

# The Genetic Structure of *Nilaparvata lugens* (Stal.) in Java Populations

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## ABSTRACT

The brown planthopper *Nilaparvata lugens* (Stal.) causes hopperburn in the rice field. The wing macropterous adults of this insect have capability for long distance flight. We aimed to analyze the genetic structure of six *N. lugens* populations in Java inferred from the combined COI-COII mitochondrial and ten loci of microsatellites markers. This study revealed low levels of nucleotides and high levels of haplotype diversity of the planthopper. The demographic test might indicate the genetic bottleneck history of *N. lugens* population in Java. The genetic diversity analyzed by using microsatellite markers also showed high levels of heterozygosity ( $H_o > H_e$ ) that indicated an isolated-breaking effect in the six populations in Java. Moreover, we found a homogenous genetic structure of *N. lugens* based on pairwise fixation indices ( $F_{ST}$ ) analyzes that appears to be maintained by high levels of gene flow and showed no correlation between genetic and geographical distance. Importantly, these studies also support accurate information of widely distributed and genetically intermixed among *N. lugens* across Asia populations. Thus, our results support the theory of long-distance migration among *N. lugens* populations. The genetic structure information of *N. lugens* in Java could support regional management, such as the new controlling strategies based on forecasting systems.

## 1. Introduction

*Nilaparvata lugens* (Hemiptera: Delphacidae) is an important insect planthopper of the rice ecosystem in three regions of Asia, i.e. South, East, and Southeast (IRRI 2009). The large number of *N. lugens* can destroy crops, which the condition called hopperburn. The *N. lugens* population is showed susceptible rice varieties and wing form response (Iwanaga *et al.* 1987). Imago of *N. lugens* has two forms of wings, i.e. brachypterous (short wings) and macropterous (long wings). The long wings form is triggered by high number of individual in the population, which causes long-distance movement behavior to survive their generations (Denno and Roderick 1990; Denno *et al.* 1991).

The movement of *N. lugens* in South, East, and Southeast Asia (not including Indonesia) has been investigated by using meteorological and molecular

approaches. The meteorological approach showed that the population of *N. lugens* has long-distance migration from Vietnam to China and Japan (Sogawa 1992; Otuka *et al.* 2008). The molecular approaches, i.e. mitochondrial and microsatellite markers were used to analyze the genetic structure of *N. lugens*. The mitochondrial sequences were analyzed by using cytochrome oxidase I (COI) and COII of *N. lugens* from 31 locations and indicated lack of genetic structure among Asian populations, except southern Philippines and Papua New Guinea (Matsumoto *et al.* 2013). Microsatellite marker was used to analyze the movement of this insect based on 30 polymorphic from two populations of *N. lugens* in China. However, this microsatellite marker is not clear to reveal the *N. lugens* movement pattern yet, because the numbers of the samples are low (Jing *et al.*, 2012).

The spatial distribution of *N. lugens* was investigated at five study sites in West Java (Indonesia), i.e. Cirebon, Indramayu, Jatisari, Karawang, and Subang. The result of this method revealed the movement among those

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areas, however, could not show the movement pattern (Kusmayadi *et al.* 1990; Sawada *et al.* 1992, 1993). Therefore, the potential approach, i.e. genetic structure of *N. lugens* in Java has been challenged to elucidate this insect movement. The objective of this study was to analyze the genetic structure of *N. lugens* populations in Java (Indonesia) using molecular approach. This study is the first genetic structure data of *N. lugens* populations in Java (Indonesia) that inferred from mitochondrial (combined of COI and COII genes) and microsatellite markers. This study could reveal the migration among *N. lugens* populations in Java and provide an ecological foundation for developing a better pest management strategy.

## 2. Materials and Methods

### 2.1. Insect Collection

Samples of *N. lugens* macropterous imago were collected from six rice fields in Java, i.e. Lebak, Bogor, Karawang, Boyolali, Pemalang, and Lamongan. Thirty of *N. lugens* were collected from each location and were used for DNA extraction.

### 2.2. DNA Extraction and Amplification

Thirty individuals from each location were used for DNA source from the thorax of *N. lugens* by using a Genomic DNA mini kit for tissue (Geneaid). Those were used to amplify the mitochondrial and microsatellite DNA.

Mitochondrial DNA amplifications used forward primer NLCOIF10: 5'AGAT-TCTGACTTTACCCCATC'3 and reverse primer NLCOIR8:5'CTATTGGAGGATTAACA GGTGTA'3 for COI gene and forward primer NLCOIFF1: 5'TCTAATATGGCAGAACATGTGA'3 and reverse primer NLCOIR1: 5'CTATTGGAGGATTAACAGGTGTA'3. The primers of COI and COII genes were designed based on *N. lugens* mitochondrial genome (Zhang *et al.* 2013). The amplification of this mitochondrial marker were performed in 30 cycles: denaturation at 95°C for 1 min, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec. Total of 72 amplicon of COI and COII genes were sequenced by using same primer pairs as in the amplifications.

Microsatellite DNA amplifications were selected from ten polymorphic microsatellite loci based on Jing *et al.* (2012) of the *N. lugens* in China and were amplified using fluorescently labeled primers. The PCR cycling program was 94°C (5 min), followed by 35 cycles of 94°C (15 sec), and 72°C (30 sec), with a

final elongation step of 72°C (10 min). We genotyped about 81 of *N. lugens* in a Genescan facilities Applied Biosystems Genetic Analyzer and interpreted using the GeneMapper® v 4.0 analysis software (Applied Biosystems).

### 2.3. Statistical Analysis

Genetic diversity. The total 32 combined of COI-COII mitochondrial sequences of *N. lugens* were analyzed by using CLUSTAL X(1.83)(Thompson *et al.* 1997) and MEGA 6 (Tamura *et al.* 2013). Genetic diversity of combined COI-COII sequences were conducted by using DnaSP version 5 (Rozas *et al.* 2003) to estimate the number of polymorphic sites (S), nucleotide diversity ( $\pi$ ), and haplotype diversity (h) of the *N. lugens*.

The microsatellite data were assembled using the GENEMAPPER software and determined for stuttering, large allele dropout and null alleles using MICRO-CHECKER version 2.2.3 (van Oosterhout *et al.* 2004). The genetic diversity was analyzed by using MICROSATELLITE ANALYZER (MSA) version 4.05 (Dieringer and Schlötterer 2003) to test the allele frequency, the number of alleles, observed and expected heterozygosity. The tests for deviations from Hardy-Weinberg equilibrium for each population were performed by using GENPOP version 4.0.11 (Raymond and Rousset 1995).

Demographic analysis. This method was conducted to test the neutrality of each population that examined from combined COI-COII sequences. The demographic history of *N. lugens* populations in Java were analyzed by the Tajima's D and Fu's F statistics. The distributions of pairwise differences between individual sequences were analyzed using mismatch distribution analysis. Demographic analysis was performed using the program ARLEQUIN version 3.5 (Excoffier and Lischer 2010).

Population structure. The COI-COII genes and the microsatellites DNA were investigated using the pairwise fixation indices (FST) approach implemented in the program ARLEQUIN version 3.5. It was used to analyze the genetic structure based on the pairwise FST values between each pair of the six populations. The values of fixation indices among populations within populations ( $F_{ST}$ ) were compared with different group numbers within each dataset. Unrooted neighbor-joining (NJ) trees were constructed based on FST values of microsatellite by using TreeFit (Kalinowski 2009).

Isolation by distance. The pairwise  $F_{ST}$  values based on the COI-COII genes and the microsatellites DNA were analyzed to establish whether any isolation-by-distance effect occurred, matrices of genetic distance

data [ $F_{ST} / (1 - F_{ST})$ ] and the logarithms of geographical distance ( $\ln \text{Km}$ ) between all the sampling sites were constructed. The  $\ln \text{Km}$  value was conducted by using Geographic Distance Matrix Generator version 1.2.3 (Ersts 2006). These matrices were analyzed to determine the degree of correlation using a Mantel test (Mantel 1967) implemented in the software IBD version 1.53 (Bohonak 2002).

Gene flow. The gene flows among populations were examined using Migrate version 3.2.16 (Beerli and Felsenstein 2001). This analysis was conducted to test the asymmetric dispersal between populations, the mutation-scaled population size ( $\Theta = N\mu$ , where  $\mu$  is the mutation rate per site per generation) and the mutation-scaled migration rate ( $M = m/\mu$ , where  $m$  is the migration rate) were calculated using Bayesian. The effective number of migrants entering and leaving each population per generation  $xNem$  is  $\Theta M$  ( $x$  is a multiplier that depends on the ploidy and inheritance of the data, and here,  $x$  is one for mitochondrial DNA).

### 3. Results

#### 3.1. The *Nilaparvata lugens* Collection

Six locations of *N. lugens* in Java sampled and identified as a central of rice field in Indonesia and those are not isolated area. All rice sampling locations were cultivated the Ciherang rice variety, except Karawang rice field that used Mikongga variety. In this field study, we observed *N. lugens* outbreak status in the rice field area in Bgr and Lmg. For genetic structure analysis, we conducted in total 36 individual of combined COI-COII sequences and 81 individual of microsatellites marker (Table 1).

#### 3.2. Genetic Diversity

Genetic diversity analysis based on combined of COI and COII genes showed highly haplotype diversity ( $h = 0.600 - 0.933$ ) but low genetic diversity ( $\pi = 0.00000 - 0.0025$ ). The demographic history, Tajima's D calculation showed no significantly positive in the four populations ( $D = 0.00000 - 0.70767$ ;  $p > 0.005$ ), except Pml and Lmg populations showed no significantly negative. Similar tendencies with Tajima's D calculation were found based on Fu's F statistics (Table 2), thus might indicate of genetic bottleneck history of this insect based on the parameters of genetic diversity and demographic history.

Based on the microsatellite loci we found the number of alleles was ranged from 71 to 83 from the six populations of *N. lugens*. The expected heterozygosity

(He) was ranged from 0.768 to 0.901 while the observed heterozygosity ( $H_o$ ) was ranged from 0.949 to 1.000. Furthermore, test of Hardy-Weinberg equilibrium resulted no significant deviation ( $P_{HWE} > 0.05$ ) among the ten loci in *N. lugens* populations (Table 3), thus *N. lugens* population is in HWE expectation. It means that the deviations from HWE (when  $H_o > He$ ) were due to the heterozygote excess. This heterozygosity analysis showed high levels of variation in general might result from an isolated-breaking.

#### 3.3. Population Structure

Pairwise fixation index ( $F_{ST}$ ) values were calculated for all combinations of six locations based on two type molecular marker. The combined COI-COII marker showed no significantly high values (0.37500;  $p > 0.05$ ) in Bogor population (Table 4). It showed that differentiation population with other population. Whereas, the pairwise  $F_{ST}$  based on microsatellites marker showed no significantly low values (less than 0.02000;  $p > 0.05$ ) (Table 5). Furthermore, the pairwise  $F_{ST}$  values of microsatellite data were used to construct the unrooted NJ tree that revealed no genetic structure among *N. lugens* populations across Java (Figure 1). Moreover, both of genetic markers showed the negative value of pairwise indicated lack of genetic structure (exclude Bogor population by using mitochondria sequences).

#### 3.4. Isolation by Distance

The Mantel test results for two type molecular marker showed high r-value (for 10 000 randomizations), i.e. 1) 0.0231 ( $P = 0.916$ ) based on combined COI-COII genes (Figure 2) and 2). 0.0494 ( $P = 0.801$ ) based on microsatellite marker (Figure 3). Those are indicating no isolation-by-distance among *N. lugens* populations in Java. Furthermore, it was supported by scatter plot analysis that showed no correlation between genetic and geographical distance among the 15 populations pairs.

#### 3.5. Gene Flow

The unidirectional estimates of gene flow ( $M$ ) ranged from 0.0 ( $\text{Krw} \rightarrow \text{Pml}$ ) to 597.4 ( $\text{Krw} \rightarrow \text{Lmg}$ ) that conducted by using the mitochondrial marker. The estimates of  $M$  analysis between each pair of the six populations showed that high gene flow values. Although, a small amount of three pairs of population showed low gene flow, i.e.:  $\text{Lbk} \rightarrow \text{Lmg}$ ,  $\text{Krw} \rightarrow \text{Pml}$ , and  $\text{Byl} \rightarrow \text{Lmg}$  (Table 6).

Table 1. Collection data of the *N. lugens* populations that used in this study

Population	Location	Date	Latitude, longitude	Total of individual	
				COI-COII mitochondrial	10 loci microsatellite
Lbk	Lebak, Banten	June 8' 2014	06°20.189"S, 106°05'465"N	6	13
Bgr	Bogor, West Java	March 10 and 11' 2014	06°25'872"S, 109°55'359"N	6	14
Krw	Karawang, West Java	April 19-21' 2014	06°05'256"S, 107°55'322"N	6	14
Byl	Boyolali, Central Java	April 9-13' 2014	07 °32'736"S, 110°43'026"N	6	14
Pml	Pemalang, Central Java	June 17' 2014	06°53.104'S, 109°27'654"N	6	13
Lmg	Lamongan, East Java	March 21-30' 2014	07°05'769"S, 112°16'628"N	6	13
Total				36	81

Table 2. Parameters of genetic diversity and demographic analysis of *N. lugens* lugens based on combined COI and COII genes

Population	<i>S</i>	$\pi$	<i>h</i>	<i>D(p)</i>	<i>Fs(p)</i>
Lbk	7	0.0024	0.0024	0.0024	0.31383 (0.51100)
Bgr	0	0.0000	0.0000	0.0000	0.00000 (1.00000)
Krw	7	0.0025	0.0025	0.0025	0.42629 (0.55300)
Pml	8	0.0022	0.0022	0.0022	-1.37181 (0.10000)
Byl	5	0.0019	0.0019	0.0019	1.41962 (0.78500)
Lmg	8	0.0025	0.0025	0.0025	-1.12062 (0.13500)
Total	48				

Number of polymorphic sites (*S*), Nucleotide diversity ( $\pi$ ), Haplotype diversity (*h*), Tajima's D (*D*), Fu's F statistics (*Fs*), Code of population refers to Table 1

Table 3. Summary statistic showing number of alleles and mean of heterozygosity of *N. lugens* lugens from 10 loci microsatellite

Population	No. of alleles	<i>Ho</i>	<i>He</i>	<i>P<sub>HWE</sub></i>
Lbk	72	0.949	0.770	0.785
Bgr	79	1.000	0.768	0.643
Krw	83	0.984	0.887	0.667
Pml	71	0.981	0.901	0.807
Byl	78	0.990	0.868	0.674
Lmg	83	1.000	0.799	0.660
Total				

*Ho* = observed heterozygosity, *He* = expected heterozygosity, HWE = hardy-weinberg equilibrium, code of population refers to Table 1

Table 4. Pairwise fixation index ( $F_{ST}$ ) values of *N. lugens* based on the combined COI and COII mitochondrial sequences

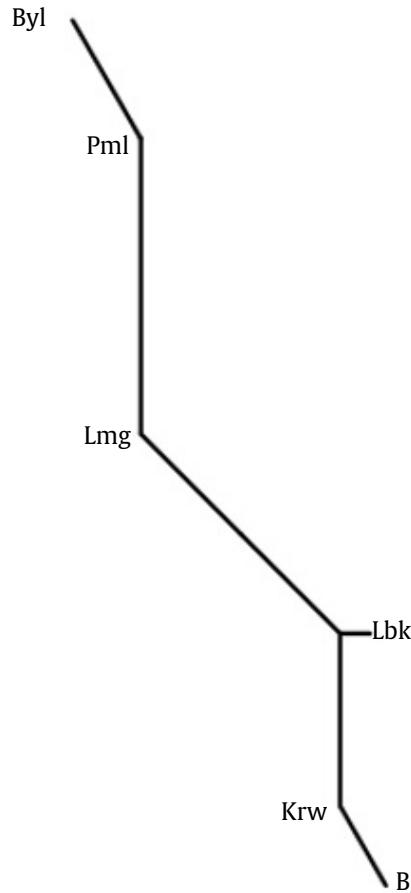
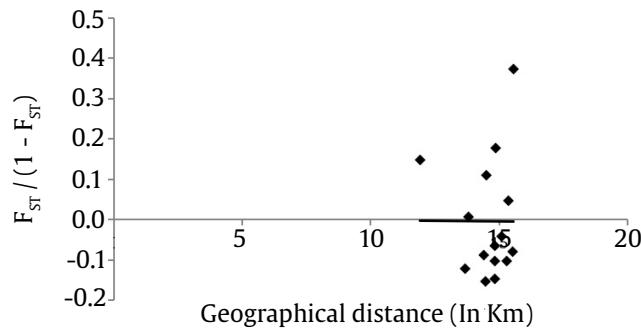
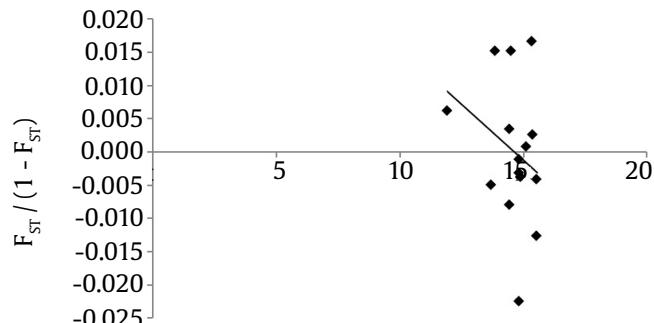
Population	Lbk	Bgr	Krw	Pml	Byl	Lmg
Lbk	0					
Bgr	0.14545	0				
Krw	-0.07778	0.37500	0			
Pml	-0.10204	0.04444	0.00357	0		
Byl	-0.14545	0.17778	-0.06531	-0.09091	0	
Lmg	-0.15200	0.10909	-0.04211	-0.10400	-0.12174	0

Code of population refers to Table 1

Table 5. Pairwise fixation index ( $F_{ST}$ ) values of *N. lugens* based on ten loci microsatellites

Population	Lbk	Bgr	Krw	Pml	Byl	Lmg
Lbk	0					
Bgr	0.00635	0				
Krw	-0.00410	-0.01268	0			
Pml	0.01662	0.00258	-0.02107	0		
Byl	-0.00108	-0.00364	-0.02241	-0.00812	0	
Lmg	0.00350	0.01523	0.00070	-0.00308	-0.00498	0

Code of population refers to Table 1

Figure 1. Unrooted NJ trees of six populations *N. lugens* in this study based on FST value of microsatelliteFigure 2. Scatter plot of genetic distance vs. geographical distance for pairwise population comparisons ( $r = 0.0231, P = 0.916, 10\,000$  randomizations), based on combined COI-COII mitochondrial sequences of *N. lugens*Figure 3. Scatter plot of genetic distance vs. geographical distance for pairwise population comparisons ( $r = 0.0494, P = 0.801, 10\,000$  randomizations), based on ten microsatellite loci of *N. lugens*Table 6. Estimates of gene flow (M) among six *N. lugens* populations based on combined COI-COII genes

Pop., i	$\Theta_i$	$Lbk \rightarrow i$	$Bgr \rightarrow i$	$Krw \rightarrow i$	$Pml \rightarrow i$	$Byl \rightarrow i$	$Lmg \rightarrow i$	Total $i$
Lbk	0.00446	-	498.7	496.6	493.0	497.2	6.2	1,991.7
Bgr	0.05008	514.9	-	507.5	489.1	492.9	485.0	1,974.5
Krw	0.04941	497.7	350.1	-	509.2	500.9	545.2	1,905.4
Pml	0.00287	502.9	502.9	0.0	-	500.4	0.1	1,003.4
Byl	0.04881	507.2	498.2	489.2	33.5	-	13.6	1,034.5
Lmg	0.04940	4.2	491.0	597.4	513.3	8.5	-	1,610.2

$\Theta$ : mutation-scaled population size, which is effective population size  $\times$  mutation rate per site per generation, M: mutation-scaled immigration rate, which is the immigration rate divided by the mutation rate

#### 4. Discussion

The genetic diversity of *N. lugens* in Java based on the combined COI and COI sequences, showed low levels nucleotide diversity ( $\pi$ ) and high levels haplotype diversity (h). Mean of heterozygosity value also showed high level of variations by using microsatellite marker. Furthermore, the demographic analyze showed in selective neutrality ( $p>0.05$ ) with a stable effective population size and thus implied genetic bottleneck history in Java population. This genetic diversity based of two types of molecular marker indicated that the *N. lugens* population in Java had reached stability in gene frequency (Sun *et al.* 2015) that was also analyzed in *Laodelphax striatelluss* (Hemiptera: Delphacidae) from China. The *L. striatelluss* has been revealed the bottleneck history and this conclusion was supported by both genetic diversity and demographic analysis by using mitochondrial and microsatellite markers.

The high haplotype diversity might due to the adaptation of this insect to crop cultivated (Wei *et al.* 2013). A high-level haplotype condition of *N. lugens* also found in *Plutella xylostella* (Lepidoptera: Plutellidae) from China by using microsatellite marker. The key factor leading to high haplotype diversity might be indicated by both of the high level of selection pressure of insecticide and local climate. Furthermore, Matsumoto *et al.* (2013) also explained that the level of insecticide resistance is related to the genetic structure of populations reflects gene flow over a longer time scale. The pairwise fixation indices ( $F_{ST}$ ) results indeed suggest no genetic structure in *N. lugens* populations across Java.

Moreover, we suggest that lack of genetic structure information of this study to be relatively similar between the outbreak and non-outbreak events. All the sampling areas consisted of two-rice field status, i.e. outbreak (Bgr and Lmg) and non-outbreak status (Lbk, Krw, Byl, and Pml), and all sampling locations are not isolated areas. It showed in *Locusta migratoria* (Orthoptera: Acrididae) as cosmopolitan insect that suggested the outbreak and non-outbreak events is not influence with the genetic structure of locust (Chapuis *et al.* 2008) by using microsatellite marker. Furthermore, this condition has been considered mostly the typical of migratory of this insect.

In the present study, the Mantel test indicated unparallel between genetic and geographical distance among the six populations of *N. lugens* in

Java. It revealed no isolation-by-distance, as in *P. xylostella* (Lepidoptera: Plutellidae) in China revealed by mitochondrial and microsatellite markers (Wei *et al.* 2013). Furthermore, the gene flow approaches indicates that the *N. lugens* populations migrates in Java. The gene flow of most pairs populations are consistent and indicate high level values. it avoids genetic drift from causing local genetic differentiation, which the species with typical of migratory abilities generally show correspondingly high levels of gene flow (Slatkin 1987). These results could be indicated the great movement abilities with the genetic mixing that occurred among *N. lugens* in Java populations. A similar condition also showed in *N. lugens* among Asia populations by using mitochondrial sequences that showed the genetically intermixed. It also could be correlated with long-distance migration theory of *N. lugens* that migrate from tropical (northern Vietnam) in April-May to temperate region (China, Korea, and Japan) in (June-July) as demonstrated based on meteorological studies (Otuka *et al.* 2008). The *N. lugens* population is long-distance migratory flights from tropical to temperate Asia regions before modern pesticides were widely used on tropical rice. Because of the infrequent pre-1960s use of insecticides in the tropics, factors other than insecticides likely triggered the movement of long wings form populations of *N. lugens* (Bottrell and Schoenly 2012).

The homogeneity genetic diversity in *N. lugens* populations of Java might be maintained by extensive migration. The rice planthopper problem is occurred since the green revolution in 1960s in Indonesia; i.e. utilizing of later improved cultivars and pesticides (IRRI 2009). Accordingly, we hope that the lack of genetic structure information of *N. lugens* could support the monitoring and tracing planthopper migration routes. The migration of the insect is confirming, than combine with findings on wing polymorphism (Iwanaga *et al.* 1987; Iwanaga and Tojo 1988) and biotype property of brown planthopper (Sogawa 1982). Thus the new controlling strategies based on forecasting systems could be developed for regional management of this insect.

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