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# *Apis laboriosa* confirmed by morphometric and genetic analyses of giant honey bees (Hymenoptera, Apidae) from sites of sympatry in Arunachal Pradesh, North East India

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**Abstract** – Two potentially different species of giant honey bees (subgenus *Megapis*) are endemic to India: *Apis dorsata*, the widespread low elevation form, and *Apis laboriosa* of the Pan-Himalayan region. Although they differ morphologically, behaviorally, and genetically, the species status of *A. laboriosa* remains unresolved. We sampled bees in three widely separated regions of Arunachal Pradesh, North East India, where these two taxa occurred sympatrically. We measured morphological characters of worker bees and conducted principal component analyses of two data sets (size-independent traits and the full set of characters excluding ratios). We also sequenced approximately 500 bp of the mtDNA cytochrome c oxidase subunit I (COI) and generated a phylogenetic tree. In all analyses, *A. dorsata* and *A. laboriosa* separated into well-defined groups, and with no bees showing traits suggestive of hybridization, regardless of where the species were collected. The a priori identifications of all specimens based on thoracic hair and abdominal color were verified by the analyses. Our results confirm that *A. laboriosa* is a valid species.

sympatry / morphometry / COI sequence / phylogenetic / *Megapis* / species

## 1. INTRODUCTION

Among the honey bees, *Apis* Linnaeus, the giant honey bees (subgenus *A. Megapis* Ashmead) are the largest. They build exposed nests, with their single comb attached to a tree branch or overhanging cliff (Ruttner 1988; Gogoi et al.

2017). Four taxa have been described (Maa 1953), hereafter referred to by the species names he used, with the number of species differing from one (Ruttner 1988; Engel 1999) to four (Maa 1953). Two of those taxa inhabit mainland Asia. In general, *A. dorsata* Fabricius, 1793 occurs at elevations less than 1000 m. a.s.l. It is replaced by *A. laboriosa* Smith, 1871 at higher elevations in the Pan-Himalayan region (Sakagami et al. 1980; Roubik et al. 1985; Kitnya et al. 2020). Where the two taxa co-occur, they have not yet been studied in detail.

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*Apis laboriosa* was described by Frederick Smith (Moore et al. 1871) from the mountainous region of southwestern Yunnan, China. It was subsequently overlooked by most mellitologists until Maa (1953) revised the honey bees. He stated that *A. laboriosa* was easily distinguished from the other giant honey bee taxa on the basis of a number of characters. However, because he excessively split taxa (e.g., he recognized 24 species of honey bees), his taxonomy was not accepted by the bee research community. Later Sakagami et al. (1980) compared more than 100 characters on numerous specimens of *A. laboriosa* (predominantly from Nepal plus several from Arunachal Pradesh, India) and of *A. dorsata* (from across its range). They concluded that *A. laboriosa* is a valid species, and that the “differences between the two taxa are as large as, probably larger than, those between the European and the Asian honeybee, *A. mellifera* and *A. cerana*.”

Despite the rigorous study by Sakagami et al. (1980), Ruttner (1988) and Engel (1999), in their reviews of the taxonomy of honey bees, failed to recognize *A. laboriosa* as a species. Rather, they considered it to be a distinct population of *A. dorsata*, with its large size, long hairs, and dark body serving as adaptations for life at high elevations and cool temperatures.

Male reproductive structures of insects are complex and frequently used in insect taxonomy (Eberhard 1985). They have proven to be definitive in confirming several but not all recently recognized species of honey bees (Koeniger and Koeniger 2000 and references therein). McEvoy and Underwood (1988) reported that *A. laboriosa* and *A. dorsata* do not differ in the structure of the male endophallus, implying that cross-mating between the two might be possible. However, Koeniger et al. (1990) pointed out several apparent differences in drawings of the endophalli of these two taxa. Until the everted endophallus of *A. laboriosa* has been examined again, this structure remains unhelpful for assessing the species status of this taxon.

Most subsequent research has focused on mitochondrial and nuclear DNA differences between these taxa. In all of those studies, *A. laboriosa* has emerged as a well-resolved clade

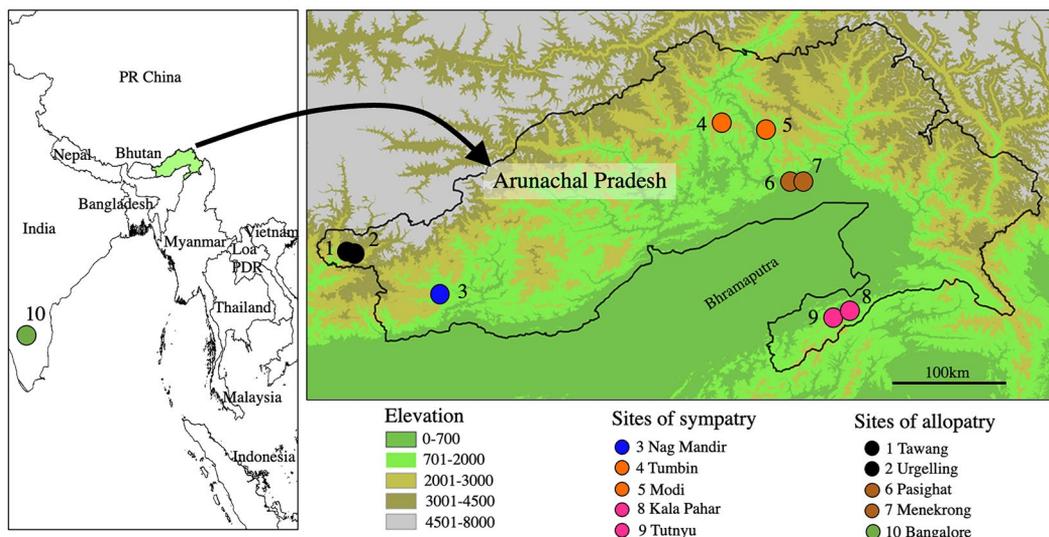
separate from the other giant honey bee taxa (Arias and Sheppard 2005; Raffiudin and Crozier 2007; Lo et al. 2010; Cao et al. 2012b), supporting the inference that it is a distinct species.

Early reports suggested that *A. dorsata* and *A. laboriosa* have non-overlapping distributions, which is the case in Nepal (Sakagami et al. 1980; Roubik et al. 1985). Sakagami et al. (1980), however, reported a site in western Arunachal Pradesh, India, in the Eastern Himalayas, where they were both collected in early May. Subsequently, others have reported temporary geographic overlap of the ranges of *A. laboriosa* and *A. dorsata* in Nepal (during winter; Underwood 1990), northern India (Joshi et al. 2008; and unpubl. data, Joshi et al. 2016), and northern Vietnam (C. H. Phung, unpubl. data). Recently, Kitnya et al. (2020) reported the co-occurrence of two morphologically distinct forms of giant honey bees that they identified as *A. dorsata* and *A. laboriosa*, at five sites in four districts of Arunachal Pradesh. Here we present the results of morphological, morphometric, and genetic (based on COI mtDNA gene) analyses of specimens from those two bee populations. These results show these taxa are distinct in morphology and mitochondrial DNA sequences, in both allopatry and sympatry, strongly supporting *A. laboriosa* as a valid species distinct from *A. dorsata*.

## 2. MATERIALS AND METHODS

### 2.1. Sample sites and collection methodology

Fieldwork was conducted from October 2017 to October 2020 (Table S1). Giant honey bee workers were collected while they foraged on flowers or collected human urine, a behavior we observed frequently and which was briefly mentioned by Butler (1940). In total, we collected bees from five sites of sympatry (e.g., where the two taxa co-occurred) in four different districts of Arunachal Pradesh, North East India (Figure 1). In addition, we collected specimens from Tawang and Urgelling, Tawang district (*A.*



**Figure 1.** Map of sample collection sites. The two taxa occurred sympatrically at sites 3–5, 8, and 9. Only *A. laboriosa* was collected in sites 1 and 2. Only *A. dorsata* was collected in sites 6, 7, and 10. Details of collection sites are presented in Table S1.

*laboriosa* only) and Pasighat and Menekrong (Taki Lalung), East Siang district (*A. dorsata* only). We also included several *A. dorsata* specimens from Bangalore (Peenya, and the campuses of the International Centre for Theoretical Sciences and National Centre for Biological Sciences), Karnataka, India, in our analyses.

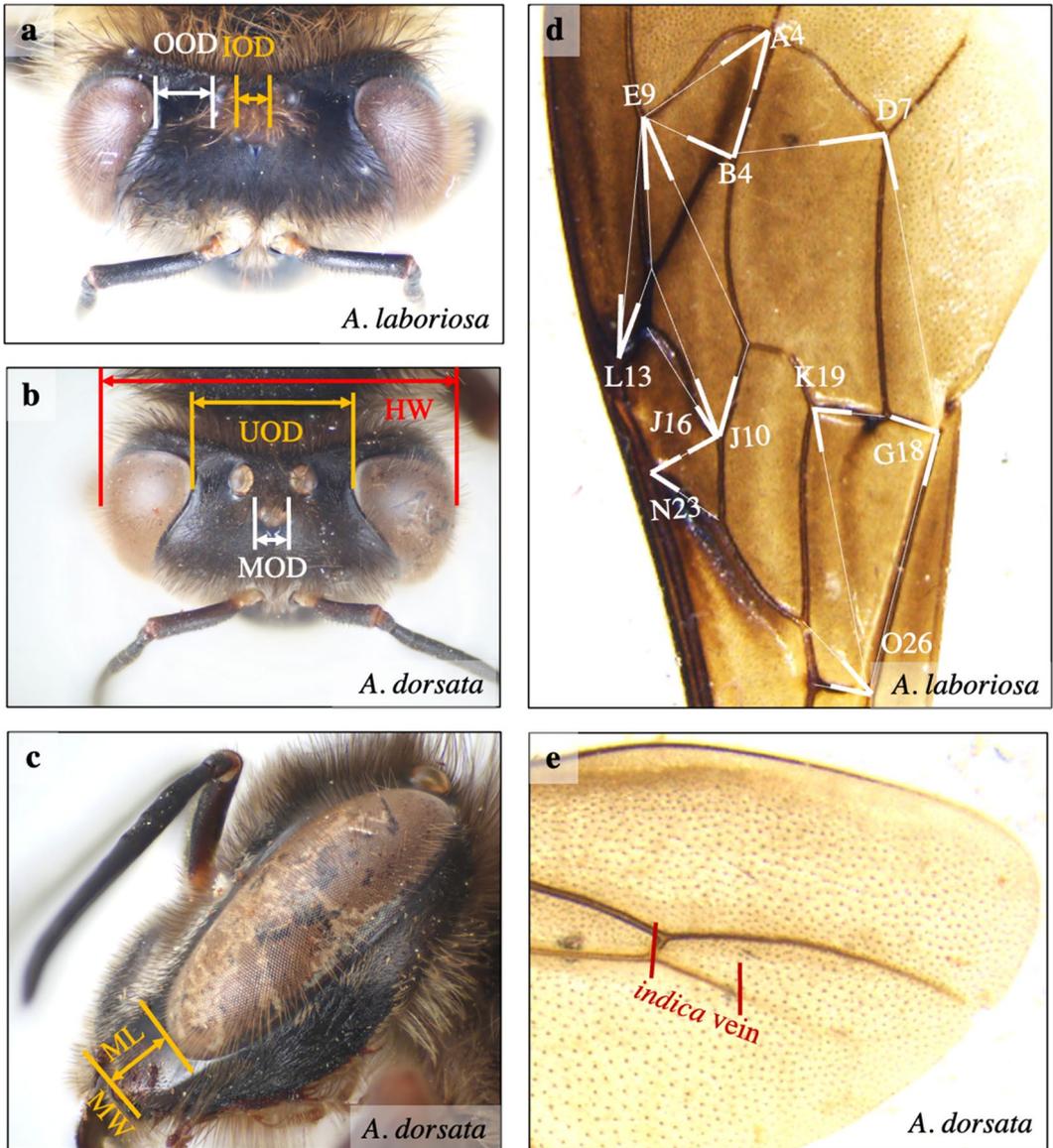
In the field, the bees were sorted based on the color of the metasomal tergites (*A. laboriosa*: all tergites completely black; *A. dorsata*: first 2–3 metasomal tergites yellow-orange) and thoracic hair color (*A. laboriosa*: golden yellow; *A. dorsata*: black), as was done by Sakagami et al. (1980) and Kitnya et al. (2020). The specimens were killed and preserved in 95% ethanol. The bees were frozen (−20° C) once we returned to our laboratory. A total of 121 individuals (*laboriosa*:  $n = 57$ ; *A. dorsata*:  $n = 64$ ) were analyzed (see Table S1 for details of specimens and their collection localities). The same set of specimens was used for both morphometric and genetic analyses. The bees were dissected, with the thorax retained for genetic analysis while the head and wings were kept for morphological study. Prior to morphological examination and

measurements, body parts were transferred from 95 to 70% ethanol. For molecular study, thorax tissue remained frozen in 95% ethanol at −20° C until prepared for analysis. The voucher specimens have been deposited at the collection facility of National Centre for Biological Science-TIFR, Bengaluru, India.

## 2.2. Morphometric measurements and statistical analyses

### 2.2.1. Morphometric measurements

The right forewing and hindwing were placed on a glass slide with glycerin and a second glass slide was placed on top to ensure that the body parts remained parallel to the plane of measurement (Damus and Otis 1996). Any folding or distortion of wings due to the second slide was avoided by gently setting it in place without applying additional pressure. To take photographs of head characters, the head was held and positioned with the help of a pin at specific angles that gave the best view of each character.



**Figure 2.** The 19 morphological characters measured. **a** Ocellular distance (OOD), inter-ocellar distance (IOD). **b** Head width (HW), distance between the inner margins of the compound eyes above (UOD), and median ocellus diameter (MOD). **c** Malar length (ML) and malar width (MW). **d** Forewing venation angles: A4, B4, D7, E9, G18, J10, J16, K19, L13, N23, O26. **e** *indica* vein.

We took morphological measurements of 19 standard characters (Figure 2) commonly used in the characterization of *Apis* and other bees. The forewing characters were described by Ruttner et al. (1978) and Ruttner (1988); the *indica* vein of the hindwing was measured by Sakagami et al.

(1980) and Mattu and Verma (1984); several head characters were adopted from Sakagami et al. (1980). We added two new characters: the distance between the inner margins of the compound eyes (UOD) and head width (HW) in frontal view (see figure 8 of Packer 2021). Images

of the characters were taken using a stereomicroscope (Leica M125 C; Camera: Leica MC 190 HD; Software: LAS V4.12) at the following magnifications:  $0.8\times$  (forewing);  $1.25\times$  (hindwing);  $4\times$  (ocellar distances);  $2\times$  (median ocellus diameter (MOD), head width (HW), and UOD), and  $3.2\times$  (length and width of malar space). Measurements on the images were made using the software package Fiji ImageJ v1.46r (Schindelin et al. 2012).

### 2.2.2. Statistical analyses

Several well-known methods of multivariate analysis were applied (Ruttner 1978; Cao et al 2012a). First we conducted one-way ANOVA to test differences in each trait between the two giant honey bee taxa that had been tentatively identified at the time of collection on the basis of thoracic hair and metasomal color. Next, the raw data obtained from morphological measurements, without a priori assignment to taxon, were subjected to hierarchical cluster analyses (HCA). This method was used to test how the specimens group together based on the data set obtained from the morphological measurements (Figure 2). The HCA was carried out twice: (i) based on data from all 19 traits (including size-dependent traits) and (ii) based on only the 14 size-free characters.

The same morphological data sets (all characters excluding ratios and size-free characters) were subjected to principal component analysis (PCA) to determine the contribution of the different variables toward the resultant clusters. Confidence ellipses (99%) were applied. The PCA is based on a multivariate correlation matrix. All analyses were carried out on individual data points, not on mean values.

Statistical analyses were performed in R version 4.1.2 (<http://www.R-project.org/>), using the RStudio IDE (<http://www.rstudio.com>). We used R base functions: *dist()* for computing Euclidean distances; *hclust()* to generate a hierarchical tree; (<https://www.datacamp.com/tutorial/hierarchical-clustering-R>); *prcomp()* to conduct PCA, *aov()* to conduct the one-way ANOVA, and *pairwise.t.test()* to detect significant differences between character measurements for the

two taxa. The packages ggplot2 (Wickham 2016) and tidy (Wickham 2021) were used to visualize the data and results.

## 2.3. Sample preparation and data analysis for phylogenetic inference

Along with morphometric analyses, we also sequenced a ~600-bp segment of the mitochondrial DNA cytochrome oxidase subunit I.

### 2.3.1. Sample preparation

Genomic DNA was extracted from thorax muscles using the protocol of Qiagen DNeasy Blood and Tissue Kit ([www.qiagen.com](http://www.qiagen.com)), with minor modifications for honey bees as recommended by Evans et al. (2013). Tissue homogenization was carried out by agitating tissue with buffer ATL and beads (carbide beads and glass beads). The polymerase chain reaction (PCR) amplification was conducted with the following primers purchased from Eurofins Genomics India Pvt., Ltd.: forward primer—*Apis* COI 2693-F 5-CGAGCATATTTTACTTCAGC-3 and reverse primer—*Apis dorsata* COI 3315-R 5-AATTGG AGATTCAATATGTGAATGTTTC-3 (designed by S. Cluff and D. R. Smith, unpublished). The amplification was performed in a 25- $\mu$ l reaction mixture containing 10 $\times$  PCR buffer (2.5  $\mu$ l), 50 mM MgCl<sub>2</sub> (2  $\mu$ l), 10 mM forward primer (0.5  $\mu$ l), 10 mM reverse primer (0.5  $\mu$ l), 10 mM dNTPs (0.25  $\mu$ l), Taq polymerase (0.25  $\mu$ l, 1.25 U), sample DNA (2  $\mu$ l), and milli-Q water (17  $\mu$ l) per reaction. The PCR buffer, MgCl<sub>2</sub>, Taq polymerase, and dNTPs were purchased from Invitrogen. The PCR thermal cycle included an initial denaturation at 94° C for 5 min, followed by 30 cycles of denaturation at 94° C for 30 s, annealing at 58° C for 60 s, elongation at 72° C for 60 s, and final elongation at 72° C for 5 min. The PCR product quantifications were carried out on a 1.5% agarose gel (Thermo Fisher) using 1X SB buffer (20X stock solution of 0.2 M NaOH and 0.73 M of H<sub>3</sub>BO<sub>3</sub>, pH 8.0). 3  $\mu$ l of PCR product and 3  $\mu$ l of pre-mixed GelRed (Biotium)

and Orange dye (Biotium) loaded into each well. The PCR amplicons were estimated using a 100-base-pair DNA ladder (Invitrogen). The magnetic bead-based method for PCR product purification was used (<https://www.mybeckman.in/resources/technologies/spri-beads>). The purified PCR products were submitted to the Next Generation Genomics Facility at National Centre for Biological Sciences (NCBS-TIFR), Bangalore, India. Sanger sequencing was carried out using 12 ng of purified PCR product (quantified using Qubit 2.0), setting up a 10- $\mu$ l cycle sequencing reaction. The master mix contained BDT Terminator v3.1 (Applied Biosystems), 5 $\times$  sequencing buffer, and nuclease free water. PCR conditions were 95 °C for 2 min, 35 cycles of 95 °C for 10 s followed by 55 °C for 10 s and 60 °C for 2 min, followed by a holding temperature of 8 °C. Purification of cycle sequencing product was carried out as per the company's protocol. Finally the purified cycle sequencing product was loaded on a 3730 DNA analyzer (Applied Biosystems).

### 2.3.2. Data analysis

Sequence editing was carried out in Geneious R11. The chromatograms of DNA sequences were checked manually to look for presence of superimposed peaks and poor base calls. The poor-quality reads were trimmed from both ends, with the number of bases trimmed varying from specimen to specimen depending on the quality of raw sequences received. The consensus sequences obtained from pairwise alignment of the forward and reverse sequences were translated into amino acid sequences and their open reading frames were checked to eliminate any stop codons from occurring within the sequences. Later, those consensus sequences were subjected to multiple alignment using MAFFT (Katoh et al. 2002). In the analysis, several additional sequences from NCBI were included. The accession numbers of those sequences are as follows: AP018369 (*A. dorsata* from Bangkok, Thailand); NC\_036155 (*A. laboriosa*, Sidhi, Nepal); AP018039 (*A. laboriosa*,

Sidhi, Nepal); KJ601784 (Africanized *A. mellifera*, Querétaro, Mexico); NC\_001566 (Australian *A. mellifera*); KF736157 (*A. andreniformis*, Chiang Rai, Thailand, pers.comm.); NC\_039709 (*A. andreniformis*, Thailand); KC170303 (*A. florea*, Yunnan province, southwest China); and AP018491 (*A. florea*, Chulalongkorn University, Thailand). The aligned sequences were exported as FASTA. After editing, sequences of about 500 bp were used for further analysis.

The aligned sequences were imported into RAxML:2.0 for phylogenetic tree construction. The PartitionFinder V1.1.1 (Lanfear et al. 2012) was used to select the best fitting sequence evolution model for our data set. The TIM1 substitution model was used with rate heterogeneity of Gamma (mean category rates). The tree construction was conducted with 1000 bootstrap replicates to produce a consensus maximum likelihood phylogenetic tree with bootstrap values shown along branches. Fig Tree v1.4.3 (online at <http://tree.bio.ed.ac.uk/software/figtree/>) was used to view and edit the phylogenetic tree. The *p*-distance estimations were carried out in MEGAX (Kumar et al. 2018). The sequences we obtained have been submitted to GenBank under the following accession numbers: *A. dorsata*: OM541597; OM541598; OM648212-OM648217; OM648261-OM648273; OM662251-OM662263; and OM841522-OM841533; and *A. laboriosa*: OM662234-OM662263; OM841715-OM841731; and OM842819-OM842834.

## 3. RESULTS

### 3.1. Morphometric analyses

The means, standard deviations, ranges, and *p*-value of all 19 morphological characters and 3 ratios are presented in Table I. These data were subjected to one-way ANOVA. Figure 3 shows four easily observed head characters with values that did not overlap between *A. laboriosa* and *A. dorsata* of India and differed significantly at  $p < 0.0001$ : malar length, IOD/OOD, MOD, and UOD/HW. Other traits that differed significantly were as follows: ocellocular distance (OOD),

**Table I** Means (mm), standard deviations, ranges, and sample sizes of the 19 characters measured and 3 ratios of pairs of measurements. Sample size, *A. laboriosa* = 75; *A. dorsata* = 64

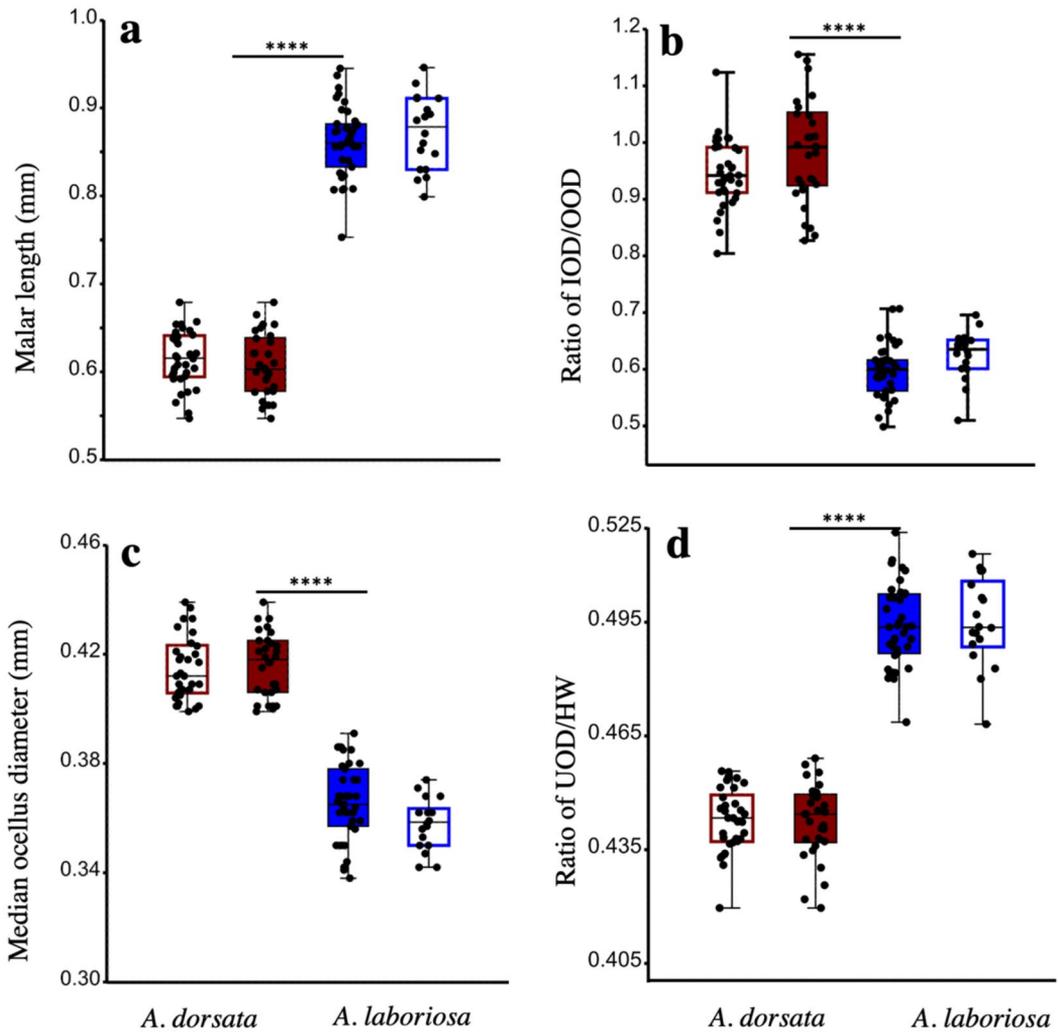
No	Characters	<i>A. dorsata</i>			<i>A. laboriosa</i>			<i>P</i> -value
		Mean	SD	Range	Mean	SD	Range	
1	Head width (HW)	4.36 mm	0.077	4.19–4.52 mm	4.56 mm	0.07	4.34–4.75 mm	0.0001
2	Distance: inner margins of the compound eyes above (UOD)	1.93 mm	0.053	1.78–2.01 mm	2.26 mm	0.067	2.03–2.41 mm	<0.0001
3	Inter-ocellar distance (IOD)	0.43 mm	0.033	0.37–0.51 mm	0.37 mm	0.013	0.29–0.47 mm	0.001
4	Ocellocular distance (OOD)	0.45 mm	0.025	0.38–0.51 mm	0.62 mm	0.041	0.49–0.73 mm	<0.0001
5	Median ocellus diameter (MOD)	0.42 mm	0.011	0.40–0.43 mm	0.36 mm	0.013	0.34–0.39 mm	<0.0001
6	Malar length (ML)	0.61 mm	0.032	0.55–0.68 mm	0.87 mm	0.04	0.75–0.95 mm	<0.0001
7	Malar width (MW)	0.67 mm	0.035	0.59–0.77 mm	0.73 mm	0.03	0.65–0.78 mm	0.001
8	<i>Indica</i> vein	0.55 mm	0.097	0.30–0.75 mm	0.97 mm	0.169	0.59–1.30 mm	0.001
9	Angle A4 (A4)	37.57°	1.79	34.2–41.7°	34.59°	2.12	29.7–38.5°	0.001
10	Angle B4 (B4)	76.93°	3.33	69.9–84.8°	85.73°	3.89	79.9–94.1°	0.001
11	Angle D7 (D7)	90.79°	2.8	84.0–97.2°	93.43°	2.81	87.8–99.9°	<0.05
12	Angle E9 (E9)	18.55°	1.54	10.2–21.0°	19.2°	0.89	17.4–21.2°	0.05
13	Angle G18 (G18)	98.29°	2.82	91.8–108.6°	93.14°	3.63	86.7–100.1°	0.001
14	Angle J10 (J10)	36.8°	1.96	32.6–41.9°	38.45°	1.77	34.6–42.2°	0.01
15	Angle J16 (J16)	91.21°	3.01	81.4–97.0°	101.6°	2.08	96.2–107.1°	0.0001
16	Angle K19 (K19)	67.48°	2.23	61.6–74.6°	69.46°	2.32	64.5–74.4°	0.05
17	Angle L13 (L13)	11.92°	0.917	10.0–14.0°	10.8°	0.88	8.4–12.8°	0.05
18	Angle N23 (N23)	75.59°	3.29	66.9–81.8°	87.06°	3.13	80.0–93.8°	0.0001
19	Angle O26 (O26)	37.13°	3.01	32.3–46.2°	29.92°	2.5	24.5–34.8°	0.0001
20	UOD/HW	0.44	0.008	0.419–0.457	0.50	0.009	0.480–0.518	<0.0001
21	IOD/OOD	0.97	0.079	0.804–1.156	0.61	0.047	0.490–0.713	<0.0001
22	ML/MW	0.91	0.064	0.064–1.041	1.19	0.062	1.050–1.310	<0.0001

UOD, *indica* vein length, and wing venation angles O26; G18; A4; J16; N23; B4 ( $P \leq 0.001$ ), J10 ( $P \leq 0.01$ ), and D7, E9, K19, and L13 ( $P \leq 0.05$ ) (Table I and Figure S1).

The data set of size-independent characters and dimensionless ratios was subjected to hierarchical cluster analysis (HCA) without a priori grouping. The HCA generated two distinct clusters (Figure 4) that correspond exactly to the taxa preliminarily identified as *A. laboriosa* and *A. dorsata* based on thoracic hair and body color. All *A. dorsata*, whether collected from the sites where they are sympatric with *A. laboriosa* or where they are the only giant honey bee species present (e.g., from low elevation sites in both

Arunachal Pradesh and Karnataka), clustered together. The same was true for *A. laboriosa*: all bees clustered together irrespective of sympatry with *A. dorsata*. The HCA of 19 size-based characters and wing vein angles yielded similar results (Figure S2).

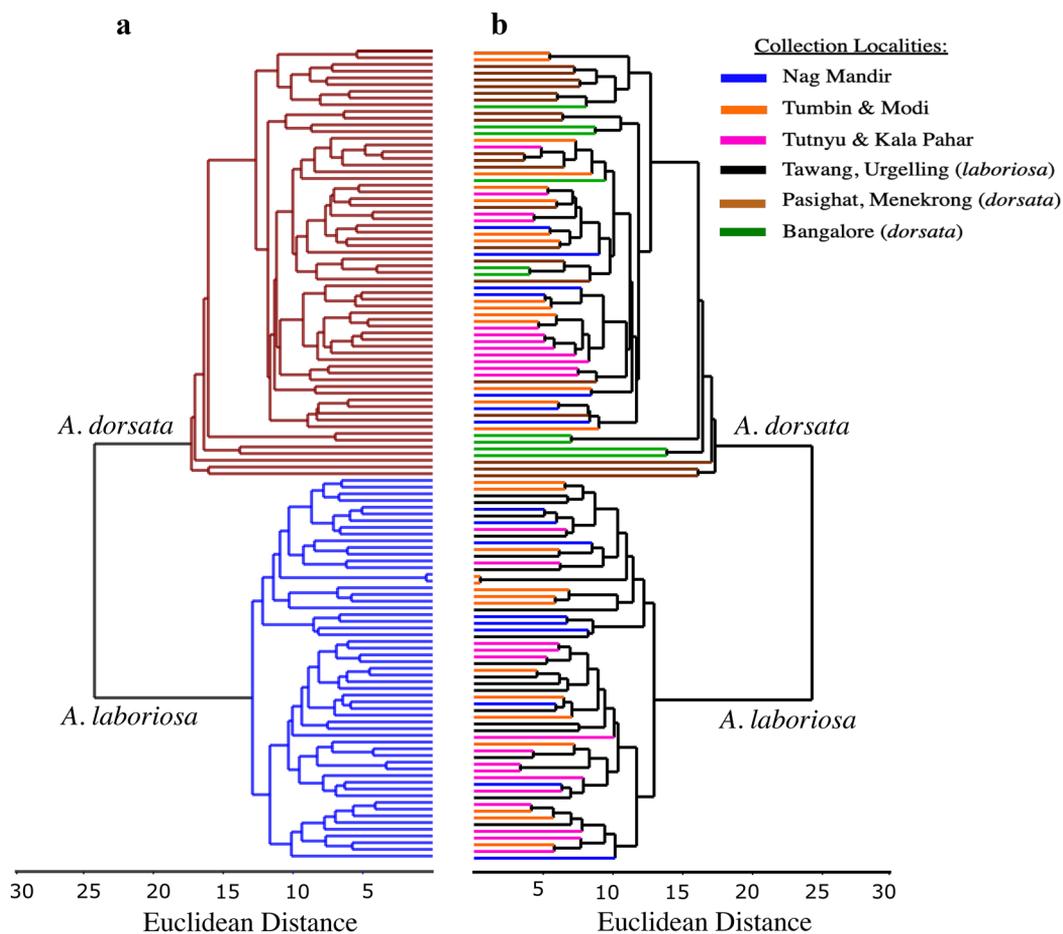
In the principal component analysis of the morphological data set that included only the size-free wing venation angles and ratios between pairs of characters (Figure 5), the first principal component (PC1) accounted for 52.91% of the variance of the total variation with an eigenvalue of 7.39. PC2 and PC3 accounted for 7.72% (eigenvalue of 1.08) and 7.43% (eigenvalue of 1.04) of the total variation, respectively. These



**Figure 3.** Four readily quantifiable head characters of *A. laboriosa* and *A. dorsata* that have non-overlapping distributions and enable identification of these species. **a** Malar length. **b** Ratio of IOD/OOD. **c** Median ocellus diameter. **d** Ratio of UOD/HW. Bees from sites of sympatry are shown with colored boxes; sites of allopatry with white boxes. *P*-value of *t*-test: \*\*\*\*  $\leq 0.0001$ . Refer to Figure 2 for abbreviations.

three principal components together accounted for 68.06% of the total variation. The other PCs had eigenvalues less than 1 and are not discussed further. The loading values of all variables on all principal components are given in Table II. The major variables that separated the two clusters in PC1 (loading value > 0.5) were as follows: (i) IOD/OOD, the ratio of the spacing between lateral ocelli (IOD) to the ocellular distance

(OOD), with negative loading value of  $-0.92$ ; (ii) the proportions of the malar space (ML/MW), with loading value 0.89; (iii) ratio of UOD to HW (loading value 0.86); and (iv) several forewing venation angles, namely B4, J16, and N23 with positive loading values and A4, G18, and O26 with negative loading values (Table S2). The 99% confidence ellipses representing the two taxa are widely separated (Figure 5).

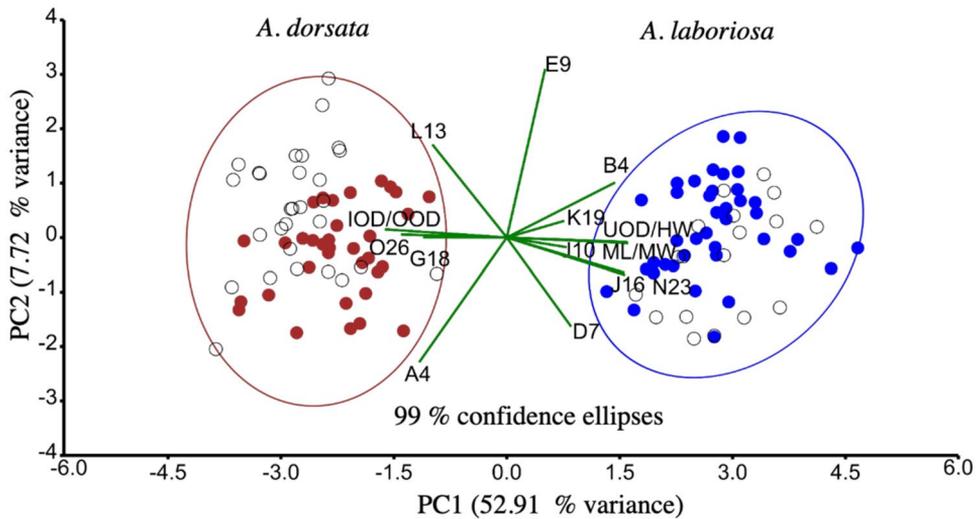


**Figure 4.** Dendrograms derived from hierarchical cluster analysis (HCA) of 14 size-free characters and based on Euclidean distances, generated by R functions *dist()* and *hclust()*. **a** Grouping the samples based on taxa (*A. dorsata* = maroon; *A. laboriosa* = blue). **b** Groupings showing the collection localities in different colors. Sites of sympatry: Nag Mandir (blue), Tumbin and Modi (orange), Tutnyu and Kala Pahar (pink); sites of allopatry: Tawang and Urgelling (black, *A. laboriosa*), Pasighat and Menekrong (brown, *A. dorsata*), and Bangalore (green, *A. dorsata*).

A second PCA conducted on the 19 morphological characters (including size-related traits and excluding ratios of two traits) also revealed two distinct clusters widely separated in the plot of PC1 and PC2 (Figure S3). This PCA shows that MOD, OOD, and wing angles B4 and O26 with negative loading values and IOD, ML, the *indica* vein, and wing angles N23 and J16 with positive loading values (Table S2) contributed strongly (i.e., absolute value of loadings > 0.5) to the formation of two non-overlapping clusters (99% CI) on PC1.

### 3.2. Phylogenetic analysis

COI mtDNA sequences were generated from 99 specimens (*laboriosa*: *n* = 50; *dorsata*: *n* = 49). Sequences obtained from *A. mellifera*, *A. florea*, and *A. andreniformis* served as outgroups to root the phylogeny. The COI amplicon length was about 500 bp. The phylogenetic tree shows *A. laboriosa* and *A. dorsata* separated into two distinct clades (Figure 6a), regardless of whether the bees were collected in sympatry or allopatry with the other taxon (Figure 6b).



**Figure 5.** Scatter plot of PC1 vs. PC2 from the principal component analysis of 14 size-independent characters (11 forewing angles between veins and 3 dimensionless ratios of characters). The 99% confidence ellipses are shown (plotted using ggplot2 package in R). Bees from sites of sympatry are shown with colored circles; from sites of allopatry with open circles. The PCA biplot (green lines) shows the magnitude of the influence of each trait in separating the two clusters. For example, IOD/OOD and ML/MW, with longer lines along the horizontal plane of PC1, were highly responsible for separating the two clusters in PC1. Wing angle E9 had a strong influence on PC2. The abbreviations of the traits are given in Table I. The built-in R function in *prcomp()* was used to conduct the PCA.

Within *A. dorsata*, the southern Indian specimens formed a clade separate from the clade representing the bees collected in North East India and the single specimen from Thailand. In Table II, the *p*-distance between *A. dorsata* and *A. laboriosa* is 0.07 and between *A. dorsata* from North East India and South India is 0.04.

## 4. DISCUSSION

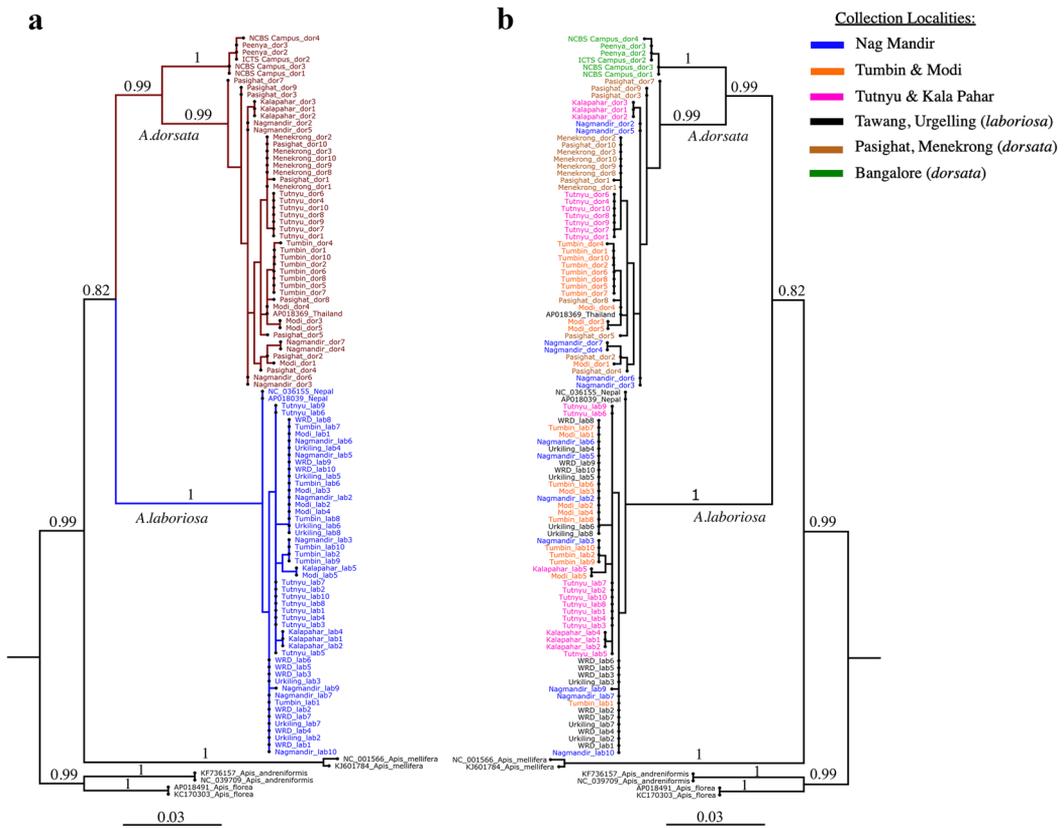
### 4.1. Co-occurrence of two giant honey bee taxa in North East India

Early studies of *Apis laboriosa* suggested it inhabits relatively high elevations of the Himalayas, with no overlap in its distribution with *A. dorsata* (Sakagami et al. 1980; Roubik et al. 1985). To those reports were added others that documented populations of *A. laboriosa* in northern India (Batra 1996) and northern Vietnam (Trung et al. 1996), living at relatively high elevations in allopatry from *A. dorsata*.

Contrasting with those studies were a handful of specimens of *A. laboriosa* and *A. dorsata* reported on by Sakagami et al. (1980) that were collected in the month of May at the “Denling Forest, Kameng Division” (western Arunachal Pradesh) at an elevation of only 229 m. This report spurred more intensive search and our discovery of five sites of sympatry of the two taxa of giant honey bees in three widely dispersed regions (East, Central, and West) of Arunachal Pradesh (Fig. 1; see also Kitnya et al. 2020). We collected the two species together at lower elevations (356 m and 534 m) in the months of March and May. Later, in October, they co-occurred at elevations > 1000 m. More data are needed to understand the elevational changes in their distribution throughout the year, but our observations suggest that both species migrate up river valleys toward Tibet in the summer months, causing their zone of sympatry to shift seasonally. Partial sympatry also has been documented in Uttarakhand, India (Joshi et al. 2008, 2016; unpubl. data) and in northern Vietnam (C. H. Phung,

**Table II** A table of loadings of all the variables for each of the principal components. The loading values that had high influence in separating the clusters (i.e., those with absolute value greater than 0.5) in each PC are highlighted in bold font

Character	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7	PC 8	PC 9	PC 10	PC 11	PC 12	PC 13	PC 14
A4	<b>-0.67</b>	<b>0.50</b>	-0.16	-0.01	0.08	0.37	-0.27	-0.04	0.01	0.11	0.11	-0.17	0.04	0.01
B4	<b>0.83</b>	-0.14	0.17	0.06	-0.21	-0.10	0.31	0.04	-0.17	0.06	0.17	-0.20	0.07	0.03
D7	0.47	<b>0.60</b>	0.13	-0.22	-0.44	0.22	0.28	0.11	0.04	0.04	-0.05	0.10	-0.02	0.00
E9	0.28	-0.14	<b>0.87</b>	-0.17	-0.07	0.07	-0.28	-0.03	0.15	0.02	0.03	-0.02	0.00	0.00
G18	<b>-0.66</b>	0.23	0.28	0.13	0.29	-0.08	0.32	-0.46	0.02	0.12	-0.03	0.01	-0.01	0.00
J10	0.45	0.27	0.28	<b>0.62</b>	0.39	0.05	0.02	0.32	-0.08	0.01	-0.01	0.04	-0.01	0.01
J16	<b>0.90</b>	0.05	-0.18	0.02	0.12	-0.01	0.02	-0.07	0.29	-0.05	0.05	0.01	-0.02	0.22
K19	0.43	-0.03	0.02	<b>-0.65</b>	<b>0.57</b>	0.09	0.11	0.14	-0.13	0.08	0.01	0.02	-0.01	0.01
L13	<b>-0.55</b>	-0.51	0.01	0.14	0.01	0.57	0.27	0.06	0.11	-0.01	0.00	-0.01	-0.04	0.00
N23	<b>0.89</b>	0.09	-0.13	0.02	0.17	-0.02	0.07	-0.04	0.29	-0.09	0.14	0.00	0.04	-0.19
O26	<b>-0.81</b>	-0.05	-0.04	-0.03	-0.04	-0.27	0.08	0.28	0.24	0.35	-0.01	-0.03	0.00	-0.01
IOD/OOD	<b>-0.92</b>	-0.01	0.05	-0.01	-0.05	-0.04	0.00	0.03	-0.06	-0.02	0.31	0.20	0.01	0.04
UOD/HW	<b>0.86</b>	-0.18	-0.11	0.11	-0.04	0.16	-0.11	-0.13	-0.06	0.26	-0.03	0.13	0.22	0.00
ML/MW	<b>0.89</b>	-0.08	-0.13	0.10	-0.07	0.05	-0.13	-0.12	-0.07	0.23	0.07	0.02	-0.27	-0.03



**Figure 6.** Phylogenetic relationships of *A. dorsata* and *A. laboriosa* from sites of sympatry and sites of allopatry with respect to outgroups (*A. mellifera*, *A. florea*, and *A. andreniformis*), built via the maximum likelihood method using the TIM1+G (mean) model. The tree is based on COI sequences of about 500 bp. The numbers on the branches indicate bootstrap support values (1000 bootstrap replicates). **a** Colors represent the two species, *A. dorsata* (maroon) and *A. laboriosa* (blue). **b** Colors indicate the sites where the bees were collected.

unpubl. data), indicating that sympatry between these taxa is not limited to North East India.

### 4.2. Morphological characters and morphometric analysis

Sakagami et al. (1980) referred to *A. laboriosa* as the world’s largest honey bee. It has black metasomal tergites and long golden yellow hairs on the thorax, in contrast to *A. dorsata* that has orange anterior metasomal tergites with black hairs on the thorax (Maa 1953; Sakagami et al. 1980). Ruttner (1988) and Engel (1999)

considered the large size, dark color, and long body hairs of *A. laboriosa* to be adaptations to life at high elevations, as seen in *A. mellifera* of the high mountains of east Africa (Ruttner 1988; Hepburn et al. 2000). In the absence of sympatry between the two taxa having been reported at the time of their reviews, they adopted the conservative view that *A. laboriosa* is a high elevation morph of *A. dorsata*. Animals at high altitudes are known to have darker and larger bodies than conspecifics from low elevations (Bergmann 1847; Trullas et al. 2007). We found that the aforementioned color differences were consistent for all specimens of

the two taxa even when they were collected in sympatry.

In addition to those two obvious characters, many other characters visibly differ between the two taxa. Several authors have pointed out that *A. laboriosa* is generally larger than *A. dorsata* (Maa 1953; Sakagami et al. 1980; Trung et al. 1996; Cao et al. 2012a). For example, Sakagami et al. (1980) found that the forewing length of *A. laboriosa* is on average 11.4% larger than that of *A. dorsata*. We have presented data for four characters that seem to define the two taxa. Specifically, malar length, IOD/OOD, MOD, and UOD/HW separate the two taxa without overlap in the data values (Figure 3). Our results for malar length, IOD/OOD, and MOD (Figure 3a, b, and c, respectively) echo the measurements obtained by Sakagami et al. (1980) for these characters. UOD/HW is new character that also differentiates the two taxa. We also detected differences in forewing venation angles between the two giant honey bees (Figure S1); these results agree with the previous report by Cao et al. (2012a). None of the wing venation angles proved to be adequate characters individually to distinguish the two taxa because of overlap in values, although all of them did differ significantly.

The hierarchical cluster analysis (HCA) based on measurements of 14 size-free morphological characters sorted the bees into two clusters that agreed in all cases with our preliminary field identifications based on thoracic hair and abdominal color, irrespective of geographic location (Figure 4). For example, *A. laboriosa* from Nag Mandir (in blue) did not cluster with *A. dorsata* from that site, but instead clustered together with *A. laboriosa* from Modi (in orange) and Tutnyu (in pink) located ~300 km away. The additional specimens from sites of allopatry (Tawang and Urgelling = only *A. laboriosa*; Pasighat and Menekrong (Arunachal Pradesh) and Bangalore (Karnataka, South India) = only *A. dorsata*) (Figure 1) did not influence the hierarchical clustering pattern. A similar pattern of clusters was generated when HCA was conducted with the full data set of 19 morphological characters (size-related traits and wing vein angles) (Figure S2). There was no evidence of morphological

differentiation between *A. dorsata* from North East India and South India (Figure 4).

The results of the principal component analysis conducted on size-free characters also suggest that *A. laboriosa* and *A. dorsata* are two non-interbreeding populations. As with the HCA, all the *A. laboriosa* clustered together irrespective of their collection localities or consideration of sympatry/allopatry; the same was true for *A. dorsata* (Figure 5). The 99% confidence ellipses for the two populations were widely separated and no bees with intermediate morphology were detected. This demonstrates that *A. laboriosa* is not simply a larger, darker, hairier bee adapted to a cool climate, but has a suite of characters unrelated to size (e.g., forewing vein angles, head proportions, size and position of ocelli) that define it. The PCA based on the data set of 19 size-dependent characters and wing venation angles yielded essentially the same result: two widely separated clusters that correspond to *A. laboriosa* and *A. dorsata* (Figure S3 and Table S2).

Despite its comparatively smaller size, *A. dorsata* has a larger space between the lateral ocelli (IOD; Figure 2). The large, strongly convex ocelli, positioned on a raised platform that is slightly bulged out toward the compound eyes, result in larger IOD in *A. dorsata* than in *A. laboriosa*. Additionally, it appears that the compound eyes of *A. dorsata* extend further toward the middle of the head than is the case for *A. laboriosa*. This arrangement leads to distinctly smaller OOD in *A. dorsata* than in *A. laboriosa*. Also, the shorter malar length and smaller ratio of ML/MW suggests that the compound eyes in *A. dorsata* might be larger than those of *A. laboriosa*. *Apis dorsata* workers are known to forage at low light conditions during twilight and on moonlight nights (Dyer 1985; Young et al. 2021). The exceptional modifications in the size and shape of the ocelli are likely adaptations that enhance detection of dim light required for nocturnal foraging (Kerfoot 1967; Somanathan et al. 2009; Berry et al. 2011). Consequently, the smaller ocelli and likely smaller compound eyes in *A. laboriosa* suggest it forages diurnally as do the dwarf and cavity-nesting honey bee species

(Somanathan et al. 2009; Streinzer et al. 2013). These differences contribute to our conclusion that *A. laboriosa* and *A. dorsata* are taxonomically distinct species.

A more complete morphological analysis of *A. laboriosa* and *A. dorsata* from their entire ranges will determine if these characters remain diagnostic.

### 4.3. Phylogenetic analysis

Phylogenetic relationships among the bees are becoming better resolved due to the advances in DNA sequencing and improved methods for creating phylogenies (Danforth et al. 2013). Our phylogenetic reconstruction resulting from maximum likelihood tree building of COI sequences confirms the three main lineages of *Apis*: the giant honey bees, the dwarf honey bees, and the medium-sized cavity-nesting *Apis mellifera* (Figure 6). These three subgenera—*A. Megapis*, *A. Micrapis*, and *A. Apis*—form separate clades with strong bootstrap values. The separation of these subgenera and their relationships to each other are now well established with high confidence based on morphology (Alexander 1991) and several molecular analyses (Smith 1991, 2021; Arias and Sheppard 2005; Raffiudin and Crozier 2007; Lo et al. 2010; Takahashi et al. 2018).

Additionally, our results show that the clades representing *A. laboriosa* and *A. dorsata* are strongly diverged, with high bootstrapping support, regardless of whether the bees were from sympatric or allopatric populations. The estimated *p*-distance of 0.07 that indicates the

degree of sequence divergence between *A. laboriosa* and *A. dorsata* is greater than mean divergence between *A. florea* and *A. andreniformis* (*p*-distance = 0.04) based on the COI sequences we compared (Table III). Others have reported similar divergence between *A. laboriosa* and *A. dorsata* in molecular phylogenies using mitochondrial genes (Takahashi et al. 2018) and both nuclear and mitochondrial genes (Arias and Sheppard 2005; Raffiudin and Crozier 2007; Lo et al. 2010). These congruent phylogenies based on different segments of DNA strongly support the conclusion that *A. dorsata* and *A. laboriosa* are two distinct taxa.

Wang et al. (2013) constructed a genealogical tree of alleles of the complementary sex determination locus (*csd*) from *A. mellifera*, *A. cerana*, *A. dorsata*, and *A. laboriosa*, which showed that while alleles from *A. mellifera* and *A. cerana* formed two separate branches on the tree, alleles from *A. laboriosa* and *A. dorsata* formed a single branch. This suggests that if *A. dorsata* and *A. laboriosa* are separate species, they are relatively young, such that balancing selection maintains alleles that predate the speciation event (Cho et al. 2006; Wang et al. 2013).

In addition, *A. dorsata* from South India formed a distinct sister clade to that of *A. dorsata* of North East India, with *p*-distance value of 0.04, confirming a pattern previously reported by Smith (2021). The mean divergence between *A. dorsata* from North East India and *A. dorsata* from South India is approximately the same as that between *A. florea* and *A. andreniformis* (4%). The isolation and subsequent divergence of these two Indian populations of *A. dorsata*

**Table III** Estimation of genetic distances (*p*-distance) between populations. The *p*-distances between *A. laboriosa*, and *A. dorsata*, from North East India, and *A. dorsata* from South India are in bold font

	<i>dorsata</i> , North East India and Thailand	<i>dorsata</i> , South India	<i>laboriosa</i>	<i>florea</i>	<i>andreniformis</i>
<i>dorsata</i> , South India	<b>0.04</b>	—			
<i>laboriosa</i>	<b>0.07</b>	<b>0.07</b>	—		
<i>florea</i>	0.08	0.07	0.08	—	
<i>andreniformis</i>	0.08	0.08	0.08	0.04	—
<i>mellifera</i>	0.09	0.10	0.09	0.09	0.10

may have been caused by disruption of gene flow caused by the same unknown geographic circumstances that led to the speciation of the dwarf honey bees *A. florea* and *A. andreniformis* (Smith 2021). Sampling to date has been inadequate to determine where the two mitochondrial types of *A. dorsata* occur, the extent of their sympatry, and their degree of inter-mating. Further analyses will determine if South Indian *A. dorsata* deserves to be considered a distinct species in its own right.

## 5. CONCLUSIONS

Our results strongly suggest that *A. laboriosa* is a species distinct from *A. dorsata*. They have overlapping ranges in at least three different regions in Arunachal Pradesh, India. From morphological comparisons of specimens from these regions of sympatry, bees were easily distinguished on the basis of thoracic hair and abdominal color as Sakagami et al. (1980) reported. Several morphological characters showed no overlap in values, even from regions of sympatry. Our morphometric analyses, especially the principal component analyses, provided no evidence that these two taxa hybridize. Finally, the COI sequences separated *A. laboriosa* and *A. dorsata* into well-differentiated clades. Combined, these consistent differences, particularly from within a broad region of partial sympatry, provide robust evidence that *A. laboriosa* is a valid species distinct from *A. dorsata*. Further research from zones of sympatry will clarify differences in foraging preferences as well as the nature of seasonal migrations. Comparative study of drone mating flight times within zones of sympatry and re-examination of male endophalli may elucidate mechanisms of reproductive isolation of *A. laboriosa* and *A. dorsata* (Koeniger and Koeniger 2000).

## SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at <https://doi.org/10.1007/s13592-022-00956-z>.

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## AUTHOR CONTRIBUTION

AB, JC, and GWO conceived and guided the study. AB and JC obtained funding. AB provided access to facilities at NCBS-TIFR. NK conducted the field work, measurements of bees, statistical analyses, and molecular work. DRS provided guidance for the molecular work and interpretation of phylogenetic reconstruction. NK and GWO drafted the manuscript. All the authors edited and have approved of the final draft.

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## DATA AVAILABILITY

The data sets generated or analyzed during the current study are available in the supplementary files (Supplementary file 2: raw data). The nucleotide sequence generated in this study have been deposited to GenBank ([www.ncbi.nlm.nih.gov/genbank](http://www.ncbi.nlm.nih.gov/genbank)) with assigned the accession numbers: *A. dorsata*: OM541597; OM541598; OM648212-OM648217; OM648261-OM648273; OM662251-OM662263; and OM841522-OM841533; and *A. laboriosa*: OM662234-OM662263; OM841715-OM841731; and OM842819-OM842834.

## CODE AVAILABILITY

Not applicable.

## DECLARATIONS

**Ethics approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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