



Phylogenetic Utility of the Nuclear cMDH Gene in Asian Honeybee (Genus *Apis*)

Thitipan Meemongkolkiat

Biochemistry Unit, Department of Biomedical Science, Faculty of Science, Rangsit University,
Pathum Thani 12000, Thailand
E-mail: thitipan.m@rsu.ac.th

Abstract

Mitochondrial markers are the primary tool for honeybee (*Apis* spp.) identification but only reflect maternal lineages. To provide a more comprehensive evolutionary perspective, this study evaluates the phylogenetic utility of the nuclear cytoplasmic malate dehydrogenase (cMDH) gene compared to the mitochondrial cytochrome b (cytb) marker. We analyzed eight sequences of cMDH from five bee species and nine sequences of cytb from six bee species using an integrated approach comprising biochemical allozyme analysis (native-PAGE) and DNA sequencing. Phylogenetic relationships were reconstructed using the Neighbor-Joining method, selected for its efficiency in resolving interspecific genetic distances and clustering stability. Analysis of the 1,002 bp cMDH and 500 bp cytb alignments revealed high congruence between the nuclear and mitochondrial markers. Both markers successfully resolved the species into distinct, well-supported clades with bootstrap support ranging from 41 to 100%, wherein interspecific nodes exhibited the highest stability (near 100%) compared to intraspecific branches. While *A. mellifera* exhibited significant intraspecific polymorphism (S, M, and F alleles, representing slow, medium, and fast electrophoretic migration rates, respectively), the native Asian species remained genetically uniform within the sampled populations. Furthermore, amino acid alignments revealed that functional sites, including NAD⁺ binding and catalytic domains, are highly conserved across all taxa. This indicates strong selection to maintain metabolic efficiency, reinforcing the reliability of cMDH as a robust nuclear biomarker for *Apis* molecular taxonomy and evolutionary studies.

Keywords: *Cytoplasmic Malate Dehydrogenase (cMDH), honeybee, molecular phylogeny, cytochrome b, enzyme polymorphism*

1. Introduction

Honeybees in the genus *Apis* play an essential role in pollination, agriculture, and ecosystem stability across Asia. Understanding how these species differ genetically is vital for conservation, species identification, and tracing their evolutionary history. While modern phylogenomics increasingly utilizes multi-locus or whole-genome approaches to resolve complex evolutionary relationships, the targeted analysis of specific nuclear loci remains a crucial tool for connecting phenotypic traits to genetic variation. Most studies rely on mitochondrial markers, such as the COI and cytochrome b regions, which work well for separating species but represent only the maternal lineage (Bawn et al., 2025; Meemongkolkiat et al., 2019). To gain a more comprehensive perspective, nuclear markers are necessary to complement the maternal signal, accounting for the different coalescent histories and slower substitution rates typically found in the nuclear genome.

Before DNA sequencing became commonplace, allozyme electrophoresis was widely used to study genetic variation in insects (Mateus & Sene, 2007). One enzyme frequently examined is cytoplasmic malate dehydrogenase (cMDH), a central metabolic enzyme capable of displaying multiple electrophoretic forms (Dong & Somero, 2009). In *Apis mellifera*, distinct MDH allozyme patterns have been reported, suggesting meaningful underlying genetic variation. The cMDH polymorphism (Del Lama et al., 1990) has been extensively utilized in honeybee (*A. mellifera*) population studies due to three common forms of cMDH, designated by their migration rates during electrophoresis: fast (F), medium (M), and slow (S) (Badino et al., 1983). Focusing on this single-locus marker is highly justified, as it provides a method to connect these



classical enzyme variants with the specific nucleotide and amino acid changes responsible for them, a functional link often missed in large-scale genomic scans.

Linking allozyme patterns with gene sequences allows researchers to examine how much evolutionary information cMDH carries and whether it can help distinguish *Apis* species at the nuclear DNA level. Comparing cMDH sequence relationships with mitochondrial gene (cytochrome b, or *cytb*) phylogeny also helps reveal how nuclear and mitochondrial histories align or differ among these species. This integrative approach is essential because mitochondrial DNA reaches coalescence approximately four times faster than nuclear DNA, which can occasionally lead to discrepancies in phylogenetic reconstruction.

In this study, we combined MDH allozyme analysis with MDH cytoplasmic gene sequences and *cytb*-based phylogenetic reconstruction to investigate four *Apis* species collected across Thailand. These species (*A. mellifera*, *A. cerana*, *A. dorsata*, and *A. florea*) were selected for their significant economic roles in regional apiculture and their vital ecological functions as keystone pollinators. By including both commercial (cavity-nesting) and wild (open-nesting) lineages, we can better evaluate the diagnostic power of the cMDH marker across diverse evolutionary histories. Our goals were to (i) document cMDH allozyme variation using native-PAGE, (ii) examine cMDH sequence differences among species, and (iii) compare cMDH and mitochondrial *cytb* phylogenetic trees to evaluate the usefulness of cMDH as a complementary marker for species identification and evolutionary studies in honeybees.

2. Objectives

- 1) Test whether cMDH allozyme patterns exhibit sufficient variability for both interspecific and intraspecific differentiation among *Apis* species in Thailand.
- 2) Investigate whether the nuclear cMDH gene resolves species-level relationships congruently with the mitochondrial *cytb* marker to confirm its phylogenetic utility.
- 3) Evaluate the hypothesis that functional domains of the cMDH gene remain highly conserved across the genus despite amino acid substitutions in other regions.

3. Materials and Methods

3.1 Sample Collection

Samples from four honeybee species, including *Apis mellifera*, *Apis florea*, *Apis cerana*, and *Apis dorsata*, were collected from northern, northeastern, and southern Thailand in previous studies (Coordinates: 8°46'42.2"N to 19°10'35.0"N latitude and 98°48'12.4"E to 103°00'06.5"E longitude) (Meemongkolkiat et al., 2019). To ensure genetic independence, individuals were sampled from at least five distinct colonies per species.

3.2 Allozyme Analysis

Thorax tissues from 10 bees per species were ground in 100 μ L of water in a microcentrifuge tube using a micropestle. The mixture was kept on ice during homogenization. While the sample size (N=10) focused on species-level characterization, we maximized diversity by sampling from multiple colonies. The samples were then subjected to a quick spin for 30 seconds at room temperature (RT). For the homogenates, 10 μ L of each sample was loaded into the wells of 8% polyacrylamide gels (native-PAGE). Electrophoresis runs were conducted at a constant voltage of 150 V in Tris-borate buffer (500 mM Tris, 650 mM boric acid, 16 mM EDTA, pH 8.0). After electrophoresis, the gel was removed and placed in a weigh boat. Hot agar (1%) warmed to 65 °C was added to an MDH staining mixture (600 μ L of 1 M Tris-HCl, pH 8.0; 200 μ L of NAD; 400 μ L of malic substrate; 100 μ L of phenazine methosulfate or PMS; and 100 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to make a final volume of 2 mL. The mixture was immediately poured over the gel, and active bands became visible after 5 minutes. Differences in banding patterns (observed genotypes) between individuals were interpreted based on (Meemongkolkiat et al., 2022).

For the phylogenetic analysis of the partial cMDH nuclear gene and partial cytochrome b (*cytb*) gene among *Apis* spp., partial cMDH sequences of the four *Apis* species were retrieved from the NCBI GenBank nucleotide database (<https://www.ncbi.nlm.nih.gov/gene>) (Accession Nos. MK967647, MK967648, MK967649,

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XM017053998.2, XM003694595.3, XM006624180.2, XM050717545.1, XM050717546.1). Selection criteria for GenBank sequences included: (i) high sequence quality scores, (ii) coverage of at least 90% of our target alignment, and (iii) verified species identification from published studies. *Bombus affinis* and *Tetragonula pagdeni* were used as cMDH sequence outgroups. To represent the mitochondrial gene data for comparison, partial cytochrome b sequences were retrieved from the database with the following accession numbers: MG548325.1, MG548332.1, MG542690.1, MG542685.1, MG548334.1, MG548316.1, MG548336.1, MG548335.1, and FJ012146.1. The obtained sequences were aligned using the ClustalX program. Finally, amino acid sequences were derived from the DNA sequences using the ORF Finder program. The 334 amino acid sequences of the three cMDH alleles of *A. mellifera* and the cMDH of *A. cerana*, *A. dorsata*, and *A. florea* inferred from the database DNA sequences were aligned using the ClustalW algorithm (Larkin et al., 2007), an add-on in MEGA 11 (Tamura et al., 2021). Phylogenetic relationships were inferred using partial cytoplasmic malate dehydrogenase (cMDH) and cytochrome b (*cytb*) DNA sequences. For the nuclear marker, approximately 1,002 bp of cMDH sequences from *A. mellifera*, *A. cerana*, and *A. florea* were analyzed, with *Bombus affinis* included as an outgroup. For comparison with a mitochondrial marker, a 500 bp fragment of the *cytb* gene from *A. mellifera*, *A. cerana*, *A. florea*, and *A. andreniformis* was used, with *Tetragonula pagdeni* serving as the outgroup. All sequences were aligned in MEGA 11, and phylogenetic trees were constructed using the Neighbor-Joining method based on the Maximum Composite Likelihood model, which provides an estimate of genetic distances (Tamura et al., 2021). Codon positions were included (1st+2nd+3rd+Noncoding), and pairwise deletion was applied to handle missing data. Branch lengths are reported as the number of substitutions per site. Outgroups (*Bombus* and *Tetragonula*) were selected based on their close phylogenetic proximity to *Apis* within the corbiculate bees to ensure proper tree rooting. The robustness of tree topologies was evaluated using 5,000 bootstrap replicates, and bootstrap values are shown at the corresponding nodes. Tree topologies with bootstrap values (BV) of 70% or greater were regarded as sufficiently resolved.

4. Results and Discussion

4.1 cMDH Allozyme Patterns in Asian Honeybee Population in Thailand

Using the cMDH allozyme interpretation criteria established by Nunamaker and Wilson (1981), identical parameters were applied in this study to identify cMDH genotypes. The electrophoretic patterns of cMDH in Asian honeybees from Thailand are depicted as phenotypes in Figure 1. Although cMDH isoenzymes have been studied in *A. mellifera*, there is limited data concerning this enzyme in other *Apis* species. Therefore, the cMDH isozyme patterns of *A. florea*, *A. dorsata*, and *A. cerana* were investigated. The cMDH activity of each Asian species across different locations in Thailand exhibited consistent patterns. The cMDH activity in *A. florea* contained a single major band. The position of *A. florea* cMDH activity in an electric field is nearly identical to the heterozygous SF form of *A. mellifera*. For *A. dorsata* and *A. cerana*, their cMDH activity is located significantly lower than the *A. florea* band. Interestingly, the cMDH activities in *A. dorsata* and *A. cerana* were electrophoretically identical under the utilized conditions, displaying a single intense band. However, in *A. dorsata*, an additional faint band appeared above the intense band. This result implies that the overall net charge of cMDH in *A. dorsata* and *A. cerana* has a more positive charge and a higher pI than that of *A. mellifera* and *A. florea*, based on migration distance during electrophoresis. This outcome aligns with findings by Nunamaker et al. (1984), which showed that *A. florea*, *A. dorsata*, and *A. cerana* from Pakistan exhibited a single band, indicating a lack of genetic variability or polymorphism within the specific populations sampled.

The observed lack of intraspecific genetic variation in Asian honeybee populations could be influenced by the high level of environmental stability within their native ranges. It has been suggested that the regulated microclimate of the hive serves as a physiological buffer, protecting the bees from external fluctuations and reducing the selective pressure to maintain high levels of genetic polymorphism (Chen et al., 2018). In contrast, *A. mellifera* has a wide geographical distribution, leading to greater adaptability across a broader range of climatic conditions. Over centuries, beekeepers have transported *A. mellifera* hives into various regions (encompassing both cold and temperate climates) where natural selection may have



influenced the current display of enzyme polymorphism (Dogantzis et al., 2021; Panziera et al., 2022) While this domestication pressure is a plausible driver for the diversity seen in *A. mellifera*, the relative uniformity within the sampled populations of Asian species likely reflects different evolutionary trajectories or narrower niche specializations (Requier et al., 2019).

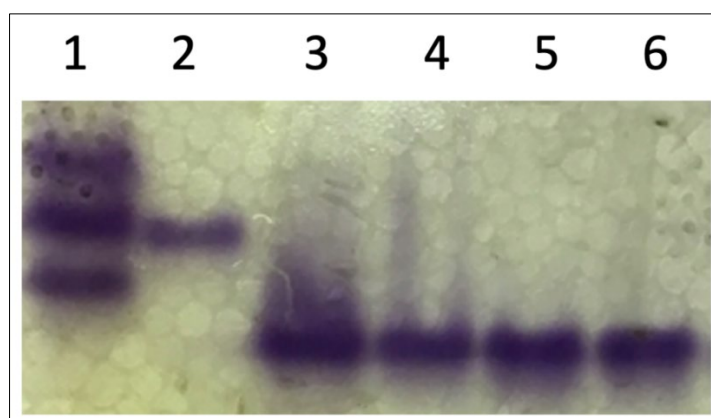


Figure 1 An 8% polyacrylamide gel stained with an MDH staining mixture to illustrate the differing cMDH forms of honeybees. Lane 1 contains cMDH (SF) of *A. mellifera* (Uttaradit province). Lane 2 contains cMDH of *A. florea* (Ratchaburi province). Lane 3 contains cMDH of *A. dorsata* (Samut Songkhram province). Lane 4 contains cMDH of *A. cerana* (Samut Songkhram province). Lane 5 contains cMDH of *A. cerana* (Nakhon Si Thammarat province). Lane 6 contains cMDH of *A. cerana* (Ratchaburi province)

4.2 Phylogenetic Analysis

The phylogenetic analysis reveals a clear consensus between the cMDH and mitochondrial cytochrome b (*cytb*) markers, as both consistently resolved species-level relationships among *Apis* taxa (Figure 2). Within the cMDH phylogeny, the three *A. mellifera* alleles (S, M, and F) clustered into a single, unified clade with robust support (BS = 98%) (Figure 2A). The use of *Bombus affinis* as an outgroup is justified by its position as a closely related corbiculate bee, allowing for the proper polarization of the *Apis* lineage. This demonstrates that these variants represent internal genetic diversity within the species rather than deep evolutionary divergence. *A. mellifera* was positioned closest to *A. cerana*, followed by the lineages of *A. dorsata* and then *A. florea*, while the inclusion of *Bombus affinis* as an outgroup (BS = 100%) further validates the phylogenetic accuracy of the MDH gene. The close relationship between *Apis mellifera* and *Apis cerana* observed in the cMDH tree is consistent with previous nuclear marker-based studies, specifically those utilizing the EF1- α nuclear gene or nuclear genome, which identify these two species as a distinct "cavity-nesting group" within the *Apis* genus (Arias & Sheppard, 2005; Chen et al., 2018). This trend confirms that nuclear markers are highly reliable for grouping these taxa together, providing a stable evolutionary signal that clearly separates them from more ancestral open-nesting bees like *A. dorsata* and *A. florea* (Danforth et al., 2013).

The *cytb*-based phylogeny mirrored these results, providing separation among species with bootstrap support ranging from 41% to 100% (Figure 2B). We note that support for the node separating *A. mellifera* and *A. cerana* was relatively low (41%), likely due to the high sequence saturation often observed in mitochondrial markers. This gene tree discordance suggests that while *cytb* is excellent for species identification, deep nodes should be interpreted with caution. Notably, the mitochondrial marker successfully resolved the relationship between the closely related sister species *A. florea* and *A. andreniformis* (BS = 80%), while correctly positioning *Tetragonula pagdeni* as a distinct outgroup (BS = 100%). The high sequence variation of *cytb* proved effective for resolving species-level boundaries, particularly in *Apis* spp., which is consistent with recent findings that mitochondrial markers, such as the *tRNA^{Leu}-cox2* region or



mitochondrial genome, are ideal for identifying closely related honeybee taxa (Ferreira, 2017; Yang et al., 2019). Although *cytb* exhibited higher sequence divergence, a typical characteristic of mitochondrial markers, the overall clustering patterns remained entirely consistent with our MDH findings.

This topological analysis suggests that both nuclear and mitochondrial markers are tracking a shared evolutionary history. Crucially, we found that the cMDH gene, despite its relatively slower rate of change, is sensitive enough to distinguish intraspecific variation from species-level divergence. This reinforces the reliability of MDH as a versatile nuclear biomarker. The preference for nuclear genes in modern research is largely due to their biparental inheritance, providing a more complete picture of a species' history than only the female lineage of mitochondrial DNA (Zimmer & Wen, 2012). Furthermore, because nuclear markers generally change at a steadier pace, they are less likely to suffer from evolutionary overwriting, a process where rapid mutations pile up and obscure the true genetic relationships between species. The preference for nuclear genes like cMDH in modern research is justified by their biparental inheritance and slower evolutionary rate, which minimizes the risk of "long-branch attraction" or saturation that can affect mitochondrial phylogenies (Lin & Danforth, 2004). By aligning molecular DNA data with classical allozyme evidence, MDH offers a valuable bridge for researchers, particularly in studies that integrate biochemical and molecular approaches to identify and classify *Apis* species.

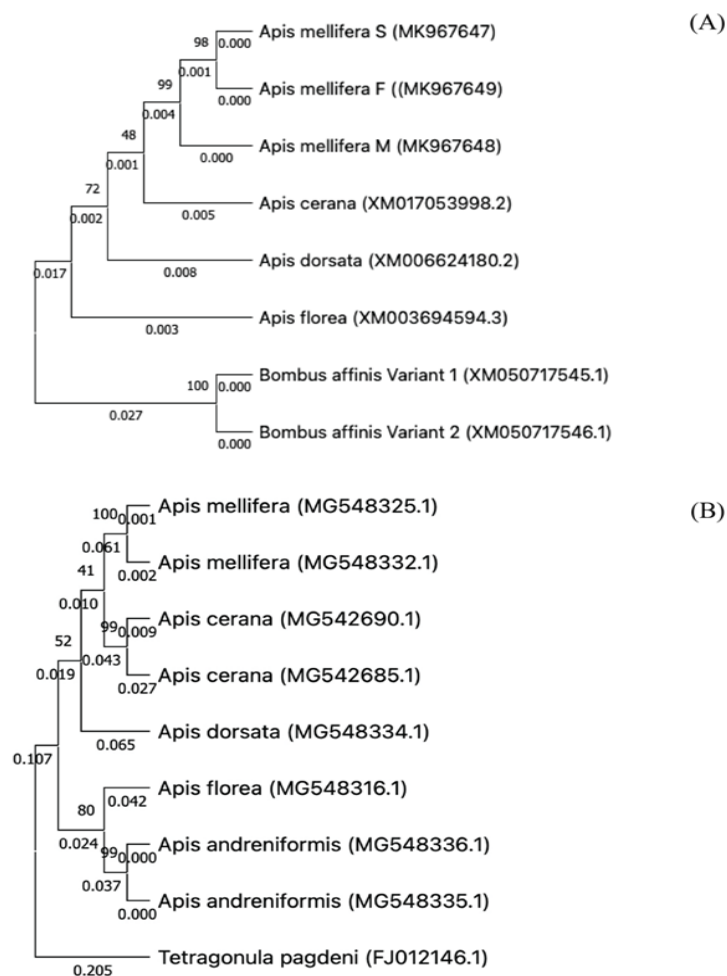


Figure 2 Phylogenetic relationships among cytoplasmic malate dehydrogenase (cMDH) (A) and cytochrome b (*cytb*) (B) sequences based on neighbor-joining (NJ). *Bombus affinis* and *Tetragonula pagdeni* were utilized as outgroups for cMDH and *cytb* sequences, respectively



4.3 cMDH Amino Acid Sequence Differences Among Species

The amino acid differences among the four *Apis* species are summarized in Figure 3. Within *Apis mellifera*, four amino acid differences exist between the S and M alleles at residues 114, 209, 215, and 259. Only one substitution (at residue 114) separates the S and F alleles, while three substitutions (at residues 209, 215, and 259) distinguish the M and F alleles.

Regarding interspecific variation, the cMDH of *A. cerana* exhibits 12 amino acid differences compared to *A. mellifera*. Furthermore, 11 and 16 differences were identified in *A. dorsata* and *A. florea*, respectively, when compared to *A. mellifera* (Figure 3). Notably, the amino acid sites involved in subunit-subunit interaction, NAD⁺ binding, and catalytic substrate binding were conserved across all four species. The prevalence of conservative rather than radical substitutions in these functional domains indicates strong purifying selection ($dN/dS < 1$). This conservation is expected, as these sites are essential for maintaining the normal biological function of the enzyme in bees. The structural stability provided by these conserved domains ensures metabolic efficiency, reinforcing the role of cMDH as a reliable marker for evolutionary studies despite millions of years of species divergence (Chapman et al., 1999; McCue & Finzel, 2022). Furthermore, the high conservation of the highlighted NAD⁺ binding and catalytic domains suggests these regions are under strong purifying selection. Given the fundamental role of cMDH in cellular respiration, this marker likely holds similar diagnostic utility for other bee species.

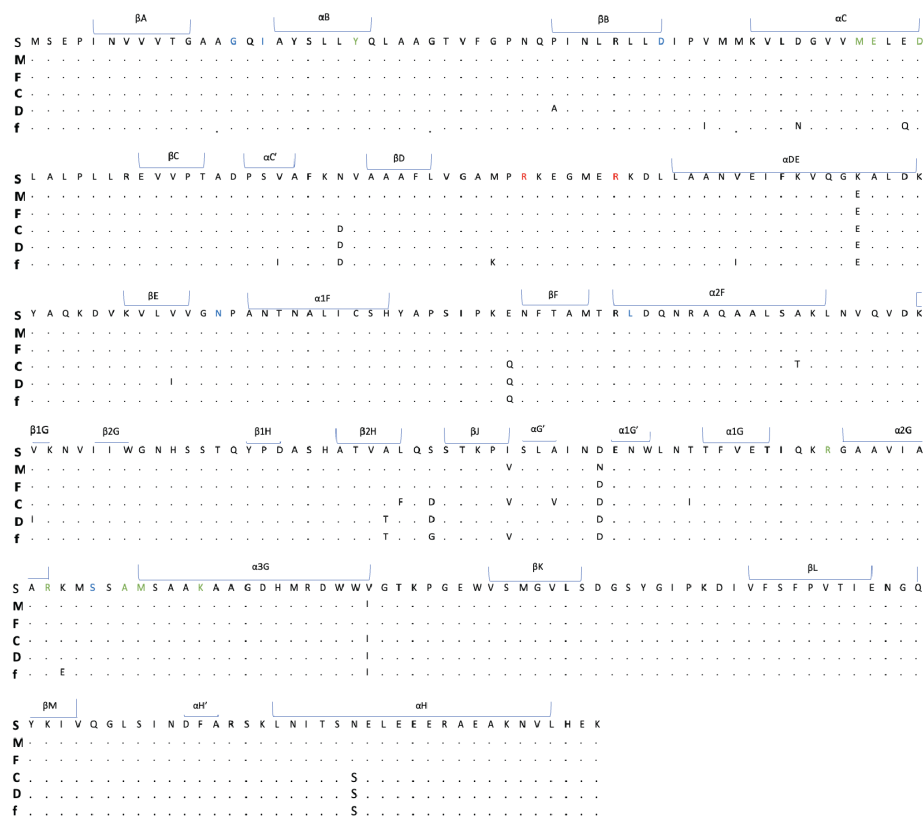


Figure 3 Amino acid sequence alignment of three cytosolic malate dehydrogenase alleles (F, M, and S) from *A. mellifera*, *A. cerana* (C), *A. dorsata* (D), and *A. florea* (F). The inferred secondary structure was calculated using Swiss-Model software based on porcine cMDH (PDB database) (Chapman et al., 1999). 5mdhA was indicated above each sequence segment by α (α -helices) or β (β -strands) followed by a number and/or a letter. The residues involved in substrate binding, cofactor binding, and subunit-subunit interactions are shown in red, blue, and green, respectively



5. Conclusion

This study demonstrates that the nuclear cMDH gene is a valuable complementary marker to mitochondrial markers for evaluating *Apis* phylogenetic relationships. The strong congruence between our nuclear and mitochondrial trees validates cMDH as a reliable tool for species-level identification and molecular taxonomy within an integrative framework.

While mitochondrial DNA remains the standard for maternal lineage tracking, cMDH provides insight into biparental evolutionary history. The contrast between the genetic uniformity of native Asian honeybees and the high polymorphism in *A. mellifera* highlights the sensitivity of this locus to capture varying levels of genetic diversity, though the drivers of these patterns require further investigation. Furthermore, the strict conservation of functional domains across all four species highlights its stability as a molecular marker under purifying selection. We acknowledge that as a single-locus study using distance-based and likelihood methods, these results represent a targeted snapshot of the *Apis* genome. Future research utilizing multi-locus or whole-genome sequencing will be essential to provide a broader genomic validation of these evolutionary signals. These findings establish cMDH as a useful nuclear biomarker, offering a more integrated perspective on honeybee genetic diversity in Southeast Asia.

6. Acknowledgements

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