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Submission date: 30-Dec-2021 09:43AM (UTC+0700)
Submission ID: 1736372302
File name: COIgeneofN.nigropictus.pdf (227.95K)
Word count: 3467
Character count: 18998

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International Journal of Entomology Research
International Journal of Entomology Research
ISSN: 2455-4758; Impact Factor: RJIF 5.24
Received: 03-08-2019; Accepted: 05-09-2019
www.entomologyjournals.com
Volume 4; Issue 6; October 2019; Page No. 12-15

Genetic analysis of rice green leafhopper *Nephotettix nigropictus* (Stål) from Samosir island-Indonesia using partial DNA sequence of cytochrome oxidase sub unit I (COI) gene

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Abstract
The green leafhopper *Nephotettix nigropictus* (Stål) (Auchenorhyncha: Hemiptera) is insect transmitter of rice tungro virus that cause damage and death on rice plants. There is none genetic analysis of *N. nigropictus* that be isolated from Samosir island-North Sumatra Indonesia by using partial DNA sequence of mitochondrial citochrome oxidase sub unit I (COI) DNA gene. This study aimed to identify and to know the nucleotide composition of *N. nigropictus* based on mt COI or DNA barcoding. Genetic analysis of that leafhopper consisted of four steps, namely: Leafhopper sample catching on rice field by using insect net, DNA extraction by using Zymo Tissue & Insect DNA Mini Prep, DNA amplification by using PCR and My Taq™ HS Red Mix and DNA sequence analysis by using ABI PISM 3730XL Genetic Analyzer. Primer cocktail tRWF-Mlep was used in DNA amplification step. The research result showed that mt COI DNA fragment of *N.nigropictus* has length 448-458 bp. This mt COI DNA sequence was dominated by A and T bases (74.5%). The concentration of T, C, A and G nucleotides in the COI sequence were 38.60%, 13.10%, 35.80% and 12.40%, respectively. Identification of *N.nigropictus* based on this mt COI DNA sequence confirmed the identification result based on its morphological characters.

Keywords: DNA barcoding, COI gene, *N. nigropictus*, Samosir

1. Introduction
Green leafhopper, *Nephotettix nigropictus (Stål)* is a member of Cicadellidae family, Hemiptera ordo that could be found in rice ecosystem. It has head rounded and vertex with submarginal black band, anterior margin of pronotum and inner margin of claves usually marked in back colour. Furthermore, it has aedagus with 8 or 9 pairs of spines (Gnaneswaran *et al*., 2008; Chowdhury *et al*., 2011) [6, 2]. In agriculture sector especially in rice ecosystem, green leaf hopper has economic importance because it plays an important role as a transmitter of rice tungro virus. This hopper species belongs to the second importance to *N. virescens* as vector of tungro virus. Therefore, the occuring of this leaf hopper direct and indirect on paddy field could cause damage, death on paddy plants and at the end causes yield loss.
The green leafhopper *N. nigropictus* is widely distributed in Asia and known from Philippines, Malaysia, Indonesia, Burma, Sri Lanka, Australia, New Guinea, China, Hong Kong, South Vietnam, Pakistan, Nepal, India and Thailand (Wilson & Claridge, 1991) [30]. The occurence of this green hopper species in rice field at Tapanuli region of North Sumatera-Indonesia especially in Samosir island has been reported. Its abundance was higher compare to *N.virescens* (Manurung *et al*., 2017) [16].
In order to achieve the effective management of pest species damaging rice plants, the accurate identification is needed. The using of morphological approach in identification of leafhopper has been used for long time and wellknown (Wilson & Claridge, 1991; Chowdhury *et al*., 2011) [30, 2], whereas the using of molecular or genetic approach especially throughout DNA analysis is still new, just since 2000 years (Hebert & Gregory, 2005; Gopurenko *et al*., 2013; Foottit *et al*., 2014) [9, 7, 5]. Gnaneswaran *et al*., (2008) [6], has succes to describe the species diversity of *Nephotettix* Matsumura based on morphology features. Furthermore, the using of morphometric approach for identification completeness of white leafhopper *Cofana spectra* has been reported by Manurung *et al* (2019) [17] and for zigzag leafhopper *M. dorsalis* has been carried out by Faruq *et al*., (2017) [4].
The using of morphological approach in leafhopper identification has many difficulties and problems. This approach could be used mainly just towards adult stage, on male and be based on aedagus forms. Morphological analysis cannot be used for leafhopper that occur in polymorphism form. Meanwhile, the using of genetic or molecular approach in identification of leafhopper could to overcome that problems (Sreejith & Sebastian, 2014) [25].
Genetic analysis, especially DNA barcoding has become one of the major tools in the last 10 years that has been used by biology experts both for animal and plant identifications and also their phylogenes with related species (Hebert & Gregory, 2005; Asokan *et al*., 2007; Lakra *et al*., 2010; Roslim, 2017; Peninal *et al*., 2017; Sitompul *et al*., 2018; Kunde *et al*., 2018) [9, 1, 15, 22, 21, 24, 13]. The using of DNA barcoding in order to identify leaf-and planthopper has been carried out some researchers (Matsumoto *et al*., 2013; Gopurenko *et al*., 2013; Foottit *et al*., 2014; Zhang *et al*., 2014; Du *et al*., 2017) [20, 7, 5, 31, 3].
This work regarding into the using of DNA barcoding, especially through mitochondrial COI Sub Unit I gene in order to identify and to study the nucleotide composition and also to find out the molecular phylogenetic of N. nigropictus with its related species that come from Samosir island-Indonesia that untill now has never been attemped.

2. Materials and Methods

2.1. Study Area

There was two sampling sites at Samosir island on non-irrigated rice field. The first sampling site was at Ambarita village (N:2o40'50''; E:98o49'57") and the second sampling site was at Siogung-ogung, Pangururan village (N:2o36'41.73''; E:98o41'37.34"). The catching of leafhopper was done in May 2019 in conventional rice cultivation field.

2.2. Collecting and Identification of Samples

The hopper was catched by sweeping with help of an insect net and aspirator (Manurung *et al*., 2004; Yi *et al*., 2012) [18, 29]. The catching was done in the western, eastern and winward sides of the paddy field (Manurung *et al*., 2005) [19]. Hopper samples were deposited directly in 96% alcohol, labeled, and transported to the laboratory for curation and identification. Morphologically species identification was done under stereo binocular microscope in taxonomy laboratory of Biology Department of Universitas Negeri Medan and be consulted on Wilson & Claridge (1991) [30]. The validation of identification have been done by aedagus assesing. The leafhopper samples were stored at - 20oC until the working of the DNA extraction.

2.3. DNA extraction, Amplification and Sequencing

DNA genomic was extracted from the tissue of one individual of leafhopper with Zymo Tissue and Insect DNA Mini Prep (Zymo Research, D6016). This extraction consisted of preparing, lysis cell, DNA binding, washing and DNA elution steps. The DNA isolated was confirmed using 1% TBE agarose. The amplification of mitochondrial genomic DNA was done with My Taq HS Red Mix (Bioline, Bio-25047). The coctail primer tRWF-Mlep was used to amplify the COI gene in Touch Down PCR condition (Foottit *et al*, 2014) [5]. This PCR profile consisted of initial denaturation at temperature of 95oC for 3 min followed by 5 cycles with denaturation reaction conditions at 94oC for 40 sec, annealing at 45oC for 40 sec, extension at 72oC for 1 min and then followed by 35 cycles with denaturation reaction conditions at 94oC for 40 sec, annealing at 51oC for 40 sec, extension at 72oC for 1 min and ending with a final phase of extension terminal at 72oC for 1 min. The purification of PCR product was done by using the Zymoclean Gel DNA Recovery Kit (Zymo Research, D4002). The PCR product was assessed by electrophoresis with 1% TBE agarose. The running agarose was done at 100 volt for 60 min (Wealtec). Furthermore, the purified PCR product was sequenced with Bi-directional Sequencing using an ABI PRISM 3730 XLGenetic Analyzer at genetic lab of PT Genetika Science Indonesia, Jakarta.

2.4. Alignment and Analyses

The obtained sequences data were aligned or assembled by using ClustalW and consensus was taken for the analysis. The combination of mtDNA sequence of COI data was analyzed by sequencing homolo using BLAST program which can be accessed at the National Center for Biotechnology Information (NCBI) website. Sequences homology analysis was performed by comparing COI sequence of green leafhopper sample with NCBI GenBank Data base. The maximum composite probability estimate of the pattern of nucleotide substitution was based on Tamura-Nei model (Kimura, 1980, Tamura & Nei, 1993) [11, 28]. Molecular Evolutionary Genetic Analysis (MEGA-X) sofware was used for phylogenetic tree construction and evolutionary analyses (Kumar *et al*., 2018) [12]. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987) [23].

3. Results and Discussion

Leafhopper sample that has been previously identified based on morphology characters and followed by assesing on the aedagus form and spines pairs numbers as Nephotettix nigropictus (Wilson & Claridge, 1991; Gnaneswaran *et al*., 2008; Chowdhury *et al*., 2011) [30, 6, 2], its analysis was then continued with genetic or molecular marker. In the working with this molecular approach, the mitochondrial cytochrome oxidase I (COI) region of the sample was succesfully amplified using tRWF-Mlep primer in Touch Down PCR condition. This result confirmed the using of that primer in Touch Down PCR condition in the study of leafhopper (Hemiptera: Auchenorrhyncha) taxonomy through DNA Barcoding approach (Gopurenko *et al*., 2013; Foottit *et al*., 2014) [7, 5]. The PCR of COI gene fragment for sample N.nigropictus (A1 and P1 samples) yielded product of 448-458 bp (Table 1). The length of this COI gene fragment is longer compared to white leafhopper Cofana spectra (305 bp) (Sreejith & Sebastian, 2014) [25] but it is still shorter compared to orange headed leafhopper Thaia subrufa (466 bp) (Sreejith & Sebastian, 2015b) [27]. The lenght of COI gene fragment of N. nigropictus of Samosir sample is shorter from the same species that be isolated from India (634 bp, Gen Bank Accession No. KX351394.1) (Table 1).

The composition of nucleotide of N.nigropictus samples showed clear bias to nucleotide AT (74.50%). The occuring of nucleotide AT bias on N. nigropictus is in line with leafhopper C. Spectra, N.virescens and T. subrufa (Sreejith & Sebastian, 2014, 2015a&b) [25, 26, 27]. The composition of T (U), C, A and G nucleotides in the COI sequence of Samosir sample were 38.60%, 13.10%, 35.80% and 12.40%, respectively (Table 1). This finding showed that thymine content was highest than three others nucleotides and be followed by adenine, cytosine and guanine.

Table 1: The length and percentage of nucleotide composition of the COI sequence of *N. nigropictus* sample from Samosir island (sample A1 and P1) and related species

Species (Gen Bank Accession No)	T(U)	C	A	G	Total
Nephotettix cincticeps COI (MF716882.1)	36.9	15.5	33.9	13.7	658
Nephotettix nigropictus COI (KX351394.1)	36.8	15.6	32.6	15.0	634
Nephotettix virescens COI (KU324167.1)	37.0	15.2	34.1	13.6	689
Sample A1 (Ambarita)	38.6	12.7	35.9	12.7	448
Sample P1 (Pangururan)	38.6	13.1	35.8	12.4	458
Average	37.4	14.7	34.3	13.6	577.4

The NCBI BLAST database result revealed that partial COI gene sequence of *N.nigropictus* population isolated from Samosir showed 96.40% similarity with *N. nigropictus* (KX351394.1) from Mid Hills of Meghalaya, India (Table 2). It means there was just a 3.60% difference is observed between the both populations. The result of this investigation stated that molecular identification has corroborated the morphological identification and also

become a valuable tool in animal taxonomy (Asokan *et al*., 2007; Karimi *et al*., 2010; Kurniawaty *et al*., 2017) [1, 10, 14]. Regarding into the high similiratity of COI gene sequences of *N. nigropictus* leafhopper between Indonesia (Samosir island) and Meghalaya-India population, this research finding stated that genetic variation between the both leafhopper populations were very low and therefore geographical distance and ecological differences between two countries may be don't have significant contribution on the creating of their gene variation. The result of this research furthermore also pointed out that the both leafhopper populations probably have the same ancestor.

Table 2: The Result of BLASTN analysis on Sample A1 and P1

Species	Accession	Query Cover	Percent Indentity
Nephotettix cincticeps COI	(MF716882.1)	60%	92.47%
Nephotettix nigropictus COI	(KX351394.1)	60%	96.40%
Nephotettix virescens COI	(KU324167.1)	65%	92.72%

The maximum composite probability estimate of the pattern of nucleotide substitution is presented in Table 3. In this case, each entry is the probability of substitution (r) from one base (row) to another base (colum). Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics (Kimura, 1980) [11]. Based on that values could be stated that highest transitional substitution occured between T(U) and C, whereas highest transversional substitution took place between T(U) and A or between T(U) and G.

Table 3: Maximum composite probability estimate of the pattern of nucleotide substitution

	A	T/U	C	G
A	-	5.70	2.23	9.01
T/U	5.22	-	10.64	2.07
C	5.22	27.20	-	2.07
G	22.70	5.70	2.23	-

Phylogenetic tree among *N.nigropictus* isolated from Samosir with other leafhopper that belongs to member of Cicadellidae familiy is displayed in Figure 1. This finding confirmed again that COI gene mitochondria could elucidate the molecular evolution and phylogenetic relationship of leafhopper. The result of this phylogenetic tree pointed out that *N.nigropictus* isolated from Samosir island-Indonesia is the nearest relative of *N. nigropictus* from Meghalaya-India, therefore the both leafhopper populations have the evolutionary similarity. In this case, Gurney *et al*., (2000) [8] and Sreejith and Sebastian (2014) [25] added that the distantly connected species will show less than 90% within the same sequence, whereas the closely connected species shows more than 90% similarity.

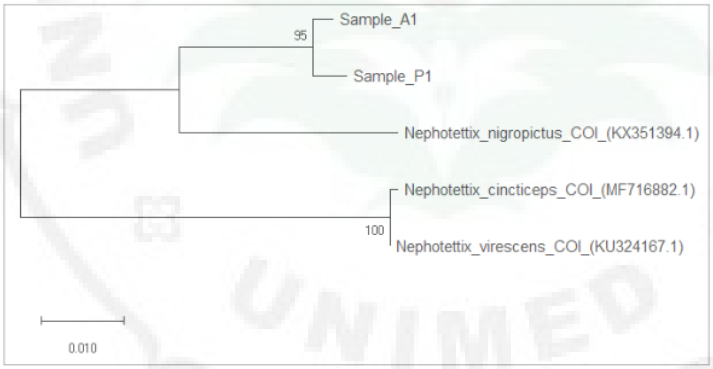

Fig 1: Phylogenetic Tree-Neighbor Joining of *N. nigropictus* from Samosir (Sample A1 and P1) based on DNA fragment mitochondria COI sequence with related species

4. Conclusion

Identification of green leafhopper *N. nigropictus* sample from island Samosir-Indonesia based on DNA barcoding marker could confirm the result of identification based on morphology characters. The leafhopper has the length of DNA of mt COI gene fragment about 448-458 bp and bias on nucleotide AT (74.50%). The composition of T (U), C, A and G nucleotides were about 38.60%, 13.10%, 35.80% and 12.40%, respectively.

Acknowledgements

The funding for doing this research was provided by Ministry of Research and Technology and Higher Education Republic of Indonesia under the research grant "Penelitian Dasar" with grant number 190/SP2H/LT/DRPM/2019.

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