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Morphological examination and DNA barcoding of the *COI* gene of *Eysarcoris ventralis* and *Scotinophara coarctata* found in Northern Thailand

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Abstract

Insect pests are significant challenge to rice production, with over 20 species recognized as major insect pests, including some stink bugs (Hemiptera: Pentatomidae). The rice black bug, *Scotinophara coarctata* (Fabricius), is an invasive insect species firstly reported in southern Thailand, which later spread northward throughout rice-growing regions. Recently, our research team firstly discovered another insect species that has not been reported in Thailand, the white-spotted stink bug, *Eysarcoris ventralis* (Westwood), that infests rice fields and causes similar damage to that of the rice black bug. However, distinguishing between these two insect pests in the field is challenging due to their similar characters. We, therefore, pursue the differentiation between these two insects using both morphological characters and barcoding of *COI* genes, including investigation of the population status. The results showed that these insects display some differences in morphological characters. DNA barcoding of the partial *COI* gene separated them into different clades. The sample of *S. coarctata* is arranged in the same clade with *S. lurida*. All of the *E. ventralis* samples studied were placed in the same clade with the sample from China, indicating that they were related, but separated from other species of the genus *Eysarcoris*. The outcomes confirmed that these rice stink bugs are separate species and can be distinguished using both morphological examination and DNA barcoding of the *COI* gene. The analysis suggested a relatively high degree of genetic similarity among geographically diverse populations of *Eysarcoris*, with some interspecific differences still detectable within the genus.

Key Words

White-spotted stink bug, *Eysarcoris ventralis*, rice black bug, S*cotinophara coarctata*, rice insect pest, *COI* gene

Introduction

Rice (*Oryza sativa* L.) is a major crop around the World, whose consumption dates back to 4000BC, and it was originally cultivated in India and China (The rice association 2025). So far, it is mostly grown in tropical Asia, Africa, and other areas with warm and humid climate; India is currently the largest rice producing country (Glauber and Mamun 2024). Growing rice needs good agricultural practices, in order to gain higher yields and good quality products. Rice production has to face many threats, among them insect pests are major cause of yield loss (Heinrichs 1994). Over 800 insect species have been reported as pests in rice fields; however, only 20 species are recognized as major pests (Dale 1994). Many species of sucking bugs of the order Hemiptera are considered insect pests in rice fields, for example, brown planthopper, *Nilaparvata lugens*

(Stal), which have been recorded as a major pest of rice in rice-growing countries, including Thailand (Rice Research and Development Division, Rice Department 2023). In addition, an invasive insect species, *Scotinophara coarctata* (Fabricius) (Hemiptera: Pentatomidae) or rice black bug, was firstly reported from the southern provinces of Thailand, as it spread from a neighboring country like Malaysia (Ruayaree 2001; Joshi et al. 2007; Lawanprasert 2017; Rice Research and Development Office, Rice Department 2023). They later moved northward and were reported in several areas of Thailand (Rice Research and Development Office, Rice Department 2007). Recently, our team documented the new record of another member of this family in Thailand, the white-spotted stink bug, *Eysarcoris ventralis* (Westwood), which infests rice fields, especially during the panicle development stage up to the grain stages and causes damage to rice plants and grains (Wattanachaiyingcharoen et al. 2025). Both of these sucking insects feed on different parts of the rice plants, but *E. ventralis* seem to be more serious threats than *S. coarctata*. Their infestations were found to overlap at different rice stages, from tillering until grain stages. However, as stated in our previous study, the morphological similarity of the two species may lead to misidentification, especially in field surveys. Accurate species identification is a fundamental for developing targeted pest management strategies and for conducting rigorous studies on systematics and biogeography (Kürzel et al. 2022). Traditionally, taxonomic classification has relied on morphological characters. However, those traits can be subjective and difficult to interpret. In many taxa, the morphological identification may not accurately reflect evolutionary relationships, particularly in cases of cryptic species or pronounced phenotypic plasticity (Wiens 2004; Anjum et al. 2025). While traditional morphological identification often faces limitations due to subtle intraspecific variations, molecular markers offer an alternative approach by providing more precise genetic information to resolve ambiguities (Park et al. 2011). However, it is important to note that molecular marker–based taxonomy also has limitations. For example, the lack of a clear barcoding gap in a single marker can make species delimitation difficult (Memon et al. 2006). The mitochondrial cytochrome c oxidase subunit I (*COI*) gene has become a cornerstone of molecular taxonomy and DNA barcoding (Hebert et al. 2003). Its rapid rate of evolution at a species level, coupled with its highly conserved nature at higher taxonomic levels, makes it an ideal marker for species discrimination, phylogenetic reconstruction, and the clarification of genetic boundaries between genera (Zhang and Hewitt 2003; Foottit et al. 2008). Several studies have demonstrated that the *COI* gene is effective for identifying uncertain or morphologically ambiguous insect species (Hebert et al. 2003; Smith et al. 2006). *COI* barcoding provides species-level resolution across diverse insect groups and is widely used to confirm species identity when morphological characters are insufficient, for example as reported by Hajibabaei et al. (2006), Kaila and Ståhls (2006), and Kundu et al. (2012). Moreover, the genetic variation of stink bugs in the genus *Halys*, from Pakistan, was studied using the genetic analysis of the portion of *COI* gene and a larger fragment encompassing the *COI*, *tRNALeu*, and *COII* genes. The results showed that molecular evidence collaborated the morphological observations, thereby revealed the identification of a new species of the stink bug (Memon et al. 2006). Furthermore, DNA barcoding of the *COI* gene served as a key tool for identifying species of Heteroptera (Park et al. 2011). In Egypt, leafhoppers (Hemiptera: Cicadellidae) were identified, and their phylogenetic relationships were assessed using DNA barcoding of the mitochondrial *COI* gene from eight genera (Emam et al. 2020).

Recently, both stink bug species, *S. coarctata* and *E. ventralis*, have been found throughout several rice-growing areas. These two insect genera slightly differ in morphological characters, although they can feed on different parts of rice plants with varying degrees of damage (Wattanachaiyingcharoen et al. 2025). Field observations, particularly superficial assessment by non-specialized researchers, may lead to misidentification and further imprecise management strategies. Unambiguous identification is necessary to position the right target in the pest management strategy. This study aims to clarify the identification of the stink bugs, *E. ventralis* and *S. coarctata*, through both morphological and genetic analysis using a molecular marker. The DNA barcoding of partial *COI* gene is employed to investigate genetic divergence between these two insects through multiple sequence alignment and phylogenetic reconstruction. Concurrently, by assessing intraspecific variation in populations of *E. ventralis*, we aim to provide insights into its genetic homogeneity or differentiation. The results of this study are expected to provide a robust molecular foundation for taxonomic separations, demonstrate the utility of the *COI* gene in Pentatomidae systematics, and thereby will facilitate subsequent research, particularly in the field of population genetics of *E. ventralis* and *S. coarctata*. Ultimately, this contributes to a comprehensive understanding of evolutionary relationships within this group, vital for advancing both taxonomic research and applied entomological practices, leading to precise pest management.

Material and method

Insect surveys and collections

We conducted surveys throughout rice growing areas in the North of Thailand and the insect samples were collected from five provinces (Table 1). The collected samples were preserved in 70 and 95% ethanol in plastic tubes.

Table 1. Geographical sampling locations, coordinates, and NCBI submission details for insect specimens.

Sample code	Insect pest identification	Sampling site	Sample locations		NCBI accession No.
			District	Province	
PL_RRC01	*S. coarctata*	16.829566,100.377575	Wang Thong	Phitsanulok	PX672271
PL_WTH01	*E. ventralis*	16.835258,100.444538	Wang Thong	Phitsanulok	PX686375
UT_NCH04	*E. ventralis*	15.318014, 99.646098	Nong Chang	Uthaithani	PX686373
NS_BPS06	*E. ventralis*	16.129101,100.073742	Ban Phot Phisai	Nakhonsawan	PX686374
PH_DCH01	*E. ventralis*	17.976009,100.074252	Den Chai	Phrae	PX686376
CM_SKP01	*E. ventralis*	18.755669,99.076399	San Kam Phaeng	Chiangmai	PX686377

Morphological study

Preserved insect samples were examined under a stereomicroscope (Olympus SZ40 Stereo Microscope, Olympus Co., Tokyo, Japan), and pictures of each character were taken. Characters examined were body size, color, pronotum, scutellum, wings, legs, and abdomen. Each character was thoroughly studied according to the references, i.e., Wood and McDonald 1984; Barrion and Litsinger 1994; Barrion et al. 2007; Biswas et al. 2014; Salini and Viraktamath 2015; Salini 2019; Pal et al. 2023.

DNA extraction

Preserved insect samples from different rice-growing areas were removed from their tubes and gently dried. Each tube contained three or four insects collected from the same location. For DNA extraction, five individuals of *E. ventralis* and one individual of *S. coarctata* were selected for sequencing. The samples were then thoroughly ground in 100 µl of Buffer CL using a disposable pestle. Total DNA was extracted using the G-spin™ extraction kit (iNtRON Biotechnology, Gyeonggi-do, South Korea), modified from Yong et al. (2015) for insect samples. The homogenized tissue was transferred to a 1.5 ml microcentrifuge tube containing 100 µl of Buffer CL, 20 µl of Proteinase K, and 5 µl of RNase A solution. The mixture was vortexed gently and incubated at 56 °C in a heat block for two hours. Following incubation, 200 µl of Buffer BL was added to the lysate, and the mixture was incubated at 70 °C for five minutes. The solution was then centrifuged at 12,000 rpm for ten minutes to separate the supernatant. The supernatant was transferred to a new 1.5 ml tube, and 200 µl of absolute ethanol was added before transferring the mixture to a spin column. The spin column was centrifuged at 12,000 rpm for two minutes. Next, 700 µl of Buffer WA was added, followed by another centrifugation. The flow-through was discarded, and the spin column was centrifuged again to remove residual wash buffer. Subsequently, 700 µl of Buffer WB was added to the column, followed by centrifugation at 12,000 rpm for two minutes. For DNA elution, 30 µl of nuclease-free water was added to the center of the membrane. The column was then incubated at room temperature for one minute and centrifuged at 12,000 rpm for one minute (Yong et al. 2015).

DNA amplification

Manual primer design was conducted using the Primer3 software, informed by the multiple sequence alignment performed via MultAlin program (Corpet 1988, http://multalin.toulouse.inra.fr/multalin/). The design process involved comparing the complete mitochondrial *COI* gene sequence of *E. ventralis* (NCBI. No. ON041148.1) with the corresponding region of *S. coarctata*. The resulting primer pair was specifically targeted to amplify a region between the nucleotide positions 1240 and 1940. The forward primer (1240F: 5'-TAAACTATTAACCTTCA AAGTTAA-3') and the reverse primer (1940R: 5'-ATTATACCTTCTGGTCG-TATA-3') were then employed to amplify the target region. Primers designed and used in this study based on the *COI* sequence were deposited under accession no. ON041148.1. Polymerase chain reaction was conducted to amplify the extracted DNA using the 2X PCR Master Mix i-StarMAX™ II (iNtRON Biotechnology, Gyeonggi-do, South Korea), which includes a high-fidelity DNA polymerase with proofreading activity. Each reaction was conducted in a final volume of 20 µl. This included 10 µl of the 2X Master Mix, 1 µl of forward primer and 1 µl of reverse primer (10 pmol/µl), and 1 µl of DNA template. The volume was adjusted with nuclease-free distilled water to 20 µl. The PCR reactions were performed in a thermal cycler with an initial denaturation step at 94 °C for four minutes. The 30 amplification cycles consist of denaturation at 94 °C for 30 seconds, annealing at temperatures at 58 °C for 30 seconds, and extension at 72 °C for 30 seconds. A final extension step was performed at 72 °C for ten minutes. Following amplification, PCR products were directly analyzed on an electrophoresis with 2% agarose gel using 1× TAE buffer with Trackit™ 1 kb and DNA Ladder (Invitrogen, Massachusetts, U.S.A). The electrophoresis was run at 100 V for 40 minutes, and the visualization of DNA fragments was performed under UV light. The PCR amplicons underwent Sanger sequencing at Macrogen (Seoul, South Korea). Sequencing was performed in both forward and reverse directions to ensure full coverage of the amplicon sequence.

DNA sequencing analysis and phylogenetic reconstruction

Raw DNA sequences were initially inspected for quality and then visually edited using GeneStudio software

(GeneStudio, Inc., Georgia, U.S.A). Forward and reverse sequences for each sample were assembled into contigs to generate a consensus sequence, further ensuring accuracy and minimizing sequencing errors. The DNA sequences obtained from the collected insect specimens have been deposited in the NCBI database (Table 1). Multiple sequence alignment of the newly generated *COI* gene sequences was performed using the MultAlin program (Corpet 1988, http://multalin.toulouse.inra.fr/multalin/) with default parameters. Subsequently, the aligned sequences were subjected to rigorous quality control, which included visual inspection and editing of the alignment. To confirm the identity of the sequences and assess their relatedness to known organisms, all sequences were aligned against the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLASTn) application (http://www.ncbi.nlm.nih.gov/BLAST/). Partial *COI* sequences of *Eysarcoris* and related *Scotinophara* species were retrieved from the NCBI database, with *Scotinophara lurida* was treated as the outgroup. The *COI* gene sequence from our study (NCBI No. ON041148.1) was included as a reference for comparison (Table 2). The target region and the positions shown in Table 2 refer to the nucleotide positions of the *COI* gene used for alignment and phylogenetic analysis. For phylogenetic reconstruction, the aligned *COI* gene sequences from the samples and selected sequences of *Eysarcoris* and *Scotinophara* listed in Table 2 were analyzed using MEGA X (Kumar et al. 2018). The most appropriate evolutionary model for nucleotide substitution was determined using jModelTest2 (Darriba et al. 2012) based on the Bayesian Information Criterion (BIC). Phylogenetic trees were constructed using Neighbor-Joining (NJ) methods, assessed by bootstrap analysis with 1000 pseudoreplicates for the NJ methods.

Table 2. Accession numbers of selected *Eysarcoris* and *Scotinophara*.

Sample	NCBI number	Nucleotide Position*
Eysarcoris ventralis (*E. ventralis* I collected from China)	MT165688.1	1269-1932
Eysarcoris ventralis (*E. ventralis* II)	ON041148.1	1262-1925
Eysarcoris rosaceus	MT165687.1	1279-1942
Eysarcoris sp.	PP198280.1	1266-1922
Eysarcoris aeneus	MK841489.1	1263-1916
Eysarcoris montivagus (isolate collected from China)	MW846867.1	1310-1972
Eysarcoris guttigerus	NC_047222.1	1263-1915
Scotinophara lurida	NC_042815.1	1271-1927

*The numbers represent the nucleotide positions of each sequence that were aligned and compared with the reference sequence ON041148.1.

Results

Morphological study

Morphological examination revealed distinct characteristics between *E. ventralis* and *S. coarctata* insect samples (Figs 1, 2) The morphological characteristics of both species are different and described as follows:

Eysarcoris ventralis: body length ranged 5.5–6.5 mm, width ranged 3.7–3.8 mm.

Body color: all parts brownish to dark brown with a slight yellowish tint.

Pronotum: hexagonal, anterior margins not pointed, yellowish-brown with two transverse black bands and scattered tiny black spots, hairless.

Scutellum: round apex with two yellowish-white round spots at each basal angle, scattered small black spots.

Wings: forewing hemelytra with scattered brown pits, cuneus membranous apex transparent with brown spots, hind wings fully membranous.

Legs: femur slender, yellowish-brown with scattered brown spots, tibia pale yellowish-brown with slightly brown spots, tarsus dark brown with claw.

Abdomen: black sternum with pale-yellow lateral margin, one red band on the middle of the first and second abdominal segments.

Scotinophara coarctata: male body length 6.8- 7.7 mm., female 8.5–9.1 mm.

Body color: dark brown with black head, thorax, and abdomen.

Pronotum: hexagonal, lateral margins smooth, anterior and posterior margins pointed, scattered pits, anterior part without humps, invisible transverse furrow.

Scutellum: tip rounded, slightly shorter than abdominal tip, with shallow concave along the middle part, scattered yellow mottles.

Wings: forewing hemelytra with scutellar pit at basal angle, dense scattered pits, cuneus membranous apex transparent, hind wings fully membranous.

Legs: slender, yellowish-brown tibia and claws, black femur.

Abdomen: black sternum, smooth and slightly rough laterally, anterior margin of terminal sternite widely dome-shaped, ventral one-half lined with concave ridges.

Phylogenetic analysis

Comparative alignment presented in Table 3 identified multiple nucleotide positions conserved between the outgroups (*S. coarctata* and *S. lurida*) and the ingroup taxa of *E. ventralis* and related samples. Comparative sequence analysis against the reference sequence (*E. ventralis* NCBI No. ON 041148.1) revealed distinct levels of genetic divergence among the collected samples. The *E. ventralis* samples obtained from the surveyed rice growing areas exhibited a low percentage of divergence, averaging 0.45. In contrast, the *S. coarctata* sample displayed higher divergence percentage, reaching 14% when compared to the *E. ventralis* reference.

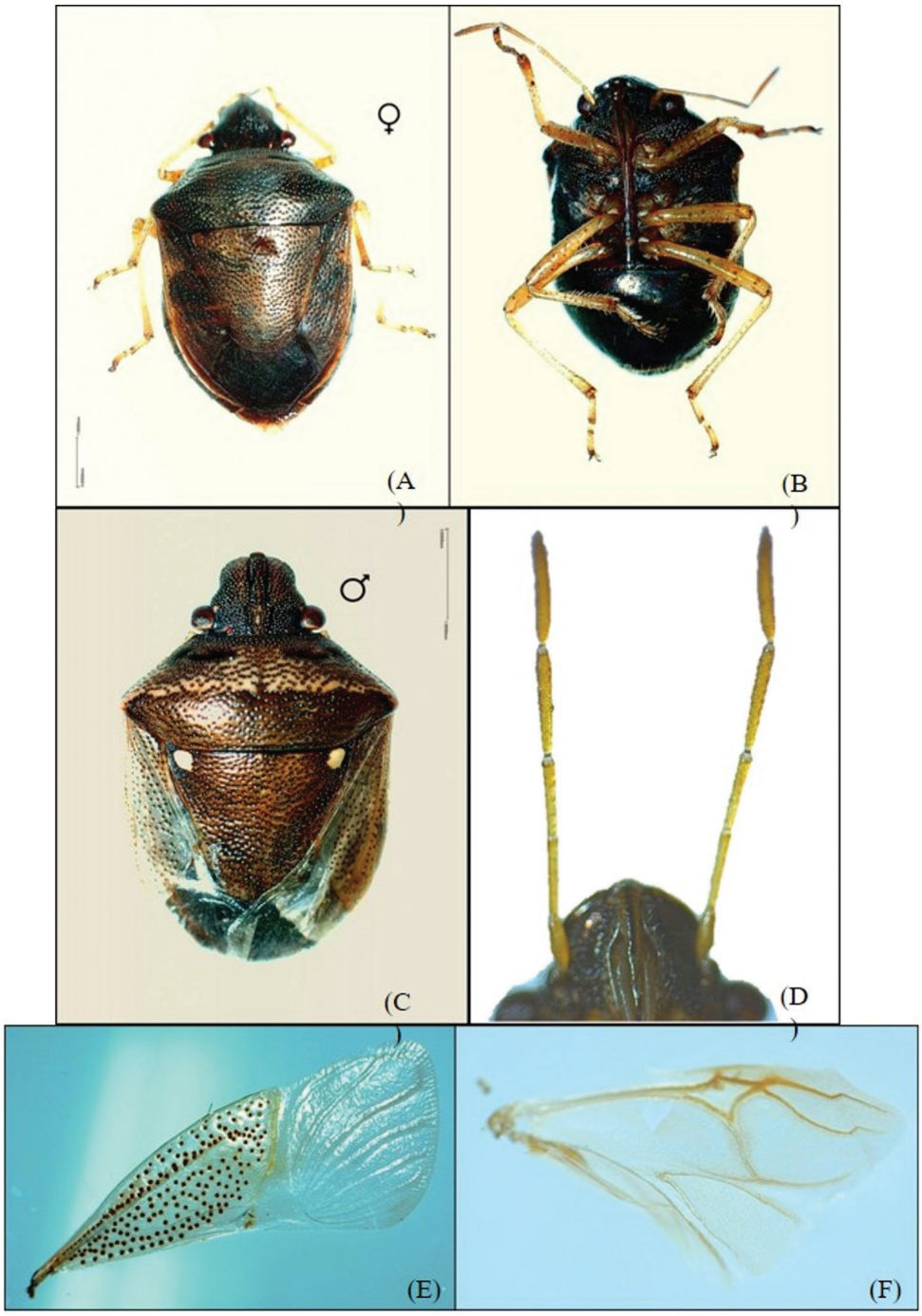

Figure 1. *Eysarcoris ventralis*. **A.** Female habitus in dorsal view; **B.** Female habitus in ventral view; **C.** Male habitus in dorsal view; **D.** Antennae; **E.** Forewing; **F.** Hindwing.

The pairwise mismatch heatmap shows the number of nucleotide differences between sequence pairs (Fig. 3). Ingroup samples of *E. ventralis* have very low mismatch values among replicates and only minimal variation within the species. This pattern differs from the greater variation found in other species, where pairwise comparisons revealed higher mismatch values across samples (Fig. 3). The phylogenetic reconstruction (Fig. 4) elucidates the evolutionary relationships among the sampled taxa. A basal divergence with full bootstrap support (100%) clearly separates the *Scotinophara* clade (*S. lurida* and *S. coarctata* (PL_RRC01)), placing it as a distant relative of the genus *Eysarcoris*. Within *Eysarcoris*, two major evolutionary clades are resolved. The first clade includes *Eysarcoris* sp., *E. aeneus*, and *E. guttigerus*. These three taxa form a well-supported monophyletic group (88%), with *E. aeneus* and *E. guttigerus* exhibiting a strong sister-group relationship (100%), while *Eysarcoris* sp. represents a distinct diverging lineage. The second clade comprises of *E. montivagus*, *E. rosaceus*, and an additional sampled examined. *E. montivagus* and *E. rosaceus* appear as early branching lineages within this group,

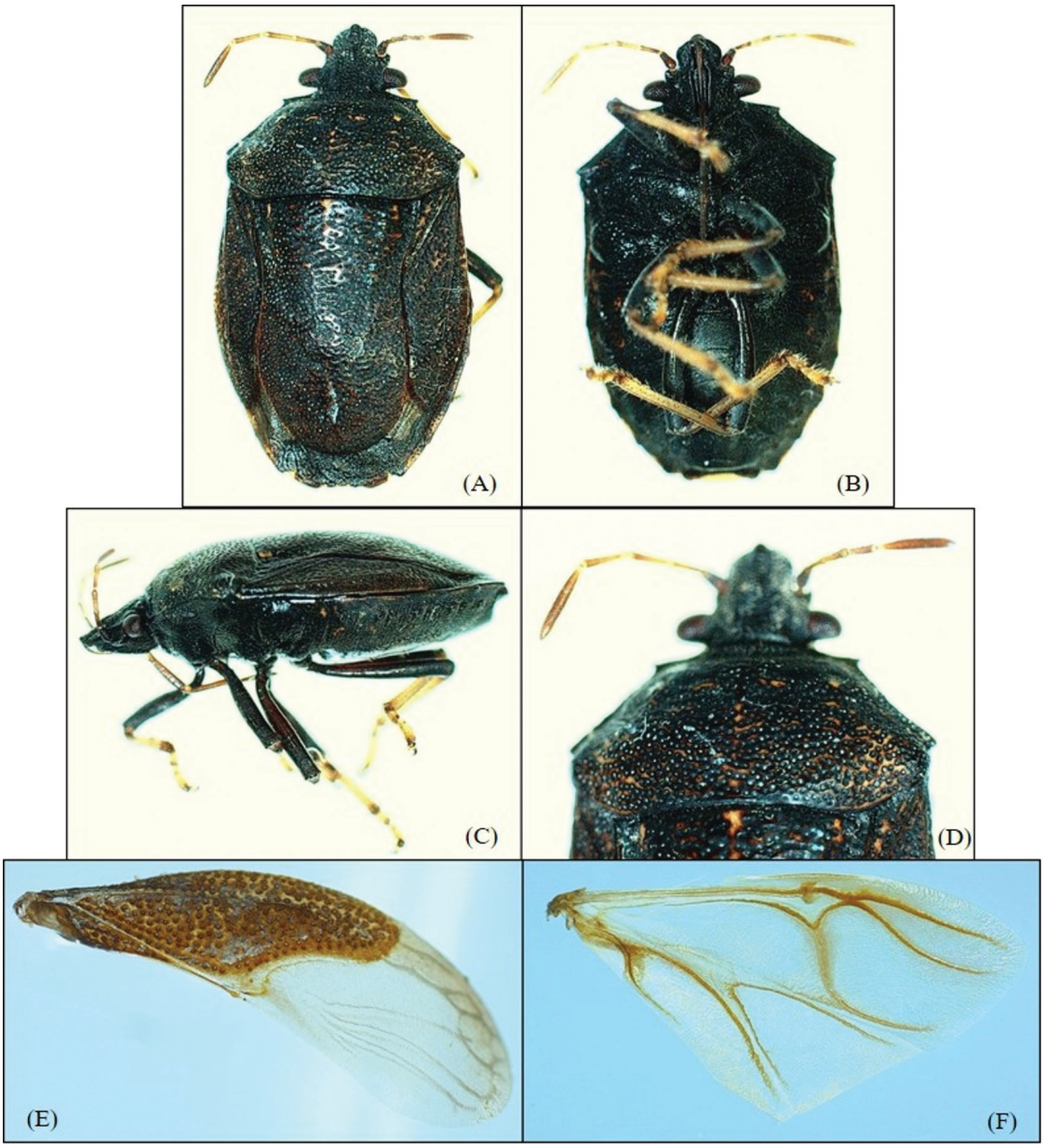

Figure 2. *Scotinophara coarctata*. **A.** Male habitus in dorsal view; **B.** Male habitus in ventral view; **C.** Male habitus in lateral view; **D.** Antennae and pronotum; **E.** Forewing; **F.** Hindwing.

although their nodal support is relatively modest (53%). A moderately supported subclade (58%) further unites *E. montivagus* and *E. rosaceus* with a more nested assemblage that includes *E. ventralis*.

The second *Eysarcoris ventralis* clade includes *E. ventralis* I (samples collected from China), *E. ventralis* II (collection source unknown), and five collected samples from our study (UT_NCH04, NS_BPS06, PL_WTH01, PH_DCH01, CM_SKP01) forming a highly supported lineage (100%). Within this clade, *E. ventralis* I and *E. ventralis* II form a closely related pair, which in turn clusters directly with all sample sequences (UT_NCH04, NS_BPS06, PL_WTH01, PH_DCH01, CM_SKP01) at 97%. Notably, the short branch lengths throughout this clade, particularly among the environmental accessions, suggest high genetic homogeneity and recent divergence. Overall, posterior probability values provide strong support for most primary relationships, reinforcing the inferred topology, while lower values at certain nodes highlight areas of phylogenetic ambiguity.

A multiple sequence alignment of the *COI* gene, spanning nucleotide positions 1262 to 1930 (compared to the reference ON041148.1, Suppl. material 1: fig. S1), was performed to investigate genetic variation within the genus *Eysarcoris*, and comparison with the genus *Scotinophara* (Suppl. material 1: fig. S1). The dataset included sequences from various samples from different sampling locations of *E. ventralis* (UT_NCH04, NS_BPS06, PL_WTH01, PH_DCH01, CM_SKP01), along with other *Eysarcoris* species, and two species of the genus *Scotinophara* (*S. lurida* and *S. coarctata*). The *COI* sequences of *S. lurida* and *S. coarctata* exhibited multiple indel

Table 3. Comparative nucleotide alignment showing conserved positions between outgroups (S. *coarctata* and *S. lurida*) and other samples.

Sequence	Nucleotide position									
	Pos_15	**Pos_46**	**Pos_47**	**Pos_49**	**Pos_51**	**Pos_72**	**Pos_106**	**Pos_139**	**Pos_160**	**Pos_188**	**Pos_230**
UT_NCH04	–	A	T	A	T	T	A	C	T	C	T
NS_BPS06	–	A	T	A	T	T	A	C	T	C	T
PL_WTH01	–	A	T	A	T	T	A	C	T	C	T
PH_DCH01	–	A	T	A	T	T	A	C	T	C	T
CM_SKP01	–	A	T	A	T	T	A	C	T	C	T
E. ventralis I	A	A	T	A	T	T	A	C	T	C	T
E. ventralis II	A	A	T	A	–	T	A	C	T	C	T
E. rosaceus	A	T	T	A	–	T	–	C	–	C	T
E. montivagus	A	C	C	A	–	–	A	T	–	C	C
Eysarcoris sp.	G	T	A	T	–	T	A	C	–	C	A
E. aeneus	G	A	T	T	–	T	A	C	–	C	A
E. guttigerus	G	A	T	–	–	T	A	C	–	T	A
S. lurida	T	T	A	T	A	A	G	A	–	T	A
S. coarctata (PL_RRC01)	T	T	A	T	A	A	G	A	G	A	G

Sequence	Nucleotide position										
	Pos_239	**Pos_245**	**Pos_248**	**Pos_269**	**Pos_278**	**Pos_281**	**Pos_284**	**Pos_317**	**Pos_329**	**Pos_342**	**Pos_356**
UT_NCH04	A	A	C	C	T	A	A	T	A	A	C
NS_BPS06	A	A	C	C	T	A	A	T	A	A	C
PL_WTH01	A	A	C	C	T	A	A	T	A	A	C
PH_DCH01	A	A	C	C	T	A	A	T	A	A	C
CM_SKP01	A	A	C	C	T	A	A	T	A	A	C
E. ventralis I	A	A	C	T	T	A	A	T	A	A	C
E. ventralis II	A	A	C	C	T	A	A	T	A	A	C
E. rosaceus	A	T	A	T	A	A	G	T	A	A	T
E. montivagus	A	A	T	T	G	A	A	T	A	A	C
Eysarcoris sp.	A	A	T	T	A	A	G	T	A	A	T
E. aeneus	A	T	T	T	A	A	T	T	A	A	A
E. guttigerus	A	C	T	T	A	A	T	T	A	A	A
S. lurida	T	T	A	T	A	G	G	C	T	G	T
S. coarctata (PL_RRC01)	G	G	A	G	C	C	G	G	G	T	A

Sequence	Nucleotide position										
	Pos_379	**Pos_380**	**Pos_386**	**Pos_398**	**Pos_399**	**Pos_421**	**Pos_473**	**Pos_492**	**Pos_510**	**Pos_512**	**Pos_518**
UT_NCH04	G	T	C	A	G	C	A	G	T	T	T
NS_BPS06	G	T	C	A	G	C	A	G	T	T	T
PL_WTH01	G	T	C	A	G	C	A	G	T	T	T
PH_DCH01	G	A	C	A	G	C	A	G	T	T	T
CM_SKP01	G	T	C	A	G	C	A	G	T	T	T
E. ventralis I	G	T	C	A	G	C	A	G	T	T	T
E. ventralis II	G	T	C	A	G	C	A	G	T	T	T
E. rosaceus	G	A	T	A	G	C	A	G	T	A	A
E. montivagus	G	A	T	A	G	C	A	G	T	A	A
Eysarcoris sp.	G	T	C	A	A	C	A	A	T	A	A
E. aeneus	T	G	G	G	T	T	T	T	C	G	G
E. guttigerus	T	G	G	G	T	T	T	T	C	G	G
S. lurida	G	A	T	T	G	C	T	A	G	A	C
S. coarctata (PL_RRC01)	A	G	G	G	T	A	C	T	C	G	G

Sequence	Nucleotide position										
	Pos_524	**Pos_542**	**Pos_545**	**Pos_551**	**Pos_558**	**Pos_575**	**Pos_578**	**Pos_581**	**Pos_584**	**Pos_599**	**Pos_632**
UT_NCH04	A	G	C	T	A	A	A	A	T	A	A
NS_BPS06	A	G	C	T	A	A	A	A	T	A	A
PL_WTH01	A	G	C	T	A	A	A	A	T	A	A
PH_DCH01	A	G	C	T	A	A	A	A	T	A	A
CM_SKP01	A	G	C	T	A	A	A	A	T	A	A
E. ventralis I	A	G	C	T	A	A	A	A	T	A	A
E. ventralis II	A	C	A	T	A	A	A	A	T	A	A
E. rosaceus	A	A	T	T	A	T	A	A	T	C	A
E. montivagus	A	G	T	T	A	T	A	T	T	C	G
Eysarcoris sp.	T	C	A	C	A	A	A	A	T	T	G
E. aeneus	G	C	T	A	T	T	G	G	T	T	C
E. guttigerus	G	C	C	A	T	T	G	G	T	T	C
S. lurida	C	T	T	A	C	C	T	T	C	T	T
S. coarctata (PL_RRC01)	G	C	T	A	T	T	G	G	A	C	G

patterns that were not detected in *Eysarcoris* (Suppl. material 1: fig. S1). These differences were distributed across several positions within the alignment, distinct indel was observed near nucleotide position 1270 between *Scotinophara* and *E. ventralis*. Within the genus *Eysarcoris*, while overall high conservation was observed, specific regions showed variations among different *Eysarcoris* species; for instance, *E. montivagus* displayed a series of distinct nucleotide changes relative to *E. guttigerus* and *E. ventralis* in several regions, particularly within the segment from position 1262 to approximately 1391 (Suppl. material 1: fig. S1). Conversely, analysis of the partial *COI* sequences of *E. ventralis* collected from different sites revealed a remarkably high degree of sequence identity (Figs 3, 4).

Discussion and conclusion

Our findings clarified morphological and revealed molecular marker distinction between the two species that invaded rice fields in Northern Thailand: *E. ventralis* and *S. coarctata*. Under thorough morphological analysis, we distinguished key characteristics that can differentiate the two bug species. They differ in body size and body color, pronotum shape, forewings, and characters of legs. However, rough observations might not be able to identify these distinct appearances; they need to be characterized by specialists. Hence, DNA analysis using the *COI* gene seems to be reliable, as it can discriminate between these two stink bugs. Since intra- and interspecific genetic diversity among Hemipteran insects overlap, Memon et al. (2006) proposed that only using DNA barcoding for genetic variation among these insects may not be reliable. Sonnekus et al. (2022) employed barcoding of the *COI* gene along with morphological analysis to identify the stink bug species in the macadamia orchards in South Africa. Twenty-one species of stink bugs were recorded, with four cryptic species of the *Pseudatelus raptorius* and an unidentified *Boerias* species. Another example was provided by Mariey et al. (2023) who verified that *E. ventralis* was a key pest attacking Egyptian barley by using both morphological identification and DNA barcoding of the *COI* gene. They conducted a comparative analysis of the *COI* sequences from the collected specimens with the reference sequences of *Nezara viridula*, *Eysarcoris* sp., *E. rosaceus*, and *Stagonomus bipunctatus* to evaluate phylogenetic relatedness. The results indicated that the examined sequence exhibited the highest similarity to *Eysarcoris* sp., while showing greater divergence from *N. viridula* and clear separation from both *E. rosaceus* and *S. bipunctatus*, despite their close genetic records in GenBank.

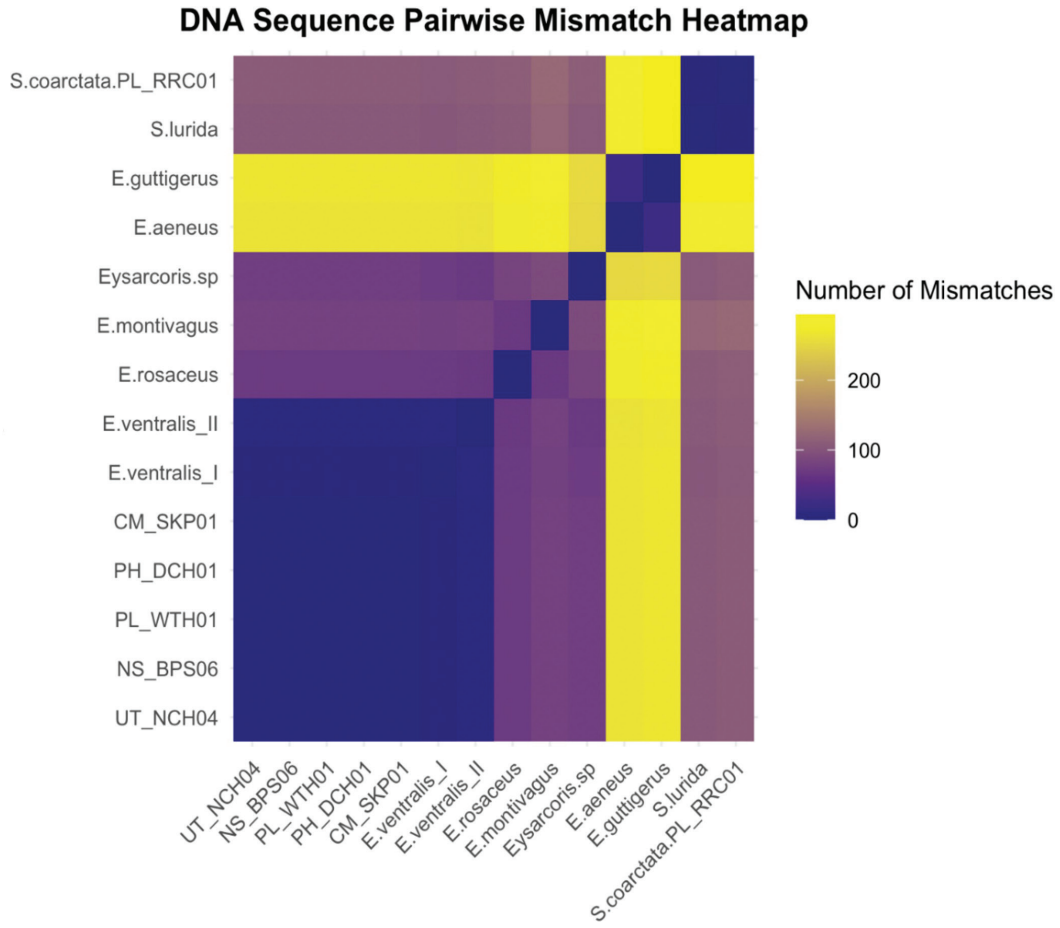

Figure 3. Heatmap of Pairwise Nucleotide Mismatches across DNA Sequences

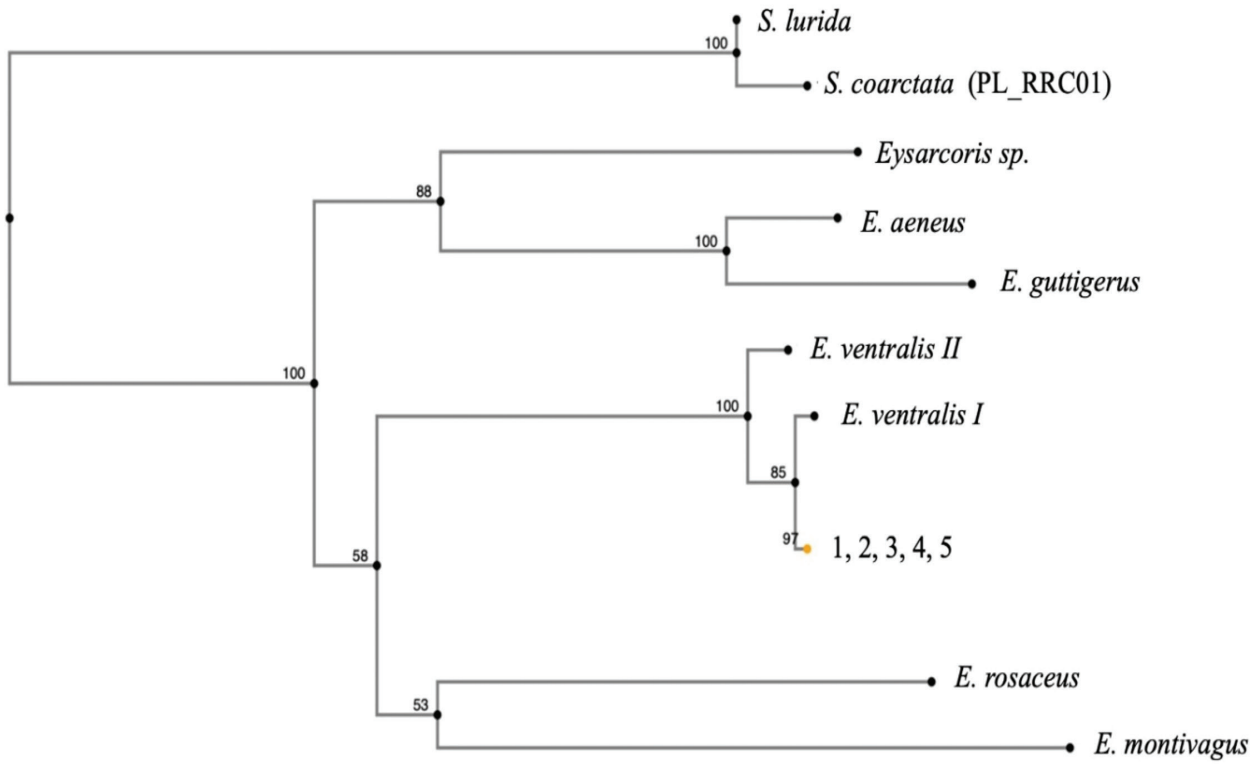

Figure 4. Phylogenetic analysis of *Eysarcoris* and *Scotinophara* specimens based on DNA sequence data. Samples 1–5 represent field-collected specimens: 1 = UT_NCH04, 2 = NS_BPS06, 3 = PL_WTH01, 4 = PH_DCH01, 5 = CM_SKP01.

The phylogenetic analysis presented in this study clarifies the evolutionary relationships within and between the genera *Eysarcoris* and *Scotinophara*, as inferred from the *COI* gene. The *COI* gene represents one of the most extensively utilized molecular markers in arthropod systematics due to its optimal balance of conservation and variability (Pentinsaari et al. 2016). This approximately 600 base pair gene fragment functions as the standard DNA barcode for animal species identification and has proven particularly effective for genus-level discrimination in Hemiptera (Kim and Jung 2018). The results supported a distinct and deep evolutionary divergence between these two genera, as demonstrated by full bootstrap support (100%) that separates the *Scotinophara* clade from the genus *Eysarcoris*. Bootstrap values exceeding 70% are generally considered statistically supported for phylogenetic inference (Soltis and Soltis 2003), making the 100% support exceptionally strong evidence for monophyly. BLAST analysis obviously identified the sequence as *E. ventralis*, which was also supported by the NJ phylogenetic tree topology. In addition, a DNA barcode successfully generated for this study, provided a crucial reference. The newly identified sequences align exactly with the existing ones from *E. ventralis*, thereby confirming the correct species assignation and its classification as *E. ventralis*. These finding demonstrate the barcode is effective not only in identifying *E. ventralis* but also allows to clearly differentiate it from the morphologically similar species *S. coarctata*. This finding is consistent with existing taxonomic classifications and underscores the utility of the *COI* gene as a robust molecular marker for species -level discrimination in these insect taxa. Both *E. ventralis* and *S. coarctata* samples exhibited approximately 660 nucleotide bases, excluding the primer-binding regions. Comparative analysis revealed multiple variable sites between the two species, including both deletion and insertion events. The pronounced genetic distance observed, particularly the unique indel pattern near nucleotide position 1270, consistently absent in *Eysarcoris*, serves as a powerful molecular signature further supporting this clear taxonomic separation. Within the genus *Eysarcoris*, the analysis provides valuable insight into intraspecific and interspecific relationships. The resolution of two major clades aligns with the hypothesis of complex evolutionary patterns within the genus. The high bootstrap support (88%) for the monophyletic group containing *Eysarcoris* sp., *E. aeneus*, and *E. guttigerus* suggests a shared ancestry, and the strong sister-group relationship between *E. aeneus* and *E. guttigerus* points to a more recent common ancestor for these two species. Conversely, the modest nodal support (53%) for the early branching of *E. montivagus* and *E. rosaceus* within the second clade suggests a degree of phylogenetic uncertainty. Such low bootstrap values often indicate either rapid evolutionary radiations where insufficient time has elapsed for the accumulation of synapomorphic characters, or the presence of conflicting phylogenetic signals resulting from incomplete lineage sorting or horizontal gene transfer events (Wiesemüller and Rothe 2006). The incorporation of additional mitochondrial genes (such as CYTB, ND5, or ATP6) or nuclear markers could provide enhanced resolution for these problematic nodes (Yang et al. 2016).

A particularly significant finding is the observed genetic divergence between the *E. ventralis I* sample from China and the highly conserved cluster of samples from various sites in Thailand (UT_NCH04, NS_BPS06, PL_WTH01, PH_DCH01, CM_SKP01). The near-identical *COI* sequences of the Thai samples indicate a remarkably high degree of genetic homogeneity across a wide geographic range, which is often characteristic of a recently established or highly mobile population with extensive gene flow (Zahoor et al. 2017). This indicates low intraspecific variation across the sampled populations, with almost identical *COI* sequences throughout the aligned region and only a few single nucleotide polymorphisms observed. The *COI* sequences analysis provided clear differentiation between the genera *Eysarcoris* and *Scotinophara*. At this level, our samples exhibited a high degree of genetic similarity despite their geographic diversity. This pattern suggests either recent population expansion from a single source or ongoing high levels of migration maintaining genetic flow across geographic barriers. This finding is consistent with previous molecular studies on hemipteran populations that have demonstrated the effectiveness of *COI* in revealing population structure and demographic history. The observed genetic divergence between Chinese and Thai populations of *E. ventralis* ranges within the typical intraspecific variation observed in hemipteran species (0.4–2.7%), which shows that the absence of shared haplotypes indicates restricted gene flow. Maximum pairwise divergences in *COI* sequences within species typically range from 0.4% in aphids to 2.7% in more diverse taxa, with the threshold for species-level differentiation generally set at 2–3% sequence divergence (Wongsa et al. 2017). This observed pattern of regional genetic divergence has important evolutionary and ecological implications. The high mutation rate of mitochondrial DNA makes it particularly sensitive to detecting recent population subdivision and demographic events (Pinho et al. 2007). From an ecological perspective, this differentiation could reflect local adaptation to heterogeneous environments, including variation in climate, host plant availability, and biotic interactions such as parasites and pathogens (Naini 2020; Xing et al. 2025). The distribution of *E. ventralis* across diverse rice-growing regions in Asia suggests exposure to varying selective pressures, including different rice cultivars, agricultural practices, and climatic conditions. These results suggest that the China and Thailand populations of *E. ventralis* may occupy partially distinct ecological niches, a hypothesis that warrants further investigations through comparative morphological studies, virulence assessments, and comprehensive genomic analysis. Understanding population genetic structure is crucial for predicting the spread of insecticide resistance alleles, planning biological control programs, and developing sustainable integrated pest management approaches (Acford-Palmer et al. 2025). The apparent genetic isolation between Chinese and Thai populations suggests that management strategies may need to be tailored to regional populations rather than treating *E. ventralis* as a genetically uniform species across its range.

Animal Ethical Approval

This research has been approved for the Ethics of Use of Animals for Scientific Work from Naresuan University (Approval No. 66-01-002).

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Supplementary material 1

Multiple sequence alignment of *Eysarcoris* and *Scotinophara* species, highlighting the consensus regions

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Data type: png

Explanation note: The alignment illustrates conserved and variable nucleotide positions among field samples (UT_NCH04, NS_BPS06, PL_WTH01, PH_DCH01, CM_SKP01) and reference sequences. Highly conserved bases forming the consensus sequence are shown in red.

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