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Species Richness of Leaf Roller and Stem Borers (Lepidoptera) Associated with Different Paddy Growth and First Documentation of Its DNA Barcode

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ABSTRACT

Leaf folder and stem borer are pest moths (Lepidoptera) of paddy crop and caused serious damage and significant rice yield loss. The richness, abundance, and diversity of the pest moths were calculated in one paddy planting season and sampled from a model conventional paddy field, located on the west coast of Peninsular Malaysia (Sabak Bernam, Selangor). The adult and immature stages of moths associated with paddy plants have been sampled using active sampling namely sweep net and stem cross-cutting. A total of 189 individuals belonging to five species under two families (Crambidae and Noctuidae) were recorded. Overall, the richness (R'), diversity (H'), and evenness (E') index of lepidopteran species were 0.76, 1.51, and 0.90, respectively. The richness and species abundance throughout the paddy stages were discussed. The DNA barcode of five collected species using cytochrome oxidase subunit 1 (*CO1*) viz. *Cnaphalocrocis medinalis* (Guenée) (leaf folder), *Scirpophaga incertulas* (Walker), *Chilo auricilius* Dudgeon, *Sesamia inferens* (Walker), and *Parapoynx stagnalis* (Zeller) (stem borers) were presented. This study's outcomes are very important as the initial

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stage for conservation purposes, especially in managing the strategy in handling the pest species populations in the paddy field.

Keywords: Agricultural ecosystem, *CO1*, genetic, grain insect pests, infestation, Lepidoptera, Malaysia

INTRODUCTION

Lepidoptera is the second-largest insect group comprising moths and butterflies. It plays a pivotal role as a food source for birds and small mammals, apart from being potential dispersal agents (Lee at al., 2002), and nutrient recyclers (Mercks et al., 2013). Lepidopteran is also very diverse in many agricultural ecosystems such as in paddy fields, oil palm plantations, orchards, and secondary forest ecosystems. This group of species mostly acts as pests during their larval stages such as the bagworm species, *Metisa plana* (Halim et al., 2017), fruit bunch moth, *Tirathaba* sp. (Yaakop & Abdul Manaf, 2015), and other lepidopteran pests (Ghazali et al., 2015).

Paddy fields are considered temporary aquatic and terrestrial habitats for these species. The paddy ecosystem or area is flooded with water throughout the planting season and is dried after the harvesting season. Thus conditions become an ideal environment for lepidopteran species (Bahaar & Bhat, 2011). Paddy grains are one of the most economically important products because it is a food source for human beings (Sie et al., 2008), but the grains have also been recorded to be infested by lepidopteran species (Syarifah Zulaikha et al., 2018).

DNA barcoding is a novel approach used to precisely identify any species using the short and standard gene region, the cytochrome oxidase subunit 1 (*CO1*). This approach enables taxonomists to precisely identify species and helps stakeholders to combat pests and the invasive species so as to ensure food safety and security (Hebert & Gregory, 2005). The latest findings reveal that identification using DNA barcoding helps to resolve problems related to species diversity (Hebert et al., 2004a, 2004b). This approach is found to be the most effective technique to support and reconfirm morphological identification (Halim et al., 2018; Nor Atikah et al., 2019). Moth species are very difficult and complicated to accurately identify because of damaged scale structures in the adult stage, as well as its very dull colouration. As leaf rollers and stem borers become pests during the larval stage, this approach enables species identification during their immature stages.

Few researchers work on moths (Scoble, 1992), hence many moths are still not yet identified. In addition, distributional and host studies are also lacking (Janzen, 1988). Several studies in Malaysia and other Southeast Asian countries have been indirectly conducted on leaf folder and stem borers in terms of diversity and abundance and pesticide applications, i.e. by Bhatnagar (2004), Faleiro et al. (2006), and Ooi et al. (2015). However, information is scarce and limited; none of the studies focussed

on molecular work such as DNA barcoding analysis for Malaysian species. Hence, the abundance and richness of moth pests are still unclear, making conservation efforts more difficult. This study is thus carried out to provide information on paddy-associated moth species and to measure the abundance and richness of paddy field moths, as well as to provide the DNA barcode of obtained moth species.

METHODS

Sampling Locations

This study was conducted at a conventionally cultivated paddy field in Parit 4 Timur, Sungai Panjang, Sabak Bernam, Selangor, Malaysia (3°42' 763" N 101°46'317"E), as a model sampling site. The plot size was approximately 10250 hectares (102.5 m x 100 m).

Insect Sampling

The sampling area was divided into four plots and then each plot was divided into four subplots. Two-line horizontal transects of 50 m each were selected randomly in the subplot and the adult stage sampled by using the sweep net, then larval stage specimens of leaf rollers were handpicked from the subplots. Sampling was carried out from November 2017 till March 2018 (1 season) according to paddy growth stages; vegetative, reproductive and mature stages at three time-zone replicates i.e. morning (1000-1100 hr), afternoon (1200-1300 hr), and evening (1400-1500hr) for the netting methodology. A total of 20 stems/ plants were also collected from a total of five paddy plants that were selected randomly from each subplot. Paddy stems were later cut longitudinally to examine the presence of larvae and pupae.

Laboratory Work

Field specimens were brought back to the Entomology Laboratory for the sorting and identification process. The adult and larval stages were preserved in 100% alcohol for molecular work.

Species Identification

The identification of species was based on external morphological characters (adult only) and conducted up to species level using a stereo microscope Zeiss Stemi DV5 by referring to a species key by Hampson (1896) and Khan et al. (1988, 1991). Seven (7) individual representatives (different morphology) were labelled with RC, LFC, LFM, LFG, KC, KK, and KH then identified using the molecular approach or DNA barcoding (Hebert et al., 2003). The DNA barcoding procedure involves DNA extraction, polymerase chain reaction (PCR), DNA purification, DNA sequencing, and DNA sequence analysis.

DNA Extraction and Polymerase Chain Reaction (PCR)

The DNA of the moth specimens was extracted using appropriate protocols by QIAGEN DNeasy® Blood and Tissue Kit (Germany). Extraction procedures were conducted using the manual provided by the

manufacturer. An Eppendorf machine was used to perform polymerase chain reaction (PCR) while cytochrome oxidase subunit 1 (*CO1*) was amplified using a set of primers, forward: LCO1490 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3' reverse: HCO22198 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3' (Hebert et al., 2003). The PCR condition is as follows; initial denaturation at 95°C for 3 min followed by denaturation at 95°C for 30 s, annealing at 52.2°C for 1 min and extension at 72°C for 30 s, and the final extension at 72°C for 10 min in a total of 30 cycles. For PCR reagents, 25 µL consisted of 12.5 µL of 1× GoTaq® Green Master Mix, 7.5 µL of ddH2O, 1-3 µL of 10 ng DNA template, 1.0 µL of 200 nm forward and reverse primers. The PCR product was later purified using the GF-1 PCR Clean-Up kit (Vivantis) to remove dNTP, primers, and buffer. The end product was then used to perform electrophoresis gel at 90V and 30 min using agarose gel 1.5%.

Sequencing Analysis, Editing, and Alignment of DNA

The purified PCR product was sent to First Base Sdn. Bhd. Shah Alam, Selangor, Malaysia for sequencing analysis to determine the variation and identity of the base sequence. DNA sequence editing was done using Sequencher software. The alignment was done on both forward and reverse alignments to ensure the validity of the base.

Alignment, Basic Local Alignment Search Tool Analysis (BLAST) and Phylogenetic Analysis

The online software, Basic Local Alignment Search Tool (BLAST) was used to ensure that the amplified sequence is the sequence of choice. BLAST showed a maximum hit for the respective species only, as available in GenBank (http://www.ncbi.nlm.nih.gov/genbank). PAUP* 4.0 was implemented to construct the neighbour joining (NJ) tree using Kimura's two parameter algorithm model with bootstrap analysis (1,000 replications).

Data Analysis

Ecological indexes such as the Shannon diversity index (H'), evenness index (E'), and Margalef's richness index (R') were evaluated using paleontological statistical software (PAST) version 2.17c.

RESULTS

Overall Composition of Lepidoptera

A total of 189 individuals belonging to five species under two families were collected from Sabak Bernam, Selangor. Species *Chilo auricilius* Dudgeon were recorded with the highest abundance with 33% (62 individuals) (Figure 3b), followed by 29% of *Parapoynx stagnalis* (Zeller) (54 individuals) (Figure 3a), 15% (29 individuals) of *Scirpophaga incertulas* (Walker) (Figure 3e), 14% (27 individuals) *Cnaphaphalocrocris medinalis*

(Guenée) (Figure 3c), and the lowest abundance *Sesamia inferens* (Walker) with 9% abundance (17 individuals) (Figure 3d). All species were stem borers except for *Cnaphalocrocis medinalis* which is a leaf roller. The stem borers belonged to family Crambidae with one species (*Sesamia inferens*) from Noctuidae.

Richness and Diversity of Moths

Shannon index (H'), evenness index (E'), and Margalef's richness index (R') counted for moths obtained in Sabak Bernam, Selangor were 1.51, 0.90, and 0.76, respectively. Pielou (1975) stated that the Shannon index was at the lowest if its range is between 0.0-2.5. Moth diversity in the paddy field is therefore low as H'= 1.518, in addition to evenness and richness at 0.90 and 0.76, respectively.

The Abundance of Lepidoptera Species at Different Paddy Growth Stages

Lepidoptera sampling in the Sabak Bernam, Selangor paddy field was carried out at three different stages i.e. vegetative, reproductive, and mature stages. Based on Figure 1, 5 moth species were recorded in all paddy growth stages with 189 individuals and the mature stages had the highest number of moths with 84 individuals, followed by 71 and 34 for the reproductive and vegetative stages, respectively. At the vegetative stage, *Parapoynx stagnalis* (Walker) had the highest number with 32 individuals followed by *Chilo auricilius* and *Cnaphalocrocis medinalis* with one individual each. At the vegetative stage, there were no records of *Sesamia inferens*. At the reproductive stages, four moth species were recorded i.e. *Chilo auricilius* (46 individuals), followed by

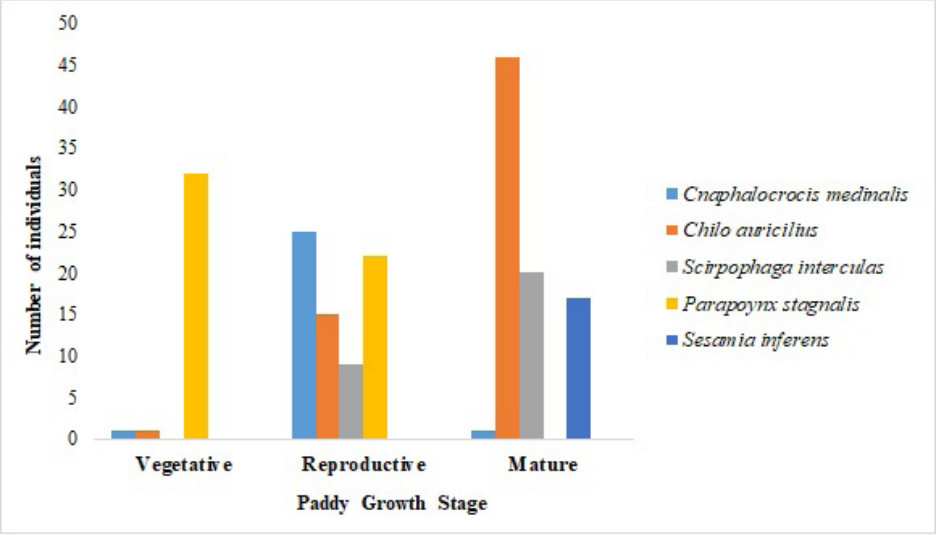

Figure 1. Lepidoptera species richness at different stages of paddy growth

Scirpophaga incertulas (20 individuals), *Sesamia inferens* (17 individuals), and *Cnaphalocrocis medinalis* (1 individual).

DNA Barcode and Genetic Distance

A total of five species were sequenced and matched with online BLAST to detect the percentage of similarities with available data in the GenBank. Samples of LFM and LFG were referred to a different species, *Cnaphalocrocis medinalis* morphologically but molecular work determined that they were the same species. All the other species showed a high percentage of similarity with the GenBank data (between 97-99%) (Table 1) and all species were located at specific lineages, showing that they belonged to different species (Figure 2). The genetic distance analysis (also presented in Table 2) shows the distance between genera between 0.112-0.166 (Table 2).

Table 1

Percentage similarity of the sequences deposited in the GenBank using BLAST analysis

Sample code	Species	Percentage of similarity (%) (compared to the GenBank data)	Accession no. (sequences submitted to GenBank)
LFM	Cnaphalocrocis medinalis	99 (*Cnaphalocrocis medinalis)*	MT357089
LFC	Chilo auricilius	98 (*Chilo auricilius*)	MT357091
LFG	Cnaphalocrocis medinalis	99 (*Cnaphalocrocis medinalis)*	MT357090
KH	Chilo auricilius	98 (*Chilo auricilius)*	MT357092
KK	Scirpophaga incertulas	98 (*Scirpophaga incertulas*)	MT357093
KC	Sesamia inferens	99 (*Sesamia inferens*)	MT357094
RC	*Parapoynx stagnalis* (= *Nymphula depunctalis*	94 (*Parapoynx stagnalis*)	MT357095

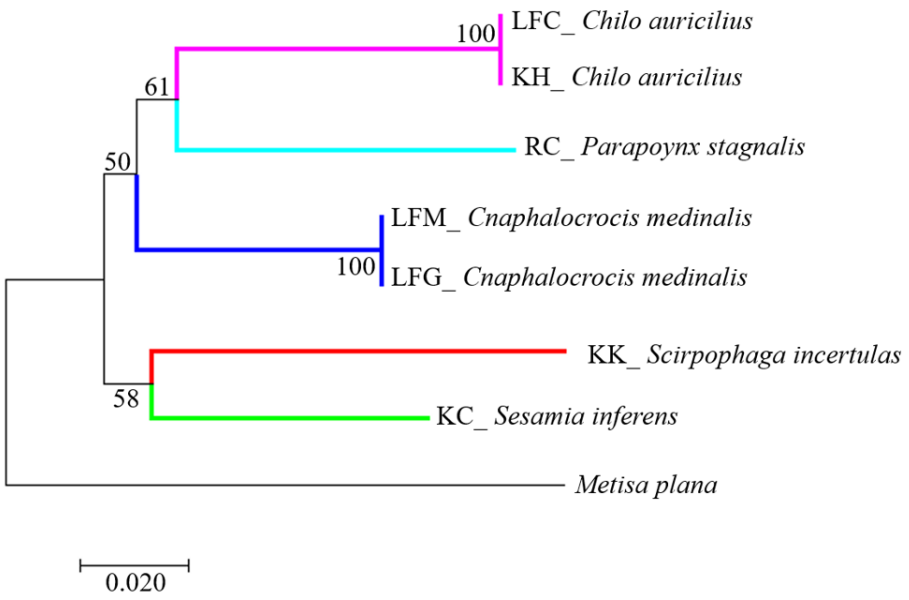

Figure 2. Neighbour joining tree resulting from the *CO1* sequences on five species

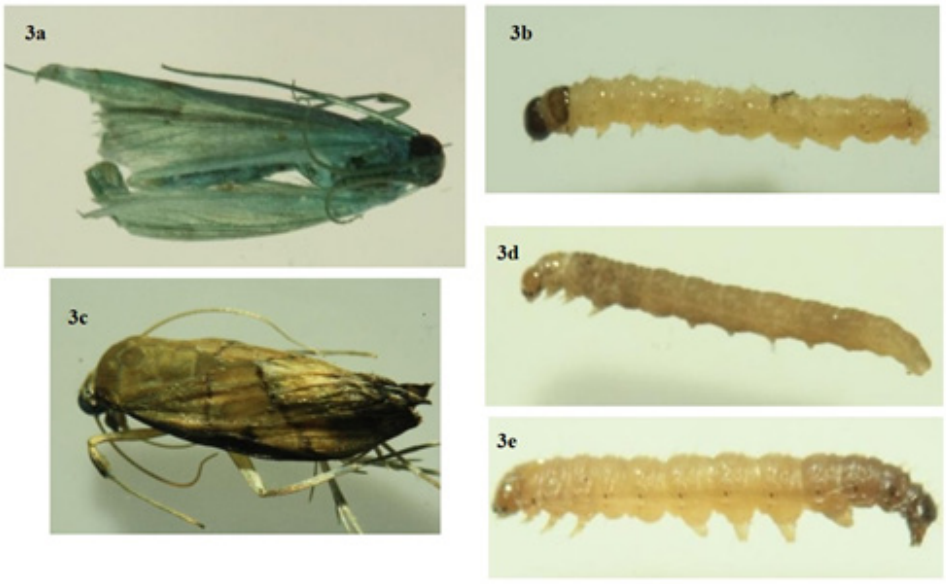

Figure 3. Photos of larval and adult stages of lepidopteran species: (a) *Parapoynx stagnalis*; (b) *Chilo auricilius*; (c) *Cnaphalocrocis medinalis*; (d) *Sesamia inferens*; (e) *Scirpophaga incertulas*

Table 2

Genetic distance between species implemented in the phylogenetic analysis

	[1]	[2]	[3]	[4]	[5]	[6]
[1] *Cnaphalocrocis medinalis*						
[2] *Chilo auricilius*	0.112					
[3] *Scirpophaga incertulas*	0.142	0.164				
[4] *Sesamia inferens*	0.116	0.120	0.135			
[5] *Parapoynx stagnalis*	0.121	0.119	0.166	0.131		
[6] *Metisa plana*_Outgroup	0.914	0.909	0.942	0.925	0.909	

DISCUSSION

This is an extensive study on pest moth species in a conventional paddy ecosystem. To date, no study has been specifically conducted on pest moth species in Malaysia and her neighbouring countries. Studies by Babendreier et al. (2020), Norela et al. (2013), Ooi (2015), Razali et al. (2015), and Siregar et al. (2017), were conducted with myriad aims and different parameters. In addition, stem borer and leaf folder species are difficult to study due to their behaviour of living inside the stem and leaf roll during their immature and pest stages (Razali et al., 2015). The model sampling site in Sabak Bernam was thus chosen to understand the richness and abundance of pest moth species in conventional rice ecosystems.

According to Norela et al. (2013), the System of Rice Intensification (SRI) supports a high diversity of insects especially on Lepidoptera, rather than conventional planting. In this study, five species were successfully collected from the conventional paddy ecosystem, consisting of two families (189 individuals) of pest moths. However, in Norela et al. (2013), there were only eight species (140 individuals) of pests and non-pests of Lepidoptera. Bahaar and Bhat (2011) stated that the paddy field was an example of an appropriate ecosystem for insect species, especially Lepidoptera due to adaptation to the disturbed habitat. According to Norela et al. (2013), the pest species collected in that study are not sampled from the SRI ecosystem. It is possible that natural enemies played a part in pest control as nitrogenous fertilizers had not been applied extensively to SRI-cultivated paddy. A paddy field is highly influenced by constant physical, chemical, and biological changes, therefore organisms that inhabit paddy fields are regarded as opportunists that are able to undergo extreme physiological and behavioural adaptations

A study by Praveen (2017) also recorded moth diversity in paddy fields in Palakkad, India, reporting results that were more or less similar to the Shannon diversity index, in terms of the number of family and species with 0.995, 0.90; 4, 2; 9, 5 respectively. In

their study, specimens from the families Noctuidae, Pyralidae, Saturniidae, and Sphingidae were collected, of which *Cnaphalacrocis medinalis* and *Spodoptera mauritia* were common species. However, in this study, only two families (Noctuidae and Crambidae) as well as *Chilo auricilius* were found to be the most abundant species. All species, both leaf folder and stem borers are widely recorded in Malaysia (Cuong & Cohen, 2002).

Referring to Nasiruddin and Roy (2012), *Cnaphalocrocis medinalis* and *Chilo auricilius* showed the highest infestation rate at the reproductive and mature stages, while *Scirpophaga incertulas* existed in almost all growth stages of paddy, but were not collected during the vegetative stage. Their findings were similar the results of this study. At reproductive stage, *Cnaphalocrocis medinalis* showed the most abundance, with 25 individuals. According to De Kraker et al. (1999), this species of leaf roller starts to appear in the fourth week of plant shift without consistent population density. The number of eggs is highest at the late vegetative stage which is the seventh week after plant shift while the number of larvae is highest one to two weeks after the late vegetative stage. Leaf damage caused by leaf roller larvae can be seen in the fourth week of plant shift and damage on the leaves is highest during the reproductive stage then decreases during the mature stages. Destruction to paddy plants occurs because larvae fold leaves longitudinally using silky thread and eventually feed on them, resulting in white, dry leaves (Goco, 1921).

Kakde and Patel (2014) showed that the percentage of infestation of *Scirpophaga incertulas* was 5.79% and 4.93% at the mature stages in conventional and in SRI paddy fields, respectively. The level of infestation by stem borers was lower in SRI-cultivated paddy fields compared to that in conventional-cultivated paddy fields. However, in this study, the infestation of *S. interculas* was high during the maturation and vegetative stages; almost 23% and 11%. In addition, *Parapoynx stagnalis*, a winged moth, was the most dominant species at the vegetative stage. It caused maximum damage during this stage and a high level of destruction was reported during the first 4 weeks of plant shift (Pulin et al., 1998). Ramasubbaiah et al. (1978) also recorded 35-40 days as the optimum duration needed by *P. stagnalis* to cause maximum damages. Litsinger et al. (1994) documented 2-6 weeks as the duration in which maximum infestation by species larvae could occur for their survival. This thus proves that the winged moth has a high adaptability rate at the vegetative stage because of a high oviposition rate, fast larvae development, high survival rate, as well as mature and bigger larvae size.

The morphological identification of insect species is first based on adult specimens. However, a lack of taxonomic keys and availability for a particular life stage and sex causes difficulties in the identification process (Ball & Armstrong, 2006). Identification using morphology is time-consuming and tedious especially for the adult stags, but almost impossible at

the larval stage. Therefore, DNA barcoding is an effective approach to overcome time constraints and to avoid misidentification at the larval stage. Molecular identification has also been popularly applied for precise and fast identification especially in the agricultural field (Ghazali et al., 2014a, 2014b, 2015).

All sequences were blasted and were highly supported with 98-99% similarity with the data available in the GenBank. The species *Parapoynx stagnalis* was identified based on its sequenced data; however, the species presented as *P. stagnalis*, was found to be taxonomically synonymous with *Nymphula depunctalis*. The phylogenetic analysis has also proven that all five species (seven individuals) were clearly separated and supported as a single species located at specific lineages on the tree. This approach is the most popular in published barcoding papers, especially for insect species (Halim et al., 2017; Nor Atikah et al., 2019; Razali et al. 2015).

CONCLUSION

A total of 189 individuals of moth pests belonging to two families and five species were collected and barcoded in this study, namely *Cnaphalocrocis medinalis* (Guenée) (leaf folders), *Scirpophaga incertulas* (Walker), *Chilo auricilius* Dudgeon, *Sesamia inferens* (Walker), and *Parapoynx stagnalis* (Zeller). The identification was confirmed using *CO1* sequencing data with the application of BLAST analysis and the sequences were successfully deposited in the GenBank.

Even though the richness, abundance and diversity of species were quite high compared to earlier studies, they were previously measured with different parameters and aims, and are thus significantly incomparable. No final conclusion can hence be made as to whether the conventional paddy ecosystem in Sabak Bernam, Selangor has an unusually high record of pest insect species. However, it does denote that an appropriate method of pest control should be implemented.

More studies should therefore be carried out in the near future to ensure updated and credible data on richness and abundance of Lepidoptera in cultivated and conventional paddy ecosystems based on different growth stages, longer sampling durations (several seasons), and with similar sampling methods. The outcomes from this study are very important for the initial stages of conservation, especially for paddy field pest management strategies.

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