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SPECIES STATUS OF A RICE YELLOW STEM BORER, *SCIRPOPHAGA INCERTULAS* (LEPIDOPTERA: PYRALIDAE) BASED ON CO I GENE SEQUENCES

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Abstract

Study on species status of a rice yellow stem borer, *Scirpophaga incertulas* (Lepidoptera: Pyralidae) was conducted. The objectives of the study were to explore the genetic variation among populations of S. *incertulas* in Java and to clarify its species status based on CO I gene sequences. The results showed that from the entire 680-bp region of sequences from10 population in Java, 99 % were constant. The average of estimated sequence divergences in the comparisons among populations within *S.incertulas* was very low about 0.01%. The base composition was slightly A+T biased (*C*:0.15) with G+C contents was 37.09%. The result of phylogenetic analysis showed that *S.incertulas* is a single species with *S.innotata* as its sister group. The differences on the body-size, black spot at female's forewing, forewing length within *S.incertulas* are solely natural variation.

Key words: CO I gene, *Scirpophaga incertulas,* species status.

INTRODUCTION

Pyralidae stem borers are one of the main pests of rice, throughout South East Asia. They are causing chronic yield losses that are often estimated at 5%. Among them, *Scirpophaga incertulas* or a yellow stem borer (YSB) is one of the most important pests in Indonesia. The intensity of YSB epidemic increased from time to time (Siwi *et. al.*, 1999). Outbreak of stem-borers on rice in northern part of West Java and other parts of Indonesia remains to occur despite various methods of control measures have been

undertaken. Various rice plant varieties have been planted by farmer in the hope to reduce the population of rice stem borer. But there always rice borers are surviving. This situation is arising hypothesis the presence of varieties of the species in the field.

The causes of outbreak are not fully understood. This is probably initiated by a complex species of *S. incertulas*, which are present in the fields. It has been reported that this species from various locations indicate variations in body length, forewing length and other characteristic feature of the wings of male and female (Siwi *et al*., 1999). Therefore, a study on the genetic variation from different population using molecular approach to clarify species status of *S. incertulas* in Indonesia is necessary.

Not all genes can be used to identify at a specific level, only a certain gene that fulfill certain requirements. In general, the followings are requirements that must be reached by a gene for a specific identification: 1). Should be able to distinguish a species and relatively more conserve within inter-species than intra-species, 2). Should be a standard, so the same position of DNA sequence gene can be used to identify species as many as possible, 3). Should be informative for phylogeny, so the data can be used to reconstruct a relationship and to rank the taxa in groups (a family, a genus and others), 4). Should have an high amplification level, so it is easy to conduct a PCR, 5). Should be a relatively short, so it can be used to test the fragmented DNA.

COI has been chosen as one of the candidate genes to be applied in DNA barcoding (a method to identify an organism by using a gene sequence) (Herbert *et al*. 2003). Almost all requirements that are needed in DNA barcoding can be reached by this gene. This gene can be used to distinguish species in almost all animals. The length of this gene is relatively short about 648 bp. Compared with another mitochondrial gene, COI gene is more conserve. This gene is very suitable to identify a species since its sequence has a low variability (in general less than 1-2%), even for the closely related species its value is less than 1%. Another benefit of using this mitochondrial gene is that it is relatively easy to sequence than nuclear genes such as Wingless, EF-1á and ITS (Sutrisno 2003, 2006; Sutrisno *et al*. 2006). Therefore, this gene was chosen to clarify the species status of *S. incertulas* in this study.

The objectives of the study were:

1). to explore the genetic variation among populations of S. *incertulas* in Java using molecular data,

2). to clarify the species status of *S. incertulas* based on CO I gene sequences

MATERIAL AND METHOD

Sampling of adult moth

Sampling of adult moths has been conducted using light traps equipped with a 160 watt mercury vapor light and a 2 X 2.5 m white screen. The light trap is set up at the open area within the forest and paddy-field. Moths attracted to the light trap and lied at the white screen were collected into an ethyl acetate-killing bottle. All specimens collected at the night then were preserved using absolute ethanol.

Site of moth sampling

To get more comprehensive results sampling was conducted at 10 localities in Java: 1). Alas Purwa National Park, East Java, 2). Baluran National Park, East Java, 3). Magetan, East Java, 4).Wates, Yogyakarta 5). Sleman, Yogyakarta, 6). Baturaden, Banyumas, Central Java, 7). Wonosobo, Central Java, 8). Garut, West Java, 9). Gunung Halimun-Salak National Park, West Java, 10). Ciamis, West Java. In general, populations of yellow rice stem borer in the paddy field were abundance but in the national parks such as Alas Purwo, Baluran and Gunung Halimun-Salak were less abundance. The reasons to choose the conservation areas such national park and nature reserve are to see do they can use other rice plants in conservation area as their host plants and to see how they distinct genetically from the paddy field population. Its sister group of this species, a rice white stem borer, *S. innotata* was also collected from the paddy field in Nusakambangan Island.

DNA extraction, PCR and Sequencing

DNA was extracted from the thorax of each individual. The tissue was homogenized in a 1.5 ml microcentrifuge tube containing 600 µl of CTAB buffer with 4% polyvinyl pyrrolidone, and incubated at 55ºC for 2 hours. The solution was extracted using phenol saturated with TE buffer (10 mM Tris-HCL, pH 8.0, 1mM EDTA), then washed once with one volume of phenol: chloroform: iso-amyl alcohol (25:24:1) and twice with chloroform: iso-amyl alcohol (24:1). The aqueous phase was transferred to a new tube and then 1.5 volumes of isopropanol were added to precipitate the DNA. This tube was then left at -20ºC for more than 1 hour. The DNA precipitant was pelleted by centrifugation at 13,000 rpm for 20 minutes, then washed with 70% ethanol, air dried, and re-dissolved in 50 µl of TE buffer.

For PCR amplification and DNA sequencing we used Mt D-4 and Mt D-9 primers (http://www.biotech.ubc.ca) to amplify the *COI* gene for a total of 686 bp. The complete sequences of the primers used were Mt D-4: 5'-TACAATTTATCGCCTAAACTTCAG CC-3', Mt D-9: 5'-CCCGGTAAAATTAAAATATAAACTTC-3'.

All PCR reactions were performed in a 50 µl volume containing 5 pM of each primer, 2 mM dNTPs, 2.5 mM $MgCl_2$, 1 X buffer, and 0.25 U of Taq polymerases, using a Takara Thermal Cycler MP (Takara) with the following PCR protocol: one cycle of denaturation at 94°C for 10 min, followed by 35 cycles each consisting of denaturation at 92°C for 30 s, annealing at 47°C for 30 s, and extension at 72°C for 1 min 30 s. These cycles were completed by final extension at 72°C for 10 min and the PCR products were purified using the QIAquick PCR Purification Kit.

Cycle sequencing was done using 35 cycles of denaturation at 96°C for 10 min, annealing at 50°C for 5 min, and extension at 60 °C for 4 min; thereafter, the products were purified using phenol-chloroform. Sequencing of the purified PCR product was performed using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kits (Perkin-Elmer) on an ABI PRISM model 310 Genetic Analyzer (PE Applied Biosystems). The sequences were aligned using BioEdit Sequence Alignment Editor (Hall 1999)

Base composition analysis.

We used the base frequency's option in PAUP* version 4.0b.10 for 32-bit Microsoft Windows to evaluate the base composition of each sequence and the homogeneity of the base frequency data across populations.

Phylogenetic analysis.

Phylogenetic analyses were performed with PAUP* version 4.0b.10 for 32-bit Microsoft Windows based on CO I gene by using Neighbor-Joining approach. We applied Kimura two-parameter model (K80) for estimating evolutionary distance among populations (Kimura 1980).

RESULTS

Base composition

Sequences of species of *S. incertulas*, as well as its sister group, *S. innotata*, were aligned with no evidence of insertion or deletion. All sequences have been submitted to the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) under the accession numbers AB495264 – AB495274 (Table. 1).

Table. 1. Population of *S. incertulas* and *S. innotata* from different localities and their genbank accession number

No	Species	Population	Date of Collection	Name of Collector	Accession Number
1	*S. incertulas*	Alas Purwo NP	13 April 2007	Sutrisno, H & Darmawan	AB495270
2	*S. incertulas*	Baluran NP	10 April 2007	Sutrisno, H & Darmawan	AB495269
3	*S. incertulas*	Magetan	16 April 2007	Sutrisno, H & Darmawan	AB495268
4	*S. incertulas*	Wates	10 February 2006	Sutrisno, H	AB495266
5	*S. incertulas*	Wonosobo	12 June 2007	Sofyan, R	AB495265
6	*S. incertulas*	Baturaden	23 March 2006	Sutrisno, H & Darmawan	AB495272
7	*S. incertulas*	Ciamis	20 May 2006	Sutrisno, H	AB495271
8	*S. incertulas*	Garut	10 June 2005	Sutrisno, H	AB495273
9	*S. incertulas*	G. Halimun NP	28 May 2006	Sutrisno, H & Darmawan	AB495274
10	*S. incertulas*	Sleman	20 March 2007	Sutrisno, H	AB495267
11	*S. innotata*	Nusakambangan	27April 2006	Sutrisno, H	AB495264

A total of 680-bp region has been successfully sequenced. The alignment sequences showed that 99.5 % (678) of the nucleotide position within 10 populations of *S. incertulas* were constant and only 3 variants were found in which one of them is informative site (*i.e*. any variants were found in more than two sequences). Due to there is no difference within local population sequences, thus, only a single individual sequence to represent each local population was submitted to the genbank. The average of estimated sequence divergence in the comparisons between populations within *S. incertulas* was very low about 0.01%.

The bias composition of nucleotides was calculated using Irwin *et al*. (1991) and ranges in value between 0 and 1, in which zero is indicating no bias and one

indicating complete bias. The results showed that the base composition was slightly A+T biased (*C*: 0.15) with the average of G+C contents was 37.09 % (Table 2).

Moreover, interspecific variations in the base composition were very low for the total nucleotides. The chi-square test of homogeneity of base frequencies across population indicated that there was no significant different in the base frequency between population in CO I (X^2= 11.7020, *df*= 36, *P*= 0.9995).

Table 2. Proportion of each nucleotide and bias in CO I

Nucleotide	Codon position			
	1st -codon	2nd-codon	3rd-codon	Mean
A	0.3135	0.1627	0.4212	0.2991
C	0.1647	0.2660	0.1274	0.1860
G	0.2684	0.1631	0.0152	0.1849
T	0.2532	0.4140	0.4361	0.3677
Bias (*C*)				0.15

Bias in base composition is calculated as

$$C=(^2/_1)\sum_{i=1}^{4}|C_i-0.25|$$

Where C is the composition bias and C_i is the frequency of its base.

Phylogenetic analysis

Given the absence of substantive composition bias (*C*= 0.15), we estimated nucleotide divergence according to the Kimura (1980) two-paramater model. This model assume that transition occur more frequent than transversion. The NJ tree showed that all population of *S. incertulas* forming one clade with very short branches but distantly separated from the out group *S. innotata* (Figure 1). There is no significant different between population from paddy field and from the nature reserve.

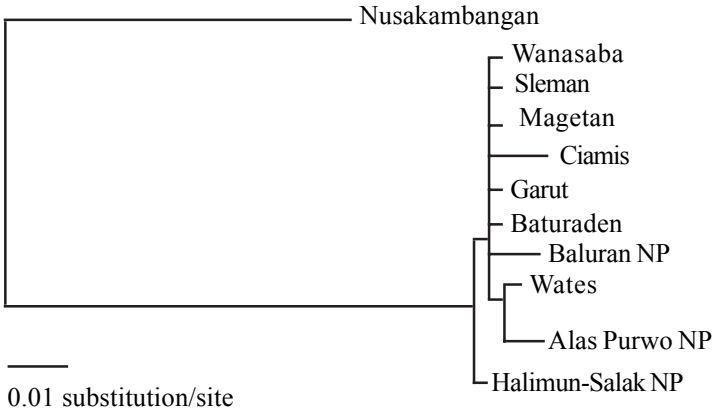

Figure 1. Neighbor Joining Tree based on Kimura two-parameter model with a bar scale to indicate the substitution/site

DISCUSSION

It has been reported that the base composition in insect genomes is biased, higher A+T biased generally is found in mitochondrial genes and less biased in nuclear genes (DeBruijn 1983, Clary & Wolstenholme 1985, DeSalle *et al*. 1987, Crozier & Crozier 1993, Goto & Kimura, 2001). The proportion of the A+T contents in CO I of *Choristoneura*, *Hemileuca* (Lepidoptera) and drosophilids (Diptera) has been reported ranging from 62% to 74% (Sperling & Hickey 1994, Goto *et al*. 2000, Rubinoff & Sperling 2002). Our previous study on genus *Glyphodes* (Lepidoptera) also showed that A+T contents of CO I ranging from 69.8% to 72.01% and indicated strong biased (*C*= 0.258) (Sutrisno 2003). The result of the study also showed a similar phenomenon, the A+T contents about 63%, which is still within a range of A+T contents that has been reported previously but it seems a low biased

Based on the phylogenetic analysis, it's clear that species of *S. incertulas* that distributed in Java is a single species. All population both from paddy field and nature reserved forms one group with very short branches in NJ tree. Study on genetic variation among populations of this species collected from 28 hotspot locations in

India using the randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) that has been conducted by Kumar *et al*. (2001) also produced almost the same results. In their study, 32 primers were used and 354 amplification products were observed. No RAPD-PCR bands diagnostic to the pest population from any specific region were identified. Cluster analysis using UPGMA showed that, with the exception of the pest population from Pattambi, all the populations cluster as one group with GD values in the range of 6–22%, suggesting that gene flow between populations is independent of geographic distance and appears to be unrestricted. The relatively high GD value of 48% exhibited by the pest population from Pattambi was the only exception.

Based on the previous study which has been conducted by Siwi *et al.* (1999), *S. incertulas* from various locations showing variations in body length, forewing length and other characteristic feature of the wings of male and female. The female *S. incertulas* found in Jatisari, Muara Baru (Karawang) and Sukamandi showed variation in size, the large size female with the forewing length around 15 mm, while the small size female with forewing length around 12-12.5 mm. All the representative females of *S. incertulas* have small black spot on the upper surface of forewings, right over the forewing discal cell. However several females of this species collected from Jatisari do not have any black spot on its upper surface of forewings. Those variations were also found in this study. It seems those variations were not different genetically based on CO I gene sequences. Those differences are solely natural variation within species of *S. incertulas*.

The host plants of this species are still in debate, whether only rice or this species has alternative hosts. In Taiwan, Shiraki (1917) studied the alternative host plants of this species by checking the stem of 15 different plants in the field every month from 1909-1911. He could not found a single larvae of the species feeding on them. The plants were *Miscanthus sinensis*, *Zizania latifolia*, *Panicum repens*, *sugar-cane*, *barley* and *teosinte*. Fletcher & Ghosh (1920) and Lewvanich (1982) stated that there is no alternative host plant of this species, but Logothetis (1950) argued that the alternative host plants are many gramineous plants, especially genus *Cyperus* (Cyperaceae).

In Java many *Cyperus* has been suspected as alternative hosts but there is no further information about its species (Siwi, SS. Biotechnology and Genetic Resources Institute, Department of Agriculture. pers.comm). Our previous study showed that in Sebangau National Park, Central Kalimantan in where there is no paddy-filed in this

park this species has an alternative host plant, *Hypolytrum nemorum* (Vahl.) Spreng. (Cyepreaceae) (Sutrisno 2007). The results of this study also indicated that the host plant of this species is not only rice since the specimens used in this study, both population from paddy-field and Baluran National Park (the grassland area), form one group with very short branches in Neighbor Joining tree.

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