., 2010b). Enhydris enhydris can thrive in various habitats, including rice paddies, canals, and artificial fishponds. Since their diet primarily consists of fish and amphibians (both adults and juveniles), the life cycle of Encyclometra can be completed with the rainbow water snake as the final host (Voris and Murphy, 2010). Based on evidence from morphological similarities, geographic localities, host characteristics, and molecular data, the specimens obtained in our study expand the known geography and host species of E. bungara, thereby affirming the presence of E. bungara in semi-aquatic snakes from Southeast Asia.

Comparing the species within the genus Encyclometra, morphological similarities are present, and key diagnostic characters such as the intestinal caeca and testes’ position are uninformative for species discrimination. Only E. asymmetrica can be successfully differentiated by having unequal intestinal caeca and a slightly larger oral sucker compared to the ventral sucker (Wallace, 1936). Furthermore, there are morphological variations within species that can complicate species identification. For instance, E. colubrimurorum was initially described as having tandem testes, but a redescription by Gupta and Mehrotra (1977) showed that E. colubrimurorum can present both tandem and diagonally testes (Rudolphi, 1819; Gupta and Mehrotra, 1977; Tkach, 2008). A recent discovery by Saito et al. (2022) also revealed morphological variations within E. japonica, including both caeca types with equal and subequal lengths (Saito et al., 2022). With the overlap of morphological characters among
E. bungara (presenting diagonal testes and equal caeca lengths), E. colubrimurorum (diagonal or tandem testes and equal caeca lengths), and E. japonica (diagonal or tandem testes and equal or subequal caeca lengths), the use of molecular data is valuable.

Previous studies have demonstrated the importance of molecular data for species discrimination among trematodes, especially among closely related and morphologically similar species (Nolan and Cribb, 2005; Thaenkham and Waikagul, 2008; Nadler and Pérez-Ponce de León, 2011; Thaenkham et al., 2011). Given the presence of morphological variations and overlap of morphological characters within Encyclometra, the use of molecular information coupled with morphology is recommended (Saito et al., 2022). Furthermore, our results also revealed that selecting appropriate genetic markers is crucial for species discrimination. As the nuclear 18S rRNA gene is highly conserved, this genetic marker may not provide sufficient sequence variation for species discrimination (Blasco-Costa et al., 2016; Chan et al., 2021). Our results showed no sequence variation between E. colubrimurorum and E. japonica using the 18S rRNA gene. Similarly, this genetic marker also provided no sequence variation between the sister species of Paragonimus heterotremus and Paragonimus pseudoheterotremus (Chan et al., 2022). However, albeit the low sequence variation of the nuclear rRNA genes, they provide robust phylogenetic inferences at higher taxonomic levels (e.g. family level and above) for Digenea, rendering them suitable markers for molecular systematics (Olson et al., 2003; Chan et al., 2021).

Molecular analysis for this study was constrained by the limited number of sequences and genetic markers employed for Encyclometra. Nevertheless, the sequences generated from the three genetic markers in this study can hopefully provide a more comprehensive insight for future studies on Encyclometra.
Conclusion

This study focused on identifying *Encyclometra* species parasitising *Enhydris enhydris* in Thailand and Cambodia using both morphological and molecular methods. Morphological analysis confirmed the specimens as *Encyclometra bungara* based on diagnostic traits like diagonally positioned testes and equal-length intestinal caeca. Molecular analysis, employing nuclear and mitochondrial genetic markers, supported differentiation from other *Encyclometra* species, with significant genetic distances. The research also revealed genetic similarity between *E. bungara* specimens from Thailand and Cambodia, affirming their conspecific status. This work underscores the importance of combining molecular data with morphology for accurate species identification within *Encyclometra* and highlights the need for selecting appropriate genetic markers, and extending the knowledge in wildlife parasites.

**Supplementary material.** The supplementary material for this article can be found at [DOI].

**Data.** The data that support the findings of this study are available from the first and corresponding authors upon reasonable request.

**Author’s contributions.** AHEC served as the principal investigator and played a pivotal role in data investigation and curation, formal analyses, and contributed significantly to methodology, conceptualization, visualization, and drafting the original article. UT, NR, and VC were actively involved in research conceptualization, methodology, specimen preparation and collection, visualization, validation, and both writing and editing of the article. AP, SB, and PN made significant contributions to specimen preparation, collection, and data investigation. PL, TT, and NPB were instrumental in host specimen collection and processing, provided valuable resources, and offered recommendations throughout the project.
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Competing interests. None

Ethical standards. All procedures performed by researchers, snake handlers, and veterinarians in handling snakes were approved by the Safety Committee of Queen Saovabha Memorial Institute (Document No. SN001). The authors confirm that the field studies did not involve endangered or protected species. The study was also approved by the Ethics Committee of Queen Saovabha Memorial Institute (Approval Protocol Number: QSMI-ACUC-11-2021).
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Rambaut, A (2009) *FigTree (Version 1.3.1).* Available at http://tree.bio.ed.ac.uk (accessed August 31, 2023)

Rudolphi CA (1819) *Entozoorum synopsis cui accedunt mantissa duplex et indices locupletissimi*. Berolini: Sumtibus A. Rücker, Berlin


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Figure 1. Morphological drawing of adult *Encyclometra bungara* obtained from *Enhydris enhydris* in a) Thailand and b) Cambodia (right).
Figure 2. Maximum likelihood phylogeny using the a) nuclear 18S rRNA gene (K2 + G + I), b) nuclear 28S rRNA gene (GTR + G), c) concatenated nuclear 18S with 28S rRNA genes (GTR + G + I), and d) mitochondrial COI gene (HKY + G). Numbers at nodes indicate bootstrap values. Representative sequences generated from this study are indicated with an ‘∗’. The families in the superfamily Gorgoderoida are colour-coded.
**Table 1.** Comparison of *Encyclometra* morphological measurements

<table>
<thead>
<tr>
<th>Species</th>
<th><em>E. bungara</em></th>
<th><em>E. colubrimurorum</em></th>
<th><em>E. japonica</em></th>
<th><em>E. asymmetrica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reference</strong></td>
<td>This study</td>
<td>Scholz, 1991</td>
<td>Wongsawad, 1998</td>
<td>Yeh, 1958</td>
</tr>
<tr>
<td><strong>Host</strong></td>
<td><em>Enhydris</em></td>
<td><em>Enhydris</em></td>
<td><em>Xenochrophis</em></td>
<td>Natrix sp.</td>
</tr>
<tr>
<td></td>
<td>plumbea</td>
<td>piscator</td>
<td></td>
<td><em>Natrix piscator</em></td>
</tr>
<tr>
<td><strong>Body length</strong></td>
<td>771–2,996 (1,344)</td>
<td>4,320–8,840</td>
<td>2,900–4,900</td>
<td>2,000–6,000</td>
</tr>
<tr>
<td><strong>Body width</strong></td>
<td>267–926 (510)</td>
<td>1,750–1,630</td>
<td>900–1,500</td>
<td>493–1,918</td>
</tr>
<tr>
<td><strong>Oral sucker</strong></td>
<td>120–263 (192) x 109–257 (185)</td>
<td>600 x 520</td>
<td>450–550</td>
<td>360–630</td>
</tr>
<tr>
<td><strong>Ventral sucker</strong></td>
<td>124–316 (198) x 122–297 (199)</td>
<td>560 x 540</td>
<td>590–650</td>
<td>400–760</td>
</tr>
<tr>
<td><strong>Ratio VS: OS</strong></td>
<td>1:1–1.1: 1</td>
<td>1:1:1</td>
<td>1:1:1</td>
<td>1:1:1–1.2</td>
</tr>
<tr>
<td><strong>Testes</strong></td>
<td>56–192 (97) x 41–170 (89)</td>
<td>270–370 x 180–190</td>
<td>240–310</td>
<td>180–500</td>
</tr>
<tr>
<td><strong>Testes position</strong></td>
<td>Diagonal</td>
<td>Diagonal</td>
<td>Diagonal</td>
<td>Tandem or diagonal</td>
</tr>
<tr>
<td><strong>Cirrus sac</strong></td>
<td>NA</td>
<td>670–610–800</td>
<td>550–640</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Ovary</strong></td>
<td>NA</td>
<td>140 x 180</td>
<td>200–240</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Caeca</strong></td>
<td>Equal</td>
<td>Equal</td>
<td>Equal</td>
<td>Subequal</td>
</tr>
<tr>
<td><strong>Egg (length)</strong></td>
<td>60–78 (72) x 32–44 (39)</td>
<td>76–84 x 39–43</td>
<td>75–84 x 38–46</td>
<td>74–91 x 42–54</td>
</tr>
</tbody>
</table>

Note: measurements are represented as the minimum – maximum (mean) values in micrometers (µm). VS = ventral sucker, OS = Oral sucker.

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Table 2. Inter- and intra-species genetic distances of *Encyclometra*

<table>
<thead>
<tr>
<th>Species</th>
<th>Genetic marker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18S</td>
</tr>
<tr>
<td><em>E. colubrimurorum</em> vs <em>E. japonica</em></td>
<td>0.0</td>
</tr>
<tr>
<td><em>E. colubrimurorum</em> vs <em>E. bungara</em></td>
<td>0.2–0.4</td>
</tr>
<tr>
<td><em>E. japonica</em> vs <em>E. bungara</em></td>
<td>0.2–0.4</td>
</tr>
<tr>
<td><em>E. bungara</em> (Thailand) vs <em>E. bungara</em> (Cambodia)</td>
<td>0.0–0.13</td>
</tr>
</tbody>
</table>

The values are expressed as the percentage of nucleotide differences.