



A worthy conservation target? Revising the status of the rarest bumblebee of Europe

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Abstract. 1. Against the context of global wildlife declines, targeted mitigation strategies have become critical to preserve what remains of biodiversity. However, the effective development of conservation tools in order to counteract these changes relies on unambiguous taxonomic determination and delineation.

2. In this study, we focus on an endemic bumblebee species recorded only from the highest altitudes of the Sierra Nevada (Spain), *Bombus reinigiellus* (Rasmont, 1983). The species has the smallest range of any European bumblebee, along with a restricted diet and an inability to disperse because of its isolated montane distribution, making it an appropriate conservation target. However, through an integrative taxonomic approach including genetics, morphometrics and semio-chemistry, we demonstrate the conspecificity of this taxon with one of the most common and widespread bumblebee species of Europe, *Bombus hortorum* (L. 1761). We assign a subspecies status to this endemic taxon (*Bombus hortorum reinigiellus* **comb. nov.**) shown to be different in colour and morphology but also in wing shape and relative wing size compared to the other conspecific subspecies.

3. Following our taxonomic revision, we reassessed the IUCN conservation status of *Bombus hortorum* both at the continental and Spanish scale. We then propose how historic climatic oscillations of the last Ice age could explain such a phenotypic divergence in a post-glacial refugium and highlight the critical role of establishing unambiguous taxonomic revision prior to any conservation assessment.

Key words. Conservation status, Hymenoptera, ice ages, integrative taxonomy, rare species, Red Lists, relictual populations, wild bees.

Introduction

Wildlife is rapidly declining globally, threatening the functioning and resilience of ecosystems (Scheffers *et al.*, 2016;

Sanchez-Bayo & Wyckhuys, 2019). Against this backdrop of negative population trends and species extinctions, targeted conservation strategies have become an ever more important tool for preserving what remains of wild populations. However, it is widely acknowledged that a basic requirement for designing and enacting a conservation programme for a targeted wild organism is an unambiguous taxonomic determination and delineation (Hey *et al.*, 2003; Mace, 2004). Red Lists and climatic atlases offer a clear example of this principal, since

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complete and unequivocal species inventories and distribution records constitute their starting point in terms of conservation assessments and projections (e.g. Settele *et al.* 2008; van Swaay *et al.*, 2010; Bilz *et al.*, 2011).

This issue of problematic taxonomic delineation for conservation purposes has been well illustrated in bumblebees (genus *Bombus*), a group of globally threatened pollinators. Bumblebees constitute a diversified and widespread group of cold-adapted bees that are key components of plant-pollinator communities in temperate and cold areas of the Northern hemisphere (Hegland & Totland, 2008; Woodard *et al.*, 2015). However, a high number of species show negative population trends (Williams & Osborne, 2009; Cameron & Sadd, 2020). Among the causes of these declines are climate change, intensification of agriculture including loss of flowering resources and increased pesticide use, as well as urbanisation and pathogen spillover (Cameron *et al.*, 2011; Rasmont *et al.*, 2015a; Potts *et al.*, 2016; Rollin *et al.*, 2020; Martinet *et al.*, 2021).

Apart from a conservation perspective, bumblebees have become an increasingly popular model in the fields of evolutionary biology (Tian *et al.*, 2019), biogeography (Williams *et al.*, 2018; Ghisbain *et al.*, 2020b) and integrative taxonomy (Martinet *et al.*, 2019; Williams *et al.*, 2020). However, despite the long history of classical taxonomic work (i.e. not involving molecular tools) on bumblebees compared to other bees (Williams, 1998), their effective conservation has remained challenging due to particularly low levels of interspecific morphological differentiation (Michener, 2007; Williams *et al.*, 2012, 2020) associated with highly variable intraspecific colour patterns (Williams, 2007; Hines & Williams, 2012; Ezray *et al.*, 2019; Tian *et al.*, 2019; Williams *et al.*, 2019; Ghisbain *et al.*, 2020a), making species level identification difficult. Delineation based on regular taxonomic tools (i.e. visual examination of the specimens) often fails to uncover correct phylogenetic affinities and levels of differentiation for more widespread taxa, resulting in over-split or over-lumped species that do not accurately represent the true diversity of the group. This delineation issue is particularly problematic for peripheral or allopatric populations showing tenuous morphological dissimilarities (e.g. cryptic taxa) within widespread species (Williams *et al.*, 2020). In this context, the use of genetic and semiochemical characters to delineate species in integrative frameworks has led to profound changes in the accepted taxonomy of species and their associated distributions (Martinet *et al.*, 2019; Ghisbain *et al.*, 2020a; Lhomme *et al.*, 2021). In Europe, for instance, the consequences of such reassessments are crucial, with an increasing need to revise and update the assessments and conclusions presented in the last European Red List of Bees (Nieto *et al.*, 2014).

In this study, we examine for the first time the case of a bumblebee taxon *Bombus reinigiellus* (Rasmont, 1983) (Fig. 1), endemic to the highest altitudes of the Sierra Nevada (Spain) (Rasmont, 1983; Rasmont *et al.*, 2015a). The species was described based on the combination of a unique colour pattern along with specific morphological features (Rasmont, 1983). In its original description, *Bombus reinigiellus* was diagnosed from the two putatively closely related taxa *B. hortorum* (L., 1761) and *B. asturiensis* (Tkalců, 1974), the latter now considered

conspecific with *B. hortorum*. Following the original description of *Bombus reinigiellus*, the species status of the taxon was first contested and synonymised with *B. hortorum* based on morphological characters (Castro, 1987). A year later, however, the same author highlighted key morphological and colour differences that separated both males and females of *reinigiellus* from *hortorum* and revised *B. reinigiellus* as a valid species (Castro, 1988). The taxon *reinigiellus* has continued to generate debate, with authors considering *reinigiellus* as a synonym of *hortorum* (e.g. Williams, 1998), although a commonly held view is to consider *reinigiellus* as a separate, valid, endemic species (Ortiz-Sánchez & Ornos, 2004; Verdú & Galante, 2005; Barea-Azcón *et al.*, 2008; Nieto *et al.*, 2014; Rasmont *et al.*, 2015a; Ortiz-Sánchez *et al.*, 2018; Michez *et al.*, 2019).

Against the background of global bumblebee decline, a taxon like *B. reinigiellus* offers an excellent case study to address in a context of developing accurate conservation strategies for multiple reasons. Firstly, its highly restricted distribution range, with an area of occupancy estimated at 36 km², makes it the most localised bumblebee in the West-Palaearctic region (Rasmont *et al.*, 2015b). Its present conservation status, *Endangered* at the European level (Nieto *et al.*, 2014) and *Endangered* at the Spanish level, with its subsequent listing in important conservation inventories of Spain (e.g. Ortiz-Sánchez & Ornos, 2004; Verdú & Galante, 2005; Barea-Azcón *et al.*, 2008; Ortiz-Sánchez *et al.*, 2018) make *B. reinigiellus* of critical interest in a context of global bee decline. Finally, *B. reinigiellus* has a reported partial dietary specialisation with the genus plant *Aconitum* (Rasmont, 1983; Castro, 1988). Because plants of the genus *Aconitum* present flowers with elongate corollae that are almost exclusively pollinated by *Bombus* (Thøstesen & Olesen, 1996; Ponchau *et al.*, 2006; Gosselin *et al.*, 2013), and because *B. reinigiellus* is one of the only long tongued bumblebees found at such altitudes in southern Iberia (Rasmont *et al.*, 2015a), there could be a close relationship between both partners (Rasmont, 1983; Castro, 1988).

In this study, we aim to formally reassess the taxonomic status of this species using genetic, semiochemical, and morphometric characters (wing shape and size) and to re-assess its IUCN conservation status at both the continental and Spanish scales based on our taxonomic conclusions. We discuss how the climatic oscillations of the Quaternary might have trapped this taxon in a refugium and how this isolation might have led to a phenotypic, potentially adaptive differentiation. We finally discuss the importance of considering a strongly supported taxonomy as prerequisite for the effective implementation of a conservation status in such taxa.

Materials and methods

Data collection and identification

We sampled both male ($n = 6$) and worker ($n = 9$) specimens of *Bombus reinigiellus* in two localities of the Sierra Nevada (Spain) in August 2019 (Supporting Information file 1). We used the characters cited in the original description of Rasmont (1983) to morphologically align our freshly collected specimens with



Figure 1. Upper-left corner: lateral view of a queen of *B. hortorum hortorum*; upper-right corner: lateral view of the queen holotype of *Bombus reinigiellus* (Rasmont, 1983); bottom-left corner: facial view of a queen of *B. hortorum hortorum*; bottom-right corner: facial view of the queen holotype of *B. reinigiellus* (Rasmont, 1983). Source: Photo credit P. Rasmont. [Color figure can be viewed at wileyonlinelibrary.com]

the type series of *B. reinigiellus* (hosted in the Zoologische Staatssammlung, Germany, comprising a queen holotype and three queen paratypes), which enabled us to unambiguously attribute the name *B. reinigiellus* to our freshly collected individuals.

Examined traits

We examined a set of three informative traits to delineate bumblebee species, the first being a mitochondrial barcode fragment of the cytochrome oxidase I (*COI*), commonly used in taxonomic assessments (e.g. Martinet *et al.* 2018; Williams *et al.* 2019) as it presents a high substitution rate and shows rapid coalescence (Zink & Barrowclough, 2008; Baker *et al.*, 2009). This gene has been shown to accurately predict bumblebee species delineation in many large-scale studies (e.g. Williams *et al.*, 2012, 2019, 2020) and has been recently shown as a useful proxy of gene flow in a widespread cryptic bumblebee species complex (Ghisbain *et al.*, 2020a). It is, however, widely acknowledged that the use of *COI* must be always combined with other traits given that its high substitution rate can excessively separate taxa that are conspecific but with a strong population structuring (e.g. see examples in the *Bombus lapidarius*

complex; Williams *et al.*, 2020). Contrastingly, information provided by the *COI* fragment can also be misleading in the opposite direction, with individuals belonging to phenotypically distinct groups sharing barely distinguishable or even identical sequences for this gene (Gibbs, 2018).

As an additional line of evidence for delimiting species, we have therefore studied the cephalic labial gland secretions (CLGSs) of male bumblebees, an eco-chemical trait involved in the nuptial behaviour of most species (Ayasse *et al.*, 2001; Baer, 2003). They are widely used for both species delimitation and intraspecific variation assessment in bumblebees (Lecocq *et al.*, 2011, 2015a,b; Brasero *et al.*, 2015, 2020; Martinet *et al.*, 2018) as they constitute a main signal for pre-copulatory recognition between conspecific taxa (Baer, 2003). As far as is known, each bumblebee species produces a specific blend of these *de novo*-synthesised aliphatic compounds (Ayasse & Jarau, 2014; Bergström, 2008; Valterová *et al.*, 2019), although possible limitations in the interpretation of CLGS has been hypothesised (but not tested yet) in the case of allopatric taxa (e.g. Williams *et al.*, 2019).

Finally, geometric morphometric analyses were used as a proxy to quantify the phenotypic divergence of the examined specimens. Wing shape has been an increasingly utilised discriminant character for insect taxonomy (e.g. Grimaldi &

Engel, 2006) and especially in bumblebee taxonomy (Dehon *et al.*, 2019), although limitations of this approach have also been discussed (Lecocq *et al.*, 2015a; Gérard *et al.*, 2020). Briefly, closely related but heterospecific taxa are not always expected to present significantly distinct wing shape. However, the technique is non-destructive and has the advantage to allow quantified morphological comparisons with older material from which no genetic sequences can be obtained (Dehon *et al.*, 2019; Gérard *et al.*, 2020).

Overall, all these traits (*COI*, CLGS, wing shape) present context-dependent benefits and limits (discussed in the study by Lecocq *et al.*, 2015a; Valterová *et al.*, 2019; Williams *et al.*, 2019; Gérard *et al.*, 2020), depicting the importance to integrate multiple lines of evidence to draw more robust and resilient conclusions about the taxonomic status of bumblebees (see below).

Genetic analyses

DNA preparation and phylogenetic inference for *COI*. We followed the same DNA extraction technique, PCR methodology, and primer pair Jerry/Pat as Lecocq *et al.* (2015a). We included all subspecies of *Bombus hortorum* in our analyses: *Bombus hortorum hortorum* (distributed in most of Europe), *B. hortorum asturiensis* (Tkalčů) (restricted to Iberia and the Pyrenees) and *B. hortorum jonghei* Rasmont and Adamski (endemic to Corsica). Following the phylogeny of Cameron *et al.* (2007), we included in the analysis (i) the East Mediterranean *Bombus portchinsky* Radoszkowski; (ii) the three known subspecies of *Bombus ruderatus* (Fabricius): *Bombus ruderatus ruderatus* (distributed in Iberia), *Bombus ruderatus autumnalis* (Fabricius) (widespread across Europe) and *Bombus ruderatus corsicola* Strand (endemic to Corsica); and (iii) *Bombus argillaceus* (Scopoli). We chose *B. consobrinus* Dahlbom as an outgroup for our phylogenetic analysis. Detailed justifications of our primer choice and ingroup/outgroup selection are available in the Appendix 1 of the Supporting Information. A Bayesian phylogenetic inference analysis on the *COI* sequence was carried out under the GTR + G model, the most appropriate one according to MEGA-X (Kumar *et al.*, 2018). The model was selected following the corrected Akaike information criterion (AICc). The parameters for the analysis and check for stationarity follow Ghisbain *et al.* (2020a,b) and subsequent mixed yule-coalescent model for species delimitation (bGMYC) follows Reid and Carstens (2012) (both analyses detailed in Appendix 2 of the Supporting Information). Newly obtained genetic sequences were deposited on GenBank (accession number MZ093449).

Semio-chemical trait analyses

CLGS were extracted from male heads by submerging the latter in 400 µl of n-heptane, which was then stored at -40 °C prior to the analyses adapted from De Meulemeester *et al.* (2011). CLGS composition was determined by gas chromatography-mass spectrometry (GC/MS; Appendix 3). All samples were analysed with a gas chromatograph-flame ionisation detector

with the same chromatographic conditions as in GC/MS (Appendix 3 Supporting Information). We elaborated a data matrix as the alignment of each compound between samples performed with GCAAligner 1.0 (Dellicour & Lecocq, 2013a,b). A clustering method computed with the unweighted pair-group method with average linkage (UPGMA) based on correlation distance matrices was used (R package ape; Legendre & Legendre, 2004; Paradis *et al.*, 2004) to assess the divergence between the taxa. We transformed data [$\log(x + 1)$] to reduce the great difference of abundance between highly and lowly concentrated compounds. *P*-values calculated by multiscale bootstrap resampling with 1,000,000 bootstrap replicates (significant branch support > 0.85) were used to assess the uncertainty in hierarchical clustering (R package pvclust; Suzuki & Shimodaira, 2011). A permutational multivariate analysis of variance (perMANOVA; R package vegan; Oksanen *et al.*, 2011) using a distance matrix was performed to assess CLGS differentiation between taxa (including a total of 97 specimens, Supporting Information file 2). A pairwise multiple comparison test with Bonferroni correction (i.e. an adjustment of *P*-values to avoid type I errors) was performed when a significant difference was detected.

Phenotypic traits analyses

We used a dataset of 120 individuals including of 37 workers of *Bombus hortorum* [including the nominal subspecies (19 specimens), *jonghei* (6 specimens), and *asturiensis* (12 specimens)], 40 workers of *Bombus ruderatus* [including the nominal subspecies (20 specimens) and *corsicola* (20 specimens)], 19 workers of *Bombus argillaceus*, 15 workers of *Bombus portchinsky* and 9 workers of *Bombus reinigiellus*. The left forewing was photographed, and its shape and shape variation were captured and compared among individuals following Dehon *et al.* (2019) and Gérard *et al.* (2020) (complete protocol detailed in Appendix 4 of the Supporting Information). Prior to the assignment, shape variation within the reference dataset and species-level discrimination were assessed by linear discriminant analyses (LDA) on the projected aligned configuration of landmarks. LDA effectiveness for discriminating species was assessed by the percentage of individuals that were correctly classified to their original taxon [hit-ratio (HR)] with a leave-one-out cross-validation procedure based on the posterior probabilities (PPs) of assignment (Gérard *et al.*, 2015). LDA effectiveness for discriminating species was assessed by the percentage of individuals that were correctly classified to their original taxon (HR) with a leave-one-out cross-validation procedure based on the posterior probabilities (PPs) of assignment (Gérard *et al.*, 2015). We assessed the morphological affinity of *Bombus reinigiellus* based on the score in the predictive discriminant space of shapes. The aligned coordinates of the species from the reference dataset were used to calculate the LDA, including a posteriori *B. reinigiellus* in the computed LDA space as 'unknown' specimen and calculating its score. The Mahalanobis distance between 'unknown' and the group mean of each species in the dataset was used to estimate the assignment (Claude, 2008). PPs of assignment were calculated to confirm the assignment to each species.

Following the results of our taxonomic framework and of the analyses of wing shape (see results), we further explored the phenotypic differentiation of *reinigiellus* relative to all subspecies of *B. hortorum*. For each specimen of these taxa, we measured the intertegulae distance (ITD), a proxy of body size in bumblebees and the size of the wing centroid as a proxy of wing size (Gérard *et al.*, 2020). We investigated whether the ratio wing centroid/ITD differed between *reinigiellus* and other *hortorum* subspecies, in other words if the size of the wings relative to the size of the overall body differed, as either an adaptation to conditions in higher altitude or genetic drift. Because the data did not follow a normal distribution, we applied a Mann–Whitney–Wilcoxon test to compare the two groups.

Decision framework and taxonomic status

Although definitions of species vary widely (e.g. Mayr, 1961; De Queiroz, 2007), we follow here the unified theoretical species concept, considering them as independently evolving lineages (De Queiroz, 2007). Because the traits examined as part of species delineation are expected to have diverged at different rates in the process of speciation, we applied an integrative taxonomic practice consisting in examining multiple lines of evidence in search of corroboration (Padial *et al.*, 2010; Schlik-Steiner *et al.*, 2010). Following this framework, a species status was conferred to a taxon that is (i) a reciprocally monophyletic lineage coalescent in *COI* supported by the Bayesian implementation of the general mixed yule-coalescent model for species delimitation (bGMYC) and (ii) significantly differentiated in its semiochemical traits (i.e. a distinct cluster with a significant result in perMANOVA and bootstrap supporting value > 0.85; Hillis & Bull, 1993). Specific status was attributed only if those criteria converge. Although this strict framework could lead to an underestimation of species differentiation, it helps prevent the abuse of species status that could lead to ‘taxonomic inflation’. Hair colour could not be used as an operational criterion for delineation at the species level as colour patterns can be shared by long-separated heterospecific bumblebee taxa (Ghisbain *et al.*, 2020a; Williams *et al.*, 2020). This character is also strongly variable at the intraspecific level (Martinet *et al.*, 2018; Williams *et al.*, 2019) and can be affected by several adaptive pressures at the local level such as Müllerian mimicry (Ezray *et al.*, 2019; Ghisbain *et al.*, 2020a). Similarly, sister species can show very similar wing shape (Gérard *et al.*, 2020), and this trait is therefore used here to assess variation at a population-level only.

We recognise the concept of subspecies as an allopatric taxon not diverging in all lines of evidence but still presenting an original combination of traits, including morphological traits (e.g. wing shape and size, colour pattern) or ecological traits (e.g. unique trophic association with a particular resource, divergence in semiochemical signals to attract a conspecific mate) (Hawlitschek *et al.*, 2012; Lecocq *et al.*, 2015a; Martinet *et al.*, 2019; Brasero *et al.*, 2021; Lhomme *et al.*, 2021). Our choice to formally recognise subspecies has the combined advantage of drawing attention to distinctive populations that vary in potentially adaptive traits and to prevent an artificial

taxonomic inflation at the specific level (Isaac *et al.*, 2004). It also provides an effective short cut for future estimations of intraspecific diversity (Phillimore & Owens, 2006) and retains a legal taxonomic status as part of conservation plans (IUCN Standards and Petitions Subcommittee, 2019; CITES 2019), as already shown in other animals (Haig *et al.*, 2006; Storch *et al.*, 2006), including insects (New, 2011; Braby *et al.*, 2012) such as bumblebees (Cejas, 2021).

Reassessment of conservation status

Based on our taxonomic conclusions, we re-assessed the conservation status of the lineages of interest following the standardised protocol implemented by the International Union for Conservation of Nature (IUCN) (e.g. Nieto *et al.*, 2014). The occurrence data and maps used in the analyses were generated using distribution data published in the study by Rasmont *et al.* (2015a) and Polce *et al.* (2018) enriched with significant Iberian data from Penado *et al.* (2016). Because the extinction risk of a species can vary widely in relation with the examined scale, we focused on two geographical scopes: (i) a continental-level for geographical Europe (see framework in Nieto *et al.*, 2014) and (ii) a country-level analysis for Spain. At both spatial scales, we measured the area of occupancy (AOO) and extent of occurrence (EOO) of *B. hortorum*. The AOO is the measure of the area in which a species occurs and corresponds to the sum of the area of grids the species occupies. We defined square grids of 5 km × 5 km, as previously suggested for bumblebees (Drossart *et al.*, 2019). The EOO is a measure of the geographic range size of a species and is calculated by drawing a convex hull, which is defined as the smallest polygon containing all the sites of occurrence and in which no internal angle exceeds 180 °C.

Results

Trait differentiation

Genetic trait. The Bayesian inference conducted on the *COI* (Fig. 2) coupled with the bGMYC partition (Supporting Information Figure 1) highlights the presence of four candidate species strongly supported by the posterior probabilities of the inference (>0.95) within our ingroup: *Bombus argillaceus*, *B. hortorum* (including *reinigiellus* from the Sierra Nevada, *asturiensis* from Iberia, the nominal subspecies *hortorum* and the Corsican *jonghei*), *B. portchinsky* and *B. ruderatus* (including the currently accepted subspecies *autumnalis*, *corsicola* and *ruderatus*). The taxon of interest, *reinigiellus*, is therefore unambiguously contained as a lineage within the widespread *B. hortorum* with no evidence of population structuring or reciprocal monophyly, and with individuals of *reinigiellus* presenting identical sequences to specimens belonging to the subspecies *asturiensis* from Iberia.

Semiochemical trait. The results obtained from the analysis of CLGS are largely in agreement with those obtained with

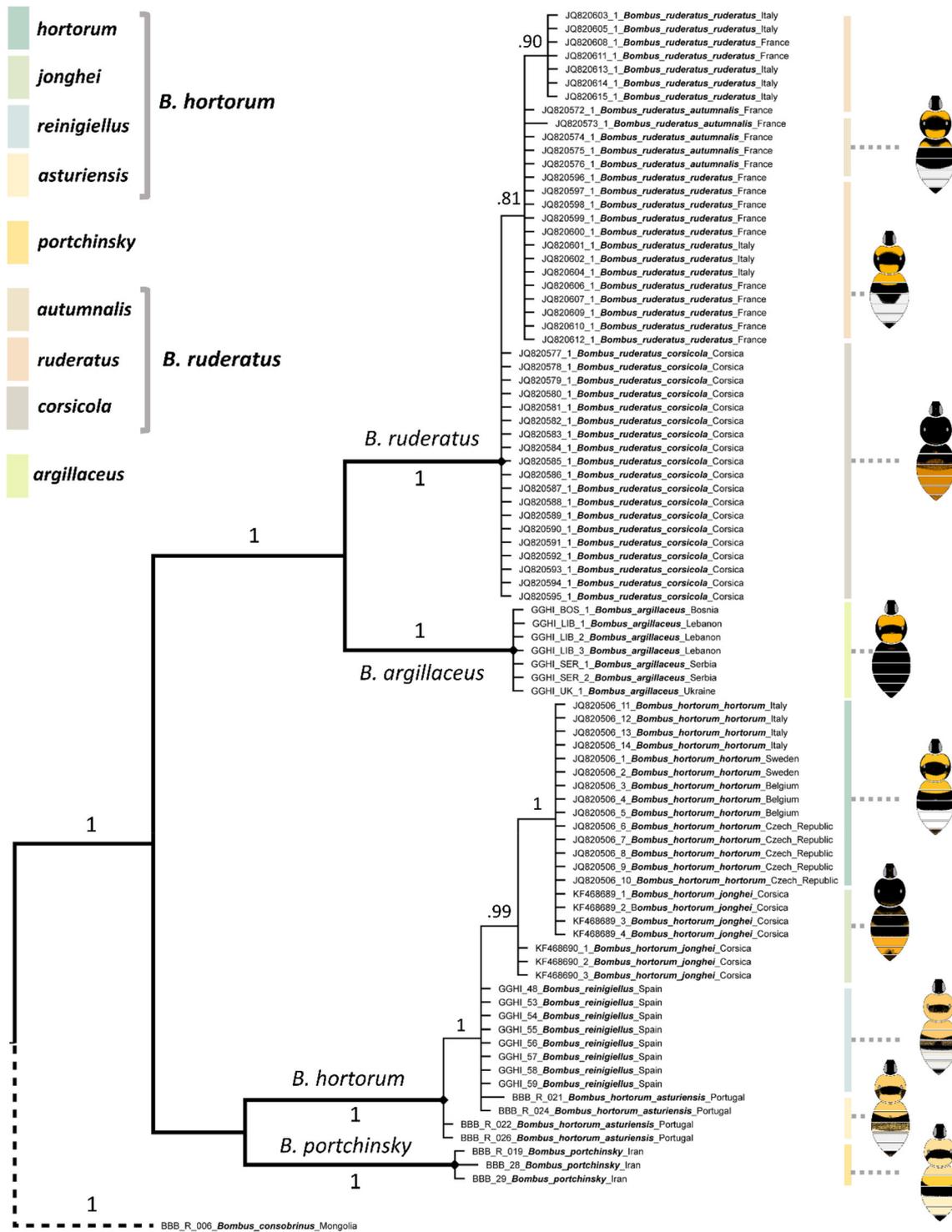


Figure 2. Bayesian phylogeny including *reiniellus* and its closely related taxa based on the mitochondrial barcode fragment of the cytochrome oxidase I (*COI*). Clade support values are the Bayesian posterior probabilities. [Color figure can be viewed at wileyonlinelibrary.com]

the phylogenetic analysis, revealing the presence of five lineages (*argillaceus*, *hortorum*, *portchinsky*, *ruderatus* and *corsicola*, but see Lecocq *et al.*, 2015 for the latter taxon differentiation)

in our ingroup (Fig. 3). The variability of the labial secretions of the taxon of interest, *reiniellus*, falls unambiguously within the broader variation displayed by *B. hortorum*, with no

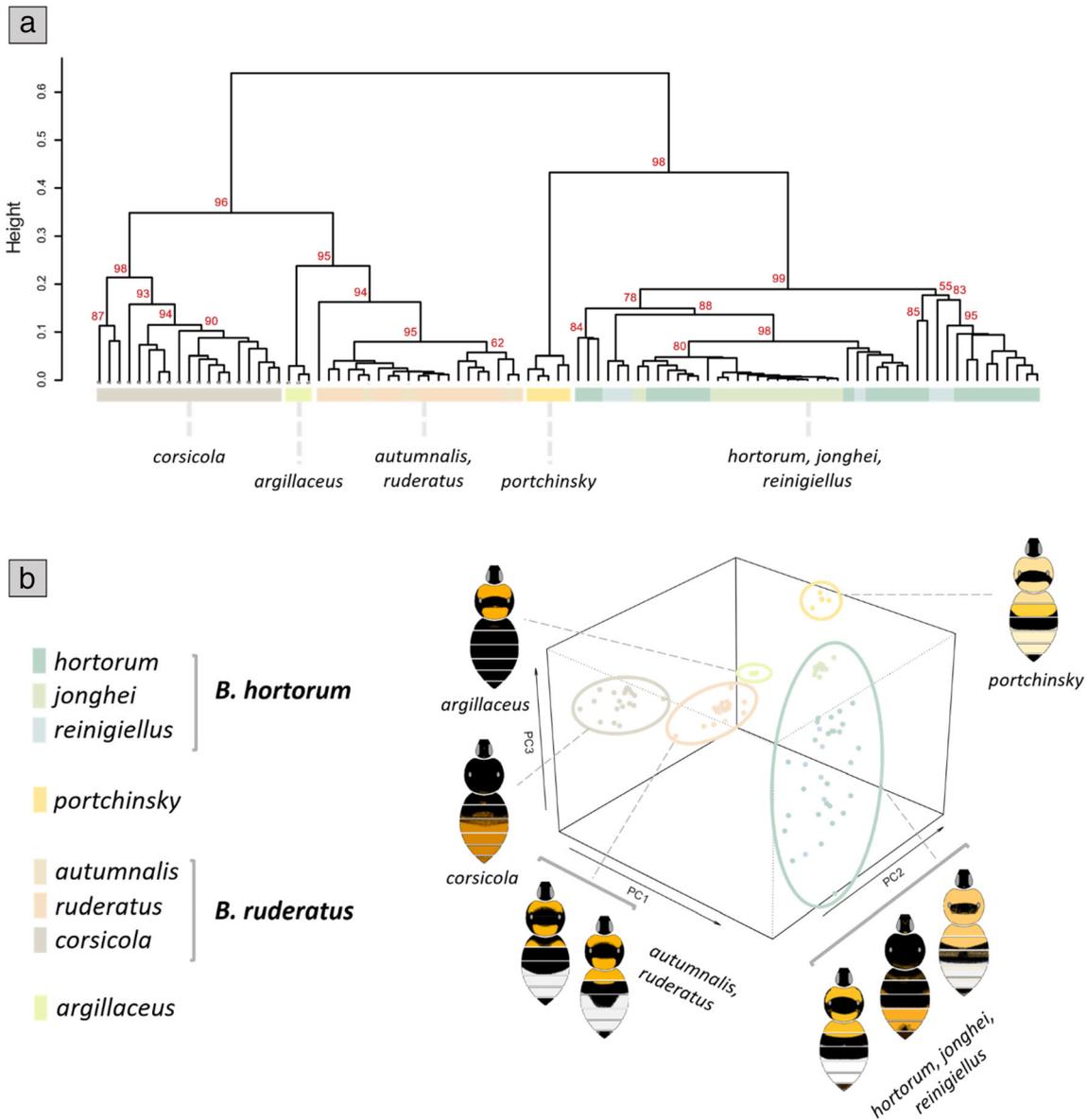


Figure 3. (a) Dendrogram of cephalic labial gland secretion (CLGS) differentiation within the subgenus *Megabombus*, including the taxon *reinigiellus* and its close relatives. This cluster was obtained by hierarchical clustering using an unweighted pair-group method with arithmetic mean (UPGMA) based on a Canberra matrix. The values near the nodes represent multiscale bootstrap resampling values. (b) PCA of CLGS differentiation within the subgenus *Megabombus* including the taxon *reinigiellus* and its close relatives. PC1, PC2 and PC3 are the first, the second and the third axes and explain 23.9%, 15.7% and 8.6% of the variance, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

evidence of population structuring. All *hortorum* taxa including *reinigiellus* share the same main compounds: the nonadec-9-ène (17.63–40.80%) (Supporting Information file 2). PerMANOVA test confirms that *reinigiellus* is not significantly different from other *hortorum* taxa ($F = 0.87$, $P = 0.65$).

Phenotypic trait. Based on the leave-one-out cross validation procedure, all taxa (*B. argillaceus*, *B. hortorum*, *B. ruderatus*, *B. reinigiellus*, *B. portchinsky*) were correctly

separated. Only one of the wings of *Bombus reinigiellus* was attributed to *Bombus hortorum*. Four taxa had HRs of 75% or higher: *B. argillaceus* (HR = 78.95%), *B. hortorum* (75.68%), *B. portchinsky* (80%) and *B. ruderatus* (85%). *Bombus reinigiellus* specimens were less correctly identified, with a hit ratio of 66.67% (Supporting Information file 3). In addition, the wing centroid size/ITD ratio was significantly different between *reinigiellus* and *hortorum* ssp. ($W = 235$, P -value = 0.0067), with *reinigiellus* presenting a larger body relative to wing size relative

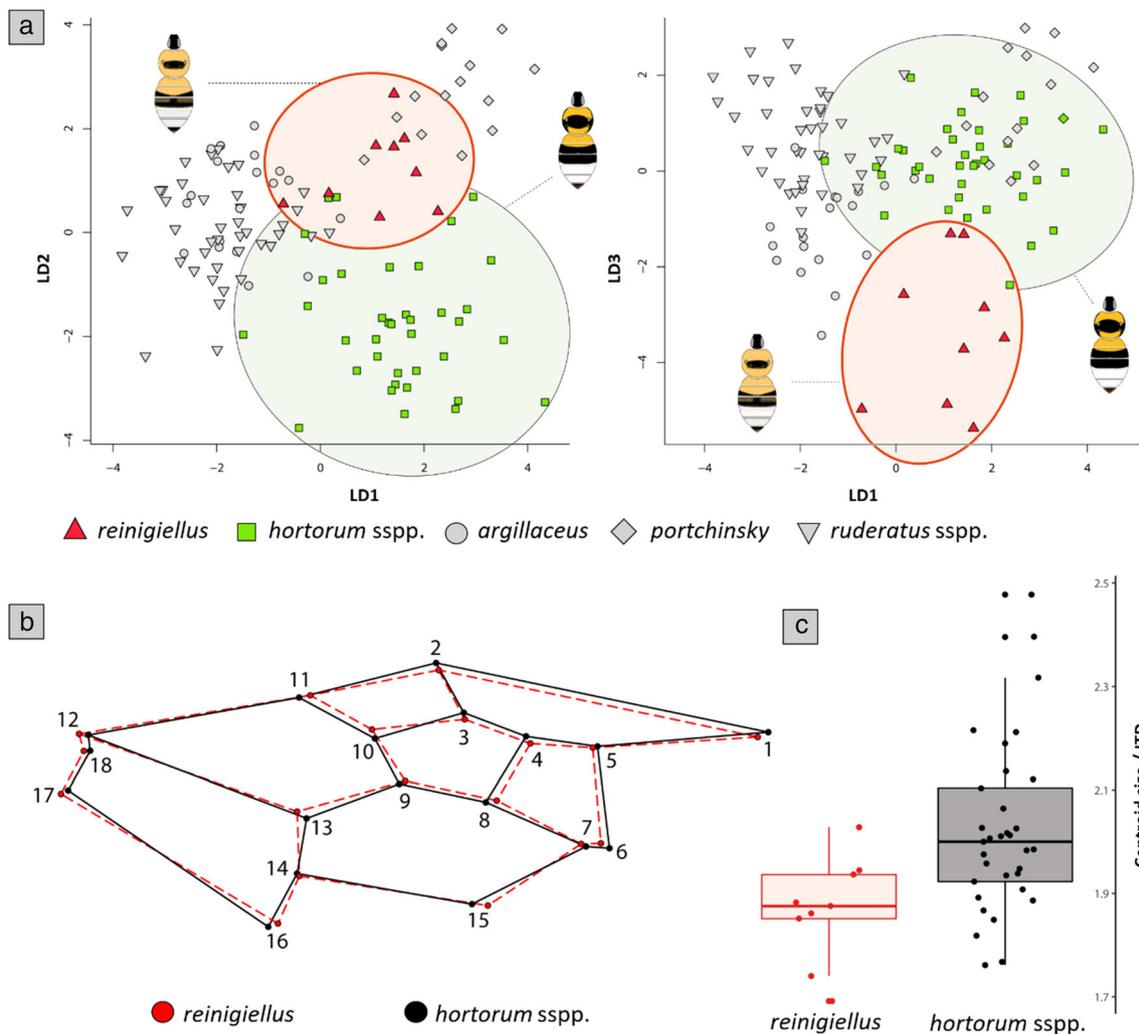


Figure 4. Wing differentiation of *reinigiellus* compared to other subspecies of *hortorum*. (a) Left: ordination of the studied taxa (total = 120 specimens) along the two first axes of the linear discriminant analysis (explaining 40.48% and 26.33% of the variance, respectively). Right: ordination of the studied taxa (total = 120 specimens) along the first and third axes of the linear discriminant analysis (explaining 40.48% and 19.21% of the variance, respectively). (b) Difference between *hortorum* spp. (black) and *reinigiellus* (red) in wing shape. (c) Difference in wing centroid size/ITD ratio between *hortorum* spp. ($n = 33$) (black) and *reinigiellus* (red) ($n = 9$). [Color figure can be viewed at wileyonlinelibrary.com]

to all subspecies of *hortorum* (Fig. 4d; Supporting Information file 4).

Decision framework of taxonomic status

Type revision and taxonomic decision. We revised the holotype (and paratypes) of *B. reinigiellus* which enabled an unambiguous identification of our freshly collected specimens with the taxon *reinigiellus* (Fig. 1). Integrative evidence based on the congruence of the examined traits (*COI*, *CLGS* and wing shape) indicates that the taxon *reinigiellus* (Rasmont, 1983) is contained within *B. hortorum* (L. 1761), the latter bearing year priority for the specific epithet. The taxon *reinigiellus*, being infraspecific to that of *hortorum*, can therefore be formally used

as a novel combination as a subspecific epithet applied to the allopatric population of the Spanish Sierra Nevada, *Bombus hortorum reinigiellus* **comb. nov.** (see discussion).

IUCN assessment

The AOO and EOO of *B. hortorum* both at the continental and Spanish scale are available in the Appendix 5 of the Supporting Information. The inclusion of *reinigiellus* in *B. hortorum* at the European scale increases the EOO by more than 70,000 km² (Fig. 5). At the Spanish scale, the inclusion of *reinigiellus* increases the EOO by more than 48,000 km². According to the criteria of the IUCN, the conservation status of *B. hortorum* is *Least Concern* both at the continental and Spanish scales. At a

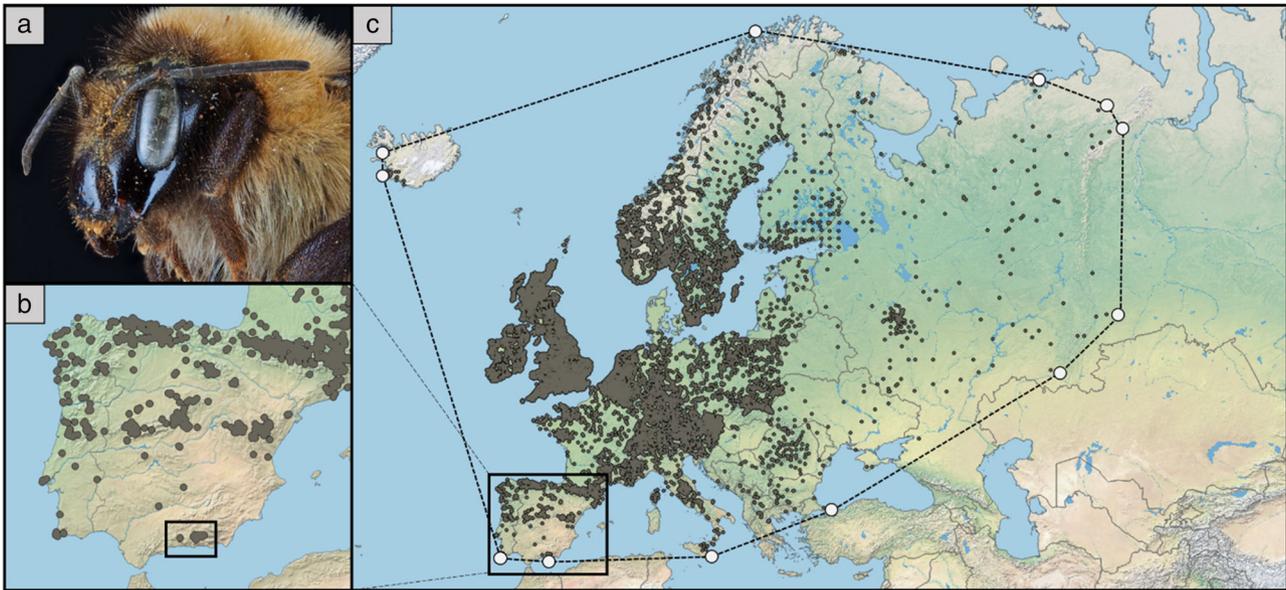


Figure 5. (a) Lateral view of the head of *Bombus reinigiellus* (Rasmont, 1983), revised here as *B. hortorum reinigiellus*, a subspecies of the widespread garden bumblebee *B. hortorum* (L. 1761). Photo credit P. Rasmont. (b) Occurrence data for *Bombus hortorum* in the Iberian Peninsula (data records belonging to *reinigiellus* are outlined with a rectangle). (c) Occurrence data for *B. hortorum* in Europe (*sensu* Nieto *et al.* 2014) with a polygon corresponding to its EOO. [Color figure can be viewed at wileyonlinelibrary.com]

subspecific level and according to our conservation framework (5 km cell width of AOO around each record), the conservation of *reinigiellus* follows the following IUCN criteria: B1 (small EOO) including B1a (severely fragmented) + B1b(iii) (continuing decline in area, extent and/or quality of habitat); B2 (small AOO) including B2a (severely fragmented) + B2b(iii) (continuing decline in area, extent and/or quality of habitat); and D (very small or restricted population). The lineage *reinigiellus* therefore constitutes an *Endangered* population.

Discussion

Trait divergence

Evidence integrating genetic, morphometric and semiochemical data does not support the species status originally conferred to the taxon *reinigiellus*. Our integrative framework unambiguously indicates its conspecificity with the widespread West Palearctic bumblebee species *B. hortorum* (Fig. 1). Based on our combination of markers, studied *reinigiellus* individuals do not constitute a coalescent based on the *COI* marker (and subsequently cannot be supported by the bGMYP analysis), are not significantly differentiated in their semiochemical secretions, but do however present a divergent wing phenotype. The lack of differentiation in the studied genetic marker might indicate that the population *reinigiellus* has only been isolated recently. Conversely, given the role of CLGS in the pre-copulatory recognition system of bumblebees (Ayasse & Jarau, 2014) and their subsequent usefulness in species delineation (Lecocq *et al.*, 2015; Martinet *et al.*, 2018, 2019), the similarity of the

latter compounds between *hortorum* and *reinigiellus* could imply maintained pre-copulative attraction between both taxa (Valterová *et al.*, 2019). Finally, our analyses of wing morphometrics support a differentiation in the wings of *reinigiellus* and showed that *reinigiellus* has significantly smaller wings relative to its overall body size (Fig. 4). Although this observation deserves to be further investigated at larger scales and across a larger set of populations, this morphological differentiation could represent either a phenotypic adaptation of the *reinigiellus* population to compensate for a lower air pressure and temperature in the high altitudes of the Sierra Nevada compared to other *hortorum* taxa living in lower elevations (Montejo-Kovacevich *et al.*, 2019), or a genetic drift following a long (near-) isolation of this population from other *hortorum* populations.

Taxonomic status

In bumblebees, a common practise to allow the recognition of similarly differentiated taxa is to formally designate subspecies. The level of differentiation of these subspecies can vary widely, ranging from (i) no genetic or semiochemical significant differentiation (e.g. based on allopatry and colour pattern, Lecocq *et al.* 2015); (ii) a low genetic differentiation accompanied with a dialectic semiochemical signal (e.g. Brasero *et al.*, 2020, 2021); (iii) a significant genetic differentiation with a dialectic semiochemical signal (Martinet *et al.*, 2018, but see Williams *et al.*, 2019). The present taxon *reinigiellus* falls in the first case where the taxon is isolated, presents tenuous but extant morphological and colour differences (Rasmont, 1983; Castro, 1988) and a distinct wing phenotype and a unique wing size/ITD ratio

but shows no molecular differentiation based on our two other traits (*COI* and *CLGS*). Lack of a such a distinctive molecular differentiation is not surprising here given the life history of the taxon *reiniellus* (see below), both in terms of mitochondrial and *CLGS* divergence. In the light of our data, we therefore formally confer a subspecies status to this high-altitude population of *Bombus hortorum* from the Sierra Nevada, *Bombus hortorum reiniellus* **comb. nov.**

Life history and biogeography

The differentiation of *B. hortorum reiniellus* relative to the widespread continental *B. hortorum hortorum* is reminiscent of the case of the insular *B. hortorum jonghei* from Corsica. In line with *reiniellus*, the Corsican population of *B. hortorum* is geographically isolated, phenotypically distinct, has raised controversy in the past over its taxonomic status, and ended up being part of the subspecific variation of the polytypic *B. hortorum* (Lecocq *et al.*, 2015a). The present phenotypic differentiation of *reiniellus* can most likely be attributed to the climatic oscillations and subsequent population shifts of the Quaternary in Europe (Castro, 1988). Colder periods might have triggered the southward expansion of *B. hortorum*, allowing the species to colonise the meridional part of the Iberian Peninsula. Later, when the climate warmed up, southernmost populations would have shifted towards the higher altitudes of the Sierra Nevada, following suitable climatic montane conditions. Progressively, the *hortorum* populations that were not able to reach higher altitudes would have disappeared from the southern part of Iberia to remain mostly in the colder mountain conditions in the north. This extended isolation of the *hortorum* population of the Sierra Nevada could explain its divergent phenotype through genetic and phenotypic drift, probably in combination with adaptations with the high-altitude conditions. Such dynamics of post-glacial isolation, often leading to differentiation or speciation (Avice, 2000; Hewitt, 2004), have previously been suggested in separate bumblebee lineages across several European mountain ranges (Reinig, 1937; Martinet *et al.*, 2018) and are likely to explain the unique phenotypic differentiation of *reiniellus*.

Overall, the present case illustrates the need to focus on allopatric, geographically restricted and phenotypically unique populations to fully comprehend both the life history of widespread, polytypic taxa (Avice, 2000; Hewitt, 2004). Understanding such phenotypic radiations driven by climatic oscillations is of central importance given that these phenotypic divergences explain much of the taxonomic confusion that have and continue to impede our accurate interpretation of the phylogenetic relationships of polymorphic species and relictual populations (Reinig, 1937; Martinet *et al.*, 2018; Williams *et al.*, 2020).

Conservation implications

Before this study, the taxon *reiniellus* was regarded as endemic to Spain, and as the rarest and most localised bumblebee species in Europe (Rasmont *et al.*, 2015a) and was listed as an Endangered species in the last Red List of European bees

(Nieto *et al.*, 2014). The assessment of Verdú and Galante (2005) included *reiniellus* and classified it as *Endangered* in Spain, in line with the later assessment of Nieto *et al.* (2014) and therefore deserving of conservation intervention as both a threatened European and Spanish endemic species.

By pooling the *reiniellus* records with those of *hortorum* in our conservation status assessments, we formally indicate that *reiniellus* must be now considered as a peripheral population of a species (*B. hortorum*) that is widespread across Europe, including in the Iberian Peninsula, common to abundant in most of its range, polytypic across its range, especially in insular conditions and categorised *Least Concern* both at the continental and Spanish scales. However, although *reiniellus* is not sufficiently well differentiated to hold a species status, its original combination of potentially adaptive morphological characters and colour pattern highlights the uniqueness of this restricted taxon, which represents one of the highest altitude populations of *B. hortorum* throughout its distribution (Rasmont *et al.*, 2015a). In addition to its alpine ecology in dry steppes (which is not the case for any other *hortorum* population), *reiniellus* is an important visitor of *Aconitum* (Ranunculaceae) species (Rasmont, 1983; Castro, 1988; G. Ghisbain & D. Cejas, field observations). *Aconitum* presents large, morphologically complex flowers that are pollinated almost exclusively by bumblebees (Thøstesen & Olesen, 1996; Ponchau *et al.*, 2006; Gosselin *et al.*, 2013). Two *Aconitum* species are found in the Sierra Nevada, *A. burnatii* and *A. vulparia neapolitanum* (Lorite, 2016), both of which are rare within Andalucía and are regionally assessed as *Vulnerable* and *Near Threatened*, respectively (Blanca *et al.*, 2011). Even if the taxonomic status of these *Aconitum* themselves is still under discussion (Raab Straube *et al.*, 2014), it is clear that in Andalucía, *Aconitum vulparia neapolitanum* populations are restricted to high elevations where they are probably pollinated almost exclusively by *reiniellus*, as one of the only long tongued bumblebee found at this altitude in southern Europe (Rasmont *et al.*, 2015a). Additional field work and pollen load analyses would be needed to formally quantify the strength of the interaction between *reiniellus* and *Aconitum* spp. in their shared ranges, and to assess whether this interaction might be endangered in near future by ongoing global changes.

Putting aside the level of differentiation and subsequent taxonomic status of *reiniellus*, it faces several threats and possesses several inherent traits that render it susceptible to global change and potentially in need of conservation. Firstly, the taxon has a small total distribution (IUCN criteria B2, AOO <500 km²), implying populations subject to isolation, as well as a weak dispersal potential due to its high-altitude ecology. Furthermore, its natural habitat is experiencing strong anthropogenic disturbance, leading to the fragmentation and destruction of numerous pieces of land where the taxon is already rare [IUCN criteria B 1b-2b (iii)], with its host plants becoming increasingly scarce (Ornosa & Ortiz-Sánchez, 2009). Much can be done to favour the resilience of these populations to future habitat and climate changes, and there is a growing consensus towards ensuring the spatial and temporal availability of sufficient floral cover and composition, a critical factor in sustaining bumblebee communities (Winfrey *et al.*, 2011; Folschweiller *et al.*, 2019; Drossart *et al.*, 2019). Diet quality and quantity are two of the

most fundamental aspects of the health and development of bumblebee colonies (Vanderplanck *et al.*, 2014, 2019; Moerman *et al.*, 2016) and their disruption is associated with severe patterns of decline at local and global scales (Williams, 1989; Williams & Osborne, 2009; Vray *et al.*, 2017, 2019). Consequently, we encourage the implementation of local conservation and mitigation strategies that ensure and continuously monitor the availability of bumblebee requirements within their flight range (resource plants) and restore small-scale habitat elements that provide targeted floral resources notably at the highest altitudes of the mountain chains in order to ensure the maintenance of population connectivity.

Overall, the present study illustrates the importance of establishing rigorous taxonomic foundations for conservation assessments in order to provide useful decision frameworks for policymakers and conservation organisations. These reassessments must incorporate a rigorous knowledge of unique ecological traits and interactions that render some populations worthy of interest for conservation purposes. Altogether, these efforts should eventually lead to allocation of the best context-dependent funding and management efforts for endangered, geographically restricted and endemic taxa.

Authors' contributions

G.G. designed the study and led the writing of the paper. B.M. and T.J.W. were involved in the writing and conceived important ideas for the study. G.G., B.M., T.J.W., K.P., D.C., and A.M. compiled critical data for the study. G.G., B.M., M.G. and I.V. analysed and interpreted the data. P.R. and D.M. contributed to reagents and materials. All authors discussed the results, edited and approved the content of the manuscript.

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DATA AVAILABILITY STATEMENT

All data supporting this manuscript are available as supplementary materials.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix 1. Detailed justifications of our primer choice and ingroup/outgroup selection (Word document).

Appendix 2. Detailed protocol for the Bayesian inference and species delineation test (Word document).

Appendix 3. Detailed protocol for the analysis of the cephalic labial gland secretions (Word document).

Appendix 4. Detailed protocol for the analysis of wing shape (Word document).

Appendix 5. Reassessment of the conservation status of *Bombus hortorum* at the continental and Spanish levels, including or not the subspecies *reinigiellus* from the Sierra Nevada (Spain).

Supplementary file 1 Summary of the sampling for both *COI* and wing shape analyses (Excel sheet).

Supplementary file 2 Matrix used for the CLGS statistical analyses (Excel sheet).

Supplementary file 3 Matrix of the Leave-One-Out analysis for wing shape (Excel sheet).

Supplementary file 4 Data for the analysis of relative wing (Excel sheet).

Supplementary figure 1 Species recognition pairwise matrix based on the maximum clade credibility tree obtained from BEAST, including *reinigiellus* and its consubgeneric closely related sister taxa.

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