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Genetic Diversity, Genetic Structure, and Demographic History of Giant Honeybee *Apis dorsata* Fabricius, 1793 (Hymenoptera: Apidae) in Thailand

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ABSTRACT. – Giant honeybee (*Apis dorsata* Fabricius, 1793) is native to Asia. It is an efficient pollinator contributing to ecosystem stability. Populations of *A. dorsata* have been decreasing continuously due to various factors; however, information on genetic diversity is very limited. Therefore, purposes of this study were to assess the genetic diversity, structure, and demographic history of *A. dorsata* based on the mitochondrial cytochrome c oxidase I (*COI*) sequences. Adult worker bees from 41 colonies were collected throughout Thailand. In addition, 24 *COI* sequences of *A. dorsata* from other countries available in public databases were also incorporated into the data analysis. Overall, high haplotype (0.958) and low nucleotide diversities (0.00536) with a maximum intraspecific genetic divergence of 1.65% were found within Thai specimens. Population pairwise F_{ST} revealed genetically significant differences among Northern, Central and Southern populations while those from Northeastern are not. Median-joining network analysis revealed a star-like shape, a characteristic of the recent expanding population. This is supported by unimodal mismatch distribution and significantly negative of Tajima's *D* and Fu's *F*s tests. Population expansion time is estimated to be 83,000 – 177,000 years ago, possibly in response to interglacial Pleistocene climatic fluctuation.

KEYWORDS: *Apis dorsata*, genetic diversity, population expansion, *COI*, Thailand

INTRODUCTION

The giant honeybee (*Apis dorsata* Fabricius, 1793) can be found across Southeast Asia, including Thailand. Based on mitochondrial gene sequences, *Apis dorsata* are classified into 3 subspecies: *A. dorsta dorsata*, *A. dorsta binghami*, and *A. dorsta breviligula* (Bhatta et al., 2024). This species differs from the western honeybee, *A. mellifera*, in that it builds a single, big, exposed comb under tree branches or cliffs rather than in cavities. *A. dorsata* developed an efficient colony defense strategy to protect their exposed combs (Ruttner, 1988; Jack et al., 2016). They play a crucial role on the natural functions of ecosystems, and they are also the most economically valuable pollinators within natural and agricultural systems. Nevertheless, honeybees are facing various threats, such as parasites, diseases, deforestation, overhunting, and climate change (Oldroyd and Nanork, 2009). To effectively conserve and manage this species, it is critical to understand its genetic diversity, genetic structure, and demographic history, as these factors determine its adaptation, resilience, and long-term viability.

Geometric morphometric on wings of *A. dorsata* populations from Thailand revealed a single group (Rattanawannee et al., 2012). Likewise, low genetic diversity of *A. dorsata* was found in specific regions. In Thailand, Insuan et al. (2007) reported low genetic variety in *A. dorsata* populations when assessing mitochondrial DNA, but increased diversity when utilizing microsatellite markers. Similarly, limited mitochondrial haplotypes diversity among *A. dorsata* populations was discovered in Borneo (Tanaka et al., 2001). In addition, Qamer et al. (2021) found minimal genetic diversity within and between Pakistani populations using RAPD markers. This underscores the need for additional research to better understand the variables that contribute to reduced biodiversity and to implement appropriate conservation strategies.

Interestingly, Paar et al. (2004) used microsatellite loci to explore the genetic structure of *A. dorsata* in Northeast India, and they discovered significant genetic heterogeneity between populations from different geographical locations. Despite this difference, gene flow was adequate to prevent significant inbreeding, indicating a complex population structure driven by both migration and local adaptation. Sahebzadeh et al. (2012) conducted research in Malaysia on the genetic structure of *A. dorsata* aggregations and discovered that different nests within the same aggregation were not closely linked. This shows that *A. dorsata* colonies in aggregations are genetically varied rather than developed through relatedness, which could be an adaptive method for maintaining genetic variability. Moreover, Rattanawannee et al. (2013) investigated the genetic variation and relatedness among *A. dorsata* nest aggregations. Microsatellite analysis of 54 nests in three aggregations revealed that no colonies were connected as mother and daughter. Thus, if reproduction happened at the research sites, the daughter colonies dispersed. This shows that rapid increases in *A. dorsata* colony numbers during general

TABLE 1. Collecting locations of *Apis dorsata* samples in Thailand.

Region	Locality (code)	Latitude/longitude	No. of colonies	Specimen ID
Northern	Chiang Mai (CM)	18º42′47″N, 99º2′10″E	2	N-Chiang Mai 1-2
	Phayao (PY)	19º35′57″N, 100º19′27″E	1	N-Phayao 1
	Nan (NAN)	19º10′27″N, 100º55′0″E	1	N-Nan 1
	Uttaradit (UTD)	17º37′31″N, 100º24′11″E	1	N-Uttaradit 1
	Tak (TAK)	16º52′11″N, 99º7′44″E	1	N-Tak 1
Northeastern	Nong Khai (NK)	18º3′51″N, 102º16′24″E	1	NE-Nong Khai 1
	Loei (LO)	16º52′38″N, 101º43′20″E	1	NE-Loei 1
	Nakhon Phanom (NPN)	17º37′43″N, 104º15′1″E	1	NE-Nakhon Phanom 1
	Mukdahan (MDH)	16º41′50″N, 104º20′49″E	10	NE-Mukdahan 1-10
	Chaiyaphum (CYP)	16º21′60″N, 102º8′1″E	1	NE-Chaiyaphum 1
	Nakhon Ratchasima (NKM)	14º42′16″N, 101º24′48″E	1	NE-Nakhon Ratchasima 1
Central	Bangkok (BK)	13º45′10″N, 100º29′37″E	1	C-Bangkok 1
	Chachoengsao (CHS)	13º40′2″N, 101º2′49″E	1	C-Chachoengsao 1
	Chanthaburi (CBR)	12º30′15″N, 102º8′30″E	2	C-Chanthaburi 1-2
	Phetchaburi (PBR)	13º4′9″N, 99º36′16″E	1	C-Phetchaburi 1
	Prachuap Khiri Khan (PJK)	11º12′34″N, 99º30′41″E	1	C-Prachuap Khiri Khan 1
	Samut Songkhram (SSK)	13º21′28″N, 99º57′43″E	3	C-Samut Songkhram 1-3
Southern	Chumphon (CHP)	10º14′12″N, 99º6′28″E	1	S-Chumphon 1
	Surat Thani (SRN)	9º33′56″N, 99º9′54″E	1	S-Surat Thani 1
	Samui Island (SMU)	9º30′26″N, 99º59′45″E	5	S-Samui Island 1-5
	Phuket (PHK)	7º56′12″N, 98º21′11″E	2	S-Phuket 1-2
	Songkhla (SK)	6º52′45″N, 100º32′60″E	2	S-Songkhla 1-2
	Total		**41**	

flowering events are more likely caused by swarms arriving from distant places than by in situ reproduction.

Cao et al. (2012) examined the phylogeography of *A. dorsata* across China and Southeast Asia, concluding that Pleistocene glaciers had a substantial impact on population distribution and divergence. This historical context is critical for comprehending the current genetic structure and diversity of *A. dorsata*. The temporal genetic structure of a drone congregation location in *A. dorsata* was determined by Kraus et al. (2005). Significant genetic differentiation across time was discovered, implying that these areas facilitate gene transfer between subpopulations. This dynamic gene flow promotes genetic variety and prevents inbreeding, demonstrating the species' ability to adapt to changing environmental conditions. Population structure, demographic history, and adaptation of giant honeybees in China have been inferred from population genomic data (Cao et al., 2023).

The genetic diversity, structure, and demographic history of *A. dorsata* provide important insights into its evolutionary history and current resilience. The aims of this study were to assess the genetic diversity, genetic structure, and demographic history of *A. dorsata* populations in Thailand based on mitochondrial cytochrome c oxidase I (*COI*) sequences. The mtDNA *COI* sequence was selected as a genetic marker because this gene is highly conserved (Hebert et al., 2003) and has been used to investigate genetic differences in honeybee species, *A. nigrocincta* (Lombogia et al., 2020) and *A. mellifera* (Alabdali et al., 2021). Understanding these processes is critical for developing conservation and management strategies for this keystone pollinator.

MATERIALS AND METHODS

Sample collections

Adult workers of 41 *A. dorsata* colonies were collected from 22 provinces throughout its distribution range in Thailand during January 2018-May 2019 (Table 1, Fig. 1). One bee per colony was used for genetic analysis. Bee samples were kept in 95% ethanol at -20°C until the next step. The research project had been reviewed and approved by the

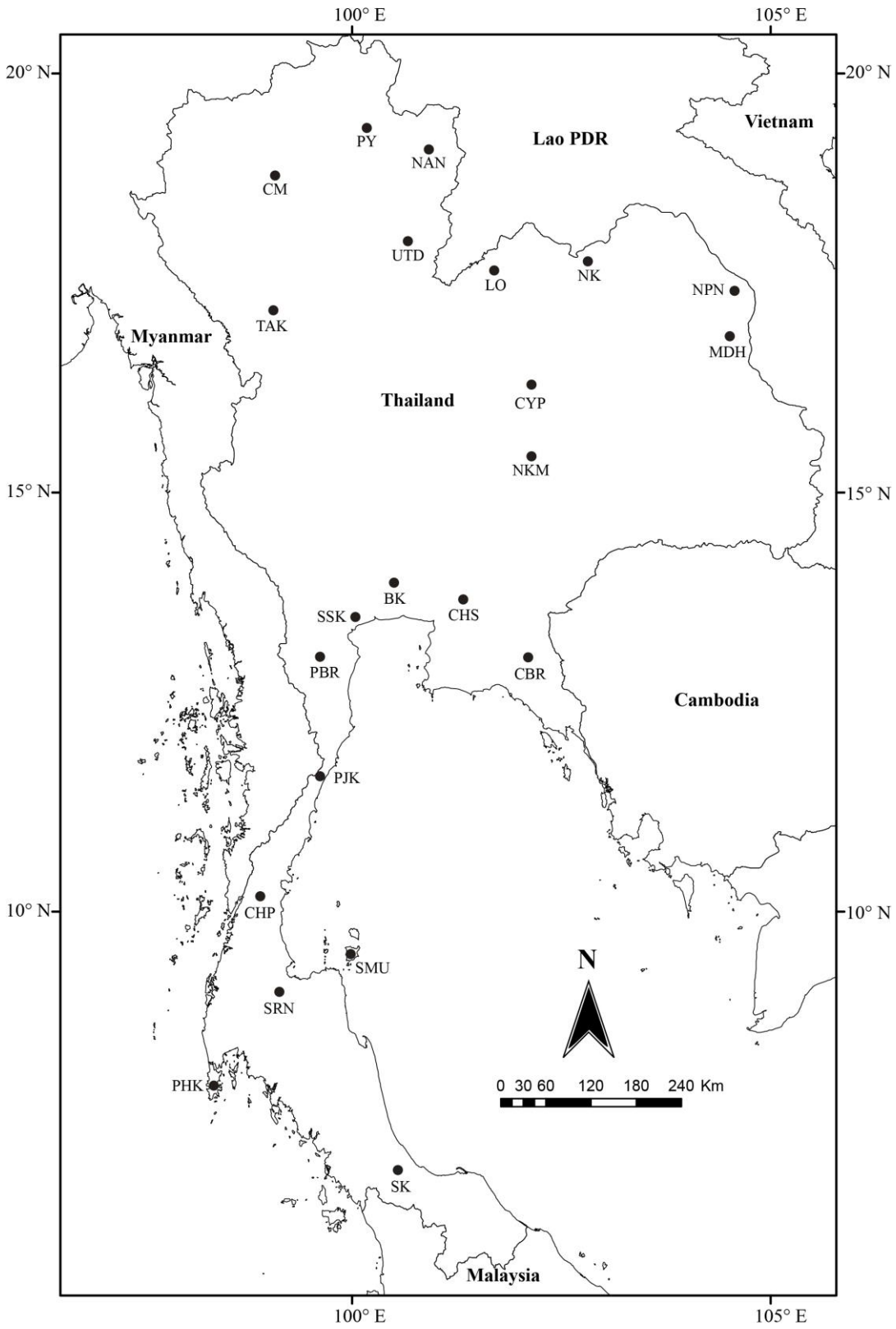

FIGURE 1. Map of study sites of *Apis dorsata* in Thailand. Details of the sampling locations were given in Table 1.

Institutional Animal Care and Use Committee, Mahasarakham University (IACUC-MSU). The approval number is IACUC-MSU-025/2018.

DNA extraction, PCR amplification and DNA sequencing

Total DNA was isolated from the thorax of each bee using E.Z.N.A. Tissue DNA Kit. DNA was preserved

TABLE 2. Accession numbers for *COI* sequences of *Apis dorsata* retrieved from GenBank used in this study.

Country	No. of samples	GenBank no.
India	6	KU752355 KT960840 KU212342-345
Myanmar	8	MT679383-388 MF804562-563
Thailand	4	MT670341-344
Vietnam	6	MT679368-373
Total	**24**	

at 4°C until needed. 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 each forward and reverse primers-LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994), 25 ng genomic DNA, and 0.04 U/µl *Taq* DNA polymerase. PCR was pre-denatured at 96°C for one min, followed by 35 cycles consisting of denaturation at 94°C for one min, annealing at 55ºC for one min, and extension at 72°C for two min. The final extension was performed at the same temperature for ten min. Agarose gel (1% w/v) electrophoresis was performed to check the PCR products. The bands were visualized under a UV transilluminator (Sambrook and Russell, 2001). Successful PCR products were sent for purification and sequencing at Macrogen, Korea using the same primers as for PCR.

Data analysis

In total, 65 *COI* sequences with a sequence length of 615 bp were used for analyses. Of these, 45 were *COI* sequences of *A. dorsata* from Thailand which included 41 sequences from this study (GenBank accession no. PQ564694-PQ564734) and four sequences retrieved from the NCBI GenBank database. The populations of Thai *A. dorsata* were grouped by geographic regions. In addition, GenBank records of *A. dorsata* from other countries were also incorporated into the data set (Table 2). The obtained sequences were subsequently aligned using the MUSCLE (Edgar, 2004) in MEGA X v.11 (Kumar et al., 2018). Genetic diversity indices were calculated for each and overall *A. dorsata* population using the software package DnaSP v.5.10.01 (Librado and Rozas, 2009). Intraspecific genetic divergence was calculated for *A. dorsata* species from Thailand in MEGA X v.11 based on the Kimura 2-parameter (K2P) model. Genetic differentiation between Thai *A. dorsata* populations was estimated using pairwise F_{ST} values computed after 1023 permutations in Arlequin v.3.5.1.2 (Excoffier and Lischer, 2010). In addition, gene flow (N_m) between pairwise populations was also estimated with the equation $N_m = (1–F_{ST})/2\ F_{ST}$ (Szalanski et al., 2016).

Neighbor-joining (NJ), maximum likelihood (ML) and Bayesian inference (BI) methods were applied to reconstruct the phylogenetic relationships in the *COI* sequences. For all phylogenetic analyses, *A. laboriosa* sequence (accession no. KX908208) was used as an out group. The ML tree was conducted in IQ-TREE v.2.0.4 (Nguyen et al., 2015) with the best model selected by ModelFinder (Kalyaanamoorthy et al., 2017) and branch support was evaluated using ultrafast bootstrapping with 1,000 replicates (Hoang et al., 2018). Bayesian inference was conducted in MrBayes 3.04b (Huelsenbeck and Ronquist, 2001) and Bayesian posterior probabilities were evaluated using Markov chain Monte Carlo analysis, which was run for 2,000,000 generations, with a sampling frequency of 100 generations, discarding the first 2,000 sampled trees as burn-in. The trees obtained were viewed using FigTree v.1.4.0 (Rambaut, 2012). Genetic relationships between the *COI* sequences were also examined using the median-joining (MJ) network analysis using PopART v.1.7 (Leigh and Bryant, 2015).

To test the hypothesis of population expansion, a mismatch distribution analysis was conducted in Arlequin v.3.5.1.2. It is expected that a unimodal distribution will typically result from expanding populations, while a multimodal distribution characterizes a stable population (Rogers and Harpending, 1992). The sum of squares deviation (SSD) and Harpending's raggedness index (rg) were applied to test deviation from the sudden expansion model. Estimation of the expansion time was calculated using

TABLE 3. Genetic diversity indices of *Apis dorsata* populations from Thailand and other countries.

Population	N	H	S	h ± SD	π ± SD
Northern Thailand (N)	10	7	9	0.867 ± 0.107	0.00376 ± 0.00080
Northeastern Thailand (NE)	15	10	18	0.895 ± 0.070	0.00715 ± 0.00151
Central Thailand (C)	9	7	9	0.944 ± 0.070	0.00370 ± 0.00069
Southern Thailand (S)	11	8	12	0.927 ± 0.066	0.00503 ± 0.00077
Mainland (N, NE, C, S)	38	22	24	0.947 ± 0.021	0.00530 ± 0.00079
Phuket and Samui Island	7	6	11	0.952 ± 0.096	0.00588 ± 0.00091
All populations from Thailand	45	27	27	0.958 ± 0.016	0.00536 ± 0.00068
Thailand	45	27	27	0.958 ± 0.016	0.00536 ± 0.00068
India	6	6	8	1.000 ± 0.096	0.00553 ± 0.00108
Myanmar	8	7	9	0.964 ± 0.077	0.00470 ± 0.00112
Vietnam	6	4	7	0.800 ± 0.172	0.00379 ± 0.00130
All populations	65	39	34	0.963 ± 0.012	0.00566 ± 0.00055

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).

the equation $\tau = 2ut$ (where $u = m_T\mu$, m_T is the length of nucleotide sequences under study, μ is the mutation rate per nucleotide, and t is the generation time) (Rogers and Harpending, 1992), assuming a divergence rate of 3.54% per million years for insect *COI* gene (Papadopoulou et al., 2010) and generation time (g) = 1 year (Cao et al., 2023).

RESULTS

Genetic diversity

Genetic diversity indices for all populations are shown in Table 3. A total of 39 haplotypes were identified from 65 *COI* sequences. High level of haplotype diversity (0.963) but low level of nucleotide diversity (0.00566) was found among overall populations. Within Thai *A. dorsata* populations, 27 haplotypes were identified from 45 *COI* sequences. The haplotype diversity was high in every population ranging from 0.867 in Northern to 0.944 in Central populations with an average of 0.958. Contrarily, the nucleotide diversity was rather low in all populations, ranging from 0.00370 in Central to 0.00715 in Northeastern populations with an average of 0.00536. Intraspecific genetic divergence based on the K2P model of Thai *A. dorsata* ranged between 0 and 1.65% with an average of 0.539%.

Genetic differentiation

Population pairwise F_{ST} among Thai *A. dorsata* populations ranged from 0.002 to 0.128. Most populations were not genetically significantly different (Table 4) except between Northern compared with Central and Southern populations. The pairwise per generation female migration rate (N_m) values among Thai *A. dorsata* populations were all more than 1 (ranging from 3.406 to infinite).

Phylogenetic relationships and haplotype network construction

All three phylogenetic analysis methods (NJ, ML, and Bayesian) revealed that Thai *A. dorsata* sequences (38 sequences from mainland, 2 sequences from Phuket Island and 5 sequences from Samui Island) clustered together and have no associated with geographical origin. The MJ network based on the *COI* haplotypes revealed a similar pattern as the phylogenetic tree. The MJ network of 65 *COI* sequences (41 sequences obtained in this study and 24 sequences obtained from NCBI GenBank database) indicated no significant deviation in the lineage. There was no connection between haplotype clusters and geographical origin. The MJ network analysis showed three main haplotypes (H1, H5 and H9) at the center of the network. The other haplotypes were located around the main haplotypes. The overall haplotype network had an approximately star-like shape (Fig. 2).

TABLE 4. Population pairwise F_{ST} values (below the diagonal) and gene flow (above the diagonal) of *Apis dorsata* in Thailand based on mitochondrial cytochrome c oxidase I (*COI*) gene sequences.

Population	Northern	Northeastern	Central	Southern
Northern	-	7.697	3.406	5.118
Northeastern	0.061	-	166.167	249.5
Central	**0.128**	0.003	-	Inf
Southern	**0.089**	0.002	-0.036	-

Note: The bold indicated statistical significance ($P < 0.05$). Inf is infinite.

Demographic history

The observed mismatch distribution showed a unimodal shape (Fig. 3), characteristic of population expansion. Both the sum of squares deviation (SSD) and Harpending's raggedness index (rg) values were not significant, indicating that the sudden expansion model could not be rejected. In addition, the results of Tajima's *D* and Fu's *F*s tests (-1.6697 and -26.1687, respectively) were also significantly negative, indicating that the tests supported the patterns of a population expansion. Population expansion time was approximated to be 148,000 years ago (95% C.I. 83,000 – 177,000 years ago).

DISCUSSION

Our study shows low genetic variation among *A. dorsata* populations in Thailand. Low nucleotide diversity values (0.00536) along with high haplotype diversity values (0.958) exhibit a large number of closely related haplotypes, indicating that this group may have recently expanded (Mendez-Harclerode et al., 2007). These findings are consistent with the earlier investigation on the genetic differentiation of the giant honeybee (*A. dorsata*) in Thailand based on PCR-RFLP of mitochondrial genes (intergenic *COI-COII*, *Cytb-tRNA*^{Ser}, *ATPase6-8*, and *lrRNA*) and microsatellites (A14, A22, and A88) analysis by Insuan et al. (2007). They indicated limited genetic diversity and a lack of genetic differentiation among *A. dorsata* populations from geographically distinct locations (Insuan et al., 2007). This homogeneity suggests a recent population bottleneck or selective sweep, affecting the whole country (Rattanawannee et al., 2013). In addition, there was no genetic differentiation among *A. dorsata* aggregations in northen Thailand, suggesting that matings may occur between aggregations (Rattanawannee et al., 2013). The low genetic diversity along with high haplotype diversity based on *COI* gene has been found in the melon fly (*Zeudodacus cucurbitae*) populations in Thailand which indicated population expansion in this species (Kunprom and Pramual, 2017).

The F_{ST} value in this study was relatively low, indicative of an overall low level of genetic structuring between *A. dorsata* populations (Luo et al., 2019). The N_m values among Thai *A. dorsata* populations were all more than one, suggesting that there is a high degree of gene flow among *A. dorsata* populations in this study. Our results are consistent with Insuan et al. (2007) which revealed the potential for substantial levels of gene flow and, so, panmixis between *A. dorsata* populations throughout Thailand. Although there were samples of *A. dorsata* from Samui Island (approximately 20 km from the mainland) and Phuket Island (approximately 1 km from the mainland), no genetic differences were found in these samples. Similarly, a high degree of gene flow was also found in Chinese *A. dorsata* populations (Qamer et al., 2021; Cao et al., 2023). A possible explanation of a high degree of gene flow among them could be because *A. dorsata* colonies are able to migrate rapidly (Hepburn, 2011; Gloag et al., 2017) and potentially move up to 200 km (Koeniger and Koeniger, 1980; Crane et al., 1993; Neumann et al., 2000). Moreover, *A. dorsata* queens are highly polyandrous (Moritz et al., 1995; Oldroyd et al., 1996) with average mating frequencies of up to 88.5 (Wattanachaiyingcharoen et al., 2003). Honeybee queens generally mate with drones from other colonies, often traveling far from their home colonies to find mates. By mating with drones from diverse genetic backgrounds, the queen facilitates the mixing of genes across colonies. This gene flow is essential for maintaining genetic diversity at the population level, as it reduces genetic differentiation between colonies and limits the effects of inbreeding (Oldroyd and Fewell, 2007). Thus, there is conceivably no considerable barrier to gene flow, and geographical constraints appear to play no significant role in directing *A. dorsata* swarming migration on mainland Thailand (Insuan et al., 2007).

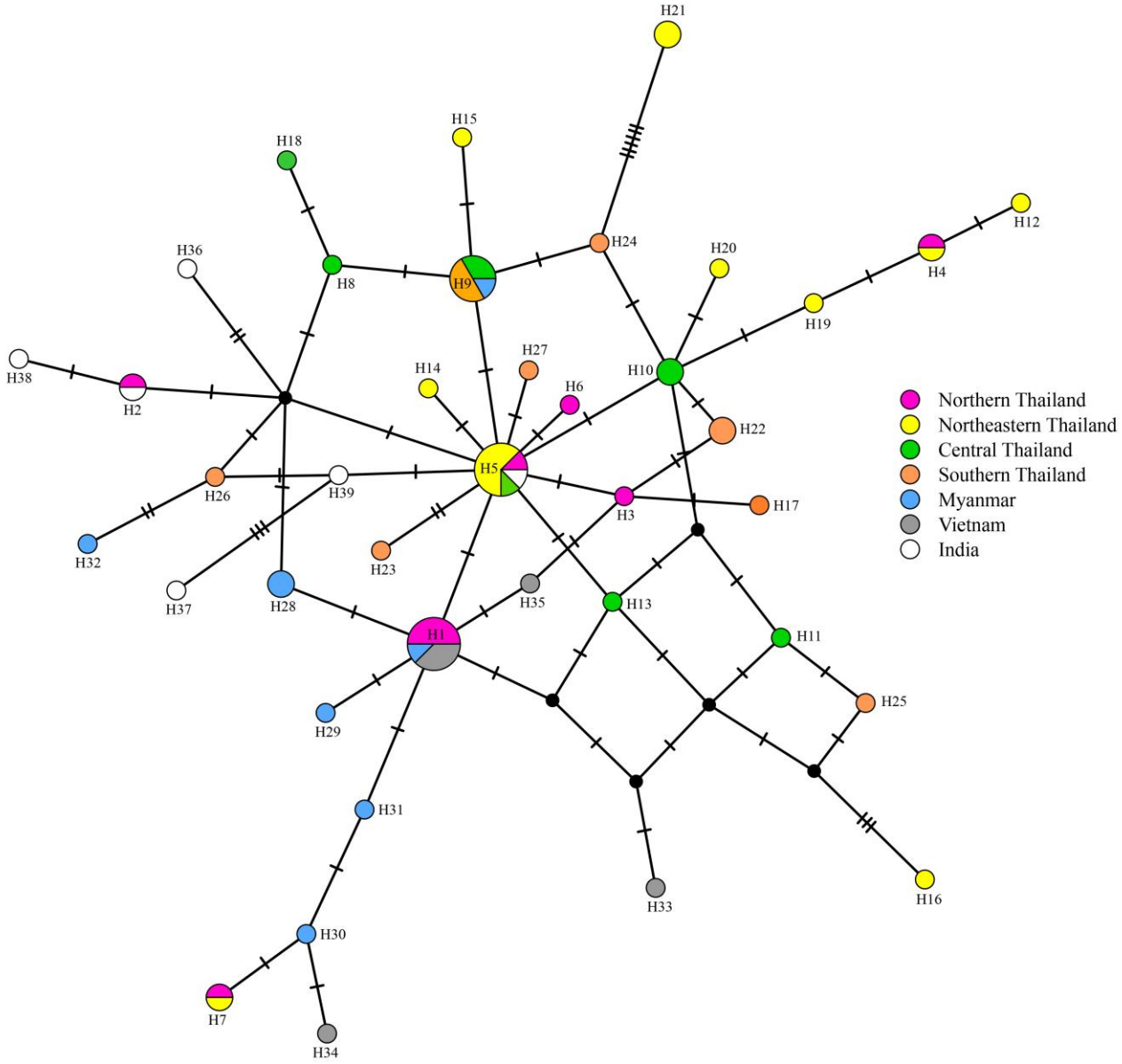

FIGURE 2. Median-joining networks representing the relationships of the haplotypes for *Apis dorsata* based on *COI* haplotypes. Small black dots indicate median vectors. Each haplotype is represented by a circle, and circle sizes are proportional to the total sequence of each haplotype. Colors indicate the geographical origin of the haplotypes and crossbars indicate mutation steps.

NJ, ML, and Bayesian trees exhibited no genetic clustering of *A. dorsata* in Thailand associated with geographic origins. This low genetic differentiation between geographically distant populations is possibly due to a sharing of recent population history. Low nucleotide but high haplotype diversities plus star-like shaped MJ network and unimodal mismatch graph indicating that the population has undergone recent demographic expansion. The population expansion time of *A. dorsata* in Thailand was estimated to be approximately 148,000 years ago, which was the time of the inter-glacial warming period of the Pleistocene (between the penultimate glaciation and the last glaciation) (Zheng et al., 2022; Cao et al., 2023). These results were consistent with the demographic history of giant honeybees in China (Cao et al., 2023) and the melon fly (*Z. cucurbitae*) in Thailand (Kunprom and Pramual, 2017). Global temperature may have led to population expansion in *A. dorsata*. The warm and humid climate may have increased host plant abundance (Lorenzen et al., 2011), resulting in an increase in honeybee populations. Given their remarkable migration abilities, historical temperature variations in the Pleistocene may have caused *A. dorsata* populations to migrate along a North-South axis (Hepburn, 2011; Cao et al., 2023). Furthermore, migration over long distances to find food plants could be a plausible explanation of *A. dorsata* population expansion (Koeniger and Koeniger, 1980; Crane et al., 1993).

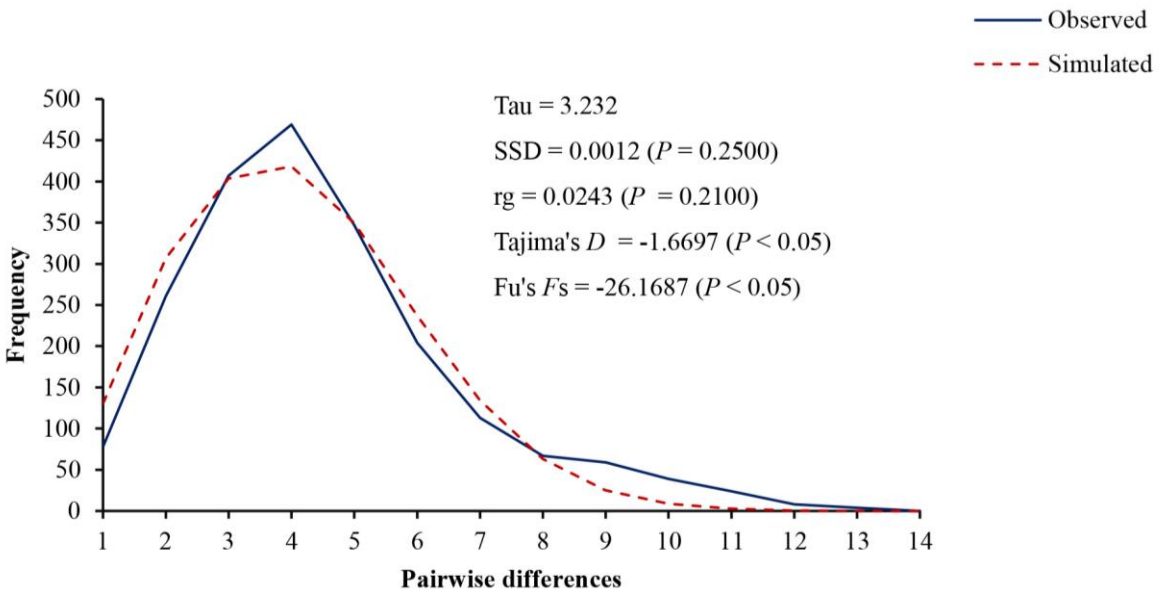

FIGURE 3. Mismatch distribution analysis based on *COI* sequences for *Apis dorsata* species. Statistics test (SSD, rg, Tajima's *D* and Fu's *F*s) are given.

In conclusion, we found that *A. dorsata* in Thailand has low nucleotide diversity but high haplotype diversity, a characteristic of the recent expanding population as revealed by mismatch distribution analysis. The population expansion time is at the late Pleistocene when the host plant increased during the climatic warm period. Although the recent population expansion can reduce genetic differentiation between populations, but we have found considerable differences between Northern with Central and Southern populations suggesting that there are some degrees of isolation which may be caused by geographic barrier. Further study using more variable markers and larger sample size will be useful in elucidating the genetic structure of this bee species.

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