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Genetic Diversity and Demographic History of Wax Moths, *Galleria mellonella* Linnaeus, 1758 and *Achroia grisella* Fabricius, 1794 (Lepidoptera: Pyralidae)

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Abstract

Wax moths, *Galleria mellonella* Linnaeus, 1758 and *Achroia grisella* Fabricius, 1794 are the key pests of honeybee colonies worldwide. They cause wax comb damages which lead to colony perishing and absconding. To control these pests, their genetic background and evolution should be considered. In this study, genetic diversity and demographic history of 2 species of wax moth were explored based on mitochondrial genes (COI). Wax moths were collected from northeatern part of Thailand for morphological and DNA analyses. *G. mellonella* and *A. grisella* sequences from GenBank were included for genetic diversity and demographic history analyses (total of 80 and 26 sequences, respectively). Overall genetic diversity for *A. grisella* samples from Northeastern Thailand was relatively low ($h = 0.467 \pm 0.132$ and $\pi = 0.00150 \pm 0.00042$). Likewise, low genetic diversity was also observed in *G. mellonella* samples ($h = 0.280 \pm 0.101$ and $\pi = 0.00046 \pm 0.00017$). Among samples from different global regions, the haplotype and nucleotide diversity of *A. grisella* were 0.834 ± 0.050 and 0.00996 ± 0.00124, respectively. The haplotype and nucleotide diversity of *G. mellonella* were 0.709 ± 0.034 and 0.01084 ± 0.00279, respectively. The ML tree revealed that these 2 species were separated into 2 major clades (*G. mellonella* clade and *A. grisella* clade). All sequences corresponding to *G. mellonella* formed to a single monophyletic clade, while all the sequences corresponding to *A. grisella* formed another single monophyletic clade. Clade *G. mellonella* was divided into 4 subclades designated as Clade Gm1, Gm2, Gm3 and Gm4, whereas *A. grisella* clade was divided into 3 subclades designated as Clade Ag1, Ag2 and Ag3. The sum of squares deviation (SSD) and Harpending's raggedness index (rg) and the results of Tajima's *D* and Fu's *F*s tests indicated population expansion was not found in either species.

Keywords: Wax moth, *Galleria mellonella*, *Achroia grisella*, Genetic diversity, Population expansion, COI, Thailand

Introduction

Beekeeping is critical to the global food supply, in terms of both honeybee products and pollination of crop plants. Beekeeping also contributes to the conservation of natural resources, especially among populations living near forest. However, habitat loss, climate change, pesticide usage, diseases, and insect pests are all threatening honeybee health and colony numbers [1,2].

Greater wax moth (Lepidoptera: Pyralidae, *Galleria mellonella* Linnaeus, 1758) or and lesser wax moth (Lepidoptera: Pyralidae, *Achroia grisella* Fabricius, 1794) are obliquitous pests of honeybee, *Apis mellifera* Linnaeus, 1758, and *Apis cerana* Fabricius, 1793 colonies worldwide [3,4]. They can be found in beehives, bumblebee nests, wasp nests, and stored waxes. Caterpillars feed on honey, pollen, beeswax, pupa skins, cocoons, and feces from the hive. The wax moth is a pervasive pest that causes severe damage to honeybee colonies by burrowing into wax combs, which leads to colony absconding, particularly in tropical and subtropical regions. Wax moth infestations are more likely to occur in weakened colonies, and the larvae of the moth can cause extensive damage, resulting in significant economic losses for beekeepers [3,6].

G. mellonella is found in all continents except Antarctica, particularly in mountain ranges, which corresponds with the presence of its host bees. It has been found in 77 countries and several islands, and it is expected that the pest will expand further, especially owing to climate change [1,7]. A new species of wax moth, *Galleria similis* Roh & Song, sp. nov. has been found in South Korea [8].

In response to economic losses of beekeeping due to wax moth, especially *G. mellonella*, several studies have examined their life history, biology, behavior, ecology, molecular biology, physiology, and control [3,6,9,10]. The complete mitochondrial genomes of *G. mellonella* [11] and *A. grisella* [12] have been reported. Other investigations have studied population structure and population expansion of other moths, the oriental fruit moth *Grapholita molesta* Busck, 1916 (Lepidoptera: Tortricidae) [13] and the soybean looper, *Chrysodeixis includens* Walker, 1858 (Lepidoptera: Noctuidae) [14]. However, the genetic diversity and evolutionary history of the wax moths remains unknown.

In this study, genetic diversity and demographic history of *A. grisella* and *G. mellonella* were explored. The genetic studies revealed significant heritable traits linked to mating signals, supporting the species' adaptive potential in varied environments, with demographic history indicating a high potential for population growth.

Materials and methods

Sample collection and identification

Wax moth samples were randomly collected from 5 provinces in Northeastern Thailand (**Table 1** and **Figure 1**). Larval wax moths were reared to adults in the laboratory. The initial identification of specimens at the species level was performed using morphological examination [3]. The identified samples were kept in 95% ethanol at −20 °C for DNA extraction.

Table 1 Sampling locations of wax moth from northeastern Thailand.

Location	Species	Code	No. of samples	Latitude/ Longitude
1) Mueang, Amnat Charoen	*G. mellonella* *A. grisella*	GA LA	5 5	15°51′55″N, 104°37′44″E
2) Chiang Khan, Loei	*G. mellonella*	GL	5	17°53′40″N, 101°39′21″E
3) Mueang, Nakhon Ratchasima	*G. mellonella*	GK	10	15°26′42″N, 102°19′52″E
4) Mueang, Mahasarakham	*G. mellonella*	GM	5	15°50′27″N, 103°12′11″E
5) Kut Chap, Udon Thani	*A. grisella*	LU	5	17°25′11″N, 102°33′58″E
Total			**35**	

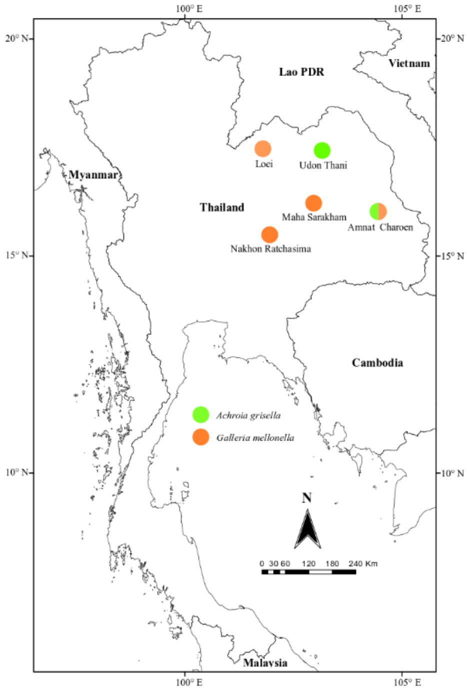

Figure 1 Map of study sites and diversity of wax moth species in Northeastern Thailand.

DNA extraction, PCR amplification and DNA sequencing

DNA was extracted from thorax using the protocols described by Buala and Sopaladawan [15] with some modifications. Polymerase chain reaction (PCR) of the Cytochrome c oxidase subunit I (COI) gene was amplified using the primers LCO1490 (5´GGTCAACAAATCATAAAGATATTGG-3´) and HCO2198 (5´-TAAACTTCAGGGTGACCAAAAAA TCA-3´) [16]. PCR reaction conditions followed those of Rivera and Currie [17]. PCR products were checked for quality evaluation using 1 % agarose gel electrophoresis. The PCR products were sent to Macrogen sequencing service (Seoul, Korea) for the purification and DNA sequencing.

Data analysis

A total of 35 COI sequences from 2 species of wax moth comprising, *A. grisella* (10 sequences) and *G. mellonella* (25 sequences) from Northeastern Thailand were obtained. In addition, GenBank records for *A. grisella* (16 sequences) and *G. mellonella* (55 sequences) from other countries were also incorporated into the data set (**Table 2**). COI sequences were aligned using the MUSCLE (Edgar, 2004) in MEGA X v.11 [18] with the default settings. Intraspecific and interspecific genetic divergences for each species from Northeastern Thailand were also performed in MEGA X v.11 [18] based on the Kimura 2-parameter (K2P) model. Calculations of COI genetic diversity indices were performed with the software package DnaSP v.5.10.01 [19].

Neighbor-Joining (NJ), Maximum likelihood (ML) and Bayesian inference (BI) methods were used to reconstruct the phylogenetic relationships in the COI sequences. For all phylogenetic analyses, *Paraphomia disjuncta* Whalley, 1964 sequences (accession no. FJ500771-73) were used as an out group to root the trees. The NJ tree was calculated in MEGA X v.11 [18] with 1,000 bootstrap replicates for branch support calculation. The ML tree was produced in IQ-TREE v.2.0.4 [20] with the best model selected by ModelFinder [21] and branch support was calculated using ultrafast bootstrapping with 1,000 replicates [22]. Bayesian inference was performed in MrBayes 3.04b [23] and Bayesian posterior probabilities were estimated using Markov chain Monte Carlo analysis, which was run for 2,000,000 generations, with a sampling frequency of 100 generations, discarding the first 2000 sampled trees as burn-in. The final phylogenetic trees were viewed and edited using FigTree v.1.4.0 [24].

A haplotype network was created using the median-joining network method in PopART v.1.7 [25] to visualize the relationships of the haplotypes for each wax moth species. To find evidence of demographic population expansion or population equilibrium of each species, mismatch distribution analysis and test statistics (SSD and rg) were computed using Arlequin v.3.5 [26]. Smooth and unimodal distributions are typical of populations expansion, whereas ragged and multimodal distributions are found in populations at demographic equilibrium [27]. Both sum of squared deviations (SSD) and Harpending's raggedness index (rg) were calculated to test for demographic expansion under the sudden expansion model using Arlequin v.3.5 [26]. The sudden expansion model is rejected at $P < 0.05$.

Table 2 GenBank accession numbers for COI sequences used in the present study.

Wax moth species	Country (Province)	Number of sequences	GenBank no.
Achroia grisella	China	1	MF509586
	Germany	5	GU706998-999 GU686750 KX040021 KX044795
	Finland	2	JF853921 KX041272
	USA	3	GU089346-347 HQ572878

Wax moth species	Country (Province)	Number of sequences	GenBank no.
	Madagascar	2	MH416068 MH416738
	Australia	3	KF397100 KF397988 KF403025
Total		**16**	
Galleria mellonella	China	3	ON823179-181
	India	6	OP164864-866 MG941001 MW488306
	Iraq	4	MN187961-964
	South Korea	17	MT439336-352
	Germany	6	GU686751 MG432719 KX041116 KX040593 KX040159 KX046439
	Finland	3	KX041204 KX041559 KT782456
	Austria	1	KP253212
	United Kingdom	1	KX044004
	Canada	4	GU091133-134 GU438917-918
	USA	4	GU087819 GU089437 KF491749-750
	Gabon	1	MK187421
	Madagascar	1	MH416635
	Australia	4	KF397592 KF398098 GU688828 JN270728
Total		**55**	

Results and discussion

Results

Genetic diversity

The level of interspecific genetic divergence based on the Kimura 2-parameter ranged from 13.20 -13.40 %, with average 13.10 %. Intraspecific genetic divergence of Thai *A. grisella* ranged from 0 - 0.32 %, with a mean of 0.15 %. Intraspecific genetic divergence of Thai *G. mellonella* ranged from 0 - 0.16 %, with a mean of 0.05 %. A summary of molecular diversity indices of COI sequences of 2 wax moth species is presented in **Table 3**. Overall genetic diversity for *A. grisella* species from Northeastern Thailand was relatively low (h = 0.467 ± 0.132 and π = 0.00150 ± 0.00042). Similarly, low genetic diversity was also observed in *G. mellonella* species (h = 0.280 ± 0.101 and π = 0.00046 ± 0.00017).

When comparing the genetic diversity of these 2 wax moth species from Thailand with the other geographic regions, it was found that the haplotype and nucleotide diversity of *A. grisella* were 0.834 ± 0.050 and 0.00996 ± 0.00124, respectively (**Table 3**). The haplotype and nucleotide diversity of *G. mellonella* were 0.709 ± 0.034 and 0.01084 ± 0.00279, respectively (**Table 3**).

Table 3 Genetic diversity indices of *Achroia grisella* and *Galleria mellonella* species from Northeastern Thailand and other countries.

Species	**Country**	**Geographic region**	**N**	**H**	**S**	***h* ± SD**	***π* ± SD**
A. grisella	Thailand	Asia	10	2	2	0.467 ± 0.132	0.00150 ± 0.00042
	China	Asia	1	1	-	-	-
	Germany	Europe	5	1	0	0.000 ± 0.000	0.00000 ± 0.00000
	Finland	Europe	2	1	0	0.000 ± 0.000	0.00000 ± 0.00000
	USA	America	3	2	1	0.667 ± 0.314	0.00112 ± 0.00053
	Madagascar	Africa	2	1	0	0.000 ± 0.000	0.00000 ± 0.00000
	Australia	Australia	3	3	14	1.000 ± 0.272	0.01761 ± 0.00773
	Total		**26**	**8**	**15**	**0.834 ± 0.050**	**0.00996 ± 0.00124**
G. mellonella	Thailand	Asia	25	2	1	0.280 ± 0.101	0.00046 ± 0.00017
	China	Asia	3	2	1	0.667 ± 0.314	0.00102 ± 0.00048
	India	Asia	6	4	43	0.800 ± 0.172	0.03817 ± 0.00793
	Iraq	Asia	4	3	3	0.833 ± 0.222	0.00299 ± 0.00109
	South Korea	Asia	17	2	1	0.309 ± 0.122	0.00063 ± 0.00025
	Germany	Europe	6	3	3	0.600 ± 0.215	0.00157 ± 0.00070
	Finland	Europe	3	2	1	0.667 ± 0.314	0.00105 ± 0.00050
	Austria	Europe	1	1	-	-	-
	United Kingdom	Europe	1	1	-	-	-
	Canada	America	4	1	0	0.000 ± 0.000	0.00000 ± 0.00000
	USA	America	4	1	0	0.000 ± 0.000	0.00000 ± 0.00000
	Gabon	Africa	1	1	-	-	-
	Madagascar	Africa	1	1	-	-	-
	Australia	Australia	4	1	0	0.000 ± 0.000	0.00000 ± 0.00000
	Total		**80**	**11**	**47**	**0.709 ± 0.034**	**0.01084 ± 0.00279**

Note: Number of sequences (N), number of haplotypes (H), number of polymorphic sites (S), haplotype diversity (*h*), and nucleotide diversity (*π*) with standard deviation (SD).

Phylogenetic relationships and haplotype network construction

All 3 phylogenetic analysis methods (NJ, ML, and Bayesian) gave similar tree topologies; thus, only the ML tree is shown (**Figure 2**). The ML tree revealed that these 2 species were separated into 2 major clades (*G. mellonella* clade and *A. grisella*), supported by perfect bootstrap values (100 %). All sequences corresponding to *G. mellonella* (80 sequences) formed to a single clade, while all the sequences corresponding to *A. grisella* (26 sequences) formed another single clade. Clade *G. mellonella* was resolved into 4 subclades designated as Clade Gm1, Gm2, Gm3 and Gm4, whereas *A. grisella* clade was resolved into 3 subclades designated as Clade Ag1, Ag2 and Ag3, supported by high bootstrap values (99 - 100 %).

The relationships of 26 sequences of *A. grisella* and 80 sequences of *G. mellonella* based on COI sequences are separately shown in median-joining haplotype networks (**Figure 3**). Based on mutation steps, the haplotype network of *A. grisella* showed 3 distinct clades (Ag1, Ag2 and Ag3), whereas the haplotype network of *G. mellonella* showed 4 distinct clades (Gm1, Gm2, Gm3 and Gm4).

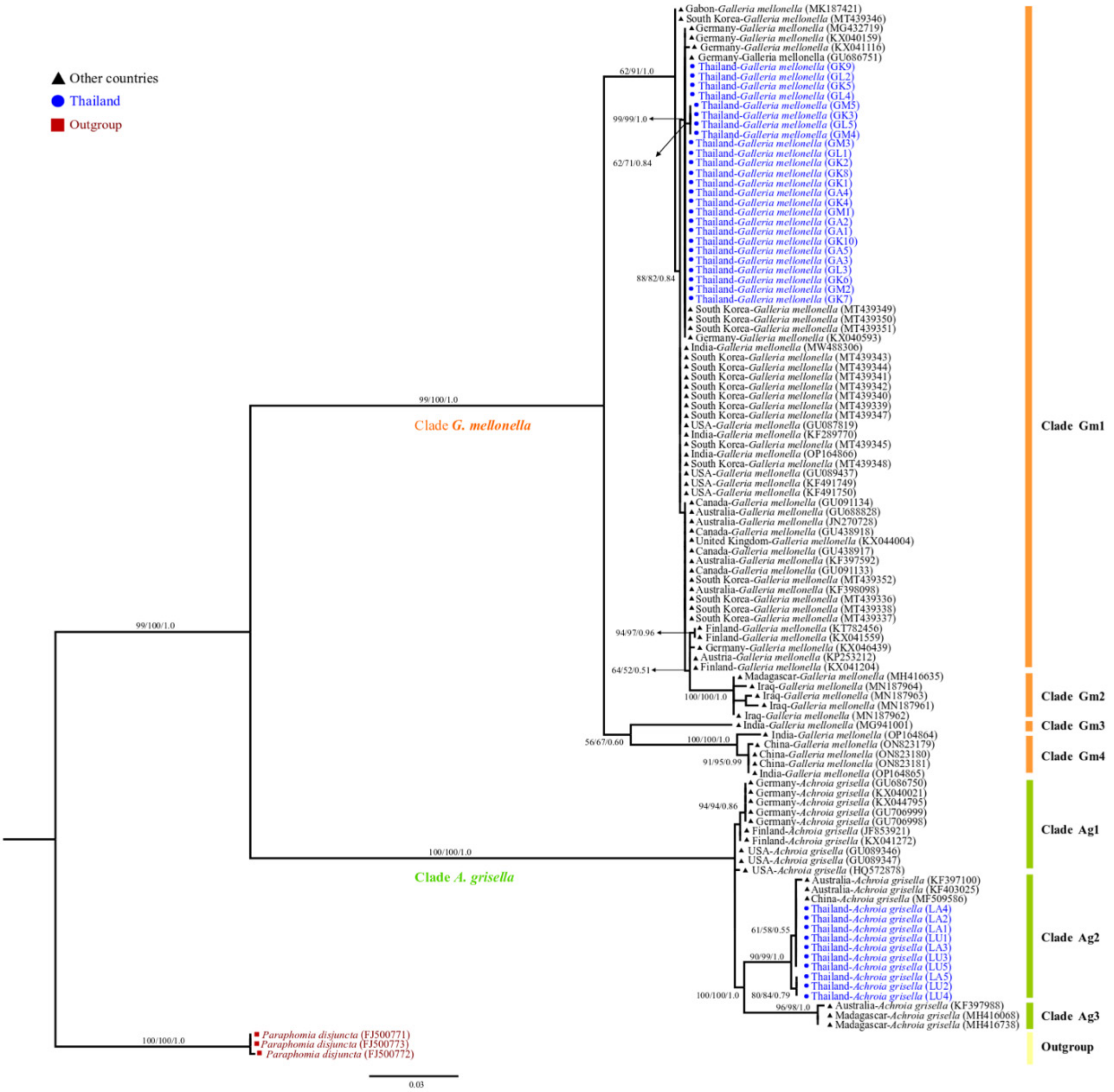

Figure 2 Maximum likelihood tree representing the relationships of haplotype between *Achroia grisella* and *Galleria mellonella*. Bootstrap values for neighbor- joining, maximum likelihood and posterior probability of Bayesian analysis are shown above or near the branches.

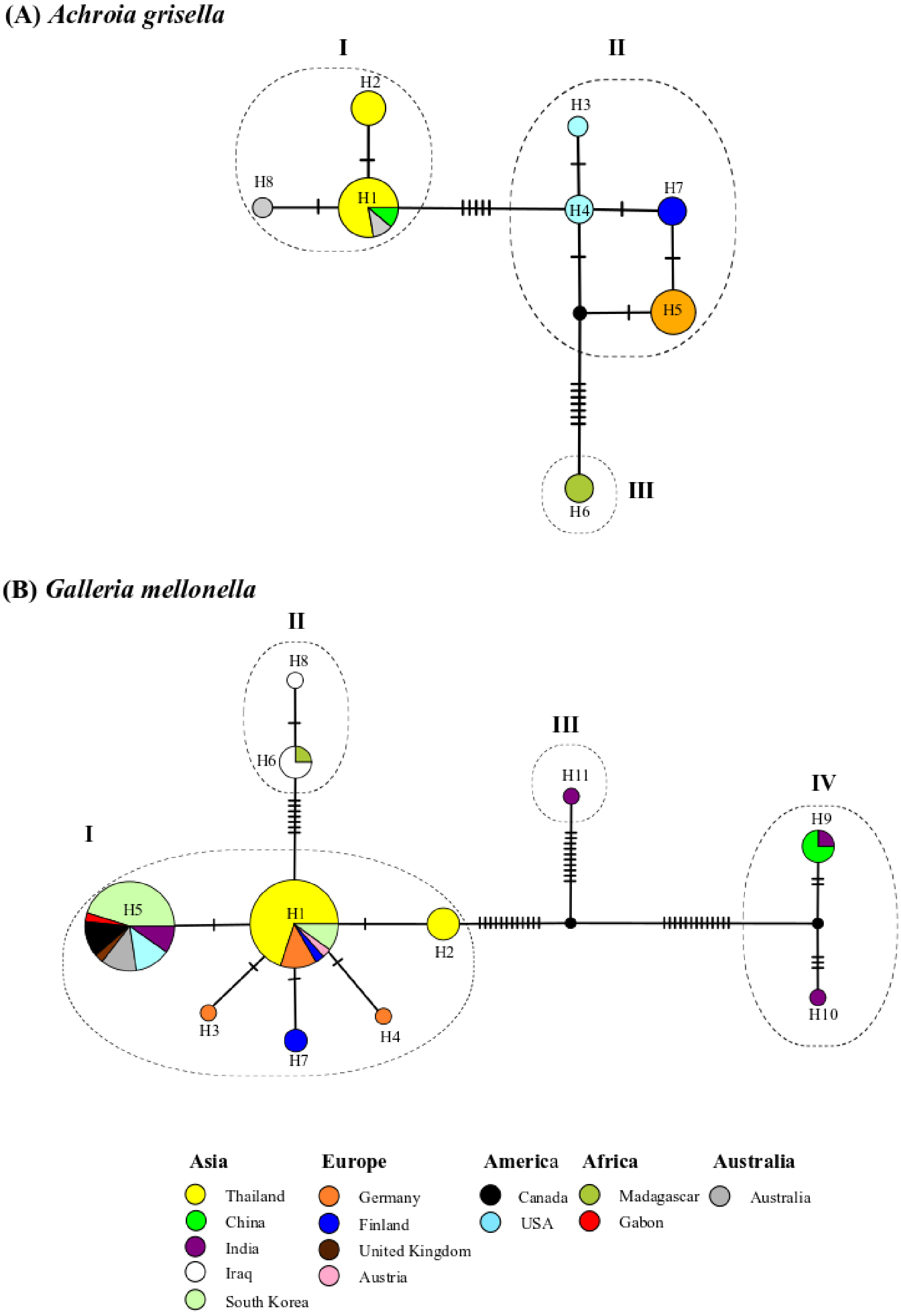

Figure 3 Median joining networks representing the relationships of the haplotypes for *Achroia grisella* (A) and *Galleria mellonella* (B) based on COI haplotypes. Small black dots indicate median vectors. Each haplotype is represented by a circle, and circle sizes are proportional to the total sequence of each haplotype. Colors indicate the geographic origin of the haplotypes and crossbars indicate mutation steps.

Demographic history

The observed mismatch distribution did not show a unimodal pattern, but the results had a multimodal pattern for both *A. grisella* and *G. mellonella* (**Figure 4**), characteristic of population in demographic equilibrium or a population decline. Both SSD and rg values showed significant difference between the observed and simulated data for both species ($P < 0.05$).

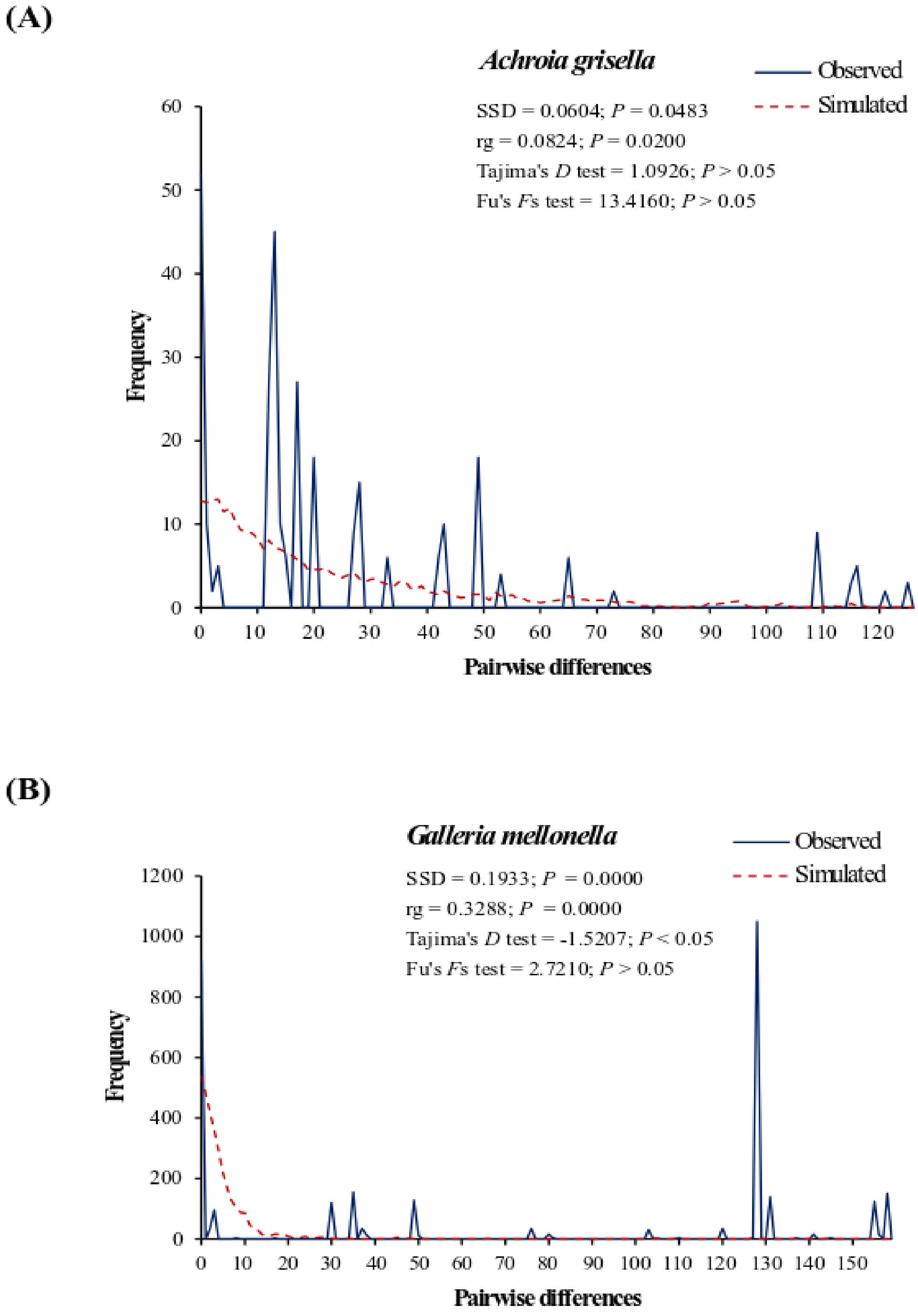

Figure 4 Mismatch distribution analysis based on COI sequences for *Achroia grisella* and *Galleria mellonella* species. Statistics test (Tajima's *D*, Fu's *F*s, SSD and rg) are given.

Discussion

Morphological and DNA analyses indicated that there were 2 species of wax moth, *A. grisella* and *G. mellonella* found in Thailand. A high level of interspecific genetic divergence was found. Low intraspecific genetic divergence and haplotype diversity were investigated in both species of wax moth in Thailand. These results may be because of the small sample size of wax moth specimens.

Relatively high haplotype diversity values with relatively low nucleotide values in both *A. grisella* (h = 0.834 and $\pi = 0.00996$) and *G. mellonella* (h = 0.709 and $\pi = 0.00279$) sequences from different part of the world indicated several closely related haplotypes, implying that these groups may have recently expanded at some level [28].

NJ, ML, and Bayesian trees (**Figure 2**) exhibited 2 major monophyletic clades (*G. mellonella* clade and *A. grisella*). The *G. mellonella* clade was divided into 4 subclades referred to as Clade Gm1, Gm2, Gm3, and Gm4. Clade Gm1 contained most of the samples from different locations, including all samples from Thailand. Interestingly, *G. mellonella* samples from India were found in Clades Gm1, Gm3, and Gm4, indicating that it was widely distributed in Indian populations.

The *A. grisella* clade retrieved ten samples from Thailand that were clustered together with sequences from Australia and China (Clade Ag2). Samples from Germany, Finland and USA were grouped together into Clade Ag1. The median joining networks (**Figure 3**) were consistent with the phylogenetic analysis. The haplotype network analysis of *A. grisella* revealed that H1 was the most common haplotypes and was shared with sequences from Thailand, China and Australia. The haplotype network analysis of *G. mellonella* showed that H1 was the central haplotype and H5 was the most common haplotype. Subclades and haplotypes *A. grisella* were associated with different geographic regions.

SSD and rg values showed significant difference between the observed and simulated data for both species ($P < 0.05$), supporting the hypothesis that the sudden expansion model was rejected. In addition, the results of Tajima's D and Fu's Fs tests were not statistically significant for both *A. grisella* and *G. mellonella* sequences, indicating that the tests did not support the patterns of a population expansion. The results suggested that population expansion of *A. grisella* and *G. mellonella* had been quite limited. This may have influenced the global distribution of wax moths *via* several factors such as the global warming, climate change and colony relocation by humans [1].

Temperature and annual precipitation are important factors influencing *G. mellonella* distribution [29, 30]. The study on modeling the potential global distribution of *G. mellonella* revealed that India, China, Thailand, USA, and Australia were highly suitable for *G. mellonella*, which threatens honeybee products industry of those countries, whereas high temperature areas such as Africa are predicted to be less suitable [1].

Life cycle and behavior of wax moths are related to their population expansion. The life cycle of *G. mellonella* contains 4 stages, egg, larva, pupa and adult, take approximately 86 days (males) and 76 days (females). The longest stage is the larval stage (7 instars, approximately 50 days) and the larvae can cause severe damage to honeybee combs. Longevity of adult wax moths is shorter than the larval stage (about 16 days in males and 6 days in females) [6], resulting less opportunity to naturally disperse to other areas. However, the spread of wax moths may be triggered by humans through beekeeping industry across the world.

Conclusions

This study revealed low genetic diversity in both *A. grisella* populations and *G. mellonella* populations in Thailand. Relatively high haplotype diversity values with relatively low nucleotide values among sequences in both *A. grisella* and *G. mellonella* from different countries. Analysis on phylogenetic relationships was consistent with haplotype network construction among DNA sequences of wax moth from different part of the world. *A. grisella* sequences were separated into 3 subclades and *G. mellonella* were grouped into 4 subclades. Population expansion was not detected in either species. However, further study using more variable markers such as microsatellite DNA will be useful to elucidating genetic structure of these wax moth species.

Ethical approval

The research project had been reviewed and approved by the Institutional Animal Care and Use

Committee, Mahasarakham University (IACUC-MSU). The approval number is IACUC-MSU-009/2020.

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