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## Following the cold: geographical differentiation between interglacial refugia and speciation in the arcto-alpine species complex *Bombus monticola* (Hymenoptera: Apidae)

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

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

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35 **ABSTRACT**

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

57 **Keywords**

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

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## 66 1. INTRODUCTION

67 Past climatic oscillations have led to significant changes in distributions of species.  
68 However, species responses to climate change depend mainly on their eco-climatic  
69 requirements and tolerances (Hewitt, 2004; Stewart *et al.*, 2010). Pleistocene and Quaternary  
70 climatic cycles triggered massive population movements resulting in periods of species range  
71 reductions (i.e. during cold periods when populations are restricted to refuge areas) for  
72 temperate species followed by periods of species range expansions (i.e. during warmer  
73 periods when populations recolonize at least portions of their initial range) (Reinig, 1937;  
74 Hewitt, 2004a; Stewart *et al.*, 2010). These population dynamics have fostered intraspecific  
75 divergence processes leading to differentiation and possibly speciation (Avice, 2000; Hewitt,  
76 2004). On the other hand, alternative demographic histories and subsequent differentiation  
77 patterns can be expected for cold-adapted species. Assessing accurately consequences of past  
78 climate change on differentiation and speciation process is a key element to better understand  
79 and predict the evolution of future biodiversity and to propose evidence-based mitigation  
80 strategies (Rasmont *et al.*, 2015).

81 While population dynamics of temperate species fostered by past climatic events and  
82 their consequences have been the focus of abundant research (Zagwijn, 1992; Taberlet, 1998;  
83 Hewitt, 1999; Stewart *et al.*, 2010), cold-adapted species have received comparatively far less  
84 attention to date (Mardulyn *et al.*, 2009). Contrary to temperate taxa, cold-adapted species are  
85 thought to have reached their largest distribution range during the Ice Ages (Hewitt, 2011).  
86 The post-glacial warming and subsequent interglacial period is thought to have led to range  
87 contraction of such cold-adapted species toward the high latitude/altitude areas (Barnes *et al.*,  
88 2007; Fedorov *et al.*, 2008; Hewitt, 2011). Such a population dynamic scenario can explain  
89 current allopatric patterns of species distributed in the Arctic and in southern mountains (i.e.  
90 arcto-alpine species) acting as interglacial refugia. These taxa have their current distribution  
91 in the relicts (refugia) of a widespread distribution fragmented by post-glacial warming  
92 (Reinig, 1937; Mardulyn *et al.*, 2009; Dellicour *et al.*, 2014). In Europe, due to interglacial  
93 periods, arcto-alpine species exhibit a strong pattern of allopatry between Southern  
94 mountains (Pyrenees, Alps, Apennines, Balkans and Caucasus) and Northern areas (arctic  
95 regions of North Scandinavia and Russia). Such allopatric patterns have fostered and still  
96 foster gene flow disruptions leading to divergence and possibly speciation of cold-adapted  
97 species (Avice, 2000; Hewitt, 2004b).

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

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

113           Among potential organisms of interest for studying climatic oscillation consequences  
114 on cold-adapted species, bumblebees (Hymenoptera, Apidae, *Bombus*) represent a relevant  
115 biological system because some of them (i) live in the coldest areas inhabited by insects, (ii)  
116 have undergone diversification processes during the Pleistocene and Quaternary climatic  
117 cycles (Hines, 2008; Duennes *et al.*, 2012; Martinet *et al.*, 2015a; Rasmont *et al.*, 2015;  
118 Dellicour *et al.*, 2016). Their interspecific and inter-population differentiations have been  
119 studied for a long time (e.g., Reinig, 1939). However, different diagnostic traits  
120 (morphological traits, DNA sequences, eco-chemical traits) have been used, resulting in  
121 conflicting biological conclusions (e.g. Gjershaug *et al.*, 2013; Williams *et al.*, 2015). Over  
122 the past few years, the efficiency of available diagnostic characters has been critically  
123 discussed and a merging of these traits in an integrative taxonomic framework has been  
124 proposed (e.g. Lecocq *et al.*, 2015c). This provides the opportunity to efficiently delimitate  
125 species for a common cold-adapted bumblebee species with a strong pattern of allopatry.  
126 Moreover, integrative taxonomy can help to define the subspecies status of allopatric  
127 populations (Lecocq *et al.*, 2015a,c). In bumblebees, subspecies definition is traditionally  
128 based on color pattern variation but this diagnostic character requires an extensive overview  
129 of the inter-individual variability (Bertsch & Schweer, 2012a). However, color pattern has  
130 been shown to be unsuitable for taxonomic delimitation (Vogt, 1909; Bertsch & Schweer,

131 2012a; Carolan *et al.*, 2012; Williams *et al.*, 2015) as well as for intraspecific variation study  
132 (Lecocq *et al.*, 2015b,c).

133 Here, we investigated the potential inter-refugium differentiation and speciation within  
134 one of the most common arcto-alpine bumblebee species in European mountains (Rasmont *et al.*,  
135 2015): *Bombus (Pyrobombus) monticola* Smith, 1849. We sampled all the allopatric  
136 regions where the species is known (infra-specific taxa). We analyzed inter-population  
137 differentiation through multiple diagnostic traits: (i) a mtDNA marker (cytochrome oxidase I,  
138 COI), (ii) a nuDNA marker (phosphoenolpyruvate carboxykinase, PEPCK), and (iii) eco-  
139 chemical traits (cephalic labial gland secretions, CLGS). Based on these traits, we developed  
140 an integrative taxonomic approach *sensu* Lecocq *et al.* (2015a,c) to assess the taxonomic  
141 status of major clades. In this approach, all taxonomic criteria used must be significantly  
142 differentiated to assign the species status.

## 143 2. MATERIAL & METHODS

### 144 2.1. Model Species

145 *Bombus (Pyrobombus) monticola* Smith, 1849 is an arcto-alpine species widespread in  
146 the alpine and sub-alpine stages of the highest mountain ranges of Europe with isolated  
147 populations in Northern Europe and Mediterranean mountains (Cantabrian Mountains,  
148 Pyrenees, Alps, Apennines, and Balkans but not Caucasus) (Svensson, 1979; Kuhlmann *et al.*,  
149 2014; Rasmont *et al.*, 2015). *Bombus monticola* was confirmed as an unique taxonomic unit  
150 by chemical (cephalic labial gland secretion and enzymology) and genetic analysis (Svensson,  
151 1979; Gjershaug *et al.*, 2013) in comparison with its most similar taxon *B. lapponicus*  
152 (Fabricius, 1793). The analysis of Hines (2008), suggested that *Bombus monticola* diverged  
153 from its sister species *B. lapponicus* about 3 Ma. The species displays geographically  
154 differentiated color patterns (Reinig, 1965) that have been used to define five phenotypically  
155 diagnosable allopatric subspecies (Tab.1 Fig 2, (Rasmont *et al.*, 2015)): (i) *B. monticola*  
156 *scandinavicus* Friese, 1912 (Fennoscandia), (ii) *B. monticola monticola* Smith, 1849 (British  
157 Islands), (iii) *B. monticola alpestris* (= *hypsophilus*, Tkalcu, 1992) Vogt, 1909 (Alps, the  
158 Balkans, and the Olympus Mount), (iv) *B. monticola rondoui* Vogt, 1909 (Cantabrian  
159 Mountains and Pyrenees), and (v) *B. monticola konradini* Reinig, 1965 (Apennine Mountains)  
160 (Figs. 1-2). We define "monticola complex" as *B. monticola* ssp. + *B. lapponicus* and only  
161 "monticola" gathering exclusively all subspecies of *B. monticola*.

### 162 2.2. Sampling

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

### 178 2.3. Genetic Differentiation Analyses

179 To investigate the potential genetic differentiation between *B. monticola* taxa, we  
180 sequenced two genes that are commonly used in bee phylogenetic and phylogeographic  
181 studies (e.g. Pedersen, 2002; Cameron *et al.*, 2007; Williams *et al.*, 2012, Dellicour *et al.*,  
182 2015): the mitochondrial gene COI and the nuclear gene PEPCK. We performed DNA  
183 extraction protocol, polymerase chain reaction (COI primers Apl2013/Aph2931, Pedersen  
184 2002; PEPCK primers FHv4/RHv4, Cameron *et al.*, 2007), sequencing procedures, and DNA  
185 sequence alignment using the method described in Lecocq *et al.* (2013). We uploaded the  
186 resulting COI (938 bp) and PEPCK (925 bp) sequences in GenBank (accession numbers  
187 Appendix S1).

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

193 In phylogenetic analyses, we analyzed each gene with maximum parsimony (MP),  
194 maximum likelihood (ML) and Bayesian (MB) methods. We carried out maximum parsimony  
195 analyses (heuristic method) using Seaview 3.2 (Galtier *et al.*, 1996) with 1,000,000 replicas.  
196 Only high quality trees and the majority rule 50% consensus tree were conserved. For ML and

197 MB, each gene was partitioned as follows: (i) the nuclear gene (PEPCK) into two exons and  
198 two introns and (ii) each nuclear exon and (iii) the mitochondrial gene (COI) by base  
199 positions (first, second and third nucleotide) to define the best substitution model with  
200 JModelTest Server 2.0 (Posada, 2008) using the corrected Akaike information criterion. Best-  
201 fitting substitution models :( i) for COI : GTR+I (1st position), TIM2+I (2nd position),  
202 TrN+G (3rd position) ; (ii) for PEPCK intron 1: TPM1 uf +I ; (iii) for PEPCK exon 1: HKY+I  
203 (1st position), JC (2nd position), TrN+I (3rd position) ; (iv) for PEPCK intron 2: TrN+I ; (v)  
204 for PEPCK exon 2 : JC (1st position), JC (2nd position), JC (3rd position). For maximum  
205 likelihood analyses (ML), we performed 10 independent runs in GARLI 2.0 for both genes  
206 (Zwickl, 2006); the topology and  $-\ln L$  was the same among replicates. Only the run with the  
207 highest likelihood was saved. We assessed statistical significance of nodes with 10,000  
208 nonparametric bootstrap replicates. We considered a topology well supported (high  
209 confidence) whenever the bootstrap value (branch supports) was greater than 85% (Hillis &  
210 Bull, 1993). We carried out Bayesian inference analyses (MB) with MRBAYES 3.1.2  
211 (Ronquist & Huelsenbeck, 2003). We achieved 10 independent analyses for each gene (100  
212 million generations, four chains with mixed models, default priors, saving trees every 100  
213 generations). Then we removed the first 10,000,000 generations as burn-in procedure. Then a  
214 majority-rule 50% consensus tree was constructed. Only branch supports (topologies) with  
215 high posterior probabilities ( $\geq 0.95$ ) were considered statistically significant (Wilcox *et al.*,  
216 2002). We (re) rooted all trees with the taxon *B. bimaculatus*.

217 To recognize species threshold, we used a Bayesian implementation of the general  
218 mixed Yule-coalescent model (bGMYC) for species delimitation based on COI tree (Reid &  
219 Carstens 2012, see an example of the use of the approach in Lecocq *et al.* 2015c). These  
220 analyses were performed with "bGMYC" R packages (Reid & Carstens 2012). The  
221 stationarity and the modal coalescent/Yule ratio have been assessed to continue the analysis.  
222 A range of probabilities  $> 0.95$  was considered as strong evidence that taxa were conspecific  
223 while a range of probabilities  $< 0.05$  suggested that taxa was heterospecific (Reid & Carstens  
224 2012). Since bGMYC required ultrametric trees, we performed a phylogenetic analysis with  
225 BEAST 1.7.4 (Drummond *et al.* 2012) using a phylogenetic clock model to generate a  
226 posterior distribution of trees (length of the MCMC chain: 1 billion generations). The first  
227 million sampled trees as burn-in, using the maximum clade credibility method and setting the  
228 posterior probability limit to 0. We based the bGMYC analysis on 1000 trees sampled every  
229 10 000 generations. For each of these 1000 trees, the MCMC was made of 100 000  
230 generations, discarding the first 90 000 as burn-in and sampling every 100 generations.



231 Posterior probability distribution have been applied against the first sample tree to provide a  
232 "heat' map".

#### 233 *2.4. Molecular Clock - Estimating Divergence Time*

234 Following the approach of Duennes *et al.* (2012) and Lecocq *et al.* (2013), we  
235 analyzed the COI dataset in BEAST v1.7.2 (Drummond & Rambaut, 2007) to estimate the  
236 divergence time among different clades. Using the GTR + I model selected by jModeltest, we  
237 ran Markov chain Monte Carlo simulations with the coalescent constant population size tree  
238 model and the relaxed clock model. Considering that no fossils of *Pyrobombus* species are  
239 available, the phylogeny is calibrated with a range date from a molecular study. We specified  
240 a range of possible substitution rates which includes the extreme rate for insect mitochondrial  
241 genes recorded in the literature (e.g. Duennes *et al.*, 2012) using a flat prior ranging from  
242  $1 \times 10^{-9}$  to  $1 \times 10^{-7}$  substitutions site<sup>-1</sup> and year<sup>-1</sup>. Simulations were run for 300 million  
243 generations, sampling every 1000 generations. Four independent runs were assessed in Tracer  
244 v1.4.1 (Rambaut & Drummond, 2016) to confirm convergence, determine burn-in, and  
245 examine the effective sample size of all posterior parameters. Log files from each run were  
246 combined in LogCombiner v1.6.1 (Rambaut & Drummond, 2016) for final parameter  
247 estimates.

#### 248 *2.5. Eco-Chemical Traits Differentiation*

249 We focused on the most studied eco-chemical trait involved in the bumblebee pre-  
250 mating recognition (Baer, 2003; Ayasse & Jarau, 2014): the cephalic labial gland secretions  
251 (CLGS). These secretions are complex mixtures of mainly aliphatic compounds synthesized  
252 *de novo* by male cephalic labial glands (Coppée *et al.*, 2008; Lecocq *et al.*, 2011; Žáček *et al.*,  
253 2013). We identified the main component as the compound that had the highest relative  
254 concentration (RA) among all compounds of CLGSs at least in one specimen of the taxon.  
255 The CLGS are species-specific blends with some inter-population variations and are,  
256 subsequently, commonly used for species discrimination and assessment of intraspecific  
257 variability in bumblebees (review in Lecocq *et al.*, 2015a). We extracted the CLGS with 400  
258  $\mu$ l of n-hexane, according to De Meulemeester *et al.*, (2011) and Brasero *et al.*, (2015).  
259 Samples were stored at - 40° C prior to the analyses.

260 We qualified the CLGS composition of each sample by gas chromatography-mass  
261 spectrometry using a Focus GC (Thermo Scientific) with a non-polar DB-5 ms capillary  
262 column [5% phenyl (methyl) polysiloxane stationary phase; column length 30 m; inner  
263 diameter 0.25 mm; film thickness 0.25  $\mu$ m] coupled to DSQ II quadrupol mass analyser  
264 (Thermo Scientific) with 70 eV electron impact ionization. We identified each compound

265 using the retention times and mass spectra of each peak, in comparison to those from the  
266 National Institute of Standards and Technology library (NIST, U.S.A) database. We  
267 determined double bond positions (C=C) by dimethyl disulfide (DMDS) derivatization  
268 (Vincenti *et al.*, 1987).

269 We quantified the CLGS compounds with a gas chromatograph Shimadzu GC-2010  
270 system (GC-FID) equipped with a non-polar SLB-5 ms capillary column [5% phenyl (methyl)  
271 polysiloxane stationary phase; column length 30 m; inner diameter 0.25 mm; film thickness  
272 0.25  $\mu\text{m}$ ] and a flame ionization detector. We quantified the peak areas of compounds in GC  
273 solution postrun (Shimadzu Corporation) with automatic peak detection and noise  
274 measurement. The relative areas (RAs, expressed in %) of compounds in each sample were  
275 calculated by dividing the peak areas of compounds by the total area of all compounds. We  
276 excluded compounds for which RA were less than 0.1% for all specimens (De Meulemeester  
277 *et al.*, 2011). The data matrix for each taxon was based (Table S2) on the alignment of each  
278 relative proportion of compound between all samples performed with GCAAligner 1.0  
279 (Dellicour & Lecocq, 2013).

280 For GC/MS and GC-FID analyses, we injected 1  $\mu\text{L}$ , using a splitless injection mode  
281 (injector temperature of 220° C) and helium as carrier gas (1 mL/min, constant velocity of  
282 50cm/s). The oven temperature (of the column)was programmed isothermal at 70° C for 2  
283 min and then rising from 70° C to 320° C at a rate of 10°C/min. The temperature was then  
284 held isothermal at 320° C for 5 min.

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

289 We performed statistical comparative analyses of the CLGSs using R environment (R  
290 Development Core Team, 2013) to detect CLGS differentiations between *B. monticola* taxa.  
291 We used a clustering method, computed with the unweighted pair-group method with average  
292 linkage (UPGMA) based on Canberra distance matrices (RA of each compound) (R package  
293 ape; Legendre & Legendre, 2004; Paradis *et al.*, 2004), to detect the divergence between taxa  
294 in the CLGS composition. We assessed the uncertainty in hierarchical cluster analysis using  
295 p-values calculated by multiscale bootstrap resampling with 100,000 bootstrap replications  
296 (significant branch supports >0.85) (R package pvclust, Suzuki & Shimodaira 2011). We  
297 assessed CLGS differentiations between taxa by performing a permutation multivariate  
298 analysis of variance using distance matrix (PerMANOVA) (R package vegan, Oksanen *et al.*,

299 2011). When a significant difference was detected, we performed a pairwise multiple  
300 comparison with an adjustment of p-values (Bonferroni correction) to avoid type I errors. We  
301 determined specific compounds of each taxon (indicator compounds) with the indicator-value  
302 (IndVal) method (Dufrêne & Legendre, 1997; Claudet *et al.*, 2006). This value is the product  
303 of relative concentration and relative occurrence frequency of a compound within a group.  
304 The statistical significance of an indicator compound (threshold of 0.01) was evaluated with a  
305 randomization procedure.

## 306 2.6. Morphological Analyses

307 To investigate diagnostic morphological character for species identification and new  
308 taxa description (not for species delimitation), a total of 60 workers were subjected to  
309 morphological analysis to discriminate *B. lapponicus*, *B. konradini* and *B. monticola*. We  
310 included only workers to have a sufficient sampling and because the differences in  
311 metabasitarsus measurements were more pronounced in females than males. We selected the  
312 maximum length and width metabasitarsus distance in reference to the work of Gjershaug *et*  
313 *al.* (2013) and we calculated the ratio (max length/max width) of these two measures to  
314 reduce the effect of body size on this morphological analysis. One picture was taken for each  
315 measurement and specimen using a binocular coupled with a digital camera (Nikon D70). The  
316 specimen was positioned in a way to maximize the focus of the metabasitarsus. The maximum  
317 metabasitarsus distance was measured on the picture with the software Imagej 1.5 (Abràmoff  
318 *et al.*, 2004) (Tab. 1, Appendix S3). Kruskal-Wallis analyses (Kruskal-Wallis test and  
319 multiple comparison test after Kruskal-Wallis, “pgirmess” R-package, Siegel & Castellan,  
320 1988) were performed using R (R Development Core Team, 2013) to compare the different  
321 studied taxa.

## 322 2.7. Data Integration and Decision Framework

323 Assuming that species diagnosis and inter-population differentiation are more efficient  
324 in a multiple evidence based approach (De Queiroz, 2007; Schlick-Steiner *et al.*, 2010), we  
325 proposed a species delimitation hypothesis according to our genetic and CLGS criteria based  
326 on the method performed by Lecocq *et al.* (2015a) derived from the approach established by  
327 Schlick-Steiner *et al.* (2010). In this method, all criteria used in the integrative approach must  
328 be convergent to assign the specific status. This strict approach could lead to underestimate  
329 the species differentiation but reduces the taxonomic inflation (Lecocq *et al.*, 2015a) We  
330 assigned the species status to a taxon (with a high degree of certainty) if this taxon: (i) was  
331 genetically differentiated in all genetic markers (unique haplotype); (ii) constituted a  
332 monophyletic group with high branch support; (iii) was significantly differentiated in CLGS

333 compositions (including IndVal indicator compounds, PerMANOVA, high bootstrap values >  
334 0.85) (Lecocq *et al.*, 2015a). We assigned the subspecies taxonomic status to phenotypically  
335 distinct allopatric populations with differentiation in some traits but not all in order to  
336 highlight those populations displaying such differentiation (originality) and to reduce the  
337 "underestimate's risk" of our strict approach to assign species status by naming them as a  
338 subspecies (Hawlitschek *et al.*, 2012; Ennen *et al.*, 2014; Lecocq *et al.*, 2016).

### 339 2.8. Identification and Type Revision

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

## 344 3. RESULTS

### 345 3.1. Inter-Taxa Differentiation

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

363 In comparison to the ML and MB analyses for COI data, the tree generated for  
364 bGMYC analysis displayed difference (not biologically significant) mainly in the branching  
365 of *mathildis* **ssp. nov.** As discussed in literature, these differences were probably due to the  
366 different parameters used in the BEAST 1.7.4 software to calculate the bGMYC model and

367 because this pairwise matrix (heat map) was plotted against a sample tree (Lecocq *et al.*,  
368 2015a; Barraclough *et al.*, 2003). The bGMYC analysis (Fig. 4) highlighted several entities  
369 with low probabilities (<0.05) to be conspecific with the other ones. These results match with  
370 the same taxa recognized in the COI tree (MP, ML, MB analyses, Fig. 3). Overall, the  
371 bGMYC suggested the delimitation of four prospective species ( $p < 0.05$ ) within the  
372 *monticola* complex (and the comparison group) as in Fig. 3 : (i) one group including all  
373 *lapponicus* (bGMYC conspecificity probabilities between individuals included in the group,  $p$   
374  $>0.98-1$ ), (ii) a group with all *konradini* from Central Apennines ( $p >0.99-1$ ), (iii) one group  
375 with all *bimaculatus* ( $p >0.98-1$ ), (iv) all *monticola* subspecies ( $p > 0.13-0.95$ ) including  
376 *rondoui* (IP  $>0.95-1$ ), *alpestris* ( $p >0.95-1$ ), *scandinavicus* ( $p >0.98-1$ ), *monticola* ( $p >0.99-$   
377  $1$ ) and *mathildis* ssp. nov. ( $p >0.99-1$ ) which are significantly conspecific. The pairwise  
378 matrix (Fig. 4) shows more structure within *B. monticola* ssp. where the group displays more  
379 different haplotypes. These intermediate values of bGMYC (Fig. 4) between the different  
380 *monticola* lineages (genetic differentiation below the species differentiation threshold) are  
381 useful to discuss of subspecies concept.

382 In chemical analyses, 103 compounds were detected; 82 in the CLGSs of *B. monticola*  
383 taxa (Appendix S2) except for *konradini* for which we detected only 50 compounds. The  
384 differentiation of CLGS composition between *B. monticola* taxa and outgroup species (*B.*  
385 *lapponicus* and *B. bimaculatus*) was conspicuous (IndVal; PerMANOVA  $F=115.63$  and  $F=$   
386  $122.52$ ,  $p$ -value  $< 0.05$ ; Fig.3). Except *konradini*, all other *B. monticola* taxa shared the same  
387 compounds with similar relative concentration (RA) (PerMANOVA  $F= 6.00 - 13.20$ ,  $p$ -value  
388  $>0.05$ ) (Appendix S2). Differences between *konradini* and other *B. monticola* taxa were  
389 particularly marked in the first half of the spectrum representing the most volatile molecules.  
390 The relative abundance of several compounds was different compared with the relative  
391 abundance in other taxa of *B. monticola*. The IndVal method highlighted several unique and  
392 diagnostic compounds of *konradini* (Tab. 3; i.e. ethyl tetradecenoate, ethyl tetradecanoate,  
393 hexadec-7-en-1-ol ethyl octadecadienoate, dotriacontane, ethyl octadec-9-enoate). In  
394 particular, *konradini* was characterized by ethyl octadec-9-enoate with a relative abundance of  
395 8.28% while it had very low relative abundance in other subspecies (median 0.57%). The  
396 discrimination between *konradini* and other *B. monticola* taxa was supported by maximal  
397 bootstrap support values (100%) (Fig.3). This differentiation was confirmed by statistical  
398 analysis (PerMANOVA  $F= 29.36$   $p$ -value  $< 0.05$ , between *konradini* and other *B. monticola*  
399 taxa).

### 400 3.2. Taxonomic Status

401 Species status was confirmed for the comparison group *B. bimaculatus* and *B.*  
402 *lapponicus*. According to the mtDNA and nuDNA divergence along with the CLGS  
403 composition differentiation (including main compounds) (Tab. 4), the species status was  
404 assigned to *konradini* (detailed information is given in Supporting information, Appendix S3).  
405 *Bombus konradini* was originally described by Reinig (1965) as a subspecies typical of the  
406 northern and central Apennines, ranging from the provinces of Genova and Parma to  
407 L'Aquila. All other taxa were included in *B. monticola* but their color pattern (Tab.1) and/or  
408 differentiation in CLGS composition (minor quantitative differences) and/or in COI marker  
409 implied their assignation to the subspecies status. It is important to note the distinction of the  
410 north Apennines (province of Genoa and Parma to the provinces of Bologna and Lucca)  
411 *monticola* population (*B. monticola mathildis* **ssp. nov**) from the central Apennines taxon (*B.*  
412 *konradini* **nov. status**) and the population from the Alps (*B. monticola alpestris*). Indeed,  
413 considering the slight differentiation in COI (0.53% of divergence from *alpestris*) and the  
414 strong divergence in coat color from *alpestris* (Tab.1, Fig.3), the north Apennines population  
415 should have a new subspecies status: *B. monticola mathildis* (detailed information is given in  
416 Supporting information, Appendix S3).

### 417 3.3. Divergence Times among Clades

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

### 427 3.4. Morphological Analysis

428 Measurements of the ratio between the maximum length and width of the metabasitarsus  
429 show significant differences (Kruskal-Wallis multiple comparison  $\chi^2 = 32.757$ ; all p-values <  
430 0.05) between *lapponicus* and *monticola alpestris*, *monticola mathildis* ssp. nov. The ratio is  
431 also significantly different between *konradini* and *monticola* ssp. but not between *monticola*  
432 *alpestris* and *monticola mathildis* (Fig. S2, Appendix S3). However, between *konradini* and  
433 *lapponicus*, although our results present a clear trend which highlight a larger ratio for  
434 *konradini*, there is no significant differentiation. According to these results, *konradini* appears

435 as intermediate between *monticola sensu stricto* (large metabasitarsus ratio) and *lapponicus*  
436 (small metabasitarsus ratio). Diagnostic morphological characters are summarized in Tab. 1.

### 437 3.4. Impact of new taxa in zoological nomenclature

438 a) *Bombus konradini sp. nov. status* (more information in Appendix S3)

439 Original taxonomic combination: *Bombus lapponicus konradini* Reinig 1965: 105

440 *Locus typicus*: Monti Sibillini, Central Apennine Mountains (Italy)

441 Syntypes: 13 queens, 93 workers, 28 males

442 Lectotype (present designation): 1 queen, labeled: 1) "Italia, Monti Sibillini, Nh. M.  
443 Vettore, Baumgrenze, 15-1600m, 14.6.61, Reinig".; 2) (on red paper) "LECTOTYPE; 3) "det.  
444 P. Rasmont 2015 *Bombus* (Pyrobombus) *monticola konradini* Reinig" (Fig.1).

445 Paralectotype: 2 queens, 41 workers, 16 males have been located, designated and  
446 labeled as paralectotypes (Tab.2). In this series, only 1 queen (lectotype) and 21 workers from  
447 Marche, Umbria, Lazio and Abruzzo have been identified as *Bombus konradini*. The  
448 remaining paralectotypes (2 queens, 20 workers, 16 males) from Liguria, Emilia-Romagna  
449 and Toscana have been assumed as *Bombus monticola mathildis*.

450 b) *Bombus monticola mathildis* Martinet, Cornalba & Rasmont **ssp. nov.** (more information in  
451 Appendix S3)

452 *Locus typicus*: North Apennines, Emilia Romagna, Reggio Emilia, Villa Minozzo, Mt Cusna  
453 (Italy).

454 Holotype (present designation): 1 male, labeled: 1) "Italy, Emilia Romagna, Reggio  
455 Emilia, Villa Minozzo, Mt Cusna, 44.283492 N 10.401028 E, 2057m, 05.VIII.2015,  
456 S/Scabiosa sp, Rec. M. Cornalba, BMAR0431"; 2) (on red paper) "Holotype"; 3) "det. B.  
457 Martinet 2016 *Bombus* (Pyrobombus) *monticola mathildis* Martinet, Cornalba & Rasmont"  
458 (Fig.1).

459 Paratype: 2 males have been located, designated and labeled as paralectotypes: "Italy,  
460 Emilia Romagna, Reggio Emilia, Villa Minozzo, Mt Cusna, 44.283492 N 10.401028 E,  
461 2057m, 05.VIII.2015, S/Scabiosa sp, Rec. M. Cornalba, BMAR0432" and "Italy, Emilia  
462 Romagna, Reggio Emilia, Villa Minozzo, Mt Cusna, 44.282288 N 10.401603 E, 2055m,  
463 12.VIII.2015, S/*Carduus carlinifolius*, Rec. M. Cornalba, BMAR0433".

## 464 4. DISCUSSION

### 465 4.1. Inter-Population Differentiation of *B. monticola*

466 The concordance between genetic differentiation, geographic distribution, and CLGS  
467 divergence of populations suggests a strong intraspecific structure between the subspecies of

468 *monticola* (Fig.3). The western subspecies (*B. monticola rondoui* from Pyrenees and *B.*  
469 *monticola monticola* from British Islands), the north Apennines population (*B. monticola*  
470 *mathildis* ssp. nov.) and the eastern-northern subspecies (*B. monticola scandinavicus* from  
471 Sweden and *B. monticola alpestris* from Alps, Balkans and Olympus Mt) constitute, with the  
472 COI marker, five differentiated groups in three main lineages which diverged recently (about  
473 40,000 -18,000 years ago based on molecular clock) during the Pleistocene/Quaternary  
474 (Fig.3). This could explain the weak divergence of PEPCK marker between *monticola*  
475 subspecies (recent divergence) because nuclear genes have a lower mutation rate than  
476 mitochondrial genes (Lunt *et al.*, 1996; Trunz *et al.*, 2016). Such time-estimation matches  
477 with the start of the last post-glacial warming. So, it appears that the geographic pattern is  
478 most likely a consequence of allopatric differentiation and genetic drift triggered by a range  
479 fragmentation subsequent to the last post-glacial warming. We speculate that, at the beginning  
480 of the current interglacial time, taxa have found refuge in southern mountainous areas (Alps  
481 and southern peninsulas of Europe) and Northern Europe by contraction of their distribution  
482 areas or range shifting (Hewitt, 1999; Hewitt & Ibrahim, 2001; Petit *et al.*, 2003; Stewart *et*  
483 *al.*, 2010), similarly to the boreo-montane leaf beetle (*Chrysomelidae*), *Gonioctena pallida*  
484 (Mardulyn *et al.*, 2009). The resulting allopatry has fostered mtDNA differentiation along  
485 with minor differentiation of chemical reproductive traits similarly to what has already been  
486 shown for insular populations of bumblebees (Lecocq *et al.*, 2013, 2015a).

487 Despite their relative geographic isolation, all other *B. monticola* allopatric taxa  
488 previously recognized by Reinig (1965) are considered as conspecific based on our diagnostic  
489 criteria with a low geographic genetic and phenotypic differentiation (decision framework  
490 Tab.4; Fig.3). They shared overall the same CLGS composition (except some low relative  
491 concentration differences) and are characterized by only a slight genetic differentiation. These  
492 low differentiations, particularly in CGLS composition, can simply be explained by the short  
493 time of divergence due to geographical isolation and intraspecific variability (Lecocq *et al.*,  
494 2011, 2016). Within *Bombus monticola alpestris*, the three sampled populations (Alps,  
495 Balkans and Olympus Mountain) are clearly consubspecific.

#### 496 4.2. Mountain Top Speciation: *Bombus konradini* nov.status.

497 Contrary to the situation within *B. monticola*, *B. konradini* nov. status displays larger  
498 genetic and chemical traits differentiations (Fig. 3). Allopatry has most likely shaped the  
499 reproductive trait (CLGS) differentiation as observed in other species (Lecocq *et al.*, 2013).  
500 The strong genetic differentiation of *B. konradini* could be explained by an earlier divergence  
501 from the common ancestor with other *B. monticola* lineages, most likely temporally close to



502 the *B. monticola* - *B. lapponicus* complex divergence. Indeed, based on genetic differences in  
503 the 16S gene, Hines (2008) suggested that *B. lapponicus* and *B. monticola* diverged from each  
504 other about 3 Ma. In temperate species, the post Ice-Age recolonization of territories by relict  
505 populations (from refugia), could lead to a new shuffling of the genetic pool by re-contacting  
506 of these populations without speciation (Coyne & Orr, 2004; Hewitt, 2004). The modification  
507 of geographical range could trigger genetic and CLGS differentiation. Indeed, it has been  
508 shown that the reproductive traits including cephalic labial glands secretions can differentiate  
509 from both sides of the physical barriers that may exist between the refuge areas (Lecocq *et al.*,  
510 2013a). The case of the new species status of *B. konradini* lends strength to the hypothesis  
511 that for cold-adapted taxa, climatic oscillations (*i.e.* interglacial periods) have led to species  
512 differentiation in mountain refuges after geographical separation. Further phylogeographic  
513 and phylogenetic studies, based on larger sampling (including additional closely related  
514 species) and other genetic markers, are needed to accurately assess these hypotheses.

515 Our integrative taxonomic decision framework supported and confirmed the species  
516 status of *B. monticola* compared with its morphologically closely related species (*B.*  
517 *lapponicus*) (Løken, 1973; Svensson, 1979; Cameron *et al.*, 2007; Gjershaug *et al.*, 2013).  
518 Our results also supported the species status of *konradini* which is endemic of high-altitudes  
519 (>1800 mt) of the Central Apennines (Manino *et al.*, 2007) (Fig. 3, Tab. 4). Concerning the  
520 eco-chemical traits (CLGS), *konradini* differed from the other *B. monticola* taxa by  
521 lightweight compounds (volatile molecules) which could have a long distance attractive role  
522 (Ayasse *et al.*, 2001). Therefore, the differentiation of these compounds may be a significant  
523 pre-mating reproductive barrier or may simply reflect divergence times and drift. Besides,  
524 according to the results of COI marker, *konradini* could be more closely related to *B.*  
525 *lapponicus* (Fig.3) than *B. monticola* taxa as suggested in the original description of Reinig  
526 (1965). However, the phylogenetic position of *konradini* is not completely resolved because  
527 of the different tree topologies between COI and PEPCK results.

528 The species status of *B. konradini* suggests that inter-glacial periods can lead to  
529 species differentiation in mountain refugia in cold-adapted taxa. Unlike the populations of the  
530 Alps, Pyrenees and Balkans, where the interconnection and thus the possibility of exchanges  
531 and conspecificity are likely, the population of the central Apennines is much more isolated  
532 from other mountain chains with a possible endemic speciation (Martin- Bravo *et al.*, 2010).  
533 Several studies have shown the presence of endemic taxa in the Central Apennines (e.g. in  
534 amphibians Mattoccia *et al.*, 2011, Canestrelli & Nascetti, 2008, 2012, in reptiles Nagy *et al.*,  
535 2002, Joger *et al.*, 2007, in turtles Fritz *et al.*, 2006, in plants Conti *et al.*, 2005; Fuente *et al.*,

536 2011, Frattaroli *et al.*, 2013; in bumblebees Lecocq *et al.*, 2013). For example, Lecocq *et al.*,  
537 (2013a) provided evidence that the population of *B. lapidarius* (a Palearctic polytypic species)  
538 from the Southern Italian refugia has experienced genetic and CLGS differentiation during  
539 Quaternary glaciations leading to an incipient speciation process. Populations inhabiting the  
540 Mediterranean mountains, (e.g. the Apennines, one of the few mountain ranges in Europe  
541 arranged on a north-south axis) are characterized by a high genetic diversity (hotspot) with  
542 endemic taxa (Ruiz-La-Bourdette *et al.*, 2012).

543 The sympatry of two different species, defined by divergent taxonomic traits,  
544 reinforces the "species" status because individuals co-inhabit in the same area without  
545 hybridization. Our results suggest that *B. monticola* (*sensu stricto*) is absent in Central  
546 Apennines unless this could be due to a sampling bias. Such absence could result in lack of  
547 sympatry between *B. monticola* and *B. konradini*. Several hypotheses could explain the  
548 potential absence of *B. monticola* (*sensu stricto*) in Central Apennines: (i) for eco-climatic  
549 constraints, historical or competition reasons, this taxon has never inhabited this region or has  
550 disappeared, (ii) despite the significant observed differences (genetic, morphological and  
551 chemical traits), a limited hybridization between *monticola* and *konradini* could be still  
552 possible. Following this second hypothesis, along their contact zone, the subspecies *mathildis*,  
553 could represent an intermediate population resulting from some introgressions of the  
554 population living in Alps (*alpestris*). Our COI results suggest that the subspecies *mathildis*  
555 (low branch support) is closer to *B. konradini* than all other subspecies of *monticola* (*sensu*  
556 *stricto*) (Fig. 3). However, phenotypic and chemical traits results do not tend towards this  
557 hypothesis (Figs. 3C, S2). Although distinct, *B. konradini* could be the "replacement species"  
558 to *B. monticola* (*sensu stricto*) with similar eco-climatic constraints and filling the ecological  
559 niche in Apennines or a relict population of a near relative of *B. lapponicus* in Italy  
560 considering COI results. Additional ethological experiments (hybridization tests) and further  
561 genetic analyses (e.g. Microsatellite, SNPs, RAD-seq) are necessary to test these hypotheses  
562 of intermediate populations or replacement species in the context of taxonomic implications.

#### 563 4.3. Conservation Remarks on *B. monticola* Complex and the Practice of Integrative 564 Taxonomy

565 Considering all taxonomic criteria in our integrative approach (Fig. 3, Tab. 4), we  
566 propose to conserve the subspecies status for five *monticola* taxa (Hawllitschek *et al.*, 2012;  
567 Lecocq *et al.*, 2015a,c, 2016): *B. monticola rondoui* from Pyrenees, *B. monticola monticola*  
568 from British Islands, *B. monticola scandinavicus* from Fennoscandia and *B. monticola*  
569 *alpestris* from Alps, Balkans, Olympus Mountains and *B. monticola mathildis* ssp. nov. from

570 North Apennines (formerly included by Reinig within *konradini*). Although the usefulness of  
571 subspecies status in bumblebees has been criticized and debated (Ebach & Williams, 2009)  
572 during recent decades (Williams, 1991; Bertsch & Schweer, 2012a), we propose that these  
573 allopatric subspecies (partially isolated lineages) represent an important component and a  
574 useful pragmatic taxonomical unit for evolutionary biology and biological conservation in the  
575 evolutionary legacy of *B. monticola* (i.e. Waples, 1995; Patten & Unitt, 2002; Phillimore &  
576 Owens, 2006; Rasmont *et al.*, 2008; Patten, 2009; Crowhurst *et al.*, 2011; Braby *et al.*, 2012;  
577 Sackett *et al.*, 2014). These differentiations could be local adaptations to particular  
578 environments (Awise, 2000; Frankham *et al.*, 2010; Braby *et al.*, 2012; Lecocq *et al.*, 2013a).  
579 Therefore, subspecies classification seems suitable to reflect the intraspecific differentiation  
580 within *B. monticola* taxa.

581         The *monticola* complex is a stunning example of the difficulty, in taxonomy, to define  
582 species or subspecies status of a population. Here the integrative taxonomy considering all  
583 criteria independently could provide strong pieces of evidence for decision. We assigned the  
584 subspecies taxonomic status to phenotypically distinct allopatric groups of populations with  
585 differentiation in some but not all criteria used in the integrative decision framework (i.e.  
586 conflict in selected criteria) (Hawlitschek *et al.*, 2012; Ennen *et al.*, 2014; Lecocq *et al.*,  
587 2015a,c, 2016). Taxonomical conclusions based only on the differentiation of one  
588 mitochondrial marker (e.g. COI barcoding) can lead to weak taxonomic hypotheses  
589 (Andriollo *et al.*, 2015; Mutanen *et al.*, 2016; Trunz *et al.*, 2016) as mitochondrial  
590 differentiation may result from sex-specific characteristics, as lower dispersion for females  
591 (Kraus *et al.*, 2009; Lepais *et al.*, 2010), or mtDNA introgression or incomplete lineage  
592 sorting (Bensasson *et al.*, 2001; Lecocq *et al.*, 2015a). Taxonomic diagnosis based on multiple  
593 evidence (integrative taxonomy) is the best approach to avoid overestimation of species  
594 diversity which would lead to taxonomic inflation. Subspecies can be considered as a simple  
595 allopatric differentiation (Mayr, 1942; Patten, 2010). This procedure allows to assign a  
596 taxonomic status to any doubtful bumblebee taxa and points out these taxa for further  
597 taxonomic studies (Lecocq *et al.*, 2015a). Moreover, despite the argument advanced by  
598 Williams *et al.* (2015), there is no case in bumblebees where the CLGS (mate recognition  
599 system) was not differentiated between two different species even when closely related  
600 bumblebee species have geographical distributions that do not overlap (e.g. *B. terrestris* (L.)  
601 and *B. ignitus* Smith, De Meulemeester *et al.*, 2011 or between *B. patagiatus* and *B. magnus*  
602 where Bertsch & Schweer, (2012b) have shown the differentiation of their cephalic labial  
603 gland secretions).

#### 604 4.4. Conservation Implication of the New Taxonomic Status of *B. konradini* nov. status

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

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#### 1049 **AUTHOR CONTRIBUTIONS**

1050 Conceived and designed the experiments: BM TL NB CU IV PR. Sampling: BM NB PB MC  
1051 PR. Analyzed the data: BM. Wrote the paper: BM TL NB PB MC CU IV JOG, DM PR.

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#### 1056 **SUPPORTING INFORMATION**

1057 **Appendix S1.** Table of sampling. Sample code refers to the sample labels used in different  
1058 analyses. COI and PEPCK are the GenBank accession numbers for each sample (when  
1059 consubspecific samples display the same gene sequence, only one of them has been submitted  
1060 to Genbank;).

1061 **Appendix S2.** Data matrix of cephalic labial gland secretions (CLGS) (relative concentration  
1062 of each compound), list of the identified compounds and IndVal analysis with specific  
1063 compounds in the *monticola* complex. Unknown x's indicate undetermined compounds.

1064 **Appendix S3.** Description of the new subspecies *Bombus monticola mathildis* and *Bombus*  
1065 *konradini* nov. status., designation of the holotype and lectotype and morphological  
1066 differentiation. **Fig. S1.** Morphology and coloration variation of the face of *Bombus konradini*  
1067 *nov. status* (Lectotype female, A) and *Bombus monticola alpestris* (female, B). Photographs  
1068 are by P. Rasmont. **Fig. S2.** Comparison of the ratio maximum length/ maximum width  
1069 metabasitarsus between workers of *B. lapponicus*, *B. konradini*, *B. monticola alpestris* and *B.*

1070 *monticola mathildis*. With n= number of used specimens; \* = significant differences (Kruskal-  
1071 Wallis multiple comparison, p-value < 0.05).

1072 **Fig. 1. Distribution map (Gall projection) of *Bombus monticola* (Rasmont & Iserbyt,**  
1073 **2014) and its traditional subspecies in Europe according to Rasmont (1983a).** A, *Bombus*  
1074 *monticola scandinavicus* queen, red area on the map; B, *Bombus monticola monticola* queen,  
1075 dark green area; C, *Bombus monticola rondoui* queen, purple area; D, *Bombus monticola*  
1076 *alpestris* queen, blue area; E, *Bombus monticola mathildis* **ssp. nov** Holotype male, pink area;  
1077 F, *Bombus konradini* **nov status** Lectotype queen, yellow area. Light green dots indicate the  
1078 occurrence of the taxon in the region. All photographs are by P. Rasmont.

1079 **Fig. 2. Photos of the studied bumblebee taxa.** A, *Bombus monticola scandinavicus* queen;  
1080 B, *Bombus monticola monticola* queen; C, *Bombus monticola rondoui* queen; D, *Bombus*  
1081 *monticola alpestris* queen; E, *Bombus monticola mathildis* **ssp. nov** Holotype male; F,  
1082 *Bombus konradini* **nov status** Lectotype queen. All photographs are by P. Rasmont.

1083 **Fig. 3. Genetic and chemical analyses within the *monticola* complex.** (A) Majority rule  
1084 (50%) consensus tree based on maximum likelihood analyses of COI. Values above tree  
1085 branches are Parsimony bootstrap values/ maximum likelihood bootstrap values/Bayesian  
1086 posterior probabilities. Only maximum likelihood and parsimony bootstrap values >70% and  
1087 posterior probabilities >0.95 are shown. (B) Majority rule (50%) consensus tree based on  
1088 maximum likelihood analyses of PEPCK. Values above tree branches are Parsimony  
1089 bootstrap values/ maximum likelihood bootstrap values/Bayesian posterior probabilities. Only  
1090 maximum likelihood and parsimony bootstrap values >70% and posterior probabilities >0.95  
1091 are shown. (C) 1) Dendrogram of cephalic labial gland secretion differentiation within  
1092 *monticola* complex and *B. bimaculatus*. This cluster was obtained by hierarchical clustering  
1093 using an unweighted pair-group method with arithmetic mean (UPGMA) based on a Canberra  
1094 matrix calculated from the cephalic labial gland secretion matrix of *B. bimaculatus* (red), *B.*  
1095 *lapponicus* (dark blue), *B. konradini* nov. status (green), *B. m. rondoui* (pink), *B. m.*  
1096 *scandinavicus* (yellow), *B. m. monticola* (light blue), *B. m. alpestris* (black), *B. m. mathildis*  
1097 *ssp. nov.* (orange). The values near nodes represent multiscale bootstrap resampling values  
1098 (only values >80 of main groups are shown except nodes between *B. monticola* subspecies).  
1099 2) Principal component analysis (PCA) of cephalic labial gland secretion differentiation  
1100 within *monticola* complex and *B. bimaculatus*: *B. bimaculatus* (red circles), *B. lapponicus*  
1101 (dark blue circles), *B. konradini* nov. status (green circles), *B. m. rondoui* (pink circles), *B. m.*  
1102 *scandinavicus* (yellow circles), *B. m. monticola* (light blue circles), *B. m. alpestris* (black

1103 circles), *B. m. mathildis* ssp. nov. (orange circles). PC1, PC2 and PC3 are the first, the second  
 1104 and the third axes.

1105 **Fig 4. Species recognition pairwise matrix.** Species recognition pairwise matrix based on  
 1106 ultrametric tree of cytochrome oxidase 1 (COI) sequences with bGMYC pairwise probability  
 1107 of conspecificity plotted on a sample tree from BEAST. The colored matrix corresponds to  
 1108 the pairwise probabilities of conspecificity returned by the bGMYC method (color scale on  
 1109 the right of the figure). Black spots show the coalescent node for each species.

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1112 Tab.1. Range, conservation status and main morphological and colour pattern differences  
 1113 (male and female) between *B. konradini* nov. status, *monticola* subspecies including *mathildis*  
 1114 ssp. nov. and the similar species *B. lapponicus* according to Gjershaug *et al.*, (2013), Løken  
 1115 (1973), Pittioni (1939) and original observations.

	<i>scandinavicus</i>	<i>monticola</i>	<i>rondoui</i>	<i>alpestris</i>	<i>mathildis</i> <i>ssp.nov.</i>	<i>konradini</i> <i>status</i>	<i>lapponicus</i>
Range	Fennoscandia	British Islands	Pyrenees	Alps, Balkans, Olympus Mounts	North Apennines	Central Apennines	Fennoscandia
Conservation status	No regression was mentioned	In decline (Fitzpatrick <i>et al.</i> , 2006; Evans & Potts 2013)	In decline (Iserbyt & Rasmont 2012)	Few data show a decline in Italy (Manino <i>et al.</i> , 2007)	No regression was mentioned	Rare and localized (Ricciardelli & Piatti 2003)	Stable (Nieto <i>et al.</i> , 2014)
<b>Female</b>							
<b>Morphology</b>							
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 <i>monticola</i>	The surface between the punctures on vertex is rugose and dull and the furrow is distinct, nearly reaching the compound eye
Hind meta-basitarsus	Slight pubescence and the maximal width of the basitarsus is high ( <i>sensu</i> Gjershaug <i>et al.</i> , 2013). The length of the metabasitarsus of these taxa is large (Appendix S3)					Strong pubescence and the maximal width of the basitarsus is low ( <i>sensu</i> Gjershaug <i>et al.</i> , 2013) as in <i>lapponicus</i> . The ratio maximum length/maximum	Strong pubescence and the maximal width of the basitarsus are low ( <i>sensu</i> Gjershaug <i>et al.</i> , 2013). The length of the metabasitarsus of this taxon is short (Appendix S3).

						width of the metabasitarsus of this taxon is intermediate (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)
<b>Color pattern</b>							
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 sometimes yellow (few)	Yellow	Yellow
<b>Male</b>							
<b>Color pattern</b>							
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

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1122 Tab.2. Summary of sampling table with genetic and eco-chemical criteria used in this study.

1123 PEPCK: Phosphoenolpyruvate carboxykinase gene, COI: Cytochrome oxidase 1 gene, CLGS:

1124 Cephalic labial gland secretions, M: male and F: female.

1125

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

1126

1127 Tab.3. List of indicator compounds (IndVal method, compounds &gt; 70%, \*) and main

1128 compound (1) identified for *B. konradini nov. status* within cephalic labial gland secretions.

1129 With MW= the molecular weight, n= number of used specimens, M= median of compound

1130 relative concentration (%), 2 = main compound identified for *B. lapponicus*, 3 = main1131 compound identified for *B. bimaculatus*. Absent compounds are noticed by "-". The full

1132 matrix is presented in Appendix S2.

1133

Compounds	MW	<i>alpestris</i> (n=13)	<i>monticola</i> (n=10)	<i>rondoui</i> (n=7)	<i>scandinavicus</i> (n=11)	<i>mathildis ssp. nov</i> (n=2)	<i>konradini nov status</i> (n=2)	<i>lapponicus</i> (n=10)	<i>bimaculatus</i> (n=10)
		M	M	M	M	M	M	M	M
Citronellol*	156	-	-	-	-	0.08	0.17	-	-
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	-
<b>Hexadec-9-enyl acetate</b> <b>1</b>	282	52.34	55.15	57.05	53.96	35.27	51.53	0.08	32.95
<b>Geranyl citronellol</b> <b>2</b>	292	-	-	-	-	-	-	71.32	-
Ethyl octadecadienoate1*	308	-	-	-	-	-	0,37	-	-
<b>Ethyl octadec-9-enoate*</b>	<b>310</b>	<b>0,68</b>	<b>0,35</b>	<b>0,57</b>	<b>0,46</b>	<b>1,73</b>	<b>8,28</b>	-	-
<b>Geranyl geranyl acetate</b> <b>3</b>	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

1134

1135 Tab.4. Taxonomic decision table with all criteria used for species delimitation. CLGS,  
1136 cephalic labial gland secretions; COI, cytochrome oxidase 1; PEPCK, phosphoenolpyruvate  
1137 carboxykinase. Morphology indicates if a taxon has a diagnostic morphological character (+/-  
1138 means that morphology is/is not diagnostic). Private haplotypes indicate if a taxon has a  
1139 specific haplotype (+/- means that the taxon has/has not only private haplotype (s). When the  
1140 taxon shares haplotype with other ones, the letters group together taxa that share haplotypes).  
1141 CLGS indicates if the taxon has/has not diagnostic composition of CLGSs with different main  
1142 compounds (+/- means that the taxon has/ has not a specific CLGS composition. When the  
1143 taxon shares CLGS composition with other ones, the letters group together taxa that share  
1144 similar CLGS. COI and PEPCK columns indicate if a taxon forms a strongly supported  
1145 monophyletic group (+/- means that the taxon is/is not a monophyletic group) with MP, ML  
1146 and MB methods . When the taxon is not a distinct monophyletic group, the letters group  
1147 together taxa included in the same monophyletic group). LS= low supported differentiation.

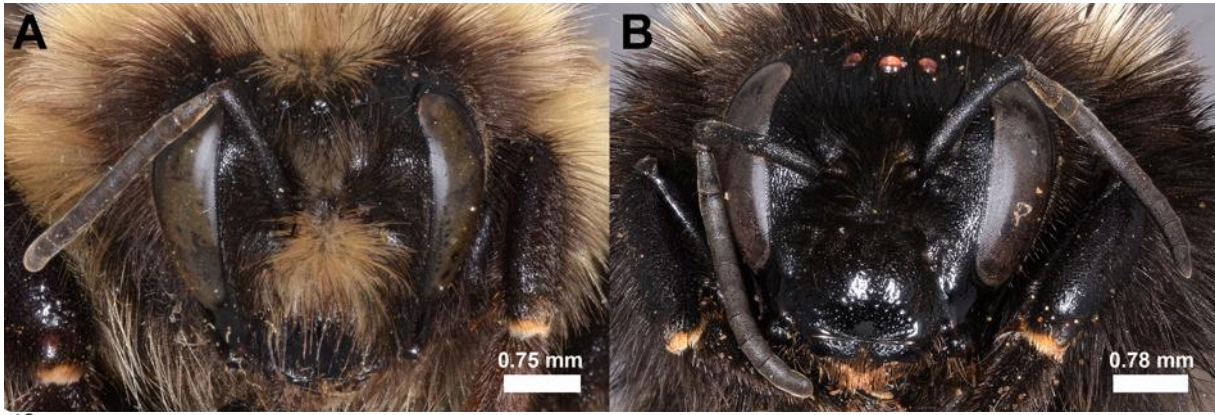
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Former taxonomic status	Morphology (Gjershaug <i>et al.</i> , 2013)	Private haplotypes (COI/PEPCK)	CLGS	COI gene / bGMYC	PEPCK gene	Proposed taxonomic status
<i>B. monticola scandinavicus</i>	-	+/-	- (B)	- LS (B) / -	-	<i>B. monticola scandinavicus</i>
<i>B. monticola monticola</i>	-	+/-	- (B)	- LS (A) / -	-	<i>B. monticola monticola</i>
<i>B. monticola rondoui</i>	-	+/-	- (A)	- LS (A) / -	-	<i>B. monticola rondoui</i>
<i>B. monticola alpestris</i>	-	+/-	- (C)	- LS (B) / -	-	<i>B. monticola alpestris</i>
<i>B. monticola konradini</i> (North Apennines)	-	+/-	- (C)	- LS (C) / -	-	<b><i>B. monticola mathildis</i> ssp. nov.</b>
<i>B. monticola konradini</i> (Central Apennines)	+	+/+	+	+ / +	+	<b><i>B. konradini</i> nov. status</b>
<i>B. bimaculatus</i>	+	+/+	+	+ / +	+	<i>B. bimaculatus</i>
<i>B. lapponicus</i>	+	+/+	+	+ / +	+	<i>B. lapponicus</i>

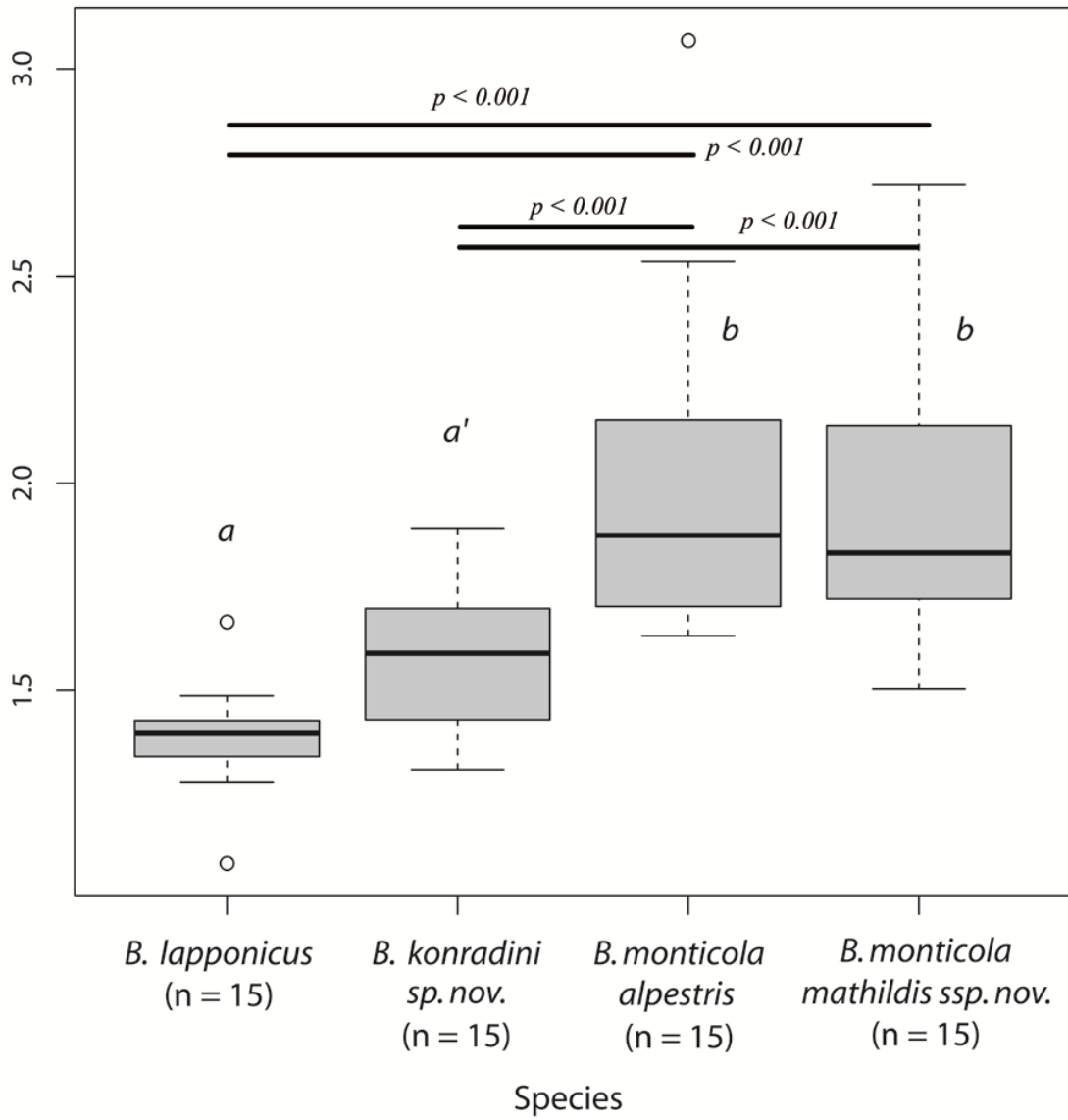
1150

1151



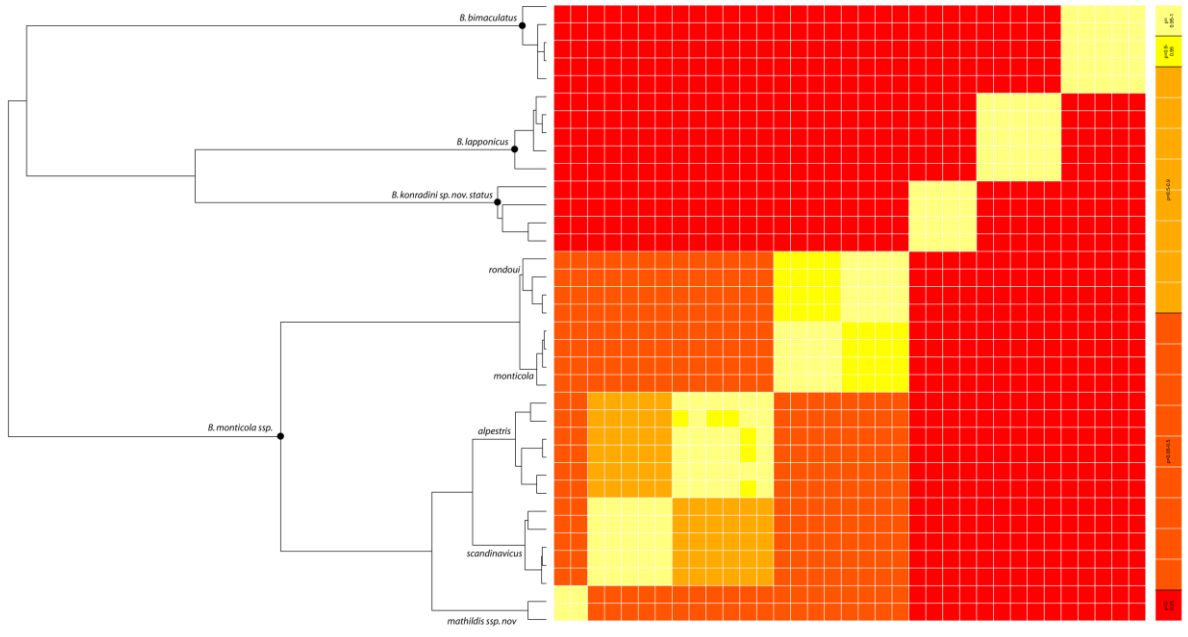
1152

Ratio maximum length / maximum width of metabasarsus of workers

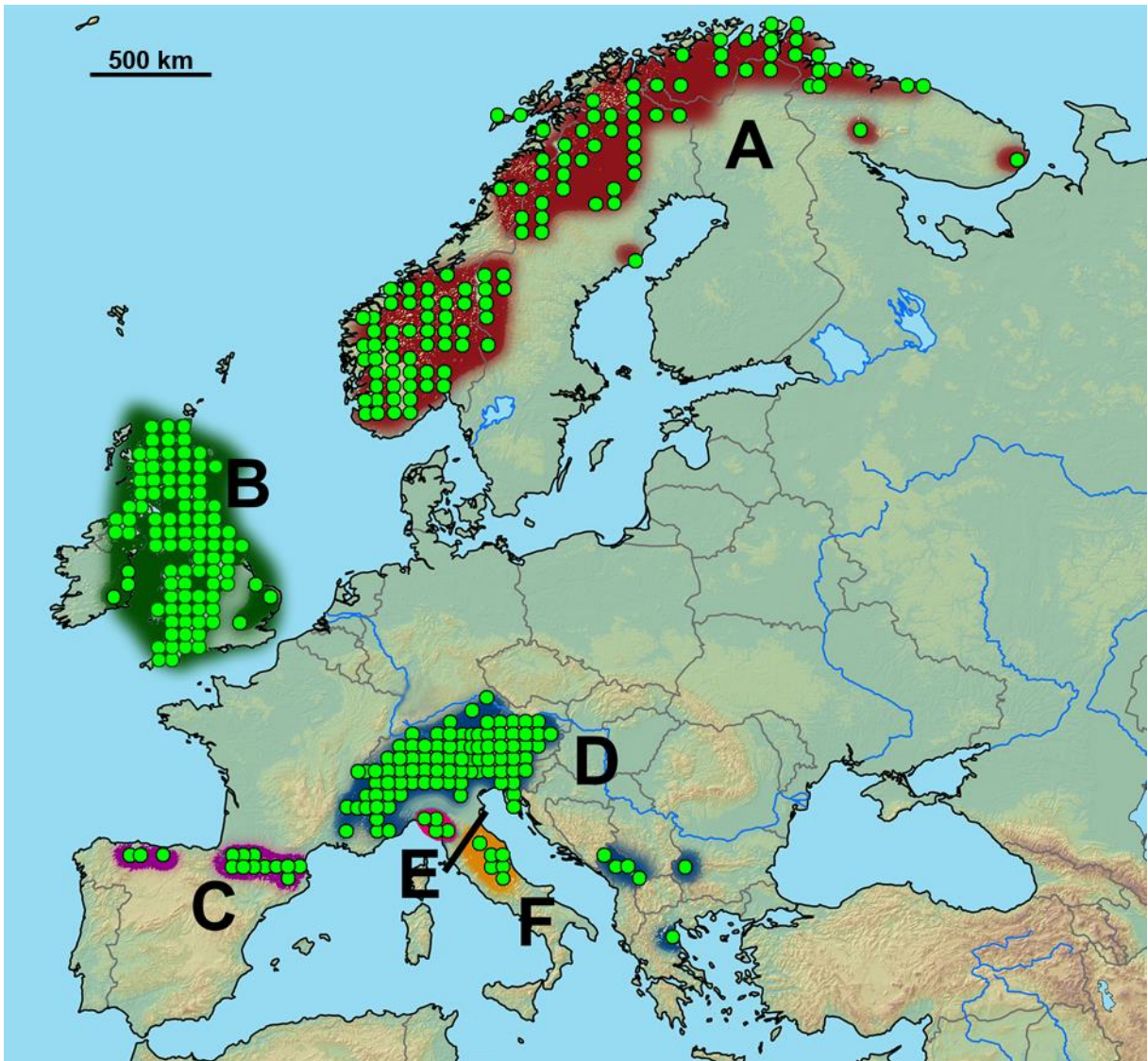


1153





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