Following the Cold: Geographic Differentiation between Interglacial Refugia and Speciation in Arcto-Alpine Species Complex *Bombus monticola* (Hymenoptera: Apidae)

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Short running Title: Following the cold: speciation in *B. monticola*

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ABSTRACT

Cold-adapted species are expected to reach their largest distribution range during a part of the Ice Ages while the post-glacial warming leads to their range contracting toward high latitude and high altitude areas. This results in extant allopatric distribution of populations and possibly to trait differentiations (selected or not) or even speciation. Assessing an inter-refugium differentiation or speciation remains challenging for such organisms because of sampling difficulties (several disjunct population) and disagreement on species concept. Here, we assessed post-glacial inter-refugium differentiation and potential speciation among populations of the most common arcto-alpine bumblebee species in European mountains, *Bombus monticola* Smith 1849. Based on mtDNA/nuDNA markers and eco-chemical traits, we performed integrative taxonomic analyses to evaluate alternative species delimitation hypothesis and to assess geographic differentiation between interglacial refugia and speciation in arcto-alpine species. Our results show that trait differentiations occurred between most of South European mountains (i.e. Alps, Balkan, Pyrenees and Apennines) and Arctic regions. We suggest that. *monticola* complex actually includes three species: *B. konradini* sp. nov. status distributed in Italy (Central Apennine Mountains), *B. monticola* with five subspecies, including *B. monticola mathildis* ssp. nov. distributed in North Apennine Mountins and *B. lapponicus*. Our results support that post Ice-Age periods can lead to speciation in cold-adapted species through distribution range contraction. We underline the importance of integrative taxonomy approach for rigorous species delimitation and for evolutionary study and conservation of taxonomically challenging taxa.

Keywords

Arcto-alpine regions, *Bombus*, cold-adapted species, integrative taxonomic approach, inter-refugium differentiation, post-glacial warming

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1. INTRODUCTION

Past climatic oscillations have led to significant changes in distributions of species. However, species responses to climate change depend mainly on their eco-climatic requirements and tolerances (Hewitt, 2004; Stewart *et al.,* 2010). Pleistocene and Quaternary climatic cycles triggered massive population movements resulting in periods of species range reductions (i.e. during cold periods when populations are restricted to refuge areas) for temperate species followed by periods of species range expansions (i.e. during warmer periods when populations recolonize at least portions of their initial range) (Reinig, 1937; Hewitt, 2004a; Stewart *et al.,* 2010). These population dynamics have fostered intraspecific divergence processes leading to differentiation and possibly speciation (Avise, 2000; Hewitt, 2004). On the other hand, alternative demographic histories and subsequent differentiation patterns can be expected for cold-adapted species. Assessing accurately consequences of past climate change on differentiation and speciation process is a key element to better understand and predict the evolution of future biodiversity and to propose evidence-based mitigation strategies (Rasmont *et al.*, 2015).

While population dynamics of temperate species fostered by past climatic events and their consequences have been the focus of abundant research (Zagwijn, 1992; Taberlet, 1998; Hewitt, 1999; Stewart *et al.,* 2010), cold-adapted species have received comparatively far less attention to date (Mardulyn *et al.,,* 2009). Contrary to temperate taxa, cold-adapted species are thought to have reached their largest distribution range during the Ice Ages (Hewitt, 2011). The post-glacial warming and subsequent interglacial period is thought to have led to range contraction of such cold-adapted species toward the high latitude/altitude areas (Barnes *et al.,* 2007; Fedorov *et al.,,* 2008; Hewitt, 2011). Such a population dynamic scenario can explain current allopatric patterns of species distributed in Arctic and southern mountains (i.e. arcto-alpine species) acting as interglacial refugia. These taxa have their current distribution in the relicts (refugia) of a widespread distribution fragmented by post-glacial warming (Reinig, 1937;

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90 Mardulyn *et al.*, 2009; Dellicour *et al.*,, 2014). In Europe, due to interglacial periods, arcto-
91 alpine species exhibit a strong pattern of allopatry between Southern mountains (Pyrenees,
92 Alps, Apennines, Balkans and Caucasus) and Northern areas (arctic regions of North
93 Scandinavia and Russia). Such allopatric patterns have fostered and still foster gene flow
94 disruptions leading to divergence and possibly speciation of cold-adapted species (Avise, 2000;
95 Hewitt, 2004b).

96 However, assessing species delimitation remains challenging because it requires to
97 arbitrarily select variable traits whose accuracy continues to be debated (Mayr, 1942; De
98 Queiroz, 2007; Lecocq *et al.*,, 2015a). Moreover, it is quite difficult to comprehensively sample
99 specimens for phylogeographic or speciation studies across vast inhospitable areas such as high
100 altitude mountains and arctic areas (Hewitt, 2011). This could lead to the underestimation of
101 the variability within each allopatric population and to misunderstand the allopatric
102 differentiation process.

103 The integrative taxonomy based on the unified species concept (De Queiroz, 2007) aims
104 to overcome limitations due to unsettled adequacy of selected diagnostic traits and limited
105 sampling. First, the approach considers multiple independent lines of evidence to evaluate inter-
106 population differentiation processes and taxonomic statuses (Schlick-Steiner *et al.*, 2010;
107 Lecocq *et al.*, 2015a,b). This reduces the likelihood of false taxonomic conclusions driven by
108 single trait. Second, analyzing multiple traits to investigate inter-population differentiation
109 allows to increase the amount of information available despite a limited sample size (Lecocq *et*
110 *al.*, 2011).

111 Among potential organisms of interest for studying climatic oscillation consequences
112 on cold-adapted species, bumblebees (Hymenoptera, Apidae, *Bombus*) represent a relevant
113 biological system because some of them (i) live in the coldest areas inhabited by insects, (ii)
114 have undergone diversification processes during the Pleistocene and Quaternary climatic cycles
115 (Hines, 2008; Duennes *et al.*, 2012; Martinet *et al.*, 2015a; Rasmont *et al.*, 2015; Dellicour *et*
116 *al.*, 2016). Their interspecific and inter-population differentiations have been studied for a long
117 time (e.g., Reinig, 1939). However, different diagnostic traits (morphological traits, DNA
118 sequences, eco-chemical traits) have been used, resulting in conflicting biological conclusions
119 (e.g., Gjershaug *et al.*, 2013; Williams *et al.*, 2015). Over the past few years, the efficiency of

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available diagnostic characters has been critically discussed and a merging of these traits in an integrative taxonomic framework has been proposed (e.g. Lecocq *et al.*, 2015a). This provides the opportunity to efficiently delimitate species for a common cold-adapted bumblebee species with a strong pattern of allopatry. Moreover, integrative taxonomy can help to define the subspecies status of allopatric populations (Lecocq *et al*., 2015a). In bumblebees, subspecies definition is traditionally based on color pattern variation but this diagnostic character requires an extensive overview of the inter-individual variability (Bertsch & Schweer, 2012a). However, color pattern has been shown to be unsuitable for taxonomic delimitation (Vogt, 1909; Bertsch & Schweer, 2012a; Carolan *et al.*, 2012; Williams *et al*., 2015) as well as for intraspecific variation study (Lecocq *et al.*, 2015b,c).

Here, we investigated the potential inter-refugium differentiation and speciation within the most common arcto-alpine bumblebee species in European mountains (Rasmont *et al.*, 2015): *Bombus* (*Pyrobombus*) *monticola* Smith 1849. We sampled all the allopatric regions where the species is known (infra-specific taxa). We analyzed inter-population differentiation through multiple diagnostic traits: (i) a mtDNA marker (cytochrome oxidase I, COI), (ii) a nuDNA marker (phosphoenolpyruvate carboxykinase, PEPCK), and (iii) eco-chemical traits (cephalic labial gland secretions, CLGS). Based on these traits, we developed an integrative taxonomic approach *sensu* Lecocq *et al.* (2015a,c) to assess the taxonomic status of major clades. We aim to exemplify the consequences of climate change on the evolution of cold-adapted bumblebees with a common mountainous species.

2. MATERIAL & METHODS

2.1. Model Species

Bombus (*Pyrobombus*) *monticola* Smith 1849 is an arcto-alpine species widespread in the alpine and sub-alpine stages of the most highest mountain ranges of Europe with isolated populations in Northern Europe and Mediterranean mountains (Cantabrian Mountains, Pyrenees, Alps, Apennines, and Balkans but not Caucasus) (Svensson, 1979; Kuhlmann *et al.*, 2014; Rasmont *et al.*, 2015). *Bombus monticola* was confirmed as an unique taxonomic unit by chemical (cephalic labial gland secretion and enzymology) and genetic analysis (Svensson, 1979; Gjershaug *et al.*, 2013) in comparison with its most similar taxon *B. lapponicus* (Fabricius, 1793). The analysis of Hines (2008), suggested that *Bombus monticola* diverged

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from its sister species *B. lapponicus* about 3 Ma (Hines, 2008). The species displays geographically differentiated color patterns (Reinig, 1965) that have been used to define five phenotypically diagnosable allopatric subspecies (Tab.1 Fig 2, (Rasmont *et al.*, 2015)): (i) *B. monticola scandinavicus* Friese 1912 (Fennoscandia), (ii) *B. monticola monticola* Smith 1849 (British Islands), (iii) *B. monticola alpestris* (= *hypsophilus*, Tkalcu 1992) Vogt 1909 (Alps, the Balkans, and the Olympus Mount), (iv) *B. monticola rondoui* Vogt 1909 (Cantabrian Mountains and Pyrenees), and (v) *B. monticola konradini* Reinig 1965 (Apennine Mountains) (Figs. 1-2). We define "*monticola* complex" as *Bombus monticola* ssp. + *Bombus lapponicus* and only "*monticola"* gathering exclusively all subspecies of *Bombus monticola.*

2.2. Sampling

We sampled 70 specimens including all *B. monticola* taxa (Appendix S1) from all mountain ranges of Europe: *B. monticola scandinavicus* (*n*= 11) from North Scandinavia, *B. monticola monticola* (*n*=10) from the British Islands, *B. monticola rondoui* (*n*= 9) from the Pyrenees, *B. monticola alpestris* from the Alps (*n*=9), Balkans (*n*=3) and the Olympus Mountain (*n*=1) and *B. monticola konradini* (*sensu* Reinig, 1965) from the Central Apennines (Sibilini Mountains) (*n*= 5) and from the North Apennines (*n*=2). The North Apennines population, whose geographic distribution includes the highest peaks in the Apuan Alps, is separated by wide gaps not only from the Central Apennines populations but also from alpine *alpestris* (almost 230 km). We used the phylogenetically closely related species *B. (Pyrobombus) lapponicus* (*n*=10) for comparison (see Cameron *et al.*, 2007) and *B. bimaculatus* (Cresson 1863) (*n*= 10) to root trees in our genetic analyses. All specimens were killed by freezing at -20°C. We considered all taxa without *a priori* taxonomic status and referred to them as *scandinavicus*, *monticola*, *rondoui*, *alpestris*, *konradini*, *lapponicus*, and *bimaculatus* (Tab.2). We further split *konradini* into *konradini*-N to indicate the Northern Apennines population and *konradini*-C to indicate the Central Apennines population.

2.3. Genetic Differentiation Analyses

To investigate the potential genetic differentiation between *B. monticola* taxa, we sequenced two genes that are commonly used in bee phylogenetic and phylogeographic studies (e.g. Pedersen, 2002; Cameron *et al.*, 2007; Williams *et al.*, 2012, Dellicour *et al.*, 2015): the mitochondrial gene COI and the nuclear gene PEPCK. We performed DNA extraction protocol, polymerase chain reaction (COI primers Apl2013/Aph2931, Pedersen 2002; PEPCK primers

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FHv4/RHv4, Cameron *et al.*, 2007), sequencing procedures, and DNA sequence alignment using the method described in Lecocq *et al.* (2013). We uploaded the resulting COI (938 bp) and PEPCK (925 bp) sequences in GenBank (accession numbers Appendix S1).

We investigated the potential genetic differentiation within *B. monticola* through haplotype network analyses and phylogenetic inference. We carried out the analyses for each gene individually. We used the median-joining method to produce haplotype networks with *Network* 4.6.1.0 (www.fluxus-engineering.com). We weighted transversions twice as high as transitions to reconstruct the network (Lecocq *et al.*, 2015).

In phylogenetic analyses, we analyzed each gene with maximum parsimony (MP), maximum likelihood (ML) and Bayesian (MB) methods. We carried out maximum parsimony analyses (heuristic method) using Seaview 3.2 (Galtier *et al.*, 1996) with 1,000,000 replicas. Only high quality trees and the majority rule 50% consensus tree were conserved. For ML and MB, each gene was partitioned as follows: (i) the nuclear gene (PEPCK) into two exons and two introns and (ii) each nuclear exon and (iii) the mitochondrial gene (COI) by base positions (first, second and third nucleotide) to define the best substitution model with JModelTest Server 2.0 (Posada, 2008) using the corrected Akaike information criterion. Best-fitting substitution models :(i) for COI : GTR+I (1st position), TIM2+I (2nd position), TrN+G (3rd position) ; (ii) for PEPCK intron 1: TPM1 uf +I ; (iii) for PEPCK exon 1: HKY+I (1st position), JC (2nd position), TrN+I (3rd position) ; (iv) for PEPCK intron 2: TrN+I ; (v) for PEPCK exon 2 : JC (1st position), JC (2nd position), JC (3rd position). For maximum likelihood analyses (ML), we performed 10 independent runs in GARLI 2.0 for both genes (Zwickl, 2006); the topology and−ln L was the same among replicates. Only the run with the highest likelihood was saved. We assessed statistical significance of nodes with 10,000 nonparametric bootstrap replicates. We considered a topology well supported (high confidence) whenever the bootstrap value (branch supports) was greater than 85% (Hillis & Bull, 1993). We carried out Bayesian inference analyses (MB) with MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). We achieved 10 independent analyses for each gene (100 million generations, four chains with mixed models, default priors, saving trees every 100 generations). Then we removed the first 10,000,000 generations as burn-in procedure. Then a majority-rule 50% consensus tree was constructed. Only branch supports (topologies) with high posterior probabilities (≥ 0.95) were

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considered statistically significant (Wilcox *et al.*, 2002). We (re) rooted all trees with the taxon *B. bimaculatus*.

To check the robustness of the genetic analyses, we applied the bGMYC method to the monticola complex (COI gene) to recognize species (threshold) following the protocol of Lecocq *et al*. (2015c). These analyses were performed with "bGMYC" (Reid & Carstens 2012) R packages. The stationarity and the modal coalescent/Yule ratio have been assessed to continue the analysis. A range of probabilities > 0.95 was considered as strong evidence that taxa were conspecific while a range of probabilities < 0.05 suggested that taxa was heterospecific (Reid & Carstens 2012). For bGMYC, ultrametric trees have been generated with BEAST 1.7.4 (Drummond et al. 2012) with a phylogenetic clock model to generate a posterior distribution of trees (length of the MCMC chain: 1 billion generations). The first million sampled trees as burn-in, using the maximum clade credibility method and setting the posterior probability limit to 0. Following the method of *Lecocq et al*. (2015c), we based the bGMYC analysis on 1000 trees sampled every 10 000 generations. For each of these 1000 trees, the MCMC was made of 100 000 generations, discarding the first 90 000 as burn-in and sampling every 100 generations. Posterior probability distribution have been applied against the first sample tree to provide a "heat' map".

2.4. Molecular Clock - Estimating Divergence Time

Following the approach of Duennes *et al.* (2012) and Lecocq *et al.* (2013), we analyzed the COI dataset in BEAST v1.7.2 (Drummond & Rambaut, 2007) to estimate the divergence time among different clades. Using the GTR + I model selected by jModeltest, we ran Markov chain Monte Carlo simulations with the coalescent constant population size tree model and the relaxed clock model. Considering that no fossils of *Pyrobombus* species are available, the phylogeny is calibrated with a range date from a molecular study. We specified a range of possible substitution rates which includes the extreme rate for insect mitochondrial genes recorded in the literature (e.g. Duennes *et al.*, 2012) using a flat prior ranging from 1×10-9 to 1×10-7 substitutions site-1 and year-1. Simulations were run for 300 million generations, sampling every 1000 generations. Four independent runs were assessed in Tracer v1.4.1 (Rambaut & Drummond, 2016) to confirm convergence, determine burn-in, and examine the

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effective sample size of all posterior parameters. Log files from each run were combined in LogCombiner v1.6.1 (Rambaut & Drummond, 2016) for final parameter estimates.

2.5. Eco-Chemical Traits Differentiation

We focused on the most studied eco-chemical trait involved in the bumblebee pre-mating recognition (Baer, 2003; Ayasse & Jarau, 2014): the cephalic labial gland secretions (CLGS). These secretions are complex mixtures of mainly aliphatic compounds synthesized *de novo* by male cephalic labial glands (Coppée *et al.,* 2008; Lecocq *et al.,* 2011; Žáček *et al.,* 2013). We identified the main component as the compound that had the highest relative concentration (RA) among all compounds of CLGSs at least in one specimen of the taxon. The CLGS are species-specific blends with some inter-population variations and are, subsequently, commonly used for species discrimination and assessment of intraspecific variability in bumblebees (review in Lecocq *et al.,* 2015a). We extracted the CLGS with 400 µl of n-hexane, according to De Meulemeester *et al.,* (2011) and Brasero *et al.,* (2015). Samples were stored at - 40° C prior to the analyses.

We qualified the CLGS composition of each sample by gas chromatography-mass spectrometry 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 ionization. We identified each compound using the retention times and mass spectra of each peak, in comparison to those from the National Institute of Standards and Technology library (NIST, U.S.A) database. We determined double bound positions (C=C) by dimethyl disulfide (DMDS) derivatization (Vincenti *et al.,* 1987).

We quantified the CLGS compounds with a gas chromatograph Shimadzu GC-2010 system (GC-FID) equipped with a non-polar SLB-5 ms capillary column [5% phenyl (methyl) polysiloxane stationary phase; column length 30 m; inner diameter 0.25 mm; film thickness 0.25 µm] and a flame ionization detector. We quantified the peak areas of compounds in GC solution postrun (Shimadzu Corporation) with automatic peak detection and noise measurement. The relative areas (RAs, expressed in %) of compounds in each sample were calculated by dividing the peak areas of compounds by the total area of all compounds. We excluded compounds for which RA were less than 0.1% for all specimens (De Meulemeester *et al.,* 2011). The data matrix for each taxon was based (Table S2) on the alignment of each

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relative proportion of compound between all samples performed with GCAligner 1.0 (Dellicour & Lecocq, 2013).

For GC/MS and GC-FID analyses, we injected 1 µL, using a splitless injection mode (injector temperature of 220° C) and helium as carrier gas (1 mL/min, constant velocity of 50cm/s). The oven temperature (of the column)was programmed isothermal at 70° C for 2 min and then rising from 70° C to 320° C at a rate of 10°C/min. The temperature was then held isothermal at 320° C for 5 min.

To facilitate the alignment of compounds and their identification, before each sample injection, a standard (Kovats) was injected containing a mix of hydrocarbons (alkanes) from C10 (decane) to C40 (tetracontane). Kovats indices were calculated with GCKovats 1.0 according to the method described by Dellicour & Lecocq (2013).

We performed statistical comparative analyses of the CLGSs using R environment (R Development Core Team, 2013) to detect CLGS differentiations between *B. monticola* taxa. We used a clustering method, computed with the unweighted pair-group method with average linkage (UPGMA) based on Canberra distance matrices (RA of each compound) (R package ape; Legendre & Legendre, 2004; Paradis *et al.*, 2004), to detect the divergence between taxa in the CLGS composition. We assessed the uncertainty in hierarchical cluster analysis using p-values calculated by multiscale bootstrap resampling with 100,000 bootstrap replications (significant branch supports >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 type I errors. We determined specific compounds of each taxon (indicator compounds) with the indicator-value (IndVal) method (Dufrêne & Legendre, 1997; Claudet *et al.*, 2006). This value is the product of relative concentration and relative occurrence frequency of a compound within a group. The statistical significance of an indicator compound (threshold of 0.01) was evaluated with a randomization procedure.

2.6. Morphological Analyses

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A total of 60 specimens were subjected to morphological analysis to discriminate *B. lapponicus*, *B. konradini* and *B. monticola*. We includes only workers to have a sufficient sampling and because the differences in metabasitarsus measurements were more pronounced in females than males. We selected the maximum length and width metabasitarsus distance in reference to the work of Gjershaug *et al.* (2013) and we calculated the ration (max length/max width) of these two measures to reduce the effect of body size on this morphological analysis. One picture was taken for each measurement and specimen using a binocular coupled with a digital camera (Nikon D70). The specimen was positioned in a way to maximize the focus of the metabasitarsus. The maximum metabasitarsus distance was measured on the picture with the software Imagej 1.5 (Abràmoff *et al.*, 2004) (Tab. 1, Appendix S3). Kruskal-Wallis analyses (Kruskal-Wallis test and Multiple comparison test after Kruskal-Wallis, "pgirmess" R-package, Siegel & Castellan, 1988) were performed using R (R Development Core Team, 2013) to compare the different studied taxa.

2.7. Data Integration and Decision Framework

Assuming that species diagnosis and inter-population differentiation are more efficient in a multiple evidence based approach (De Queiroz, 2007; Schlick-Steiner *et al.*, 2010), we proposed a species delimitation hypothesis according to our genetic and CLGS criteria based on the method performed by Lecocq *et al.*(2015a) derived from the approach established by Schlick-Steiner *et al.* (2010) . In this method, all criteria used in the integrative approach must be convergent to assign the specific status. This strict approach could lead to underestimate the species differentiation but reduce the taxonomic inflation (Lecocq *et al*., 2015a) We assigned the species status to a taxon (with a high degree of certainty) if this taxon: (i) was genetically differentiated in all genetic markers (unique haplotype); (ii) constituted a monophyletic group with high branch support; (iii) was significantly differentiated in CLGS compositions (including IndVal indicator compounds, PerMANOVA, high bootstrap values > 0.85) (Lecocq *et al.*, 2015a). We assigned the subspecies taxonomic status to phenotypically distinct allopatric populations with differentiation in some traits but not all in order to highlight these populations displaying of a such differentiation (originality) and to reduce the "underestimate's risk" of our strict approach to assign species status by naming them as a subspecies (Hawlitschek *et al.*, 2012; Ennen *et al.*, 2014; Lecocq *et al.*, 2016).

2.8. Identification and Type Revision

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The type series of *Bombus lapponicus konradini* Reinig 1965 are presently at the Zoologische Staatssammlung München and have been revisited for this study. The identification of other studied taxa was checked with traditional identification keys such as Løken (1973) and Gjershaug *et al.* (2013).

3. RESULTS

3.1. Inter-Taxa Differentiation

Haplotype network analysis revealed six unique haplotypes for COI and two for PEPCK (Fig.3) within *B. monticola* taxa complex. *konradini*-C was the only taxon displaying unique COI (6.8 % sequence difference from *monticola alpestris* and 5.3% from *lapponicus*) and PEPCK (0.7% sequence difference from *monticola* and 0.97% from *lapponicus*) haplotypes in the ingroup. All phylogenetic analyses (MP, ML and MB) of each single gene showed a similar topology with clades corresponding to haplotype groups found in the networks. Analyses showed strong support for all groups, but the position of *konradini*-C was variable in the clade in our phylogenetic analyses, and hence remains uncertain (Fig.3). Phylogenetic analyses on PEPCK showed two main lineages within *"monticola"* (Fig.3): the central Apennines lineage (*konradini*-C, hereafter referred to simply as *konradini*) and the main lineage (all other taxa). COI-based trees resolved *konradini* as the sister group to the outgroup *B. lapponicus* rather than to other lineages of *B. monticola*. Among these last ones, COI phylogenetic trees underlined some geographic subgroups within "*monticola*" (Fig.3): (i) the northern Apennine lineage of "*monticola"* (described hereafter as *mathildis* **ssp. nov**.), (ii) a western group including taxa from Pyrenees (*rondoui*) and Scotland (*monticola*); (iii) an eastern-northern group including specimens from Sweden (*scandinavicus*) and Alps + Balkans + Olympus Mt. (*alpestris*).

Compare to the maximum likelihood and Bayesian methods for COI data, the tree generated for bGMYC analysis displays significant difference mainly in the branching of *mathildis* ssp. nov. These differences are probably due to the different parameters used in the BEAST 1.7.4 software to calculate the bGMYC model and because this heat map is plotted against on a sample tree and not on the consensus tree. The bGMYC analysis (Fig. 4) highlights several entities with low probabilities (<0.05) to be conspecific with the other ones which match with the same taxa recognize in the COI tree (MP, ML, MB methods, Fig. 3). This threshold leads us to consider three prospective species within the monticola complex and the comparison group *B. bimaculatus* as in Fig. 3 where the monophyly of these species is highly supported: (i)

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one group including all *lapponicus* (bGMYC conspecificity probabilities between individuals included in the group, IP >0.98–1), (ii) a group with all *konradini* from Central Apennines (IP >0.99–1), (iii) one group with all *bimaculatus* (IP >0.98–1), (iv) all *monticola* subspecies (IP > 0.13–0.95) including rondoui (IP >0.95–1), alpestris (IP >0.95–1), scandinavicus (IP >0.98–1), monticola (IP >0.99–1) and *mathildis* nov. spp. (IP >0.99–1) which are significantly conspecific. The "heap map" (Fig. 4) shows more structure within *B. monticola* ssp. where the group displays more different haplotypes.

In chemical analyses, 103 compounds were detected; 82 in the CLGSs of *B. monticola* taxa (Appendix S2) except for *konradini* for which we detected only 50 compounds. The differentiation of CLGS composition between *B. monticola* taxa and outgroup species (*B. lapponicus* and *B. bimaculatus*) was conspicuous (IndVal; PerMANOVA F=115.63 and F= 122.52, p-value < 0.05; Fig.3). Except *konradini*, all other *B. monticola* taxa shared the same compounds with similar relative concentration (RA) (PerMANOVA F= 6.00 - 13.20, p-value >0.05) (Appendix S2). Differences between *konradini* and other *B. monticola* taxa were particularly marked in the first half of the spectrum representing the most volatile molecules. The relative abundance of several compounds was different compared with the relative abundance in other taxa of *B. monticola*. The IndVal method highlighted several unique and diagnostic compounds of *konradini* (Tab. 3; i.e. ethyl tetradecenoate, ethyl tetradecanoate, hexadec-7-en-1-ol ethyl octadecadienoate, dotriacontane, ethyl octadec-9-enoate). In particular, *konradini* was characterized by ethyl octadec-9-enoate with a relative abundance of 8.28% while it had very low relative abundance in other subspecies (median 0.57%). The discrimination between *konradini* and other *B. monticola* taxa was supported by maximal bootstrap support values (100%) (Fig.3). This differentiation was confirmed by statistical analysis (PerMANOVA F= 29.36 p-value < 0.05, between *konradini* and other *B. monticola* taxa).

3.2. Taxonomic Status

Species status was confirmed for the comparison group *B. bimaculatus* and *B. lapponicus*. According to the mtDNA and nuDNA divergence along with the CLGS composition differentiation (including main compounds) (Tab. 4), the species status was assigned to *konradini* (detailed information is given in Supporting information, Appendix S3). *Bombus konradini* was originally described by Reinig (1965) as a subspecies typical of the

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northern Apennines, ranging from the provinces of Genova and Parma to L'Aquila. All other taxa were included in *B. monticola* but their color pattern (Tab.1) and/or differentiation in CLGS composition (minor quantitative differences) and/or in COI marker implied their assignation to the subspecies status. It is important to note the distinction of the north Apennines (province of Genoa and Parma) *monticola* population (*B. monticola mathildis* **ssp. nov)** from the central Apennines taxon (*B. konradini* **nov. status**) and the population from the Alps (*B. monticola alpestris*). Indeed, considering the slight differentiation in COI (0.53% of divergence from *alpestris*) and the strong divergence in coat color from *alpestris* (Tab.1, Fig.3), the north Apennines population should have a new subspecies status: *B. monticola mathildis* (detailed information is given in Supporting information, Appendix S3).

3.3. Divergence Times among Clades

Based on the COI data, the divergence between *B. konradini* (Central Apennines) and *B. lapponicus* was estimated with a median of 0.79 Ma (min 0.25 - max 1.9 Ma) at the end of the Günz-Mindel inter-glacial period. The divergence time between the outgroup *bimaculatus* and the clade "*monticola-lapponicus-konradini"* was estimated with a median of 2.40 Ma (min 1.14 - max 3.88 Ma). In contrast, the divergence time between *lapponicus-konradini* and the clade "*monticola"* was estimated with a median of 2.30 Ma (min 1.23 - max 4.11 MA. These last two divergence times correspond approximately to the onset of glaciation events and the formation of the Bering Strait. The other *monticola* subspecies have diverged recently with a estimated time of 40,000-18,000 (min 7,500 - max 548,000) years ago.

3.4. Morphological Analysis

Measurements of the ratio maximum length and width metabasitarsus show significant differences (Kruskal-Wallis multiple comparison $chi^2 = 32.757$; all p-values < 0.05) between *lapponicus* and *monticola alpestris*, *monticola mathildis* ssp. nov. The ratio is also significantly different between *konradini* and *monticola* ssp. but not between *monticola alpestris* and *monticola mathildis* (Fig. S2, Appendix S3). However, between *konradini* and *lapponicus*, although our results present a clear trend which highlight a larger ratio for *konradini*, there is no significant differentiation. According these results, *konradini* appears as intermediate between *monticola sensu stricto* (large metabasitarsus ratio) and *lapponicus* (short metabasitarsus ratio). Diagnostic morphological characters are summarized in Tab. 1.

4. DISCUSSION

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425 *4.1. Inter-Population Differentiation of B. monticola*

426 The concordance between genetic differentiation, geographic distribution, and CLGS
427 divergence of populations suggests a strong intraspecific structure between the subspecies of
428 *monticola* (Fig.3). The western subspecies (*B. monticola rondoui* from Pyrenees and *B.*
429 *monticola monticola* from British Islands), the north Apennines population (*B. monticola*
430 *mathildis* ssp. nov.) and the eastern-northern subspecies (*B. monticola scandinavicus* from
431 Sweden and *B. monticola alpestris* from Alps, Balkans and Olympus Mt) constitute, with the
432 COI marker, five differentiated groups in three main lineages which diverged recently (about
433 40,000 -18,000 years ago based on molecular clock) during the Pleistocene/Quaternary (Fig.3).
434 This could explain the weak divergence of PEPCK marker between *monticola* subspecies
435 (recent divergence) because nuclear genes have a lower mutation rate than mitochondrial genes
436 (Lunt *et al.*, 1996; Trunz *et al.*, 2016). Such time-estimation matches with the start of the last
437 post-glacial warming. So, it appears that the geographic pattern is most likely a consequence of
438 allopatric differentiation and genetic drift triggered by a range fragmentation subsequent to the
439 last post-glacial warming. We speculate that, at the beginning of the current interglacial time,
440 taxa have found refuge in southern mountainous areas (Alps and southern peninsulas of Europe)
441 and Northern Europe by contraction of their distribution areas or range shifting (Hewitt, 1999;
442 Hewitt & Ibrahim, 2001; Petit *et al.*, 2003; Stewart *et al.*, 2010), similarlyto the boreo-montane
443 leaf beetle (*Chrysomelidae*), *Gonioctena pallida* (Mardulyn *et al.*, 2009). The resulting
444 allopatry has fostered mtDNA differentiation along with minor differentiation of chemical
445 reproductive traits similarly to what has already been shown for insular populations of
446 bumblebees (Lecocq *et al.*, 2013, 2015a).

447 Despite their relative geographic isolation, all other *B. monticola* allopatric taxa
448 previously recognized by Reinig (1965) are considered as conspecific based on our diagnostic
449 criteria with a low geographic genetic and phenotypic differentiation (decision framework
450 Tab.4; Fig.3). They shared overall the same CLGS composition (except some low relative
451 concentration differences) and are characterized by only a slight genetic differentiation. These
452 low differentiations, particularly in CGLS composition, can simply be explained by the short
453 time of divergence due to geographical isolation and intraspecific variability (Lecocq *et al.*,
454 2011, 2016). Within *Bombus monticola alpestris*, the three sampled populations (Alps, Balkans
455 and Olympus Mountain) are clearly consubspecific.

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4.2. Mountain Top Speciation: Bombus konradini nov.status.

Contrary to the situation within *B. monticola*, *B. konradini* nov. status displays larger genetic and chemical traits differentiations (Fig. 3). Allopatry has most likely shaped the reproductive trait (CLGS) differentiation as observed in other species (Lecocq *et al.*, 2013). The strong genetic differentiation of *B. konradini* could be explained by an earlier divergence from the common ancestor with other *B. monticola* lineages, most likely temporally close to the *B. monticola - B. lapponicus* complex divergence. Indeed, based on genetic differences in the 16S gene, Hines (2008) suggested that *B. lapponicus* and *B. monticola* diverged from each other about 3 Ma. In temperate species, the post Ice-Age recolonization of territories by relict populations (from refugia), could lead to a new shuffling of the genetic pool by re-contacting of these populations without speciation (Coyne & Orr, 2004; Hewitt, 2004). The modification of geographical range could trigger genetic and CLGS differentiation. Indeed, it has been shown that the reproductive traits including cephalic labial glands secretions can differentiate from both sides of the physical barriers that may exist between the refuge areas (Lecocq *et al.*, 2013a). The case of the new species status of *B. konradini* lends strength to the hypothesis that for cold-adapted taxa, climatic oscillations (*i.e.* interglacial periods) have led to species differentiation in mountain refuges after geographical separation. Further phylogeographic and phylogenetic studies, based on larger sampling (including additional closely related species) and other genetic markers, are needed to accurately assess these hypotheses.

Our integrative taxonomic decision framework supported and confirmed the species status of *B. monticola* compared with its morphologically closely related species (*B. lapponicus*) (Løken, 1973; Svensson, 1979; Cameron *et al.*, 2007; Gjershaug *et al.*, 2013). Our results also supported the species status of *konradini* which is endemic of alpine levels of the Central Apennines (Manino *et al.*, 2007) (Fig. 3, Tab. 4). Concerning the eco-chemical traits (CLGS), *konradini* differed from the other *B. monticola* taxa by lightweight compounds (volatile molecules) which could have a long distance attractive role (Ayasse *et al.*, 2001). Therefore, the differentiation of these compounds may be a significant pre-mating reproductive barrier or may simply reflect divergence times and drift. Besides, according to the results of COI marker, *konradini* could be more closely related to *B. lapponicus* (Fig.3) than *B. monticola* taxa as suggested in the original description of Reinig (1965). However, the phylogenetic

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position of *konradini* is not completely resolved because of the different tree topologies between COI and PEPCK results.

The species status of *B. konradini* suggests that inter-glacial periods can lead to species differentiation in mountain refugia in cold-adapted taxa. Unlike the populations of the Alps, Pyrenees and Balkans, where the interconnection and thus the possibility of exchanges and conspecificity are likely, the population of the central Apennines is much more isolated from other mountain chains with a possible endemic speciation (Martin- Bravo *et al.,* 2010). Several studies have shown the presence of endemic taxa in the Central Apennines (e.g. in amphibians Mattoccia *et al.,* 2011, Canestrelli & Nascetti, 2008, 2012, in reptiles Nagy *et al.,* 2002, Joger *et al.,* 2007, in turtles Fritz *et al.,* 2006, in plants Conti *et al.,* 2005; Fuente *et al.,* 2011, Frattaroli *et al.,* 2013; in bumblebees Lecocq *et al.,* 2013). For example, Lecocq *et al.,* (2013a) provided evidence that the population of *B. lapidarius* (a Palearctic polytypic species) from the Southern Italian refugia has experienced genetic and CLGS differentiation during Quaternary glaciations leading to an incipient speciation process. Populations inhabiting the Mediterranean mountains, (e.g. the Apennines, one of the few mountain ranges in Europe arranged on a north-south axis) are characterized by a high genetic diversity (hotspot) with endemic taxa (Ruiz-La-Bourdette *et al.,* 2012). This ascertainment questions the conspecificity of cold-adapted species with an allopatric distribution with genetic diversity.

These results suggest that *B. monticola* (*sensu stricto*) could be absent in Southern Italy unless this absence could be due to a sampling bias. Such absence results in lack of sympatry between *B. monticola* and *B. konradini*, which strikingly contrasts with the sympatry between *B. lapponicus* and *B. monticola* in Scandinavia. We cannot exclude the hypothesis that *B. konradini* could be, although distinct, the "replacement" species of *B. monticola* in Apennines. Although the results do not tend towards this hypothesis (morphological analysis Fig. 3, Fig. S2), we may also question the status of *monticola mathildis* and the (phenotypically) intermediate position of this population between *konradini* and *monticola alpestris* (Alps). Further genetic analyses (e.g. *Microsatellite, SNPs, RAD-seq) are necessary to explore this hypothesis.4.3. Conservation Remarks on B. monticola Complex and the Practice of Integrative Taxonomy*

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Considering all taxonomic criteria in our integrative approach (Fig. 3, Tab. 4), we propose to conserve the subspecies status for five *monticola* taxa (Hawlitschek *et al.*, 2012; Lecocq *et al.*, 2015a,c, 2016): *B. monticola rondoui* from Pyrenees, *B. monticola monticola* from British Islands, *B. monticola scandinavicus* from Fennoscandia and *B. monticola alpestris* from Alps, Balkans, Olympus Mountains and *B. monticola mathildis* ssp. nov. from North Apennines (formerly included by Reinig within *konradini*). Although the usefulness of subspecies status in the bumblebee has been criticized and debated (Ebach & Williams, 2009) during recent decades (Williams, 1991; Bertsch & Schweer, 2012a), we propose that these allopatric subspecies (partially isolated lineages) represent an important component and a useful pragmatic taxonomical unit for evolutionary biology and biological conservation in the evolutionary legacy of *B. monticola* (i.e. Waples, 1995, Phillimore & Owens, 2006; Rasmont *et al.*, 2008; Patten, 2009; Crowhurst *et al.*, 2011; Braby *et al.*, 2012; Sackett *et al.*, 2014). These differentiations could be local adaptations to particular environments (Avise, 2000; Frankham *et al.*, 2010; Braby *et al.*, 2012; Lecocq *et al.*, 2013a). Therefore, subspecies classification seems suitable to reflect the intraspecific differentiation within *B. monticola* taxa.

The *monticola* complex is a stunning example of the difficulty, in taxonomy, to define species or subspecies status of a population. Here the integrative taxonomy considering all criteria independently could provide definitive pieces of evidence for decision. We assigned the subspecies taxonomic status to phenotypically distinct allopatric groups of populations with differentiation in some but not all criteria used in the integrative decision framework (i.e. conflict in selected criteria) (Hawlitschek *et al.*, 2012; Ennen *et al.*, 2014; Lecocq *et al.*, 2015a,c, 2016). Taxonomical conclusions based only on the differentiation of one mitochondrial marker (e.g. COI barcoding) can lead to weak taxonomic hypothesis (Andriollo *et al.*, 2015; Mutanen *et al*,; 2016; Trunz *et al.*, 2016) as mitochondrial differentiation may result from sex-specific characteristics, as lower dispersion for females (Kraus *et al.*, 2009; Lepais *et al.*, 2010), or mtDNA introgression or incomplete lineage sorting (Bensasson *et al.*, 2001; Lecocq *et al.*, 2015a). Taxonomic diagnosis based on multiple evidence (integrative taxonomy) is the best approach to avoid overestimation of species diversity which would lead to taxonomic inflation. Subspecies can be considered as a simple allopatric differentiation (Mayr, 1942; Patten, 2010). This procedure allows to assign a taxonomic status to any doubtful bumblebee taxa and points out these taxa for further taxonomic studies (Lecocq *et al.*, 2015a). Moreover, despite the

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argument advanced by Williams *et al.* (2015), there is no case in bumblebees where the CLGS (mate recognition system) was not differentiated between two different species even when closely related bumblebee species have geographical distributions that do not overlap (e.g. *B. terrestris* (L.) and *B. ignitus* Smith, De Meulemeester *et al.*, 2011 or between *B. patagiatus* and *B. magnus* where Bertsch & Schweer, (2012b) have shown the differentiation of their cephalic labial gland secretions).

4.4. Conservation Implication of the New Taxonomic Status of B. konradini nov. status

The new taxonomical status has implication for the red list assessments of the here studied European bumblebees according to the IUCN criteria (Nieto *et al.*, 2014). While Rasmont *et al.* (2015) assess all taxa lumped into *B. monticola*, the new taxonomic status of *B. konradini* implies an evaluation of its conservation status independently from other *B. monticola taxa*. *Bombus konradini* was described as a rare, geographically very restricted taxon endemic to the central Apennines of Marche, Umbria, Lazio, Abruzzo and mostly occurring exclusively at elevations over 1800m (Reinig, 1965; Ricciardelli & Piatti, 2003; Manino *et al.*, 2007, Rasmont *et al.*, 2015). The apparent scarcity of *B. konradini* could lead to significant genetic drifts (Ricciardelli & Piatti, 2003; Frankham *et al.*, 2010) that might significantly increase the species extinction risk (Rasmont *et al.*, 2015). Indeed, according Frankham *et al.* (2010), small and isolated populations of a taxon are inherently more vulnerable to local extinction due to environmental and demographic stochasticity. It is therefore important to consider this new taxonomic status in our models and in our future backup plans (mitigation measures).

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986 **AUTHOR CONTRIBUTIONS**

987 Conceived and designed the experiments: BM TL NB CU IV PR. Sampling: BM NB PB MC
988 PR. Analyzed the data: BM. Wrote the paper: BM TL NB PB MC CU IV JOG, DM PR.

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993 **SUPPORTING INFORMATION**

994 **Appendix S1**. Table of sampling. Sample code refers to the sample labels used in different
995 analyses. COI and PEPCK are the GenBank accession numbers for each sample (when
996 consubspecific samples display the same gene sequence, only one of them has been submitted
997 to Genbank;).

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Appendix S2. Data matrix of cephalic labial gland secretions (CLGS) (relative concentration of each compound), list of the identified compounds and IndVal analysis with specific compounds in the *monticola* complex. Unknown x's indicate undetermined compounds.

Appendix S3. Description of the new subspecies *Bombus monticola mathildis* and *Bombus konradini* nov. status., designation of the holotype and lectotype and morphological differentiation. Fig. S1. Morphology and coloration variation of the face of *Bombus konradini* ***nov. status*** (Lectotype female, A) and *Bombus monticola alpestris* (female, B). Photographs are by P. Rasmont. Fig. S2. Comparison of the ratio maximum length/ maximum width metabasitarsus between workers of *B. lapponicus*, *B. konradini* and *B. monticola scandinavicus*.

Fig. 1. Distribution map (Gall projection) of *Bombus monticola* (Rasmont & Iserbyt, 2014) and its traditional subspecies in Europe according to Rasmont (1983a). A, *Bombus monticola scandinavicus* queen, red area on the map; B, *Bombus monticola monticola* queen, dark green area; C, *Bombus monticola rondoui* queen, purple area; D, *Bombus monticola alpestris* queen, blue area; E, *Bombus monticola mathildis* **ssp. nov** Holotype male, pink area; F, *Bombus konradini* **nov status** Lectotype queen, yellow area. Light green dots indicate the occurrence of the taxon in the region. All photographs are by P. Rasmont.

Fig. 2. Photos of the studied bumblebee taxa. A, *Bombus monticola scandinavicus* queen; B, *Bombus monticola monticola* queen; C, *Bombus monticola rondoui* queen; D, *Bombus monticola alpestris* queen; E, *Bombus monticola mathildis* **ssp. nov** Holotype male; F, *Bombus konradini* **nov status** Lectotype queen.

Fig. 3. Genetic and chemical analyses within the *monticola complex*. (A) Majority rule (50%) consensus tree based on maximum likelihood analyses of COI. Values above tree branches are Parsimony bootstrap values/ maximum likelihood bootstrap values/Bayesian posterior probabilities. Only maximum likelihood and parsimony bootstrap values >70% and posterior probabilities >0.95 are shown. **(B)** Majority rule (50%) consensus tree based on maximum likelihood analyses of PEPCK. Values above tree branches are Parsimony bootstrap values/ maximum likelihood bootstrap values/Bayesian posterior probabilities. Only maximum likelihood and parsimony bootstrap values >70% and posterior probabilities >0.95 are shown. **(C)** a) Dendrogram of cephalic labial gland secretion differentiation within *monticola* complex and *B. bimaculatus*. This cluster was obtained by hierarchical clustering using an unweighted

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pair-group method with arithmetic mean (UPGMA) based on a Canberra matrix calculated from the cephalic labial gland secretion matrix of *B. bimaculatus, B. lapponicus, B. konradini* nov. status, *B. m. rondoui*, *B. m. scandinavicus, B. m. monticola*, *B. m. alpestris, B. m. mathildis* ssp. nov. The values near nodes represent multiscale bootstrap resampling values (only values >80 of main groups are shown except nodes between *B. monticola* subspecies). b) Principal component analysis (PCA) of cephalic labial gland secretion differentiation within *monticola* complex and *B. bimaculatus*. PC1, PC2 and PC3 are the first, the second and the third axes.

Fig 4. Species recognition heat map. Species recognition heat map based on ultrametric tree of Cytochrome oxidase 1 (COI) sequences with bGMYC pairwise probability of conspecificity plotted on a sample tree from BEAST. The colored matrix corresponds to the pairwise probabilities of conspecificity returned by the bGMYC method (color scale on the right of the figure). Black spots show the coalescent node for each species.

Tab.1. Range, conservation status and main morphological and colour pattern differences (male and female) between *B. konradini* nov. status, *monticola* subspecies including *mathildis* ssp. nov. and the similar species *B. lapponicus* according to Gjershaug *et al.*, (2013), Løken (1973), Pittioni (1939) and original observations.

	scandinavicus	*monticola*	*rondoui*	*alpestris*	*mathildis* ***ssp.nov.***	*konradini* ***nov status***	*lapponicus*
Range	Fennoscandia	British Islands	Pyrenees	Alps, Balkans, Olympus Mounts	North Apennines	Central Apennines	Fennoscandia
Conservation status	No regression was mentioned	In decline (Fitzpatrick *et al.*, 2006; Evans & Potts 2013)	In decline (Iserbyt & Rasmont 2012)	Few data show a decline in Italy (Manino *et al.*, 2007)	No regression was mentioned	Rare and localized (Ricciardelli & Piatti 2003)	Stable (Nieto *et al.*, 2014)
Female							
	Morphology						
Furrow of gena	The surface between the punctures on vertex is shiny, and there is a slight depression with some punctures near the compound eye					Similar to *monticola*	The surface between the punctures on vertex is rugose and dull and the furrow is distinct, nearly

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							reaching the compound eye
Hind meta-basitarsus	Slight pubescence and the maximal width of the basitarsus is high (*sensu* Gjershaug *et al*., 2013). The length of the metabasitarsus of these taxa is large (Appendix S3)					Strong pubescence and the maximal width of the basitarsus is low (*sensu* Gjershaug *et al*., 2013) as in *lapponicus*. The ration maximum length/maximum width of the metabasitarsus of this taxon is intermediate (Appendix S3).	Strong pubescence and the maximal width of the basitarsus are low (*sensu* Gjershaug *et al*., 2013). The length of the metabasitarsus of this taxon is short (Appendix S3).
Coat color variation	Dark	Dark	Light	Relatively dark	Light and colorful	Large and light (Reinig, 1965)	Varies from very light and colorful in Northern Fennoscandia, to rather dark in Southern Fennoscandia (Southern Norway)
	Color pattern						
Face	Black	Black	Yellow	Black	Yellow or sometimes black (Fig. S1)	Yellow (Fig.S1)	Black
Collare and scutellare	Small dark yellow	Small dark yellow and black	Light yellow/yellow	Small dark yellow / dark yellow	Wide light yellow with a black line near the tegulae/ yellow	Wide yellow band to the tegulae/ yellow	Yellow
Tergite 1	Black/Red	Black	Yellow	Yellow/black	Yellow/black (center of tergite)	Yellow/ Red/black	Yellow/Red/Black
Tergite 4	Dark red	Dark red	Red	Light R-red	Dark R-red with sometimes yellow (few)	Yellow	Yellow
Tergite 5	Dark red	Light red	Light red	Light red	Dark red with	Yellow	Yellow

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					sometimes yellow (few)		
Male							
	Color pattern						
Face	Dark yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Collare/ scutellare	Dark yellow /NO	Yellow / dark yellow	Yellow / large yellow	Yellow / large yellow	Yellow / large yellow	Yellow / large yellow	Yellow / large yellow
Tergite 1	Black and Red	Black and Red	Yellow and Black	Yellow and Black	Yellow and Black	Yellow	Yellow
Tergite 4	Dark red	Dark red	Dark red	Dark red	Light red	Red/yellow	Yellow/Red
Tergite 5	Dark red	Red	Red	Red	Light red	Red/yellow	Yellow/Red

Tab.2. Summary of sampling table with genetic and eco-chemical criteria used in this study. PEPCK: Phosphoenolpyruvate carboxykinase gene, COI: Cytochrome oxidase 1 gene, CLGS: Cephalic labial gland secretions, M: male and F: female.

Taxa	**Sampling site**	**PEPCK**	**COI**	**CLGS**
***B. lapponicus* (Fabricius 1793)**	North Sweden	5 M	5 M	10 M
***B. bimaculatus* (Cresson 1863)**	East Canada	3 M	5 M	10 M
***B. monticola scandinavicus* Friese 1911**	North Sweden	5 M	5 M	11 M
***B. konradini nov. status* Reinig 1965**	Italy (Central Apennines)	3 M, 2 F	2 M, 2F	2 M
***B. monticola mathildis ssp. nov.* Martinet, Cornalba & Rasmont 2016**	Italy (North Apennines)	2M	2M	2M
***B. monticola alpestris* Vogt 1909**	Alps, Balkans, Olympus Mts	6 M	6 M	13 M
***B. monticola monticola* Smith 1849**	Scotland	5 M	4 M	10 M
***B. monticola rondoui* Vogt 1909**	France (Pyrenees)	2F	4 M	7 M

Tab.3. List of indicator compounds (IndVal method, compounds > 70%, *) and main compound (1) identified for *B. konradini* nov. status within cephalic labial gland secretions. With MW= the molecular weight, n= number of used specimens, M= median of compound relative concentration (%), 2 = main compound identified for *B. lapponicus*, 3 = main compound identified for *B. bimaculatus.* Absent compounds are noticed by "-". The full matrix is presented in Appendix S2.

Compounds	**MW**	***alpestris* (n=13)**	***monticola* (n=10)**	***rondoui* (n=7)**	***scandinavicus* (n=11)**	***mathildis ssp. nov* (n=2)**	***konradini nov status* (n=2)**	***lapponicus* (n=10)**	***bimaculatus* (n=10)**
		M	M	M	M	M	M	M	M
Citronellol*	156	-	-	-	-	0.08	0.17	-	-

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Ethyl tetradecenoate2*	254	-	-	-	-	-	0,13	-	-
Ethyl tetradecanoate*	256	-	-	-	-	-	0,18	-	-
Hexadec-7-en-1-ol*	240	0,11	0,08	-	-	0.25	1,84	-	-
Ethyl hexadec-9-enoate*	282	-	-	1.00	-	-	1.41	0,03	-
Hexadec-9-enyl acetate [1]	282	52.34	55.15	57.05	53.96	35.27	51.53	0.08	32.95
Geranyl citronellol [2]	292	-	-	-	-	-	-	71.32	-
Ethyl octadecadienoate1*	308	-	-	-	-	-	0,37	-	-
Ethyl octadec-9-enoate*	**310**	**0,68**	**0,35**	**0.57**	**0,46**	**1.73**	**8,28**	-	-
Geranyl geranyl acetate [3]	332	-	-	-	-	-	-	-	31.61
Dotriacontane*	451	-	-	-	-	-	0,09	-	-
Hexadecyl hexadecanoate2*	480	0,04	0,07	0,09	0,22	0.25	0,94	0.19	0.09

Tab.4. Taxonomic decision table with all criteria used for species delimitation. CLGS, cephalic labial gland secretions; COI, cytochrome oxidase 1; PEPCK, phosphoenolpyruvate carboxykinase. Morphology indicates if a taxon has a diagnostic morphological character (+/- means that morphology is/is not diagnostic). Private haplotypes indicate if a taxon has a specific haplotype (+/- means that the taxon has/has not only private haplotype (s). When the taxon shares haplotype with other ones, the letters group together taxa that share haplotypes). CLGS indicates if the taxon has/has not diagnostic composition of CLGSs with different main compounds (+/- means that the taxon has/ has not a specific CLGS composition. When the taxon shares CLGS composition with other ones, the letters group together taxa that share similar CLGS. COI and PEPCK columns indicate if a taxon forms a strongly supported monophyletic group (+/- means that the taxon is/is not a monophyletic group) with MP, ML and MB methods . When the taxon is not a distinct monophyletic group, the letters group together taxa included in the same monophyletic group). LS= low supported differentiation.

Former taxonomic status	Morphology (Gjershaug *et al.*, 2013)	Private haplotypes (COI/PEPCK)	CLGS	COI gene / bGMYC	PEPCK gene	Proposed taxonomic status
B. monticola scandinavicus	-	+/-	- (B)	- LS (B) / -	-	*B. monticola scandinavicus*
B. monticola monticola	-	+ /-	- (B)	- LS (A) / -	-	*B. monticola monticola*
B. monticola rondoui	-	+/ -	- (A)	- LS (A) / -	-	*B. monticola rondoui*
B. monticola alpestris	-	+/-	- (C)	- LS (B) / -	-	*B. monticola alpestris*

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B. monticola konradini (North Apennines)	-	+/-	- (C)	- LS (C) / -	-	***B. monticola mathildis* ssp. nov.**
B. monticola konradini (Central Apennines)	+	+/+	+	+ / +	+	***B. konradini nov. status***
B. bimaculatus	+	+/+	+	+ / +	+	*B. bimaculatus*
B. lapponicus	+	+/+	+	+ / +	+	*B. lapponicus*

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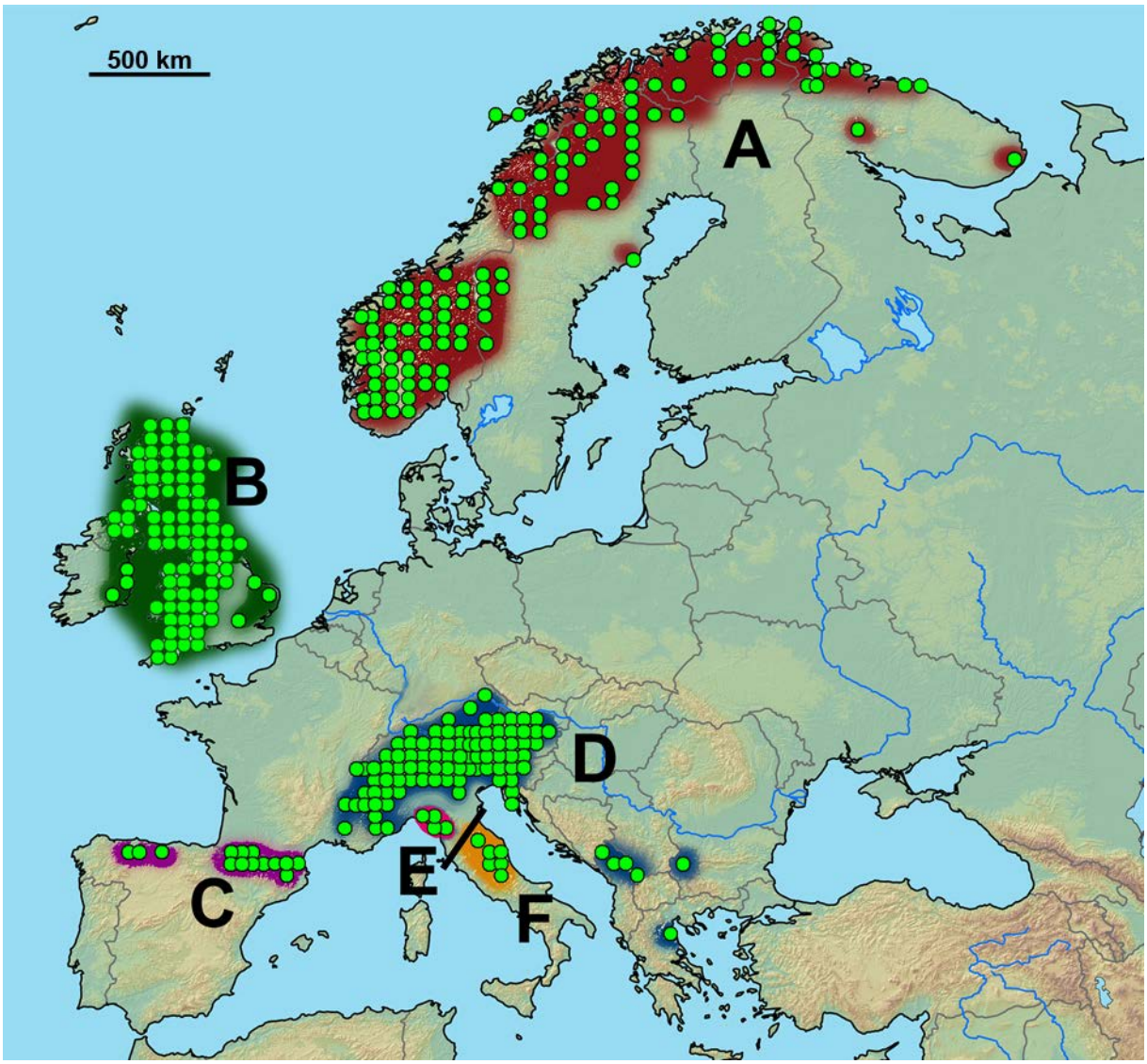

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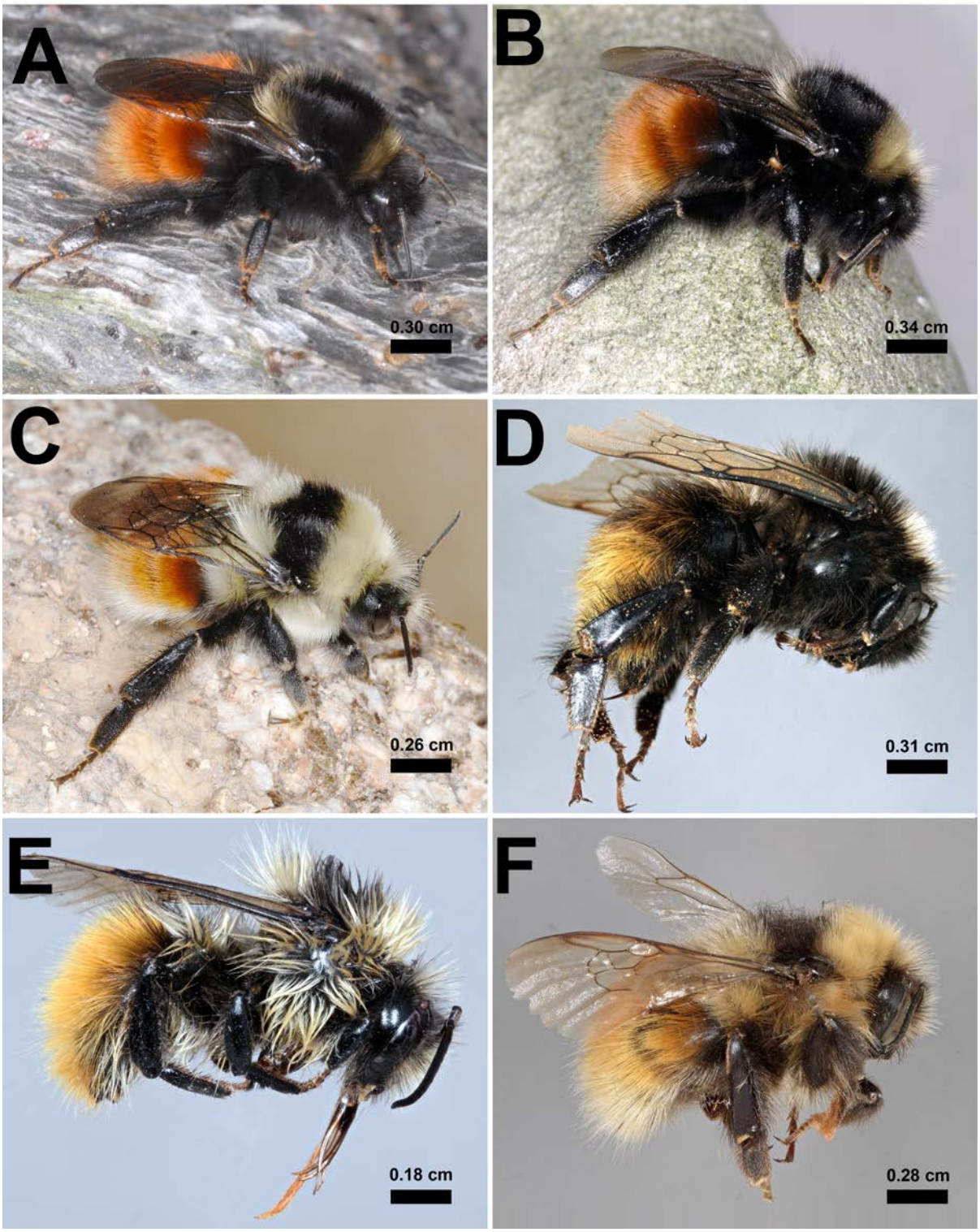

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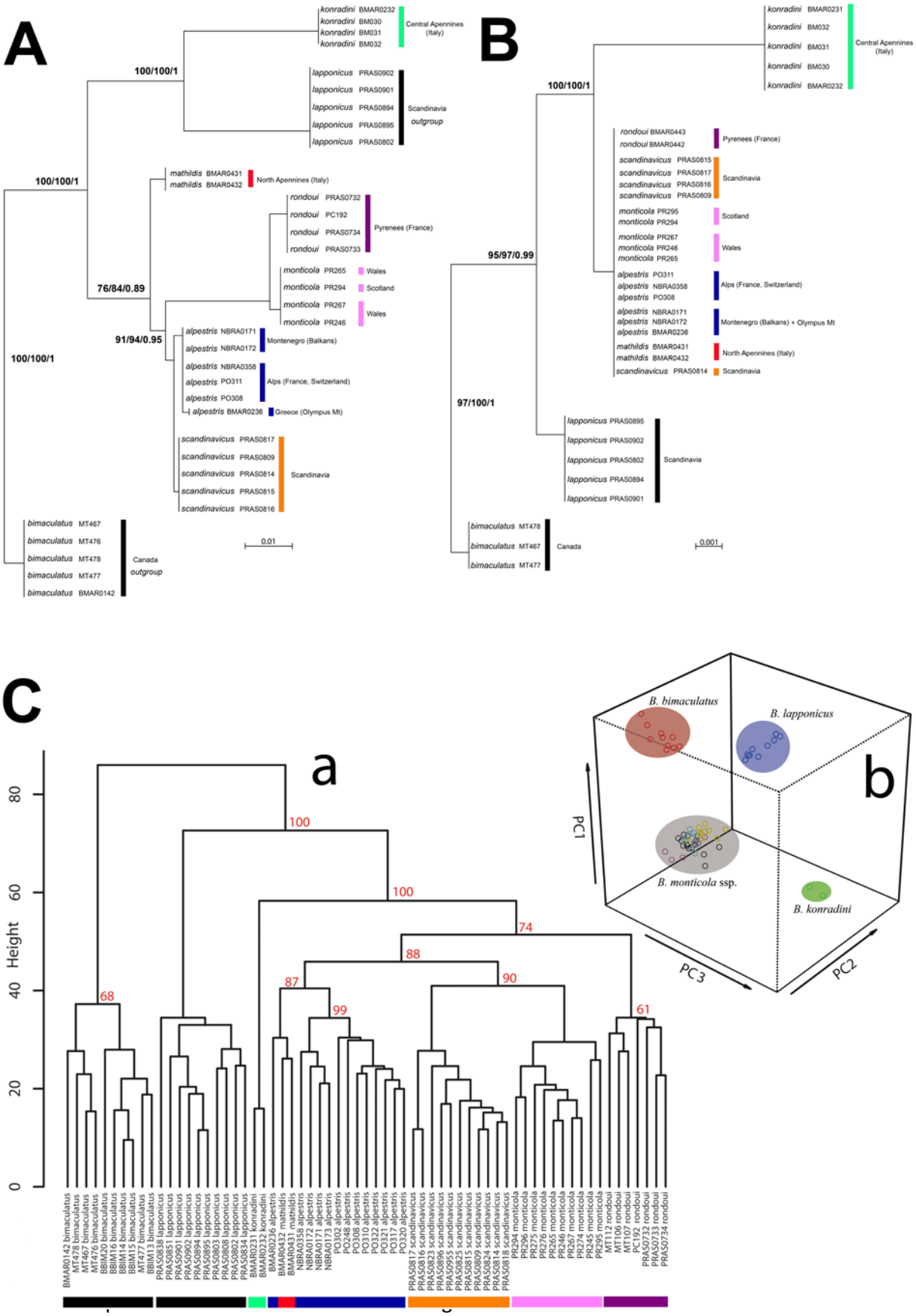

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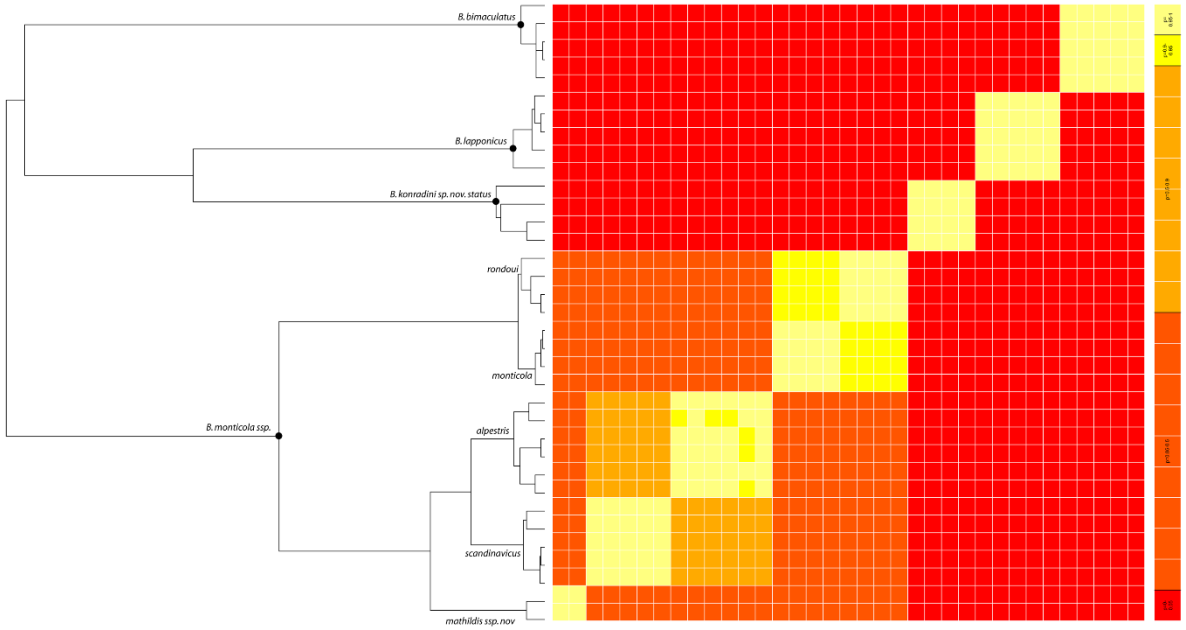

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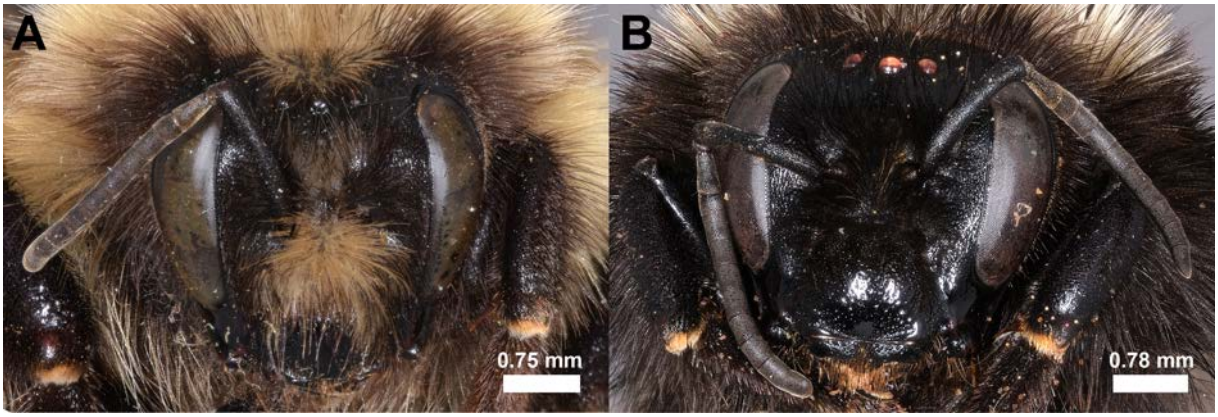

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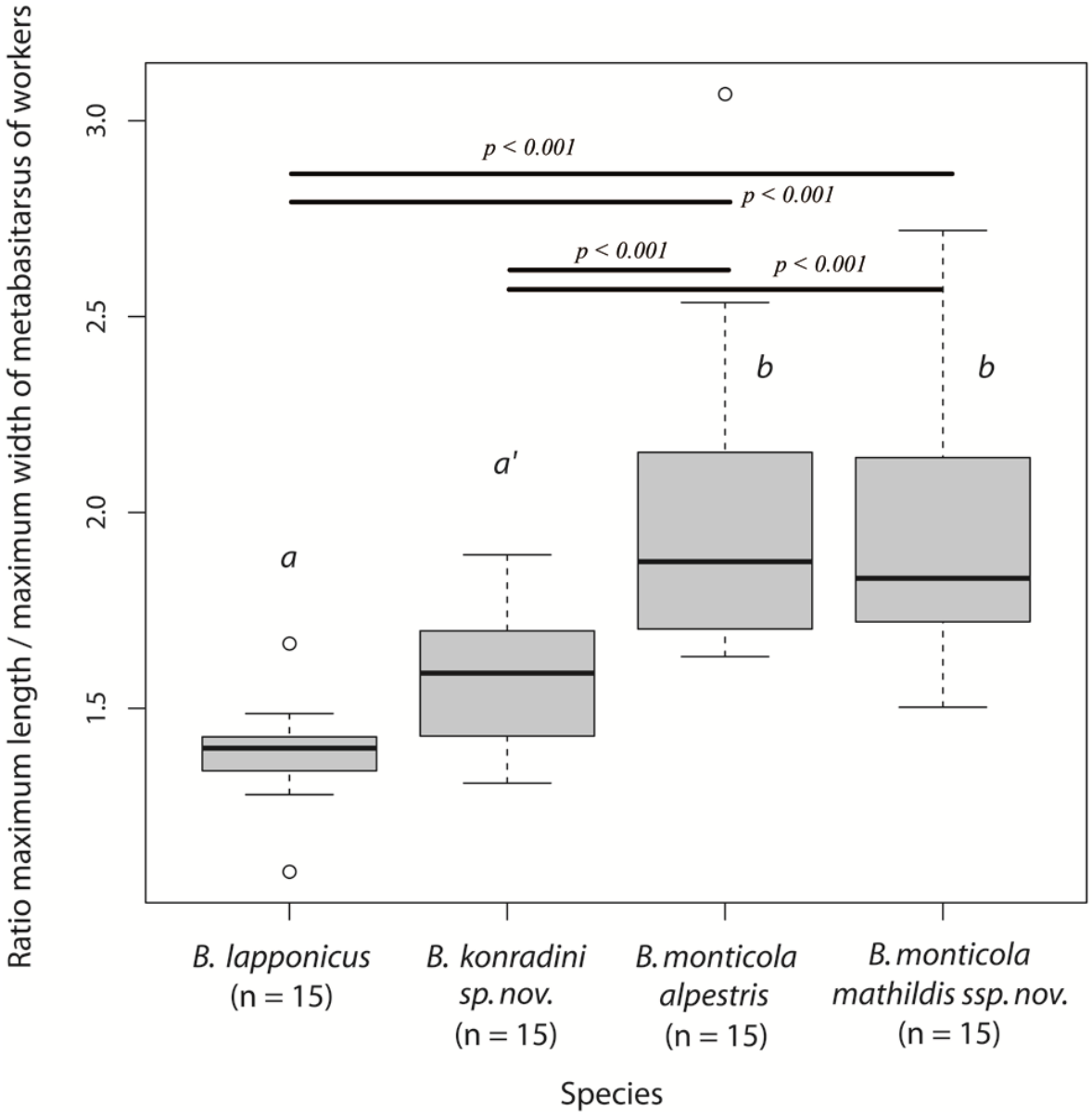

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