

Resolving the species status of overlooked West-Palaeartic bumblebees

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Abstract

Multisource approaches in taxonomy gather different lines of evidence in order to draw strongly supported taxonomic conclusions and constitute the basis of integrative taxonomy. In the case of overlooked taxa with disjunct distributions for which sampling is more challenging, integrative approaches help to propose stable hypotheses at the species and subspecies levels. Here, based on genetic and semio-chemical traits, we performed an integrative taxonomic analysis to evaluate species delimitation hypotheses within a monophyletic group of bumblebees (Hymenoptera, Apidae, *Bombus*) including the formerly recognised subgenera *Eversmannibombus*, *Laesobombus* and *Mucidobombus* which are now included in the subgenus *Thoracobombus*. Our results demonstrate the conspecificity of several polytypic taxa, and we formally recognise the subspecies *Bombus laesus alicae* comb. nov. Cockerell, 1931, endemic to North Africa, based on its allopatry, unique mitochondrial haplotype and divergent cephalic labial gland secretions. This highlights the need to maintain studying polytypic complexes of bumblebee taxa for which phylogenetic relationships could be still entangled and eventually implement conservation strategies for taxonomically differentiated lineages.

KEYWORDS

Bombus, cryptic species, DNA sequences, integrative taxonomy, male marking secretion

1 | INTRODUCTION

The importance of the species status in biology makes its accurate definition essential (Mayr, 1969). However, criteria for delimiting species are still controversial (Agapow, 2005; De Queiroz, 2007). These disagreements are exemplified by the numerous species delimitation approaches using alternative diagnostic criteria (De Queiroz, 2007). While traditional taxonomy is mainly based on discrete morphological traits (Cipola et al., 2014; Ji & Du, 2014; Rampini et al., 2012), such traits can fail to detect species in taxon groups with low or no morphological differentiation (i.e., cryptic species) or in groups exhibiting large morphological variability at the intraspecific level (e.g., in some bumblebee species complexes: Carolan et al., 2012; Ghisbain, Lozier, et al., 2020; Williams et al., 2012, 2020). Subsequently, many attempts have been made to improve species delimitation by using alternative features such as shapes (Aytekin et al., 2007; Gérard et al., 2020), genetic markers (White et al., 2014), or semiochemical markers (Martin et al., 2008). Nevertheless, each of these approaches presents its own limitations (for bumblebees see review in Lecocq, Dellicour, et al., 2015; Williams et al., 2015). One solution is to use a multisource approach to gather different lines of evidence of speciation to robustly test taxonomic hypotheses (Arribas et al., 2013; Lecocq, Dellicour, et al., 2015; Roe & Sperling, 2007 a, b). The development of integrative taxonomy based on the unified species concept (USC) provides a methodological framework for this taxonomic approach (De Queiroz, 2007; Schlick-Steiner et al., 2010). Many biologists now agree with the USC, recognising that species are evolving fragments of metapopulation lineages where delimitation criteria do not evolve at the same rate (De Queiroz, 2007). Therefore, multiple operational criteria must be considered independently to evaluate taxonomic status (Schlick-Steiner et al., 2010).

When using a set of multiple independent traits, Padial et al., (2010) discussed the limitations of two commonly used frameworks called “integration by cumulation” and “integration by congruence”. The integration by cumulation assumes that all taxonomic characters are contingent and even one single character could be the basis for species delimitation Padial et al., (2010). This approach might, however, overestimate biodiversity by detecting intraspecific variation as species status (Padial et al., 2010). On the contrary, the integration by congruence is a strict approach in which two or more criteria must be divergent across taxa, although this method might underestimate biodiversity, being unable to detect recent domestication processes or cryptic species. Assigning subspecies taxonomic status to distinct allopatric lineages where differentiation is highlighted in at least one (but not all) criteria can be used as a rational alternative option to reduce the underestimate risk of the congruence approach (Hawllitschek et al., 2012; Lecocq, Brasero, et al., 2015).

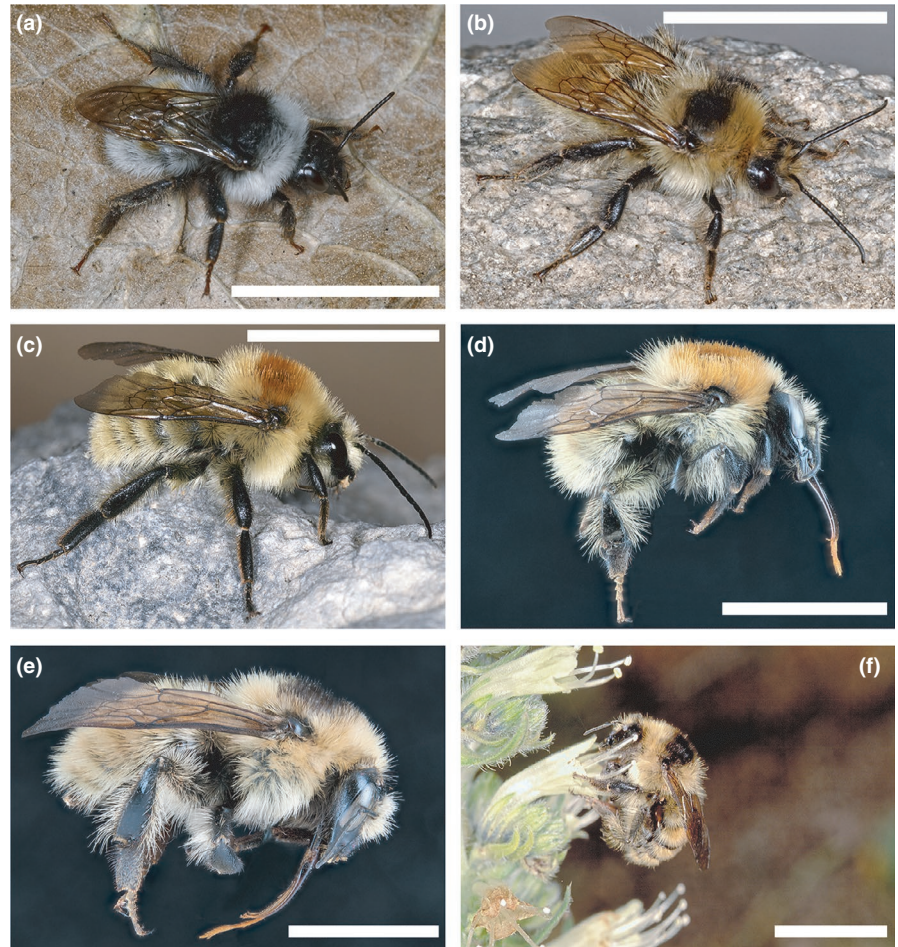
Here, we revise the taxonomic status inside a monophyletic group of bumblebees (Apidae, *Bombus*) including the formerly recognised subgenera *Eversmannibombus* Skorikov 1938, *Laesobombus* Krüger 1920 and *Mucidobombus* Krüger 1920, now all included in the subgenus *Thoracobombus sensu lato* (Cameron et al., 2007; Williams et al., 2008). The *Eversmannibombus* group includes a single taxon: *Bombus persicus* Radoszkowski 1881. Two subspecies that are phenotypically diagnosable by the coat colour have been described: (a) *B. persicus eversmanniellus* Skorikov 1923 with tergites 1 to 4 white mixed with brown on tergite 2 (Figure 1a) and (b) *B. persicus persicus* Radoszkowski 1881 with tergites 1 to 4 white fringed with yellow or completely yellow hairs. This species is geographically restricted to mountainous steppes in eastern Turkey, Northern Iran and the Caucasus. Most of this range corresponds to the subspecies *eversmanniellus* while the subspecies *persicus* is restricted to Northern Iran (Rasmont, Franzen, et al., 2015; Rasmont & Iserbyt, 2014).

The *Mucidobombus* group includes a single taxon: *Bombus mucidus* Gerstaecker 1869, located in the highest subalpine and alpine stage mountain ranges of Western Europe (Cantabrian, Pyrenees, Alps, Apennines, Balkan Mountains, and the Carpathians) (Rasmont, Franzen, et al., 2015). Three subspecies, phenotypically diagnosable by coat colour, are currently recognised: (a) *B. mucidus mollis* Pérez 1879 (Figure 1b) from the Cantabrian, Pyrenean and Western Alps mountains; (b) *B. mucidus* Gerstaecker 1869 from Central and Eastern Alps, Apennines, and Balkan Mountains; and (c) *B. mucidus pittioniellus* Tkalců 1969 from the mountains of the Balkan Peninsula (Delmas, 1976; Grandi, 1957; Rasmont & Iserbyt, 2014; Tkalců, 1960) (Figure 2).

Species status in *Laesobombus* have been the centre of a major debate (refer Table 1), although the common opinion is to consider two distinct species inside this former subgenus: *B. laesus* Morawitz 1875 (Figure 1c,d) and *B. mocsaryi* Kriechbaumer 1877 (Figure 1e,f), with a ginger and a black spot on the mesosoma, respectively. However, many taxa were previously described: *B. laesus* Morawitz 1875 from Turkestan (Semiretschensk region), later described as *B. laesus mocsaryi* from *maculidorsis* Skorikov 1922; *B. mocsaryi* Kriechbaumer 1877 from Hungary; and *Bombus mocsaryi aliciae* Cockerell 1931 from Morocco. Currently, despite their variation in coat colour, *B. laesus* Morawitz 1875 and *B. mocsaryi* Kriechbaumer 1877 are sometimes interpreted as a single species named *B. laesus* Morawitz, 1875 (Rasmont, 1983; Reinig, 1971; Williams, 1998; Williams et al., 2009) (Table 1). These taxa are found in a large part of the Palaearctic steppes and dry grasslands but have declined recently and are becoming extremely rare and localised in some areas (Rasmont, Franzen, et al., 2015) (Figure 3).

In this study, we apply an integrative taxonomic approach by congruence to the previously cited taxon by combining three operational and independent criteria commonly used in

FIGURE 1 Pictures of some bumblebee taxa examined as part of this study: (a) *Bombus persicus eversmanniellus* female from Turkey; (b) *B. mucidus mollis* male from Pyrenees, France; (c) *B. laesus* male from Turkey; (d) *B. laesus* female from Kyrgyzstan; (e) *B. mocsaryi* female from Kyrgyzstan; (f) *B. mocsaryi* female from Morocco. All white lines correspond to a scale of 1 cm. Photo credit P. Rasmont



bumblebee taxonomy: a mitochondrial DNA marker (*COI*), a nuclear DNA marker (*PEPCK*) and a semio-chemical trait (cephalic labial gland secretions of males, herein referred to as CLGS) and resolve the taxonomic affinities of these rare and poorly known West-Palaeartic taxa.

2 | MATERIAL AND METHODS

2.1 | Sampling

We sampled male and female specimens of all clades of interest across the West-Palaeartic region between 2001 and 2016 (Appendix S1, Table 2) and determined them based on their morphology and colour patterns (Pittioni, 1939). We attributed a taxon name to the specimens without a priori hypothesis as to their species status. The in-group comprises *eversmanniellus* from Turkey and Iran ($n = 5$); *persicus* from Iran ($n = 4$); *laesus* from Iran, Kyrgyzstan and Turkey ($n = 19$); *mocsaryi* from France, Hungary, Mongolia and Kyrgyzstan ($n = 20$); *aliceae* from Morocco ($n = 5$); *mollis* from France, Andorra and Spain ($n = 8$); *mucidus* from Switzerland, Austria and Italy ($n = 24$); and *pittioniellus* from Montenegro, Albania and Macedonia ($n = 10$).

We complemented the in-group with the six species from the *sylvarum*-group of bumblebees (Brasero et al., 2020), the sister group of our examined clades with the subgenus *Thoracobombus* (Cameron et al., 2007). The outgroup includes the related species *B. (Thoracobombus) mesomelas* ($n = 1$) (formerly included in the *Rhodobombus* subgenus) as well as the more distant species *B. vestalis* ($n = 1$), which belongs to the sister subgenus *Psithyrus*.

2.2 | Genetic trait analyses

We sequenced two genes commonly used in bumblebee phylogeny (Cameron et al., 2007; Williams et al., 2019): the barcode fragment of the fast-evolving cytochrome oxidase 1 (hereafter referred to as *COI*) mitochondrial gene (from 100 specimens), and the nuclear slow-evolving protein-coding gene phosphoenolpyruvate carboxykinase (hereafter referred to as *PEPCK*) (Appendix S1, Table 2). We carried out polymerase chain reaction (PCR) amplifications with primer pair LepF1/LepR1 (Hebert et al., 2003) for *COI* and FHv4-RHv4 (Cameron et al., 2007) for *PEPCK*. We performed sequencing procedures described in Lecocq et al., (2013). Sequences were aligned with BioEdit version 7.2.5 (Hall, 1999). The

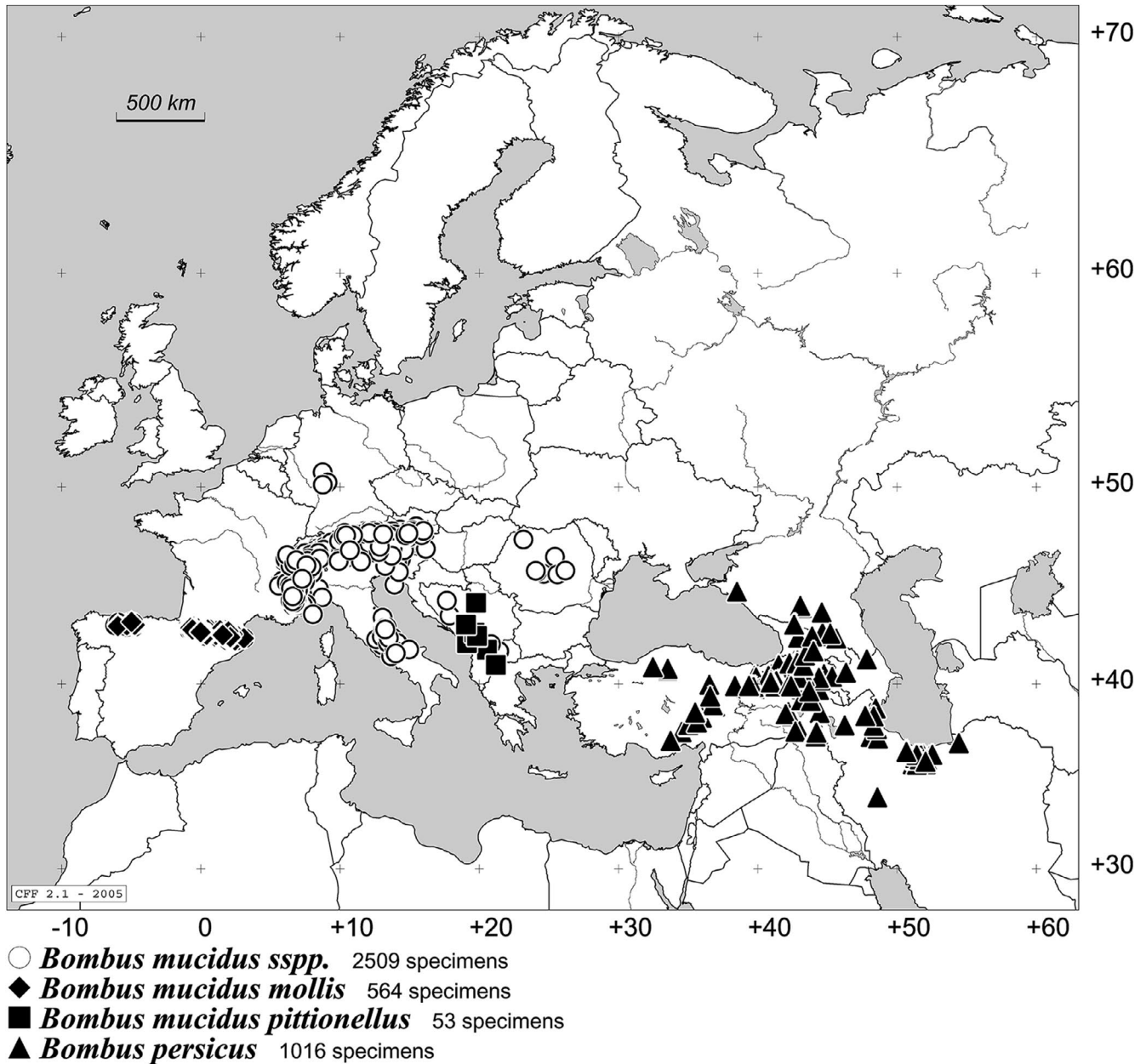


FIGURE 2 West-Palaearctic distribution of *Bombus persicus* and *Bombus mucidus* including *B. mucidus mucidus* (white circles), *B. mucidus mollis* (black diamonds) and *B. mucidus pittingiellus* (black squares). Adapted from Rasmont, Franzen, et al. (2015) with the taxonomic conclusions of the present study

final molecular datasets spanned 660 bp from *COI* and 889 bp from *PEPCK*. Sequences are available on GenBank (accession numbers in Appendix S1).

The phylogenetic analyses were performed for each gene independently with a Bayesian method (MB) to explore the genetic divergence as well as to define lineages within the different groups. We partitioned each gene to explore the best substitution model: (a) *PEPCK* into two exons and two introns; (b) *COI* and *PEPCK* exons by base position (1st, 2nd and 3rd). Each dataset was submitted to the Akaike information criterion corrected for small sample sizes (Hurvich & Tsai, 1989) to choose the best-fitting substitution models

with JModelTest Server version 2.0 (Posada, 2008). The chosen models were: (a) For *COI*: GTR + G (1st), HKY (2nd) and GTR + G (3rd); (b) for *PEPCK*, exon 1: F81 (1st), JC (2nd) and K80 + I (3rd); *PEPCK*, exon 2: JC (1st), TrN (2nd), and JC + I (3rd); *PEPCK*, intron 1: TPM uf + I; *PEPCK*, intron 2: TPM1 uf. For the MB method, we performed Bayesian inference analyses with MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). Ten independent analyses were achieved for each gene (100 million generations, four chains with mixed models, default priors, saving trees every 1,000 generations). We assessed convergence by examining (a) likelihood plots (for stationarity) and convergence statistics in

TABLE 1 List of taxa of the *Laesobombus* from previous taxonomic revisions

| Morawitz (1875) | Kriechbaumer (1877) | Radoszkowski (1888) | Skorikov (1922) | Cockerell (1931) | Panfilov (1956) | Williams (1998) |
|------------------|---------------------|---------------------|---|-----------------------------|---|------------------|
| - | - | <i>B. sidemii</i> | - | - | - | <i>B. laesus</i> |
| <i>B. laesus</i> | - | - | <i>B. laesus mocsaryi</i> <i>Form maculidorsis</i> | - | <i>B. tianschanicus</i> <i>B. laesus</i> | - |
| - | <i>B. mocsaryi</i> | - | - | <i>B. mocsaryi alicaeae</i> | <i>B. mocsaryi</i> | - |
| - | - | - | - | - | <i>B. maculidorsis</i> | - |

MrBayes version 3.1.2 and (b) ESS values in Tracer version 1.7.1 (Rambaut et al., 2018), which led us to conservatively discard the first 10 million generations as a burn-in procedure. A majority rule 50% consensus tree was constructed. We only considered as statistically significant the clades supported branch supports with high posterior probabilities (≥ 0.95) (Wilcox et al., 2002).

We applied bGMYC methods to the *COI* dataset by using R Package “bGMYC” (Reid & Carstens, 2012). A range of probabilities < 0.05 was considered as strong evidence that taxa were heterospecific while a range of probabilities 0.05–1 suggested that taxa were conspecific (Reid & Carstens, 2012). BEAST version 1.7.4 (Drummond et al., 2012) was used to generate ultrametric trees (required for bGMYC method) with a phylogenetic clock model to calculate a posterior distribution of trees (length of the MCMC chain: 1 billion generations). The first million sampled trees were burned-in using the maximum clade credibility method and setting the posterior probability limit to 0. The bGMYC analysis was based on 1,000 trees sampled every 10,000 generations. For each of these 1,000 trees, the MCMC was made of 100,000 generations, discarding the first 90,000 as burn-in and sampling every 100 generations. In order to provide a “heat map” of species delineation probability, posterior probability distribution has been applied against the first sample tree.

We also applied the Poisson Tree Processes (PTP) model, complementary to the GMYC model to avoid potential biases of the time-calibrating procedure and only relies on the number of DNA-nucleotide substitutions using the branch lengths from a metric gene tree (Zhang et al., 2013). We used the online implementation of the bPTP server (<https://species.hits.org/>) to recognise coalescent as evidence for candidate species in our dataset applying default PTP options, as already performed in integrative taxonomic approaches applied to bumblebee taxonomy in the past (Potapov et al., 2017; Williams et al., 2019).

2.3 | Eco-chemical trait analyses

We focused on the main reproductive trait involved in the bumblebee pre-mating recognition (Ayasse et al., 2001; Valterová et al., 2019): the cephalic labial gland secretions (CLGS) of males. The CLGS constitute a semio-chemical species-specific trait (Calam, 1969) providing efficient diagnostic characters for species delimitation (Martinet et al., 2018, 2019). They are complex mixtures of mainly aliphatic compounds synthesised de novo (Žáček et al., 2013) in the head of bumblebee males. By main compounds, we mean the compounds that have the highest relative amounts (RA) among all compounds of CLGS at least in one individual of the taxon. All specimens were killed by freezing at -20°C and the CLGS were extracted with 400 μl of heptane (method described in De Meulemeester

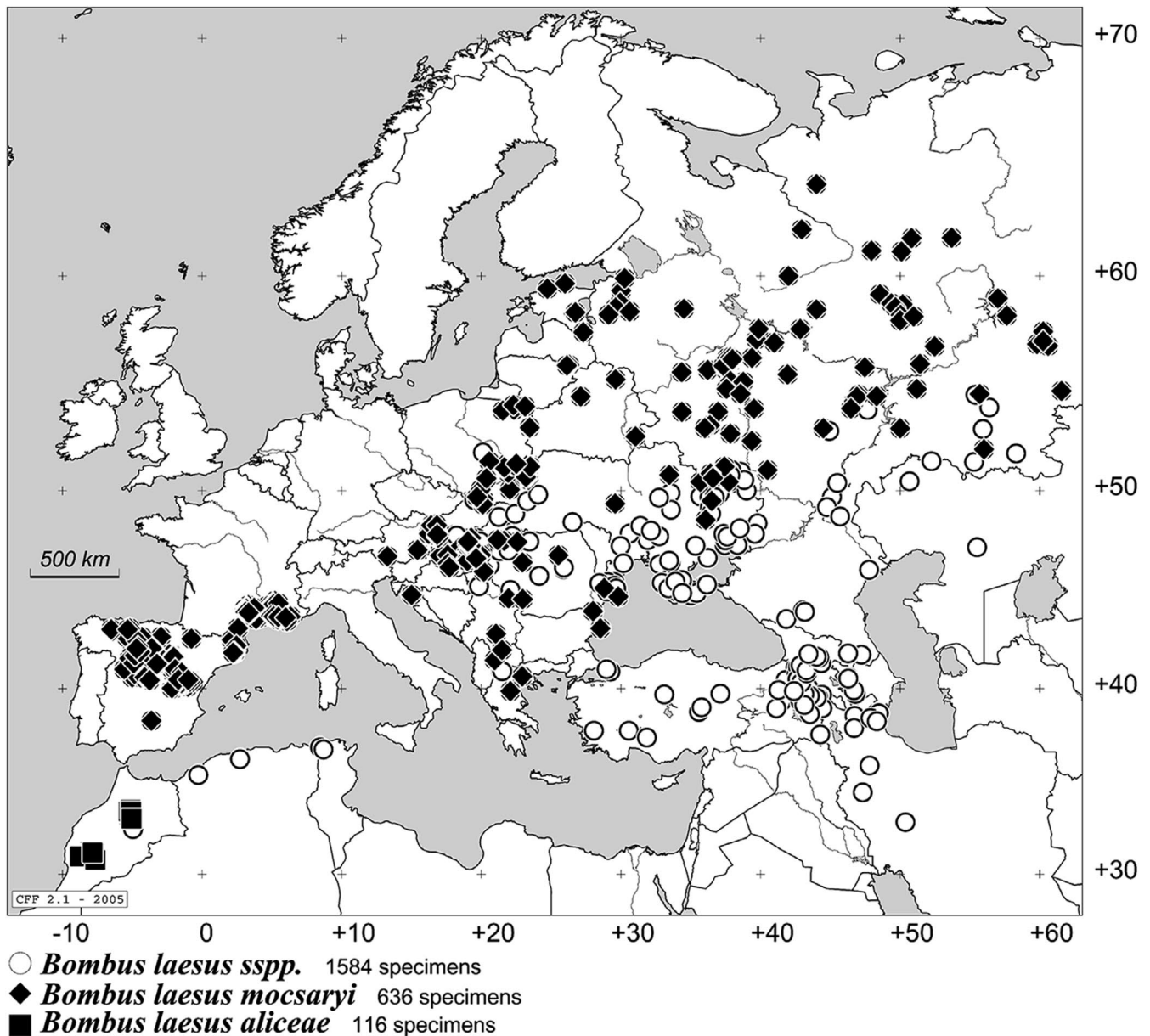


FIGURE 3 West-Palaearctic distribution of *Bombus laesus* including *B. laesus laesus* (white circles), *B. laesus mocsaryi* (black diamonds) and *B. laesus alicaeae* (black squares). Adapted from Rasmont, Franzen, et al. (2015) with the taxonomic conclusions of the present study

et al., 2011). Samples were stored at -40°C prior to analysis. In total, we were able to sample 35 specimens belonging to the three groups *Eversmannibombus*, *Laesobombus* and *Mucidobombus* (Appendix S1, Table 2).

We qualified the CLGS composition by gas chromatography–mass spectrometry (GC/MS) using a Focus GC (Thermo Scientific) with a non-polar DB-5 ms capillary column [5% phenyl (methyl) polysiloxane stationary phase; column length 30 m; inner diameter 0.25 mm; film thickness 0.25 μm] coupled to DSQ II quadrupol mass analyser (Thermo Scientific) with 70 eV electron impact ionisation. We used a splitless injection mode (220°C) and helium as a carrier gas (1 ml/min). The temperature program of the oven was set to 70°C for 2 min and then heated up at a rate of $10^{\circ}\text{C}/\text{min}$ to

320°C . The temperature was then held at 320°C for 5 min. Compounds were identified in XcaliburTM using the retention times (t_r) and mass spectra of each peak, in comparison with those at National Institute of Standards and Technology library (NIST, USA). Double-bond positions ($\text{C} = \text{C}$) were determined by dimethyl disulfide (DMS) derivatisation (Cvacka et al., 2008). We quantified the CLGS of all samples by a gas chromatograph–flame ionisation detector Shimadzu GC-2010 with a SLB-5 ms non-polar capillary column (5% phenyl (methyl) polysiloxane stationary phase; 30-m column length; 0.25-mm inner diameter; 0.25- μm film thickness) with the same chromatographic conditions as in GC/MS. Peak areas of compounds were detected in GCsolution Postrun (Shimadzu Corporation) with automatic peak detection and

TABLE 2 Number of *Bombus* females (F) and males (M) sampled for each operational criterion

| Taxa | | | nuDNA marker | mtDNA marker | |
|-------------------------|------------------------|--|--------------|--------------|------|
| Group | Taxon | Sampling country | (PEPCK) | (COI) | CLGS |
| <i>Eversmannibombus</i> | <i>persicus</i> | Iran | 3F | 4F | - |
| | <i>eversmanniellus</i> | Iran, Turkey | 2 M, 1F | 2 M, 2F | 3 M |
| <i>Laesobombus</i> | <i>laesus</i> | Iran, Kyrgyzstan, Turkey | 5 M, 13F | 6 M, 13F | 6 M |
| | <i>mocsaryi</i> | France, Hungary, Kyrgyzstan, Mongolia | 3 M, 12F | 5 M, 15F | 2 M |
| | <i>aliceae</i> | Morocco | 5 M | 5 M | 5 M |
| <i>Mucidobombus</i> | <i>mollis</i> | Andorra, France and Spain | 2 M, 4F | 3 M, 4F | 4 M |
| | <i>mucidus</i> | Austria, France, Italy and Switzerland | 17 M, 7F | 14 M, 4F | 15 M |
| | <i>pittioniellus</i> | Albania, Macedonia and Montenegro | 10F | 5F | - |
| Outgroups | <i>mlokosievitzi</i> | Brasero et al. (2020) | 3F | 3F | - |
| | <i>sylvarum</i> | Brasero et al. (2020) | 3 M | 3 M | - |
| | <i>veteranus</i> | Brasero et al. (2020) | 3 M | 3 M | - |
| | <i>inexpectatus</i> | Brasero et al. (2020) | 3 M | 3 M | - |
| | <i>runderarius</i> | Brasero et al. (2020) | 3F | 3F | - |
| | <i>velox</i> | Brasero et al. (2020) | 1F | 1F | - |
| | <i>mesomelas</i> | Brasero et al. (2020) | 1F | 1F | - |
| | <i>vestalis</i> | Data from Genbank and BOLD | 1F | 1F | - |

noise measurement. We calculated RA (in %) of compounds in each sample by dividing the peak areas of compounds by the total area of compounds. We discarded all compounds for which RA was recorded as <0.1% for all specimens (De Meulemeester et al., 2011). The data matrix (Appendix S2) for each taxon was based on the alignment of each relative proportion of the compound between all samples performed with GAligner version 1.0 (Dellicour & Lecocq, 2013). Before each sample injection, a standard (Kováts) containing a mix of hydrocarbons (alkanes) from C10 (decane) to C40 (tetracontane) was injected to facilitate the alignment and the identification of compounds. Kováts indices were calculated with GC Kováts version 1.0 (Dellicour & Lecocq, 2013). Clustering method was performed using R version 3.3.2 (R Development Core Team, 2020) to detect CLGS differentiation between taxa. We transformed data ($\log(x + 1)$) to reduce the great difference of abundance between compounds (De Meulemeester et al., 2011). A Pearson r correlation distance matrix based on the CLGS data matrix (RA of each compound) was computed. An unweighted pair group method with arithmetic mean (UPGMA) was used as a clustering method (R-package ape, Suzuki & Shimodaira, 2011). We assessed the uncertainty in hierarchical cluster analysis using p -values calculated via multiscale bootstrap resampling with 10,000 bootstrap replications (significant branch support > 0.85) (R-package pvclust, Suzuki & Shimodaira, 2011). We assessed CLGS differentiations between taxa by performing a permutation multivariate

analysis of variance using distance matrix (PerMANOVA) (R package vegan; Oksanen et al., 2011). When a significant difference was detected, we performed a pairwise multiple comparison with an adjustment of P -values (Bonferroni correction) to avoid the type I errors.

To determine the indicator compounds (IC) of each taxon, we used the indicator value (IndVal) method (Claudet et al., 2006; Dufrene & Legendre, 1997). The value given is the product of relative abundance and relative frequency of occurrence of a compound within a group. We evaluated the statistical significance of a compound as an indicator at the 0.01 level with a randomisation procedure.

2.4 | Decision framework of taxonomic status

We followed the decision framework proposed by Lecocq, Brasero, et al. (2015). This approach follows the USC (De Quieroz, 2007) and corresponds to the strictest commonly used framework called “integration by congruence” by Padiál et al., (2010). We thus assigned a specific status to a taxon (a) which is genetically differentiated in all genetic markers (i.e., unique haplotypes); (b) which constitutes a reciprocally monophyletic group with a highly supported branch support; and (c) with a significant differentiation in CLGS composition (including IndVal IC, PerMANOVA

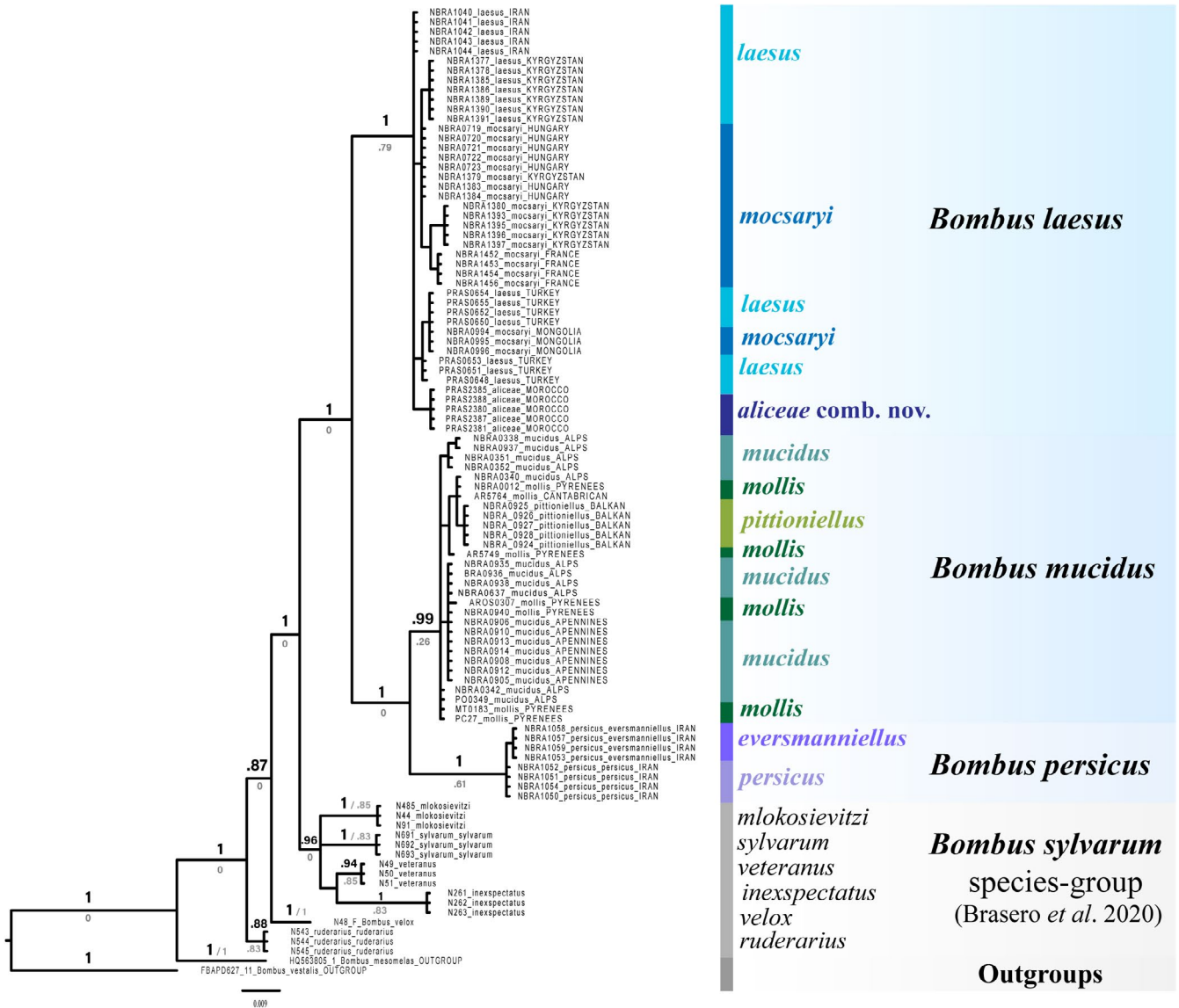


FIGURE 4 Genetic differentiation within the three former *Bombus* subgenera *Laesobombus*, *Mucidobonus* and *Eversmannibombus*, now included in the broader subgenus *Thoracobombus*. Majority rule (50%) consensus tree based on Bayesian analyses of *COI* (cytochrome oxidase 1). Values above tree branches are Bayesian posterior probabilities/bPTP (Poisson Tree Process) values. The tree is rooted with the most distant outgroup *Bombus (Psithyrus) vestalis*

and high bootstrap values > 0.85). We assigned a subspecies status to those allopatric populations which were not diverging in all lines of evidence but exhibiting original phenotypic features (Hawlitschek et al., 2012) such as a divergent morphology or a derived CLGS signal (Lecocq, Dellicour, et al., 2015; Martinet et al., 2019). Hair colour was not used as an operational criterion for species delineation as colour patterns can be shared by long-separated heterospecific taxa (Ghisbain, Lozier, et al., 2020; Williams et al., 2012); this character is a strong variable at the intraspecific level (Martinet et al., 2018; Williams et al., 2020) and can be strongly influenced by evaluative pressures from Müllerian mimicry at a regional level (Ezray et al., 2019; Ghisbain, Lozier, et al., 2020).

2.5 | Conservation

Based on our taxonomic conclusions, we proposed an updated conservation status for *Bombus laesus* following the standardised protocol implemented by the International Union for Conservation of Nature (IUCN) (e.g., Nieto et al., 2014). Occurrence data used in the analyses are those published in Rasmont, Franzen, et al. (2015) and Polce et al. (2018). We evaluated the conservation status following Nieto et al. (2014), measuring the area of occupancy (AOO) and extent of occurrence (EOO) of *B. laesus*. The AOO is a measure of the area in which the species occurs and corresponds to the sum of the area of grids the species occupies. For this purpose, we defined square grids of 5 × 5 km, as previously suggested in

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. The final conservation status was proposed following the criteria used in Nieto et al. (2014).

3 | RESULTS

3.1 | Genetic trait analyses

The phylogenetic analyses showed the expected differentiation between outgroup and in-group and highlighted similar relationship between taxa. Both *COI* and *PEPCK* phylogenetic

analyses detected three monophyletic bootstrap-supported groups inside our ingroups (Figures 4 and 5): (a) a first clade comprising the taxa *laesus* + *mocsaryi* + *aliceae*; (b) a second clade comprising *mollis* + *mucidus* + *pittioniellus* and (c) a third clade comprising *eversmanniellus* + *persicus*.

The tree generated by bGMYP analyses on *COI* sequences split off the tree in several groups with low probabilities (<0.05) to be conspecific with the other ones (Figure 6). The tree generated by the bGMYP analysis showed the delimitation of three prospective species within our in-group ($p < .05$). Three groups were highlighted using this threshold: (a) *mocsaryi* + *laesus* + *aliceae* (bGMYP conspecificity probabilities between individuals included in the group, $p > .09-1$); (b) *mucidus* + *mollis* + *pittioniellus* ($p > .38-1$) and (c) *persicus* + *eversmanniellus* ($p > .08-1$). The complementary PTP

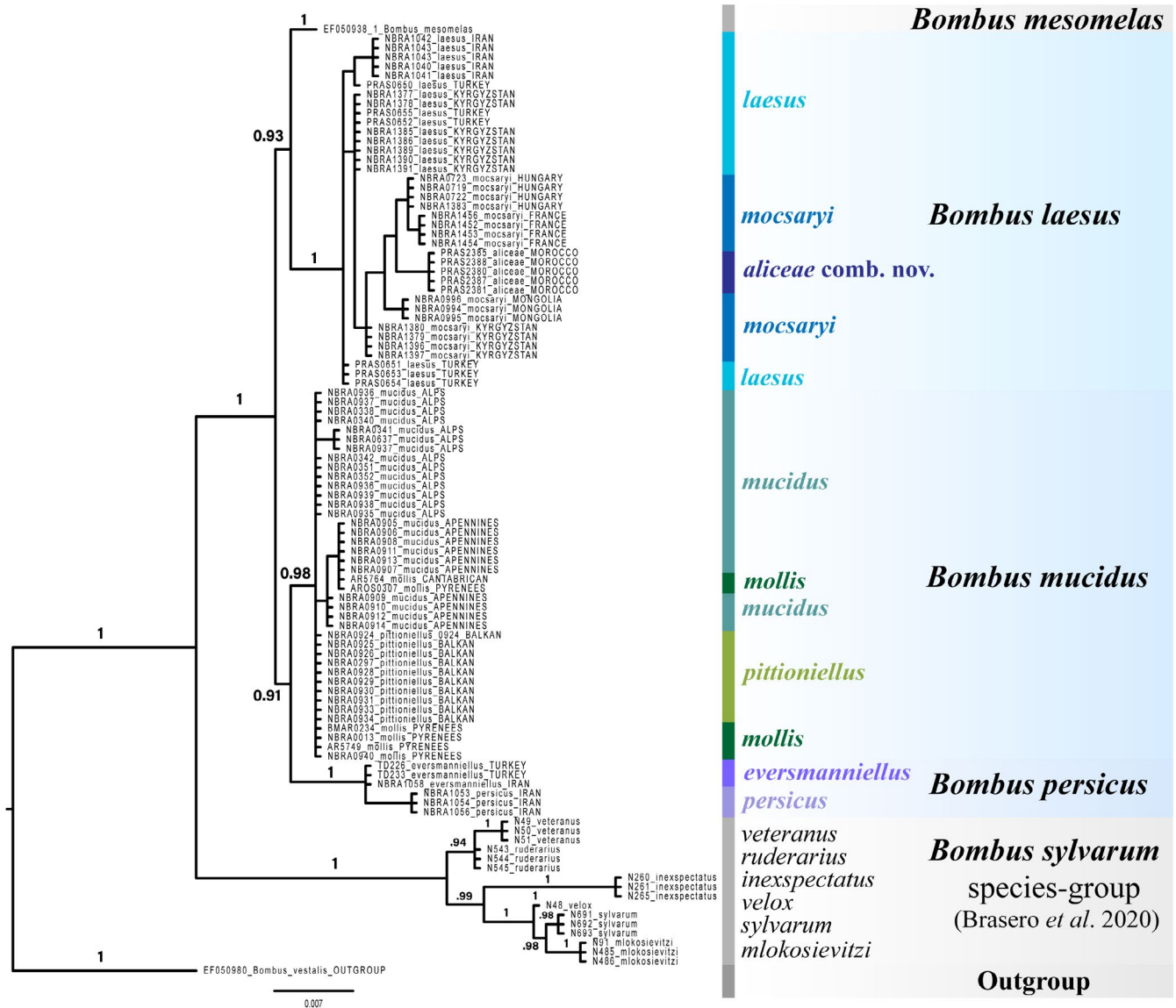


FIGURE 5 Majority rule (50%) consensus tree based on Bayesian analyses of *PEPCK* (phosphoenolpyruvate carboxykinase). Values above tree branches are Bayesian posterior probabilities/bPTP (Poisson Tree Process) values. The tree is rooted with the most distant outgroup *Bombus* (*Psithyrus*) *vestalis*

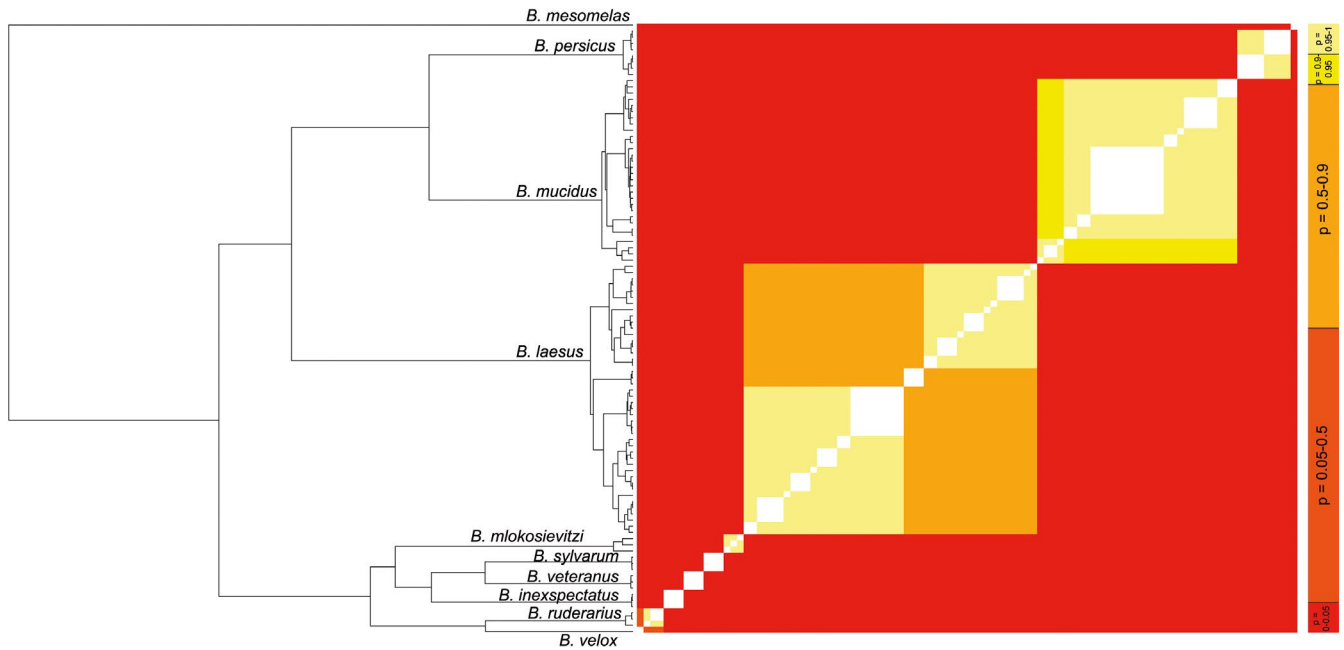


FIGURE 6 bGMYC results based on the *COI* (cytochrome oxidase 1) phylogenetic tree. The vertical and horizontal lines correspond to the three different groups shown on the tree. The scale corresponds to the probability to be conspecific

analysis with the most support and performed on the same dataset corroborated the results of the bGMYC by recognising the same candidate species with high support (refer the grey values accompanying the posterior probabilities in the Figures 4 and 5).

3.2 | Eco-chemical trait analyses

The cluster analysis revealed three strongly supported groups (bootstrap > 85%) (Figure 7): *eversmanniellus* (Turkey); *mucidus* (Alps, Apennines) + *mollis* (Pyrenees); and *mocsaryi* (Hungary), *aliceae* (Morocco) + *laesus* (Turkey). In total, 113 compounds were detected by chemical analyses (37 from *eversmanniellus*, 53 from *mucidus* + *mollis* and 93 from *mocsaryi*, *aliceae* + *laesus*, respectively; see Appendix S2). Main compounds were detected for each group: octadec-9-en-1-yl acetate (38%–51%) for *eversmanniellus* group; octadec-9-en-1-ol (7%–61%) and octadec-9-en-1-yl acetate (23%–68%) for *mucidus* group; and tetradecyl acetate (0%–47%), octadec-9-enyl acetate (4%–64%) and octadec-9-enoic acid (2%–27%) for *laesus* group (Figure 7). Despite the separation we observed between *mocsaryi* from Hungary, *laesus* from Turkey and *aliceae* from Morocco, our statistical analysis did not significantly support this differentiation (bootstrap < 85%). Moreover, the IndVal method revealed several significant indicator compounds (IC) (IndVal value > 70) for the *eversmanniellus* group (IC = 9), *mucidus* group (IC = 2) and *laesus* group (IC = 24) (Appendix S2). PerMANOVA test confirmed the differentiation between (a) *eversmanniellus* and *laesus* + *mocsaryi* + *aliceae* group ($F = 11.8$; $p < .05$); (b) *eversmanniellus*

and *mucidus* ($F = 14.007$; $p < .05$); and (c) *B. mucidus* and *B. laesus* + *B. mocsaryi* group ($F = 49.458$; $p < .05$).

3.3 | Decision framework of taxonomic status

Based on the genetic traits and supported by CLGS (for the sampled taxa), three species are supported in the studied in-group following the methodological framework of Lecocq, Brasero, et al., (2015): *B. laesus*, *B. mucidus* and *B. persicus* (Table 3). The genetic traits *COI/PEPCK* show no significant difference among the subspecies of *Bombus persicus* (*eversmanniellus* and *persicus*) and of *Bombus mucidus* (*mollis*, *mucidus* and *pittioniellus*). These results also confirm that the lineage *mocsaryi* is subspecific to *Bombus laesus*. Moreover, based on the combination of its private *COI* haplotype, its allopatry and tenuous divergence in CLGS, we formally recognise a subspecies status for the North African lineage *aliceae* Cockerell 1931 within *Bombus laesus*. We also provide the first description of the males of this subspecies. We designated a lectotype and a series of paralectotypes that are deposited in the collection of the Laboratory of Zoology of the University of Mons (Belgium). We also provide the first description of the males of this subspecies. We designated a lectotype and a series of paralectotypes that are hosted in the collection of the Laboratory of Zoology of the University of Mons (Belgium).

First description of the males: Body length measured in lateral aspect from the base of the antenna to the posterior edge of tergite 7: 12.4 mm \pm 0.3 mm (SE), head width: 3.9 \pm 0.2 mm.

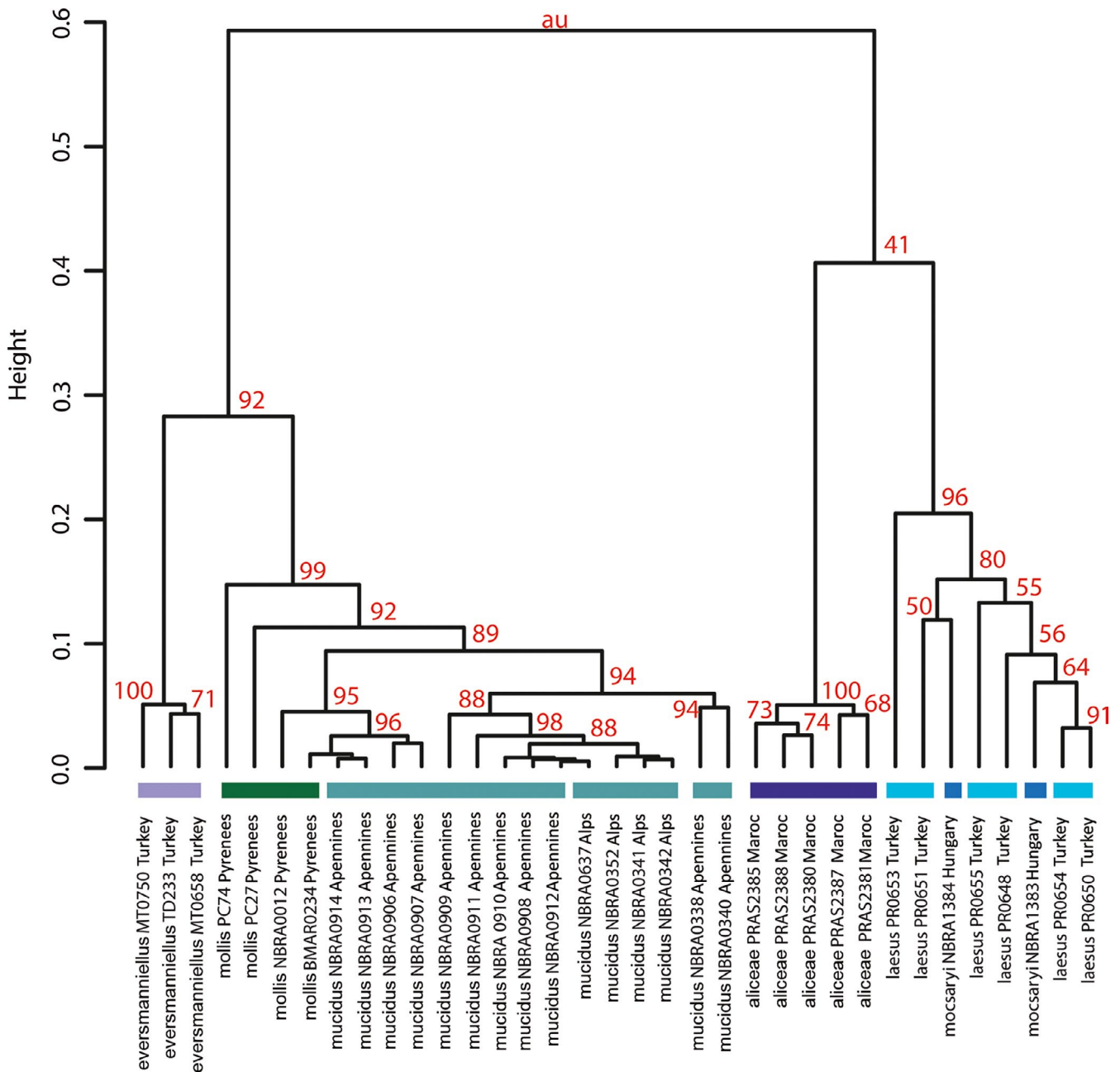


FIGURE 7 Unweighted pair group method with arithmetic mean cluster based on a Pearson's r correlation distance matrix calculated from the cephalic labial gland secretions matrix of six *Bombus* taxa: *eversmanniellus* (in purple), *mollis* (dark green), *mucidus* (light green), *mocsaryi* (blue), *aliceae* (dark blue) and *laesus* (light blue). The values near the node are multiscale bootstrap resampling

Coloration: (a) Head: large patch of yellow hairs on the clypeus reaching the inner border of compound eyes. The antennal socket is covered with yellow hairs intermixed with some black hairs. The inner border of compound eyes has some long black setae. The vertex is covered with a large patch of yellow hairs. (b) Mesosoma: yellow-haired collare on the whole pronotum, extending to the pleurae and reaching the scutellum under the tegulae. Wide and yellowish collare on the pronotum and the mesonotum reaching the middle of the tegulae. Black hairs under the tegulae on the mesonotum. The scutellum is yellow haired. (c) Metasoma: Terga 1–7 are

yellow-haired; the tergum 7 has black-haired intermixed with some long yellow hairs at the lateral extremities. (d) Legs: coxa, trochanter and femur yellow haired intermixed with black hairs. Tibia bordered with yellowish setae.

3.4 | Conservation status

Our spatial analysis showed an EOO of 7,654,628.492 km² and an AOO of 10,675.000 km² at the European scale. Based on these values only, *B. laesus* could be considered as “Least

TABLE 3 Decision framework including former (according to Rasmont *et al.* 2008 and Williams *et al.* 2012b) and proposed taxonomic status

| Former taxonomic status | | COI/bGMYC | PEPCK | CLGS | Proposed taxonomic status |
|-------------------------|------------------------------------|-----------------------|-------------------|------|-----------------------------------|
| <i>Laesobombus</i> | <i>mocsaryi alicae</i> (Morocco) | +(A) ^a / - | +(A) ^a | - | <i>B. laesus alicae</i> comb. nov |
| | <i>laesus</i> (Turkey, Kyrgyzstan) | - (A) / - | - (A) | - | <i>B. laesus</i> |
| | <i>laesus</i> (Iran) | - (A) / - | - (A) | / | |
| | <i>mocsaryi</i> (Hungary) | - (A) / - | - (A) | - | |
| | <i>mocsaryi</i> (France) | - (A) / - | - (A) | / | |
| | <i>mocsaryi</i> (Kyrgyzstan) | - (A) / - | - (A) | / | |
| | <i>mocsaryi</i> (Mongolia) | - (A) / - | - (A) | / | |
| <i>Mucidobombus</i> | <i>mollis</i> (Pyrenees) | - (B) / - | - (B) | - | <i>B. mucidus</i> |
| | <i>mollis</i> (Cantabrian) | - (B) / - | - (B) | / | |
| | <i>mucidus</i> (Alps) | - (B) / - | - (B) | - | |
| | <i>mucidus</i> (Apennines) | - (B) / - | - (B) | - | |
| | <i>pittioniellus</i> (Balkans) | - (B) / - | - (B) | / | |
| <i>Eversmannibombus</i> | <i>eversmanniellus</i> (Turkey) | - (C) / - | - (C) | - | <i>B. persicus</i> |
| | <i>eversmanniellus</i> (Iran) | - (C) / - | - (C) | / | |
| | <i>persicus</i> (Iran) | - (C) / - | - (C) | / | |

COI (cytochrome oxidase 1) and PEPCK (phosphoenolpyruvate carboxykinase) columns indicate if a taxon is strongly supported as monophyletic group (\pm means that the taxon is/is not a monophyletic group). When the taxon is not a distinct monophyletic group, the latter group together with taxa included in the same monophyletic group. CLGS (cephalic labial gland secretions) indicates if the taxon has/has not specific composition of cephalic labial gland secretions (\pm means that the taxon has/has not a specific CLGS composition; ++ means that the specific composition involved main compounds).

^aSpecimens from Morocco are well supported but do not constitute a reciprocal monophyletic group with the other specimens.

Concern.” Nevertheless, reductions of the populations across the range of the species have been observed (criterion A2c in Nieto *et al.*, 2014) and population reductions can be suspected in a near future (Rasmont, Franzen, *et al.*, 2015) with a clear decline in habitat quality, calling the IUCN criterion A3c. Altogether and awaiting for more studies on the conservation of the species, we propose a “Near Threatened” categorisation of *Bombus laesus* (A2c + 3c).

4 | DISCUSSION

The highly polytypic nature of bumblebees makes their taxonomy especially complex (Williams, 1998). Although the increasing use of genetic markers (Ghisbain, Lozier, *et al.*, 2020; Williams *et al.*, 2020), semio-chemical traits (Martinet *et al.*, 2018, 2019) sometimes combined with other tools (e.g., geometric morphometrics on the wings, Gérard *et al.*, 2020) is significantly refining our global comprehension of this diverse group of bees, some taxa have remained overlooked. Here, we clarified the taxonomic status of several uncommon bumblebee taxa belonging to the former subgenera *Eversmannibombus*, *Laesobombus* and *Mucidobombus*, now gathered in the monophyletic genus *Thoracobombus* (Williams *et al.*, 2008).

4.1 | Taxonomic implication for the *Thoracobombus* group

Firstly, our integrative taxonomic approach highlighted the conspecific status of the two taxa *eversmanniellus* and *persicus*, as part of the species *Bombus persicus* Radoszkowski 1881 (refer decision framework Table 3). The distribution of *B. persicus* is limited to the north by the Caucasus but no geographical barrier seems to occur to the east between the different populations from Turkey to Iran.

Secondly, our decision framework concluded that all taxa within *Mucidobombus* are conspecific despite their geographic isolation and marked differences in colour pattern (Cantabrian Range, Pyrenees, Alps, Apennines, and Balkans) (Table 3). Although we have not been able to analyse the CLGS of specimens from the Balkans and Cantabrian mountain ranges, the genetic structure observed in our markers (Figure 4) and the analysed CLGS strongly support the conspecificity of all studied populations, as part of a single species, *Bombus mucidus* Gerstaecker 1869. As for many other bumblebee species, the current disjoint distribution of *B. mucidus* can be explained by past climatic oscillations of the Quaternary in Europe. During these most recent ice ages, a plain of permafrost as well as tundra and cold steppe extended between the mountains of southern Europe to

the Urals. These habitats have facilitated the movement of cold-adapted species like *B. mucidus* as well as a constant gene flow between populations (Hewitt, 1999; Steward et al., 2003). Following the global climate warming of the inter-glacial period, the lineage *mucidus* has been trapped in the mountains of Southern Europe, a pattern also observed in some plants, birds and mammals (Angus, 1983; Steward et al., 2003, 2010). Because ice age periods are longer than inter-ice age periods (Hansen, 2004), the genetic homogeneity found among all populations of *B. mucidus* could be explained by these long periods of continuous gene flow. This pattern has been already observed in another mountain bumblebees (*B. mendax*) from an analysis combining (a) dating of mountain orogeny, (b) modelling of regional climate change, (c) modelling of evolution of climate preferences along species' lineages, and (d) estimation of species' dispersal/establishment potential (Williams et al., 2017).

Finally, our integrative taxonomic decision framework supports the conspecificity of all lineages within the group *Laesobombus* (Table 3). This result confirms the hypothesis proposed by Williams (1998, 2009) who did not find discrete morphological differences between *laesus* and *mocsaryi*, except an obvious difference in colour pattern. The genetic divergences based on *COI* and *PEPCK* sequences did not reflect the current separation between the taxa *laesus* and *mocsaryi* based on coat colour pattern (Figures 4 and 5). Although no genetic structuring was found in *COI* analyses (MB and bGMYC), the analyses of the *PEPCK* fragment highlighted a well-supported clade including all *mocsaryi*, therefore making the *laesus* group paraphyletic (Figures 4 and 5).

The eco-chemical traits bring new information about Moroccan populations originally described by Cockerell (1931) (Figure 7). While non-significant at the specific level, the CLGS of the Moroccan population differed from other populations encountered in Turkey and Hungary in light weight compounds (e.g., octadec-9-enyl acetate and octadec-9-enoic acid; Appendix S2). These compounds are known to have a long-distant attractive effect (Ayasse et al., 2001). This differentiation could, therefore, lead to the establishment of a reproductive isolation barrier as previously already suggested in other subgenera including *Megabombus*, *Psithyrus*, *Pyrobombus* and *Thoracobombus* (reviewed in Valterová et al., 2019). Unfortunately, we could not analyse the CLGS composition of the French population exhibiting unique haplotypes in both *COI* and *PEPCK* markers (Figures 4 and 5). Since allopatric taxa of this species are found between southern France and Hungary, further analyses are needed to assess the hypothesis whether the French population is closer or not to that of Morocco.

Even if unique haplotypes stand out from our analyses (*laesus* from Iran; *mocsaryi* from France, Kyrgyzstan, Mongolia and *aliceae* from Morocco), they are not strongly supported as reciprocal monophyletic groups in our phylogenies (Figures 4

and 5). All taxa within the *Laesobombus* group can, therefore, be considered as infraspecific to *B. laesus* Gerstaecker 1869. Based on its unique haplotype, a subspecies status is proposed for the population of Morocco. The name *aliceae* was originally used by Cockerell (1931) to describe what he called a “variety” of the taxon *mocsaryi* as *Bombus mocsaryi* var. *aliceae* Cockerell 1931, based on a single worker collected in 1930 in Asni, Morocco. Given that the oldest available name for the species is *Bombus laesus*, the Moroccan subspecies must rather be regarded as *Bombus laesus aliceae* Cockerell 1931.

As previously mentioned, some populations could not be analysed in the light of their attractive chemical secretions. We can, however, safely propose species delineation hypotheses according to our decision framework on the basis of the unambiguous genetic data. Overall, we can conclude that the CLGS data does not conflict with the genetic data for the slightly reduced set of samples for which it was collected. A broader sampling of CLGS would be still useful to further investigate any eco-chemical differentiation within these uncommon species at the population level, notably for *B. persicus persicus* and *B. mucidus pittioniellus*.

4.2 | Implications for conservation

All species studied here are known to be restricted to open-field environments (either steppes, sub-alpine or alpine meadows) (Rasmont, Franzen, et al., 2015). It appears clear that the current fragmentation of their populations is the result of multiple threats including climate change, intensification and extension of agricultural practices to the detriment of open-field environments (Iserbyt & Rasmont, 2012; Manino et al., 2007). Among the studied species, only *B. mucidus* has been assessed to the International Union for Conservation of Nature Red List as “Near Threatened” (Rasmont et al., 2015). Based on the taxonomic knowledge at that time, *Bombus laesus* and *B. mocsaryi* had been assessed as two different species by Nieto et al. (2014), with *B. laesus* assessed as “Near Threatened” and *B. mocsaryi* as “Endangered”. Both subspecies *mocsaryi* and *laesus* had also been assessed as separate species by Rasmont, Franzen, et al. (2015), and the current status of *B. laesus* as a species including all taxa *laesus*, *mocsaryi* and *aliceae* implies the need for a re-evaluation of the species' conservation status. Based on our results, we propose such a re-evaluation of the conservation status of *B. laesus* as “Near Threatened” according to the same methodology of Nieto et al. (2014).

The effective conservation of bumblebees ultimately relies on both the precise knowledge of their distribution and an unambiguous identification (Ghisbain et al., 2020). Although bumblebees constitute one of the most studied groups of bees worldwide, much work remains to clarify the taxonomy of the genus. New species are continuously described all over

the world (Martinet et al., 2019; Williams et al., 2020), highly polymorphic lineages thought to be conspecific can hide multiple species (Ghisbain, Lozier, et al., 2020; Martinet et al., 2018; Williams et al., 2019) and in contrast taxa thought to be distinct can result in being conspecific (Williams et al., 2020; present study). Much work is still required to disentangle the phylogenetic relationships and taxonomic status of widespread, polymorphic bumblebee taxa to ensure their correct identification and eventually implement geographically and taxonomically adapted conservation strategies.

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AUTHOR CONTRIBUTIONS

NB, GG, TL, IV, PR and BM: conceived and designed the experiments. NB, AM, PR, PB and BM: performed sampling. NB, GG and BM: analysed the data. NB, GG and BM: wrote the paper. All authors discussed the results, edited and approved the content of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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