JHR 96: 507–541 (2023)
doi: 10.3897/jhr.96.104715
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

A taxonomic re-assessment of the widespread oriental bumblebee *Bombus flavescens* (Hymenoptera, Apidae)

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Academic editor: Michael Ohl | Received 11 April 2023 | Accepted 8 June 2023 | Published 22 June 2023

https://zoobank.org/2AC09EB7-1B86-4818-A803-8B1170FE8373

Citation: Thanoosing C, Orr MC, Warrit N, Vogler AP, Williams PH (2023) A taxonomic re-assessment of the widespread oriental bumblebee *Bombus flavescens* (Hymenoptera, Apidae). Journal of Hymenoptera Research 96: 507–541. https://doi.org/10.3897/jhr.96.104715

Abstract

Bombus flavescens Smith is one of the most widespread bumblebee species in the Oriental region. Due to colour polymorphisms, this species or species-complex has been a challenge for taxonomy. This study aims to assess the taxonomic status of the *flavescens*-complex using evidence from COI barcodes and morphology. We then reconstruct its biogeographic history from a phylogenetic analysis of populations across the current range, combining COI with 16S and nuclear PEPCK data. Despite a large range of polymorphisms across its distribution, the results show that *B. flavescens* is a single species based on algorithmic species delimitation methods, and it is clearly separated from its sister species, *B. rotundiceps* Friese. We suggest that *B. flavescens* diverged from its sister lineage in the Himalaya and dispersed into Southeast Asia in the Pleistocene. Conservation of the widespread *B. flavescens* will need to consider its several unique island populations.

Keywords

COI, Museum specimens, Polymorphism, *Pyrobombus*

Introduction

Bumblebees (genus *Bombus* Latreille) are well-studied pollinators, especially in temperate regions (Williams et al. 2014; Rasmont et al. 2021). They can also be found in both subtropical and tropical areas in Central and South America, and Asia (Williams 1998), although taxonomic knowledge gaps and the relative rarity of bumblebees in these areas still constrain our understanding of their biogeography and ecology.

The taxonomic studies of bumblebees in Asia, based on morphological evidence only, have been considered highly problematic, due to colour pattern polymorphism within the same species (Huang et al. 2015b; Ding et al. 2019) and cryptic species (Williams et al. 2020, 2022a). Moreover, the same colour pattern is also observed from different species which are co-existed in same locality (Hines and Williams 2012). Therefore, these confusions of taxonomic delimitation of Asian bumblebees require molecular evidence (Williams et al. 2012). Taxonomic status of bumblebees has been revised and various new species have been recently described in Asia, based both morphology and molecular evidence (Williams et al. 2020, 2022a, c; Williams 2022).

Bombus flavescens Smith is a Southern Asian species of the subgenus *Pyrobombus* Dalla Torre (Williams 1998). While the type locality of *B. flavescens* is Zhoushan, China (Smith 1852), this taxon is found in many other countries in the Himalaya and Southeast Asia with tropical low montane habitats, including Nepal, India, Bhutan, Myanmar, Thailand, Laos, Vietnam, Malaysia, and the Philippines (Starr 1992; Williams 1998, 2022; Williams et al. 2009; Koch and General 2019).

Most frequently, *B. flavescens* is a predominantly black pubescence (or hair) bumblebee with an orange tail (the area of hairs covering posterior part of metasoma) and legs in workers and queens. Males often (e.g., in parts of China and in the Himalaya) show a predominantly pale yellow (flavescent) hair pattern with orange legs (Fig. 1), although they sometimes exhibit the widespread dark pattern of the females. However, the colour pattern of hair varies across its wide area of distribution (Fig. 2), which has caused taxonomic problems for more than a century and led to the formal description of local variants (Frison 1934). For example, the name *B. alienus* Smith in China (Fig. 2E; morphologically synonymised by Williams (2022)) was applied to a specimen with an anterior yellow band on its metasoma (terga 1–2 or T1–2) whereas specimens of from the Philippines (Fig. 2G), with yellow hair covering the side of the mesosoma and metasoma (T1–2) but without the orange tail, have been described as *B. baguionensis* Cockerell (morphologically synonymised by Williams (1998)). In addition, a form with an entirely body, covered with uniformly orange hairs, has been described as *B. rufoflavus* Pendlebury (Fig. 2J; provisionally synonymised by Williams (1998), based on morphology). Further names were published for colour patterns resembling the typical *B. flavescens*: *B. mearnsi* Ashmead from Mindanao in the Philippines (morphologically synonymised by Pittioni (1949)); *B. bakeri* Cockerell from Negros (Fig. 2H; morphologically synonymised by Frison (1925)); and *B. tahanensis* Pendlebury from the Tahan mountains of Malaysia (Fig. 2I; morphologically synonymised by Frison (1934)). Apart from this high level of colour variation, other morphological characters, including shape of labrum, punctures on clypeus, and male genitalia, are relatively similar

Figure 1. Holotype male of *B. flavescens*, deposited in the entomological type collection at the Natural History Museum, London (NHMUK 014025381). Scale bar: 5 mm.

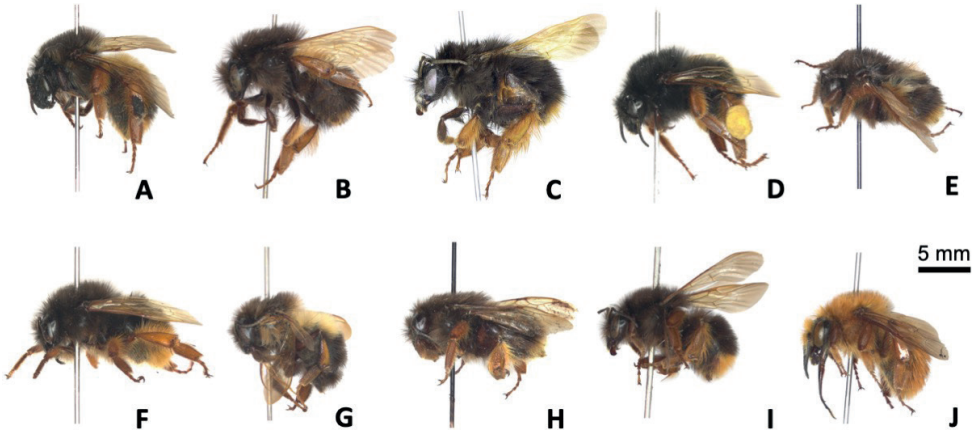

Figure 2. Colour variation of *B. flavescens* workers with their unique specimen identifiers **A** India (FLA#5) **B** Nepal (NHMUK 010814958) **C** Thailand (CT#1024) **D** Kunming, China (FLA#6) **E** *alienus*; Fujian, China (NHMUK 010815023) **F** Taiwan (FLA#4) **G** *baguionensis*; Luzon, the Philippines (CT#932) **H** *bakeri*; Negros, the Philippines (ZMA.INS.758600) **I** *tahanensis*; Gunung Tahan, Malaysia (NHMUK 010820651) **J** *rufoflavus*; Cameron highlands, Malaysia (NHMUK 010814574). Some images are reversed. Scale bar: 5 mm.

(Williams 1998; Thanoosing 2022). Yet, at various times through the 20th century different intraspecific names have been applied to describe minor *B. flavescens* variants (Frison 1925, 1928, 1934; Bischoff 1936; Chiu 1948; Pittioni 1949; Table 1) although they are clearly grouped together because of their morphological similarity (Williams 1998).

Table 1. List of *Bombus flavescens* names.

Author	Name
Smith (1852)	*Bombus flavescens*
Smith (1854)	*Bombus alienus*
Ashmead (1905)	*Bombus mearnsi*
Friese (1905)	*Bombus rufocaudatus*
Cockerell (1917)	*Bombus geei*
Cockerell (1920)	*Bombus irisanensis* var. *baguionensis*, *Bombus bakeri*
Pendlebury (1923)	*Bombus tahanensis*, *Bombus rufoflavus*
Frison (1925)	*Bremus mearnsi*, *Bremus mearnsi* var. *bakeri*, *Bremus irisanensis* var. *baguionensis*
Hedicke (1926)	*Bombus imuganensis*
Frison (1928)	*Bremus* (*Pratobombus*) *baguionensis*, *Bremus* (*Pratobombus*) *baguionensis* var. *imuganensis*
Dover (1929)	*Bremus rufoflavus*
Skorikov (1933)	*Pratobombus flavescens*
Frison (1934)	*Bremus mearnsi* var. *deflectus*, *Bremus mearnsi* var. *ditutus*, *Bremus mearnsi* var. *bakeri*, *Bremus mearnsi* var. *geei*
Bischoff (1936)	*Bombus* (*Pratobombus*) *mearnsi* ssp. *chekiangensis*
Chiu (1948)	*Bombus mearnsi* var. *deflectus*, *Bombus mearnsi* var. *ditutus*, *Bombus mearnsi* var. *bakeri*, *Bombus mearnsi* var. *geei*, *Bombus mearnsi* var. *subrufus*, *Bombus mearnsi* var. *luteus*
Pittioni (1949)	*Bombus flavescens* f. *dilutior*

Colour-pattern variation of bumblebees can be controlled by differential gene expression of, e.g., the *Abd-B* and *nubbin* genes affecting pigment generation during pupal development (Rahman et al. 2021). However, the evolutionary forces determining variation in colour pattern among *B. flavescens* populations remain unclear. Selection for mimicry might take a major role in driving evolutionary differences because the colour pattern of *B. flavescens* locally matches other bumblebee species in the same areas (Williams 2007). For example, on Luzon lsland, *baguionensis* shows a similar pattern to *B.* (*Megabombus*) *irisanensis* Cockerell, and in the Malay Peninsula, *rufoflavus* resembles *B.* (*Megabombus*) *montivagus* Smith with a similar predominantly orange pattern of both pubescence and underlying sclerites, which had been named *B. maxwelli* Pendlebury (Hines and Williams 2012).

Orange body colour (pubescence and sclerites) and enlarged ocelli in Hymenoptera are often associated with a nocturnal or crepuscular lifestyle, for example, *Megalopta* bees and *Apoica* wasps in Central and South America (Roubik 1989; Williams 2007; Warrant 2008). Interestingly, male and female nocturnal carpenter bees, *Xylocopa* (*Nyctomelitta*) *myops* Ritsema, show a pattern closely similar to the orange pattern bumblebees. This carpenter bee is found in Malaysia, Singapore, and Borneo, based on specimens in the Natural History Museum, London (NHMUK) collection and records by Ascher et al. (2022). Similarly, the two orange pattern bumblebees in Malaysia, *rufoflavus* (*B. flavescens*) and *maxwelli* (*B. montivagus*), might be active nocturnally also, at least in part (Williams 2007). Species of the subgenus *Nyctomelitta* Cockerell display relatively large ocelli compared to day-flying carpenter bees (Cockerell 1929; Michener 2007). However, the ocelli of the orange pattern bumblebees remain to be assessed in this regard. Observation of a night-flying bumblebee had been claimed in Myanmar (Doria 1886), but this record is a misidentification of the carpenter

bee, *X.* (*Nyctomelitta*) *tranquebarica* (Fabricius) (Cameron 1910). Therefore, genuine evidence of nocturnal bumblebees has yet to be recorded in Southeast Asia.

The colour-pattern diversity of *B. flavescens* must be seen in light of the complex biogeography of its distribution range across the Southeast Asian mainland and the Philippines. The most recent common ancestor (MRCA) of the *flavescens* species complex has been placed into the Miocene epoch around eight million years ago (Ma), presumably on the mainland (Hines 2008) with two possible dispersal routes to the Philippines (Starr 1989): 1) through the Sundaland route, via Borneo–Sulawesi from the South; or 2) across the Luzon strait via Taiwan from the North. When global temperatures increased after the last glacial maximum (LGM), bumblebees likely became restricted to highlands (e.g., the Cameron Highlands, Malaysia) and diverged in allopatry. At the same time, as the sea level rose and submerged land bridges across Sundaland (Voris 2000; Sathiamurthy and Voris 2006; Woodruff 2010), populations of bumblebees on the Philippines were even further isolated.

This study seeks to clarify genetic patterns within the *flavescens*-complex, to establish the species status of geographically separated lineages and to infer biogeographic scenarios for bumblebees in Southeast Asia more generally. Species-level entities in bumblebees have been delimited by morphological, molecular (e.g., Williams et al. (2019, 2020)), and chemical approaches, the latter using Cephalic Labial Gland Secretions or CLGS (e.g., Brasero et al. (2021), Ghisbain et al. (2021)). Given the challenges of morphology for taxonomic assessment of the *flavescens*-complex, molecular data are required to resolve the species status of sub-lineages and their relationships. In addition, this study aims to clarify the question about the nocturnal lifestyle in *B. flavescens*, using morphological examination of their ocelli.

Methods

Sampling

Museum and institutional specimens of the *flavescens*-complex (Table 1) were examined for morphological analysis. Collecting information was recorded and made available at the NHMUK Data Portal (https://doi.org/10.5519/qd7f4uuw and https://doi.org/10.5519/isxh6saw). Additional records were obtained from reliable sources, including: 1) published literature (Williams et al. 2009, 2010); 2) major natural history collections, including the Naturalis Biodiversity Center (NMNL; Bakker F, Creuwels J), the Smithsonian Institution (USNM; Orrell T, Informatics Office), the University of Illinois at Urbana-Champaign (INHS; McElrath T), and the Taiwan Forestry Research Institute (TFRI; Lu S), available on the Global Biodiversity Information Facility (GBIF) (GBIF.org 2021). Records with unclear locality information and clear geographic outliers were ignored. For example, there is a record of *B. flavescens* from Japan (e.g., GBIF occurrence 3801818589; McElrath 2022), far outside the established range of *B. flavescens*, based on the specimen records by Williams (1998, 2022).

COI barcode data of *B. flavescens* and relatives were obtained from public databases (Suppl. material 1) and newly sequenced from pinned or fresh specimens (Table 2).

Table 2. List of specimens included, species name, ID, deposit place (KKIC = Kasetsart Kamphaeng Sean Insect Collection, PHW = Paul H. Williams Research Collection, CUNHM = Chulalongkorn University Natural History Museum Collection, NMNL = Naturalis Biodiversity Center Collection), sex/caste (w = worker, m = male), locality, collecting date, and COI GenBank accession number.

Species	Project ID	Collection (specimen ID)	Sex/caste	Locality	Collecting date	GenBank accession
B. flavescens	CT#662	KKIC	w	Thailand, Nakorn Pathom?	7/5/2017	OP355718
	CT#669	KKIC	m	Thailand, Loei	13/4/2016	OP355719
	CT#926	PHW	w	Malaysia, Gunung Tahan	NA	OP355720
	CT#1018	NMNL (ZMA.INS.758598)	w	Philippines, Negros	4/5/1953	OP355722
	CT#1023	CUNHM (BSRU-AB-9609)	w	Thailand, Chiang Mai	15/2/2021	OP355723
	CT#1024	CUNHM (BSRU-AB-9610)	w	Thailand, Chiang Mai	15/2/2021	OP355724
	FLA#2	PHW	w	Philippines, Luzon	27/4/1986	OP355725
	FLA#3	PHW	w	Taiwan, Tai Chung	5/5/1980	OP355726
	FLA#4	PHW	w	Taiwan, Nantou	24/6/1989	OP355727
	FLA#5	PHW	w	India, Uttar Pradesh	6/5/1990	OP355728
	FLA#6	PHW	w	China, Kunming	8/4/2018	OP355729
	FLA#7	NMNL (ZMA.INS.773029)	w	Bhutan, Lungtenphu	10/6/1996	OP355730
	FLA#10	PHW	w	Philippines, Luzon	27/4/1986	OP355731
B. rotundiceps	CT#960	CUNHM (BSRU-AB-1268)	m	Thailand, Chiang Mai	17/6/2019	OP355721
	ROT#1	PHW	w	India, Uttar Pradash	6/5/1990	OP355732
	ROT#2	PHW	w	India, Uttar Pradash	6/5/1990	OP355733
	ROT#3	PHW	w	China, Guangxi	3/6/2016	OP355734
	ROT#4	PHW	w	China, Guangxi	5/6/2016	OP355735

The samples were selected to represent the geographical distribution and all major colour pattern variants of *B. flavescens* (Fig. 3). *Bombus flavescens* belongs to the *pratorum*-group (Williams 1998). Thus, closely related species in the *pratorum*-group (Cameron et al. 2007; Williams et al. 2009, 2010), were included in the dataset: *B. ardens* Smith, *B. pratorum* (Linnaeus), *B. pyrenaeus* Pérez, *B. modestus* Eversmann, *B. nursei* Friese, *B. biroi* Vogt, *B. wangae* Williams et al., and *B. rotundiceps* Friese. The status of *B. nursei* as a distinct species was suggested in Williams (2022). Thirty COI sequences (> 600 bp) of *B. flavescens* and the relatives were downloaded from the Barcode of Life Data System (BOLD; Ratnasingham and Hebert 2007) and the National Center for Biotechnology Information (NCBI) database (GenBank; Sayers et al. 2022) (Suppl. material 1). Nine *B. flavescens* sequences from China were provided by co-author MO, sequenced from freshly collected specimens. Thirteen further *B. flavescens* and five *B. rotundiceps* specimens from museum and institution collections were selected for sequencing (Table 2). The identifier numbers were applied to the studied specimens: 1) "CT#*n*" for Southeast Asian bumblebee specimens 2) "FLA#*n*" and "ROT#*n*" refer to *B. flavescens* and *B. rotundiceps* specimens from outside Southeast Asia, respectively.

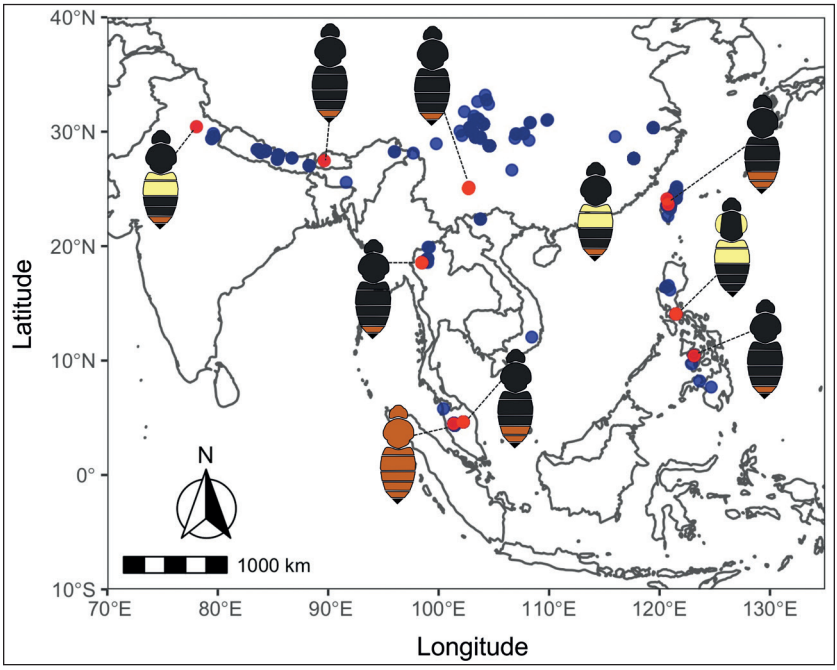

Figure 3. Map showing the distribution of *B. flavescens* with the pattern of hair colour in the dorsal view. The blue dots represent the records from museum collections, literature, and GBIF database (n = 702). The red dots represent the localities of barcoded specimens.

DNA extraction

Pinned and fresh specimens of *B. flavescens* and *B. rotundiceps* were selected for DNA extraction. For the recent specimens (< 20 years old), the tissue sources were a right front leg or a whole body for high DNA concentration yield. Each leg sample was ground using a small pestle in a microcentrifuge tube, whereas the whole-body sample was directly put in a microcentrifuge tube without specimen damages. The tissue samples were incubated at 56 °C for 24 hours in the ATL buffer with Proteinase K enzyme. Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit, following the kit protocol. DNA quality and quantity were assessed using the Nanodrop spectrophotometer (Thermo Scientific ND8000) and the Agilent 2200 TapeStation system (Agilent Technologies, Inc.).

For older specimens (> = 20 years old), genomic DNA was likely to be degraded and all DNA extraction steps were performed inside a laminar flow chamber using dedicated UV sterilised equipment and consumables different from those used with modern bumblebee DNA. The DNA extraction method followed Sproul and Maddison (2017) using the Qiagen QIAamp Micro Kit, but without the use of carrier RNA. For each specimen, the right front leg was cleaned with sterile water, frozen with liquid nitrogen, and then ground with a small pestle. The samples were incubated in ATL buffer with Proteinase K at 55 °C for 24 hours. A blank sample (only the ATL buffer

with the enzyme) was included for each extraction batch as a contamination control. Samples were eluted from the column twice with 15 µl AE buffer and incubated at room temperature for 10 minutes. The DNA concentration was measured using the Qubit fluorometer high sensitivity (Invitrogen) and samples were stored at -20 °C.

Primer design, amplification, and sequencing

For the recent specimens, PCR was performed to amplify the full length of COI amplicons (658 bp) using primers LepF1 and LepR1 (Hebert et al. 2004) and an annealing temperature at 50 °C. For older specimens, due to the DNA degradation, three pairs of primers were newly designed for amplifying partial COI contigs (150–300 bp), using Primer3 software (Untergasser et al. 2012). The COI sequence of *B. flavescens* (GenBank accession number: GU085209) was used as a reference sequence for the first two pairs of primers. The third pair was designed based on the partial mitogenome of *B. pratorum* (GenBank accession number: KT164684). There are three pairs of new primers for partial COI amplification (contig) in this study (Table 3): 1) FLA1: ParCOI_246_FLA_F1 (Tm° = 66.2 °C) and ParCOI_416_FLA_R1 (Tm° = 63.7 °C), 2) FLA2: ParCOI_349_FLA_F2 (Tm° = 64.2 °C) and ParCOI_662_FLA_R2 (Tm° = 66.6 °C), and 3) PRA1: ParCOI_1816_PRA_F1 (Tm° = 61.7 °C) and ParCOI_1994_PRA_R1 (Tm° = 65.5 °C). These primers were tested for amplification efficiency (Suppl. materials 2–4). The annealing temperature for the pair FLA1 and FLA2 was set at 65 °C and PRA1 was set at 63 °C.

The PCR products were sequenced in both forward and reverse directions using ABI technology at the NHMUK's sequencing facility. The sequences were edited using MEGA version 7.0.26 (Kumar et al. 2016: https://www.megasoftware.net/), to trim low-quality bases near the ends of the traces. The COI barcodes have been deposited in GenBank (Table 2).

Phylogenetic analysis and species delimitation

The aligned dataset was analysed to determine the best-fitting nucleotide substitution model using jModelTest software version 2.1.6 (Darriba et al. 2012). Phylogenetic trees were constructed using Bayesian Inference (BI) with MrBayes version 3.2.2 (Ron-

Table 3. Newly designed primers were used in this study, including gene, primer name, strand (F = forward, R = reverse) and primer sequence.

Gene	Primer name	Strand	Primer sequence
Partial COI	ParCOI_246_FLA_F1	F	5'– CCTGACATAGCTTTCCCACGA –3'
	ParCOI_416_FLA_R1	R	5'– TGCAATATCAACTGAAGGTGATG –3'
	ParCOI_349_FLA_F2	F	5'– CAGGATGAACTGTTTACCCTCCT –3'
	ParCOI_662_FLA_R2	R	5'– TGGATCACCTCCTCCTATTGGA –3'
	ParCOI_1816_PRA_F1	F	5'– TTCGTATAGAATTAAGTCATCCTGGT –3'
	ParCOI_1994_PRA_R1	R	5'– TCGTGGAAAAGCTATATCAGGTGAT –3'

quist and Huelsenback 2003). Markov Chain Monte Carlo (MCMC) chains were run for 10 million generations and sampled every 1000th generation. The first 10% was discarded as burn-in. The phylogenetic trees were visualised using FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Bumblebee species from closely related subgenera (Cameron et al. 2007) were used as outgroups, including *B.* (*Bombus*) *terrestris* (Linnaeus) and *B.* (*Alpinobombus*) *polaris* Curtis. The outgroup sequences were generated by Thanoosing (2017).

Poisson Tree Process (PTP) analysis is used to identify likely species coalescents. The test establishes the transition from long branches defining between-species diversification to short branches within species (estimated from the number of substitutions on branches) (Zhang et al. 2013; Kapli et al. 2017). The proportion of between- and within-species sampling was in a range where the PTP analysis performs well (Luo et al. 2018). The PTP analysis was run on the bPTP server (https://species.h-its.org; accessed 2021) under default parameters. The analysis was restricted to unique haplotype sequences in order to avoid introducing misleading 'zero-length' branches. The input phylogenetic tree was estimated using MrBayes rooted with *Bombus terrestris*. Results from the PTP analysis were compared to the Generalised Mixed Yule Coalescent (GMYC), which uses the branching rate rather than the number of nucleotide changes to establish the species-to-population transition (Pons et al. 2006). The required ultrametric tree was obtained with BEAST (Bouckaert et al. 2019) using the same dataset as for the PTP analysis. The clock rate was set at 0.0177 using the divergence rate from tenebrionid beetle COI (Papadopoulou et al. 2010). The GMYC analysis was run in the splits package of R (Ezard et al. 2009; https://r-forge.r-project.org/projects/splits/).

Biogeographic analysis

To reconstruct phylogenetic trees for biogeographic scenarios of *B. flavescens*, more genetic markers were added to the dataset, including mitochondrial 16S rDNA (16S) and nuclear phosphoenolpyruvate carboxykinase (PEPCK). Due to a lack of fresh specimens, most of 16S and PEPCK sequences for relatives of *B. flavescens* were retrieved from GenBank, especially from the dataset compiled by Cameron et al. (2007) (Suppl. material 5). 16S and PEPCK were not available from existing sources for three species; *B. rotundiceps*, *B. wangae*, and *B. nursei*. *Bombus nursei* is rare in inaccessible areas of the western Himalaya (Williams, 1991, 2022), and *B. wangae* also is a rare bumblebee from Sichuan (Williams et al. 2009). For *B. rotundiceps* and additional *B. flavescens*), the 16S and PEPCK were amplified using primers 16SWb (Dowton and Austin 1994) /874–16SIR (Cameron et al. 1992), and FHv4/RHv4 (Cameron et al. 2007) and sequenced in both directions (Table 4).

An ultrametric tree was constructed based on the combined dataset of COI (unique haplotypes), 16S and PEPCK (Suppl. material 6), using *BEAST (Ogilvie et al. 2017). If not available from the same individual, sequences in these three datasets were concatenated for different representative from the same area of distribution. For PEPCK, the sequence was partitioned for introns and exons. The closely related

Table 4. List of 16S and PEPCK sequences, included species, ID, and the 16S and PEPCK GenBank accession numbers, generated in this study.

Taxa	Project ID	16S	PEPCK
B. flavescens	CT#926	OP354521	-
	CT#1023	OP354523	OP382631
	FLA#6	OP354524	OP382632
B. rotundiceps	CT#960	OP354522	OP382630

B. (*Pyrobombus*) *lepidus* Skorikov, based on Cameron et al.'s (2007) tree, was used as an outgroup. The nucleotide substitution model for each fragment was selected according to the BIC criterion. The BEAUti software (Bouckaert et al. 2019) was used to generate an input file for BEAST. Trees were estimated under a strict clock and a Calibrated Yule Model as a species tree prior with the birth rate prior drawn from a Gamma distribution. Due to a lack of fossil evidence for the subgenus *Pyrobombus*, the clock was calibrated from molecular rate estimates based on five genes by Hines (2008), placing the origin to approximately 8.5 +/- 1 Ma. Accordingly, the prior distribution was 10.1–6.86 Ma (5% tails). The MCMC algorithm was run for 500 million generations and sampled every 5000th generation. The trace file was visualilsed using Tracer (Rambaut et al. 2018). A 20% burn-in was discarded using TreeAnnotator (Bouckaert et al. 2019) to construct the maximum clade credibility tree.

S-DIVA analysis (Yu et al. 2010) and BioGEOBEARS with DIVALIKE+J model (Matzke 2013a, 2013b, 2014) in the RASP package (Yu et al. 2020) were used to estimate biogeographic scenarios for the *flavescens*-complex and their relatives. The biogeographical distributions were divided into 11 principal areas, based on bumblebee global biogeography (Williams 1996), areas of endemism in Southeast Asia and the distributions of *flavescens*-complex and their relatives (Table 5): 1) Europe (A), 2) Central Asia (B), 3) North and Central China and North Asia (C), 4) Korean peninsula and Japan (D), 5) South China (E), 6) Himalaya (F), 7) Thailand (G), 8) Peninsular Malaysia (H), 9) Taiwan (I), 10) Luzon (J), and 11) Negros (K). These areas were chosen so that the maximum range size for any species was limited to three of these areas, due to acknowledged problems with the methods in inheriting broader distributions (Lamm and Redelings 2009). Then, the dispersal-corridor model was created with possible bumblebee permitted dispersal routes (Fig. 4). According to this model, the possible dispersal areas in this study are AB, ABC, ABF, AC, ACD, ACE, BEF, BF, BFG, CD, CDE, CE, CEF, CEG, CEI, EF, EFG, EFI, EG, EGH, EGI, EI, EIJ, FG, FGH, GH, GHK, HJK, HK, IJ, IJK, and JK. The burnin was set at 20%. Then, the *BEAST trees were used as the input trees.

Morphological study of ocelli in the *flavescens*-complex

The idea of the nocturnal lifestyle in the orange pattern bumblebees, *rufoflavus* (*B. flavescens*) and *maxwelli* (*B. montivagus*) from Peninsular Malaysia, has been introduced by

Table 5. Principal areas of endemic distribution of the *flavescens*-complex and their relatives in this study.

Area	Principal ranges included	Species recorded
A	Europe	*B. pratorum*, *B. pyrenaeus*
B	Central Asia	*B. biroi*
C	North China, and North Asia	*B. modestus*
D	Korea peninsula and Japan	*B. ardens*, *B. modestus*
E	South China	*B. flavescens*, *B. lepidus*, *B. rotundiceps*, *B. wangae*
F	Himalaya	*B. flavescens*, *B. lepidus*, *B. nursei*, *B. rotundiceps*
G	Thailand	*B. flavescens*, *B. rotundiceps*
H	Peninsular Malaysia	*rufoflavus* (*B. flavescens*), *tahanensis* (*B. flavescens*)
I	Taiwan	*B. flavescens*
J	Luzon	*baguionensis* (*B. flavescens*)
K	Negros	*bakeri* (*B. flavescens*)

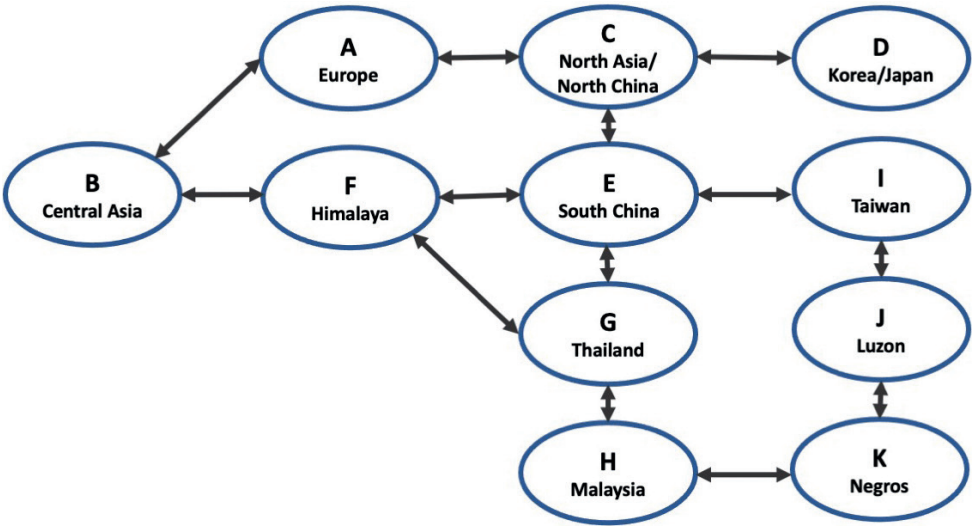

Figure 4. A corridor-dispersal model diagram of the *flavescens*-complex and their relatives in this study.

Williams (2007). Although orange hair pattern can be recognised in diurnal bumblebees (e.g., *B. humillis* Illiger, *B. pascuorum* (Scopoli), and *B. muscorum* (Linnaeus)), *rufoflavus* and *maxwelli* show the uniformly orange pattern both of pubescence and sclerites uniquely. This orange pattern can be recognised in the female specimens of nocturnal carpenter bees, *X. myops*, mentioned in Williams (2007), which can be also found in Peninsular Malaysia. For this reason, we selected the female specimens of *X. myops* and their related species, *X. tranquebarica* from the NHMUK collection, included in this study to test the idea.

Due to the sexual dimorphism bias and the availability of male specimens, only female or worker specimens were chosen in this study. Female *flavescens*-complex specimens (n = 107) together with its relative, *B. rotundiceps* (n = 12), outgroups including the diurnal *B. irisanensis* (n = 4), the orange pattern *B. montivagus* (taxon *maxwelli*) (n = 3), and the nocturnal carpenter bees, *X. myops* (n = 20) and *X. tranquebarica* (n = 12), were selected for

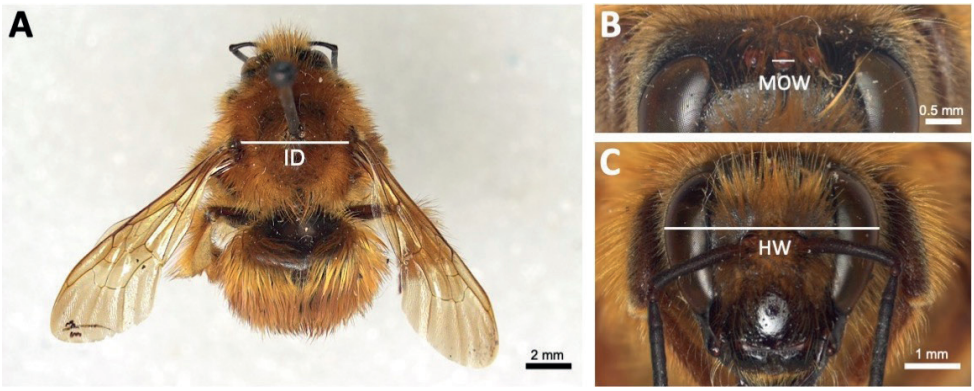

Figure 5. The measurement of morphological characters **A** dorsal aspect: intertegular distance (ID) **B** frontal aspect: median ocellus width (MOW) **C** frontal aspect: head width (HW). The specimen shown is *B. flavescens*, NHMUK 010814574, from the Cameron Highlands, Malaysia.

morphological character measurement, including median ocellus width (MOW), intertegular distance (ID), and head width (HW) (Fig. 5), under a Wild Heerbrugg M4A stereomicroscope, calibrated with ocular and stage micrometres. The specimens of the *flavescens*-complex were divided into eight groups: 1) Thailand (n = 4), 2) *baguionensis* (n = 15), 3) Himalaya (n = 16), 4) China (n = 20), 5) Taiwan (n = 6), 6) *bakeri* (n = 6), 7) *rufoflavus* (n = 20), and 8) *tahanensis* (n = 20). The data are available in the Suppl. material 8.

The morphological values, including MOW, the ratio between MOW and ID (MOW:ID), and the ratio between MOW and HW (MOW:HW), were visualized in box plots using R package ggplot2 (Wichham 2016). To test for differences between species, and within the *flavescens*-complex, statistical tests were performed in R (R core team 2021). First, a Shapiro-Wilk normality test was used to examine the distribution of data of each of the *flavescens*-complex taxa and each bee species. Where the data showed a normal distribution, an ANOVA test and Tukey test were then performed. A Kruskal-Wallis test and Dunn's test were used instead if the data were not normally distributed. The Dunn's test was conducted using the R package *FSA* (Ogle et al. 2022).

Results

DNA extraction, amplification, and sequencing

Genomic DNA was extracted successfully for most specimens of the *flavescens*-complex and *B. rotundiceps*. Most of the old samples were degraded and had relatively low DNA concentrations (< 10 ng/µl). The three amplicons obtained with new primer pairs PRA1, FLA1, and FLA2 were assembled into a single contig. However, there was a 25-base pairs gap between the first (PRA1) and second (FLA1) fragments, and the 5' part of the COI barcode region (88 base pairs) was not amplified at all. COI sequences were generated for 18 collected specimens with top BLAST hits to the subgenus *Pyrobombus*.

Phylogenetic analysis and species delimitation

The final dataset of the *flavescens*-complex and relatives comprised 57 COI sequences, including 25 unique haplotypes. The resulting 575 bp alignment was used to construct the BI phylogenic tree under the GTR+Γ model selected by jModelTest (BIC = 4596) (Fig. 6). *Bombus rotundiceps*, sampled from China, Nepal, India, and Thailand, was the reciprocally monophyletic sister group of the *flavescens*-complex. The latter included four regional clades: 1) Thailand and Himalaya, 2) China, 3) Malaysia, and 4) the Philippines and Taiwan.

The unique haplotype COI dataset was used to estimate the input tree for the species-delimitation analysis. The nucleotide substitution model of this dataset was GTR+Γ (BIC = 3877). Species delimitation using PTP returned each of the nine species as a separate entity, with Bayesian support values between 0.14–1.00 (Fig. 6). In contrast, the GMYC lumped the *flavescens*-complex and *B. rotundiceps*, as well as the *B. modestus* and *B. wangae* species pair (Fig. 6). All analyses recovered the *flavescens*-group, including *B. flavescens s. str.*, *B. baguionensis*, *B. bakeri*, *B. rufoflavus*, and *B. tahanensis*, as a single species.

Biogeographic analysis

The phylogenetic analysis was based on three loci (see supplement for missing data in 16S and PEPCK; Suppl. material 6) and 26 terminals, including the outgroup. Tree searches were conducted under partitioning and separate model choice for COI (575 bp), 16S (551 bp), PEPCK introns (483 bp), and PEPCK exons (369 bp). The resulting maximum clade credibility tree showed a deep separation of the *B. flavescens*/*B. rotundiceps* clade from their closest relatives, *B. pratorum* and *B. ardens*, estimated at 6 +/- 2 Ma (Fig. 7). Within the *flavescens*-complex the early split in was occupied by the Malaysian group. The extant members of the *flavescens*-complex diversified during a time interval approximately 1–2 Ma. However, there is some uncertainty about relationships among the four regional groups, as in the COI tree the Thai and Himalayan groups were sister to the China, Malaysia, the Philippines and Taiwan groups.

Although DEC model was the best fit model for the BioGEOBEARS analysis in this study (the highest AICc wt; Suppl. material 7), we selected DIVALIKE+J model instead (the second high AICc wt; Suppl. material 7). The DIVALIKE+J model supports widespread founder-event speciation at nodes (Yu et al. 2020) which fits the distribution changes of bumblebees (Williams et al. 2020, 2022b).

Results from S-DIVA and DIVALIKE+J showed that there was uncertainty in ancestral areas of *B. flavescens* and relatives, with more than one possible ancestral area suggested in both scenarios (Figs 8, 9). There were only four nodes (III, XI, XIV, and XVI; Fig. 8) for which we identified a single ancestral area in S-DIVA, whereas the DIVALIKE+J result (Fig. 9), only node XI showed a single ancestral area. The results suggested that the ancestral area for the MRCA of the *pratorum*-group was likely to be in area covered by Europe, Central Asia, and the Himalaya (ABF) in S-DIVA, and Central Asia (B) in DIVALIKE+J with low probability. This group diversified in the late Miocene.

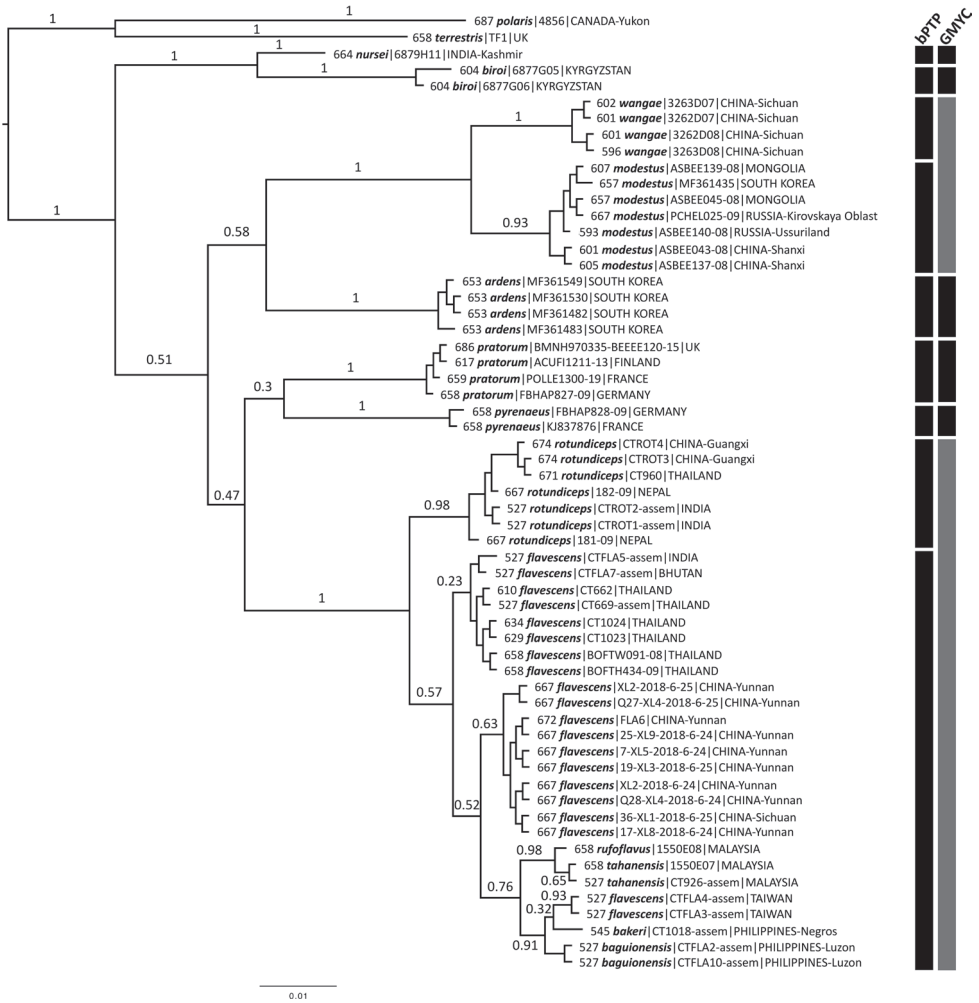

Figure 6. Bayesian inference (BI) phylogenetic tree based on a 575 bp fragment of COI in MrBayes. MCMC chains were run for 10 million generations, sampled every 1000th generation. Burn-in fraction is 10%. The posterior probabilities are shown above the branches. The tip of the tree shows the sample label including the sequence length, a taxon name, an identifier code from the database, and its geographic origin. The results of species delimitation methods are shown in the right-hand side columns. The black and grey bars within the same columns indicate the same species.

For the *flavescens*-complex, the S-DIVA biogeographic scenario illustrated that the MRCA of *B. rotundiceps* and *B. flavescens* (Node IX; Fig. 8) might have occurred in the Himalaya (F) around 1.5 Ma during the Pleistocene epoch. Next, the MRCA of *B. flavescens* in the S-DIVA analysis was inferred to be in the FGH areas, including Himalaya, Thailand, and Peninsular Malaysia (Node X; Fig. 8) at around 1 Ma. However, the DIVALIKE+J biogeographic scenario suggested that the group might

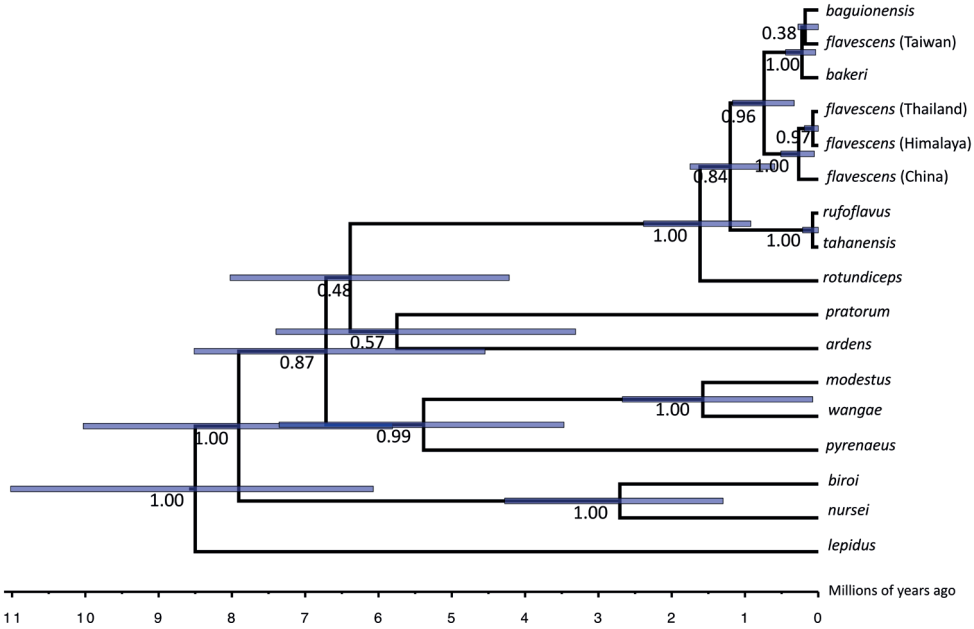

Figure 7. The maximum clade credibility tree of *B. flavescens* and their closely related species, reconstructed with *BEAST from gene trees for the COI, 16S, and PEPCK genes and calibrated using estimate time from Hines (2008). The MCMC chains were run for 500 million generations, sampled every 5000th generation. Burn-in fraction is 20%. The posterior probabilities are shown under the nodes. Blue bars represent the 95% confidence limits on the estimate dates of divergence. *Bombus lepidus* is the outgroup.

have originated in Malaysia (H), but with low probability. The DIVALIKE+J showed higher support for a Peninsular Malaysian origin (Node X; Fig. 9).

The diversification of the *B. flavescens* populations began after 1 Ma, resulting in three distinct clades: 1) *rufoflavus+tahanensis* from Malaysia (Node XI, Figs 8, 9); 2) *flavescens s. str.* from Thailand, Himalaya and China (Node XIII, Figs 8, 9); 3) *baguionensis+bakeri+flavescens s. str.* from Taiwan (Node XV, Figs 8, 9). According to our corridor-dispersal model diagram (Fig. 4), the S-DIVA showed that the *B. flavescens* used all possible corridors between E–K, except the corridor H–K, the Sundaland route, whereas the DIVALIKE+J included the corridor H–K as a permitted route.

Morphological study of *Bombus flavescens* ocelli

The carpenter bees, *Xylocopa tranquebarica* and *X. myops*, had a significantly larger median ocellus than the bumblebees (Fig. 10). *Bombus irisanensis* clearly showed the smallest median ocellus and *B. flavescens*, *B. rotundiceps*, and *B. montivagus* exhibited similar median ocellus sizes (Fig. 10).

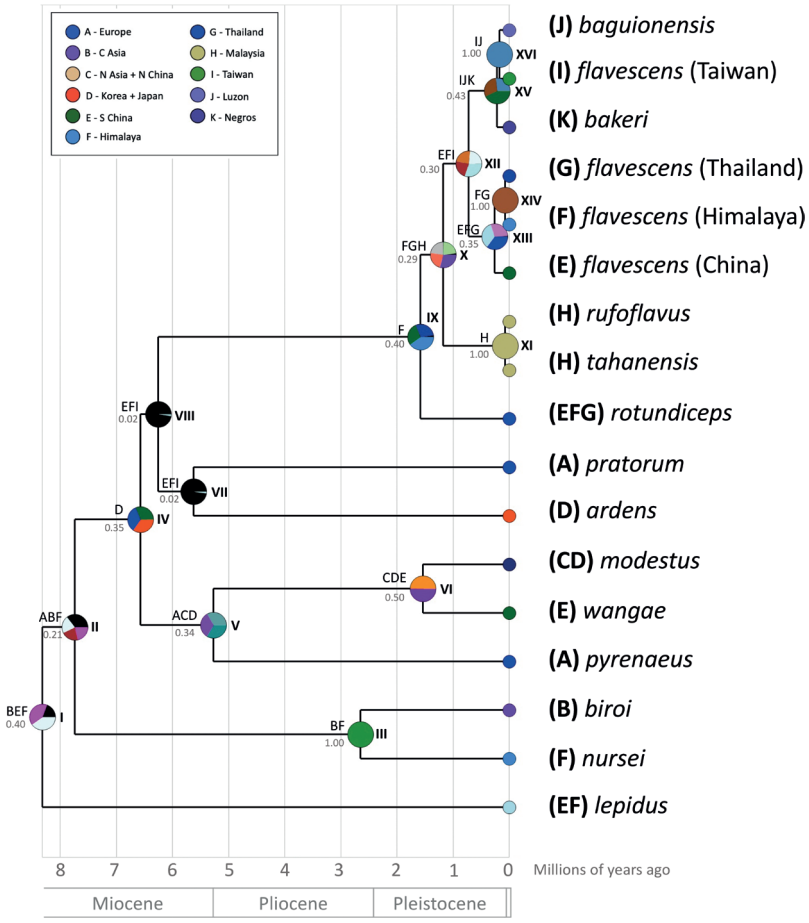

Figure 8. Biogeographic scenarios for the *flavescens*-complex and its close relatives by S-DIVA analysis. The input tree is reconstructed with *BEAST from gene trees for the COI, 16S, and PEPCK genes and calibrated using estimate time from Hines (2008). The internal nodes are represented in Roman numerals. The colours of the nodes represent the possible ancestral areas. The area codes used are given in Table 5. The most likely ancestral areas are represented above the nodes with the probabilities below.

For the *flavescens*-complex (Fig. 11), the Shapiro-Wilk normality test suggested that the MOW was not normally distributed (W = 0.93, p-value < 0.05), whereas the MOW: ID and MOW: HW did not differ significantly from a normal distribution (W = 0.99, p-value = 0.28 and W = 0.99, p-value = 0.45 respectively). There was a significant difference within the MOW of the *flavescens*-complex (Kruskal-Wallis test, chi-squared = 36.217, p-value < 0.05; Dunn's test, p-value < 0.05), including the taxa *baguionensis-rufoflavus*, *baguionensis-tahanensis*, Himalaya-*rufoflavus*, and Himalaya-*tahanensis*. For the MOW: ID, nine pairs were significantly different (ANOVA, F-value = 6.781, p-value < 0.05; Tukey test, p-value < 0.05): *bakeri*-China, *rufoflavus*-China, *tahanensis*-China, *bakeri*-Himalaya , *rufoflavus*-Himalaya, *tahanensis*-Himalaya, Taiwan-*bakeri*, Taiwan-*rufoflavus*, and Taiwan-*tahanensis*. In addition, only two pairs

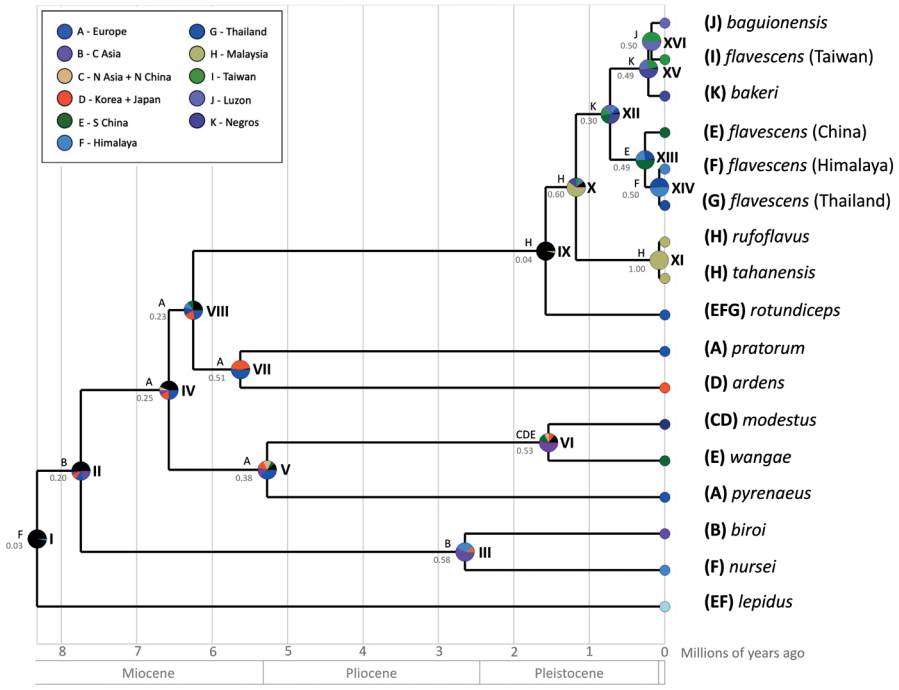

Figure 9. Biogeographic scenarios for the *flavescens*-complex and its close relatives by BioGeoBEARS with DIVALIKE+J analysis. The input tree is reconstructed with *BEAST from gene trees for the COI, 16S, and PEPCK genes and calibrated using estimate time from Hines (2008). The internal nodes are represented in Roman numerals. The colours of the nodes represent the possible ancestral areas. The area codes used are given in Table 5. The most likely ancestral areas are represented above the nodes with the probabilities below.

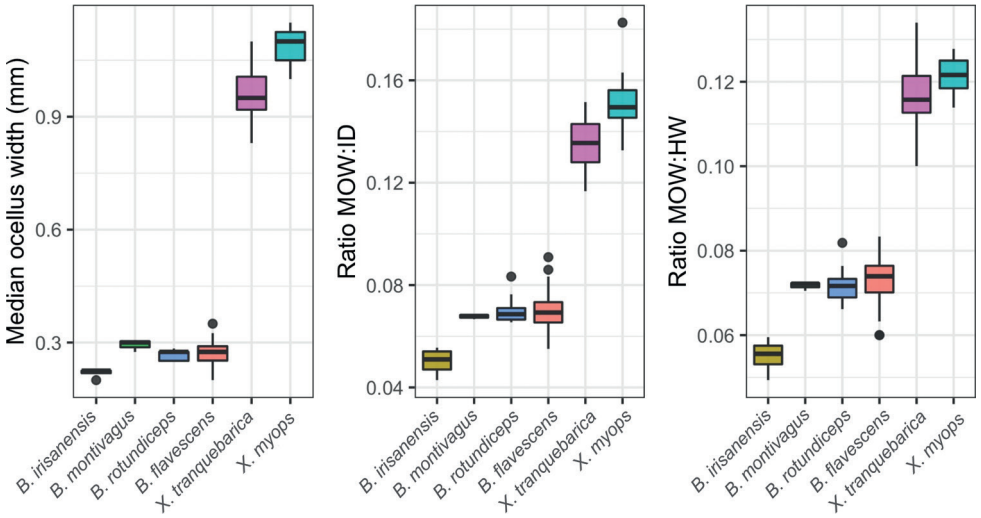

Figure 10. Box plots showing the median ocellus width (MOW), the ratio between MOW and inter-tegular distance (MOW:ID), the ratio between MOW and the head width (MOW:HW) of *B. irisanensis*, *B. rotundiceps*, *B. flavescens*, *B. montivagus* taxon *maxwelli*, *Xylocopa tranquebarica*, and *X. myops*.

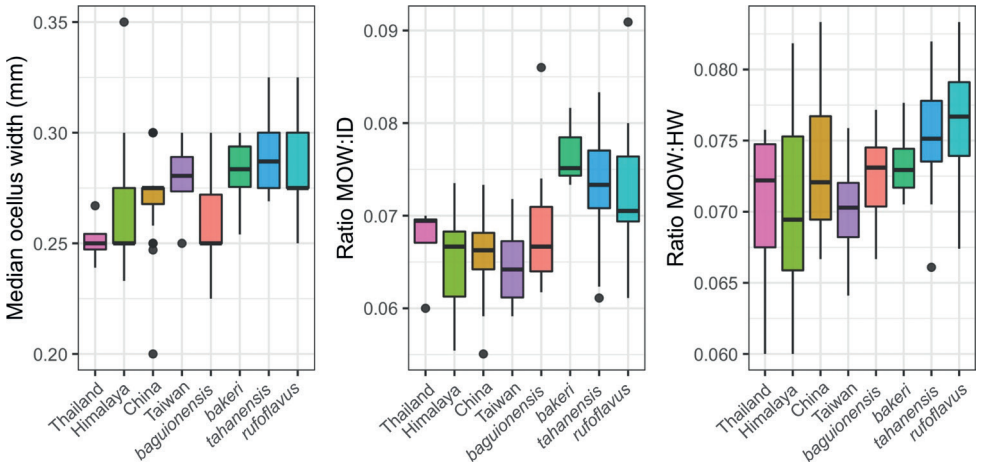

Figure 11. The box plots of the median ocellus width (MOW), the ratio between MOW and intertegular distance (MOW:ID) and the ratio between MOW and head width (MOW:HW) among *Bombus flavescens* population: Thailand, China, Himalaya, Taiwan, *baguionensis*, *bakeri*, *rufoflavus*, *tahanensis*.

of taxa were significantly different in MOW:HW (ANOVA, F-value = 3.701, p-value < 0.05; Tukey test, p-value < 0.05), *rufoflavus*-Himalaya, and *tahanensis*-Himalaya.

Discussion

Species status of the *flavescens*-complex

The status of species within the *flavescens*-complex taxa has been debated for nearly a century. Theodore Frison (1895–1945), an American entomologist wrote in his work in 1934, "*This species of bumblebee* [*B. flavescens*] *has been a problem to most persons who have attempted to determine…*" (Frison 1934). Our results for the COI-barcode tree (Fig. 6) support that the *flavescens*-complex is a monophyletic group, including the populations in China, the Himalaya, and Southeast Asia. The PTP and GMYC results group the *flavescens*-complex together as one species. This demonstrates that *B. flavescens s. l.* includes high intraspecific variation in colour pattern across its distributional range. The species-delimitation analyses confirm the conspecific status of the various geographical and morphological variants named as *B. baguionensis*, *B. bakeri*, *B. tahanensis* and *B. rufoflavus* by previous authors. Although this study did not include the taxon *mearnsi* from Mindanao, the taxon *mearnsi* also is expected to be a synonym of *B. flavescens* as morphologically suggested by Pittioni (1949), similar to the taxon *bakeri* from Negros, another population from the southern Philippines islands.

There is a discrepancy between the number of species recognised by PTP and GMYC. The GMYC analysis lumped 1) *B. flavescens* and *B. rotundiceps* 2) *B. modestus* and *B. wangae*, as single species, whereas PTP split them into four species. GMYC

requires an ultrametric tree, which distorts the tree and branch lengths (Fujisawa and Barraclough 2013). For this, the closely related sister taxa which establish their own short clade might be identified as the same species. In contrast, the PTP keeps the original tree shape, and should be considered more reliable between the two approaches (Williams 2021), in this instance.

Nevertheless, when we investigate the morphological evidence of these four taxa, their morphological characters are unique (Williams 1998; Williams et al. 2009, 2010). The males of *B. wangae* and *B. modestus* are distinct, especially in their genitalia, which the gonostylus of *B. modestus* is broader than *B. wangae*. For the *B. flavescens* and *B. rotundiceps*, their male genitalia are also different: the gonostylus of *B. flavescens* is broad with round inner margin, whereas the gonostylus of *B. rotundiceps* is narrower with straight inner margin. In this case, the PTP results show congruence with the morphological characters whereas GMYC does not. Then, we suggest that the PTP analysis corroborates more consistently morphologically recognisable species for these bumblebees. For further study, if fresh male specimens of *B. flavescens* are available, chemical evidence, including, CLGS, is recommended as an alternative approach for defining the species recognition, whether it shows agreement with the molecular delimitation in this study.

Fresh specimens of *B. flavescens* and their relatives are not available from enough samples because of the rarity of these bees. Bumblebees in subgenus *Pyrobombus* are active particularly early in the year (Williams et al. 2014), so subgenus *Pyrobombus* sampling can only be conducted in a relatively short period. The accessibility of their habitats in Southeast Asia is also relatively limited and no records have been reported during recent decades, especially for *B. flavescens* from Gunung Tahan, Malaysia, and from Negros and Mindanao in the Philippines. Moreover, although the *flavescens*-complex is widely distributed, recorded in at least eleven countries, it is difficult to obtain specimens from some countries, because of limits imposed by local or international regulations. Therefore, old museum specimens are the best available option for clarifying genetic relationships within this group. However, these specimens were collected between 1980 and late 1990. The DNA of those specimens has become degraded through time (Pääbo 1989). It is difficult to extract the DNA from old specimens with standard protocols. However, numerous DNA extraction techniques for old insect specimens have been developed (Gilbert et al. 2007; Thomsen et al. 2009; Ballare et al. 2019). Next generation sequencing would help to obtain DNA sequences from historical specimens (Nazari et al. 2016; Prosser et al. 2016; Sproul and Maddison 2017; Call et al. 2021), but a high DNA quantity is required (Goodwin et al. 2016). According to our results, the DNA concentration of the samples in this study was not high enough for the sequencing requirement. The newly designed primers in this study were successful for the *flavescens*-complex and *B. rotundiceps*. The primers can amplify the DNA from specimens up to 70 years old. From our results, fresh specimens or pinned specimens less than 20 years old are recommended for use as DNA sources as the first option. Museum specimens are an alternative way if fresh specimens are not available. However, using museum specimens requires additional molecular processes, for example, extra-sterilisation of equipment and laboratory space, and conducting

negative extraction and PCR controls. This is the first time that at least the partial COI barcodes of the *flavescens*-complex populations from the Philippines from both North and South islands have been retrieved. This information is invaluable because this is the key to resolving cryptic status among this group, but it also provides a workflow for approaching other difficult bee groups for which only old specimens are available.

Orange pattern bumblebees in Peninsular Malaysia

In the Cameron Highlands, Malaysia, several orange pattern bumblebees have been recorded, including the *rufoflavus* form of *B. flavescens* (Fig. 2J) and the *maxwelli* form of *B. montivagus*. Their fulvous hair and sclerite colour might relate to the nocturnal lifestyle of hymenopterans, similar to the nocturnal carpenter bees in subgenus *Nyctomelitta* (Williams 2007). In this study, the ocelli of *B. flavescens* populations were similar in size (Fig. 11) which did not match the hypothesis of enlarged ocelli, whereas the nocturnal carpenter bees have much more distinctively enlarged ocelli (Fig. 10). However, in the context of the variation among *B. flavescens* populations, the median ocellus of both Malaysian populations, *B. flavescens* taxon *rufoflavus* and taxon *tahanensis*, were significantly larger than the populations at higher latitudes, including from Himalaya and Luzon Island (Fig. 11). The results showed a gradient of ocellar size within the *B. flavescens* populations from high latitude (smaller ocellus) to low latitude (larger ocellus): MOW, MOWID, and MOWHW differed significantly between low latitude (0°–20°N) and high latitude (>20°N) group (MOW: Mann-Whitney U test, p-value = 0.037; MOWID: ANOVA, F-value = 30.49, p-value < 0.05; MOWHW: ANOVA, F-value = 8.573, p-value < 0.05).

Although there is a significant difference between low-latitude and high-latitude populations of *B. flavescens*, intraspecific variation of ocellar size in bumblebee populations has been reported before for *B. terrestris* (Kapustjanskij et al. 2007), without any evidence for nocturnal behavior in this well-known species. This also suggests that ocellar size might not be a good diagnostic character to distinguish species in bumblebees; it may be that it is relatively plastic to local selection regimes. The ocelli are visual organs that detect polarized light in low-light conditions (Wellington 1974; Roubik 1989). Ocellar size shows a negative correlation with light intensity (Kerfoot 1967; Warrant et al. 2006; Kapustjanskij et al. 2007), and there are a high number of nocturnal and crepuscular bees in tropical or low latitude areas (Dorey et al. 2020). The trend seen here towards larger ocellar size in the tropics might be evidence that bumblebees are more active in low-light conditions, for example, in the early morning or late afternoon, to avoid heat stress, as is suggested for *B. breviceps* Smith and *B. haemorrhoidalis* Smith (Williams 1991; Thanoosing 2022). Larger ocelli can also be observed in other tropical forest bees, for example, stingless bees, because sunlight is filtered by the tree canopy, thereby reduced in the understory (Streinzer et al. 2016).

Numerous light traps were run at night on the Cameron Highlands recently by researchers (Musthafa et al. 2021) and by tourist services (e.g., Cameron Service: https://cameronservice.blogspot.com/), but no bumblebees have been observed at traps. Con-

sequently, a nocturnal or crepuscular lifestyle of the orange pattern bumblebees in the Cameron Highlands remains unsupported.

Apart from orange *B. flavescens*, another bumblebee, *B. montivagus* taxon *maxwelli*, and a variation of the diurnal hornet *Vespa velutina* Lepeletier taxon *divergens*, in the same locality, are also orange in colour (Hines et al. 2012; Perrard et al. 2014). In addition, not only the orange pattern but also the black hair with a red tail pattern of *B. flavescens* in South China (Fig. 2D) is recognised in the colour variation of *V. velutina* taxon *nigrithorax* (Perrard et al. 2014). For this reason, the colour patterns of *B. flavescens* might potentially be a result of Müllerian mimicry.

Biogeography of *Bombus flavescens*

Pyrobombus is a bumblebee subgenus in which five species groups are found in the Old and New World (Williams 1998). *Bombus flavescens* and its relatives are in the '*pratorum*-group'. This group is the sister group to the '*lepidus*-group' (Cameron et al. 2007). The groups originated in the Palearctic and Oriental regions, in the late Miocene (~11 Ma), and the *lepidus*-group is entirely restricted to the Oriental region (Williams 1998). In this study, biogeographic analyses show that the origin of the *pratorum*-group is likely to be in the Oriental region, specifically around the Qinghai-Tibetan Plateau (QTP) or Himalaya. Then, during the late Miocene and early Pliocene, the diversification of this group occurred. This is coincident with the period when the monsoon climate in this area intensified (Zhou et al. 2018), which also accelerated the diversification of bumblebee food plants in the Oriental region (Yu et al. 2015; Ma et al. 2016; Matuszak et al. 2016). Our results show that there were dispersal events between the Oriental (E, F, G, H, I, J, and K; Fig. 8) and the Palearctic region (A, B, C, and D; Fig. 8), for example, the clade of *biroi+nursei*, *pyrenaeus+modestus+wangae*, and *pratorum+ardens*. In addition, there are the lineages of *B. nursei* and *B. wangae* that reinvaded the Oriental Region: *B. nursei* from the west and *B. wangae* from the east. *Bombus nursei* prefers subalpine meadow habitats similar to *B. biroi* (Williams 1991; Williams 2022). This dispersal pattern between the Oriental and Palearctic regions during the Miocene-Pliocene can be observed in other bumblebee subgenera, for example, in subgenera *Mendacibombus* (Williams et al. 2018) and *Melanobombus* (Williams et al. 2020).

Only the group *rotundiceps+flavescens* has been restricted to the Oriental until now. The divergence between *B. rotundiceps* and *B. flavescens* was in the Pleistocene (ca 1.5 Ma). The climate in Southeast Asia during the late Pliocene and Pleistocene was cooler than the present day (Morley 2012). This might have given the opportunity for temperate and montane flora to colonise these areas. At the same time, the MRCA of *B. flavescens* dispersed southward into Southeast Asia or Sundaland, following suitable habitat (A; Fig. 12). *Bombus flavescens* is likely to have dispersed to and colonised the Philippines islands when the islands or the Pleistocene Aggregate Island Complex (PAIC), including Luzon, Negros-Panay or Visayan, and Mindanao, were connected in the LGM (Brown et al. 2013). This study supports that *B. flavescens* dispersed to the Philippines by the

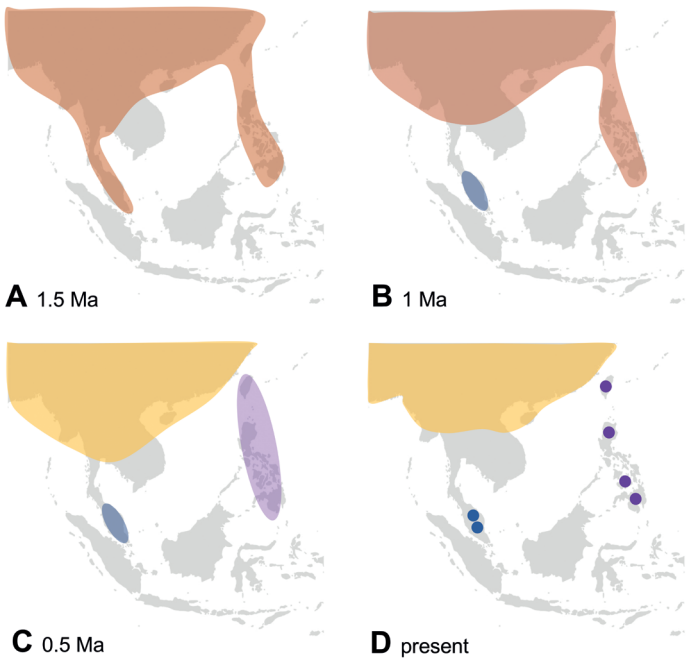

Figure 12. Biogeographic scenarios of *Bombus flavescens* through time **A** the most recent ancestor of *B. flavescens* dispersed through Southeast Asia or Sundaland around 1 Ma (orange) **B** the Malaysian population was isolated on the highland of the peninsula due to the temperature rising around 1 Ma (blue), at that time, the populations in Taiwan and Philippines were connected to the mainland populations (orange) **C** the bridge between the mainland and Taiwan was submerged, the Taiwan-Philippines populations were isolated around 0.5 Ma (purple) **D** at present day, *B. flavescens* populations distribute in the subtropical of Southeast Asia, China and Himalaya (yellow), on the Highlands of Malay peninsula (blue), and the islands of Taiwan and the Philippines (purple).

Taiwan-Luzon route in the same way as *B. irisanensis*, the other species in the Philippines. The oscillation of sea level and raising of global temperature in the late Pleistocene facilitated the population isolation and higher latitude immigration of *B. flavescens* (B, C; Fig. 12). First, the populations from the North (Luzon) and the South (Negros and Mindanao) were separated, because the South population (taxon *bakeri*) is the sister of the North population (taxon *baguionensis*) and the Taiwan group (Fig. 7). Then, the populations on the North of Philippines and Taiwan were isolated when the sea submerged the bridges, so that the populations in Southeast Asia were captured in montane refuges as biological islands until the present, similar to the populations on the Cameron Highlands and Gunung Tahan mountain in Malaysia (D; Fig. 12).

Biogeography of bumblebees in Southeast Asia

Many lineages of bumblebees in Southeast Asia might are hypothesised to have originated around the QTP, then dispersing into the region via the Himalaya–Hengduan corridor (Williams 1985; Hines 2008; Williams et al. 2022). The bumblebee fauna

at the Northern border and highlands of the Southeast Asian region is similar to the fauna of the Himalaya and Southern China, adjacent neighbours both spatially and genetically (Williams et al. 2009; Williams et al. 2010; Hines and Williams 2012; Streinzer et al. 2019; Williams 2022). The subgenera *Orientalibombus* Richards and *Alpigenobombus* Skorikov are mainly restricted to the North of the Southeast Asian region, whereas the subgenera *Megabombus* Dalla Torre, *Melanobombus* Dalla Torre, and *Pyrobombus* are more widely distributed throughout the region (Williams 1998).

The long-faced subgenus *Megabombus* crown group might have originated around 13 Ma (Hines 2008). Then, two independent lineages of subgenus *Megabombus* diverged in Southeast Asia approximately between 4.25–1.5 Ma, based on molecular dating (Huang et al. 2015a). The lineages are 1) a *trifasciatus*-group: *B. montivagus*, *B. albopleuralis* Friese, *B. burmensis* (Skorikov) in the North of Southeast Asia and the Malay Peninsula, and 2) a *senex*-group: *B. irisanensis* on Luzon Island, in the Philippines. However, due to a lack of genetic information on *B. senex* Vollenhoven and *B. melanopoda* Cockerell, the phylogenetic relationship between mainland and Sumatran subgenus *Megabombus* remains relatively unresolved (Huang et al. 2015a). Consequently, the biogeography of Sumatran subgenus *Megabombus* bumblebees remains unclear.

Nonetheless, the link between the Southeast Asian mainland and the Indonesian islands can be explained by the phylogenetic relationship of two subgenus *Melanobombus* sister species, *B. eximius* Smith and *B. rufipes* Lepeletier of the *rufipes*-group. The MRCA of the *rufipes*-group lineage diverged from the other subgenus *Melanobombus* lineages around 16 Ma and was distributed in the Himalaya and QTP (Williams et al. 2020). Speciation within the *rufipes*-group is likely to have occurred around 1 Ma in the Pleistocene epoch, after the Sundaland land bridge was submerged, isolating part of the *rufipes*-group on Sumatra and Java (Williams et al. 2020).

Southeast Asian bumblebees in the subgenus *Pyrobombus* are also distributed both on the mainland and on the islands of the Philippines, for example, *B. flavescens*. There is no record of subgenus *Pyrobombus* on the islands of Indonesia and adjacent areas. A member of subgenus *Pyrobombus* had been recorded on the Andaman Islands, named *B. andamanus* Gribodo (Gribodo 1882). However, this is a mislabeled specimen of a Nearctic bumblebee, *B. bifarius* Cresson (Williams 1998).

Conclusion

Genetic information proved crucial for the study of bumblebees in Southeast Asia. This is the first gene-based study to address the taxonomic status of *B. flavescens*, which is highly variable in hair colour. This study confirms that the populations of *B. flavescens* on the Asian mainland and on the islands are parts of the same species. Despite this, a trend among *B. flavescens* populations can be observed towards larger ocelli at lower latitudes. This might only reflect local selection based on foraging in the low-light conditions within dense forest, or in more early morning or twilight activity in warmer environments. *Bombus flavescens* originated in the Himalaya and dispersed to Southeast Asia during the Pleistocene. Its constituents, various regional colour forms,

diversified through an allopatric divergence process. *Bombus flavescens* is a useful model for studying the biogeography of bumblebees in Southeast Asia, many of which are less known. Nevertheless, more genetic information is required to investigate the conservation of endemic populations of *B. flavescens*.

Acknowledgements

We would like to thank K. Atthasopa and K. Klaithin (ACMU), N. Badruddin (FRIM), F. Bakker and W. van Bohemen (NMNL), N. Chatthanabun and P. Nalinrachatakan (CUNHM), N. Likhitrakarn (MJUMZ), K. Lee (Entopia), D. Notton and J. Monks (NHMUK), N. Pinkaew (KKIC), B. Santos and A. Touret-Alby (MNHN) for their generous help in accessing collections. Thanks are extended to T. Srimaneeyanon for collecting fresh Thai specimens; to I. Barnes and S. Brace (NHMUK) for their suggestion on ancient DNA technique; to M. Musthafa for sharing information on light trapping in the Cameron Highlands; and to P. Nalinrachatakan and S. Yendee for facilitating specimen imaging at CUNHM. Finally, we thank N. Meeprom and J. Satthaphorn for helping with bioinformatic software. This research was supported by a postgraduate scholarship of the Royal Thai Government to CT.

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Supplementary material 1

List of COI sequences from databases, research and collaboration, including the accession number or ID, the original sequence length and country

Authors: Chawatat Thanoosing, Michael C. Orr, Natapot Warrit, Alfried P. Vogler, Paul H. Williams

Data type: Sequence ID (word document)

Link: https://doi.org/10.3897/jhr.96.104715.suppl1

Supplementary material 2

Primer testing

Authors: Chawatat Thanoosing, Michael C. Orr, Natapot Warrit, Alfried P. Vogler, Paul H. Williams

Data type: Text (word document)

Explanation note: Testing newly designed primers in this study.

Link: https://doi.org/10.3897/jhr.96.104715.suppl2

Supplementary material 3

Primer testing with gradient annealing temperatures

Authors: Chawatat Thanoosing, Michael C. Orr, Natapot Warrit, Alfried P. Vogler, Paul H. Williams

Data type: Experimental (word document)

Explanation note: The samples were *B. breviceps* (CT#552) and *B. flavescens* (CT#662). The brightness of gel electrophoresis is presented in symbols: *** = strong, ** = medium, * = weak, NS = unsuccessful.

Link: https://doi.org/10.3897/jhr.96.104715.suppl3

Supplementary material 4

Polymerase chain reaction temperature profile of different pairs of primers in this study

Authors: Chawatat Thanoosing, Michael C. Orr, Natapot Warrit, Alfried P. Vogler, Paul H. Williams

Data type: Experimental (word document)

Link: https://doi.org/10.3897/jhr.96.104715.suppl4

Supplementary material 5

List of 16S and PEPCK sequences from GenBank with the accession number, the original sequence length, and country

Authors: Chawatat Thanoosing, Michael C. Orr, Natapot Warrit, Alfried P. Vogler, Paul H. Williams

Data type: Sequence ID (word document)

Link: https://doi.org/10.3897/jhr.96.104715.suppl5

Supplementary material 6

The accession number or ID of the parsing dataset of COI, 16S, and PEPCK sequences for *BEAST analysis

Authors: Chawatat Thanoosing, Michael C. Orr, Natapot Warrit, Alfried P. Vogler, Paul H. Williams

Data type: Sequence ID (word document)

Link: https://doi.org/10.3897/jhr.96.104715.suppl6

Supplementary material 7

Ancestral area reconstruction model selection, estimated in BioGeoBEARS

Authors: Chawatat Thanoosing, Michael C. Orr, Natapot Warrit, Alfried P. Vogler, Paul H. Williams

Data type: Model test (word document)

Link: https://doi.org/10.3897/jhr.96.104715.suppl7

Supplementary material 8

List of specimens, including species, collection, specimen ID, origin country and latitude group, and measurement of intertegular distance (ID), head width (HW), median ocelli width (MOW) in millimetres (mm)

Authors: Chawatat Thanoosing, Michael C. Orr, Natapot Warrit, Alfried P. Vogler, Paul H. Williams

Data type: Morphological (word document)

Explanation note: List of specimens, including species, collection (CUNHM = Chulalongkorn University Natural History Museum, Bangkok, Thailand; KKIC = Kasetsart Kamphaeng Saen Insect Collection, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom, Thailand; NHMUK = Natural History Museum, London, UK; NMNL = Naturalis Biodiversity Center, Leiden, the Netherlands; PHW = Paul H. Williams research collection, UK), specimen ID, origin country and latitude group (Low = 0°–20°N; High = > 20°N), and measurement of intertegular distance (ID), head width (HW), median ocelli width (MOW) in millimetres (mm) (available at the NHM Data Portal: https://doi.org/10.5519/wknn9sd2).

Link: https://doi.org/10.3897/jhr.96.104715.suppl8