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Genetic structure of the rice leaf folder, *Cnaphalocrocis medinalis* (Lepidoptera: Crambidae) in Taiwan

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ABSTRACT: *Cnaphalocrocis medinalis* (Lepidoptera: Crambidae) is an economically important rice pest in Taiwan. The population genetic structure of *C. medinalis* is not clear and thus was studied in this research. *Cnaphalocrocis medinalis* samples were collected from six sampling locations in Taiwan using white sticky paper traps coated with sex pheromone. Genetic diversity was analyzed by mitochondrial and nuclear markers. Phylogenetic analysis revealed that all specimens of *C. medinalis* in Taiwan belonged to a single clade. Population genetic analysis of *C. medinalis* indicated no significant population structure because of a lack of significant genetic differentiation among the six sampling sites. Population genetic analysis also revealed no significant genetic differentiation among *C. medinalis* populations in Taiwan and neighboring regions, suggesting a common origin. The extremely low genetic diversity of *C. medinalis* in East Asia might be due to a population expansion after a recent founder effect. This study provides molecular information for understanding the population genetic structure of *C. medinalis* in Taiwan and East Asia.

KEY WORDS: Molecular marker, pest, population genetics, rice, sex pheromone trap

INTRODUCTION

Cnaphalocrocis medinalis Guenée (Lepidoptera: Crambidae) is an important rice pest found in Taiwan that feeds on many graminaceous crops and weeds, with rice as its primary host plant. The larvae of *C. medinalis* avoid pesticides when hidden within the leaves. At high levels of infestation, this species caused more than 50% of grain to be unfilled, directly resulting in a 30–80% reduction in yield (Padmavathi *et al*., 2013a; Fu *et al*., 2014). *Cnaphalocrocis medinalis* is widely distributed across southern and eastern Asia (Kisimoto, 1984). It has been proposed that this species makes annual long-distance aerial migrations from Southeast to Northeast Asia via air currents (Liao, 2010). The origin of invasive *C. medinalis* in Taiwan could be neighboring regions, such as the Philippines or China.

A female sex pheromone has been developed to monitor the distribution and population density of *C. medinalis* in southern and eastern Asia (Ramachandran *et al*., 1990; Ganeswara Rao *et al*., 1995; Kawazu *et al*., 2000). Three sex pheromone blends, the Philippine blend, the Indian blend, and the Japanese blend, were developed separately based on *C. medinalis* populations in the Philippines, India, and Japan, respectively (Ramachandran *et al*., 1990; Ganeswara Rao *et al*., 1995; Kawazu *et al*., 2000). The variations in the female sex pheromone of *C. medinalis* suggested geographic variations and genetic differentiation. Monitoring of *C. medinalis* is essential for formulating and implementing effective pest control strategies against this pest. White sticky paper traps coated with the three female sex pheromone blends, have been used in Taiwan to monitor *C. medinalis* populations, with only the Japanese pheromone blend proving successful in attracting adult moths (Liao and Hung, 2008).

Molecular markers are valuable tools for population genetic studies. Cytochrome c oxidase subunit I (COI) gene has been applied to study the phylogenetic systematics of stemborers, including *C. medinalis* (Lee *et al*., 2019). Nuclear internal transcribed spacer 2 (ITS2), a spacer between the small-subunit and large-subunit ribosomal RNA genes, has been used to confirm the phylogenetic relationships between *C. medinalis* and *Marasmia patnalis* (Yang *et al*., 2017). Comparisons of the ITS2 and mitochondrial AT-rich regions of DNA from *C. medinalis* populations in China and Korea showed low levels of genetic variation, indicating high levels of gene flow and no isolation by distance. (Wan *et al*., 2011). The application of population genetic analysis can clarify the invasion events of *C. medinalis*.

Cnaphalocrocis medinalis populations have continued to grow in Taiwan since 1995, especially during the second rice cropping season each year (Huang *et al*., 2010). In this study, adult *C. medinalis* were collected using female sex pheromone traps. Mitochondrial AT-rich and nuclear ITS2 markers were used to analyze the population genetic structure of *C. medinalis* in Taiwan.

Table 1. List of samples used in this study and their GenBank accession numbers.

Species	Location	Acronym	GenBank accession no.	
			AT	ITS2
Cnaphalocrocis medinalis	Changhua	CH01- CH10	MK690797- MK690806	MK690864- MK690872
Cnaphalocrocis medinalis	Miaoli	ML02- ML19	MK690808- MK690825	MK690874- MK690890
Lepidoptera sp	Miaoli	ML01	MK690807	MK690873
Chilo suppressalis	Miaoli	ML03	MK690809	MK690875
Cnaphalocrocis medinalis	Pingtung	PT01- PT06	MK690826- MK690831	MK690891- MK690895
Cnaphalocrocis medinalis	Tainan	TN01- TN10	MK690832- MK690841	MK690896- MK690900
Cnaphalocrocis medinalis	Taipei	TP01- TP14	MK690842- MK690855	MK690901- MK690910
Cnaphalocrocis medinalis	Taitung	TT01- TT07	MK690856- MK690862	MK690911- MK690913
Chilo suppressalis	Taitung	-	MK690863	MK690914

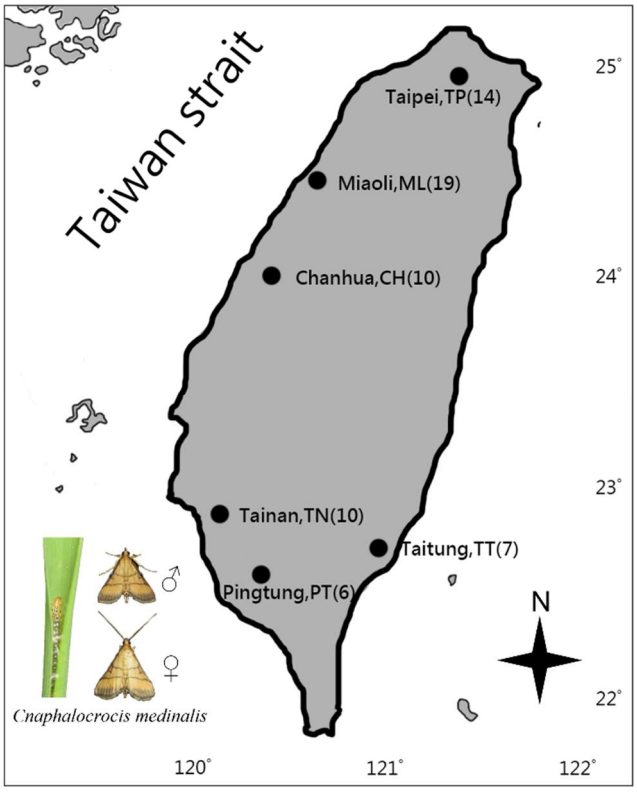

Fig. 1. Location of the six ***Cnaphalocrocis medinalis*** sampling sites in Taiwan. Numbers in parentheses are sample sizes.

MATERALS AND METHODS

Sampling with the pheromone trap

White sticky paper traps coated with the Japanese pheromone blend were used to attract adult moths of *C. medinalis*. The Japanese pheromone blend, which is composed of (Z)-11-octadecenal (Z11-18:Ald), (Z)-13-octadecenal (Z13-18:Ald), (Z)-11-octadecen-1-ol (Z11-18:OH), and (Z)-13-octadecen-1-ol (Z13-18:OH) (Kawazu *et al*., 2000), was obtained from the Taichung District Agricultural Research and Extension Station, COA, Changhua, Taiwan. In September 2015, these pheromone traps were placed in rice paddy fields at six collection sites spread across Taiwan (Taipei (TP), Miaoli (ML), Changhua (CH), Tainan (TN), Pingtung (PT) and Taitung (TT)) (Fig.1, Table 1). A total of 66 adult moths were collected from Taiwan. All specimens were identified to the species level based on morphology. The trapped specimens were stored at -80 °C until DNA extraction and analysis.

DNA extraction, amplification, and sequencing

Whole insect genomic DNA was isolated using a modified CTAB-based protocol (Doyle, 1987). Three molecular markers were used for genetic analysis, including two noncoding regions (mitochondrial AT-rich region and nuclear internal transcribed spacer 2 (ITS2) region) and one coding gene (mitochondrial cytochrome c oxidase subunit I (COI) gene). Three sets of primers were used for PCR analysis: the forward primer 5′-AATAATAGGGTATCTAATCCTAG-3′ and the reverse primer 5′-AATTTATCCTATCAGAATAATCC-3′ (Wan *et al*., 2011) located between the 12S rRNA and $tRNA^{Met}$, respectively, for the mitochondrial AT-rich region (~650 bp), the forward primer 5′-GGTCAACAAATCATAAAGATATTGG -3′ and reverse primer 5′- TAAACTTCAGGGTGACCAAAAAATCA -3′ (Ji *et al*., 2003) for the partial mitochondrial COI gene sequence (ca. 700 bp) (Folmer *et al*., 1994), and the forward primer 5′-ATGAACATCGACATTTCGAACGCACAT-3′ and reverse primer 5′-TTCTTTTCCTCCGCTTAGTAATATGCTTAA-3′ (Ji *et al*., 2003) for the nuclear ITS2 region (~500 bp). The PCRs were carried out in a 50 μL solution containing 50 ng of template DNA, each primer at 10 mM, and 25 μL of G-Taq DNA polymerase MasterMix (2X, GeneTeks BioScience Inc, Taiwan). The PCR amplification of the AT-rich region included an initial denaturation for 7 min at 94 °C, 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55.6 °C, elongation for 1 min at 72 °C, and a final extension at 72 °C for 70 s; PCR amplification of the ITS2 region included an initial denaturation for 4 min at 94 °C, 35 cycles of denaturation for 40 s at 95 °C, annealing for 20 s at 64 °C, and elongation for 40 s at 72 °C, and final extension at 72°C for 2 min and 40 s (Yang *et al*., 2017). All reactions were performed with a FlexCycler2 PCR thermal cycler (Analytik Jena, Germany). The PCR products were

separated in 2% agarose gel using electrophoresis, stained with an EDTA-free dye, and then purified using the GenepHlow Gel/PCR Kit (Geneaid Biotech Ltd, Taiwan). Sequencing was performed on an ABI 3730 DNA Analyzer (Applied Biosystems, CA, USA) using an ABI PRISM Terminator Cycle Sequencing Ready Reaction Kit, ver. 3.1 (Applied Biosystems, CA, USA), and sequencing reactions were carried out by the Genomics Company, New Taipei City, Taiwan. Forward and reverse sequences were visualized, edited, and assembled using SeqMan II software (Lasergene version 5; DNA Star, Inc., Madison, WI.).

Phylogeny and population genetics analysis

A molecular phylogeny was generated to analyze the genetic differences of *C. medinalis*. A typical rice pest in Taiwan, *Chilo suppressalis* (Lepidoptera: Pyralidae), was selected as an outgroup. To compare sequences of *C. medinalis* from different locations, we downloaded 13 AT-rich and ITS2 sequences from China and Korea available in GenBank (Wan *et al*., 2011; Yang *et al*., 2017). Mitochondrial COI gene sequences of *C. medinalis* and outgroups from multiple Asian countries were also downloaded for analysis. Multiple sequence alignments were performed using the default settings in Muscle in MEGA (version 7.0). The best nucleotide substitution model for phylogenetic analysis was estimated using ModelTest in MEGA (version 7.0) (the Tamura 3-parameter model for the AT-rich region and the Kimura 2-parameter model for the ITS2 region) (Kumar *et al*., 2016). Phylogenetic trees were reconstructed using maximum likelihood (ML) with 1000 bootstrap replications for nodal supports in MEGA (version 7.0). Bayesian inference was also used to reconstruct the phylogenetic tree based on the general time reversible model using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001). Two runs of four independent Metropolis-coupled Markov chain Monte Carlo (MCMC) analyses were run for $1x10^6$ generations and sampled every 1000 generations with a burn-in length of the initial 10% generations. According to the sampling sites, genetic distance was used to assess the population's genetic differences. Based on the Kimura 2-parameter model, the genetic distance was calculated in MEGA (version 7.0) (Kumar *et al*., 2016).

Population genetic parameters were applied to analyze the populations' genetic structure. Genetic diversity, including the number of haplotypes (h), haplotype diversity (H), nucleotide diversity (π), number of segregating sites (S), and population demography, was analyzed with Tajima's D and Fu's Fs neutrality tests using DnaSP (version 6.11.01) software (Rozas *et al*., 2017). Analysis of molecular variance (AMOVA) and genetic differences (F_{ST}) were used to analyze population structure and estimate genetic variance components among and within populations using Arlequin software version 3.5.2.2 (Excoffier and Lischer, 2010). The relationship between genetic data and geographic information was also examined by haplotype network analysis. The haplotype network was used to analyze haplotype relationships among the six geographic samples using HapView (Salzburger *et al*., 2011). A mismatch distribution analysis implemented in Arlequin software version 3.5.2.2 (Excoffier and Lischer, 2010) was used to estimate demographic events. A multimodal distribution reflects demographic equilibrium, and a unimodal distribution indicates recent demographic expansion (Slatkin and Hudson, 1991; Rogers and Harpending, 1992). The sum of square deviations (SSD) and Harpending's raggedness index (r) were used to estimate demographic expansion, again in Arlequin software (Excoffier and Lischer, 2010).

RESULTS

A total of 66 adult moths were captured, from which 66 AT-rich (621 bp) and 50 ITS2 (428-503 bp) sequences were recovered (Fig. 1; Table 1). Phylogenetic analysis revealed three distinct clades, ML03 clustered with *Ch. suppressalis* as Clade III, ML01 formed the independent Clade II, and the remaining 64 specimens belonged to Clade I with *C. medinalis* (Fig. 2). Phylogenetic analysis also showed that Clade I from Taiwan clustered with the sequences of *C. medinalis* from China and Korea (Fig. 1 & 2).

The genetic distances within Clade I were 0.001 for the AT-rich region and 0.0002 for ITS2. The genetic distance between the three clades ranged from 0.147 to 0.202 for the AT-rich region and from 0.323 to 0.770 for ITS2. The short genetic distances within Clade I, which included *C. medinalis*, supported Clade I as *C. medinalis*. Similarly, the results supported ML03 as *Ch. suppressalis*. ML01 represented an unknown species.

Pairwise genetic distances of the six *C. medinalis* samples collected from Taiwan ranged from 0.001 to 0.004 (with a mean of 0.001) for the AT-rich region and 0.003 to 0.005 (with a mean of 0.003) for the ITS2 region. The results indicated that there is no significant genetic differentiation among populations in Taiwan. Pairwise genetic distances of *C. medinalis* specimens from Taiwan, Korea, and China ranged from 0.002 to 0.003 for the partial AT-rich region and 0.007 to 0.01 for the partial ITS2 regions (Table 2). Among-population distances of the AT-rich region ranged from 0.002 to 0.003, whereas within-population distances ranged from 0.002 to 0.003; the distances of the partial ITS2 region ranged from 0.007 to 0.01 among the three populations, whereas the within-population distances ranged from 0.004 to 0.01 (Table 2). The results suggested that there was no significant genetic differentiation among populations from Taiwan, Korea, and China.

We performed AMOVA to examine the genetic variation between collection sites in Taiwan (Table 3). For the AT-rich region, approximately 86% of the genetic variation was shared within populations (F_{ST}=0.14, p<0.001). For the ITS2

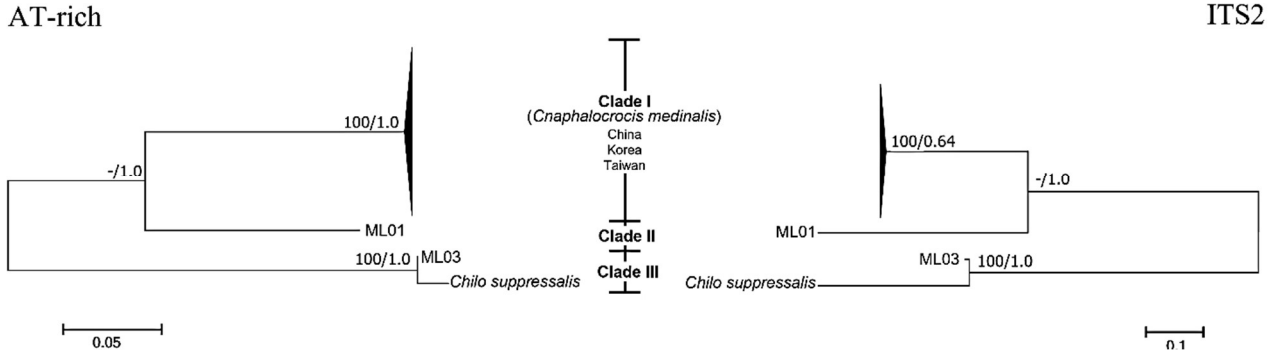

Fig. 2. Maximum-likelihood phylogeny of ***Cnaphalocrocis medinalis*** based on mitochondrial AT-rich and nuclear ITS2 sequences. Sequences of ***Chilo suppressalis*** were used as an outgroup. Numbers at the nodes are maximum-likelihood bootstrap values and Bayesian posterior probabilities.

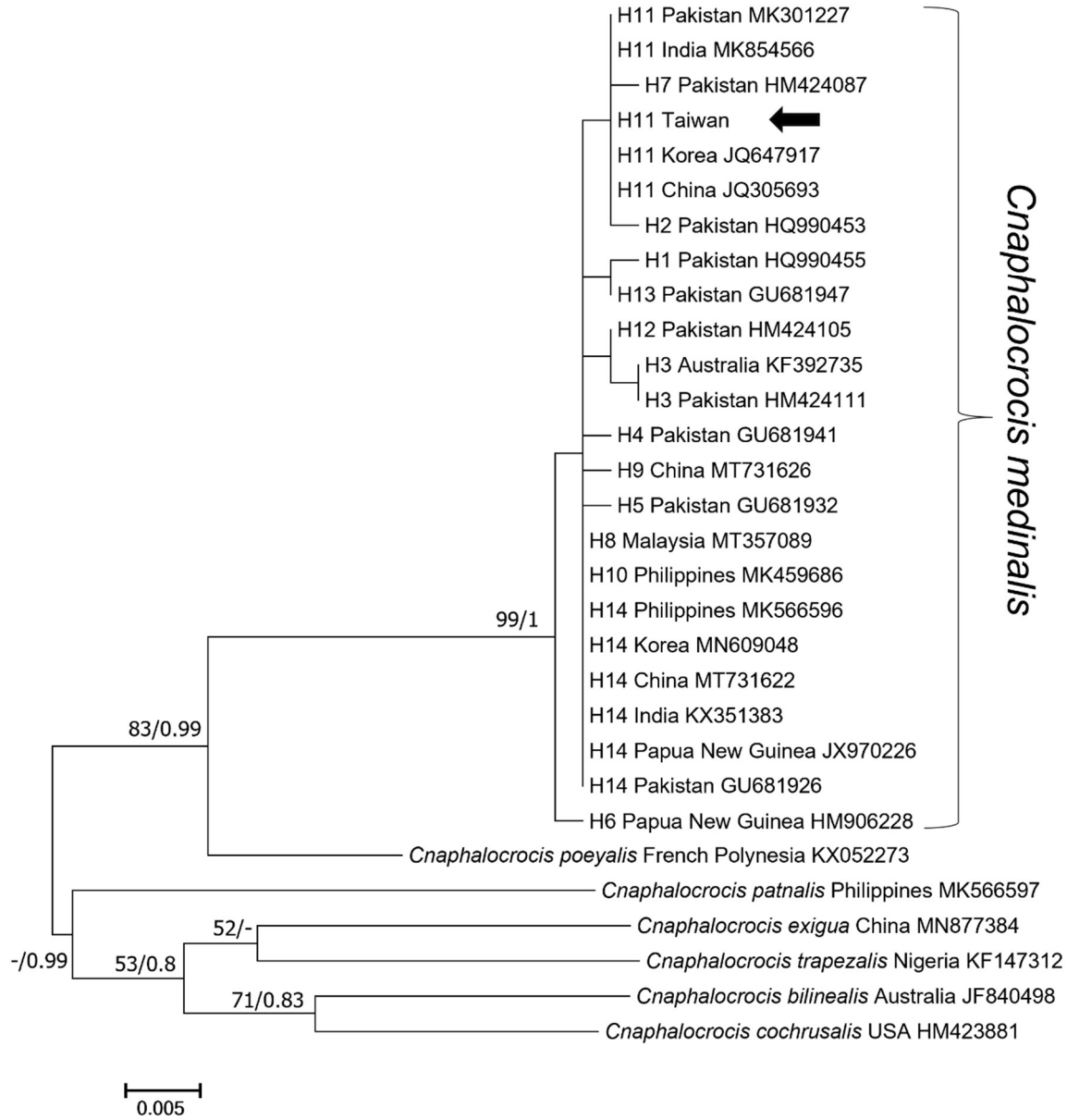

Fig. 3. Maximum-likelihood phylogeny of ***Cnaphalocrocis medinalis*** based on mitochondrial COI sequences. Numbers at the nodes are maximum-likelihood bootstrap values and Bayesian posterior probabilities.

Table 2. Genetic distances between populations of ***Cnaphalocrocis medinalis*** in East Asia based on the mitochondrial AT-rich region (lower) and nuclear ITS2 region (upper).

		1	2	3	AT-rich	ITS2
1	Taiwan		0.007	0.007	0.003	0.004
2	Korea	0.003		0.01	0.002	0.01
3	China	0.003	0.002		0.002	0.01

Table 3 Analysis of molecular variance and genetic differences in ***Cnaphalocrocis medinalis*** in Taiwan.

	Source of variation	d.f.	Sum of squares	Variance components	% of variation	F_{ST}
AT-rich	Among populations	5	6.908	0.08418a	14.25	
	Within populations	58	29.389	0.50670b	85.75	0.14246*
	Total	63	36.297	0.59088		
ITS2	Among populations	5	2.775	-0.01356a	-2.11	
	Within populations	42	27.600	0.65714b	102.11	-0.02107
	Total	47	30.375	0.64358		

Statistical significance: * $p < 0.001$

Table 4. Genetic diversity and neutrality tests of ***Cnaphalocrocis medinalis***.

Location	Marker	N	h	H	π	S	Tajima's *D*	Fu's *Fs*
Taiwan	AT	64	20	0.64	0.002	20	-2.29**	-20.64***
	ITS2	48	13	0.73	0.004	9	-1.23	-8.06***
East Asia	AT	77	21	0.59	0.003	21	-2.35**	-23.39***
	ITS2	61	18	0.78	0.005	20	-1.97*	-13.07***

h: haplotype number; H: haplotype diversity; π: nucleotide diversity; S: segregating sites. Tajima's D and Fu's Fs tests at * $p < 0.001$. ** $p < 0.01$; *** $p < 0.001$.

region, 100% of genetic variation was shared within populations (F_{ST}=-0.02, p>0.05), indicating that all specimens belonged to a single population. The haplotype diversities (H) among AT-rich and ITS2 regions were 0.64 and 0.73, respectively, and the lowest nucleotide diversities (π) were 0.002 for the former and 0.004 for the latter (Table 4). Tajima's D values were negative for both the AT-rich (-2.29, $p < 0.01$) and ITS2 (-1.23, $p > 0.05$) regions. In addition, Fu's Fs values were significant and negative for both the AT-rich (-20.64, $p < 0.001$) and ITS2 (8.06, $p < 0.001$) regions. When the sequences from Korea and China were included in the calculations, significant negative Tajima's D and Fu's Fs values (Table 4) were found for both the partial AT-rich and ITS2 regions.

High-resolution molecular marker sequences of the AT-rich and ITS2 regions were only available from populations in East Asia. More mitochondrial COI gene sequences are available for download from GenBank from South Asia, Southeast Asia, and East Asia. COI gene sequences of *C. medinalis* from multiple Asian countries were divided into 14 haplotypes (Fig. 3). Phylogenetic analysis of COI revealed a polytomy and ambiguous relationships between haplotypes. The haplotype network of the COI region showed that most populations shared two

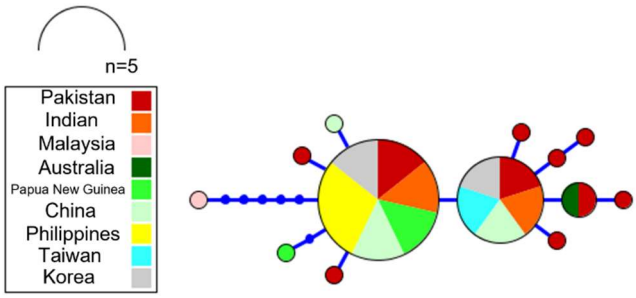

Fig. 4. Haplotype network of ***Cnaphalocrocis medinalis*** based on the mitochondrial COI gene.

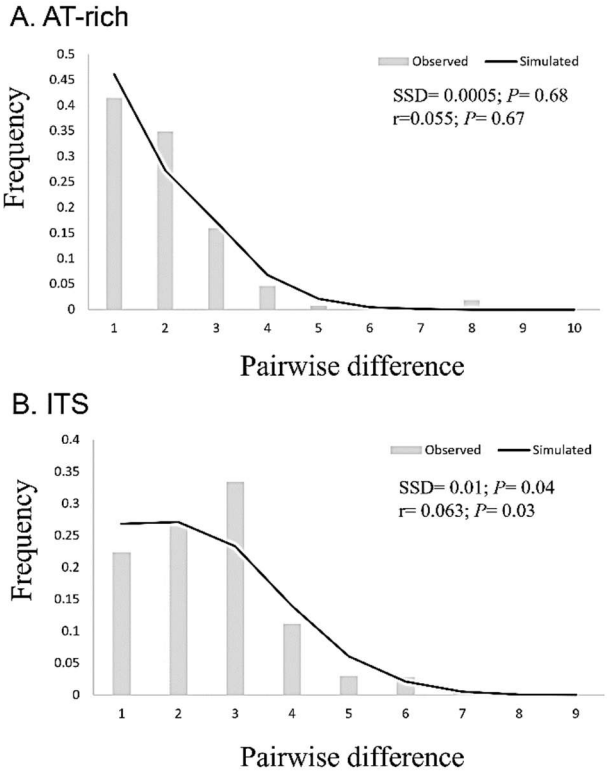

Fig. 5. Pairwise mismatch distributions of ***Cnaphalocrocis medinalis*** in East Asia based on mitochondrial AT-rich (A) and nuclear ITS2 (B) regions.

dominant haplotypes (Fig. 4). Taiwanese samples shared the haplotype with samples from India, Pakistan, China, and Korea. The haplotype network of both molecular markers indicated that most populations shared dominant haplotypes and displayed no significant geographic differentiation.

The population demography of *C. medinalis* in East Asia was also analyzed in this study. There was a unimodal mismatch distribution (Fig. 5), and the SSD and Harpending's raggedness index (r) based on AT-rich region genetic data were nonsignificant. The ITS2 region showed a unimodal mismatch distribution (Fig. 5) and a significant SSD and Harpending's raggedness index (r). The unimodal mismatch distribution revealed a recent population expansion.

DISCUSSION

Phylogenetic trees indicated that all specimens of *C. medinalis* in Taiwan grouped into one clade. The low intraspecific genetic distance and low population differentiation of *C. medinalis* suggested a lack of divergence among the six sampling sites. AMOVA showed that only a few genetic variations were shared among sampling sites, suggested no significant population structure. The results revealed that *C. medinalis* in Taiwan might be considered a single genetic population with no restricted gene flow between geographic sites.

Adult *C. medinalis* can be found across Taiwan throughout the year, except for winter. The intrinsic optimum temperatures for different developmental stages of *C. medinalis* are between 23.7 and 28.9 °C (Padmavathi *et al*., 2013b). The average lowest temperature based on 30 years (1991–2020) at the southernmost point of Taiwan was 18.6 °C (Central Weather Bureau of Taiwan). Due to low winter temperatures, *C. medinalis* encounters obstacles in completing its life cycle in Taiwan. In addition, the assistance of wind in the cross-sea migration of *C. medinalis* has been reported (Miyahara *et al*., 1981). *Cnaphalocrocis medinalis* might migrate from neighboring regions to Taiwan every year. However, molecular analysis of the AT-rich and ITS2 regions in *C. medinalis* from East Asia (Taiwan, Korea, and China) indicated no significant genetic differentiation, despite the limited geographical information available for these genes. The COI gene of *C. medinalis* can be obtained from a wider range of locations and provides more geographic information. The higher haplotype diversity of the mitochondrial COI gene in Pakistan suggested that *C. medinalis* may have originated in South Asia. The haplotype network of COI revealed that most *C. medinalis* specimens from South and Southeast Asia shared two dominant haplotypes (H11 and H14). The H11 haplotype was shared among Taiwan, Korea, China, Pakistan, and India. The lack of genetic divergence in populations of *C. medinalis* between Taiwan and neighboring regions suggested that they shared the same origin, although our molecular data are insufficient to trace the migration route of *C. medinalis* into Taiwan.

Extremely low genetic diversity of *C. medinalis* based on AT-rich and ITS2 DNA sequences was observed in East Asia in this study. Negative Tajima's D values from the neutrality tests revealed an excess of low-frequency mutations indicating recent population expansion (Tajima, 1989). Negative Fu's Fs values are the best explained by a recent population expansion (Fu, 1997). The unimodal mismatch distribution and lack of significance of SSD and r indices also provide evidence for a recent population expansion (Slatkin and Hudson, 1991; Rogers and Harpending, 1992). Therefore, low genetic diversity is due to the loss of genetic variation when a subset of individuals separate from a sizeable original population. Furthermore, the genetic distance data showed that there was no significant genetic differentiation between the populations in East Asia. The scenario of population expansion after a recent founder effect for *C. medinalis* in East Asia was possible, suggesting a single origin. However, the migration routes of *C. medinalis* are still unclear and more information and exploration are needed in the future.

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