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Phylogeography of the giant honey bees based on mitochondrial gene sequences

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Our goal was to resolve phylogenetic relationships among *Apis laboriosa*, and the *Apis dorsata* subspecies *A. d. dorsata*, *A. d. binghami*, and *A. d. breviligula*, the last two of which have been proposed as full species by several authors. We carried out a phylogenetic analysis of the giant honey bees using mitochondrial *cox1* and *cox2* gene sequences analyzed with maximum likelihood methods. We obtained strong support for four clades within *A. dorsata* in the broad sense: the three subspecies or species mentioned above, and a fourth lineage from south India. However, our analysis did not resolve the phylogenetic relationships among the four lineages. The presence of two genetically distinguishable groups of *"A. dorsata"* in India parallels the presence there of two cavity-nesting honey bees, *A. cerana cerana* and *A. c. indica* (the black hill bees and yellow plains bees, respectively). This suggests that past climatic or geological events may have temporarily isolated Indian populations from populations of the Asian mainland, leading to divergence and possibly speciation of Indian giant and cavity-nesting bees, followed by recolonization of India by eastern Asian forms. Recognition of these distinct lineages is important for conservation planning, so that their individual distributions, ecologies, and migration patterns can be considered, and so that the genetic diversity they represent can be maintained.

KEYWORDS

phylogeny, *Apis dorsata*, *Apis laboriosa*, cox1 gene, cox2 gene, species discrimination

Introduction

The giant honey bees have a geographic range centered on south and southeast Asia, extending northwest into Pakistan, eastwards through India, Bangladesh, Nepal, Bhutan, Myanmar, Thailand, southern China, and southeast Asia, and through the islands of Malaysia, Indonesia, and the Philippines (Otis, 1996; Kitnya et al., 2020; Huang et al., 2022; Kitnya et al., 2024; Otis et al., 2024; Voraphab et al., 2024; Warrit et al., 2024). Several earlier writers including Maa (1953) and Ruttner (1988) pointed out diversity among giant honey bee populations based on morphological and morphometric data. In particular they noted that the giant honey bees of the Himalayan region, the Indonesian island of Sulawesi, and the oceanic

Philippine islands (i.e., those islands never connected to the Asian mainland) differed from one another and from the more widespread form found elsewhere. Maa divided honey bees into three genera—*Micrapis*, the dwarf honey bees, *Megapis*, the giant honey bees, and *Apis*, the cavity-nesting honey bees—and recognized four giant bee species: *Megapis breviligula* from the Philippines, *M. binghami* from Sulawesi and smaller nearby islands, *M. laboriosa* from high altitude Himalayan regions, and the more widespread *M. dorsata*. Ruttner, like most subsequent authors, recognized just one genus, *Apis*, and only one species of giant honey bee, *Apis dorsata*. He and many subsequent authors (e.g., Engel, 1999, 2002) considered the Himalayan form a subspecies, *A. d. laboriosa*, but noted that additional information might confirm it as a distinct species.

The taxonomic status of *A. laboriosa* remained contentious for many years despite numerous studies. Sakagami et al. (1980) made detailed morphological comparisons of *A. laboriosa* from Nepal and *A. dorsata* collected from many parts of its range, documenting "distinct and stable differences between them" supporting species status of *A. laboriosa*. McEvoy and Underwood (1988) reported that they could find no morphological differences between male genitalia (the everted endophallus) of *A. laboriosa* and *A. dorsata*, but nonetheless supported species status of *A. laboriosa* on the basis of other morphological differences, habitat, the presence of two species of braulid parasites (Diptera: Bruaulidae, *Megabraula*) in nests of *A. laboriosa* but (apparently) not those of *A. dorsata*, and genetic differences revealed by allozyme electrophoresis.

However, some authors argued that the characters used to support species status of *A. laboriosa*—including habitat, color patterns, and morphometric characters—could represent intraspecific variation and adaptation to different habitats, and thus took the conservative position that more data were needed, particularly concerning reproductive isolation of populations occurring in sympatry (e.g., Ruttner, 1988; Engel, 1999). Cao et al. (2012a) carried out morphometric comparisons of *A. laboriosa* and *A. dorsata* collected from Yunnan, Guangxi and Hainan provinces in China and again found significant differences between them. Collection sites for the two were in relatively close proximity (on the order of 200-300 km) but not strictly sympatric, and they were found at different elevations (*A. laboriosa* 1500 m and above, *A. dorsata* 1300 *m* and lower, though all but one collection was made at 700 m or lower).

More recently, new distributional records for *A. laboriosa* (Kitnya et al., 2020) reported *A. dorsata* and *A. laboriosa* foraging sympatrically at sites in Arunachal Pradesh, India and in northern Vietnam. Kitnya et al. (2022), found distinct morphological, morphometric, and genetic differences between Indian populations of *A. dorsata* and *A. laboriosa*, both in sympatry and in allopatry, providing convincing support for the species status of *A. laboriosa*.

Until recently the species status of *A. d. breviligula* and *A. d. binghami* have received much less attention. Arias and Sheppard (2005) included *A. laboriosa*, *A. dorsata* from Thailand and Sri Lanka, and *A. binghami* in a larger phylogenetic analysis of *Apis* species using both nuclear (*EF-1α* intron) and mitochondrial (*ND2*) sequence data. The giant honey bees were recovered as a monophyletic group and *A. laboriosa* was consistently recovered as a clade distinct from *A. dorsata* and *A. d. binghami*; however, *A. dorsata* and *A. d. binghami* were not consistently resolved as separate lineages. Raffiudin and Crozier (2007) used both mitochondrial (*cox2, ND2*, and the large (16S) ribosomal subunit or *rrnL*) and nuclear (inositol 1,4,5-triphosphate receptor or *itpr*) gene sequences in their phylogenetic analysis of *Apis* taxa, also including the giant honey bees *A. dorsata* from Sabah, Malaysia, *A. d. binghami*, and *A. laboriosa*. Their analyses consistently recovered *A. laboriosa* as sister to *A. dorsata* and *A. d. binghami*. Lo et al. (2010) carried out a more comprehensive coverage of giant honey bees, including *A. laboriosa*, *A. dorsata* from Sabah, Malaysia and Palawan Island, the Philippines, *A. d. binghami* and *A. d. breviligula* in their phylogenentic analysis of *Apis* species, using the same set of genes as Raffiudin and Crozier (2007) minus the mitochondrial *ND2*. Their results strongly supported the species status of *A. d. breviligula* from the Philippines, though the placement of *A. d. binghami* remained unresolved.

Kitnya et al. (2024) carried out a taxonomic study of giant honey bees using morphological characters. Their study included *A. laboriosa*, *A. dorsata*, and the island lineages *A. d. binghami* and *A. d. breviligula*. They found that *A. dorsata* from mainland Asia differs morphologically from *A. d. binghami* and *A. d. breviligula* but concluded that the latter two represent a single morphological species, *A. binghami*, with two subspecies, *A. b. binghami* and *A. b. breviligula*.

In this paper, we accept the species status of *A. laboriosa*. We use the names *A. dorsata dorsata*, *A. d. breviligula* and *A. d. binghami* for the other distinctive populations of giant honey bees because the species status of the latter two is still subject to investigation. We use the name "A. d. SouthIndia" to refer to a population that appears to be a cryptic unnamed species or subspecies (Smith, 1991; Kitnya et al., 2022). "*Apis dorsata* in the broad sense" will refer to all giant honey bees excluding *A. laboriosa*.

The objective of this study is to carry out a phylogenetic analysis for populations of giant honey bees, including representatives from as much of their range as we could obtain, to test whether the lineages within *A. dorsata* in the broad sense are monophyletic, and to determine relationships among them. Samples include *A. laboriosa* [Nepal], *A. d. dorsata* [multiple populations], and the distinctive island populations *A. d. binghami* [Sulawesi and smaller nearby islands] and *A. d. breviligula* [the oceanic islands of the Philippines]. We also include the dwarf honey bees, *A. florea* and *A. andreniformis*, and the cavity-nesting honey bees *A. mellifera* and *A. cerana* as outgroups. We generated partial sequences of the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) and cytochrome c oxidase subunit 2 (*cox2*) genes and used Maximum Likelihood methods in MEGA7 to construct phylogenetic trees.

Methods

Field methods

Samples used in this study were collected by multiple researchers from 1989 to 2018 using a variety of collection and preservation techniques. Table 1 gives locality and collection

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TABLE 1 Bee samples used in this study.

Taxa	ID code (see Figure 1)	Country	Locality	Genbank Accession #s	
				cox1	*cox2*
Apis laboriosa					
A. laboriosa	DNA-13232 NEPAL	Nepal	Baglung	PP833006	**
A. laboriosa	DNA-13233 NEPAL	Nepal	Kaski	PP833007	**
A. laboriosa	DNA-13235 NEPAL	Nepal	Kaski	PP833008	**
A. laboriosa	GB-AP018039 NEPAL	Nepal	unknown	AP018039	AP018039
Apis dorsata dorsata					
A. d. dorsata	DNA-14b PAKISTAN	Pakistan	Islamabad	PP832985	PP842828
A. d. dorsata	DNA-12956 NEPAL	Nepal	Kanchanpur	PP832988	PP842831
A. d. dorsata	DNA-12958 NEPAL	Nepal	Kanchanpur	PP832989	PP842832
A. d. dorsata	DNA-13129 NEPAL	Nepal	Banke	PP832990	PP842833
A. d. dorsata	DNA-13131 NEPAL	Nepal	Bardiya	PP832991	PP842834
A. d. dorsata	DNA-BPO18 INDIA- Assam	India (northeast)	Assam	PP832987	PP842830
A. d. dorsata	DNA-BPO19 INDIA- Assam	India (northeast)	Assam	PP832986	PP842829
A. d. dorsata	DNA-1a INDIA-Andaman Is.	India	Andaman Is.	PP832992	PP842835
A. d. dorsata	DNA-HP88-N09THAILAND-Chiangmai	Thailand	Chiang Mai	PP832993	PP842836
A. d. dorsata	GB-AP018369 THAILAND-Bangkok	Thailand	Bangkok	AP018369	AP018369
A. d. dorsata	DNA-GWO89-07 MALAYSIA-peninsula	Malaysia	Peninsula	PP832994	PP842837
A. d. dorsata	DNA GWO89-97A MALAYSIA-Borneo	Malaysia	Borneo	PP832995	PP842838
A. d. dorsata	DNA-12906 INDONESIA-Timor	Indonesia	Timor	PP832998	PP842841
A. d. dorsata	DNA-12907 INDONESIA-Timor	Indonesia	Timor	PP832997	PP842840
A. d. dorsata	DNA-12910 INDONESIA- Flores	Indonesia	Flores	PP832999	PP842842
A. d. dorsata	DNA-SR92-007 PHILIPPINES- Palawan	Philippines	Palawan Island	PP832996	PP842839
Apis dorsata (South India)					
A. d. dorsata -Sindia	SI DNA-08b S-INDIA	India (south)	Karnataka	PP832980	PP842823
A. d. dorsata -Sindia	SI DNA-12127 S-INDIA	India (south)	Tamil Nadu	PP832984	PP842827
A. d. dorsata -Sindia	SI DNA-12128 S-INDIA	India (south)	Karnataka	PP832982	PP842825
A. d. dorsata -Sindia	SI DNA-12129 S-INDIA	India (south)	Tamil Nadu	PP832981	PP842824
A. d. dorsata -Sindia	SI DNA-10240 S-INDIA	India (south)	Karnataka	PP832983	PP842826

(Continued)

TABLE 1 Continued

Taxa	ID code (see Figure 1)	Country	Locality	Genbank Accession #s	
				cox1	*cox2*
Apis dorsata breviligula					
A. d. breviligula	DNA-12905 PHILIPPINES-Mindanao	Philippines	Mindanao Island	PP833000	PP842843
A. d. breviligula	DNA-13217 PHILIPPINES- Luzon	Philippines	Luzon Island	PP833001	PP842844
A. d. breviligula	DNA-13222 PHILIPPINES- Luzon	Philippines	Luzon Island	PP833002	PP842845
Apis dorsata binghami					
A. d. binghami	DNA-12912 INDONESIA-Sulawesi	Indonesia	S. Sulawesi	PP833003	PP842846
A. d. binghami	DNA-GWO89-81B INDONESIA-Sulawesi	Indonesia	S. Sulawesi	PP833005	**
A. d. binghami	DNA-GWO89-80A INDONESIA-Sulawesi	Indonesia	S. Sulawesi	PP833004	**
Short *cox1* sequences included in phylogenetic analysis					
A. d. dorsata		India	Mizoram	KU212344.1	**
A. d. dorsata		India	Mizoram	KU212345.1	**
A. d. dorsata		Myanmar		MFBO4562.1	**
A. d. dorsata		Myanmar		MF804563.1	**
Outgroups					
A. mellifera ligustica	ligustica L06178.1	Australia		L06178.1	L06178.1
A. cerana	NC014295 CHINA	China		NC014295	NC014295
A. andreniformis	DNA-4668 THAILAND	Thailand	Surat Thani	PP832976	PP842821
A. andreniformis	DNA GWO89-113 MALAYSIA-Borneo	Malaysia	Borneo	PP832977	PP842822
A. florea- East	DNA-10435 THAILAND	Thailand	Ratchaburi	PP832975	PP842820
A. florea- East	DNA-10434 THAILAND	Thailand	Ratchaburi	PP832974	PP842819
A. florea- East	DNA-7014 CAMBODIA	Cambodia	Kampong Spoe	PP832971	PP842816
A. florea- East	DNA-10246 CAMBODIA	Cambodia	Kampong Spoe	PP832972	PP842817
A. florea- East	DNA-10248 CAMBODIA	Cambodia	Kampong Spoe	PP832973	PP842818
A. florea- West	DNA-9744 SAUDI ARABIA	Saudi Arabia	Hasa	PP832965	PP842810
A. florea- West	DNA-COLONY 1 ISRAEL	Israel	Eilat	PP832966	PP842811
A. florea- West	DNA-10264 INDIA	India (south)	Karnataka	PP832967	PP842812
A. florea- West	DNA-10265 INDIA	India (south)	Karnataka	PP832968	PP842813
A. florea- West	DNA-10222 INDIA	India (south)	Karnataka	PP832970	PP842815
A. florea- West	DNA-10224 INDIA	India (south)	Karnataka	PP832969	PP842814

Taxa and DNA ID code are used in the phylogenetic tree presented in the Figure 1. More detailed collection information is presented in Supplementary File 1. ** indicates no *cox2* sequences obtained in our work, or no *cox2* sequences in data obtained from GenBank.

information, and sample IDs corresponding to those used in Figure 1. Most specimens were collected directly from colonies, though some bees were collected while they were foraging. Most specimens are adult worker bees, while a few are pupae collected directly from nests. Individual bees or bee thoraces were preserved in the field in liquid nitrogen (1988–1990) or in 95% ethanol (1991 onwards). Frozen specimens were later stored at −80°C. Ethanol-preserved specimens were stored at 4° to −20°C.

Laboratory methods

Genomic DNA was extracted from the mitochondrion-rich thoracic flight muscle tissue using DNA spin-columns, primarily the Qiagen DNEasy Blood and Tissue kit (www.Qiagen.com, Ann Arbor, MI USA) and the GenElute Mammalian Genomic DNA Miniprep kit (www.sigmaaldrich.com, St. Louis, MO USA) following the manufacturers' recommendations. Extracted DNAs were stored at −20°C. Portions of the mitochondrial genome were amplified using the primers shown in Table 2. These sequences included a large portion of *cox1*, leucine $tRNA_{UUR}$, a short non-coding sequence, and a portion of *cox2*. Figure 2 shows the relative position of the primers on the honey bee *cox1* to *cox2* sequences. Sanger sequencing was carried out at the Idaho State University Molecular Research Core Facility, Pocatello, ID. As only protein-coding sequence was included in the phylogenetic analysis, the tRNA and non-coding sequences were removed after alignment (see below) and the *cox1* and *cox2* sequences were concatenated. Some of the sequences were also obtained from Genbank (Table 1). The total number of sequences for each taxon and their geographic origins are summarized in Table 3.

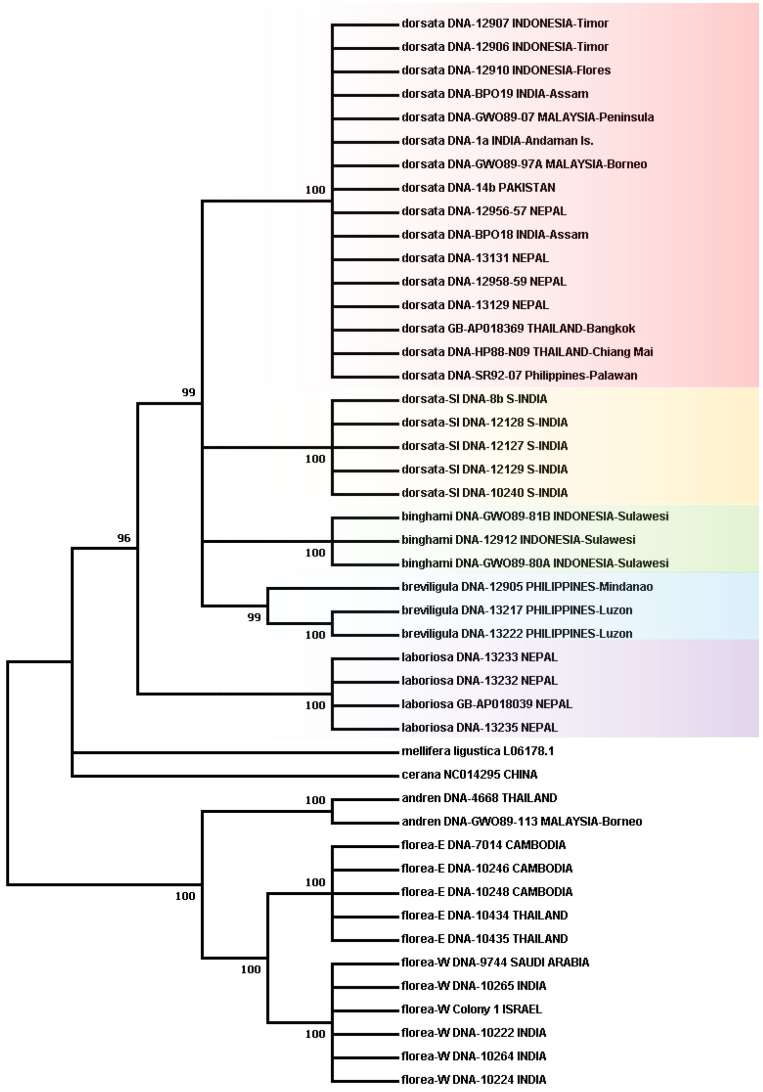

FIGURE 1
Phylogenetic tree obtained using the Maximum Likelihood method based on the General Time Reversible model. Numbers on branches indicate bootstrap support; partitions with less than 95% support were collapsed. See text for more detailed information on analysis methods.

TABLE 2 PCR primer sequences used in this study.

NAMES of primer pairs	GENE	SEQUENCE 5′ to 3′	PRODUCT SIZE	Reference
Apis COI 3090-F Apis COII 3937-R	*cox1* *cox2*	5-TCTATACCACGACGTTATTC-3 5-GATCAATATCATTGATGACC-3	273 bp *cox1*, 324 bp *cox2*, plus tRNA & non-coding sequence	Hall and Smith, 1991
Apis LEU-tRNA 3363-F Apis COII 3937-R	*LEU-tRNA* *cox2*	5-GGCAGAATAAGTGCATTG-3 5-GATCAATATCATTGATGACC-3	tRNA & non-coding sequence plus 324 bp *cox2*	Cornuet et al., 1991 Hall and Smith, 1991
Apis COI 1908-F Apis dorsata COI 3315-R	*cox1*	5-TTAAGATCCCCAGGATCATG-3 5-AATTGGAGATTCAATATGTGAATGTTC-3	1407 bp	Hall and Smith, 1991 Smith, unpublished
Apis COI 1908-F Apis COI 2715-R	*cox1*	5-TTAAGATCCCCAGGATCATG-3 5-CCTCTAGGAACGGCAATAATTATTG-3	807 bp	Hall and Smith, 1991 Smith, unpublished
Apis COI 2693-F Apis dorsata COI 3315-R	*cox1*	5-CGAGCATATTTTACTTCAGC-3 5-AATTGGAGATTCAATATGTGAATGTTC-3	622 bp	Smith, unpublished
Apis COI 2005-F Apis COI 2715-R	*cox1*	5-TTTTTAATTGGAGGATTTGG-3 5-CCTGTAGGAACGGCAATAATTATTG-3	710 bp	Smith, unpublished

"GENE" indicates the gene the primer binds to. Numbers in the primer name refer to the position of the 5' end of the primer on the complete mitochondrial genome of *Apis mellifera ligustica* (Genbank Accession #L06178.1; Crozier and Crozier, 1993). Exact sizes of some products cannot be specified as the primers span the intergenic non-coding sequence (see Figure 2), which varies dramatically in size among *Apis* species and populations (e.g., Cornuet et al., 1991; Hall and Smith, 1991; Smith and Hagen, 1996).

Phylogenetic analysis

Sequences were aligned manually with *cox1* and *cox2* sequences from *Apis mellifera ligustica* (Crozier and Crozier, 1993; Genbank accession L06178) in MEGA7 (Kumar et al., 2016). Sequences were screened for missing bases and correct reading frame by translating DNA sequences to amino acid sequences. In total, 46 sequences were used in the phylogenetic analysis and another four Genbank sequences of *A. dorsata dorsata* from India (Mizoram) and Myanmar (Table 1) that were too short to include in the phylogenetic analysis were aligned with the larger data set to determine which sequences they matched most closely.

The best model of sequence evolution was selected using MEGA "Model Selection" analysis and the following conditions: maximum likelihood statistical methods, partial deletion of sites with missing data, coverage cutoff of 75%, all codon positions used, moderate branch swapping filter. The model of sequence evolution with the lowest Bayesian Information Criteria (BIC) score was selected for use in the phylogenetic analysis. This model (BIC score 13302.38) was a general time reversible model with non-uniform rates of evolution among sites (gamma distributed) and a fraction of sites seemingly invariable (GTR+G+I).

Phylogenetic trees were constructed using Maximum Likelihood methods in MEGA7 with the following settings: model of evolution gamma distributed with invariant sites (GTR+G+I) with 5 gamma categories, partial deletion of sites with missing data, 75% site coverage cutoff, all codon positions used, maximum likelihood heuristic method Subtree-Pruning-Regrafting-Fast, initial tree generated by Neighbor-Joining, moderate branch swap filter, 3 threads. Support for the branching patterns was evaluated with 1000 bootstrap replicates. Branches with less than 95% bootstrap support were collapsed. A coverage cutoff of 75% was chosen during model choice and tree-building to ensure that inclusion of shorter sequences did not result in elimination of informative data.

Results

Figure 1 presents the phylogenetic tree obtained showing partitions with 95% bootstrap support or better. As has been found in other recent studies, *A. laboriosa* constitutes a well-supported lineage separate from and sister to all *A. dorsata* in the broad sense, further supporting its status as a distinct species.

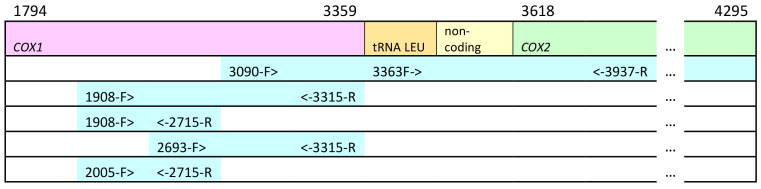

FIGURE 2
Relative position of primers on the mitochondrial *cox1*, *cox2* and leucine tRNA genes. Numbers above *cox1* and *cox2* indicate starting and ending position of the genes in the complete mitochondrial genome of *Apis mellifera ligustica*: numbers in the primer names refer to the position of the 5' end of the primer in the *A. m. ligustica* mitochondrial genome (Genbank Accession #L06178.1; Crozier and Crozier, 1993). Not drawn to scale.

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TABLE 3 Summary of the number of sequences used for each taxon and their geographic origins.

Species	# sequences	Country of origin
Giant bees		
A. laboriosa	4	Nepal
A. d. dorsata	16	Pakistan, India (northeast, Andamans), Nepal, Thailand, Malaysia (peninsula and Borneo), Indonesia, Philippines (Palwan)
A. d. SouthIndia	5	India (south)
A. d. breviligula	3	Philippines
A. d. binghami	3	Indonesia (Sulawesi)
Outgroups		
A. andreniformis	2	Thailand, Malaysia (Borneo)
A. florea- East	5	Cambodia, Thailand
A. florea- West	6	India, Saudi Arabia, Israel
A. mellifera ligustica	1	Australia
A. cerana	1	China

Within *A. dorsata* in the broad sense, we found four distinct lineages: *A. d. breviligula* from the oceanic Philippine islands, *A. d. binghami* from the Indonesian island of Sulawesi, A. d. SouthIndia, a genetically distinct population so far known only from southern India, and a more narrowly defined *A. dorsata dorsata*, represented by our samples from Pakistan, Nepal, northeastern India (Assam and the Andaman Islands), Thailand, Malaysia (Peninsular and Sabah, Borneo), the Philippine island of Palawan, and the Indonesian islands of Timor and Flores. The short sequences from Mizoram, India and Myanmar most closely matched those of the *A. dorsata dorsata* group and were clearly distinct from the A. d. SouthIndia group (Table 4).

Unfortunately, although this analysis shows four well-supported lineages within *A. dorsata* in the broad sense, it does not resolve branching patterns among the four lineages.

Discussion

In this study we support the species status of *A. laboriosa* and show that *Apis dorsata* in the broad sense includes four genetically distinguishable lineages: *A. dorsata dorsata*, *A. d. binghami*, *A. d. breviligula* and A. d. SouthIndia, though our data do not resolve branching patterns among the four lineages. Regardless of whether these four lineages merit species status, recognition and continued investigation of these groups are important for the study of honey bee biogeography, for maintenance of existing diversity within the giant honey bees, and even for conservation of the Asian bee fauna.

Honey bee biogeography

Although color, morphometric and morphological differences among *A. d. dorsata*, *A. d. binghami* and *A. d. breviligula* have been reported (e.g., Maa, 1953, but see Kitnya et al., 2024), there are no obvious morphological differences between A. d. SouthIndia and the widespread *A. d. dorsata* (Kitnya et al., 2024). In their study of *A. laboriosa* and *A. dorsata* in India, Kitnya et al. (2022) examined specimens of *A. dorsata* from Arunachal Pradesh in the extreme northeast of India and Karnataka (specifically Bangalore) in south India. In a dendogram displaying morphometric similarity of the samples, the south India specimens did not form a discrete cluster but were mixed in among the specimens from northeast India. However, in their phylogenetic analysis of the same collections (using a 500 bp fragment of the mitochondrial *cox1* gene), the samples from Arunachal Pradesh and south India formed two separate clades with 99% and 100% bootstrap support, respectively. To the best of our knowledge, there is no information on the geographical distributions of *A. d. dorsata* and A. d. SouthIndia. Although giant honeybees have been collected at points between southern and northern India, at the moment the only way to tell the two apart is by genetic testing.

The distinctive nature of *A. d. breviligula* and *A. d. binghami* compared to the more widespread *A. d. dorsata* has long been recognized (e.g., Maa, 1953; Ruttner, 1988). *A. d. breviligula* and *A. d. binghami* are primarily black, with white stripes on metasomal

TABLE 4 Comparison of sequence similarity between samples of *A. dorsata* from Myanmar and Mizsoram, India (see Table 1) and the giant bee lineages *A. dorsata dorsata*, *A. breviligula* (or *A. d. breviligula*), *A. binghami* (or *A. d. binghami*) and a mitochondrially distinct giant bee found in southern India (A. d. SouthIndia).

	Number of sequences	Myanmar & Mizoram	*dorsata*	SouthIndia	*breviligula*	*binghami*
Myanmar & Mizoram	4		**0.001**	0.012		
dorsata	16	**0.004**		0.012	0.014	0.016
SouthIndia	5	0.052	0.052		0.013	0.015
breviligula	3	0.085	0.085	0.069		0.016
binghami	3	0.079	0.078	0.070	0.084	

All sequences were truncated to match the length of the sorter sequences from the giant bees of Mizoram, India and Myanmar, for a total of 435 positions in the final dataset. The chart shows the number of base substitutions per site from averaging over all sequence pairs between groups (below diagonal, indicated by shaded boxes), and standard error estimates (above the diagonal). The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Codon positions included were 1st+2nd+3rd. All ambiguous positions were removed for each sequence pair. Analyses were conducted using the Tamura-Nei model (Tamura and Nei, 1993) in MEGA7 (Kumar et al., 2016). The samples from Mizoram and Myanmar are most similar to samples of *A. dorsata dorsata* (as indicated by bold-faced values for average number of base substitutions per site and standard error of the estimate) by an order of magnitude and are considered members of that lineage.

tergites 3,4, and 5 formed by short white hairs, while the metasomal terga 1-3 (and sometimes tergites 4-5)and sterna 1-2 are yellow to brown in *A. dorsata dorsata* (Kitnya et al., 2024). In addition to differences in coloration, *A. dorsata dorsata* of mainland Asia differs from the two island taxa based on ocellus size and the spacing of the compound eyes and ocelli (Kitnya et al., 2024). Whether the two island forms constitute separate species remains to be determined. Kitnya et al. (2024) found no morphological basis for separating the two, and considered them a single species, distinct from *A. d. dorsata*, with two subspecies: *A. binghami binghami* and *A. b. breviligula*. Evidence from mitochondrial gene sequences presented here retrieves *A. d. binghami* and *A. d. breviligula* as genetically distinct but is insufficient determine species status.

The fact that isolated island populations show traits distinct from those of mainland populations is not surprising. What is more surprising is the presence of a genetically distinct giant honey bee in southern India, along with the more widespread *A. dorsata dorsata* in northern India. However, a broader view of Indian *Apis* shows that this pattern has appeared more than once. India is also home to two cavity-nesting bees, the yellow or plains bee, and the hill or black bee (Ruttner, 1988 and references cited therein; Bhatta et al., 2020). According to Engel (2002) the yellow or plains bee corresponds to *A. cerana indica* Fabricius, 1798 while the black or hill bee corresponds to *A. cerana cerana* Fabricius, 1793. Genetic evidence collected over the past three decades (e.g., mitochondrial *cox1*, *cox2* and non-coding sequences, Smith, 1991; Smith and Hagen, 1996; mitochondrial and nuclear gene sequences, Lo et al., 2010; and genomic SNPs, Su et al., 2023) support species status of the yellow Indian bee, as proposed by Lo et al., 2010; Smith, 2011, and Su et al., 2023. The dwarf honey bee, *Apis florea*, also consists of two distinct groups revealed by mitochondrial *cox1-cox2* sequences and nuclear SNPs (Smith, 2011; Su et al., 2023, and Figure 1 of this study). These are an eastern lineage including populations from Thailand eastwards, and a western lineage including populations from India westward, including the invasive dwarf honey bee populations in Jordan and Israel, and probably those in east Africa as well. The "switchover" from East to West is apparently in the poorly sampled region from northeastern India through Bangladesh and Myanmar.

Why does India have a distinct variety of cavity-nesting bee, *A. cerana indica*, along with *A. cerana cerana*, and a distinct south Indian variety of giant honey bee along with *A. dorsata dorsata* in northern India? And why does it have a variety of *A. florea* different from that in eastern Asia? Answering these questions requires (1) information on the ranges of the species and putative species of *Apis* in India, particularly the distributions of the yellow and black cavity-nesting bees, and A. d. SouthIndia and *A. dorsata dorsata*, and (2) a phylogeny that resolves the branching patterns of the four lineages within *A. dorsata* in the broad sense. A robust phylogeny would provide information on the order and timing of diversification events. A time calibrated phylogeny could suggest specific geological and climatic events that could have promoted diversification, and help us determine if the dwarf, giant, and cavity nesting lineages responded to historical events with similar patterns of diversification.

Maintenance of diversity in the giant bees

At least three of the four lineages within *A. dorsata* in the broad sense exhibit migratory behavior. The vast majority of giant honey bee migration research has been carried out on populations that our study would place in *A. d. dorsata* (for example, Dyer and Seeley, 1994; Kahono et al., 1999; Neumann et al., 2000; Paar et al., 2000; Itioka et al., 2001; Rattanawannee et al., 2013), which is not surprising, as it is the most widespread. Koeniger and Koeniger (1980) investigated giant honey bee migration in Sri Lanka; though we have not sampled any giant bees from Sri Lanka, we predict that they are part of the A. d. SouthIndia clade, based on the fact that the south Indian plains bee, *A. c. indica*, is also found in Sri Lanka. At least one set of observations has been made on migration by *A. d. binghami* in Sulawesi (Nagir et al., 2016). Morse and Laigo (1969, cited in Robinson, 2021) reported that *A. d. breviligula* in the Philippines does not migrate.

Migration is typically a predictable annual response to seasonal patterns of rainfall and resource availability (e.g., Dyer and Seeley, 1994) or a response to erratically occurring masting events in which forest trees produce a superabundance of blossoms and resources (Itioka et al., 2001). Migrating bees appear to show fidelity to their nesting sites at either end of the migratory route (Neumann et al., 2000; Paar et al., 2000). This alone means that protecting a giant honey bee's nesting and foraging habitat means protecting more than one location. Migrating colonies of giant honey bees may travel distances that require "rest stops" to forage. Recent work by Robinson, (2012, 2021) has shown that migrating *A. d. dorsata* in Thailand make use of "traditional" rest stops, where they forage for food and water for variable lengths of time before continuing their journey. These rest stops are likely to be crucial for successful migration. To maintain the genetic diversity represented by linages within *A. dorsata* in the broad sense, it will be necessary to maintain not only the endpoints of their migratory routes, but quite probably sufficient rest stops along the route too, in conditions that provide the bees with the forage, resting sites and nesting sites they need. This would require tracking migration routes and their timing and noting changes in migration timing or route due to climate change or habitat destruction.

Conservation

Warrit et al. (2024) discuss the challenges facing bee research and bee conservation in Asia, noting, "If we do not know the species present, their distribution and threats, we cannot protect them." They point to the eusocial bees as "flagship species" for bee conservation measures, as their economic value to humans—through pollination services and honey production—is generally known to the public. In particular, "the honey bee" is likely the only bee most people know, especially in urban populations. Although the giant bees are large, conspicuous, and widespread across the Asian continent, we are still discovering new diversity (at the species or subspecies level) and still lack basic information on the ranges of some lineages such as the south Indian giant bee.

Giant honey bees are not just major pollinators in Asian ecosystems. With their large, conspicuous open-air combs, large aggregations of nests, and migratory behavior, plus the well-publicized harvesting of cliff-side *A. laboriosa* nests, they are arguably the most charismatic of the Asian social bees. Public support for protection of giant honey bees would also have the effect of protecting habitat for the many other social and solitary Asian bee species.

Our results suggest avenues for additional research, particularly regarding Indian populations. What is the range of the South Indian giant honey bee, and what are its migration patterns? Is the range of the southern Indian cavity-nesting "plains bee" (currently *A. cerana indica*) congruent with the range of the southern Indian giant honey bee, suggesting similar biogeographic history? Does the south Indian giant honey bee differ in behavior or ecology from *A. d. dorsata*? And of course, will behavioral and genetic study of giant honey bees from a greater portion of their ranges (e.g., as in Cao et al., 2012b) reveal more diversity?

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

Ethical approval was not required for the study as no human subjects, other vertebrates, or higher invertebrates were used. This study used preserved insect specimens collected from 1989 to 2018.

Author contributions

CB: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. SZ: Investigation, Methodology, Writing – review & editing. DS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frbee.2024.1401851/full#supplementary-material

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