

GENETIC DIVERSITY AND DEMOGRAPHIC HISTORY OF DWARF HONEYBEE *Apis florea* FABRICIUS, 1787 POPULATIONS IN THAILAND

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Abstract

The dwarf honeybee, *Apis florea* Fabricius, 1787, is native to Southeast Asia, with its primary habitats in Thailand, Pakistan, India, Sri Lanka, Myanmar, and Laos. This species plays a crucial role in pollinating a wide variety of plants and economically important crops. In this study, we examined the genetic diversity and demographic history of *A. florea* in Thailand using partial sequences of mitochondrial cytochrome c oxidase I (COI) gene. The analysis was conducted using samples from 59 *A. florea* colonies across Thailand, combined with 13 COI sequences of *A. florea* from various countries obtained from GenBank. Overall, low nucleotide diversity (0.00321) coupled with high haplotype diversity (0.858) were observed within the Thai populations. The low genetic variation in *A. florea* suggests that this species occupies a wide range of environments and maintains temporally continuous gene flow, possibly due to its reproductive season. Median-joining network analysis revealed a star-like shape, indicating recent population expansion. This finding was further supported by a unimodal mismatch distribution and significantly negative results for Tajima's D and Fu's F_s tests. Demographic history analyses suggested that *A. florea* may have experienced a population expansion approximately 55,000-243,000 years ago.

Keywords: *Apis florea*; Genetic variation; Pleistocene; Population expansion; MtCOI

Introduction

Honeybees are the most economically important insect, producing honey, beeswax, royal jelly, pollen, and bee venom. Honeybee products are becoming increasingly significant as health foods and in apitherapy. Bee venom is commonly used to treat arthritis and other autoimmune illnesses. The most essential contribution of honeybee is pollinating numerous economically important crops and natural plants (Oldroyd, 2021).

Dwarf honeybee *Apis florea* Fabricius, 1787 (order Hymenoptera, genus *Apis*), is native to Southeast Asia. It plays a crucial role in pollinating wild plants, supporting global agriculture, and maintaining ecosystems. *A. florea* exhibits non-selective foraging behavior on flowers, contributing to pollination, enhancing genetic diversity in plants, and increasing agricultural productivity (Oldroyd, 2021). *A. florea* is a wild bee species that is unique

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due to its small size (Hepburn and Radloff, 2011). It commonly builds single nests on shrubs, trees, buildings, or caves, and can be found in both urban and rural localities. The body size of *A. florea* is generally smaller and they are less aggressive than other *Apis* species. They are adaptable and can tolerate hot climates (Kevan, 2021). Furthermore, they successfully compete for foraging resources with other bee species (El-Niweiri *et al.*, 2019). Its geographical distribution has been documented from Persian Gulf region to Himalayas, extending to the Asia Pacific region, including Thailand (Hepburn and Radloff, 2011; Salem *et al.*, 2020). Furthermore, their spread into various countries by humans has been documented, including Saudi Arabia, Jordan, Sudan, Ethiopia, Egypt (Salem *et al.*, 2020), Indonesia, Taiwan, Singapore and Israel (Silva *et al.*, 2020).

Asadi *et al.* (2016) studied the geographic diversity of *A. florea* populations in southern Iran using microsatellite markers. They identified 2 distinct clusters among the populations. However, genetic diversity and population structure of *A. florea* populations in India were observed using COII region, revealing 6 distinct clusters (Lazar *et al.*, 2013). In Thailand, *A. florea* nests are commonly found in various habitats, both in rural and urban areas. Research on *A. florea* in Thailand has primarily focused on behavior, ecology, distribution, and morphology (Deowanish *et al.*, 2000). Chaiyawong *et al.* (2004) conducted multivariate morphometric analyses in Thai *A. florea* populations using 22 characters. The results showed that the populations clustered into a single group. Similarly, PCR-RFLP analysis using mitochondrial DNA between the COI-COII regions of *A. florea* in Thailand demonstrated lower genetic variation was observed (Nanork, 2001).

The honey and nest products of *A. florea* have contributed to improving villagers' incomes in Thailand (Oldroyd, 2021). 'Bee hunter' is a well-known profession, and a single person may harvest hundreds of *A. florea* nests per week and sell them at the local market. However, because honey is obtained by destroying the brood, *A. florea* populations in Thailand have rapidly declined (Deowanish *et al.*, 2000; Oldroyd and Nanork, 2009). *A. florea* populations are likely to exhibit significant genetic structure, as the flight range of swarming, absconding and migration is relatively small, ranging from a few to several hundred meters (Ruttner, 1988). Generally, *A. florea* nests are found on plains up to 500 metres above sea level. However, seasonal migrations occur to elevations of up to 1,500 meters above sea level, and even

higher in northern Thailand and southern China (Deowanish *et al.*, 2000). These migrations may represent an adaptation to seasonal variations and food scarcity (Ruttner, 1988).

The genetic diversity and demographic history of *A. florea* provide crucial insights into its evolutionary history and current resilience. The purpose of this study was to analyze the genetic diversity and demographic history of *A. florea* populations in Thailand using mitochondrial cytochrome c oxidase I (COI) sequences. Understanding these mechanisms is vital for designing conservation and management plans for this important pollinator.

Materials and Methods

Sample Collection

Adult workers of *A. florea* were collected from 21 localities across Thailand (Table 1; Figure 1); a total of 59 *A. florea* colonies were collected for this study. Bee specimens were stored in 95% ethanol at -20°C, a bee per colony was used for genetic analysis. This research was conducted with approval from the Institutional Animal Care and Use Committee at Mahasarakham University (ACUC-MSU), with the approval number IACUC-MSU-0019/2017.

DNA extraction, PCR amplification and DNA sequencing

Total DNA was extracted from the thorax of each bee using E.Z.N.A. Tissue DNA Kit and stored at 4°C. The COI gene fragment was amplified in a 30 µL reaction volume containing 20 mM Tris-HCl, pH 8.4, 1.0 mM MgCl₂, 100 µM of each dNTP, 0.4 µM of both forward and reverse primers-LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.*, 1994), 25 ng of genomic DNA, and 0.04 U/µl *Taq* DNA polymerase. The PCR included an initial denaturation at 96°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. A final extension was performed at 72°C for 10 min. The PCR products were verified using 1% (w/v) agarose gel electrophoresis and visualized under a UV transilluminator. Successfully amplified PCR products were sent to Macrogen in Korea for purification and sequencing using the same primers.

Table 1. Collecting locations of *Apis florea* samples in Thailand

Region	Locality (code) Latitude/Longitude	No. of COI sequence	Specimen ID	GenBank no.
Northern	Chiang Rai (CRI) 20°17'20"N, 99°48'35"E	2	N-Chiang Rai	OK350400-01
	Nan (NAN) 18°53'58"N, 100°27'23"E	1	N-Nan	OK350406
	Phetchabun (PBN) 16°42'21"N, 101°03'03"E	1	N-Phetchabun	OK350399
	Phrae (PRE) 18°07'26"N, 100°10'44"E	4	N-Phrae	OK350402-05
Northeastern	Ubon Ratchathani (UBN) 15°58'06"N, 105°16'38"E	7	NE-Ubon Ratchathani	OK350421-27
	Kalasin (KSN) 16°14'37"N, 103°27'13"E	4	NE-Kalasin	OK350416-19
	Loei (LEI) 17°26'55"N, 101°28'41"E	1	NE-Loei	OK350420
	Maha Sarakham (MKM) 16°15'07"N, 103°14'21"E	4	NE-Maha Sarakham	OK350412-15
	Khon Kaen (KKN) 16°28'21"N, 102°49'14"E	3	NE-Khon Kaen	OK350409-11
	Buri Ram (BRM) 15°17'24"N, 103°11'24"E	1	NE-Buri Ram	OK350408
	Amnat Charoen (ACR) 15°51'55"N, 104°33'38"E	1	NE-Amnat Charoen	OK350407
	Ratchaburi (RBI) 13°30'59"N, 99°57'14"E	1	C-Ratchaburi	OK350385
	Kanchanaburi (KRI) 14°14'07"N, 99°01'23"E	3	C-Kanchanaburi	OK350382-84
	Chanthaburi (CTI) 12°57'51"N, 102°24'24"E	3	C-Chanthaburi	OK350386-88
Central	Phetchaburi (PBI) 12°42'15"N, 99°57'28"E	2	C-Phetchaburi	OK350380-81
	Nakhon Pathom (NTP) 13°47'53"N, 100°14'00"E	1	C-Nakhon Pathom	OK350379
	Samut Songkhram (SKM) 13°25'32"N, 99°38'41"E	1	C-Samut Songkhram	OK350378
	Saraburi (SRI) 14°48'19"N, 101°18'56"E	1	C-Saraburi	OK350377
	Phra Nakhon Si Ayutthaya (AYA) 14°21'34"N, 100°35'34"E	2	C-Phra Nakhon Si Ayutthaya	OK350375-76
	Lop Buri (LRI) 14°46'47"N, 100°40'42"E	6	C-Lop Buri	OK350369-74
	Samui Island, Surat Thani (SMI) 9°34'12"N, 100°03'49"E	10	S-Surat Thani	OK350389-98
	Total	59		

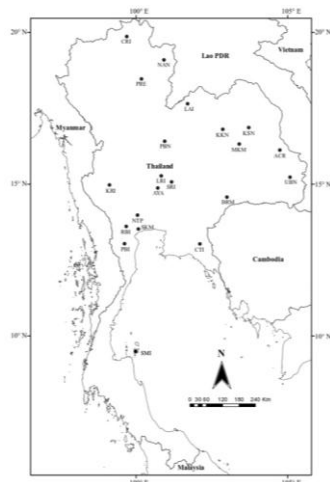


Figure 1. Map of study sites of *Apis florea* in Thailand. The sampling locations details were given in Table 1.

Table 1. Map of study sites of *Apis florea* in Thailand. The sampling locations details were given in Table 1.

Country	No. of samples	GenBank no.
India	2	KT960841 KU752356
Myanmar	5	MT679389-393
Thailand	6	MT670331-336
Total	13	

Data Analysis

Sixty-five COI sequences of *A. florea* from Thailand were analyzed, 59 of which were obtained in this study (GenBank accession numbers: OK350369–OK350427), while six sequences were retrieved from the NCBI GenBank database (Table 2). Additional seven GenBank records of *A. florea* from other countries were also included in the data

set (Table 2). In total, 72 COI sequences were aligned using MUSCLE (Edgar, 2004) in MEGA X v.11 (Kumar *et al.*, 2018), resulting in a final sequence length of 619 bp. Genetic diversity indices were calculated for individual and overall *A. florea* populations using DnaSP v5.10.01 (Librado and Rozas, 2009). Intraspecific genetic divergences of *A. florea* from Thailand were assessed using the Kimura 2-parameter (K2P) model in MEGA X v.11 (Kumar *et al.*, 2018).

Phylogenetic relationships of the COI sequences were reconstructed using Neighbor-Joining (NJ), Maximum Likelihood (ML), and Bayesian Inference (BI) methods. *Apis andreniformis* sequences (accession numbers: KU212333–KU212335) were used as the outgroup to root the trees. The NJ tree was constructed in MEGA X v.11 (Kumar *et al.*, 2018) with 1,000 bootstrap replicates to assess branch support. The ML tree was generated using IQ-TREE v.2.0.4 (Nguyen *et al.*, 2015), with ModelFinder used to identify the best-fit model (Kalyaanamoorthy *et al.*, 2017). Branch support was calculated using ultrafast bootstrapping with 1,000 replicates (Hoang *et al.*, 2018). Bayesian inference was conducted in MrBayes 3.04b (Huelsenbeck and Ronquist, 2001) with Bayesian posterior probabilities estimated using Markov Chain Monte Carlo (MCMC) analysis, run for 2,000,000 generations with a sampling frequency of 100 generations, and the first 2,000 trees discarded as burn-in. Final phylogenetic trees were visualized and edited using FigTree v.1.4.0 (Rambaut, 2012). A haplotype network was constructed using the median-joining network method in PopART v.1.7 (Leigh and Bryant, 2015) to visualize haplotype relationships.

To test the hypothesis of population expansion, mismatch distribution analysis was conducted using Arlequin v.3.5.1.2 (Excoffier and Lischer, 2010). A unimodal distribution typically

indicates expanding populations, while a multimodal distribution suggests stable populations (Rogers and Harpending, 1992). The sum of squared deviations (SSD) and Harpending's raggedness index (rg) were calculated to test for demographic expansion under the sudden expansion model, with significance tested at $P < 0.05$ using Arlequin v.3.5.1.2 (Excoffier and Lischer, 2010). The expansion time was estimated using the equation $\tau = 2ut$, where $u = mT\mu$ (mT = sequence length, μ = mutation rate per nucleotide), and t = generation time (Rogers and Harpending, 1992), assuming a divergence rate of 3.54% per million years for insect COI gene (Papadopolou *et al.*, 2010) and generation time (g) = 1 (Cao *et al.*, 2023).

Results and Discussion

Results

Genetic diversity

Genetic diversity indices of *A. florea* populations based on COI sequences are presented in Table 3. Analysis of 72 COI sequences identified a total of 25 haplotypes. The overall average haplotype diversity was 0.880. The average nucleotide diversity was 0.00440, with the nucleotide diversity of individual populations ranging from 0 in Myanmar to 0.00405 in Northeastern Thailand. Among the 65 COI sequences from Thai *A. florea* populations, a total of 22 haplotypes were identified. High haplotype diversity was observed within each population, ranging from 0.705 to 0.848. In contrast, nucleotide diversity was relatively low across all populations, ranging from 0.00165 to 0.00405. Intraspecific genetic divergence based on the K2P model for Thai *A. florea* ranged from 0 to 0.975%, with an average of 0.322%.

Table 3. Genetic diversity indices of *Apis florea* populations from Thailand and other countries

Populations	Sample size	No. of haplotype	No. of polymorphic sites	Haplotype diversity $\pm$ SD	Nucleotide diversity $\pm$ SD
Northern Thailand	14	7	5	0.802 $\pm$ 0.094	0.00225 $\pm$ 0.00046
Northeastern Thailand	21	10	14	0.848 $\pm$ 0.059	0.00405 $\pm$ 0.00057
Central Thailand	20	6	6	0.705 $\pm$ 0.070	0.00165 $\pm$ 0.00036
Southern Thailand	10	4	4	0.711 $\pm$ 0.117	0.00226 $\pm$ 0.00055
All Thai populations	65	22	23	0.858 $\pm$ 0.029	0.00321 $\pm$ 0.00033
India	2	2	1	1.000 $\pm$ 0.500	0.00162 $\pm$ 0.00081
Myanmar	5	1	0	0.000 $\pm$ 0.000	0.00000 $\pm$ 0.00000
All populations	72	25	32	0.880 $\pm$ 0.024	0.00440 $\pm$ 0.00069

This study revealed low genetic diversity among *A. florea* populations in Thailand. Low nucleotide diversity (0.003), coupled with high haplotype diversity (0.858), suggests the presence of many closely related haplotypes, indicating that Thai *A. florea* populations may have undergone expansion (Mendez-Harclerode *et al.*, 2007). A previous study on the genetic differentiation of *A. florea* in Thailand, focusing on the COI-COII intergenic region using PCR-RFLP, also reported limited genetic diversity and a lack of genetic differentiation across *A. florea* populations from geographically distinct locations (Nanork, 2001).

Phylogenetic Relationships and Mitochondrial Genealogy

The phylogenetic relationships reconstructed using three methods displayed similar topologies. The ML tree, shown with bootstrap support values

for all three analyses, is presented in Figure 2. In the ML tree, *A. florea* populations from Thailand did not group according to their geographical origin. The MJ network of 72 COI sequences (59 sequences from this study and 13 sequences retrieved from the NCBI GenBank database) revealed no major divergent lineages (Figure 3). The overall haplotype network displayed a star-like pattern, a characteristic indicative of population expansion. Nine haplotypes differed by one mutation step, while the remaining haplotypes differed by two or more mutation steps. Sequences from all geographic regions, except Myanmar and India, shared the central haplotype (H2). All COI sequences from Thailand and other countries consisted of 25 haplotypes, and none of the haplotypes from Thailand were shared with sequences from other countries.

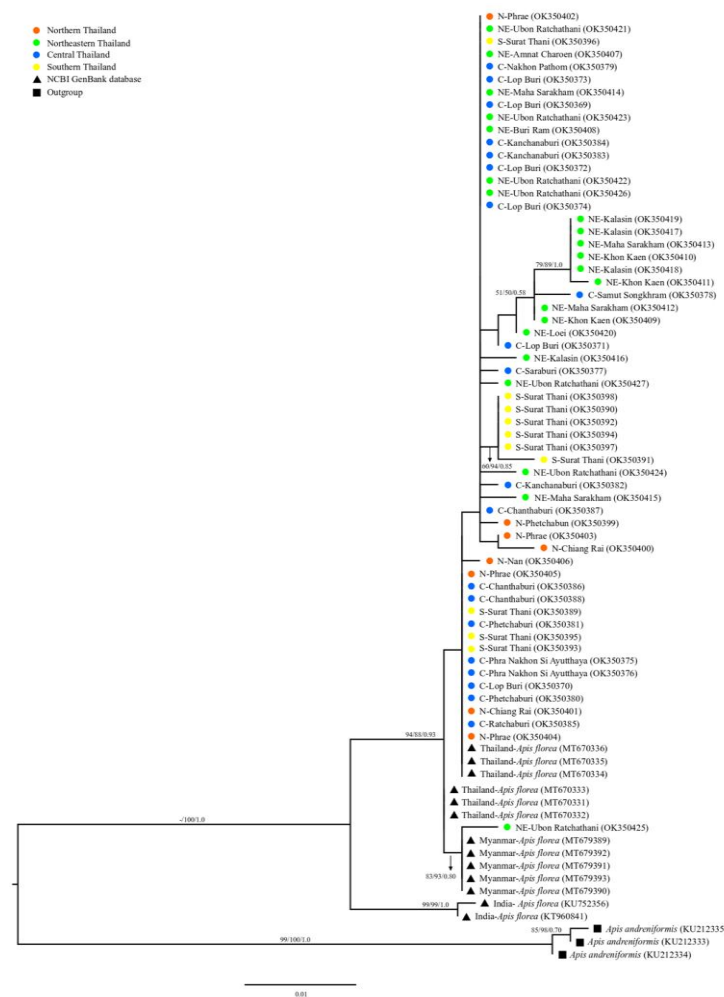


Figure 2. Maximum likelihood tree representing the relationships of *Apis florea* sequences. Bootstrap values for neighbor- joining, maximum likelihood and posterior probability of Bayesian analysis are shown above or near the branches

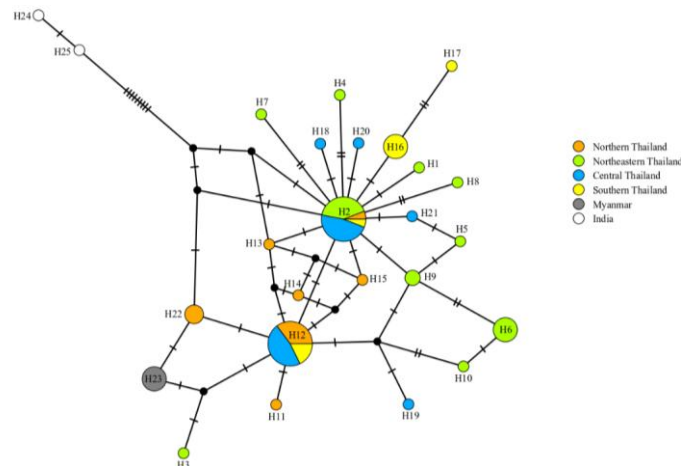


Figure 3. Median-joining networks representing the relationships of the haplotypes for *Apis florea* based on COI haplotypes. Small black dots indicate median vectors. Each haplotype is represented by a circle, and circle sizes are proportional to the total sequence of each haplotype. Colors indicate the geographical origin of the haplotypes and crossbars indicate mutation steps

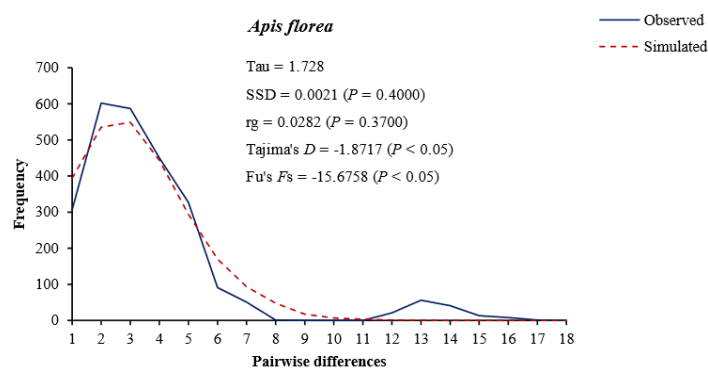


Figure 4. Mismatch distribution analysis based on COI sequences for *Apis florea* species. Statistics test (SSD, rg, Tajima's *D* and Fu's *F_s*) are given

Demographic History

The mismatch distribution graph exhibited a unimodal shape, characteristic of Figure 4 population expansion. Both the sum of squares deviation (SSD = 0.0021; $P = 0.400$) and Harpending's raggedness index (rg = 0.0282; $P = 0.3700$) were not significant, indicating that the sudden expansion model could not be rejected. Additionally, the results of Tajima's *D* (-1.8717; $P < 0.05$) and Fu's *F_s* (-15.6758; $P < 0.05$) tests were significantly negative, supporting patterns consistent with population expansion. The estimated time of population expansion was 79,000 years ago (95% C.I. 55,000-243,000 years ago).

Discussion

Low genetic diversity among *A. florea* populations in Thailand was observed in this study.

Low nucleotide diversity (0.003), combined with high haplotype diversity (0.858), suggests the presence of many closely related haplotypes, indicating that Thai *A. florea* populations may have undergone recent expansion (Mendez-Harclerode *et al.*, 2007). A previous study on genetic differentiation of the dwarf honeybee (*A. florea*) in Thailand, based on the COI-COII intergenic region using PCR-RFLP, also reported limited genetic diversity and a lack of genetic differentiation among *A. florea* populations from geographically distinct locations (Nanork, 2001). Our findings further highlight the potential for a high degree of gene flow between populations.

No genetic clustering associated with the geographic origin of *A. florea* in Thailand was found based on Neighbor-Joining (NJ), Maximum Likelihood (ML), and Bayesian trees. For *A. florea*

in Thailand, this suggests that the bees readily interbreed and exchange genetic material across different regions, resulting in a lack of significant population structure. The low genetic divergence between geographically distant populations may be attributed to a shared recent population history. Recent demographic expansion of *A. florea* populations in Thailand is indicated by low nucleotide diversity, high haplotype diversity, a star-like shaped MJ network, and a unimodal mismatch distribution. The estimated population expansion time for *A. florea* in Thailand ranged from 55,000 to 243,000 years ago (approximately 79,000 years ago), coinciding with an interglacial warming period during the Pleistocene (between the Penultimate Glaciation and the Last Glaciation) (Cao *et al.*, 2023). These results align with the demographic history of giant honeybees in China (Cao *et al.*, 2023) and the melon fly (*Zeudodacus cucurbitae*) in Thailand (Kunprom and Pramual, 2017). The rising global temperatures during this period may have facilitated the growth of *A. florea* populations by increasing the abundance of host plants in a warmer and more humid climate. Additionally, Hepburn and Radloff (2011) proposed that *A. florea* underwent speciation during the early Pleistocene and subsequently achieved widespread distribution.

Furthermore, *A. florea* colonies are reported to migrate over short distances, ranging from a few meters to several hundred meters. This migration is part of their swarming activity, during which a new colony is established at a new location (Nagaraja, 2020). Swarming is a natural reproductive behavior of honeybee colonies, where a new queen and a group of worker bees leave the original colony to establish a new one. *A. florea* colonies can swarm up to eight times per year, with periods of resource scarcity having the most significant impact on their seasonal migration behavior in Thailand (Deowanish *et al.*, 2000). These colonies migrate seasonally in response to a lack of resources such as nectar and pollen. When these resources become scarce, colonies may relocate to areas with higher food availability to ensure their survival and sustainability. Temperature fluctuations also play a crucial role in determining suitable breeding sites for *A. florea*.

In addition to natural factors, human activities such as disturbances and habitat changes can influence *A. florea*'s nesting site selection. The anthropogenic movement of honeybee colonies between regions tends to homogenize the gene pool, potentially reducing the biodiversity of *A. florea* populations in Thailand (Oldroyd and Nanork, 2009).

Conclusions

We observed high haplotype diversity coupled with low nucleotide diversity in populations of *A. florea* in Thailand, a pattern characteristic of a recently expanded population, as supported by mismatch distribution analysis. The population expansion likely occurred during the late Pleistocene, a climatically warm period when the abundance of host plants increased. Further research using more variable genetic markers, such as microsatellites, will provide deeper insights into the genetic structure of this bee species.

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