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Genetic Variations of the Asian Honey Bee *Apis cerana* Fabricius, 1793 in Kalimantan - Indonesia and Their Relationships Across Asia

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Abstract

The vast distribution of *Apis cerana* (F.) across the Asian continent is a source of high genetic variations, particularly in areas experiencing geological formation and isolation, such as the Sundaland, including Kalimantan, Indonesia. This study investigated the genetic variations of *A. cerana* in the five provinces in Kalimantan and their relationships across Asia. We utilized mitochondrial DNA of COI, COII, and igs COI/COII sequences for genetic studies of 29 colonies from 12 locations in the five provinces of Kalimantan. The trees and network data confirmed the two lineages of *A. cerana* in Kalimantan, i.e., the Indo-Malayan (Borneo haplotype) and Indonesian (Java haplotype), with a slightly higher population of the latter. The high Java haplotype in Kalimantan might be due to the anthropogenic impact that transferred this honey bee from Java to Kalimantan. This finding agreed with the previous studies of geometric morphometrics using the same colonies as this study. We identified the common Borneo1 haplotype in the Indo-Malayan lineage of *A. cerana*, confirming previous COI findings. Furthermore, three specific haplotypes and putative amino acids of *A. cerana* were found in East and South Kalimantan. Those differed among the Asian population and thus could serve as the markers for these regions. Based on the sequences studied, the phylogenetic tree showed distinct clusters of *A. cerana* in Kalimantan and Sundaland from other *A. cerana* in the Asian regions. This study revealed that the mitochondrial DNA sequences showed the origin of the *A. cerana* population in Kalimantan and among islands in Sundaland.

Introduction

Mitochondrial DNA (mtDNA), a powerful tool in animal evolutionary studies, has been extensively utilized to investigate population genetics and evolution due to its high mutation rate (Ladoukakis & Zouros, 2017). Each mtDNA gene exhibits different mutation rates (Xu et al., 2012; Konrad et al., 2017). The cytochrome c oxidase 1 (COI) gene is highly effective in distinguishing closely related species (Hebert et al., 2003) and detecting intra-species DNA variation in animals (Goodall-Copestake et al., 2012; Feng et al., 2017). Intra-species DNA variations are the outcome of behavioral isolation (Groot et al., 2010; Pardy et al., 2021), temporal isolation (Boumans et al., 2016), and geographic isolation (Wang et al., 2017).

The geographic isolation in Sundaland, Southeast Asia, separated the islands of Sumatra, Java, and Borneo from the

mainland due to rising sea levels in the Pleistocene epoch (Hall, 2013). Borneo is one of the major evolutionary hotspots for biodiversity in Southeast Asia (de Bruyn et al., 2014). Geographic barriers in the form of mountains and rivers play a significant role in shaping the biodiversity and separating populations of Coleoptera *Darlingtonea kentuckensis* (Boyd et al., 2020), Lepidoptera *Atrijuglans hetaohei* (Qiqi et al., 2022), and Hymenoptera of the Asian honey bee *Apis cerana* (F.) (Zhu et al., 2017).

The wide distribution of *A. cerana* in South, East, North, and Southeast Asia is composed of eight subspecies, namely, *A. c. cerana*, *A. c. indica*, *A. c. japonica* (Ruttner, 1988), *A. c. koreana* (Ilyasov et al., 2018), *A. c. ussuriensis* (Ilyasov et al., 2019), *A. c. heimifeng, A. c. skorikovi*, and *A. c. javana* (Engel, 1999). Based on traditional morphometrics, Radloff et al. (2010) revealed six morphoclusters of *A. cerana*, one of which is the Indo-Malayan cerana morphocluster distributed in the Sundaland, including Sumatra, Java, and Borneo. Apart from morphological characters, molecular characters are also essential markers in the study of genetic variation and populations of *A. cerana*. Seven complete mtDNA of *A. cerana* was published for *A. cerana* in China (Tan et al., 2011), Japan (Takahashi et al., 2016; Okuyama et al., 2017a), Taiwan (Shinmura et al., 2017), Korea (Ilyasov et al., 2018), Russia (Ilyasov et al., 2019), and Borneo (Sabah) (Okuyama et al., 2017b).

Based on the COI gene of mtDNA, the population of *A. cerana* in Sundaland was separate from other parts of Asia (Zhao et al., 2014). This population was further divided into Indo-Malayan and Indonesian lineage (Tanaka et al., 2001a, 2001b, 2003; Zhao et al., 2014). The Indo-Malayan lineage is distributed across regions on Borneo Island, i.e., Sabah, Sarawak, Brunei, as well as West, East, and South Kalimantan (Tanaka et al., 2001a, 2001b, 2003; Okuyama et al., 2017b). The Indonesian lineage covers *A. cerana* in East (Raffiudin & Shullia, 2020) and West Java, Banten, Belitung (Raffiudin et al., 2022), and also found in South and Central Kalimantan (Tanaka et al., 2003).

In addition to the COI gene, population studies of *A. cerana* have also been previously conducted on the mtDNA cytochrome c oxidase 2 (COII) gene in Java (Raffiudin et al., 2022) and Sumatra (Simanjuntak et al., 2024). Furthermore, on mtDNA non-coding intergenic spacer (igs) COI/COII region in Borneo (Sabah), Java, and Nusa Tenggara (Smith & Hagen, 1996).

This study investigated the molecular character of *A. cerana* in Kalimantan, covering a wide area. We hypothesize that the geographic isolation within the island has led to the development of specific haplotypes in *A. cerana* in Kalimantan. Therefore, this study aims to reveal the genetic variations of *A. cerana* using mtDNA COI, COII, and igs sequences in five provinces in Kalimantan and their relationships across *A. cerana* in Asia. These findings could have profound implications for our understanding of genetic variation in the Asian honey bee.

Materials and Methods

Sample collection

Samples of *A. cerana* were collected from 29 colonies from 12 locations in five provinces of Kalimantan, Indonesia (Table 1; Fig S1), part of Borneo Island. The samples were preserved in absolute ethanol and stored at 4 ºC. For each colony, we used two individuals for DNA sequences.

DNA extraction, amplification, and sequencing

The thorax of *A. cerana* was used for DNA extraction using the Tissue Genomic DNA Mini Kit (GT300; Geneaid Biotech Ltd., New Taipei City, Taiwan), following the manufacturer's instructions. Two pairs of primers were used. The first pair of primers: forward primer Am_cox1b_F (5'-**AGG AGG TGG AGA TCC AAT TC**- 3') and reverse primer Am_cox1b_R (5'-**TGG ATA GTC TGA ATA ACG TCG TG**-3') (Raffiudin et al., 2022) were used to amplify the COI gene. The second pair of primers: forward primer E2 (5'-**GGC AGA ATA AGT GCA TTG**-3') (Cornuet et al., 1991) and reverse primer H1 (5'-**GTT CAT GAA TGA ATT ACA TCT G**-3') (Estoup et al., 1996) were used to amplify two sequential regions, i.e., of the igs and COII gene. The reaction of the total of 25 µL PCR mixture was consisted of 1.0 µL forward primer 10 µM, 1.0 µL reverse primer 10 µM, 12.5 µL MyTaq™ Red Mix (Bioline Reagents Ltd, United Kingdom), 1.5 µL MgCl2 25 mM, 2 µL DNA template <250 ng/µL. The PCR process consisted of pre-denaturation at 95 °C for 2.5 minutes, 35 cycles of denaturation for 15 seconds at 95 °C, annealing for 15 seconds at 47 °C (COI)/55 °C (igs-COII), and elongation for 1 minute at 72 °C, post elongation for 2 minutes at 72 °C and final elongation for 5 minutes at 15 °C. PCR products were sequenced at 1stBASE, Selangor, Malaysia.

Similarity search, genetic variation, and relationship analysis

The DNA homology analysis of the Asian honey bee was implemented using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The *A. cerana* COI, COII, and igs sequences were aligned with mtDNA *A. cerana* AP018149 (Okuyama et al., 2017b) utilizing Clustal X 2.1 (Larkin et al., 2007). The haplotypes of the three sequences and their concatenation, genetic diversity (JI and Hd), and genetic differentiations (F_{ST}) were analyzed with DNAsp 5.10 (Rozas et al., 2003). Median-joining networks were created using Network 10.2 (Forster, 2020). Putative amino acid variation, pairwise genetic distance, and nucleotide-based phylogenetic tree were analyzed in MEGA11 (Kumar et al., 2018) using 58 sequences of each COI, COII, igs, and concatenation of the three sequences from the current study. The analysis was also performed using 58, 36, and 67 sequences of COI, COII, and igs, respectively, and 30 concatenation sequences of COI-igs-COII from *A. cerana* across Asia from GenBank (Table S1a-d). The maximum likelihood method with the best-suggested model (Tamura 3-parameter) performed in MEGA11 with 1,000 bootstrap replications was used to construct the phylogenetic trees.

Table 1. Samples, number of colonies, locations, and coordinates of *Apis cerana* and the outgroup species in the current study.

No	Sample ID	No. of Colonies	District, Regency/City	Coordinate
Ingroup				
A. West Kalimantan				
1	Ac_SnP_Mmp	5	Sungai Pinyuh, Mempawah	0°18'0.8"N 109°6'33.8"E
B. East Kalimantan				
2	Ac_EsB_Blk	2	East Balikpapan, Balikpapan	-1°7'54.84513"S 116°59'40.29938"E
3	Ac_MrB_KtK	2	Muara Badak, Kutai Kartanegara	-0°15'51.72281"S 117°14'53.22473"E
4	Ac_LKl_KtK	2	Loa Kulu, Kutai Kartanegara	-0°29'39.86271"S 117°0'15.62659'E
5	Ac_TnS_KtK	1	Tenggarong Seberang, Kutai Kartanegara	-0°14'49.23869"S 117°7'0.3317"E
C. South Kalimantan				
6	Ac_SnL_TnB	2	Sungai Loban, Tanah Bumbu	3°39'31.4"S 115°44'44.8"E
7	Ac_Png_Bnj	2	Pangaron, Banjar	3°17'05.9"S 115°04'54.8"E
8	Ac_Tks_TnL	2	Takisung, Tanah Laut	3°50'48.5"S 114°38'40.3"E
9	Ac_Plh_TnL	2	Pelaihari, Tanah Laut	3°48'22.8"S 114°44'10.5"E
D. Central Kalimantan				
10	Ac_Sbg_PlR	3	Sebangau, Palangka Raya	-2°16'30,658"S 114°1'4,558"E
11	Ac_BkB_PlR	3	Bukit Batu, Palangka Raya	-1°59'53.688"S 113°44'35.832"E
E. North Kalimantan				
12	Ac_TnP_Bln	3	Tanjung Palas, Bulungan	2°49'59.72892'N 117°20'26.3403'E
Outgroup				
13	*Apis dorsata* Riau	1	Gunung Salihan, Kampar, Riau	00°07'38.8"N 101°19'83.7"E
14	*Heterotrigona itama* Banten	1	Cileles, Lebak, Banten	6°29'03.0"S 106°04'47.6"E

Results

Haplotype and putative amino acid variations of Apis cerana mitochondrial DNA in Kalimantan

The mitochondrial DNA sequences of *A. cerana* in Kalimantan revealed 645 bp, 474 bp, and 88 bp for the COI, COII, and igs, respectively (Genbank accession numbers COI LC788820 - LC788877 and igs-COII LC788878 - LC788935 (Table S1a-d)). We found that almost 50% of the COI genes of *A. cerana* populations in Kalimantan were aligned with *A. cerana* from Borneo, Sabah (AP0181149), the Indo-Malayan lineage. A similar percentage was aligned with *A. cerana* from Java (LC596969, LC596978, LC461196) from the Indonesian lineage with a high similarity of over 98% (Table S2a).

In the five provinces in Kalimantan, a total of 28 (4.3%), 26 (5.5%), and seven (7.95%) nucleotide variations for *A. cerana* were found for COI, COII, and igs, respectively (Table S3a, c, e) and resulted in 10, nine, and seven haplotypes for the three sequences. In agreement with the BLAST results, approximately 14 out of 29 (48%) *A. cerana* colonies found in our collected samples in Kalimantan were detected as the Borneo haplotype, the Indo-Malayan lineage (Fig 1). In COI and COII, the substitutions mainly occurred in the third codon (Fig S2a-f), having AT-rich 74.5 and 82.1%, respectively. Most changes in putative amino acids in both genes resulted from substituting nucleotides in the first codon. We found that the transition exceeded the transversion in both genes and igs (Fig S2a-f).

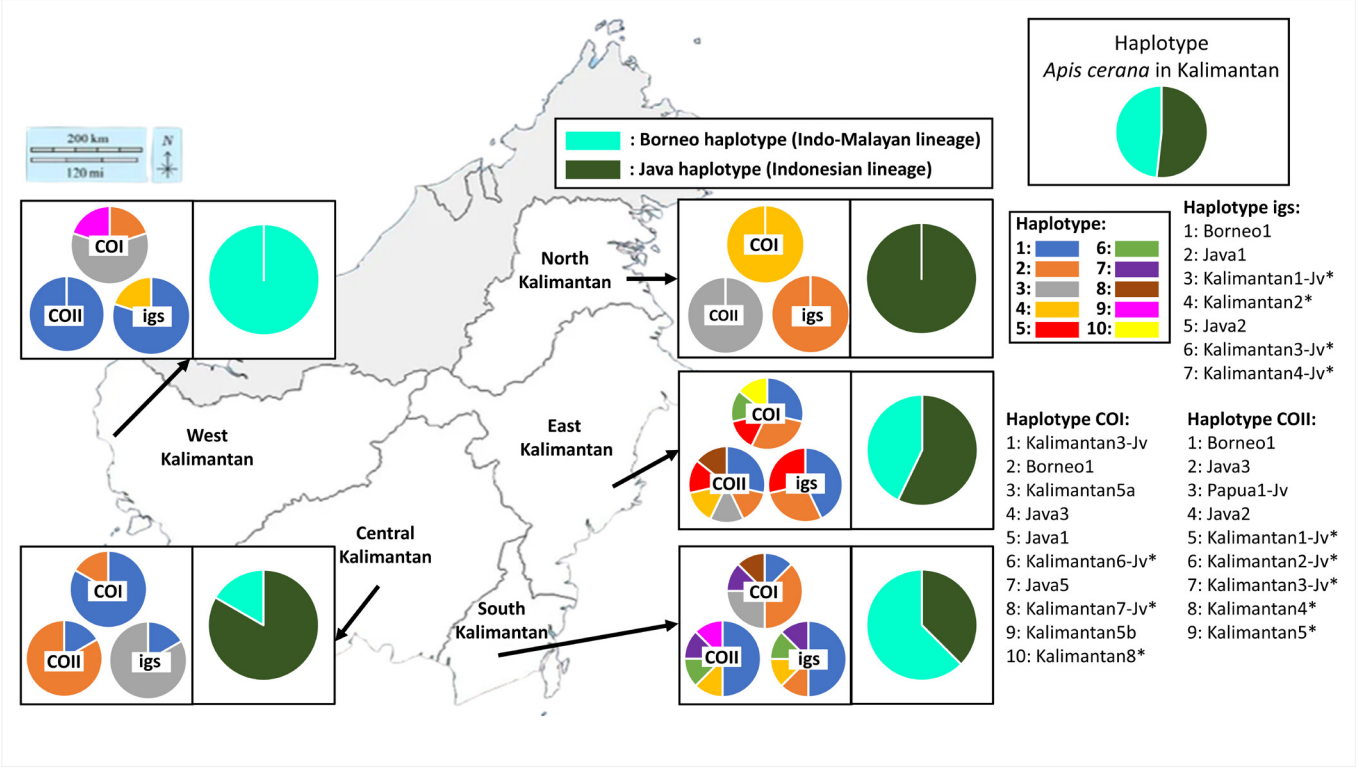

Fig 1. The haplotypes distribution of *Apis cerana* from Kalimantan based on the COI, COII, and igs. The haplotypes correspond to Table S3a, c, e. Asterisk (*) indicates the new haplotypes.

Our study revealed that the Borneo1 haplotype was the common haplotype in the Indo-Malayan lineage in Kalimantan *A. cerana* with a percentage of 24, 41, and 41 for each COI, COII, and igs, respectively (Fig 2a-c; Table S3a, c, e). The Borneo1A and Kalimantan2 haplotypes of Indo-Malayan lineage in Kalimantan *A. cerana* have the highest number of Median-joining network branches and were the two most common haplotypes based on the three sequences concatenation but are not dominant (Fig 2d; Table S3f).

Our research has uncovered four, three, and two haplotypes for the COI, COII, and igs in the Indo-Malayan lineage in Kalimantan *A. cerana*. Further analysis of the homology of COI, COII, and igs of the Asian honey bee across Asia using 528 bp, 464 bp, and 91 bp revealed 49 (9.3%), 52 (11.2%), and 35 (38.5%) nucleotide variations, respectively (Table S4a, c, e). The new haplotype of Indo-Malayan lineage in Kalimantan *A. cerana* COI-Kalimantan8, East Kalimantan, stands out with specific variations at nucleotide site 85 (G → A) (Table S4a). The new haplotype COII is Kalimantan4, East Kalimantan, with specific variations at nucleotide site 202 (A → G), and haplotype Kalimantan5, South Kalimantan, that varies at the sites 150 and 203 (T → C) (Table S4c). These specific variations can be a molecular marker of *A. cerana* Indo-Malayan lineage from East and South Kalimantan among all Asian populations. New haplotype igs-Kalimantan2 in West and South Kalimantan has variations at nucleotide site 31 (G → A) but can not be used as a marker (Table S4e). Each new haplotype of COI and COII genes of the Indo-Malayan lineage in Kalimantan *A. cerana* derived from one colony. However, igs derived from two colonies.

Several specific variations were found in the Indo-Malayan lineage of *A. cerana* in Borneo populations. Those can be used as markers to distinguish this population from all Asian populations. In the COI gene, two specific variations occurred in the substitution of T → C at base number 252 for several and 255 for all Indo-Malayan lineage of *A. cerana* in Borneo. (Table S4a; Tanaka, 2001a, b, 2003; Okuyama et al., 2017b). In the igs, the specific variations occurred in the substitution of T → A at base number 22 for several Indo-Malayan lineage of *A. cerana* in Borneo (Table S4e; Smith & Hagen, 1996; Okuyama et al., 2017b). No specific variation of the Indo-Malayan lineage of *A. cerana* in Borneo was found in COII (Table S4c).

Our analysis of COI, COII, and igs revealed that 52% of *A. cerana* in Kalimantan is Indonesian lineage, which is the Java haplotype. We found that the Java haplotype of *A. cerana* was concentrated in East, Central, and North Kalimantan at 57, 83, and 100%, respectively. Interestingly, West Kalimantan has no Java haplotype of *A. cerana* (Fig 1). We discovered six Java haplotypes for each COI and COII gene and five for igs. Among these, three haplotypes were newly described as Java haplotypes found in Kalimantan COII (Kalimantan 1-Jv, Kalimantan 2-Jv, Kalimantan 3-Jv) and Kalimantan igs (Kalimantan 1-Jv, Kalimantan 3-Jv, Kalimantan 4-Jv). At the same time, COI had two (Kalimantan 6-Jv, Kalimantan 7-Jv) (Table S3a, c, e).

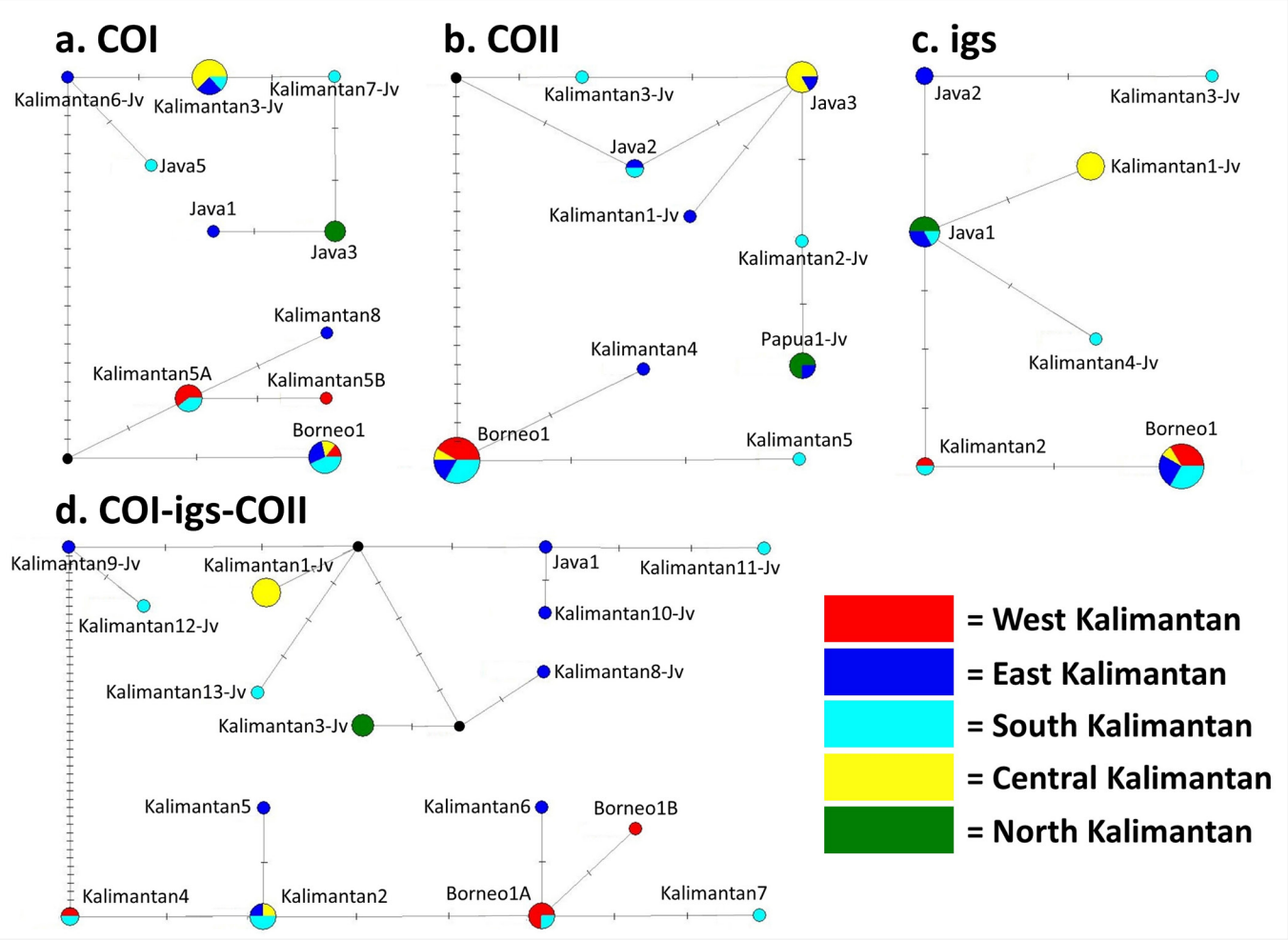

Fig 2. Median-joining network *Apis cerana* from Kalimantan based on the COI (a), COII (b), igs (c), and the combined COI-igs-COII (d). Black circles show hypothesized sequences that are not represented in the current study. The size of the circle indicates the number of individuals. The dashes on line show the number of mutations.

Genetic diversity and genetic differentiation of COI, COII, *and* igs *of Apis cerana in Kalimantan*

Genetic diversity was assessed based on nucleotide diversity (JI) (Fig S3a) and haplotype diversity (Hd) (Fig S3b). Similar patterns include nucleotide diversity (JI) and haplotype diversity (Hd) of *A. cerana* from Kalimantan. Based on the COI and COII genes, *A. cerana* in East Kalimantan has the highest genetic diversity, while for igs, the highest was in South Kalimantan (Fig S3a-b). These two provinces comprise 57 and 38% Java haplotype (Fig 1). These results parallel the genetic differentiations in all sequences that showed weak fixation index value (F_{ST}: 0-0.5 Wright, 1978) between the East and South Kalimantan populations, meaning the *A. cerana* populations of East and South Kalimantan have no differentiation (Table S5). This phenomenon could be because many Java haplotypes of *A. cerana* were found in both provinces. Overall, there are very significant genetic differentiations (F_{ST}: > 0.25 Wright, 1978) of *A. cerana* populations between all Kalimantan provinces for all sequences (Table S5).

Genetic distance of all sequences of Apis cerana from Kalimantan, network analysis, and phylogenetic relationship across Asia

The highest genetic distance is within *A. cerana* from the five provinces in Kalimantan based on COI, COII, igs, and concatenation of COI-igs-COII sequences was 4.08%, 5.92%, 6.27%, and 4.79%, respectively (Table S6a-d). These relatively high numbers were due to 52% of the samples being Java haplotypes (Fig 1).

Within the Indo-Malayan lineage in Kalimantan, the highest genetic distance of COI was 0.58% between the Kalimantan8 haplotype, Muara Badak, East Kalimantan, and the common Borneo1 haplotype (Table S6a). The common Borneo1 haplotype also showed the highest genetic distance of igs (1.18%) with Kalimantan2 haplotype Sungai Pinyuh, West Kalimantan (Table S6c). The highest genetic distance in COII (0.66%) occurs between Kalimantan4 haplotype, East Balikpapan, East Kalimantan, with Kalimantan5 haplotype, Sungai Loban, South Kalimantan (Table S6b). Based on the concatenation of three sequences (Table S6d), the highest genetic distance (0.47%) was found between Sungai Loban, South Kalimantan, and Sungai Pinyuh, West Kalimantan.

The median-joining network (Fig 2a-d) displays a pattern corresponding to the phylogenetic tree construction of *A. cerana* from Kalimantan and across Asia (Fig 3a-d). The results were similar for COI, COII, igs, and the concatenation of the three sequences. The population of *A. cerana* in Sundaland has distinct clusters with *A. cerana* from other regions of Asia, forming two main lineages (Fig 3a-d). The Indo-Malayan lineage consists *A. cerana* colonies from Kalimantan (both from the current study and Tanaka et al., 2003), as well as *A. cerana* from other parts of Borneo (Smith & Hagen, 1996; Tanaka et al., 2001a,b, 2003; Raffiudin & Crozier, 2007; Okuyama et al., 2017b), Sumatra (Simanjuntak et al., 2024) and Selangor (Smith & Hagen, 1996). The Indonesian lineage includes *A. cerana* colonies from Kalimantan (both from the current study and Tanaka et al., 2003), Java (Smith & Hagen, 1996; Raffiudin & Shullia, 2020; Raffiudin et al., 2022), Lesser Sunda (Smith & Hagen, 1996), North Sumatra (Simanjuntak et al., 2024), and Belitung (Raffiudin et al., 2022), as well as from non-native regions such as Sulawesi (Smith & Hagen, 1996; Tanaka, unpublished data), Ambon, and Manokwari (Raffiudin et al., 2022).

The phylogenetic tree shown by the COI gene separates the Indo-Malayan lineage of *A. cerana* from Borneo and Sumatra (Fig 3a). Current studies show that COI, COII, and igs can reveal the lineage of *A. cerana* populations in Sundaland, particularly in Kalimantan (Fig 2a-d; Fig 3a-d).

Discussion

This study aimed to explore the genetic variations of *A. cerana* from Kalimantan using mtDNA COI, COII, and igs. We discovered that 52% of *A. cerana* colonies in Kalimantan belong to the Java haplotype, with representation across all provinces except West Kalimantan (Fig 1). Our result agreed with Tanaka et al. (2003) that within 20 years after the first finding, we found five additional Java haplotypes of the COI gene and new records on East and North Kalimantan (Fig 1; Table S3a).

Most of the sampling sites are located in the southern and eastern parts of Kalimantan (Table 1; Fig S1). The southern part is geographically closer to Java, while the eastern part is near Sulawesi. Although Sulawesi is not a native range for *A. cerana*, previous studies (Tanaka, Unpublished data; Smith & Hagen, 1996) have reported the presence of *A. cerana* colonies in South and Central Sulawesi, all of which belong to the Java haplotype. The presence of the Java haplotype in southern and eastern parts of Kalimantan is presumably due to anthropogenic factors such as colony trading between these locations with Java and Sulawesi Islands. Colony trading between islands has been observed for stingless bee species such as *Tetragonula sapiens* between Java (Yogyakarta) and Lombok Island (Trianto & Purwanto, 2020), and also between provinces for *T. laeviceps* in Banten and West Java (Sayusti et al., 2023). In contrast, the sampling sites in West Kalimantan are farther to the west (Fig S1), closer to Sumatra, which harbors only about 3% of the *A. cerana* Java haplotype (Simanjuntak et al., 2024). The absence of the Java haplotype in West Kalimantan may be explained by its greater geographic distance from Java and Sulawesi, which likely limited anthropogenic factors.

Apis cerana from Java was also introduced to Papua in the late 1970s (Anderson, 1994). Java haplotype was then found in West Papua and Moluccas (Raffiudin et al., 2022). Furthermore, a single Java haplotype of the COI gene (Sun et al., 2022) and mitogenome (Dogantzis et al., 2014) was found in all invasive *A. cerana* populations in Australia. Phenomena of intra-species introduction also occurred in the Africanized honey bee (*A. mellifera scutellata*) found in East and Central Europe (Oleksa et al., 2021). It warrants our attention because introducing intra-species can negatively impact the native population. For instance, in Southern Africa, *A. m. scutellata* faced colony collapse disorder due to the introduction of parasitic *A. m. capensis* (Martin et al., 2002). The genetic introgression of *A. cerana* from Java can also lead to the loss of the native (Indo-Malayan lineage/Borneo haplotype) *A. cerana* from Kalimantan genetic identity. The phenomenon of genetic introgression had been identified in *A. mellifera* C-lineage into *A. m. mellifera* (M-lineage) in northeastern Poland (Oleksa et al., 2011).

Apis cerana is hardly found during our exploration in Kalimantan, although it is one of the favorite bees for beekeeping in other islands in Indonesia (Buchori et al., 2022). People in Kalimantan prefer to keep stingless bees in meliponiculture (Buchori et al., 2022) and bee hunting for forest giant honey bee *A. dorsata* (Kahono et al., 2018).

Our study revealed the low genetic distance (0 - 0.47%) within the native *A. cerana* in the five provinces in Kalimantan based on the concatenated sequences (Table S6d). However, a high genetic distance of 4.00 - 4.79% was found when we compared those native bees with the Java haplotype of *A. cerana* in Kalimantan. We found a similar genetic distance (4.21 - 4.67%) between *A. cerana* native from Kalimantan and *A. cerana* native from Java (Raffiudin et al., 2022) (Table S6d). This number is higher than the genetic distance of complete mtDNA *A. c. koreana* in Korea compared to *A. c. cerana* in China (2.58%) and *A. c. japonica* in Japan (2.57%) (Ilyasov et al., 2018).

Due to the high genetic introgression of the Java haplotype in *A. cerana* populations in East and South Kalimantan (Fig 1), these provinces had the highest genetic diversity (Fig S3a-b). Studies of geometric morphometrics from the same colony using 19 landmarks of wing venations also showed the highest diversity in South Kalimantan (Latif et al., in review). This phenomenon may be facilitated by the easy access from Java to East and South Kalimantan, urban areas with the highest Human Development Index (HDI) in Kalimantan (National Development Planning Agency, 2024). This high HDI may also cause high colony trading between these two provinces, making *A. cerana* populations in East and South Kalimantan have no differentiation (F_{ST}: 0-0.5

Wright, 1978) (Table S5). Colony trading between both provinces also found for stingless bee *Tetragonula biroi* (Purwanto et al., 2022).

Borneo1 was the common haplotype for the Indo-Malayan lineage in Kalimantan *A. cerana* for the three sequences (Fig 2a-c; Table S3a, c, e), confirming previous findings on the COI gene (Tanaka et al., 2003). Despite the low genetic distance among this lineage in Kalimantan (Table S6d), we found three new specific haplotypes in East and South Kalimantan (Table S4a, c) among the Asian *A. cerana* population. These new specific haplotypes form specific putative amino acid substitutions (Table S4b, d). In the site 29 COI gene Kalimantan8 haplotype (East Kalimantan), glycine (G) was substituted for serine (S) (Table S4b). Substitution of isoleucine (I) to valine (V) and threonine (T) (site 68) was found in COII of *A. cerana* haplotypes Kalimantan4 (East Kalimantan) and Kalimantan5 (South Kalimantan), respectively (Table S4d). Both amino acid substitutions of G→S and I→T change the chemical properties of amino acids from nonpolar aliphatic to polar (Nelson & Cox, 2005). Changes in these amino acids can alter the changes in protein structure and function and thus can be used as markers of evolution (Nelson & Cox, 2005; Schaefer & Rost, 2012). These specific haplotypes and putative amino acids have the potential to be markers for native *A. cerana* from East and South Kalimantan.

The use of three mtDNA sequences (Fig 3a-d) shows that the Sundaland population of *A. cerana* is separated from other Asian populations and forms two distinct lineages, i.e., the Indo-Malayan lineage, which includes Borneo and Sumatra, and the Indonesian lineage, which includes Java. This finding supports the results from the COI gene (Zhao et al., 2014) and genome-wide single nucleotide polymorphisms (SNPs) (Su et al., 2023). In contrast to the molecular data that group Borneo and Sumatra together, traditional morphometrics using measurements of 12 morphological characters revealed three distinct subclusters corresponding to Borneo, Sumatra, and Java (Radloff, 2010). The morphology of *A. cerana* can also vary with altitude, as demonstrated by studies in the basins and highlands of southwestern China (Zhu et al., 2017). Moreover, studies of geometric morphometrics using 19 landmarks of wing venation from the same colonies showed that *A. cerana* in this study is more closely related to Java than to Sumatra (Latif et al., in review), which is consistent with our findings.

Conclusions

Median-joining network and haplotype analysis show that *A. cerana* from Kalimantan was almost the same 50% for Indo-Malayan and Indonesian lineage. Most Indonesian lineage (Java haplotype) was found in East, Central, and North Kalimantan but none in West Kalimantan. The Borneo1 haplotype was the common haplotype in the native (Indo-Malayan) *A. cerana* in Kalimantan. Native *A. cerana* from East Kalimantan has two new specific haplotypes of COI-Kalimantan8 and COII-Kalimantan4, while one new specific haplotype found in South Kalimantan, i.e., COII-Kalimantan5. These new haplotypes could be markers of *A. cerana* from these provinces among *A. cerana* from all of Asia. Populations of *A. cerana* from East and South Kalimantan populations have no differentiation (F_{ST}: 0-0.5). The populations of *A. cerana* in these two provinces also have the highest genetic diversity. The highest genetic distance within the native *A. cerana* in Kalimantan based on COI-igs-COII sequences is 0.47%. The maximum likelihood phylogenetic tree from all sequences shows that the population of *A. cerana* in Sundaland has distinct clusters with *A. cerana* from other regions of Asia. The Sundaland clusters consist of the Indo-Malayan (Borneo and Sumatra) and the Indonesian lineage (Java). The COI gene phylogenetic tree distinguishes the Indo-Malayan lineage *A. cerana* from Borneo and Sumatra. This study shows that mtDNA can determine the origin of the *A. cerana* population, particularly in Kalimantan.

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Disclosure statement

The authors report no potential conflict of interest.

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Authors' Contributions

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List of Supplementary captions

Table S1. List of ingroup of *Apis cerana* and outgroup species used for bioinformatics and phylogenetic analysis based on COI, COII, and igs.

Table S2. BLASTN analysis result of *Apis cerana* from Kalimantan based on COI & igs-COII gene.

Table S3. The nucleotide, haplotype, and amino acid of *Apis cerana* from Kalimantan based on COI, COII, and igs.

Table S4. The nucleotide, haplotype, and amino acid of *Apis cerana* from Kalimantan and across Asia based on COI, COII, and igs.

Table S5. Genetic differentiation of *Apis cerana* populations in Kalimantan based on Fixation index value (F_{ST}) on the COI (left), COII (right, below diagonal), and igs (right, above diagonal).

Table S6. The genetic distances of *Apis cerana* from Kalimantan and across Asia based on COI, COII, and igs.

Fig S1. Sampling locations of *Apis cerana* in Kalimantan.

Fig S2. The number of transitions and transversions of *Apis cerana* from Kalimantan in each codon position of the COI (a-c), COII (d-f), and igs (g).

Fig S3. Genetic diversity of *Apis cerana* from Kalimantan population based on the COI, COII, and igs sequences. (a) nucleotide diversity (Л). (b) haplotype diversity (Hd). Ac: *Apis cerana*, WK: West Kalimantan, EK: East Kalimantan, SK: South Kalimantan, CK: Central Kalimatan, NK: North Kalimantan. The genetic diversity among the COII gene in Ac_WK and COI, igs, and COII sequences in Ac_NK is 0.00.

Appendix

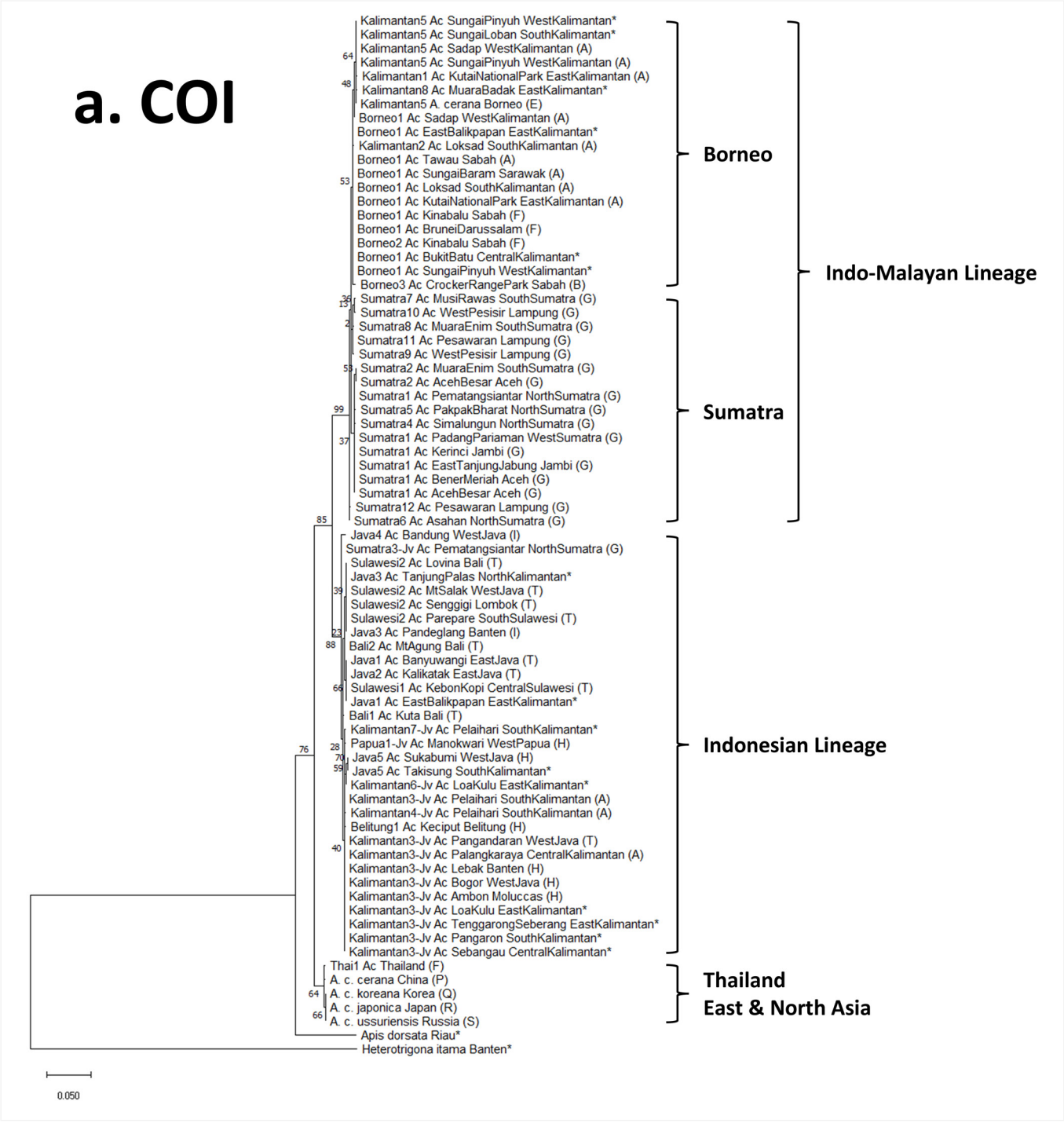

Fig 3a. The maximum likelihood phylogenetic tree of *Apis cerana* is based on the COI. Asterisk (*) indicates the current study haplotype that refers to Table S3a. The letter in parentheses indicates the reference of the sample. A: Tanaka et al. (2003), B: Tanaka et al. (2001b), E: Okuyama et al. (2017b), F: Tanaka et al. (2001a), G: Simanjuntak et al. (2024), H: Raffiudin et al. (2022), I: Raffiudin & Shullia (2020), P: Tan et al. (2011), Q: Ilyasov et al. (2018), R: Takahashi et al. (2016), S: Ilyasov et al. (2019), T: Tanaka (Unpublished data).

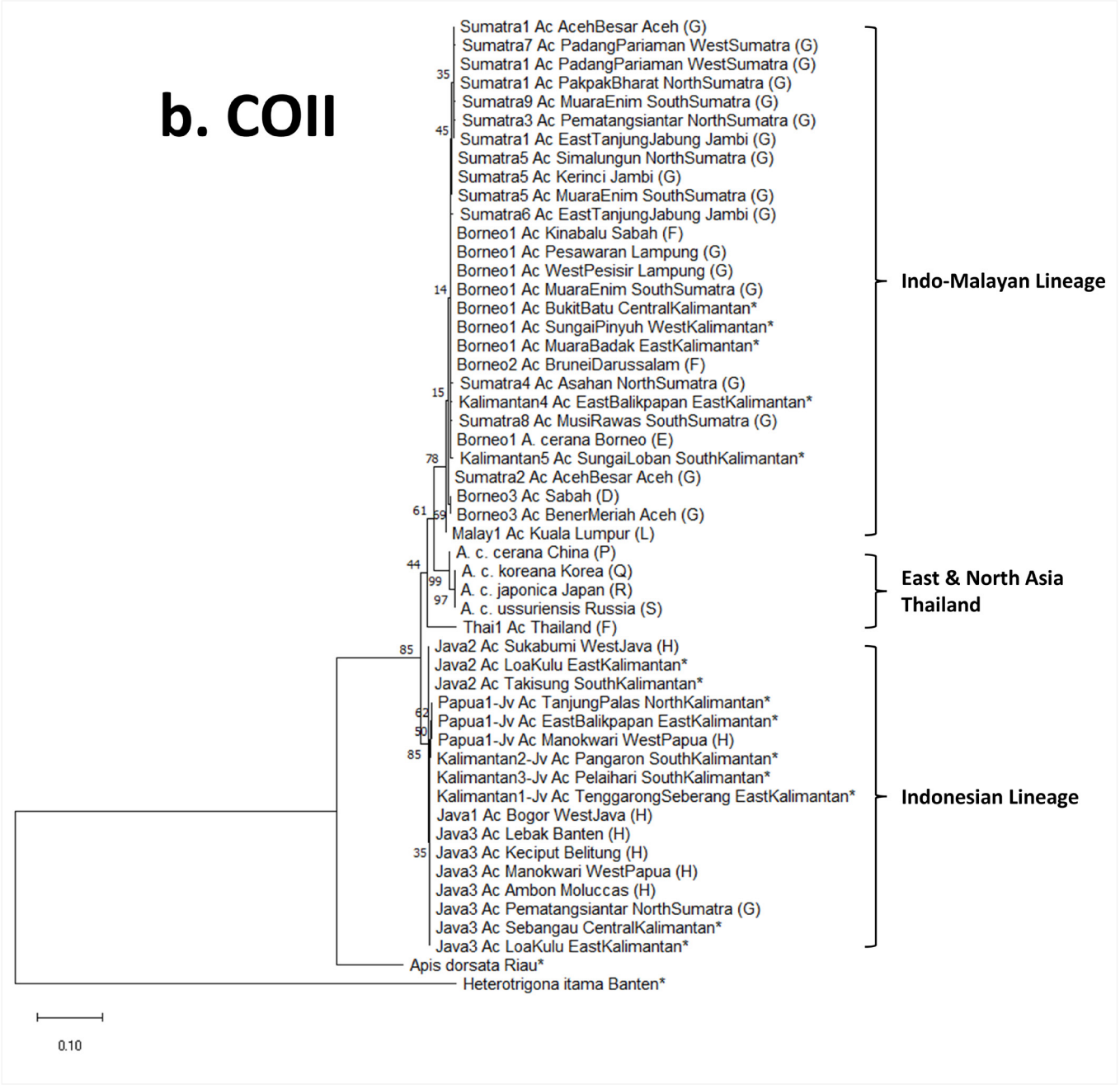

Fig 3b. The maximum likelihood phylogenetic tree of *Apis cerana* is based on the COII. Asterisk (*) indicates the current study haplotype that refers to Table S3c. The letter in parentheses indicates the reference of the sample. D: Raffiudin & Crozier (2007), E: Okuyama et al. (2017b), F: Tanaka et al. (2001a), G: Simanjuntak et al. (2024), H: Raffiudin et al. (2022), L: Willis et al. (1992), P: Tan et al. (2011), Q: Ilyasov et al. (2018), R: Takahashi et al. (2016), S: Ilyasov et al. (2019).

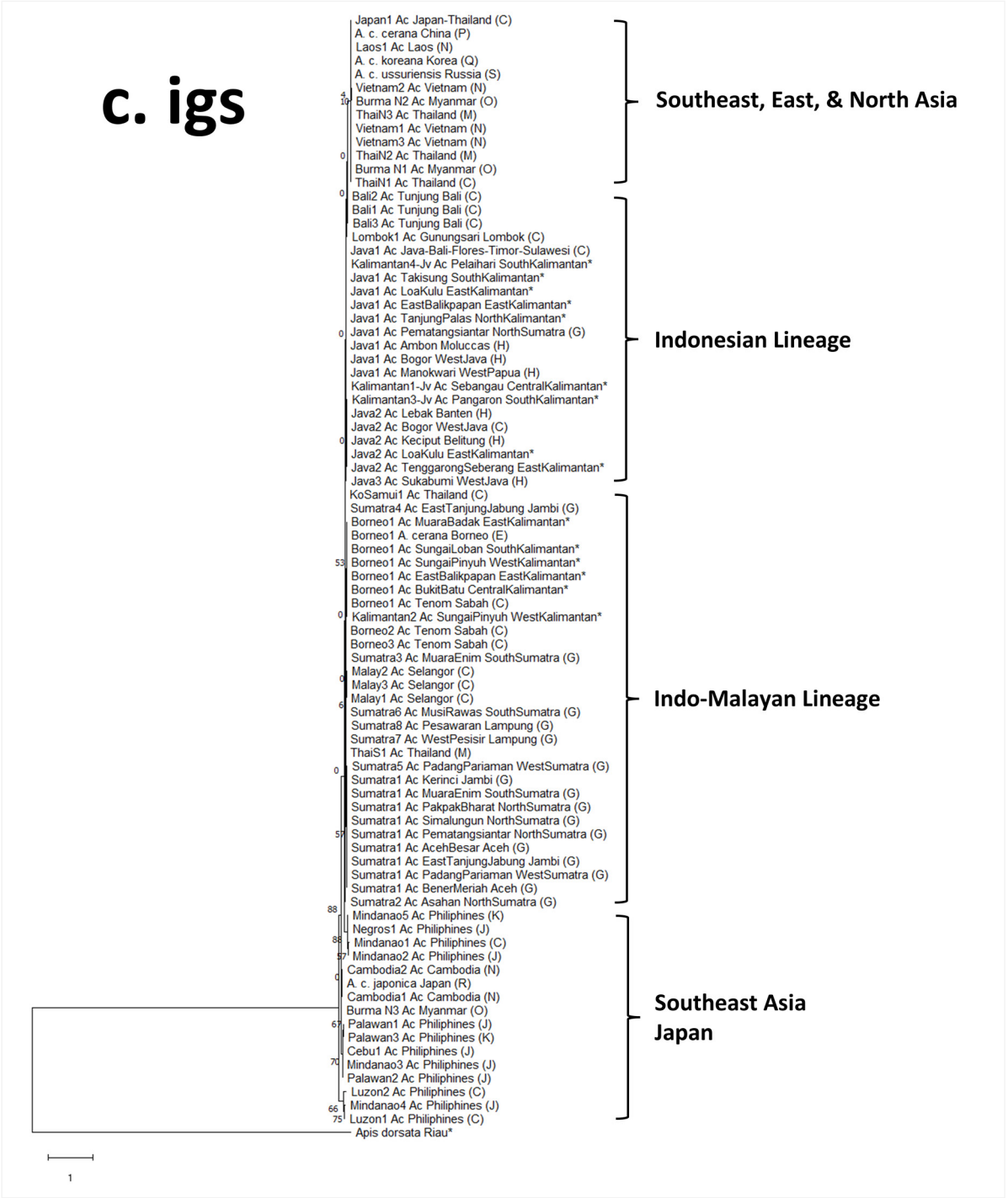

Fig 3c. The maximum likelihood phylogenetic tree of *Apis cerana* is based on the igs. Asterisk (*) indicates the current study haplotype that refers to Table S3e. The letter in parentheses indicates the reference of the sample. C: Smith & Hagen (1996), E: Okuyama et al. (2017b), G: Simanjuntak et al. (2024), H: Raffiudin et al. 2022, J: Smith et al. (2000), K: de la Rúa et al. (2000), M: Warrit et al. (2006), N: Smith et al. (2005), O: Smith et al. (2004), P: Tan et al. (2011), Q: Ilyasov et al. (2018), R: Takahashi et al. (2016), S: Ilyasov et al. (2019).

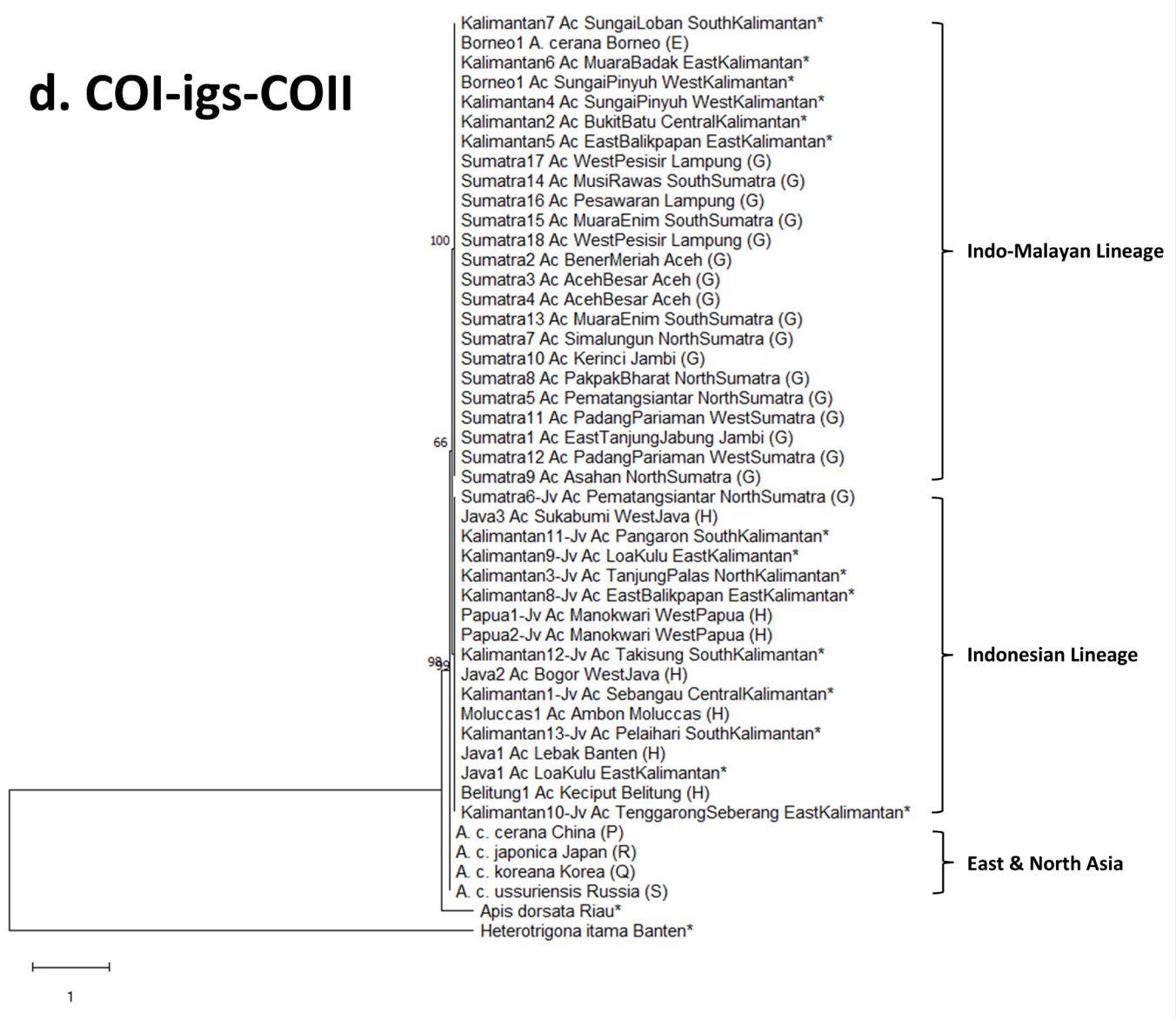

Fig 3d. The maximum likelihood phylogenetic tree of *Apis cerana* is based on the COI-igs-COII. Asterisk (*) indicates the current study haplotype that refers to Table S3f. The letter in parentheses indicates the reference of the sample. E: Okuyama et al. (2017b), G: Simanjuntak et al. (2024), H: Raffiudin et al. (2022), P: Tan et al. (2011), Q: Ilyasov et al. (2018), R: Takahashi et al. (2016), S: Ilyasov et al. (2019).