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Mitochondrial *cox* sequences of *Nilaparvata lugens* and *Sogatella furcifera* (Hemiptera, Delphacidae): low specificity among Asian planthopper populations

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Abstract

The brown planthoppers (BPH) *Nilaparvata lugens* (Stål) and the white-backed planthoppers (WBPH) *Sogatella furcifera* (Horváth) annually migrate from tropical and subtropical regions to temperate regions in Asia, including Japan, Korea and northern China. To elucidate the genetic divergence based on geography of planthoppers and to estimate their migration route on the basis of molecular data, we analysed a part of their mitochondrial genome sequences. Sequences of cytochrome oxidase subunit I (*cox1*) – transfer RNA for Leu (*trnL2*) – *cox2* were determined for 579 BPH (1,928 bp) and 464 WBPH (1,927 bp) individuals collected from 31 and 25 locations, respectively, in East and Southeast Asia. Thirty and 20 mitochondrial haplotypes were detected for BPH and WBPH, respectively. Single populations of both planthoppers included multiple haplotypes, and many haplotypes were shared in some populations and areas. The most frequently detected haplotypes accounted for approximately 50% of all BPH and WBPH individuals. To evaluate gene flow among planthoppers in different regions in Asia, pairwise fixation index (*Fst*) values were calculated. For BPH, high *Fst* values (0.580–0.926) were shown between planthoppers in Papua New Guinea (PNG) and the other areas and moderate *Fst* values (0.176–0.362) were observed between those in southern Philippines and other areas. For WBPH, the *Fst* value was the highest between Taiwan and southern Vietnam (0.236), and low among the other areas. AMOVA indicated no genetic structure among eight areas, excluding southern Philippines and PNG, for BPH, and among ten areas for WBPH. These data indicate that both planthoppers do not show much differentiation of local populations and/or have genetically intermixed Asian populations. These data also indicate that it may be difficult to distinguish regional planthopper populations on the basis of differences in mitochondrial sequences.

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Introduction

Three species of rice planthoppers are economically important in rice production in Asia: the brown planthopper (BPH) *Nilaparvata lugens*, the white-backed planthopper (WBPH) *Sogatella furcifera*, and the small brown planthopper (SBPH) *Laodelphax striatellus*. These three species are vectors of rice plant viruses (Hibino, 1979; Nemoto *et al*., 1994; Zhou *et al*., 2008). The former two species, BPH and WBPH, also cause sucking damage to rice plants; BPH, in particular, causes a severe form of damage, which is called hopperburn. Unlike SBPH, BPH and WBPH cannot overwinter in temperate Asian countries, including Japan, Korea and northern China. They annually migrate into Japan and Korea during *Bai-u* rainy season (June–July) after travelling long distances from the subtropical and tropical areas in lower altitudes, where rice is cultivated throughout the year (Kisimoto, 1976; Watanabe & Seino, 1991; Sogawa, 1992, 1995; Kisimoto & Sogawa, 1995; Otuka et al., 2005*a*, 2008). The migration of the species has been studied by such methods as observation on the ships (Asahina & Turuoka, 1968), meteorological analyses (Kisimoto, 1976; Seino *et al*., 1987) and mark-recapture experiments (Kiritani & Hirai, 1987). At present, BPH and WBPH migrating from northern Vietnam or southern China are considered as the primary sources of planthoppers in northeastern China, Korea and Japan. Furthermore, on the basis of meteorological computer simulations, Otuka *et al*. (2005*b*, 2008) suggested that BPH also migrated from northern Philippines to Taiwan and Japan.

For planthopper management, it is very important to know the characteristics of various biotypes in insect populations, such as insecticide susceptibility levels (Matsumura *et al*., 2008; Matsumura & Sanada-Morimura, 2010), tolerance profile against resistant rice varieties (Sogawa, 1992; Myint *et al*., 2009; Naeemullah *et al*., 2009) and wing-dimorphic traits (Morooka & Tojo, 1992). Matsumura *et al*. (2008) reported substantial degradation of susceptibility to neonicotinoids and phenylpyrazole, which are the insecticides most commonly used against BPH and WBPH in paddy fields of East and Southeast Asia. Resistance to these modern insecticides is becoming a serious problem in East and Southeast Asia. To determine the insecticide susceptibility of various phenotypes, potency assays of various insecticides in each population are necessary. However, such assays are time-consuming and labour-intensive. The virulence tests of planthoppers against planthopper-resistant rice varieties are similarly tedious. Therefore, simple methods for differentiation of phenotypes or biotypes need to be developed. In addition, prediction of planthopper occurrence and timely measures for planthopper control in East Asian countries require rapid determination of biotypes of immigrant planthopper populations.

In this regard, molecular markers linked with biotypes or representing regional populations will be extremely useful for planthopper management. Genome sequences can be handled easily with recent technology, because DNA is stable and can be isolated from individual planthoppers with ease. We had previously studied the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene of WBPH to distinguish regional populations in Southeast and East Asia (Fu *et al*., 2012). However, we did not find population-specific nucleotide sequence features; heterogeneity was found among rRNA gene copies of even single planthoppers. Since the ITS region showed too high a variation to be used as a molecular marker to discriminate local populations, we selected mitochondrial gene sequences as alternative candidates. Mitochondrial DNA sequences are often used as molecular markers for interspecies and inter-population differences. The population genetic structure of various species has been revealed using mitochondrial DNA sequences (Yoshida *et al*., 2001; Nobre *et al*., 2006; Cai *et al*., 2008; Meraner *et al*., 2008). Mun *et al*. (1999) reported genetic variation of BPH and WBPH populations among Asian populations within an 850 bp region of the mitochondrial cytochrome oxidase I (*cox1*). That pioneering study, involving 71 BPH and WBPH individuals, created the expectation that planthopper populations could be distinguished on the basis of mitochondrial molecular sequences by utilising longer sequences and larger samples from local populations.

In this paper, we extended the size of the mitochondrial sequence and increased the number of samples to further explore the utility of molecular markers in distinguishing subpopulations of BPH and WBPH, and furthermore, we analysed the genetic structure of East and Southeast Asian rice planthopper populations on the basis of their mitochondrial sequences.

Materials and methods

Insect samples

Thirty-one populations of *N. lugens* (Stål) (BPH) and 25 populations of *S. furcifera* (Horváth) (WBPH) were collected from ten areas [Japan, China, Taiwan, Laos, Thailand, northern and southern Vietnam (Vn and Vs), northern and southern Philippines (Pn and Ps), and Papua New Guinea (PNG)] from 1966 to July 2009 (fig. 1 and table 1). These populations were collected in the field or obtained from insects maintained in the laboratory for several generations (1 month to 40 years) after collecting adults (table 1).

DNA purification, polymerase chain reaction (PCR) and sequencing

Total DNA was extracted from individual insects by using DNeasy (Qiagen Inc., Valencia, CA, USA), and was eluted in 100–200 µl. Three to 21 individuals of respective populations were obtained for DNA extraction (table 1).

Mitochondrial genes were amplified by PCR using the primer pairs B_C1-J-1718 and B_C2-N-3665 for BPH, and C1-J-1718 and B_C2-N-3665 for WBPH (table 2). The basic PCR mixture (30 µl) consisted of 0.15 µM deoxynucleotides, 0.5 µM forward primer, 0.5 µM reverse primer, 1 µl of the PCR template, and 0.5 U of *Taq* DNA polymerase (TAKARA BIO Inc., Tokyo, Japan) in PCR buffer (TAKARA BIO Inc.).

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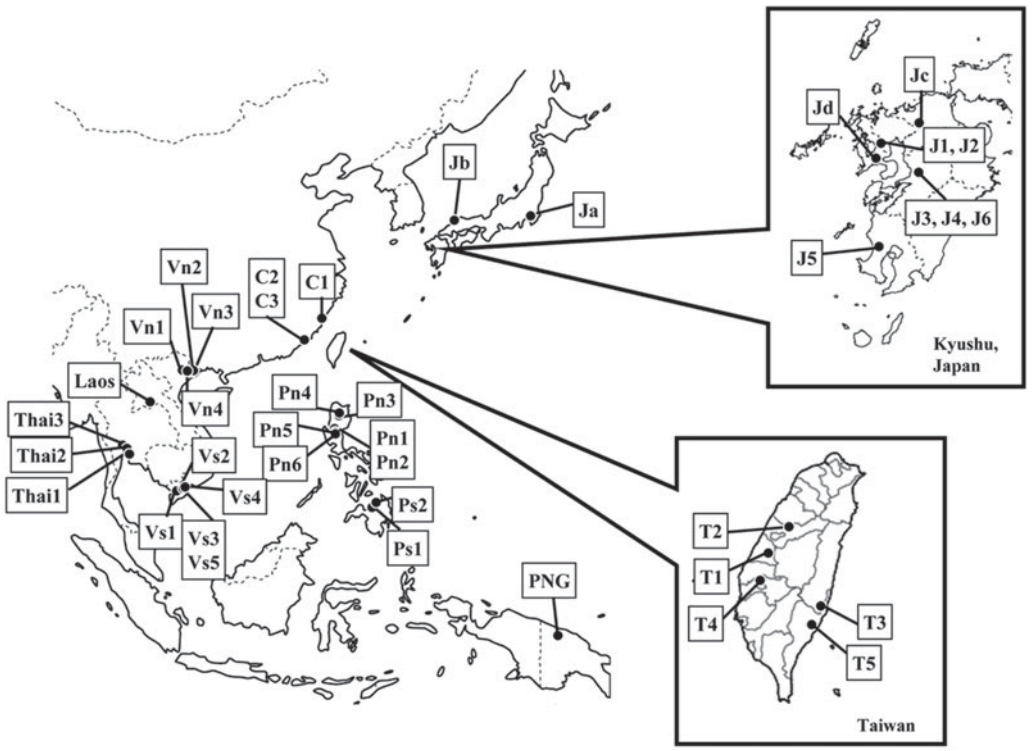

Fig. 1. Sampling sites of rice planthoppers in East and Southeast Asia; Japan (Ja–Jd and J1–J6), Taiwan (T1–T5), China (C1–C3), Vietnam (Northern area: Vn1–Vn4 and Southern area: Vs1–Vs5), Laos, Thailand (Thai1–Thai3), the Philippines (Northern area: Pn1–Pn6, Southern area: Ps1 and Ps2) and Papua New Guinea (PNG).

The PCR temperature profile was 94°C for 2 min, followed by 94°C for 30 s, 52°C for 30 s, 72°C for 2 min for 30 (for BPH) or 35 cycles (for WBPH), with a final extension at 72°C for 5 min. Electrophoresis in 1% (w/v) agarose/TAE gel was used to confirm the identity of the PCR-amplified products. The amplified fragments were purified using Sephacryl S-300 HR (GE Healthcare UK Ltd, Buckinghamshire, England) spin columns.

Using a sequencing kit (ABI PRISM Dye Primer Cycle Sequencing Kits; Applied Biosystems Division, Perkin-Elmer Inc.), we performed sequencing reactions with the DNA amplification products and the following primers designed for this study: B_f1, B_f2 and B_r1 for BPH, and W_f1, W_f2 and W_r1 for WBPH (table 2). The reaction products were sequenced using a DNA Sequence System (model 3700; Perkin-Elmer Inc.). Nucleotide sequences with lengths of 1,928 bp (BPH) or 1,927 bp (WBPH) encoding cytochrome oxidase I (*cox1*)–*trnL2*–*cox2* were determined.

Data analyses

The *trnL2* region was predicted using tRNAscan-SE 1.21 (Lowe & Eddy 1997, http://lowelab.ucsc.edu/tRNAscan-SE/). The default search mode was used (cove score cut-off ≥15), specifying mitochondrial/chloroplast DNA as the source and using the invertebrate mitochondrial genetic code for tRNA isotype prediction.

Median-joining (MJ) was performed using Network 4.5.1.0 (Bandelt *et al.*, 1999, http://www.fluxus-engineering.com/index.htm) with default conditions. The pairwise fixation index *Fst*, analysis of molecular variance (AMOVA) and Mantel test were estimated using ARLEQUIN 3.11 (Excoffier *et al.*, 2005). To test significance, the number of permutations was 1000. Unrooted neighbour-joining (NJ) trees were constructed based on *Fst* values using TreeFit (Kalinowski, 2009).

Migration rate between areas (2NM) were calculated using Isolation-with-Migration model (IMa2) (Hey, 2010). Mitochondrial mutation rate per locus per year were estimated 0.001434432 (BPH, 1,928 bp/locus) and 0.001433688 (WBPH, 1,927 bp/locus), under the conditions that mitochondrial mutation rate was 6.2×10^{-8}/single site/fly generation (Haag-Liautard *et al.*, 2008) and rice planthoppers would produce 12 generations per year. We used the HKY model, burnin period steps 100,000 run duration 150,000. Parameters were set based on population mutation rates ($4N_eu$) estimated using Arlequin3.11 (Excoffier *et al.*, 2005). Migration prior value was 0.56 and 0.76, maximum time of population splitting 2.24 and 3.02, and maximum for population size parameter 5.6 and 7.55 for BPH and WBPH (random number seed was 1234).

Results

Nucleotide sequences of the *cox1*–*trnL2*–*cox2* region of the mitochondrial genome were determined in 31 BPH populations (579 individuals) and 25 WBPH populations (464 individuals). There were no insertions or deletions in the sequences; the sizes were 1,928 bp and 1,927 bp for BPH and WBPH, respectively. There were no unexpected stop codons in the mitochondrial *cox1* and *cox2* genes of all haplotypes

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Table 1. Sampling data for BPH and WBPH populations.

Abbr.	Location	Latitude, longitude	Collection date	Sampling date	Sample number		No. [1]
					BPH	WBPH	
	Japan						
Ja	Kanagawa	35°22′29″N, 139°13′11″E	1966	15 February 2007	19		
Jb	Izumo, Shimane	35°22′1″N, 132°45′16″E	1987	26 October 2008	20	20	
Jc	Chikugo, Fukuoka	33°12′45″N, 130°30′8″E	1989	15 February 2007	20	20	
Jd	Nagasaki	32°50′38″N, 130°3′11″E	1999	15 February 2007	20		
J1	Ureshino, Saga	33°5′4″N, 129°56′4″E	4 September 2006	3 November 2006	20		2
J2	Ureshino, Saga	33°5′4″N, 129°56′4″E	15 June 2006	15 June 2006		12	
J3	Koshi, Kumamoto	32°52′26″N, 130°44′25″E	16 July 2005	16 July 2005	15		
J4	Koshi, Kumamoto	32°52′26″N, 130°44′25″E	26 June 2006	26 June 2006		20	3
J5	Minamisatsuma, Kagoshima	31°28′N, 130°20′E	5 July 2006	8 September 2006 8 October 2006	20	19	
J6	Koshi, Kumamoto	32°52′26″N, 130°44′25″E	2 July 2009	2 July 2009		13	
	Taiwan						
T1	Dacun, Changhua	24°0′2″N, 120°32′17″E	16 October 2006	3 November 2006	19	16	9
T2	Daya, Taichung	22°59′5″N, 120°59′15″E	9 November 2005	9 November 2005	19		
T3	Fuli, Hualien	23°11′33″N, 121°16′50″E	18 October 2006	22 November 2006	20		11
T4	Sikou, Chiayi	23°35′8″N, 120°24′27″E	17 Oct. 2006	23 November 2006		20	10
T5	Guanshan, Taitung	23°1′3″N, 121°10′33″E	18 Oct. 2006	22 November 2006		19	12
	China						
C1	Fuqing, Fujian	25°49′84″N, 119°23′39″E	22 September 2006	3 November 2006	20	20	5
C2	Shantou, Guangdong	23°30′28″N, 116°49′5″E	27 September 2006	3 November 2006	3	20	7
C3	Shantou, Guangdong	23°30′28″N, 116°49′5″E	September 2006	13 Oct. 2006		4	
	Northern Vietnam						
Vn1	Dai Dong, Ha Tay	21°5′6″N, 105°34′21″E	31 August 2006	6 Oct. 2006	19		13
Vn2	An Lao, Hai Phong	20°48′12″N, 106°34′51″E	31 August 2006	3 November 2006	20	20	14
Vn3	An Lao, Hai Phong	20°46′24″N, 106°34′41″E	5 September 2007	12 October 2007	20		
Vn4	Tu Liem Ha Noi	21°4′N, 105°46′E	7 September 2007	12 October 2007	20	20	
	Southern Vietnam						
Vs1	Long Dinh, Tien Giang	10°24′12″N, 106°15′11″E	3 September 2006	6 October 2006	20		15
Vs2	Hoa Ninh, Tien Giang	10°26′37″N, 106°20′57″E	4 September 2006	13 December 2006		20	16
Vs3	SOFRI [2]	10°21′15″N, 106°22′0″E	26 November 2007	26 November 2007	20		
Vs4	Hoa Ninh Tien Giang	10°26′34″N, 106°21′6″E	9 March 2008	29 May 2008	20	20	
Vs5	SOFRI [2]	10°21′15″N, 106°22′0″E	17 May 2008	17 May 2008	20		
	Laos						
Laos	Viang Chan	17°57′46″N, 102°36′52″E			20	20	
	Thailand						
Thai1	Sai Noi, Nonthaburi	13°59′20″N, 100°19′19″E	19 February 2008	5 July 2008	20		
Thai2	Banlan, Nakhon Pathum	14°0′45″N, 100°12′22″E	19 February 2008	29 May 2008		20	
Thai3	Samckom<Sam Khok >, Pathum Thani	14°7′5″N, 100°33′42″E	19 February 2008	29 May 2008	20	20	
	Northern Philippines						
Pn1	Munoz, Nueva Ecija	15°40′11″N, 120°53′24″E	27 September 2006	13 November 2006	20		17
Pn2	Munoz, Nueva Ecija	15°40′11″N, 120°53′24″E	26 September 2006	28 November 2006 4 February 2007	20	20	17
Pn3	Quezon, Isabela	17°20′16″N, 121°36′14″E	18 October 2006	4 February 2007		20	20
Pn4	Solana, Cagayan	17°39′50″N, 121°41′34″E	19 October 2006	11 November 2006 28 November 2006	20 [3]	20	19
Pn5	Sta. Arcadia, Cabanatuan, Nueva Ecija	15°29′45″N, 121°00′48″E	4 May 2009	July 2009	20		
Pn6	San Isidro, Cabanatuan, Nueva Ecija	15°27′60″N, 120°58′80″E	4 May 2009	July 2009	20		
	Southern Philippines						
Ps1	Culit, Nagipit, Agusan del Norte	8°57′62″N, 125°21′28″E	6 May 2009	July 2009	20	20	
Ps2	Basilisa, Remedios T. Romualdez, Agusan del Norte	9°03′89″N, 125°34′99″E	6 May 2009	July 2009	12	20	
	Papua New Guinea						
PNG	East Sepik. Maprik	3°37′37″S, 143°3′6″E	19 January 2008 3 February 2008	19 January 2008 3 February 2008	13	21	
				Total	**579**	**464**	

[1] Population numbers are indicated in the populations studied in the previous report [table 1 of Matsumura *et al*. (2008)].
[2] Southern Fruit Research Institute in Vietnam.
[3] The collected population was derived *ca*. 20 individuals.

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Table 2. Primers list.

Primer	Sequence	References
B_C1-J-1718	5′-GGAGGTTTTGGTAATTGATTAGT-3′	This study
B_C2-N-3665	5′-CCACAGATTTCGGAGCATTG-3′	This study
C1-J-1718	5′-GGAGGATTTGGAAATTGATTAGT-3′	Dallas *et al.*, 2003
B_f1	5′-TCCTTATTCTTCCAGGATTTGGA-3′	This study
B_f2	5′-GATTAAGATTTAAACGAAAAATGGTG-3′	This study
B_r1	5′-GCAATAATTATAGTAGCTGACGT-3′	This study
W_f1	5′-TCCAGAAGTTTATATCCTGATTC-3′	This study
W_f2	5′-CTTGAGAAAGATTAGTTTATAAACG-3′	This study
W_r1	5′-CAATAATTATTGTCGCTGAAGT-3′	This study

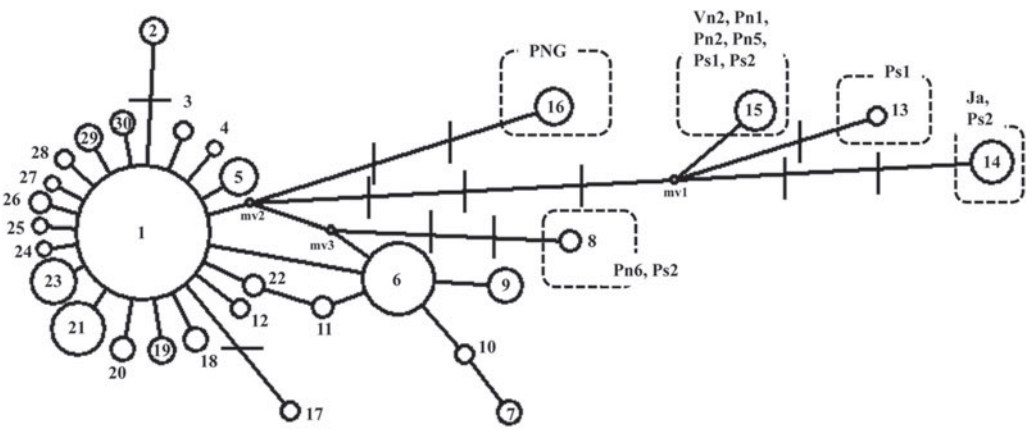

Fig. 2. Haplotype network for BPH (n = 579). Circle sizes are proportional to frequencies of haplotype sequences (1–30). Single line shows a predicted one-nucleotide substitution. The median vectors (mv) were not present in the samples. Haplotypes 8 and 13–16 were specifically detected in the populations shown within dotted squares.

obtained in this study. While all sequences were used for haplotype network analyses, the Ja, Jb, Jc and Jd populations (table 1) were excluded for analysis of pairwise *Fst*, AMOVA, Mantel test and IMa2 because they were reared in the laboratory for a long time (8–40 years).

BPH: genetic diversity

We detected 30 haplotypes of the mitochondrial *cox1–trnL2–cox2* region in BPH (DNA database accession numbers AB572299–AB572328) (table 3; supplementary table 1). The most frequently identified haplotype (haplotype 1 of BPH) was detected in 58.9% of the individuals (341/579 individuals). Polymorphic nucleotide sites in the other haplotypes were denoted by comparing the sequences with that of haplotype 1. For example, when thymine at the 257th position in haplotype 1 was altered to adenine, the variation was shown as T257A (supplementary table 1). Haplotype 1 was shared by all populations except C2 and PNG (table 3). Population-specific haplotypes were observed in C2 (haplotype 12) and PNG (haplotype 16) populations, although the number was small in C2 (n = 3). Among the other 29 populations, 26 populations showed two to six haplotypes, and three populations, Jb, Jc and Jd, which had been reared in the laboratory for a long time, showed only haplotype 1. The numbers of variable sites were 28 in *cox1*, 12 in *cox2* and 0 in *trnL2*. Thirty-nine variable positions were binary, and only C103 was ternary (C103T and C103A). Four sites in *cox1* and one in *cox2* were non-synonymous (supplementary table 1).

BPH: haplotype networks

An MJ haplotype network was created for 30 BPH haplotypes (fig. 2). A star-like phylogeny was obtained in the BPH network, in which haplotype 1, the most frequent haplotype, was centred. Thus, in the star phylogeny, the ancestral haplotype was centred and the other derived haplotypes had close connections to the former, implying that the populations were subject to recent expansion and/or selective mutation, (Slatkin & Hudson 1991; Rogers & Harpending 1992; Avise 2000; Mousset *et al.*, 2004). Five haplotypes (haplotypes 8 and 13–16) are connected to haplotype 1 via median vectors (mv) 1, 2 and/or 3 that were not present in samples. BPH haplotypes 8, 13 and 15 were all detected only in the Philippines, except haplotype 15 that was found in one population from Vietnam (Vn2). Haplotype 16 was only detected from PNG populations, and haplotype 14 was observed in Ja and Ps2.

BPH: genetic structure

All pairwise *Fst* values (Wright, 1978) were calculated for BPH populations in the ten areas (table 4). An unrooted NJ tree was constructed based on *Fst* values (fig. 3). The *Fst* values

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Table 3. The number of haplotypes in each population of BPH.

Haplotype	Ja	Jb	Jc	Jd	J1	J3	J5	T1	T2	T3	C1	C2	Vn1	Vn2	Vn3	Vn4	Vs1	Vs3	Vs4	Vs5	Laos	Thai1	Thai3	Pn1	Pn2	Pn4	Pn5	Pn6	Ps1	Ps2	PNG	Total
1	4	20	20	20	14	12	11	10	11	12	14	–	11	11	14	15	17	15	12	16	14	14	12	10	11	1	4	4	9	3	–	**341**
2	–	–	–	–	–	–	–	–	–	6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**6**
3	–	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**3**
4	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**2**
5	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	1	1	–	–	3	7	–	–	–	–	–	–	–	–	**13**
6	–	–	–	–	2	2	3	–	3	2	–	–	3	6	1	2	1	1	–	–	–	–	–	4	6	–	5	–	3	3	–	**47**
7	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	–	–	–	–	**5**
8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	–	1	–	**4**
9	–	–	–	–	–	–	–	–	–	–	1	–	–	1	–	–	–	–	–	1	–	–	–	1	–	–	–	7	–	–	–	**11**
10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	1	–	–	**3**
11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	–	–	–	–	**4**
12	–	–	–	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**3**
13	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	–	–	**3**
14	15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	**16**
15	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	3	2	–	1	–	4	4	–	**15**
16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	13	**13**
17	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**3**
18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**5**
19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6	–	–	–	**6**
20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5	–	–	–	–	–	–	–	–	–	–	–	–	**5**
21	–	–	–	–	4	–	–	6	–	–	2	–	–	–	–	–	–	2	–	2	4	3	1	–	–	–	–	–	–	–	–	**24**
22	–	–	–	–	–	–	–	–	–	–	–	–	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**4**
23	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	19	–	–	–	–	–	**19**
24	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	**2**
25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**2**
26	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	2	1	–	–	–	–	–	–	–	–	–	–	–	**4**
27	–	–	–	–	–	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**2**
28	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	**3**
29	–	–	–	–	–	–	1	–	–	–	1	–	–	–	–	1	2	1	–	–	–	–	–	–	–	–	–	–	–	–	–	**6**
30	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	**5**
Total	**19**	**20**	**20**	**20**	**20**	**15**	**20**	**19**	**19**	**20**	**20**	**3**	**19**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**12**	**13**	**579**

Haplotypes 1–30 correspond to DNA accession numbers AB572299–AB572348, respectively.

Table 4. Pairwise fixation index (*Fst*) values in BPH populations in ten areas.

	Japan	China	Taiwan	Vietnam_N	Vietnam_S	Laos	Thailand	Philippines_N	Philippines_S
China	0.0261**	0							
Taiwan	0.0242*	0.0292^{NS}	0						
Vietnam_N	0.0129*	0.0419****	0.0422****	0					
Vietnam_S	0.0199*	0.0280*	0.0432****	0.0335****	0				
Laos	0.0441^{NS}	0.0293^{NS}	0.0278^{NS}	0.0782****	0.0472*	0			
Thailand	0.107****	0.0957****	0.0857****	0.109****	0.0887****	0.0999*	0		
Philippines_N	0.0678****	0.0735*	0.0791****	0.0562****	0.108****	0.0778****	0.125****	0	
Philippines_S	**0.303****	**0.230****	**0.278****	**0.304****	**0.362****	**0.236****	**0.301****	0.176****	0
PNG	**0.888****	**0.881****	**0.807****	**0.852****	**0.900****	**0.925****	**0.579****	**0.665****	**0.579****

*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$; NS, not significant; $Fst>0.200$ values are indicated in bold.

Table 5. The number of haplotypes in each population of WBPH.

Haplotype	Jb	Jc	J2	J4	J5	J6	T1	T4	T5	C1	C2	C3	Vn2	Vn4	Vs2	Vs4	Laos	Thai2	Thai3	Pn2	Pn3	Pn4	Ps1	Ps2	PNG	Total
1	–	–	5	8	16	11	4	17	15	10	9	2	7	14	–	8	9	13	7	9	12	13	7	19	– 10	**225**
2	–	20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**20**
3	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**3**
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	**2**
5	–	–	–	–	–	–	1	1	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	1	**4**
6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	**3**
7	–	–	–	–	–	–	–	–	–	–	–	–	9	–	–	–	–	–	–	8	–	–	–	–	–	**17**
8	–	–	3	8	1	1	2	–	–	4	2	2	4	6	15	2	–	5	8	–	–	1	7	–	4	**75**
9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	**2**
10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	**2**
11	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**2**
12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	**2**
13	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**2**
14	–	–	4	3	2	1	4	–	4	3	6	1	–	–	5	1	4	2	2	3	8	6	2	–	6	**67**
15	–	–	–	1	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**4**
16	–	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**3**
17	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5	–	–	–	–	–	–	–	–	**5**
18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	**2**
19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	–	–	–	–	–	–	–	–	–	**4**
20	20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**20**
Total	**20**	**20**	**12**	**20**	**19**	**13**	**16**	**20**	**19**	**20**	**20**	**5**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**19**	**21**	**464**

Haplotypes 1–20 correspond to DNA accession numbers AB572329–AB572348, respectively.

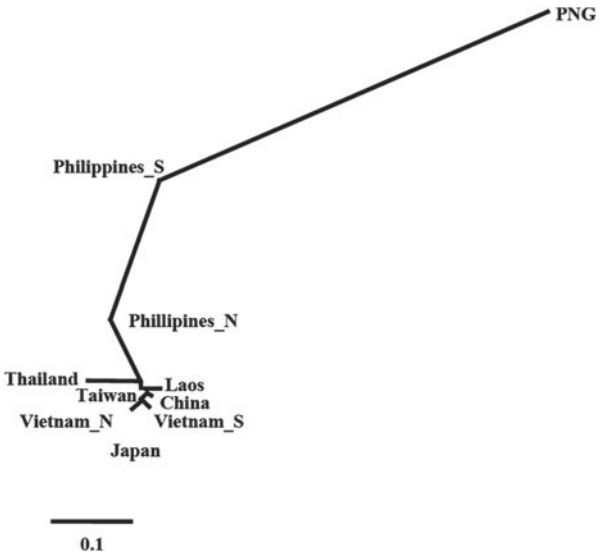

Fig. 3. Unrooted NJ trees based on *Fst* values of ten areas for BPH.

between populations from PNG and all other areas were significantly high (more than 0.579, $P<0.0001$). Significantly high *Fst* values were also shown between populations in Ps and all other areas (0.176–0.579). Although the analysis indicated differences in genetic structure among populations from other eight areas, including Japan, Taiwan, China, Vn, Vs, Laos, Thailand and Pn, *Fst* values were relatively low (<0.125, which was between Thailand and Pn). In the eight areas, a hierarchical AMOVA showed that the variation attributed much more within populations (ΦST, 79.7%), than among populations within areas (ΦSC, 19.6%) and among areas (ΦCT, 0.74%). ΦST and ΦSC were significant ($P<0.00001$), on the other hand, ΦCT was not significant ($P=0.312$) (supplementary table 3), indicating no genetic structure among the areas. Mantel test was not significant in 24 populations of the eight areas ($P=0.663$), showing no correlation between *Fst* values and geographic distance among the populations. Migration rate (2NM) among areas using IMa2 showed significant level of gene flow of BPH: Taiwan>Pn (2NM=0.873, $P<0.01$), Vn>Thai (2NM=0.708, $P<0.05$), Vs>Thai (2NM=0.739, $P<0.05$), Pn>Japan (2NM=0.853, $P<0.05$), Pn>China (2NM=0.812, $P<0.05$), Pn>Ps (2NM=0.692, $P<0.05$), Ps>Pn (2NM=1.098, $P<0.001$) (supplementary table 4).

WBPH: genetic diversity

We found 20 haplotypes in the mitochondrial *cox1–cox2* region in WBPH (DNA database accession numbers AB572329–AB572348) (table 5; supplementary table 2). The most frequent haplotype (haplotype 1 for WBPH) was found in 48.5% of all WBPH individuals tested (225/464 individuals). The WBPH haplotype 1 was observed in all populations, except Jb, Jc and Vs2. Population-specific haplotypes were shown in Jb and Jc, which have been reared in the laboratory for a long time. One to seven haplotypes were observed in each of the other 23 populations. Eighteen and two polymorphic sites were present in *cox1* and in *cox2*, respectively. All the variable positions were binary. Amino acid variable sites were two and one in *cox1* and *cox2*, respectively (supplementary table 2).

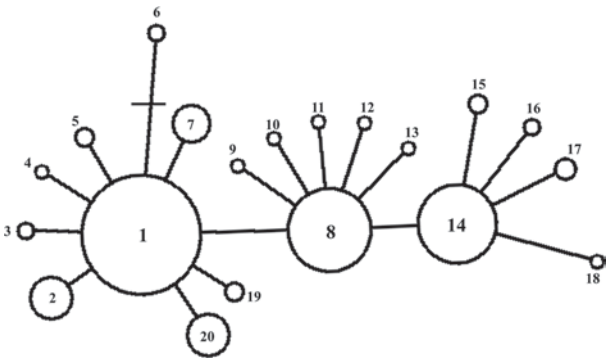

Fig. 4. Haplotype network for WBPH ($n=464$). Circle sizes are proportional to frequencies of haplotype sequences (1–20). Single line shows a predicted one-nucleotide substitution.

WBPH: haplotype network

Three major haplotypes (haplotypes 1, 8 and 14) were found in the WBPH MJ network (fig. 4). There was a single substitution between haplotypes 1 and 8 and between haplotypes 8 and 14; the other haplotypes were their satellites. The WBPH haplotypes 8 and 14 were the second (16.2%) and third (14.4%) most prevalent haplotypes, and were as widely distributed as haplotype 1. Haplotype 8 was detected in all areas except Laos, and haplotype 14 was detected in all areas except Vn (table 5).

WBPH: genetic structure

Pairwise *Fst* values were calculated for all combinations of the ten areas with WBPH. Although some values were significant, the highest value was less than 0.235 (between Taiwan and Vs) (table 6). In contrast to the findings for BPH, WBPH populations PNG and Ps populations did not show high *Fst* values against those from other areas (table 6). An unrooted NJ tree was constructed based on *Fst* values (fig. 5). For WBPH ten areas, a hierarchical AMOVA showed that the variation attributed much more within populations (ΦST, 85.01%), than among populations within areas (ΦSC, 15.13%) and among areas (ΦCT, −0.13%) (supplementary table 3), ΦST and ΦSC were significant ($P<0.00001$), on the other hand, ΦCT was not significant ($P=0.546$) (supplementary table 3), indicating no genetic structure among the ten areas. Mantel test was not significant among 23 populations of the ten areas ($P=0.384$). IMa2 showed significant migration rate (2NM) among Japan>China (2NM=0.21, $P<0.05$) (supplementary table 4).

Discussion

Our aims were to distinguish regional populations of planthoppers that show different biological properties, for example, virulence against rice plants or insecticide resistance, and to estimate the annual migratory route of BPH and WBPH using simple molecular markers. An earlier study showed that the ITS region of rRNA genes was not a good candidate as a molecular marker, because it was highly variable among the

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Table 6. Pairwise fixation index (*Fst*) values in WBPH among ten areas.

	Japan	China	Taiwan	Vietnam_N	Vietnam_S	Laos	Thailand	Philippines_N	Philippines_S
China	0.0413^{NS}	0							
Taiwan	0.00388^{NS}	0.0992**	0						
Vietnam_N	0.0596*	0.1339**	0.0610*	0					
Vietnam_S	0.158****	0.0578*	**0.235****	**0.204****	0				
Laos	0.0854*	0.0159^{NS}	0.129*	0.1684****	0.102*	0			
Thailand	0.00536^{NS}	0.0357^{NS}	0.0426*	0.0479^{NS}	0.0912**	0.0788*	0		
Philippines_N	0.0115^{NS}	0.0376^{NS}	0.0259^{NS}	0.0415^{NS}	0.153****	0.0535^{NS}	0.0318^{NS}	0	
Philippines_S	−0.00182^{NS}	0.0916*	−0.001^{NS}	0.0421^{NS}	0.199****	0.135**	0.0152^{NS}	0.0387^{NS}	0
PNG	0.00395^{NS}	−0.024^{NS}	0.0565^{NS}	0.0924*	0.0505^{NS}	0.00683^{NS}	−0.00248^{NS}	0.00549^{NS}	0.0499^{NS}

*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$; NS, not significant; *Fst* values >0.2000 are indicated in bold.

Fig. 5. Unrooted NJ trees based on *Fst* values of ten areas for WBPH.

rice planthopper individuals and did not contain features specific to populations (Fu *et al*., 2012). In the present study, mitochondrial sequences were selected as alternative candidate molecular markers. The mitochondrial cytochrome oxidase region in BPH populations in southern Philippines showed differentiation from those in other areas in pairwise *Fst* values. However, no specific haplotypes were related to regional populations, except haplotype 16 in PNG.

No genetic structure was indicated among BPH population from eight areas, excluding Ps and PNG, and major part of variance was observed at the population level (supplementary table 3). In the case of WBPH, some differentiation was shown among areas (table 6), however, no genetic structure was indicated among ten areas, and most variation was present at population level (supplementary table 3). Northern Vietnam area is considered as the primary source region of rice planthopper migrants to East Asia, including Japan, Korea and Northern China (Kisimoto 1976; Watanabe & Seino, 1991; Sogawa, 1995; Otuka *et al*., 2005*b*, 2008). Low genetic differentiation and the genetic structure among BPH and WBPH populations from most areas studied here are consistent with the hypothesis that East Asian planthoppers are derived from northern Vietnam; however, the data reported here did not conclusively support it. IMa2 analysis showed significant gene flow among some areas (supplementary table 4), however, it did not show clear migration route of rice planthoppers. To our knowledge, the present study is the first extensive study on mitochondrial sequences of Asian planthopper populations. Our analyses, which utilized a larger sample size and a longer mitochondrial genomic sequence, did not yield a novel molecular marker for

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discrimination of planthopper populations, similar to the previous report by Mun *et al*. (1999). The data indicate that respective BPH and WBPH partly share a gene pool in Asia, and that the mitochondrial *cox1–trnL2–cox2* region did not provide sufficient resolution for planthoppers populations.

The insecticide resistance in BPH and WBPH populations collected from East and Southeast Asia (Japan, China, Taiwan, Vietnam and the Philippines) has been reported (Matsumura *et al*., 2008; Matsumura & Sanada-Morimura, 2010). Examination of insecticide resistance levels in 16 populations of BPH revealed resistance against O-*sec*-butylphenyl methyl carbamate and less resistance against phenylpyrazole (fipronil). Twelve populations of BPH, collected in Japan, China, Taiwan and Vietnam, showed resistance against neonicotinoid insecticides (imidacloprid and thiamethoxam), and four populations in the Philippines were susceptible (Matsumura *et al*., 2008). Among the 16 populations of BPH previously tested for insecticide resistance (Matsumura *et al*., 2008), individuals of 11 (J1, T1, T3, C1, C2, Vn1, Vn2, Vs1, Pn1, Pn2 and Pn4) were used in the present study. We did not find a clear correlation between mitochondrial haplotypes and insecticide resistance level. For example, a majority of individuals from the Philippines populations, which showed a relatively high genetic distance from other populations (Table 4), were of haplotype 1, present also in most of the other populations. The level of insecticide resistance is usually related to the amount and/or duration for which the corresponding insecticide has been used, whereas the mitochondrial genetic structure of populations reflects genetic flow over a longer time scale. Differences in the temporal scale of development of the two biological traits might contribute to the discrepancy of their distributions within populations. An additional layer of complexity is added by the gene exchange brought about by the long-distance migration of BPH.

In WBPH, neonicotinoid resistance is not as prevalent, however, resistance to phenylpyrazole has been observed widely in East and Southeast Asia. The individuals of the same 11 populations that were used for the insecticide resistance study (J4, T1, T4, T5, C1, C2, Vn2, Vs2, Pn2, Pn3 and Pn4; Matsumura *et al*., 2008) were examined in the present study. Our analysis revealed relatively low genetic distance among these populations, indicating that simple molecular markers, such as mitochondrial sequences, may not be useful for insecticide-resistance monitoring in WBPH.

Other important issues in planthopper management are rice resistance against planthoppers, and virulence of planthoppers against the resistant rice varieties (Sogawa, 1992; Zhang, 2007). The populations of BPH Ja, Jc and Jd, which had been maintained in the laboratory, were tested for their virulence against rice varieties (Myint *et al*., 2009; Naeemullah *et al*., 2009). These studies reported that the BPH Ja population, which has been reared from 1966, showed avirulence against all the rice varieties examined. The Jc population, which was collected in 1989, was virulent against the Mudgo rice variety carrying the BPH-resistant gene *BPH1*, and Jd, which was collected in 1999, was virulent against the ASD7 rice variety carrying *bph2*. Myint *et al*. (2009) pointed out that virulence status is not affected substantially by long-term mass rearing in the laboratory. However, similar to the findings for insecticide resistance, mitochondrial haplotype analysis does not seem to be useful in estimating the biotypes for resistant rice varieties.

Mitochondrial gene sequences are useful molecular markers for distinguishing populations in some insect species (Pramual *et al*., 2005; Cai *et al*., 2008; Lohman *et al*., 2008; Meraner *et al*., 2008; Nolan *et al*., 2008). However, in long-distance-migrating rice planthopper species, BPH and WBPH, mitochondrial DNA appears to be less useful. Our interests focus on aspects of planthopper biology important to agriculture, e.g., insecticide resistance or mechanisms of virulence against resistant rice varieties. Genes responsible for these biological phenomena and other genomic regions closely linked to these genes are expected to provide more informative markers for distinguishing resistant individuals and populations.

The supplementary material for this article can be found at http://www.journals.cambridge.org/BER

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