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Integrating evidence to disentangle multiple cryptic speciation events in a cuckoo bumblebee species group

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Abstract

Integrating multiple approaches for the study of lineages is fundamental, and searching for coherent evidence is generally accepted as a way to discover new species. Bumblebee speciation is ideal for study due to taxonomic history, color mimicry, and morphological challenges. Here, we focused on the *Bombus flavidus* species group, cuckoo bumblebees, by characterizing the genetic lineages and variation, investigating morphometrics of wing shape and body parts, and studying morphology. From 553 COI sequences within the *B. flavidus* species group, *B. barbutellus* species and *B. bohemicus* group, and 265 morphological specimens, lines of evidence coherently demonstrated distinct lineages, especially within the *B. flavidus, B. norvegicus,* and *B. sylvestris* complexes. Multiple gene-based delimitation algorithms identified three new lineages, and morphology and morphometrics also confirmed this, leading to a new taxonomic status for *Bombus lutescens* **stat. nov.** in South Europe, *Bombus sichuanicus* Biella **sp. nov.** in South-East Asia, and *Bombus popovi* **stat. nov.** in East Asia. Since these new clades occur at the periphery of the species complex's range, speciation at the distribution edges is interpreted as a possible mechanism, while possible host-shifts are also discussed. This study illuminates broad zoological research questions, showing that integrating sources of evidence is operationally useful for studying speciation events in the context of cryptic lineages, while also clarifying the evolutionary history of an intricate bumblebee group.

Keywords: Apidae, Integrative zoology, New species, *Psithyrus*, Species delimitation, Taxonomy

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Introduction

In recent decades, an intensified focus on speciation and differentiation processes has taken place, thus resulting in new species descriptions, changes in taxonomic status, or the reshaping of the taxonomic hierarchy at the family, genus, species, and intraspecific levels[1]. In most cases, such advances were made possible thanks to the integration of multiple methods and criteria, such as phylogenetics, phylogeography, chemical ecology, morphometrics, and niche ecology[2]. The operational integration of several approaches has now become a prominent feature in taxonomic studies, allowing recognition of distinct species based on multiple sources of data[3], which coherently point out differences between groups and constitute evidence of speciation[4,5]. Therefore, integrative taxonomy is now a pillar in the process of testing the hypothesis of speciation and the status of taxa in the classification hierarchy, as this approach is believed to provide strongly supported decisions[6].

The integration of several independent lines of evidence to study speciation was applied to several bumblebee groups (Hymenoptera: Apidae: *Bombus*)[7,8]. These pollinating bees have a rich taxonomic history[9]; however, in many cases, the low morphological diversification of body parts between related species, the high level of regional color mimicry, and the intraspecific color variation lead to a surprisingly confusing taxonomy and unclear taxonomic status of many taxa[10–12]. In spite of that, bumblebee taxonomic studies have reflourished in the last 15 years: based on phylogenetic and subgeneric reassessments of the whole genus[13,14], integrative studies focused on a number of species groups to address unclear cases[15,16]. In contrast, the subgenus *Psithyrus* Lepeletier, 1833 received surprisingly limited attention[17–19], despite the fact that morphological similarities and the isolation of some populations would make this group a really good candidate for testing speciation events.

In this study, we investigated the species boundaries within the *Bombus flavidus* complex, cuckoo bumblebees with a nest-parasitic mode of life belonging to subgenus *Psithyrus* (previously referred to as the subgenus *Fernaldaepsithyrus* Frison, 1927). *Psithyrus* is a monophyletic clade[13] with 28 nominal species worldwide[9], consisting exclusively of social parasites of other bumblebees[20]. The variability

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in size and color[19,21], the morphological similarities among taxa, and sometimes the rarity led to many specific and subspecific names[22,23]. With more than 50 existing names for species, subspecies, forms, and varieties within the *B. flavidus* complex only, and their broad geographic distribution, this bumblebee group is one of the most interesting and intricate to investigate under an integrative approach.

This study aims to shed light on the relationships between distinct populations of the species *B. flavidus* (Eversmann, 1852), *Bombus norvegicus* (Sparre Schneider, 1918), and *Bombus sylvestris* Lepeletier, 1832 by sourcing specimens for their entire global distribution ranges. A previous study by Lhomme et al.[11] focused exclusively on *B. flavidus* but excluded some key distribution areas where geographically isolated subspecies exist[24]. Another study on the species group focused on the variation of wing shapes using morphometrics in populations from Scandinavia[25], but extending their approach to the southern European populations revealed to be problematic, possibly due to different morphologies there[26]. To date, no study has dealt with the evolutionary dynamics within the global distribution of the species within the *B. flavidus* group, although some traditional taxonomic works with a regional scope have been carried out in the past[22,27]. Therefore, here we aimed to integrate and evaluate several possible lines of evidence to shed light on this intricate case by focusing on: (1) the genetic relatedness of populations; (2) the morphological differences between distinct lineages or subspecies; and (3) the integration of existing evidence to evaluate speciation processes. Here, three species complexes within the group are considered: populations of *B. sylvestris*, *B. norvegicus,* and *B. flavidus* complexes, belonging to the *B. flavidus* group. Nevertheless, mainly for comparative purposes in the genetic analyses, we also included populations of the related taxa in the *flavidus* group (i.e., *B. quadricolor* (Lepeletier, 1832) and *B. skorikovi* (Popov, 1927)), species belonging to the *B. bohemicus* species group (previously *Ashtonipsithyrus* Frison, 1927) and *B. barbutellus* (Kirby, 1802), to provide more comprehensive species hypotheses and understanding of the putative speciation patterns.

Materials and methods

Sampling and target taxa

Specimens were retrieved from several collections and sampling events within the distribution range of the target species. Samples were obtained from a global network of collaborators, from sites that are deliberately widely scattered. Sampling effort was directed to achieve as much of the range of variation as possible. Specimens were retrieved from those stored at the University of Milano-Bicocca Collection (code MIB, including samples from the Paolo Biella research collection), the Intoppa-Piazza Collection (at the University of Milano-Bicocca, code CIP), the collection of the Federal Scientific Center of the East Asia Terrestrial Biodiversity (Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia, code FSCV), the collection of the University Museum of the Norwegian University of Science and Technology (Trondheim, Norway, code NTNU-VM), the collection of the Kunming Institute of Botany at the Chinese Academy of Sciences, (Kunming, China, code KIB), the collection of the Institute of Entomology of the Agricultural University of Georgia (Tbilisi, Georgia, code IEAUG), the collection of the Zoological Museum Amsterdam (ZMA) at the Naturalis Biodiversity Center (Leiden, the Netherlands), the collection of van Asperen de Boer at the Naturalis Biodiversity Center, the Martin Streinzer research collection (MSPC), the Johann Neumayer research collection (JNRC), the Walter Wallner research collection (WWRC), the Paul H. Williams research collection (PW), and the Alexandr Byvaltsev research collection (ABRC).

The taxa targeted in this study are listed below by species, geographically delimited subspecies following recent taxonomic studies or their frequently used synonyms. In the *B. flavidus* species group the following taxa were included: in the *B. flavidus* complex, specimens from northern Eurasia (= *B. flavidus flavidus* (Eversmann, 1852) and referred to as *flavidus* s.s., that stands for 'sensu stricto', in some sections of this study); from North America (= *B. fernaldae* (Franklin, 1911), including *B. flavidus appalachiensis* Lhomme & Hines, 2021); from the Pyrenees (= *B. flavidus lutescens* (Pérez, 1890) and referred to as *lutescens* s.s. in some sections of this study); and from the Alps in southern Europe (= *B. flavidus alpium* (Richards, 1928)). In addition, in the *B. norvegicus* complex, *B. norvegicus* (Sparre Schneider, 1918) from Eurasia, and from Japan (= *B. norvegicus japonicus* (Yasumatsu, 1937)). Moreover, in the *B. sylvestris* complex, specimens of *B. sylvestris* (Lepeletier, 1832) from western Eurasia (and referred to as *sylvestris* s.s. in some sections of this study), and specimens from East Asia (= *B. sylvestris popovi* (Yasumatsu, 1933)). In addition, specimens of *B. skorikovi* (Popov, 1927) from central Asia (Qinghai–Tibet Plateau) and of *B. quadricolor* (Lepeletier, 1832) from northern and central Europe (= *B. quadricolor globosus* (Eversmann, 1852)), from western Europe (= *B. quadricolor quadricolor* (Lepeletier, 1832)), from the Alps (*B. quadricolor meridies* Rasmont, Ghisbain, Terzo 2021, synonym of *B. quadricolor meridionalis* (Richards, 1928)), from West Asia and Caucasus (= *B. quadricolor ingermanlandicus* (Popov, 1931) syn. of *B. quadricolor rossicus* (Popov, 1931)[24]) (Supplementary Tables S1, S2). Following Cameron et al.[13], as a sister group in the genetic analyses, the *B. bohemicus* species group was included with specimens of *B. suckleyi* Greene, 1860 from North America, specimens of *B. bohemicus* Seidl, 1838 from Eurasia, and its synonym *B. ashtoni* (Cresson, 1864) from North America, specimens of *B. vestalis* (Geoffroy, 1785) from Europe, including ones from southern Europe (= *B. vestalis obenbergeri* (May, 1944)) and from the Tyrrhenian islands in Southern Europe (= *B. vestalis perezi* (Schulthess-Rechberg, 1886) and *B. vestalis sorgonis* (Strand, 1917)). Within the subgenus *Psithyrus*, we also included *B. barbutellus* (Kirby, 1802), including the synonym *B. maxillosus* (Klug, 1817), and the outgroup *B. citrinus* (Smith, 1854).

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from a mid-leg using the Qiagen DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. Before proceeding with the extraction phases, the specimens were rinsed and rehydrated in physiological solution (NaCl 0.9%) for 15 min.

Concerning DNA barcoding characterization, PCRs were carried out using PuReTaq Ready-To-Go™ PCR Beads from GE Healthcare in 25 µl final reaction volumes containing 22 µl of sterile water, 1 µl of each primer, and 1 µl of gDNA (10 ng). For each sample, the standard DNA barcode 5′-end region of the cytochrome c oxidase subunit 1 gene (COI) (658 bp) was amplified using the LCO1490-HCO2198 primer pair[28]. In case of unsuccessful amplification, five alternative COI primer pairs developed in this study and targeting shorter and partially overlapping fragments of the gene were adopted to amplify and sequence the full barcode region (Supplementary Text S1). In both cases, PCR amplification conditions were as follows: 94 °C for 5 min, 5 cycles at 94 °C for 60 s, 45 °C for 90 s and 72 °C for 90 s followed by 35 cycles at 94 °C for 60 s, 50 °C for 90 s and 72 °C for 60 s and a final extension at 72 °C for 7 min. Amplification success was verified on a 1% agarose gel. PCR products were purified and sequenced bidirectionally using an ABI 3730xl DNA Analyser (Applied Biosystems, Waltham, MA, USA).

Individual assembled sequences were obtained from each specimen by editing the forward and reverse electropherograms with BioEdit

7.2[29] and Geneious Prime (Biomatters, Auckland, New Zealand). After primer trimming, the presence of an open reading frame was verified for the obtained sequences by using the online tool EMBOSS Transeq (www.ebi.ac.uk/Tools/st/emboss_transeq), and data were checked for high divergence NUMTS or for conflicts from non-orthologous sequences[30–32]: no sequence contained insertion/deletions, stop codons, or was biased by NUMT interference. In this study, a total of 128 new COI sequences were produced (Supplementary Table S1); specimen sampling metadata and edited sequences were deposited in the BOLD System (project ZPLFP; see Supplementary Table S1 for process ID codes).

Genetic dataset assembly

Orthologous COI-5' barcode sequences and associated metadata belonging to the species *B. barbutellus*, *B. bohemicus, B. citrinus, B. flavidus, B. norvegicus, B. quadricolor, B. skorikovi, B. suckleyi, B. sylvestris*, and *B. vestalis* were downloaded from BOLD Systems[33], last accessed: 29 July 2025, using the package *BOLDconnectR*[34] in R[35]. After discarding sequences shorter than 400 bp and sequences likely taxonomically misidentified (i.e., with discordant names), a total of 426 COI barcode sequences were retrieved (Supplementary Table S1). These sequences were grouped with those obtained in the present study, aligned with MAFFT v7.409[36] using the E-INS-i option, and finally trimmed to obtain the standard 658 bp-long COI barcode region (from here on called 'dataset DS1', 553 sequences). To reduce the impact of missing data on subsequent analyses, the dataset was further filtered by removing sequences shorter than 555 bp and those with ambiguous sites, resulting in a 555 bp-long alignment of 468 sequences that were finally collapsed into unique haplotypes using FaBox v1.61[37] (from here on called 'dataset DS2', 76 sequences).

Gene tree reconstructions

Both datasets DS1 and DS2 were used to infer evolutionary relationships among the sequences. The most appropriate substitution models were determined with ModelFinder[38], using the Akaike information criterion and the Bayesian information criterion, resulting in GTR + G + I in all cases. Gene tree reconstructions were performed using two optimality criteria, namely Bayesian inference (BI) and maximum likelihood (ML). BI analyses were conducted using BEAST v1.8.2[39], setting a coalescent tree prior and an uncorrelated lognormal relaxed clock. Three replicate analyses were run for 100 million generations, sampling every 10,000th generation, and were combined using LogCombiner v1.8.2[39] with a burn-in set to 25%, after checking stationarity for effective sample size (ESS) and unimodal posterior distribution using Tracer v1.7.2[40]. Maximum clade credibility trees were computed using TreeAnnotator v1.8.2[39]. ML analyses were performed using RAxML 8.2.9[41] with 1,000 bootstrap replicates. Each tree was rooted using *B. citrinus* as an outgroup, following previous phylogenetic reconstructions[13]. The obtained trees were visualized using FigTree v.1.4.3[42], and their aesthetics customized using CorelDraw x7. Specifically, only the Bayesian topology was shown, with support values shown only when the Bayesian posterior probabilities (BPP) were higher than 0.9 and/or the maximum likelihood bootstrap support (MLBS) was higher than 75.

DNA-based species delimitation

Since missing and ambiguous data are known to negatively affect phylogenetic inference[43], only dataset DS2 was used to perform DNA-based species delimitation analyses. Different implementations of the tree-based Poisson Tree Processes 'PTP'[44,45] and Generalized Mixed Yule Coalescent 'GMYC'[46,47] were used to explore species boundaries, using the Bayesian inference tree based on dataset DS2 as input. PTP analyses were performed using both single-threshold (mlPTP and bPTP) and multiple-threshold (mPTP) approaches on the websites https://species.h-its.org/ptp and https://mcmc-mptp.h-its.org, respectively, in both cases running the analyses for 100,000 MCMC generations, with thinning value = 100 and burn-in = 0.25, and checking the trace files for convergence of the MCMC. Single-threshold GMYC analysis was performed in R using the packages *splits*[48] and *ape*[49]. Overall, we conservatively considered a delimitation to be a reliable hypothesis when all tree-based methods were in agreement, as clarified in the integrative assessment section.

Genetic distances and haplotype networks

Genetic distances among the identified species hypotheses in the dataset DS2 were computed using MEGA v11.0.1[50]. Intra- and interspecies mean K2P distances were calculated with 1,000 bootstrap replicates. The maximum intra-specific K2P distance and the minimum K2P distance to the nearest non-conspecific neighbour were calculated using the *spider* package in R. Finally, for selected species, median-joining haplotype networks were obtained with PopArt v1.7[51], using default settings and with haplotypes coloured according to the geographic provenance.

Morphological evaluation and morphometric analyses

The morphology of 265 specimens was analysed (Supplementary Table S2). In the *B. flavidus* complex, both males and females (queens) were subjected to morphometric analyses. A geometric morphometric analysis of the venation pattern of the male right forewing was performed. We digitized a total of n = 74 specimens of the *B. flavidus* complex (*fernaldae* from North America: n = 5, *flavidus* s.s. from North Eurasia: n = 26, *alpium* from the Alps: n = 25, *lutescens* s.s. from the Pyrenees: n = 18; Supplementary Table S2). Wings were flattened between two coverslips and photographed perpendicular to the wing surface, including a size reference. The same 18 homologous landmarks used by Lhomme et al.[11] were digitized manually using ImageJ2 (v2.9.0) and converted manually to the tps format. In addition, we used the digitized data from Lhomme et al. (*fernaldae*: n = 94, *flavidus* s.s.: n = 9)[11]. Data were analysed with the *geomorph* package[52,53] in R. The coordinates were superimposed using the GLS Procrustes algorithm[54], and PCA (principal component analysis) was performed to estimate variability and grouping. Here we ran the analysis both with four groups (*fernaldae* vs *flavidus* s.s. vs *alpium* vs *lutescens* s.s.) and with two groups (*fernaldae* + *flavidus* s.s. vs *alpium* + *lutescens* s.s.). To test whether the wing morphology differed significantly between taxa, a Procrustes ANOVA[55] was performed on the output of the Procrustes analysis in the *geomorph* package in R. To assess the discrimination power of the wing morphology, we performed LDA (linear discriminant analysis) following the procedures of Lhomme et al.[11] using the MASS package for R[56], followed by LOO (leave-one-out) procedures. Morphometric measures on female body parts were taken from n = 43 females (*fernaldae*: n = 11, *flavidus* s.s.: n = 10, *alpium*: n = 11, *lutescens* s.s.: n = 11, Supplementary Table S2); the statistical analysis was performed to compare the malar space ratio (length/width) across the four groups (measures taken following[24]). An analysis of variance was performed (*aov* function in R) and a post-hoc test to compare the groups (Tukey test, with single-step adjustment method). On the same females, external morphological features were studied (e.g., coloration, punctuation patterns, etc.).

In the *B. sylvestris* complex, a geometric morphometric analysis of the male right wing followed the same procedures as described above.

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We digitized 97 male specimens of the *B. sylvestris* complex (*sylvestris s.s.* from western Eurasia: $n = 76$, *sylvestris* from Eastern Asia: $n = 21$; Supplementary Table S2). On the available females (*sylvestris* s.s. from western Eurasia: $n = 12$, *sylvestris* from Eastern Asia: $n = 9$ from different areas; Supplementary Table S2), morphometric measures on female body parts were taken, and the statistical analysis was performed to compare the labral furrow width ratio (furrow width measured between the most protruding and innermost points of the labral tubercules/labrum width) and to compare the labral lamella ratio (lamella/labrum widths). An analysis of variance was performed (*aov* function in R) and a post-hoc test to compare the groups (Tukey test, with single-step adjustment method); external morphological features were studied on the same females (e.g., coloration, punctuation patterns, etc.).

In the *B. norvegicus* complex, an exhaustive morphometric analysis was not possible due to the rarity of the species in southeast China, with a resulting paucity of samples in the collections. Hence, the external morphological features (e.g., coloration, punctuation patterns, etc.) were compared across the available female specimens (*norvegicus* from Eurasia excluding Southeast Asia: $n = 12$ from different areas; *norvegicus* from Southeast Asia: $n = 5$, Supplementary Table S2). However, only one male is known to us from Southeast Asia, which therefore does not allow for a morphological and morphometric comparison of males.

Taxonomic treatment

To provide an updated view and check the appropriateness of existing taxonomic names, all available names for the target species, subspecies, and infraspecific levels were retrieved from the Catalogue of Life (but corrected using the original publications); locations of the specimens the names referred to were also noted to associate the names with a geographic entity. The validity of the names was checked using the criteria of the International Code of Zoological Nomenclature[57] (accessed on 01/09/2025). Examination of the type specimens was not necessary because they were recently revised[11,24,58], except that the type for *B. sylvestris popovi*, described by Yasumatsu[59], was not found. This publication is registered in ZooBank: http://zoobank.org/urn:lsid:zoobank.org:pub:3D9B753C-941B-478F-9FA0-AD1BA845FCAB.

Integrative assessment

Here, species are in theory considered evolutionarily independent lineages that, in practice, are recognizable by using an integrative procedure: by comparing gene tree reconstructions coupled with several species delimitation algorithms, morphology, and morphometrics. A candidate species is accepted if evidence from different sources is coherent in showing differences among groups[2]. In other words, for the target taxa (i.e., *B. sylvestris*, *flavidus*, *norvegicus* species complexes), putative species were considered valid if simultaneously showing differences in morphology (or morphometrics when examined), in gene tree-based species delimitation algorithms, and forming distinct clades in the gene trees. In the case of morphometrics, the ranges of character values are statistically tested, while for morphology, the consistency of characters within and between groups is qualitatively assessed from the analysed samples. Instead, for the other taxa included in the genetic analyses for comparative purposes, a morphological analysis was not carried out, and hence their species status is not formally evaluated here with an integrative approach; additional studies could be carried out in future targeting those taxa.

Results

All DNA analyses and morphometric and morphological analyses were concordant in delimiting the new species *B. sichuanicus* **sp. nov.**, sister to *B. norvegicus*, and the two species *B. popovi* **stat. nov.**, previously ascribed to *B. sylvestris*, and *B. lutescens* **stat. nov.**, previously ascribed to *B. flavidus*, as detailed in the following paragraphs.

Molecular results

The reconstructed BI and ML trees for datasets DS1 and DS2 were broadly concordant and showed monophyletic clades, with a few exceptions (Fig. 1a; Supplementary Figs S1, S2). Specifically, ML analyses did not recover *B. quadricolor* as monophyletic, and the ML reconstruction of dataset DS1 did not recover *B. norvegicus* as monophyletic. Other slight differences concerned the position of *B. barbutellus*, *B. vestalis*, and *B. sichuanicus* **sp. nov.**, which varied across datasets and optimality criteria (Fig. 1a; Supplementary Figs S1, S2). Despite these subtle variations, all the recovered species consistently diverged from their sister groups.

Species delimitations confirmed all the obtained major clades as species hypotheses, and the number of delimited species ranged from 14 to 15 (Fig. 1a). Single-threshold mlPTP and bPTP analyses revealed the presence of 15 species, where the *B. flavidus*, *B. sylvestris,* and *B. norvegicus* complexes are actually hypothesised to be composed of two species each, with a further subdivision of *B. quadricolor* into two groups, one composed of a single haplotype from Georgia and one composed of sequences from other localities in the Palearctic. The mPTP analysis resulted in similar delimitations, with the only exception being *B. barbutellus* recovered as a single species hypothesis. Finally, the GMYC analysis delimited the same 15 species hypotheses found with ml- and bPTP methods (Supplementary Table S3). In the sister group (*bohemicus* group), the haplotypes of *B. bohemicus* were in a rather homogeneous clade with very shallow differences across geographic regions, and similarly for *B. vestalis* and *B. suckleyi*; *B. barbutellus* resulted in two putative species by most species delimitation algorithms, although one did not confirm the split (i.e., mPTP).

Genetic distances among the species hypotheses spanned between 1.3% and 13.5%, whereas mean intra-specific distances spanned between 0.0% and 1.6 % (Fig. 1b; Supplementary Table S4). The maximum intra-specific distances spanned between 0.0% and 2.6%, whereas the minimum distances to the nearest non-conspecific neighbour spanned between 1.3% and 10.6% (Supplementary Table S5). Regarding the mean genetic distances between the newly described taxa and their sister species, *B. flavidus* and *B. lutescens* **stat. nov.** revealed a genetic distance of 3.0% ± 0.7%, *B. norvegicus* and *B. sichuanicus* **sp. nov.** a distance of 3.5% ± 0.8%, and *B. sylvestris* and *B. popovi* **stat. nov.** a distance of 2.3% ± 0.6%. On the other hand, *B. barbutellus* showed high intraspecific distance, and the two clades of *B. quadricolor* delimited by tree-based delimitation methods showed a low genetic distance of 1.3% ± 0.5% from each other.

Notably, in the haplotype networks, no clear geographic structure was observed within existing or newly recognized species, with the same haplotype being often found in multiple localities (Fig. 2). Nevertheless, *B. flavidus* specimens from west Nearctic, east Nearctic, and north Palearctic never shared the same haplotype (Fig. 2a).

Morphometric analyses

In the *B. flavidus* complex, in the PCA analysis, the specimens from the four initial groups (*fernaldae*, *flavidus* s.s., *alpium,* and *lutescens* s.s.) showed some clustering with considerable overlap (Supplementary Fig. S3a); however, the LDA revealed two groups (*fernaldae* +

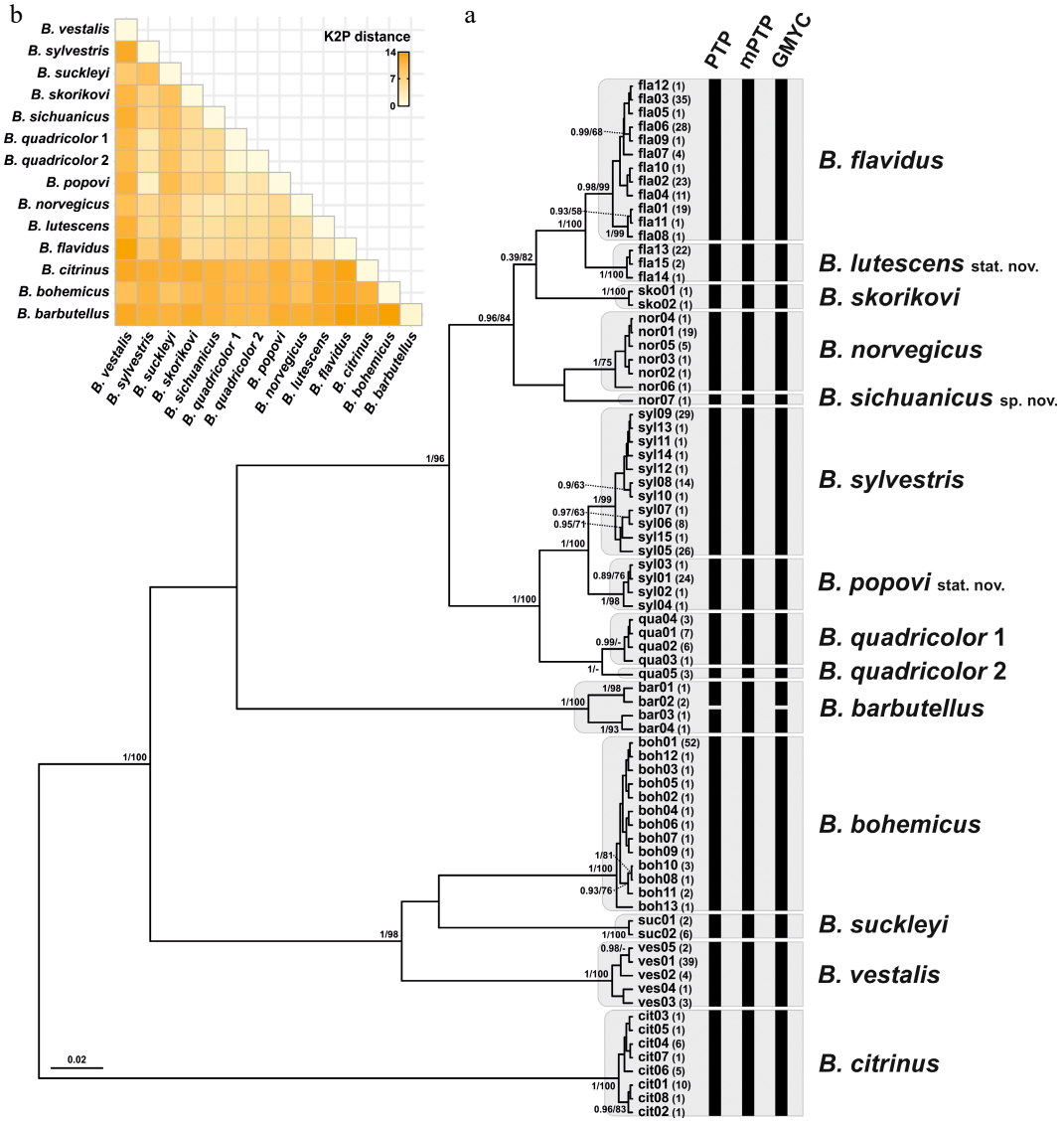

Fig. 1 Gene tree reconstruction and species delimitation of dataset DS2, based on the 555 bp-long alignment of unique haplotypes. (a) Bayesian tree, with alphanumeric codes at tips indicating the haplotype names (Supplementary Table S1), and the numbers in brackets indicating the number of sequences sharing that specific haplotype. Numbers at nodes reflect Bayesian posterior probabilities (BPP) and maximum likelihood bootstrap values (MLBS), respectively, indicated only when at least BPP > 0.9 and/or MLBS > 75. Black bars indicate the species delimitation results based on Poisson Tree Processes (PTP; single-threshold PTP including maximum likelihood mlPTP and Bayesian bPTP), and Generalized Mixed Yule Coalescent (GMYC). Species hypotheses consistently supported by multiple methods are highlighted in grey boxes. (b) Heatmap showing the mean pairwise % K2P distances within and between species, with darker colours indicating higher distances, as shown in the legend.

flavidus s.s. and *alpium* + *lutescens* s.s.) with significant within-group overlap (Supplementary Fig. S3b). Cross-validation by the LOO procedure showed medium discrimination power in all four groups (hit ratios: *flavidus* s.s.: 75%, *fernaldae*: 89%, *alpium*: 83%, *lutescens* s.s. only Pyrenees: 80%); in almost all cases, misclassification occurred between *flavidus* and *fernaldae,* or between *alpium* and *lutescens* s.s., respectively (Supplementary Table S6).

When the overlapping groups were merged into two clusters (*flavidus* = *flavidus* s.s. + *fernaldae*, *lutescens* **stat. nov.** = *alpium* + *lutescens* s.s.), the Procrustes ANOVA revealed statistically significant differences in wing shape between *B. flavidus* and *B. lutescens* **stat. nov.** ($F = 21.26$, $p < 0.001$), even though the PCA showed some clustering with overlap (Fig. 3a). The LDA revealed that the two clusters were well differentiated (Fig. 3b) and, in the cross-validation, the hit-ratio significantly increased (hit-ratios: *flavidus* = *flavidus* s.s. + *fernaldae*: 100%; *lutescens* **stat. nov.** = *alpium* + *lutescens* s.s.: 93%; Fig. 3).

A morphometric analysis of the malar space ratio (length/width) of the females showed significant differences between the two groups of the *flavidus* complex ($p < 0.01$, Fig. 4a; Supplementary Table S7).

In the *B. sylvestris* complex, the LDA showed two well-separated groups (Fig. 5b), while the PCA analysis showed some clustering with overlap (Fig. 5a), and the Procrustes ANOVA revealed statistically significant differences in wing shape between *B. sylvestris* s.s. and *B. popovi* **stat. nov.** ($F = 10.40$, $p < 0.001$). Cross-validation by the LOO procedure showed high hit ratios (hit ratios: *sylvestris* s.s.: 99%, *popovi* **stat. nov.**: 87%). A morphometric analysis of the labral furrow width relative to the labrum width of the females showed significant differences between the two taxa ($p < 0.01$, Fig. 4b; Supplementary Table S7) as well as for the labral lamella width (Supplementary Table S7; Supplementary Fig. S4).

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Fig. 2 Median-joining haplotype networks of (a) *B. flavidus* and *B. lutescens* stat. nov., (b) *B. sylvestris* and *B. popovi* stat. nov., and (c) *B. norvegicus* and *B. sichuanicus* sp. nov., based on sequences included in dataset DS2 prior to collapsing into unique haplotypes. Colours indicate geographic provenance, each cross-bar represents a single nucleotide change, small black circles represent putative missing haplotypes, and the size of each circle is proportional to the number of sequences sharing that specific haplotype, as indicated in the legend. (d) Geographic provenance of the species' sequences analysed in this work, with colours referring to the locality groups used in the haplotype networks. Points in white indicate sequences used in dataset DS1 only (i.e., sequences with missing or ambiguous data).

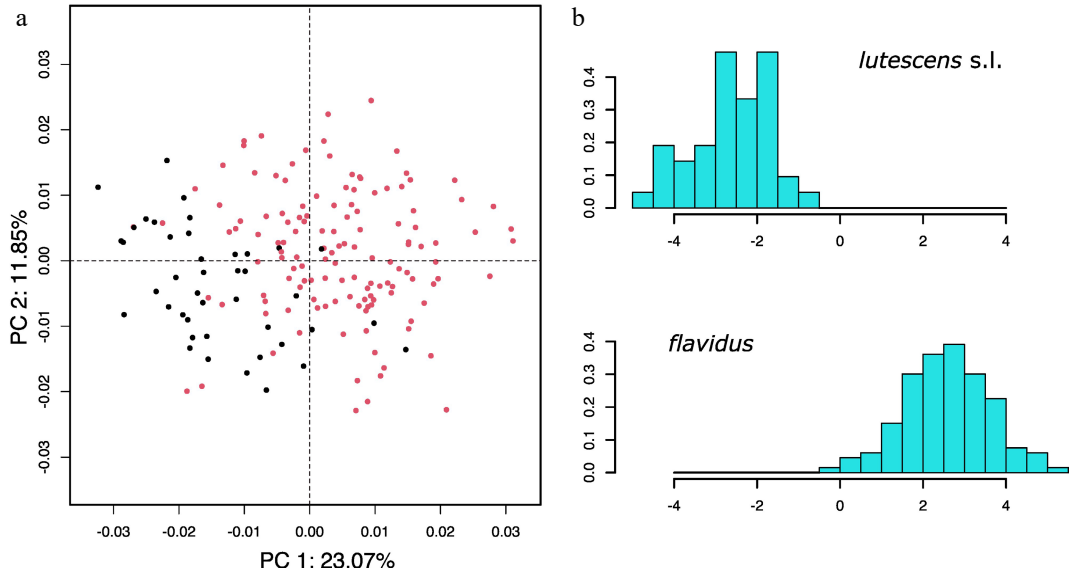

Fig. 3 Male wing morphometrics of the *B. flavidus* complex population groups. (a) PCA analysis of the wing venation landmarks showing the first two principal components (red: *flavidus* = *flavidus* s.s. + *fernaldae*, black: *lutescens* stat. nov. = *alpium* + *lutescens* s.s.). (b) LDA analysis of the first dimension of two groups: *flavidus* (*flavidus* s.s. + *fernaldae*) and *lutescens* stat. nov. (*alpium* + *lutescens* s.s.).

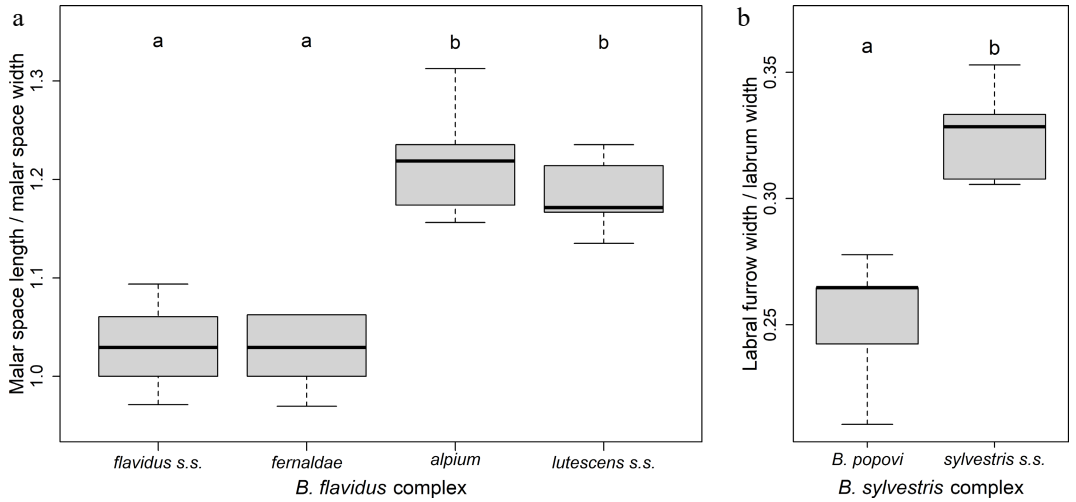

Fig. 4 Female morphometrics in the (a) *B. flavidus* complex: malar space ratio between length and width across the four *taxa*; (b) in the *B. sylvestris* complex: labral furrow width relative to labrum width between the two taxa. Significantly different groups are marked with a different letter; real values are in Supplementary Table S7.

Morphological evaluation

In the species complexes of *B. flavidus*, *B. sylvestris*, and *B. norvegicus*, the females revealed several major differences in the features of their body parts (Table 1).

Integration of evidence and taxonomic evaluation

Integrating evidence for the species complexes that are the main object of study resulted in taxonomic changes. For all species that are not the main focus of the study (e.g., *B. quadricolor*, *B. barbutellus*, etc.), no formal morphological evaluation was carried out in this study; therefore, the genetic data will need to be integrated with more evidence in future studies to test species hypotheses in those taxa.

B. sichuanicus Biella sp. nov.

In the *B. norvegicus* complex, two distinct population groups with clearly different distribution areas show remarkable differences in COI sequences, gene tree reconstruction, and morphology. Therefore, a new species is described; a novel name is given here because the other existing names refer to specimens originally collected in other parts of the world and are therefore inappropriate for the geographic extent of the new taxon (Supplementary Text S2).

ZooBank registration statement: http://zoobank.org/urn:lsid:zoobank.org:act:26105AAE-83AE-42EF-86B3-BACFBB917CF9; http://zoobank.org/urn:lsid:zoobank.org:pub:3D9B753C-941B-478F-9FA0-AD1BA845FCAB

Distribution: the populations spanning Europe, central-north Asia, and Japan belong to the species *B. norvegicus* (Sparre Schneider, 1918); the populations in southeast continental Asian mountains (Sichuan, Yunnan) belong to a new species for which no names are available for specimens from this region. *B. sichuanicus* Biella **sp. nov.** is here recognized from specimens in southeast Asian mountains in China (Sichuan, Yunnan), occurring at high elevations; it is geographically separated from the newly defined range of *B. norvegicus*.

https://doi.org/10.48130/jzser-0026-0016

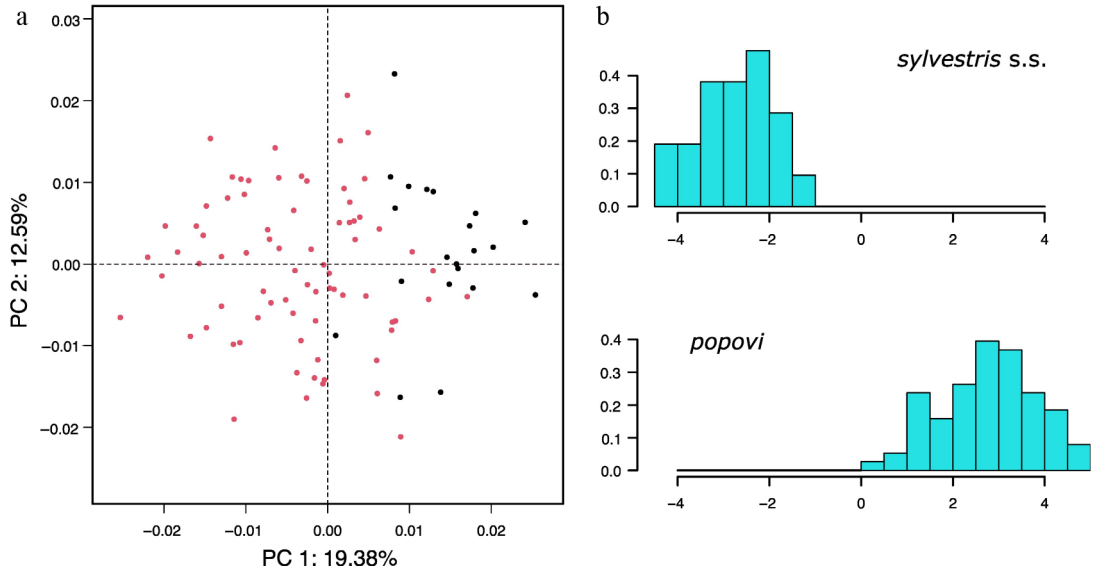

Fig. 5 Male wing morphometrics of the *B. sylvestris* complex population groups. (a) PCA analysis of the wing venation landmarks showing the first two principal components (red: sylvestris s.s., black: *popovi* stat. nov.). (b) LDA analysis of the first dimension in the two groups.

Table 1. Morphological differences in superficial characteristics of the females in the studied species complexes.

Complex	Species	Sex	Body part	Diagnostic characters
norvegicus complex	*Bombus norvegicus*	Female	Head	- Vertex with long and short yellow hairs; in dark specimens the vertex may have some black long hairs. - Labral lamella more defined from the labrum and with a semicircular profile.
			Thorax	- Collar band entirely yellow (sometimes with only a few isolated black hairs in the posterior part). - Collar more extended and with a sublinear posterior edge between the tegulae.
			Abdomen	- Callosities on S6 very swollen.
	Bombus sichuanicus **sp. nov.**	Female	Head	- Vertex with long black hairs, some short plumate hairs are light in color among long black hairs. - Labral lamella less defined from the labrum and with a more sublinear profile.
			Thorax	- Yellow collar with many long black hairs mixed with the yellow ones. - Yellow collar narrower and with a posterior edge concave (down-facing C shape).
			Abdomen	- Callosities on S6 weakly swollen.
sylvestris complex	*Bombus sylvestris* s.s.	Female	Head	- Vertex and collar band on the thorax with a dark yellow coloration, ocraceous (especially in fresh specimens). - Labrum with a wide furrow of about 1/3 (or more) of the labrum width. - Labral lamella wide (about 2/5 of the labrum width)
			Thorax	- Coat more hirsute with hairs of varying length
	Bombus popovi **stat. nov.**	Female	Head	- Vertex and collar band on the thorax with a very light yellow coloration, nearly cream-colored. - Labrum with a narrow furrow, wide about 1/4 of the labrum width. - Labral lamella narrow (about 1/3 of the labrum width).
			Thorax	- Coat comparatively more even and short
flavidus complex	*Bombus flavidus*	Female	Head	- Ocello-ocular area with sparse punctures of a smaller diameter, so that comparatively the area is less intensely dotted. - Malar space with more scattered punctuation and of smaller diameter, the dots at a distance more than half-dot diameter; At the base of the compound eye, the punctures are closer together, while they are more spaced on the rest of the malar area. - Malar space shorter, equal to its width or only slightly longer.
	Bombus lutescens **stat. nov.**	Female	Head	- Ocello-ocular area with more numerous and denser punctures of larger diameter, especially towards the forehead. - Malar space with denser and stronger punctuation, the dots at a distance less than half-dot diameter or even adjacent to each other; At the base of the compound eye (usually up to the middle of the malar area), the dots are especially coarse and nearby, while the rest of the Malar space has dense fine dots with heterogeneous diameter. - Malar space longer than wide.

Diagnosis and description: Female (Fig. 6): medium-sized species (length 19–19.5 mm). Coloration of the face black, except some short light plumose hairs on the vertex, malar area of width similar to its length and with small punctures densely covering the half near the compound eye but anteriorly fading, labrum with a labral lamella that is slightly defined from the labrum and with a sublinear distal profile;



Fig. 6 *B. sichuanicus* Biella sp. nov. holotype, female.

thorax with anterior band (collar) with a yellow coloration and with long black hairs, concave shape that slightly extends toward the episternum below the tegulae, rest of the thorax black (no posterior light band); abdomen with metasomal terga 1–2 black (occasionally a few yellow hairs at the side of T1), tergum 3 black with postero-latereally white hairs, tergum 4 with white hairs laterally; tergum 5 black, tergum 6 with short light hairs, terga 4–5 more sparsely punctate than tergum 3, tergum 6 with a longitudinal keel, sterna with orange and long black hairs, the callosities of sternum 6 are small, weakly swollen, the apex of S6 is down-curved and with a spinose projection beyond the apex of tergum 6; legs with black hairs, metatibia and metabasitarsus with the longest hairs of similar length as their longest width, wings mid brown. Comparative characters are given in Table 1. Male: body length 14–15 mm, hair of the thorax with a narrow, anterior yellow band (collar) extending toward the episternum below the tegulae, rest of the thorax black, metasomal tergum 1 yellow, tergum 2 black, tergum 3 black anteriorly and dark yellow posteriorly, tergum 4 pale yellow, tergum 5 mostly with dark hairs, last tergites black with orange hairs (especially on T7), sternites with pale hairs; legs with orange (posterior legs) and pale yellow hairs (anterior legs), genitalia described in Williams et al.[58] (pages 156, 157).

Etymology: The new name, *sichuanicus*, refers to the region where the specimens were collected by Prof. van Asperen de Boer.

Material examined: Holotype: a female from Sichuan, China: Emei Shan, 25.05.1998, ca. 2,500 m, collection by van Asperen de Boer at the Naturalis Biodiversity Center (Leiden, The Netherlands), code RMNH.INS.1620904. Paratypes: three females from Sichuan, China: Emei Shan, 25.05.1998, ca. 2,500 m, collection of van Asperen de Boer at the Naturalis Biodiversity Center. Allotype: one male; location: China, Yunnan, Lijiang: Yulong Snow Mountain, 22.07.2018, 3,760 m, 27.015 N, 100.171 E, Plant: *Ligularia vellerea*; DNA barcoded, KIB collection (China): this male specimen shares the DNA identity in the COI region with the available COI sequences from our female specimens (Supplementary Fig. S1). Other material: one female from Sichuan: Emei Shan, PW collection. All of these were examined in this study and included in Supplementary Table S2.

B. norvegicus (Sparre Schneider, 1918)

In the same species complex, and described from Norway, a female lectotype is available and designated by A. Loken in 1984 (https://arts-databanken.no/arter/takson/77996/beskrivelse); types were examined in Williams et al.[58], and the morphology was treated in other studies (Supplementary Text S2); the nomenclature was previously discussed[9]. Its distribution is from Europe to East Russia and Japan, according to DNA-barcoded specimens (Supplementary Fig. S1). Given the geographic vicinity to DNA-barcoded specimens, specimens from Mongolia and northeast China[60] could be expected to belong to this species instead of the new taxon (to be confirmed by DNA barcoding). The synonyms, the existing names, and the location where they were described are in Supplementary Text S2.

B. popovi (Yasumatsu, 1933) stat. nov.

In the *B. sylvestris* complex, the investigated lines of evidence agreed in showing two different groups based on COI sequences, gene tree reconstructions, morphometrics, and morphology. Both population groups have clearly defined distribution areas, which allows us to attribute them to existing names: the populations spanning from Europe to Siberia belong to the species *B. sylvestris*; the populations from Central Asia to East Asia belong to a previously described subspecies that is here elevated to the new status of species. The taxonomic treatment of the two groups follows. *B. popovi* (Yasumatsu, 1933) **stat. nov.** includes specimens from Siberia, the Russian Far East, and Korea, belonging to this species: in this study, the specimens sharing the COI identity are from the Korean Peninsula (Gangwon-do[61]) and Russia (Primorsky Krai, Altai Republic, Novosibirsk Oblast, Khabarovsk Krai, Jewish Autonomous Oblast, Sakhalin, and Kamchatka Krai) (Supplementary Fig. S1); they also share wing morphometry and distinctive female morphology. In addition, given the geographic vicinity, specimens from Mongolia and northeast China could be referred to as this species, as noted for specimens from Jilin Province [27], and therefore it is probably the case for those from Shanxi[60]. The name *B. popovi* is chosen because *Psithyrus sylvestris* var. *popovi* Yasumatsu, 1933 is the only taxon name of the *sylvestris*

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complex that refers to specimens originally from East Asia, and specifically from Korea[59]. That taxon was later elevated to the subspecies level by the same author[62], and therefore it is a valid name (art. 45.6, ICNZ), which we here further elevated to species status. The morphological description of this taxon is available from regional studies and the original description[27,59,62,63]. The taxon was only described from the holotype collected in 'Kongo-san' (= North Korea, Kangwon Province, Mount Kumgang [Geumgangsan], 38.656 N, 128.105 E), 10.9.1931[59]; the author stated that the type specimen is located in the Kyushu University collection. Local curators searched the Yasumatsu collection at this institution but could not locate the specimen despite thorough efforts, and they confirmed that it is currently missing (ICZN art. 75.3.4). The type is not available at other renown Institutions, to the best of our knowledge. According to ICZN Article 75.1, the original holotype is not available, and hence we designate a neotype with the express purpose of clarifying the taxonomic status (ICZN art. 75.3.1): one female labelled Russia, Ussuri-Ssutschan, Tigrojawa: 21.06.1928, Collection ZMA, code ZMA.INS.648645 (ICZN art. 75.3.3 and 75.3.7). The chosen location is appropriate because, despite lying in a region neighboring Korea, this specimen is the known one that is geographically the closest to the locus typicus (the distance to the current administrative border with Korea is not high, being nearly 200 km, ICZN art. 75.3.5 and art. 75.3.6). This collection site surely falls within the geographic range of *B. popovi* because this specimen is from a site near the locations of specimens used in the genetic analyses, where DNA barcodes of the analyzed specimens showed high similarity with each other and with other *'popovi'* specimens from Korea. The morphology of the neotype matches the available descriptions of the taxon[27,59,62,63] and bears the distinguishable characters listed in Table 1 (ICZN art 75.3.4). Other examined material: Supplementary Table S2.

B. sylvestris (Lepeletier, 1832)

In the same species complex, *B. sylvestris* (Lepeletier, 1832) was described from Europe, originally as *Psithyrus quadricolor* var. *sylvestris* Lepeletier, 1832; its distinct species status from *B. quadricolor* had been established since old times[64]. Types were recently revised[24], and the morphology was treated in other studies (Supplementary Text S2). Its distribution ranges from Europe to the Caucasus and to the south of Western Siberia according to DNA barcoding (Supplementary Fig. S1). The synonyms, the existing names, and the location where they were described are in Supplementary Text S2.

B. lutescens (Pérez, 1890) stat. nov.

In the *B. flavidus* complex, two population groups emerge with clearly defined distribution areas and consistent differences in COI sequences, gene tree reconstruction, morphometrics, and morphology. Therefore, the populations spanning northern Europe, Asia, and North America belong to the species *B. flavidus* (Eversmann, 1852); the populations from southern Europe belong to a new species. To the latter, we attribute an existing name and we change its taxonomic status: *B. lutescens* (Pérez, 1890) **stat. nov.** includes specimens from both the Pyrenees and the Alps (central and southern Europe), given that they share DNA barcodes (Supplementary Fig. S1), wing morphometry, and distinctive female morphology. Among the available names, the oldest one referring to specimens from southern Europe within the *B. flavidus* complex is *Psithyrus quadricolor* subsp. *lutescens* Pérez, 1890, described from the Pyrenees (Supplementary Text S2). Although the *'lutescens'* name could appear problematic (originally an infrasubspecific rank and potentially overlapping later defined names within *Bombus*), it is valid under art. 45 and art. 23 of the International Code of Zoological Nomenclature[57]. Therefore, it can be used for the new species; hence, *B. lutescens* (Pérez, 1890) **stat. nov.** is here elevated to the species rank. Types were revised in a recent study[24], and the morphology was described in previous studies (Supplementary Text S2); an updated list of synonyms, existing names, and comments on the location where they were described is available (Supplementary Text S2).

B. flavidus Eversmann, 1852

In the same species complex, *B. flavidus* Eversmann, 1852 was described from a male specimen from the Asian part of Russia. Its distribution ranges from North America to northern Europe and Russia. Types were revised[11,24], and the morphology was described in previous studies (Supplementary Text S2); the synonyms, the existing names, and the location where they were described are in Supplementary Text S2.

Discussion

In this study, we investigated a group of kleptoparasitic bumblebees (cuckoos) belonging to the *B. flavidus* species group. Using an integrative approach that combined multiple lines of evidence, we assessed speciation and differentiation patterns among populations from several localities throughout the entire distribution range. Specifically, we evaluated mtDNA variation, evolutionary relationships, and morphological differentiation shown by populations of three species complexes. In all cases, the combined approaches coherently pointed out differences among populations that, under an integrative taxonomic framework, can be considered evidence of the existence of distinct lineages within each species complex; therefore, three new species are described or elevated from previous subspecies status.

The morphological evaluation highlighted that female features and male wing shape pointed to the existence of distinct groups within the species complexes. Wing morphology has been successfully used to study bumblebee speciation and cryptic species in other cases[7,65] and, also in our study, it represents a reliable option for differentiating evolutionary lines, clustering together partially differentiated and conspecific lineages due to stabilizing selection on wing shape[66]. Specifically, our study shows how wing morphometry distinguishes well the two separated groups within *flavidus* and *sylvestris* complexes, hence demonstrating the existence of morphologically well-defined lineages. In the PCA, the small observed overlap in a two-dimensional view is compatible with what was observed between distinct species in a previous study[65], and our additional tests clarified that the wing groups are distinct. In a study on the *flavidus* complex, west and east Nearctic *B. flavidus* populations were not sufficiently different in wing morphology[11]: but when extending the analysis to the entire species range, our study shows that southern populations in Europe are well differentiated from the others, which is also supported by our genetic analysis. Similarly, within the *sylvestris* complex, consistent differences were observed between two geographic lineages of East vs West Eurasia, in line with genetic data. Furthermore, female morphology and morphometrics confirmed the existence of distinct groups for the three species complexes, aligning with the male wing variation when available. Interestingly, morphological features in the three species complexes were present in previous studies: in the *B. flavidus* complex, color differences between European subspecies[24,67] and male sizes between North American subspecies[11] were noticed; in the *B. norvegicus* complex, the female morphological features of Southeast Asian species were detailed[58], but without comparison to other areas; in the *B. sylvestris* complex, some authors described females of the East Asian clade pointing out the pale-yellow coloration[62,63] and narrow labral furrow in contrast to European specimens[62], features that we

coincidentally found important for differentiating the two clades of this species complex.

The morphological differentiation overlaps with the results of the DNA-based analyses, as the geographic populations were also genetically distinct, and forming clear lineages. Intra- and interspecific distances were calculated to provide an informative view of the genetic variability of the case species. In fact, ranges of genetic distance reported in the literature are variable, depending on the taxa included and the presence of possibly dubious taxa, and in some cases they show overlaps between maximum intraspecific and minimum interspecific distances[61,68,69]. Additionally, the geographical scale of sampling has been demonstrated to have a critical impact on the application of DNA barcoding[70,71]. As already shown for other taxa, distance ranges can be group-specific, depending on the taxonomic extent of the analysis[72,73], and must therefore be taken cautiously for comparative purposes. Indeed, it cannot be excluded that adding genetic variability to the dataset may slightly vary the ranges documented here for the group. The genetic distance results are aligned with the existence of distinct lineages in the gene reconstruction tree, confirmed by all species delimitation algorithms, that also overlap with the morphological grouping. The robustness of our results is particularly supported by the comprehensive extent of our sampling and by the fact that we also characterized the genetic variation of all species of the ingroup. A comprehensive characterization of populations across the geographic range of each considered species complex is a crucial aspect of reliable taxonomic delimitation processes[74]. Nevertheless, it should be acknowledged that the genetic analysis in our study includes a unique locus, the mitochondrial COI. This genetic marker is ideal because it is a fast-evolving gene that is deemed to be the most likely to be informative for relationships near the species level in bumblebees[75]. The COI gene is often used for detecting recent (shallow) divergences that could be unsignalled by slowly evolving nuclear markers[76]. In the context of integrative taxonomy of bumblebees, the COI gene is frequently employed as a single, standard marker[7], especially in relation to the use of species delimitation algorithms[75]. Although adding other genetic markers could provide additional information, given that mitochondrial genes can sometimes overestimate species boundaries or reflect historical demographic structure, if nuclear markers were to be used, then it should be considered that common nuclear genes (e.g., ITS, PEPCK, EF-1α) are lowly variable in closely related bumblebee species and may offer little resolution[8,77], as clearly noted for the *B. flavidus* group[11]. This means that these nuclear genes are much less useful than mitochondrial genes when the scope is to test recent speciation hypotheses in bumblebees and therefore were not used in our study.

Although we aimed to be taxonomically and geographically complete, it was not possible to include some populations in the *B. bohemicus* sister group because no DNA sequences of the species *B. coreanus* were available, as well as from South and East Asian populations of *B. bohemicus*. Nevertheless, the ingroup is well represented, and small changes in the sister group composition would not dramatically overturn the results for the ingroup, while it is also important to stress that different evolutionary reconstructions, including a smaller set of species delimitation algorithms, could lead to different taxonomic conclusions.

Given that taxa other than the three focus complexes were included in the genetic analyses, it is possible to briefly discuss the status of several previously recognized subspecies[24] or eventual species hypotheses that emerged from the delimitation algorithms, although a dedicated, integrative study with a broad geographical framework would still be needed. In the other two species of the *flavidus* species group, *B. skorikovi* resulted as a unique clade, which would confirm its species status; in *B. quadricolor,* two clades emerged from the gene tree, but whether these clades deserve to be a taxonomic entity depends on additional detailed evidence to be analyzed (e.g., morphology). Hence, we conservatively confirm the current taxonomic view that *quadricolor* is a unique taxon, given that the known color differences are only partially geographically consistent[24] and other different morphological characters are currently not known. In the *B. bohemicus* species group, several subspecies of *B. vestalis* seem to be conspecific based on genetic data: the Sardinian endemic subspecies *B. vestalis sorgonis*, characterized here for the first time (sequence name ZPL_BRUNCU1B_ITA in Supplementary Fig. S1), the Corsican-Tuscan *B. vestalis perezi* (sequence LPRCW634_21_FRA in Supplementary Fig. S1), the south European *B. vestalis obenbergeri* (e.g. sequence UNIFI1491_25_ITA in Supplementary Fig. S1) are either genetically identical in the COI or only a few *bp* differentiated from other *vestalis*, that hence appears to be a unique clade, in line with a previous study[78]. In the *B. bohemicus* species, the genetic differences are shallow and do not highlight the existence of independent lineages, at least based on the localities and the population pool included here. A more puzzling scenario emerged for *B. barbutellus* because some species delimitation algorithms highlighted two distinct lineages (but not all of them); hence, based on the disagreement between species delimitation algorithms, we would confirm the taxonomic conclusion of its species status from a previous study[19]. In fact, we also highlight the need for future work on this species. At present, our study could potentially be viewed as a reference base for future studies on *B. quadricolor*, the *B. bohemicus* species group, and *B. barbutellus* with a comprehensive geographical and integrative approach.

Regarding the speciation processes leading to the new species recognized here, it is interesting to hypothesize about putative underlying speciation scenarios. However, it should be clearly noted that we did not formally assess the speciation mechanisms in our study, and the exact speciation patterns are often difficult to enclose in pre-defined categories[79,80]. Hence, the following discussion is to be considered simply as a potential interpretation of the current results, or as hypotheses to be carefully tested in dedicated future studies. The first possible speciation mechanism stems from the fact that these bumblebees are nest parasites of a specific host species, even if putative inquilinism in other species was recorded[81] or inferred from distributional mismatches[82]. It is not uncommon that speciation occurred after a host switch in kleptoparasite organisms[83,84]. In the case of the cuckoo taxa we studied, the main host of *B. sylvestris* is *B. pratorum* that does not extend its European range further east than central Siberia[24]; since *B. popovi* **stat. nov.** extends from Siberia to East Asia, where *B. pratorum* does not, it is possible that *B. popovi* **stat. nov.** switched its host to another species; perhaps also *B. lutescens* **stat. nov.** speciation could be connected to a host change, considering that in northern Europe the main host of *B. flavidus* is *B. jonellus,* but in southern Europe it is *B. monticola* instead[26,85]. In the *B. norvegicus* complex, *B. norvegicus* is a kleptoparasite of *B. hypnorum*, but in southern Asia the host species has speciated into a distinct lineage[86], and therefore it would be a plausible speciation mechanism that *B. sichuanicus* **sp. nov.** speciated following the differentiation of its host. As the knowledge on host–parasite relationships, crucial for cuckoo-bumblebee evolution, is very scarce[17,18], the mentioned mechanisms are presented as an interpretative hypothesis of the results and future understanding of host–parasite relationships in the context of parasite speciation processes are needed.

As another possible driver of speciation, the current geographic distributions show that speciation might have happened in populations at the periphery of the ancestral species ranges, with isolation from the main range. In our case, for the Holarctic *B. flavidus* and the Palaearctic *B. norvegicus* complexes, the newly recognized species occur on mountains located at the southern edges of the ranges. A

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Table 2. Summary of the process of evidence integration to reach a taxonomic conclusion for the studied species (with morphology and morphometrics evaluated only in the main target taxa).

	Clades	Species delimitation algorithms	Morphology and morphometrics	Taxonomic evaluation
B. flavidus complex	2	2	Two groups	*B. flavidus* and *B. lutescens* **stat. nov.**
B. norvegicus complex	2	2	Two groups	*B. norvegicus* and *B. sichuanicus* **sp. nov.**
B. sylvestris complex	2	2	Two groups	*B. sylvestris* and *B. popovi* **stat. nov.**
B. quadricolor	2	2	Not evaluated	Needing further study
B. skorikovi	1	1	Not evaluated	Confirmed
B. barbutellus	2	2 or 1	Not evaluated	Needing further study
B. bohemicus	1	1	Not evaluated	Confirmed
B. vestalis	1	1	Not evaluated	Confirmed
B. suckleyi	1	1	Not evaluated	Confirmed
B. citrinus	1	1	Not evaluated	Confirmed

The numbers indicate how many distinct groups are found by each approach.

great geographic separation occurs between the ranges of each new species and the rest of the complex they belong to, which hints at an allopatric process. As an additional element, the isolation is located at the periphery of the range that could be compatible with a scenario of speciation by isolation at the periphery, which is advocated to be frequent[87]. Also in the *B. sylvestris* complex, *B. popovi* **stat. nov.** occurs at the easternmost periphery of the Eurasian range. Isolated populations leading to differentiated lineages between Europe and Asia were recorded in several case studies in the Palaearctic[86,88,89]. However, in the case of the *sylvestris* complex, no large geographical delimitation nowadays occurs between the ranges of the two distinct lineages, perhaps because of isolation followed by range expansion, as pointed out as very likely for another bumblebee in the Nearctic[11], and it is a view reinforced by the known ability of bumblebees to track changes in climate by adjusting their distribution[90]. However, on the other hand, the hypothesis of parapatric speciation cannot be totally ruled out, in which populations in a continuous range speciated following the geographic variation in environmental conditions[91], also given the climatic variations and regionalization across Eurasia[92–94]. Only dedicated studies might shed further light on the possible speciation mechanisms.

Conclusions

By extensively sourcing specimens and compiling several lines of evidence, our study dealt with the speciation in a socially parasitic bumblebee species group that was previously neglected. Our study strongly supports the existence of three previously unrecognized or underrecognized lineages that we identified and established as distinct species, in light of an integrative zoological and taxonomic approach, summarized in Table 2, with a synonymic list of the nominal species in the *B. flavidus* species group provided in Supplementary Text S2. The study highlighted the existence of *B. lutescens* **stat. nov.**, *B. sichuanicus* **sp. nov.,** and *B. popovi* **stat. nov.**. Given its broad taxonomic and geographic coverage, this study could also serve as a basis for a future in-depth re-evaluation of *B. quadricolor*, *B. bohemicus* species group, and *B. barbutellus*. Understanding speciation patterns in other bumblebee lineages is now a renewed priority that could be fulfilled by a sound integration of multiple approaches, with implications also in the field of species conservation, such as for making Red Lists, for instance, and for their importance as pollinators.

Ethical statements

Ethical approval was not required for this study.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design, resources: Biella P; methodology: Biella P, Galimberti A; data collection: Biella P, Streinzer M, Byvaltsev A, Ødegaard F, Ramazzotti F, Sheffield C, Japoshvili G, Neumayer J, Proshchalykin MY, Ren ZX; data curation: Biella P, Maggioni D, Ramazzotti F, Galimberti A; analysis and interpretation of results: Biella P, Maggioni D, Streinzer M; validation: Biella P, Williams PH; draft manuscript preparation: Biella P, Maggioni D, Streinzer M; review and editing of the manuscript: Biella P, Williams PH, Byvaltsev A, Ødegaard F, Ramazzotti F, Sheffield C, Japoshvili G, Neumayer J, Proshchalykin MY, Ren ZX, Galimberti A. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All relevant data are openly accessible from the supplementary files and from online repositories: BOLD, https://boldsystems.org, project ZPLFP, and see Supplementary Table S1 for process ID codes of specimens and sequences; and the online repository Bicocca Open Archive Research Data (https://board.unimib.it/research-data) at the link https://doi.org/10.17632/dpmfbkrsvf.1

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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