RESEARCH ARTICLE

Genetic variability of *Nilaparvata lugens* (Stål) from Northern Malay Peninsula using microsatellite markers

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***Nilaparvata lugens* or brown planthopper is widely known as one of the primary pests of rice capable of causing significant yield losses. Therefore, this work was geared towards assessing the genetic variability of 161 individual *N. lugens*, which were collected from six different populations located in the Northern part of the Malay Peninsula. Analysis carried out using 11 microsatellite markers revealed the presence of 196 alleles, which were characterized with 17.818 alleles per locus on average. The allele number ranged from 7 (BM435) to 34 for marker NLES33. Meanwhile, gene diversity and heterozygosity were ranged from 0.429 (BM435) to 0.918 (NLES33) and from 0.264 (BM435) to 0.795 (NLES22), respectively. The polymorphic information content (PIC) value varied from 0.387 (BM435) to 0.914 (NLES33), yielding 0.769 per locus on average. Further structure analysis delineated two structure groups present, whereas analysis of molecular variance (AMOVA) analysis suggested low genetic variation among the population and high genetic variation among the individual across the population. As such, understanding the genetic variability of *N. lugens* will significantly aid in strategizing and managing a case of its infestation, as well as preventing it.**

Keywords: Brown planthopper; genetic diversity; simple sequence repeat (SSR) marker.

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Introduction

Nilaparvata lugens, which is otherwise typically known as brown planthopper, is an economically important pest that can cause significant yield losses in the context of global rice production. In the case of severe infestations, it may result in a completely wilted and dried rice plants; this occurrence is also termed as 'hopperburn' [1]. Furthermore, the species can lead to rice destruction due to its role in disseminating the ragged stunt and grassy stunt viruses. This insect is capable of completing up to 12 generations annually when found in tropical localities in which it is prevalent throughout the year, whereas it serves as a migratory species in temperate regions and yield fewer life cycles. The history of rice cultivation has been decorated with various brown planthopper's episodic outbreaks, which have increased in frequency and intensity following the production of improved rice varieties and yield-focused farming practices in the 1960s around the time of green revolution [2].

Due to its status as a prominent migratory pest,

Table 1. Six locations of sample collection.

No	Location	GPS Point	Number of samples	Code
1	Titi Serong, Perak	5.103827,100.459924	18	Titi Serong
2	Telok Chengai, Kedah	6.102412, 100.328299	20	T Chengai
3	Sungai Limau, Kedah	5.899790, 100.369290	11	Sg Limau
4	Kubang Rotan, Kedah	6.133460, 100.286003	19	Kbg Rotan
5	MARDI Seberang Perai, Kepala Batas, Penang	5.544217, 100.467349	74	S Perai
6	Arau, Perlis	6.384027,100.246630	19	Arau

understanding the species' diversity and variability is henceforth highly significant. To this end, DNA marker has emerged as the best tools to evaluate the element of genetic diversity in which microsatellite is one of the common types of DNA typically utilized in genetic research. Microsatellite is also known as simple sequence repeats (SSR) and has remained as the most trustworthy marker for plant genotyping for longer than the past 20 years. This is attributable to its characteristics of being informative, highly polymorphic, multi-allele and co-dominant in nature, experimentally reproducible, and transferable across the species [3]. As a result, microsatellite is deemed as greatly useful and consistently employed to assess the genetic diversity, engage in cultivar or biotype identification via DNA fingerprinting, linkage and QTL analysis, and even in evolutionary studies [4]. Therefore, this work aims to evaluate the genetic variability of *N. lugen* sourced from six populations located in the Northern part of Malay Peninsula by using a microsatellite marker. By understanding their variability, it will allow further strategizing and managing of rice plant cultivation practices in order to prevent brown planthopper infestation.

Material and methods

Sample collection and DNA extraction

In this study, 161 samples of *N. lugens* were acquired from six geographic sites in Malaysia, with a focus on the Northern part of Malay Peninsula (Table 1). For preservation purposes, the samples were kept in absolute alcohol before DNA extraction was carried out. Then, DNA of the samples were extracted by using the Macherey-Nagel DNA extraction kit for insect (Macherey-Nagel, Düren, Germany) following the manufacturing protocols. Following this, DNA integrity and concentration were measured using 0.8% agarose gel and Epoch spectrophotometer (Biotek, Winooski, VT, USA).

SSR genotyping

A total of 11 microsatellite markers (Table 2) were selected from previous studies [5] and subjected to polymerase chain reaction (PCR) as described by Schuelke [6], specifically by ligating both the primer sequence (i.e., either forward or reverse) and the fluorescent dye (i.e., FAM, PET, NED, or VIC) with the M13 sequence (5'-TGTAAAACGACGGCCAGT-3'). The detail of the primer and fluorescent dye combination were described in Table 2. In particular, the PCR cocktails consisted of 10 µL sample containing 1× buffer (Invitrogen, Carlsbad, California, USA), 10 µM of each forward and reverse primers, 5 µM of fluorescence labelled M13 adaptor, 2 µM of each dNTP (Invitrogen, Carlsbad, California, USA), 0.1 µL of bovine serum albumin (BSA) as the PCR enhancer, and 1 unit of Taq polymerase (Invitrogen, Carlsbad, California, USA). The PCR amplification was carried out by employing a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, California, USA). The PCR profile was obtained via the processes of pre-denaturation for 2 min at 94°C, following by 35 cycles of 94°C for 30 s, 41–65°C for 45 s, and 72°C for 45 s. The PCR reaction was subjected to post-extension at 72°C for 5 min. Following the amplification process, the PCR products obtained were multiplexed up to four primers with different combinations of fluorescent dyes. The

Table 2. List of microsatellites used in this study.

Marker name	Repeat motif types	Forward primer (5' to 3')	Reverse primer (5' to 3')	Expected product size (bp)
BM376	(TG)10	GCATCGAATGTGAAATGAGT	CTAGCCAAGGAGGACTTGTT	246
BM410	(TA)9	TTGAACCATGGAATATCAGC	TGGAAAGATTGGAAATGGTA	270
BM435	(TA)7	CCTAACACTTAATGCGTGCT	CCAACAATACAATTCGTCGT	124
BM488	(AG)7	GCCTACAAGATCTTCCCCTA	GGATGAAGCAATGAACACAT	203
BM489	(AG)7	GCCTACAAGATCTTCCCCTA	TTTGTTAGGATGAAGCGATG	206
NLES2	(CTT)23	CACGTGCAGTCAAACCAAAG	CGAGAGCCTCGAATATTGTTCT	166
NLES22	(CT)12	AAAACAACCGCTCTATTGCTC	TCGGCATTCTCTCTGTTTCA	188
NLES24	(TC)12	AGCTCCAAGAAATGACAGGTG	CCTTTCTTTCAAACAAGGTTAACAA	213
NLES26	(TAT)12	GCTAACAGTGCAAATTTATTCTAAACA	GATGAAGCATAGCAGCAGGA	222
NLES33	(AGG)11	GCATGAATGTCACTTCGAGA	CAAGACACCTCCAGTGCATA	203

next step requiring the products to be mixed with Hi-Di formamide and GeneScan 500 LIZ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) as a standard molecular weight ladder prior to be resolved via an ABI 3130xL Genetic Analyser (Applied Biosystems, Foster City, California, USA). The GeneMapper Version 5 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to score the size of the alleles, whereby the electropherograms produced were scored and analyzed accordingly as described by Arif, *et al*. [7].

Data analysis

In this phase, PowerMarker [8] was used to calculate the number of alleles, major allele frequency, gene diversity, extent of heterozygosity, and polymorphism information content (PIC) of each microsatellite marker. Using the same software, a pairwise genetic distance based on shared alleles was also generated. Furthermore, MEGA7 was employed to visualize the neighbor joining dendrogram tree, whereby it was constructed by applying the matrices of genetic distance of the shared microsatellite alleles [9].

Next, the Principle Component Analysis (PCoA) and AMOVA was undertaken by using GenAlEx 6 [10], whereas STRUCTURE (Version 2) software [11] was employed to determine the structure group of the overall biotypes. This process involved the calculation of the K values (i.e., population number), which was obtained by varying the K values extending from 1 to 10 and with 20 independent runs per K value. This was carried out under the conditions of 50,000 burn-in period and 100,000 Markov chain Monte Carlo (MCMC) repetitions. Then, computations of the optimal K value were done by using web-based Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester/) [12] and the formulae described by Evanno, *et al*. [13].

Results

Characterization of microsatellite marker

Analysis of 11 microsatellite marker successfully revealed the presence of 196 alleles ranging from 7 (marker BM435) to 34 for marker NLES33, wherein 17.818 alleles per locus were obtained on average. Furthermore, the major allele frequencies extended from 0.154 (NLES22) to 0.736 (BM435), with an average of 0.348. Meanwhile, the gene diversity and heterozygosity values varied from 0.429 (BM435) to 0.918 (NLES33) and from 0.264 (BM435) to 0.795 (NLES22), respectively. Finally, the PIC values were in the range of 0.387 (BM435) to 0.914 (NLES33), yielding an average of 0.769 per locus. The details of each microsatellite marker are summarized accordingly in Table 3.

Table 3. Microsatellite characterization of six *N. lugens* populations.

Marker	Major Allele Frequency	Allele No	Gene Diversity	Heterozygosity	PIC
BM13	0.250	10.000	0.839	0.569	0.820
BM376	0.434	16.000	0.744	0.735	0.716
BM410	0.614	10.000	0.569	0.423	0.527
BM435	0.736	7.000	0.429	0.264	0.397
BM488	0.248	15.000	0.854	0.427	0.839
BM489	0.259	12.000	0.830	0.503	0.810
NLES2	0.174	24.000	0.906	0.579	0.899
NLES22	0.154	19.000	0.911	0.795	0.904
NLES24	0.361	22.000	0.818	0.764	0.802
NLES33	0.224	34.000	0.918	0.290	0.914
NLES26	0.370	27.000	0.836	0.536	0.828
Mean	0.348	17.818	0.787	0.535	0.769

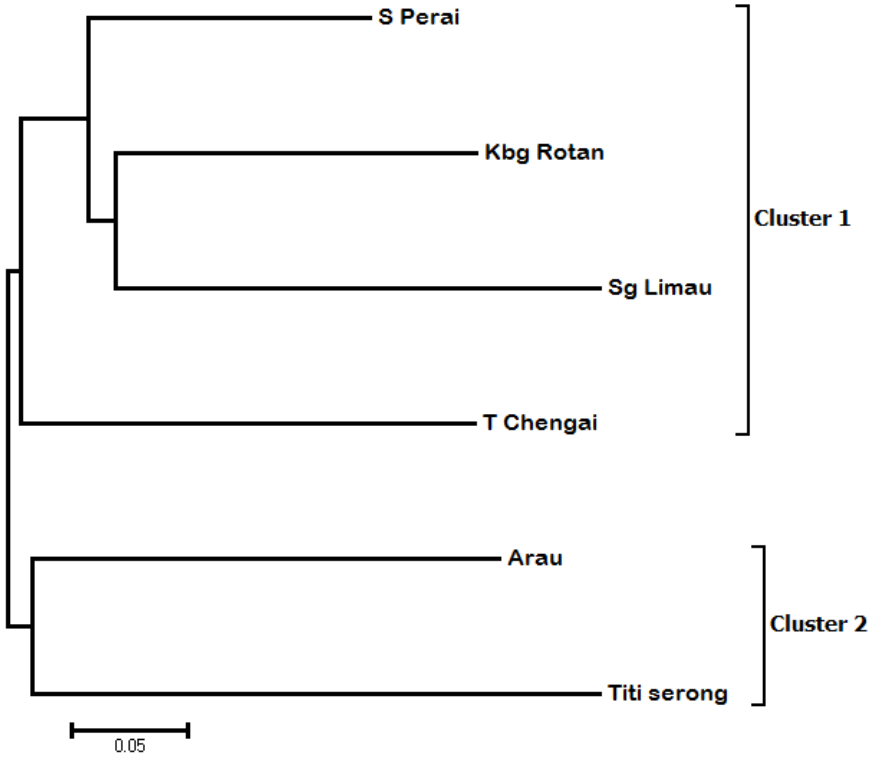

Figure 1. Neighbor joining dendrogram tree of six *N. lugens* population by using 11 microsatellite markers.

Genetic variability of *N. lugens*

The neighbor joining dendrogram (Figure 1) was generated from the shared allele genetic distance. It suggested that the populations could be demarcated into two clusters; here, Cluster 1 consisted of *N. lugens* from the populations of Kbg Rotan, Sg Limau, Seberang Perai, and Teluk Cengai, while Cluster 2 consisted of those obtained from the populations of Arau and Titi Serong. Moreover, the pairwise genetic distance

Table 4. Pairwise genetic distance of six *N. lugens* population by using 11 microsatellite markers.

OTU	Arau	Kbg_Rotan	S_Perai	Sg_Limau	T_Chengai	Titi_serong
Arau	0.000	0.441	0.394	0.418	0.435	0.453
Kbg_Rotan	0.441	0.000	0.282	0.370	0.389	0.502
S_Perai	0.394	0.282	0.000	0.357	0.342	0.417
Sg_Limau	0.418	0.370	0.357	0.000	0.471	0.497
T_Chengai	0.435	0.389	0.342	0.471	0.000	0.449
Titi_serong	0.453	0.502	0.417	0.497	0.449	0.000

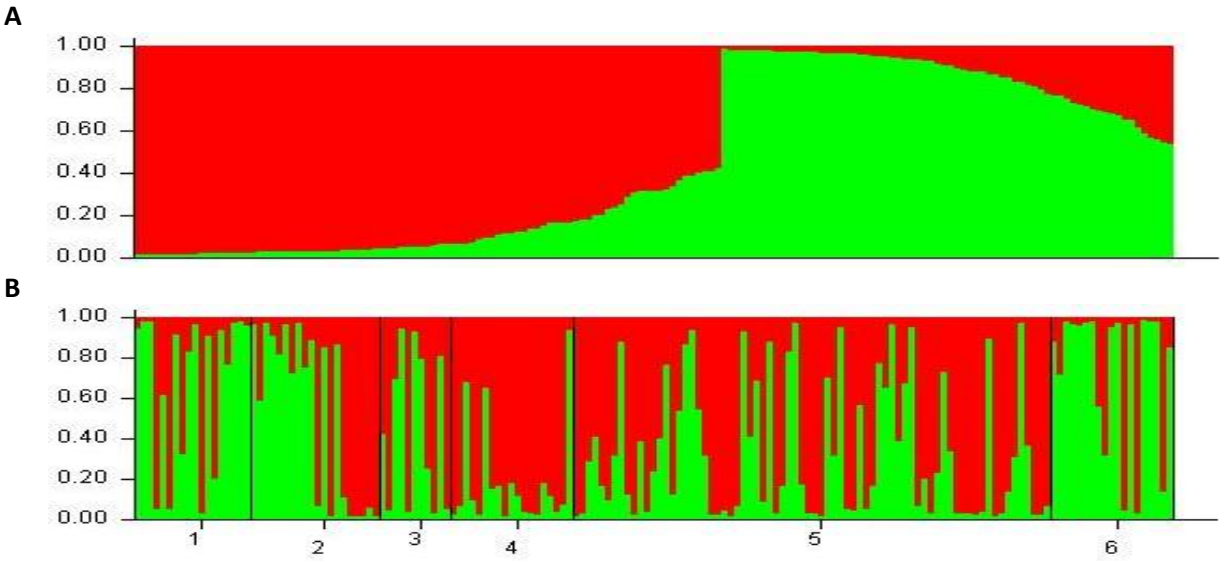

Figure 2. Population genetic structure diagram produced by the STRUCTURE program, when K = 2, based on 11 polymorphic SSR markers. **A.** The population genetic structure produced by the structure program, when K = 2 as computed by ΔK = m([L''K])/s[L(K)] based on 20 polymorphic SSR markers. The diagram indicates two genetically well-defined individual groups among the accessions. **B.** when K=2 and sorted by their respective population: 1 = Titi Serong, 2 = Telok Cengai, 3 = Sg Limau, 4 = Kbg Rotan, 5 = Seberang Perai, 6 = Arau.

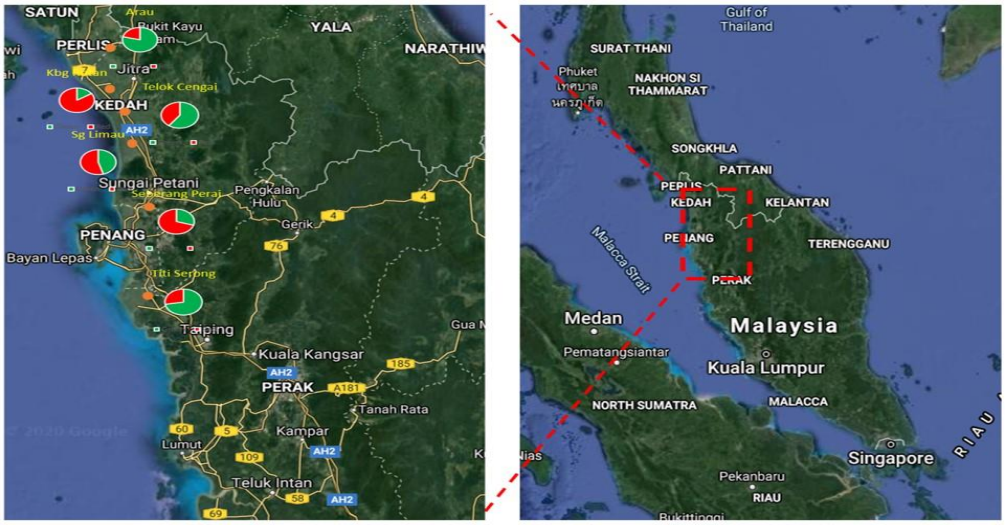

Figure 3. Structure groups based on the population localities in the Northern Peninsular Malaysia. Red color represents Red Structure Group, Green color represents Green Structure Group.

Table 5. Summary of AMOVA for 161 individuals encompassing six populations.

Source	df	The sum of squares	Mean squares	Estimate of variance	%
Among Pops	5	60.983	12.197	0.127	3%
Among Individual	155	969.055	6.252	1.815	40%
Within Individual	161	422.000	2.621	2.621	57%
Total	321	1452.037	–	4.563	100%

yielded values ranging from 0.2824 (Seberang Perai ↔ Kbg Rotan) to 0.5018 (Titi Serong ↔ Kbg Rotan). The details of the pairwise genetic distance as describe in Table 4.

Next, a plot of ΔK against the number of K as detailed by Evanno, *et al*. [13] showed that the optimal ΔK was at K=2 (Figure 1). Here, all of the *N. lugens* individuals were clustered accordingly into two major groups, namely the green and red structure groups. A bar plot when K=2 is thus described in Figure 2. Analysis by population thus revealed that the populations of Titi Serong and Arau consisted with a majority of the green structure group, whereas a dominant percentage of Kbg Rotan and Seberang Perai populations came from red structure group (Figure 3). Besides, an AMOVA analysis conducted on all six populations revealed that only 3% variance was recorded among them. In contrast, 40% variance was obtained among the individuals, while 57% variance was logged within the individual (Table 5).

Discussion

Microsatellites are persistently deemed the most favorable type of molecular marker for the purpose of plant genotyping over the past two to three decades and more. This can be correlated with their informativeness, codominant inheritance, highly polymorphic and multi-allelic in nature, experimentally reproducible, and transferable across the species [3], which make it suitable and favorable for various genetic studies [4].

In this study, however, the philosophy of Hildebrand, *et al*. [14] was adhered to and nine microsatellite markers (81.8%), excluding BM410 and BM435, were thus considered as highly informative (i.e., PIC value > 0.7). Based on the AMOVA analysis, only 3% of the total variation was found among the populations, which might be contributed by the locality of the sampling site. This study focused on the Northern part of Malay Peninsula only, rendering it easier for *N. lugens* to migrate from one field to another field. Migration is an essential process undergone by a population and a basic characteristic of insect life cycles. It is undeniable that a concerted effort to understand the dynamics and endurance of insect populations is highly crucial [15]. Besides, highly migratory insects require a deeper consideration of their population attributes, which include virulence against resistant rice varieties, insecticide resistance, and winged response to density. Collectively, these elements may extensively differ between populations acquired from dissimilar geographic settings [16]. Furthermore, Hamrick and Godt [17] and Bustamante, *et al*. [18] have expressed the ways that population genetic structure and species variability are influenced by several evolutionary elements, such as migration system and gene flow, mating system, mode of reproduction, and natural selection. In this study, this was reflected in the significantly high level of variation detected within the individual in which it contributed to 57% of the total variation. It agreed with the current study performed small brown planthopper *Laodelphax striatellus* in Northeast China, where the genetic variation mainly from within the population with 87% level of variation. Besides, the study also revealed the presence of two clusters in their clustering tree and Structure analysis. As a high variation of the planthopper was detected here, the cultivation

of mono-variety would also lead to a severe infestation of the pest [19].

Conclusion

The present study succinctly revealed the applicability and suitability of microsatellite markers in measuring the genetic variation of *N. lugens* populations, which were acquired from the Northern region of Malay Peninsula. In general, various difficulties have been perceived in using traditional approaches such as morphological assessment, which are especially attributable to their diminutive size, short life cycle, large population size, rapid aerial population dilution, and the extended distances this insect can fly. However, microsatellite markers successfully showed their potential in overcoming a lot of the hindrances, thus yielding an extensive comprehension of the species and its population relationships. Hence, the results obtained in this study provide a deeper insight for breeders, entomologists, and geneticists to produce better strategizing and managing capacity to prevent future episodes of brown planthopper infestation in the local regions.

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