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

Baptiste Martinet, Thomas Lecocq, Nicolas Brasero, Paolo Biella, Klára Urbanová, Irena Valterová, Maurizio Cornalba, Jan Ove Gjershaug, Denis Michez, Pierre Rasmont

▶ **To cite this version:**

Baptiste Martinet, Thomas Lecocq, Nicolas Brasero, Paolo Biella, Klára Urbanová, et al.. Following the cold: geographical differentiation between interglacial refugia and speciation in the arcto-alpine species complex Bombus monticola (Hymenoptera: Apidae). Systematic Entomology, 2018, 43 (1), pp.200-217. 10.1111/syen.12268 . hal-01699909

HAL Id: hal-01699909
https://hal.science/hal-01699909

Submitted on 8 Dec 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Martinet Baptiste[1*], Lecocq Thomas[1,2], Brasero Nicolas[1], Biella Paolo[3,4], Urbanová Klára[5,6], Valterová Irena[5], Cornalba Maurizio[7], Gjershaug Jan Ove[8], Michez Denis[1],Rasmont Pierre[1]

1: University of Mons, Research institute of Biosciences, Laboratory of Zoology, Place du Parc 20, 7000 Mons, Belgium

2: University of Lorraine-INRA, Research Unit Animal and Functionalities of Animal Products (URAFPA), 2 Avenue de la Forêt de Haye, BP 172, 54505, Vandoeuvre-lès-Nancy, France

3: University of South Bohemia, Department of Zoology, Faculty of Science, Branišovská 31, 37005;

4: Biology Centre of the Academy of Sciences of the Czech Republic, v.v.i., Institute of Entomology, Branišovská 31, 37005, České Budějovice, Czech Republic

5: Academy of Sciences of the Czech Republic, Institute of Organic Chemistry and Biochemistry, Flemingovo nám 2, CZ-166 10 Prague, Czech Republic (e-mail: irena@uochb.cas.cz)

6: Czech University of Life Sciences, Faculty of Tropical AgriSciences, Department of Sustainable Technologies, Kamýcká 129, CZ-165 21 Prague, Czech Republic

7: University of Pavia, Department of Mathematics, via Ferrata 5, I-27100 Pavia, Italy

8: Norwegian Institute for Nature Research, P.O. Box 5685 Sluppen, NO-7485 Trondheim, Norway.

***Corresponding author:** Baptiste Martinet (baptiste.martinet@umons.ac.be)

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

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 allopatric population) and disagreement on species concept. Here, we assessed post-glacial inter-refugia differentiation and potential speciation among populations of one of the most common arcto-alpine bumblebee species in European mountains, *Bombus monticola* Smith, 1849. Based on mitochondrial (mt) DNA/nuclear (nu) DNA markers and eco-chemical traits, we performed integrative taxonomic analyses to evaluate alternative species delimitation hypotheses 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 the *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 the North Apennine mountains 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 an 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-refugia differentiation, post-glacial warming

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

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

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

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

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 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 from its sister species *B. lapponicus* about 3 Ma. 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 *B. monticola* ssp. + *B. lapponicus* and only "*monticola"* gathering exclusively all subspecies of *B. monticola.*

2.2. Sampling

We sampled 70 specimens including all *B. monticola* taxa (Appendix S1) from the entire known distribution area : *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 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 considered statistically significant (Wilcox *et al.*, 2002). We (re) rooted all trees with the taxon *B. bimaculatus*.

To recognize species threshold, we used a Bayesian implementation of the general mixed Yule-coalescent model (bGMYC) for species delimitation based on COI tree (Reid & Carstens 2012, see an example of the use of the approach in Lecocq *et al*. 2015c). These analyses were performed with "bGMYC" R packages (Reid & Carstens 2012). 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). Since bGMYC required ultrametric trees, we performed a phylogenetic analysis with BEAST 1.7.4 (Drummond et al. 2012) using 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. 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 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 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

To investigate diagnostic morphological character for species identification and new taxa description (not for species delimitation), a total of 60 workers were subjected to morphological analysis to discriminate *B. lapponicus, B. konradini* and *B. monticola*. We included 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 ratio (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 reduces 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 those populations displaying 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

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*).

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

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

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 northern and central 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 to the provinces of Bologna and Lucca) *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 an estimated time of 40,000-18,000 (min 7,500 - max 548,000) years ago.

3.4. Morphological Analysis

Measurements of the ratio between the maximum length and width of the 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 to these results, *konradini* appears

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

3.4. Impact of new taxa in zoological nomenclature

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

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

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

Syntypes: 13 queens, 93 workers, 28 males

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

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

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

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

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

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

4. DISCUSSION

4.1. Inter-Population Differentiation of B. monticola

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

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

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

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 high-altitudes (>1800 mt) 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 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).

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

4.3. Conservation Remarks on B. monticola Complex and the Practice of Integrative Taxonomy

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 bumblebees 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; Patten & Unitt, 2002; 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 strong 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 hypotheses (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 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 to 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).

5. ACKNOWLEDGEMENTS

The authors thank the Abisko and Tarfala scientific stations (Sweden) for their welcome and their help in material collection. We acknowledge all people that helped us in our journey to the Abisko and Tarfala stations: P. Lakare and G. N. Rosqvist (University of Stockholm), M. Augner and L. Wanhatalo (Abisko Station), H. Savela (Oulu University, INTERACT project administration), J. Strand and T. Wikstrom (Lansstyrelsen i Norrbottens lan Naturvardsenheten, Lulea). The authors also thank the Parco Nazionale dei Monti Sibillini and the Parco Nazionale dell'Appennino Tosco-Emiliano for granting permission to collect in their respective territories. Special thanks go to P. Salvi (Sibillini), W. Reggioni (Appennino Tosco-Emiliano) and A. Cetkovic (University of Belgrade) for their help in the sampling. Computational resources have been provided by the *Consortium des Équipements de Calcul Intensif* (CÉCI), funded by the Belgian F.R.S.-FNRS. We thank also the two anonymous reviewers for their help to improve this manuscript. BM contributes as PhD student granted by Research Council of University of Mons and by the FRS-FNRS (Fonds de la Recherche Scientifique). PB contributes as PhD student funded by Czech Science Foundation (GAČR GP14-10035P) and by University of South Bohemia (GA JU 152/2016/P). Part of this work (I.V.) was supported by the Institute of Organic Chemistry and Biochemistry of the Academy of Sciences of the Czech Republic (project No. 61388963).

6. REFERENCES

638 Abràmoff, M.D., Magalhaes, P.J. & Ram, S.J. (2004) Image Processing with Imagej.
639 *Biophotonics*, **11**, 36-42
640 Andriollo, T., Naciri, Y. & Ruedi, M. (2015) Two mitochondrial barcodes for one biological
641 species: the case of European Kuhl's Pipistrelles (Chiroptera). *Plos One*, **10**,
642 e0134881.
643 Avise, J.C. (2000) *Phylogeography: The History and Formation of Species*. Harvard
644 University Press, Cambridge, Massachusetts.
645 Ayasse, M. & Jarau, S. (2014) Chemical ecology of bumble bees. *Annual Review of*
646 *Entomology*, **59**, 299–319.
647 Ayasse, M., Paxton, R.J. & Tengö, J. (2001) Mating behavior and chemical communication in
648 the order Hymenoptera. *Annual Review of Entomology*, **46**, 31–78.
649 Aytekin, A.M., Terzo, M., Rasmont, P. & Cagatay, N. (2007) Landmark based geometric
650 morphometric analysis of wing shape in *Sibiricobombus* Vogt (Hymenoptera :
651 Apidae : *Bombus* Latreille). *Annales de la Société Entomologique de France*, **43**,
652 95–102.
653 Baer, B. (2003) Bumblebees as model organisms to study male sexual selection in social
654 insects. *Behavioral Ecology and Sociobiology*, **54**, 521–533.
655 Bandelt, H.J., Forster, P. & Rohl, A. (1999) Median-joining networks for inferring
656 intraspecific phylogenies. *Molecular Biology and Evolution*, **16**, 37–48.
657 Barraclough, T.G., Birky, C.W. Jr. & Burt, A. (2003) Diversification in sexual and asexual
658 organisms. *Evolution (N. Y.),* **57**, 2166–2172.
659 Bardhan, A. & Sharma, T. (2000) Meiosis and speciation: A study in a speciating mus
660 terricolor complex. *Journal of Genetics*, **79**, 105–111.
661 Barnes, I., Shapiro, B., Lister, A., Kuznetsova, T., Sher, A., Guthrie, D. & Thomas, M.G.
662 (2007) Genetic structure and extinction of the woolly mammoth, Mammuthus
663 primigenius. *Current Biology*, **17**, 1072–1075.
664 Bensasson, D., Zhang, D., Hartl, D.L., Hewitt, G.M. (2001) Mitochondrial Pseudogenes:
665 evolution's miplaced witnesses. *Trends In Ecology and Evolution*, **16**, 314-321.
666 Bertsch, A. (1997) Discrimination of the bumblebee species *Bombus cryptarum* and *B.*
667 *lucorum* by means of male labial gland secretions and morphological characters
668 (Hymenoptera: Apidae). *Entomologia Generalis*, **22**, 129–145.
669 Bertsch, A. & Schweer, H. (2012a) Cephalic labial gland secretions of males as species
670 recognition signals in bumblebees: are there really geographical variations in the

671 secretions of the *Bombus terrestris* subspecies? *Beiträge zur Entomologie*, **62**,
672 103–124.

673 Bertsch, A. & Schweer, H. (2012b) Male labial gland secretions as species recognition signals
674 in species of *Bombus*. *Biochemical Systematics and Ecology*, **40**, 103–111.
675
676 Bertsch, A., Schweer, H., Titze, A. & Tanaka, H. (2005) Male labial gland secretions and
677 mitochondrial DNA markers support species status of *Bombus cryptarum* and *B.*
678 *magnus* (Hymenoptera, Apidae). *Insectes Sociaux*, **52**, 45–54.
679 Braby, M.F., Eastwood, R. & Murray, N. (2012) The subspecies concept in butterflies: has its
680 application in taxonomy and conservation biology outlived its usefulness?
681 *Biological Journal of the Linnean Society*, **106**, 699–716.
682 Brasero, N., Martinet, B., Urbanová, K., Valterová, I., Torres, A., Hoffmann, W., Rasmont, P.
683 & Lecocq, T. (2015) First chemical analysis and characterization of the male
684 species-specific cephalic labial gland secretions of South American
685 bumblebee. *Chemical & Biodiversity*, **12 (10)**, 1535-1546.
686 Cameron, S.A., Hines, H.M. & Williams, P.H. (2007) A comprehensive phylogeny of the
687 bumble bees (*Bombus*). *Biological Journal of the Linnean Society*, **91**, 161–188.
688 Carolan, J.C., Murray, T.E., Fitzpatrick, U., Crossley, J., Schmidt, H., Cederberg, B.,
689 McNally, L., Paxton, R.J., Williams, P.H. & Brown, M.J.F. (2012) Colour
690 patterns do not diagnose species: quantitative evaluation of a DNA barcoded
691 cryptic bumblebee complex. *PLoS ONE*, **7**, e29251.
692 Cederberg, B., Michez, D., Nieto, A., Radchenko, V., Rasmont, P., & al. (2013) *Bombus*.
693 IUCN 2013 IUCN Red List Threat Species Version 20132: 1. Available:
694 www.iucnredlist.org.
695 Claudet, J., Pelletier, D., Jouvenel, J.Y., Bachet, F. & Galzin, R. (2006) Assessing the effects
696 of Marine Protected Area (MPA) on a reef fish assemblage in a northwestern
697 Mediterranean marine reserve: identifying community-based indicators.
698 *Biological Conservation*, **130**, 346-369.
699 Conti, F., Abbate, G., Alessandrini, A., Blasi, C., Bonacquisti, S. & Scassellati, E. (2005) *An*
700 *annotated checklist of the Italian vascular flora.* Palombi Ed., Roma.
701 Coppée, A., Terzo, M., Valterova, I. & Rasmont, P. (2008) Intraspecific variation of the
702 cephalic labial gland secretions in *Bombus terrestris* (L.) (Hymenoptera: Apidae).
703 *Chemistry & Biodiversity*, **5**, 2654–2661.

Coyne, J.A. & Orr, H.A. (2004) *Speciation*. Sinauer Associates, Sunderland, MA, 545p.

Crowhurst, R.S., Faries, K.M., Collantes, J., Briggler, J.T., Koppelman, J.B. & Eggert, L.S. (2011) Genetic relationships of hellbenders in the Ozark highlands of Missouri and conservation implications for the Ozark subspecies (*Cryptobranchus alleganiensis bishopi*). *Conservation Genetics*, **12**, 637–646.

Dellicour, S. & Lecocq, T. (2013a) *GCALIGNER 1.0 and GCKOVATS 1.0 – Manual of a Software Suite to Compute a Multiple Sample Comparison Data Matrix from Eco-chemical Datasets Obtained by Gas Chromatography*. University of Mons, Mons.

Dellicour, S. & Lecocq, T. (2013b) GCALIGNER 1.0: an alignment program to compute a multiple sample comparison data matrix from large eco-chemical datasets obtained by GC. *Journal of Separation Science*, **36**, 3206–3209.

Dellicour, S., Lecocq, T., Kuhlmann, M., Mardulyn, P. & Michez, D. (2014a) Molecular phylogeny, biogeography, and host plant shifts in the bee genus Melitta (Hymenoptera: Anthophila). *Molecular Phylogenetics and Evolution*, **70**, 412–419.

Dellicour, S., Fearnley, S., Lombal, A., Heidl, S., Dahlhoff, E.P., Rank, N.E. & Mardulyn, P. (2014b) Inferring the past and present connectivity across the range of a North American leaf beetle: combining ecological niche modeling and a geographically explicit model of coalescence. *Evolution*, **68**, 2371–2385.

Dellicour, S., Michez, D. & P., Mardulyn. (2015) Comparative phylogeography of five bumblebees: impact of range fragmentation, range size and diet specialization. *Biological journal of the Linnean Society*, **116 (4)**, 926-939.

Dellicour, S., Kastally, C., Varela, S., Michez, D., Rasmont, P., Mardulyn, P. & Lecocq, T. (2016) Ecological niche modelling and coalescent simulations to explore the recent geographic range history of five widespread bumblebee species in Europe. *Journal of Biogeography*, doi:10.1111/jbi.12748.

De Meulemeester, T., Gerbaux, P., Boulvin, M., Coppee, A. & Rasmont, P. (2011) A simplified protocol for bumble bee species identification by cephalic secretion analysis. *Insectes Sociaux*, **58**, 227–236.

De Queiroz, K. (2005) Ernst Mayr and the modern concept of species. Proceedings of the National Academy of Sciences, **102 Suppl**, 6600–6607.

De Queiroz, K. (2007) Species concepts and species delimitation. *Systematic Biology*, **56**, 879–886.

737 Drummond, A.J. & Rambaut, A. (2007) BEAST: bayesian evolutionary analysis bysampling
738 trees. *BMC Evolutionary Biology*, **7**, 214.
739 Duennes, M.A., Lozier, J.D., Hines, H.M. & Cameron, S.A. (2012) Geographical patterns of
740 genetic divergence in the widespread Mesoamerican bumble bee *Bombus*
741 *ephippiatus* (Hymenoptera: Apidae). *Molecular Phylogenetics & Evolution*, **64**,
742 219–231.
743 Dufrene, M. & Legendre, P. (1997) Species assemblages and indicator species: The need for a
744 flexible asymmetrical approach. *Ecological Monographs*, **67**, 345–366.
745 Ebach, M.C. & Williams, D.M. (2009) How objective is a definition in the subspecies debate?
746 *Nature*, **457**, 785.
747 Ennen, J.R., Kalis, M.E., Patterson, A.L., Kreiser, B.R., Lovich, J.E., Godwin, J. & Qualls,
748 C.P. (2014) Clinal variation or validation of a subspecies? A case study of the
749 Graptemys nigrinoda complex (Testudines: Emydidae). *Biological Journal of the*
750 *Linnean Society*, **111**, 810–822.
751 Estoup, A., Solignac, M., Cornuet, J.M. & Goudet, J.S. (1996) Genetic differentiation of
752 continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in
753 Europe. *Molecular Ecology*, **5**, 19–31.
754 Evans, R.L. & Potts, S.G. (2013) *Iconic Bees: North East Bilberry Bumblebee*. Friends of the
755 earth. University of Reading. Reading. 6p.
756 Fedorov, V.B., Goropashnaya, A.V., Boeskorov, G.G. & Cook, J.A. (2008) Comparative
757 phylogeography and demographic history of the wood lemming (*Myopus*
758 *schisticolor*): implications for late Quaternary history of the taiga species in
759 Eurasia. *Molecular Ecology*, **17**, 598–610.
760 Fitzpatrick, U., Murray, T.E., Paxton, R.J. & Brown, M.J.F. (2006) *The state of Ireland's*
761 *Bees*. Northern Ireland Environment Agency.
762 Frankham, R., Ballou, J.D. & Briscoe, D.A. (2010) *Introduction to Conservation Genetics,*
763 *2nd edn*. Cambridge University Press, Cambridge, 644pp.
764 Frattaroli, A.R., Di Martino, L., Di Cecco, V., Catoni, R., Varone, L., Di Santo, M. & Gratani
765 L. (2013) Seed germination capability of four endemic species in the Central
766 Apennines (Italy): relationships with seed size. *Lazaroa*, **34**, 43-53.
767 Fuente, V., Rufo Nieto, L. & Sánchez-Mata, D. (2011) *Sarcocornia hispanica*
768 (Chenopodiaceae), a new species from the Iberian Peninsula. *Lazaroa*, **32**, 9-13.

769 Galtier, N., Gouy, M., Gautier, C. (1996). SEAVIEW and PHYLO_WIN: two graphic tools
770 for sequence alignment and molecular phylogeny. *Computer Applications in the*
771 *Biosciences*, **12**, 543-548.
772 Gibbs, J. (2009) Integrative taxonomy indentifies new (and old) species in the *Lasioglossum*
773 (Dialictus) *tegulare* (Robertson) species group (Hymenoptera, Halictidae).
774 *Zootaxa*, **2032**, 1–38.
775 Gjershaug, J., Staverløkk, A., Kleven, O. & Ødegaard, F. (2013) Species status of *Bombus*
776 *monticola* Smith (Hymenoptera: Apidae) supported by DNA barcoding. *Zootaxa*,
777 **3716**, 431–440.
778 Hawlitschek, O., Nagy, Z.T. & Glaw, F. (2012) Island evolution and systematic revision of
779 Comoran snakes: why and when subspecies still make sense. *PLoS ONE*, **7**,
780 e42970.
781 Hewitt, G. (1999) Post-glacial re-colonization of European biota. *Biological Journal of the*
782 *Linnean Society*, **68**, 87–112.
783 Hewitt, G. & Ibrahim, K. (2001) *Inferring glacial refugia and historical immigrations with*
784 *molecular phylogenies*. In: Silverton J., Antonovics J. (ed.) Fourteenth Special
785 Symposium of the British Ecological Society. British Ecological Society,
786 Blackwell Science Ltd.
787 Hewitt, G.M. (2004a) The structure of biodiversity - Insights from molecular phylogeography.
788 *Frontiers in Zoology*, **1**, 4.
789 Hewitt, G.M. (2004b). Genetic consequences of climatic oscillations in the Quaternary.
790 *Philosophical Transactions of the Royal Society B: Biological Sciences*, **359**, 183–
791 195.
792 Hewitt, G.M. (2011) Quaternary phylogeography: the roots of hybrid zones. *Genetica*, **139**,
793 617–638.
794 Hillis, D.M. & Bull, J.J. (1993) An empirical test of bootstrapping as a method for assessing
795 confidence in phylogenetic analysis. *Systematic Biology*, **42**, 182–192.
796 Hines, H.M. (2008) Historical biogeography, divergence times, and diversification patterns of
797 bumble bees (Hymenoptera: Apidae: *Bombus*). *Systematic Biology*, **57**, 58–75.
798 Hines, H.M. & Williams, P.H. (2012) Mimetic colour pattern evolution in the highly
799 polymorphic *Bombus trifasciatus* (Hymenoptera: Apidae) species complex and its
800 comimics. *Zoological Journal of the Linnean Society*, **166**, 805–826.
801 IUCN Species Survival Commission. (2012) *Guidelines for application of IUCN red list*
802 *criteria at regional and national levels Version 4.0. IUCN*, Gland, Switzerland. ii i+ 41

803 pp. p. Available:http://www.iucnredlist.org/technical-documents/categories-and-
804 criteria.
805 Iserbyt, S. & Rasmont, P. (2012). The effect of climatic variation on abundance and diversity
806 of bumblebees: a ten years survey in a mountain hotspot. *Annales de la Société*
807 *entomologique de France (N.S.)*, **48**, 261-273.
808 Kim, M.J., Yoon, H.J., Im, H.H. & Jeong, U.H. (2009) Mitochondrial DNA sequence
809 variation of the bumblebee, *Bombus ardens* (Hymenoptera: Apidae). *Journal of*
810 *Asia-Pacific Entomology*, **12**, 133–139.
811 Kraus, F.B., Wolf, S. & Moritz, R.F.A. (2009) Male flight distance and population
812 substructure in the bumblebee *Bombus terrestris*. *Journal of Animal Ecology*, **78**,
813 247-252.
814 Kuhlmann, M., Ascher, J.S., Dathe, H.H. & al. (2014) Checklist of the Western Palaearctic
815 Bees (Hymenoptera: Apoidea: *Anthophila*) [WWW document]. URL
816 http://westpalbees.myspecies.info. [accessed on 04 November 2015].
817 Lecocq, T., Lhomme, P., Michez, D., Dellicour, S., Valterova, I. & Rasmont, P. (2011)
818 Molecular and chemical characters to evaluate species status of two cuckoo
819 bumblebees: *Bombus barbutellus* and *Bombus maxillosus* (Hymenoptera, Apidae,
820 Bombini). *Systematic Entomology*, **36**, 453–469.
821 Lecocq, T., Dellicour, S., Michez, D., Lhomme, P., Vanderplanck, M., Valterova, I., Rasplus,
822 J.Y. & Rasmont, P. (2013a) Scent of a break-up: phylogeography and
823 reproductive trait divergences in the red-tailed bumblebee (*Bombus lapidarius*).
824 *BMC Evolutionary Biology*, **13**, 263.
825 Lecocq, T., Vereecken, N.J., Michez, D., Dellicour, S., Lhomme, P., Valterová, I., Rasplus,
826 J.Y., Rasmont, P. (2013b) Patterns of genetic and reproductive traits
827 differentiation in mainland vs. Corsican populations of bumblebees. *PLoS ONE*,
828 **8**, e65642.
829 Lecocq, T., Brasero, N., De Meulemeester, T., Michez, D., Dellicour, S., Lhomme, P., de
830 Jonghe, R., Valterova, I., Urbanova, K. & Rasmont, P. (2015a) An integrative
831 taxonomic approach to assess the status of Corsican bumblebees: implications for
832 conservation. *Animal Conservation*, **18**, 236–248.
833 Lecocq, T., Coppee, A., Mathy, T., Lhomme, P., Cammaerts-Tricot, M.C., Urbanova, K.,
834 Valterova, I. & Rasmont, P. (2015b) Subspecific differentiation in male
835 reproductive traits and virgin queen preferences, in *Bombus terrestris*. *Apidologie*,
836 **46 (5)**, 595-605.

837 Lecocq, T., Dellicour, S., Michez, D., Dehon, M., Dewulf, A., De Meulemeester, T., Brasero,
838 N., Valterova, I., Rasplus, J.Y. & Rasmont, P. (2015c) Methods for species
839 delimitation in bumblebees (Hymenoptera, Apidae, *Bombus*): towards an
840 integrative approach. *Zoologica Scripta*, **44**, 281–297.
841 Lecocq, T., Brasero, N., Martinet, B., Valterovà, I. & Rasmont, P. (2015d) Highly polytypic
842 taxon complex: interspecific and intraspecific integrative taxonomic assessment of
843 the widespread pollinator *Bombus pascuorum* Scopoli 1763 (Hymenoptera:
844 Apidae). *Systematic Entomology*, **40**, 881–888.
845 Lecocq, T., Coppée, A., Michez, D., Brasero, N., Rasplus, J.Y., Valterova, I. & Rasmont, P.
846 (2016) The alien's identity: consequences of taxonomic status for the international
847 bumblebee trade regulations. *Biological Conservation*, **195**, 169–176.
848 Legendre, P. & Legendre, L. (2004) *Developments in environmental modelling 20*. *Numerical*
849 *Ecology*, Second english edition. Elsevier Scientific Publication Co., Amsterdam,
850 853pp.
851 Lepais, O., Darvill, B., O'Connor, S., Osborne, J.L., Sanderson, R.A., Cussans, J., Goffe, L.
852 & Goulson, D. (2010) Estimation of bumblebee queen dispersal distances using
853 sibship reconstruction method. *Molecular Ecology*, **19**, 819-31.
854 Løken, A. (1973) Studies on Scandinavian bumble bees (Hymenoptera, Apidae). *Norsk*
855 *Entomologisk Tidsskrift*, **20**, 1–218.
856 Lunt, D.H., Zhang, D.X., Szymura, J.M. & Hewitt, O.M. (1996) The insect Cytochrome
857 Oxidase I gene: evolutionary patterns and conserved primers for phylogenetic
858 studies. *Insect Molecular Biology*, **5**, 153-165.
859 Manino, A., Patetta, A., Porporato, M., Quaranta, M., Intoppa, F., Piazza, M.G. & Friilli, F.
860 (2007) Bumblebee (*Bombus* Latreille, 1802) distribution in high mountains and
861 global warming. *Redia*, **90**, 125-129.
862 Mardulyn, P., Mikhailov, Y.E. & Pasteels, J.M. (2009) Testing phylogeographic hypotheses
863 in a euro-siberian cold-adapted leaf beetle with coalescent simulations. *Evolution*,
864 **63**, 2717–2729.
865 Martinet, B., Rasmont, P., Cederberg, B., Evrard, D., Ødegaard, F., Paukkunen, J. & Lecocq,
866 T. (2015a) Forward to the North: two Euro-Mediterranean bumblebee species now
867 cross the Arctic Circle. *Annales de la Société entomologique de France (N.S.)*, 51
868 **(4)**, 303-309.

869 Martinet, B., Lecocq, T., Smet, J. & Rasmont, P. (2015b) A Protocol to Assess Insect
870 Resistance to Heat Waves, Applied to Bumblebees (*Bombus Latreille, 1802).*
871 *PLoS ONE*, **10(3)**, e0118591.
872 Martín-Bravo, S., Valcárcel, V., Vargas, P. & Luceño, M. (2010) Geographical speciation
873 related to Pleistocene range shifts in the western Mediterranean mountains. *Taxon*,
874 **59**, 466–482.
875 Mayr, E. (1942) *Systematics and the Origin of Species*. Columbia University Press, New
876 York, New York.
877 Mayr, E. (1963) *Animal Species and Evolution*. Harvard University Press, Cambridge,
878 Massachusetts.
879 Michener, C.D. (1990) *Classification of the Apidae (Hymenoptera)*. University of Kansas
880 Natural History Museum Special Publication, **54**, 75–164.
881 Michener, C.D. (2007) *The Bees of the World. Second Edition*. Johns Hopkins University,
882 Baltimore, 1016 pp.
883 Mutanen, M., Kivelä, S.M., Vos, R.A., Doorenweerd, C., Ratnasingham, S., Hausmann, A.,
884 Huemer, P., Dincă, V., van Nieukerken, E.J., Lopez-Vaamonde, C., Vila, R.,
885 Aarvik, L., Decaëns, T., Efetov, K.A., Hebert, P.D., Johnsen, A., Karsholt, O.,
886 Pentinsaari, M., Rougerie, R., Segerer, A., Tarmann, G., Zahiri, R. & Godfray,
887 HC. (2016) Species-Level Para- and Polyphyly in DNA Barcode Gene Trees:
888 Strong Operational Bias in European Lepidoptera. *Systematic Biology*, **65**, 1025-
889 1040.
890 Nieto, A., Roberts, S.P.M., Kemp, J., Rasmont, P., Kuhlmann, M., García Criado, M.,
891 Biesmeijer, J.C., Bogusch, P., Dathe, H.H., De la Rúa, P., De Meulemeester, T.,
892 Dehon, M., Dewulf, A., Ortiz-Sánchez, F.J., Lhomme, P., Pauly, A., Potts, S.G.,
893 Praz, C., Quaranta, M., Radchenko, V.G., Scheuchl, E., Smit, J., Straka, J., Terzo,
894 M., Tomozii, B., Window, J. & Michez, D. (2014). *European red list of bees*.
895 IUCN, European Commission, Luxembourg.
896 Oksanen, F.J., Blanchet, G., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara,
897 R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E. & Wagner, H.
898 (2011) Tertiary Vegan: Community Ecology Package.
899 Paradis, E., Claude, J. & Strimmer, K. (2004) APE: analyses of phylogenetics and evolution
900 in R language. *Bioinformatics*, **20**, 289–290.
901 Patten, M.A. (2009) 'Subspecies' and 'race' should not be used as synonyms. *Nature*, **457**,
902 147.

903 Patten, M.A. & Unitt, P. (2002) Diagnosability versus mean differences of Sage Sparrow
904 subspecies. *The Auk*, **119**, 26–35.
905 Patten, M.A. (2010) Null expectations in subspecies diagnosis. *Ornithological Monographs*,
906 **67**, 35-41.
907 Pedersen, B.V. (2002) European bumblebees (Hymenoptera: Bombini) – Phylogenetic
908 relationships inferred from DNA sequences. *Insect Systematics and Evolution*, **33**,
909 361–386.
910 Pekkarinen, A. (1979) Morphometric colour and enzyme variation in bumble bees
911 (Hymenoptera, Apidae, *Bombus*) in Fennoscandia and Denmark. *Acta Zoologica*
912 *Fennica*, **158**, 1–60.
913 Petit, R. J., Aguinagalde, I., De Beaulieu, J.L., Bittkau, C., Brewer, S., Cheddadi, R., Ennos,
914 R., Fineschi, S., Grivet, D., Lascoux, M., Aparajita, M., Müller-Starck, G.,
915 Demesure-Musch, B., Palmé, A., Martin, J.P., Rendell, S. & Vendramin, G.G.
916 (2003) Glacial refugia: hotspots but not melting pots of genetic diversity. *Science*,
917 **300**, 1563–1565.
918 Phillimore, A.B. & Owens, I.P.F. (2006) Are subspecies useful in evolutionary and
919 conservation biology? *Proceedings of the Royal Society of London Series B:*
920 *Biological Sciences*, **273**, 1049–1053.
921 Pirounakis, K., Koulianos, S. & Schmid-Hempel, P. (1998) Genetic variation among
922 European populations of *Bombus pascuorum* (Hymenoptera: Apidae) from
923 mitochondrial DNA sequence data. *European Journal of Entomology*, **95**, 27–33.
924 Posada, D. (2008) jModelTest: Phylogenetic model averaging. *Molecular Biology and*
925 *Evolution*, **25**, 1253–1256.
926 Powell, J.R. (1978) The Founder-Flush speciation theory: an experimental approach. *The*
927 *society for the study of evolution*, **32 (3)**, 465-474.
928 Rambaut, A. & Drummond, A.J.(2013) Tracer version 1.4. http://beast.bio.ed.ac.uk/Tracer.
929 Rasmont, P. (1983) Catalogue commenté des Bourdons de la région ouest-paléarctique
930 (Hymenoptera, Apoïdea, Apidae). *Notes fauniques de Gembloux*, **7**, 1-72.
931 Rasmont, P., Terzo, M., Aytekin, A.M., Hines, H., Urbanova, K., Cahlikova, L. & Valterova,
932 I. (2005) Cephalic secretions of the bumblebee subgenus *Sibiricobombus* Vogt
933 suggest *Bombus niveatus* Kriechbaumer and *Bombus vorticosus* Gerstaecker are
934 conspecific (Hymenoptera, Apidae, Bombus). *Apidologie*, **36**, 571–584.

935 Rasmont, P., Coppée, A., Michez, D. & De Meulemeester, T. (2008) An overview of the
936 *Bombus terrestris* (L. 1758) subspecies (Hymenoptera: Apidae). *Annales de la*
937 *Société Entomologique de France* (n.s.), **44**, 243–250.
938 Rasmont, P., Franzen, M., Lecocq, T., Harpke, A., Roberts, S.P.M., Biesmeijer, K., Castro,
939 L., Cederberg, B., Dvorak, L., Fitzpatrick, U., Gonseth, Y., Haubruge, E., Mahé,
940 G., Manino, A., Michez, D., Neumayer, J., Odegaard, F., Paukkunen, J.,
941 Pawlikowski, T., Potts, S.G., Reemer, M., Settele, J., Straka, J. & Schweiger, O.
942 (2015) *Climatic risk and distribution atlas of European bumblebees*. BioRisk, **10**,
943 1–236.
944 Rasmont, P. & Iserbyt, S. (2014) *Atlas of the European Bees: genus Bombus. 3d Edition.*
945 STEP Project. Status Trends Eur Pollinators, Atlas Hymenoptera, Mons,
946 Gembloux. http://www.zoologie.umh.ac.be//hymenoptera/page.asp?ID=169.
947 R Development Core Team. (2013) R: A Language and Environment for Statistical
948 Computing. R Foundation for Statistical Computing, Vienna. [WWW document].
949 URL http://www.R-project.org/ [accessed on 04 November 2015].
950 Reid, N. M. & Carstens, B. C. (2012) Phylogenetic estimation error can decrease the accuracy
951 of species delimitation: a Bayesian implementation of the general mixed Yule-
952 coalescent model. *BMC Evolutionary Biology*, **12**, 196.
953 Reinig, W.F. (1937) *Die Holarktis. Ein Beitrag zur diluvialen und alluvialen Geschichte der*
954 *Cirkumpolaren Faunen- und Florengebiete*. Gustav Fischer, Jena, 124 p.
955 Reinig, W.F. (1965) Die Verbreitungsgeschichte zweier fûr die Apenninen neuer
956 boreoalpinen Hummelarten mit einem Versuch der Gliederung boreoalpiner
957 Verbreitungsformen. *Jahrbücher Abteilung fiir Systernatik*, **92**, 703-742.

958 Ricciardelli D' Albore, G. & Piatti, C. (2004) Ecology of *Bombus monticola konradini* Reinig
959 (Hymenoptera: Apidae) in the National Park of the Sibillini mountains (Central
960 Italy). *Annali della Facolta di Agraria, Universita degli Studi di Perugia*, **55**, 283-
961 291.
962 Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes 3: bayesian phylogenetic inference under
963 mixed models. *Bioinformatics*, **19**, 1572–1574.
964 Ruiz-Labourdette, D., Nogués-Bravo, D., Sáinz Ollero, H., Schmitz, M.F. & Pineda, FD.
965 (2012) Forest composition in Mediterranean mountains is projected to shift along
966 the entire elevational gradient under climate change. *Journal of Biogeography*, **39**,
967 162-176.

Sackett, L.C., Seglund, A., Guralnick, R.P., Mazzella, M.N., Wagner, D.M., Busch, J.D. & Martin, A.P. (2014) Evidence for two subspecies of Gunnison's prairie dogs (*Cynomys gunnisoni*), and the general importance of the subspecies concept. *Biological Conservation*, **174**, 1–11.

Schlick-Steiner, B.C., Steiner, F.M., Seifert, B., Stauffer, C., Christian, E. & Crozier, R.H. (2010) Integrative taxonomy: a multisource approach to exploring biodiversity. *Annual Review of Entomology*, **55**, 421–438.

Siegel, S. & Castellan, N.J. (1988) *Non parametric statistics for the behavioural sciences*. New York: MacGraw Hill Humanities.

Stewart, J.R., Lister, A.M., Barnes, I. & Dalén, L. (2010) Refugia revisited: Individualistic responses of species in space and time. *Proceedings of the Royal Society B: Biological Sciences*, **277**, 661–671.

Suzuki, R. & Shimodaira, H. (2011) Pvclust: Hierarchical Clustering with P-values via Multiscale Bootstrap Resampling. Contributed package. Version 1-1.10. R Foundation for Statistical Computing, Vienna. [WWW document]. URL http://www.R-project.org [accessed on 04 November 2015].

Svensson, B.G. (1979) *Pyrobombus lapponicus* auct., in Europe recognized as two species: *P. lapponicus* (Fabricius 1793) and *P. monticola* (Smith, 1849) (Hymenoptera, Apoidea, Bombinae). *Insect Systematics & Evolution*, **10**, 275–296.

Taberlet, P. (1998) Biodiversity at the intraspecific level: The comparative phylogeographic approach. *Journal of Biotechnology*, **64**, 91–100.

Terzo, M., Urbanova, K., Valterova, I. & Rasmont, P. (2005) Intra and interspecific variability of the cephalic labial glands' secretions in male bumblebees: the case of *Bombus* (*Thoracobombus*) *ruderarius* and *B.* (*Thoracobombus*) *sylvarum* Hymenoptera, Apidae. *Apidologie*, **36**, 85–96.

Thuiller, W. (2004) Patterns and uncertainties of species' range shifts under climate change. *Global Change Biology*, **10**, 2020–2027.

Thuiller, W., Albert, C., Araújo, M.B., Berry, P.M., Cabeza, M., Guisan, A., Hicklerf, T., Midgley, G.F., Paterson, J., Schurr, F., Sykes, M.T., Zimmermann, N.E. (2008) Predicting global change impacts on plant species' distributions: Future challenges. *Perspectives in Plant Ecologt, Evolution and* Systematics, **9**, 137–152.

Tkalcu, B. (1992) Notiz zur NOinenkiatur der Alpenpopulation von *Pyrobombus (Pyrobombus) montieola* (SMITH, 1849) (Hym. Apoidea). *Entomologische Nachrichten und Berichte*, **36**,1992/2

Trunz, V., Packer, L., Vieu, J., Arrigo, N. & Praz, C.J. (2016) Comprehensive phylogeny, biogeography and new classification of the diverse bee tribe Megachilini: Can we use DNA barcodes in phylogenies of large genera? *Molecular Phylogenetics and Evolution*, **103**, 245-259.

Tsao, W.C. & Yeh, W.B. (2008) DNA-based discrimination of subspecies of swallowtail butterflies (Lepidoptera: Papilioninae) from Taiwan. *Zoological Studies*, **47**, 633–643.

Vincenti, M., Guglielmetti, G., Cassani, G. &Tonini, C. (1987) Determination of Double Bond Position in Di unsaturated Compounds by Mass Spectrometry of Dimethyl Disulfide Derivatives. *Analytical Chemistry*, **59**, 694-699.

Vogt, O. (1909) Studien über das Artproblem. 1. Mitteilung. Über das Variieren der Hummeln. 1. Teil. *Sitzungsberichte der Gesellschaft naturforschender Freunde zu Berlin*, **1909**, 28-84.

Waples, R. (1995) *Evolutionary significant units and the conservation of biological diversity under the Endangered Species Act. Evolution and the Aquatic Ecosystem*. Ed. J. Nielsen, pp. 8–27. American Fisheries Society, Bethesda, Maryland.

Whittaker, R.J. & Fernandez-Palacios, J.M. (2007) *Island Biogeography – Ecology, Evolution, and Conservation*, 2nd edn. Oxford University Press, Oxford.

Wilcox, T.P., Zwickl, D.J., Heath, T.A. & Hillis, D.M. (2002) Phylogenetic relationships of the dwarf boas and a comparison of Bayesian and bootstrap measures of phylogenetic support. *Molecular Phylogenetics and Evolution*, **25**, 361–371.

Williams, P.H. (1991) The bumble bees of the Kashmir Himalaya (Hymenoptera: Apidae, Bombini). *Bulletin of the Natural History Museum (Entomology)*, **60**, 1–204.

Williams, P.H. (1998) An annotated checklist of bumble bees with an analysis of patterns of description (Hymenoptera: Apidae, Bombini). *Bulletin of the Natural History Museum (Entomology)*, **67**, 79–152.

Williams, P.H., Brown, M.J.F., Carolan, J.C., An, J., Goulson, D., Aytekin, A.M., Best, L.R., Byvaltsev, A.M., Cederberg, B., Dawson, R., Huang, J., Ito, M., Monfared, A., Raina, R.H., Schmid-Hempel, P., Sheffield, C.S., Sima, P. & Xie, Z. (2012) Unveiling cryptic species of the bumblebee subgenus *Bombus s. str*. worldwide with COI barcodes (Hymenoptera: Apidae). *Systematics and Biodiversity*, **10(1)**, 21–56.

Williams, P.H., Byvaltsev, A.M., Cederberg, B., Berezin, M.V., Ødegaard, F., Rasmussen, C., Richardson, L.L., Huang, J., Sheffield, C.S. & Williams, S.T. (2015) Genes

suggest ancestral colour polymorphisms are shared across morphologically cryptic species in arctic bumblebees. *PLOS One*, **10(2)**, e0144544.

Žacek, P., Prchalova-Horˇnakova, D., Tykva, R., Kindl, J., Vogel, H., Svatoš , A., Pichová, I. & Valterová , I. (2013) De novo biosynthesis of sexual pheromone in the labial gland of bumblebee males. Chem Bio Chem, **14**, 361–371.

Zagwijn, W.H. (1992) The beginning of the Ice Age in Europe and its major subdivisions. *Quaternary Science Reviews*, **11**, 583–591.

Zink, R.M. (2004) The role of subspecies in obscuring avian biological diversity and misleading conservation policy. *Proceedings of the Royal Society of London Series B: Biological Sciences*, **271**, 561–564.

Zwickl, D.J. (2006) *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criteria*. PhD Dissertation, The University of Texas, Austin, Texas.

AUTHOR CONTRIBUTIONS

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

FUNDING

The research has received funding from the European Community's Seventh Framework Program, STEP Project (Status and Trends of European Pollinators, www.step-project.net, grant agreement no 244090, FP7/2007-2013) and from the INTERACT project.

SUPPORTING INFORMATION

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

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*, *B. monticola alpestris* and *B.*

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

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. All photographs are by P. Rasmont.

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)** 1) Dendrogram of cephalic labial gland secretion differentiation within *monticola* complex and *B. bimaculatus*. This cluster was obtained by hierarchical clustering using an unweighted pair-group method with arithmetic mean (UPGMA) based on a Canberra matrix calculated from the cephalic labial gland secretion matrix of *B. bimaculatus* (red), *B. lapponicus* (dark blue), *B. konradini* nov. status (green), *B. m. rondoui* (pink), *B. m. scandinavicus* (yellow), *B. m. monticola* (light blue), *B. m. alpestris* (black), *B. m. mathildis* ssp. nov. (orange). The values near nodes represent multiscale bootstrap resampling values (only values >80 of main groups are shown except nodes between *B. monticola* subspecies). 2) Principal component analysis (PCA) of cephalic labial gland secretion differentiation within *monticola* complex and *B. bimaculatus*: *B. bimaculatus* (red circles), *B. lapponicus* (dark blue circles), *B. konradini* nov. status (green circles), *B. m. rondoui* (pink circles), *B. m. scandinavicus* (yellow circles), *B. m. monticola* (light blue circles), *B. m. alpestris* (black

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

Fig 4. Species recognition pairwise matrix. Species recognition pairwise matrix 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 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 ratio maximum length/maximum	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).

						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)
	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 sometimes yellow (few)	Yellow	Yellow
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	-	-
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.

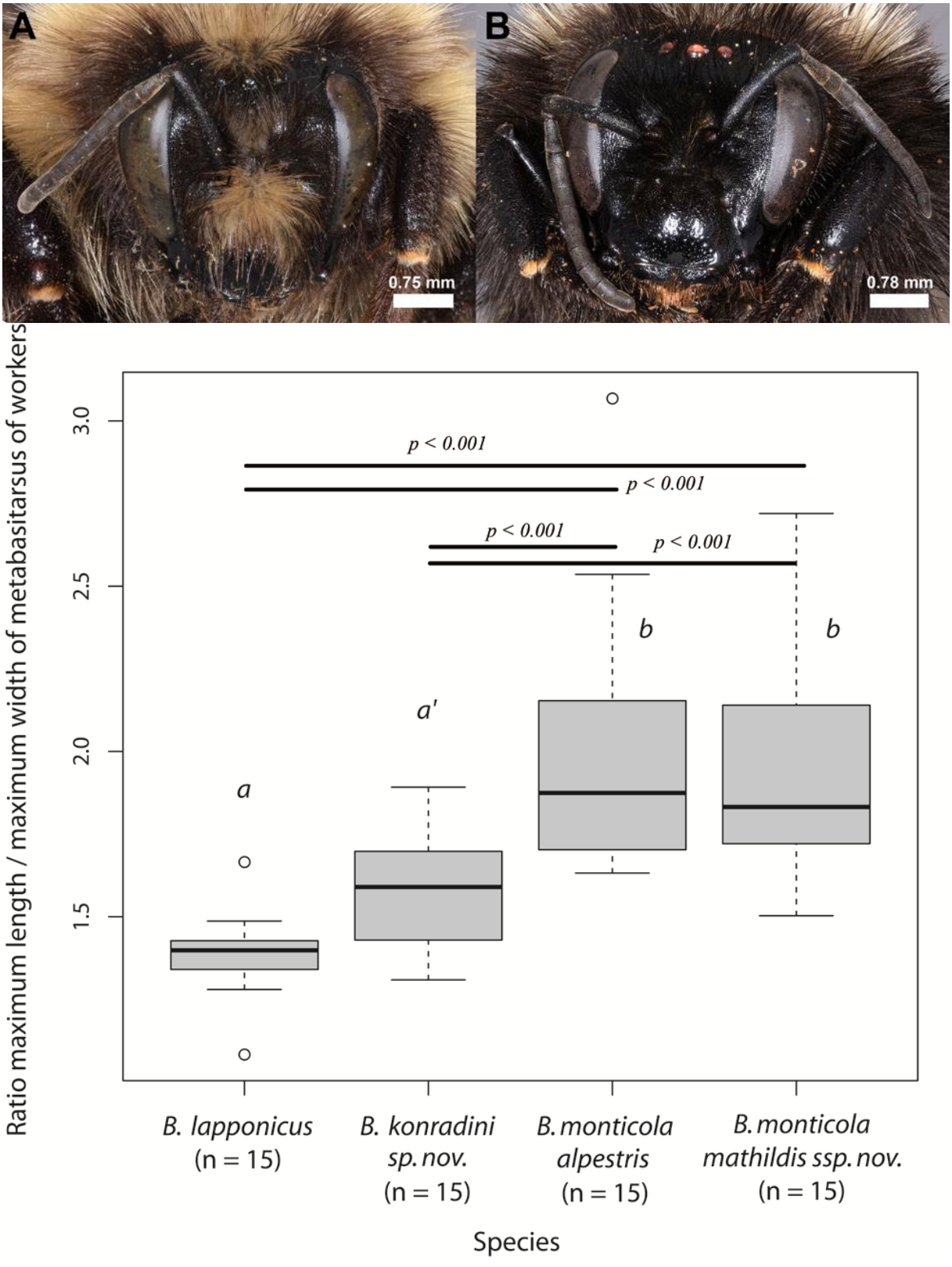
1149

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*
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*

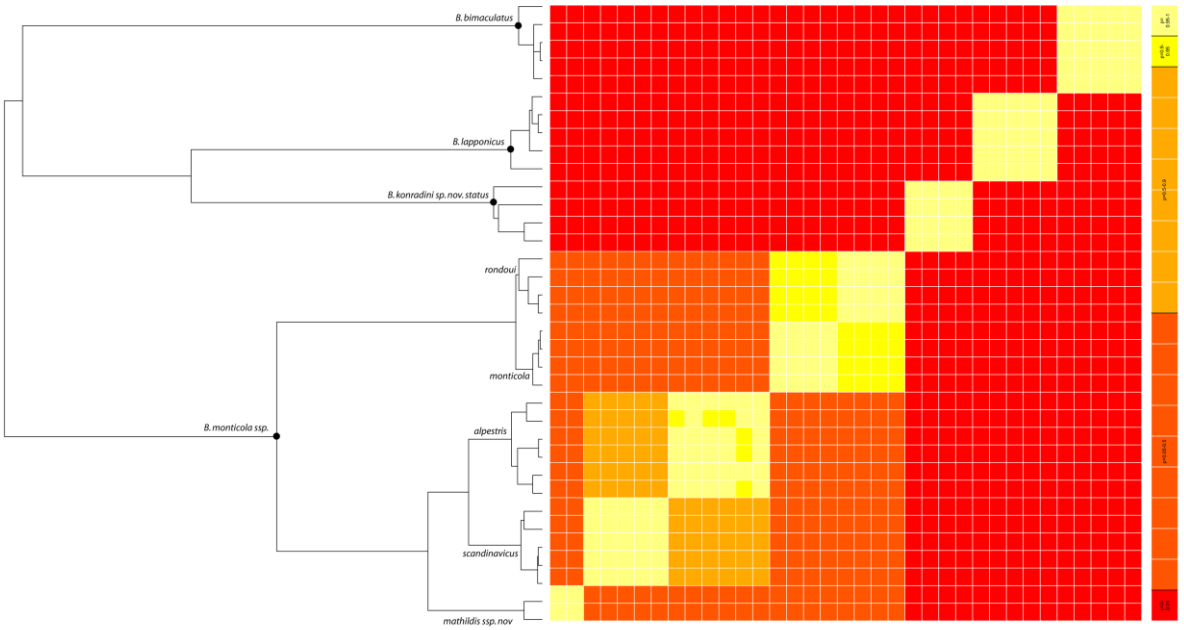
1150

1151

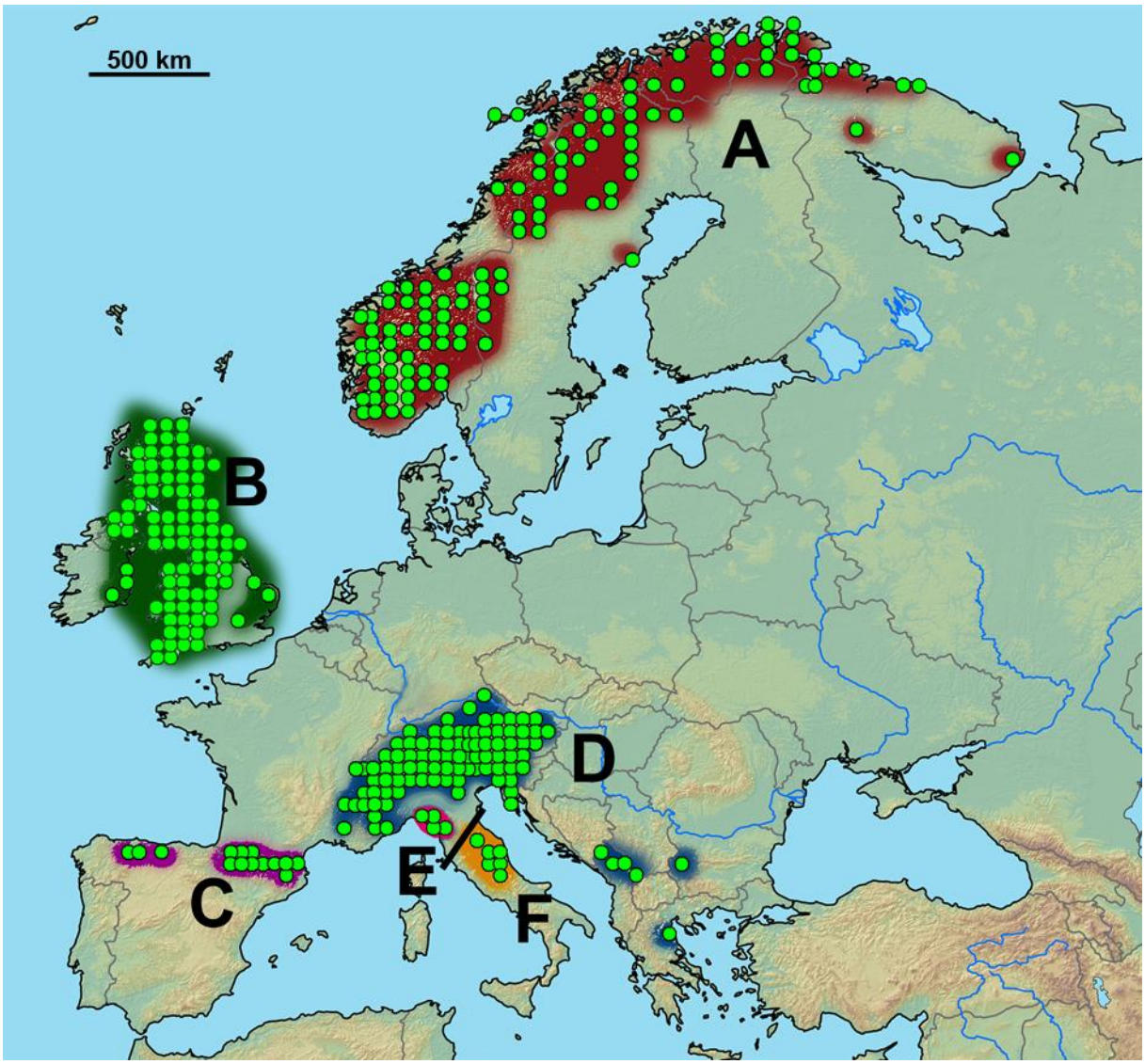
1152


A
0.75 mm
B
0.78 mm
Ratio maximum length / maximum width of metabasitarsus of workers
3.0
2.5
2.0
1.5
p < 0.001
p < 0.001
p < 0.001
p < 0.001
a
a'
b
b
B. lapponicus
(n = 15)
B. konradini
sp. nov.
(n = 15)
B. monticola
alpestris
(n = 15)
B. monticola
mathildis ssp. nov.
(n = 15)
Species

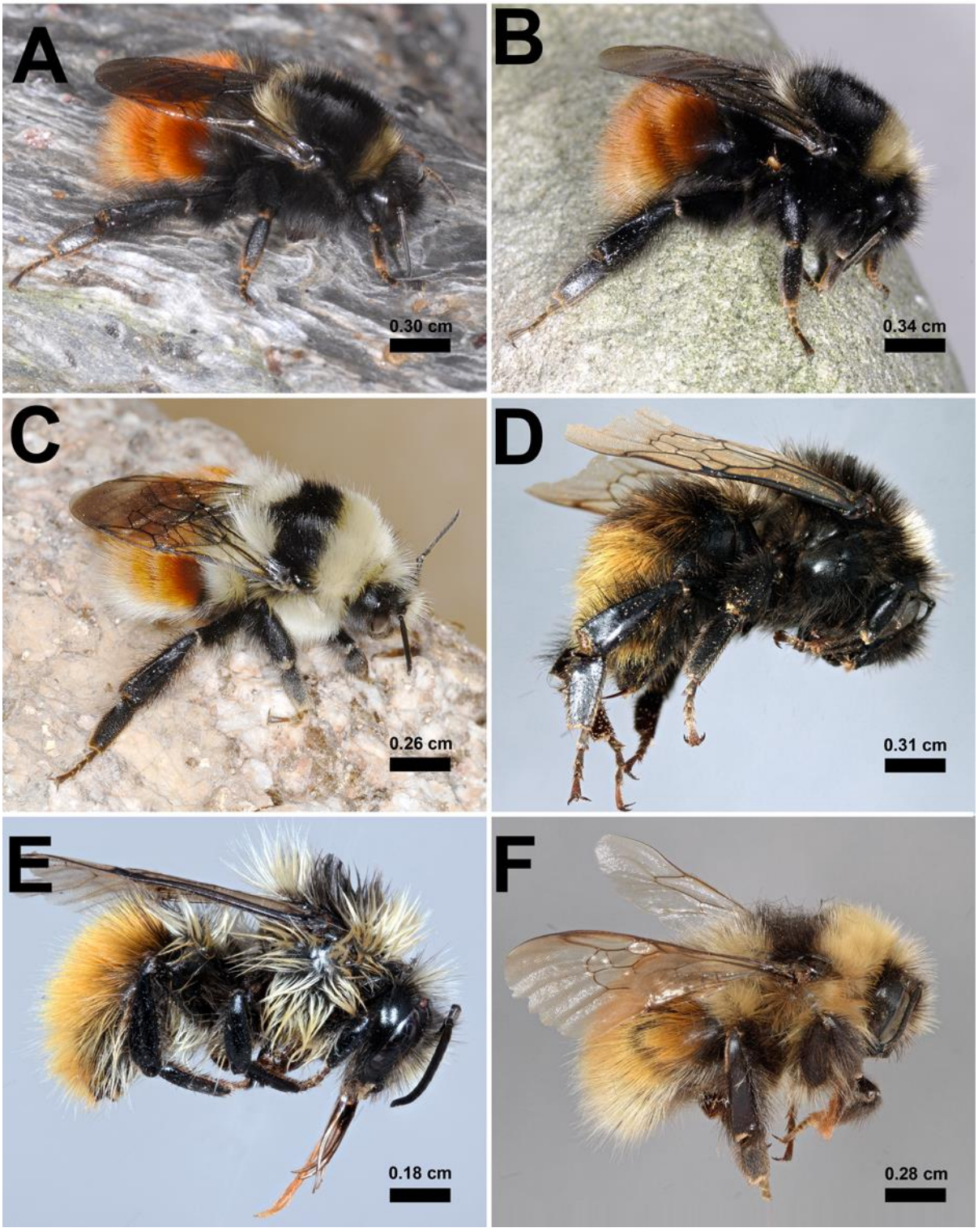
1153


B. bimaculatus
B. lapponicus
B. konradini sp. nov. status
rondoui
monticola
B. monticola ssp.
alpestris
scandinavicus
mathildis ssp. nov

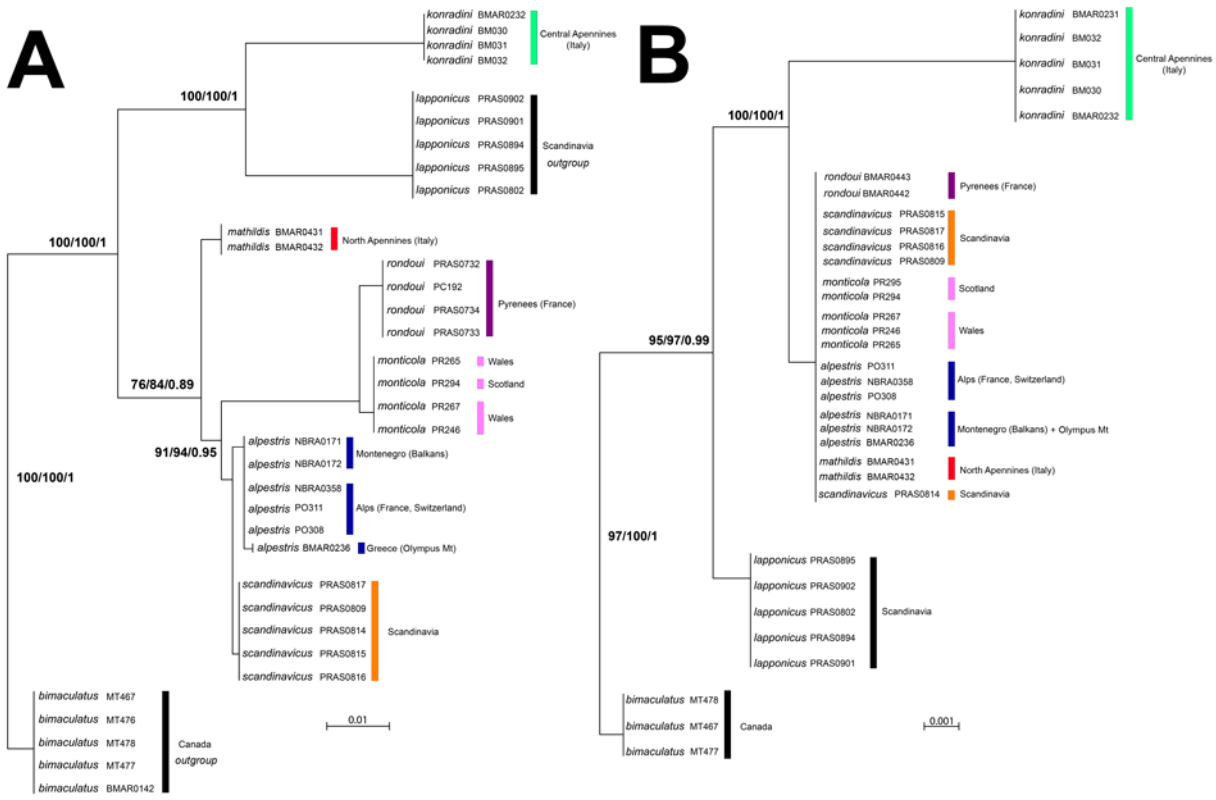
1154

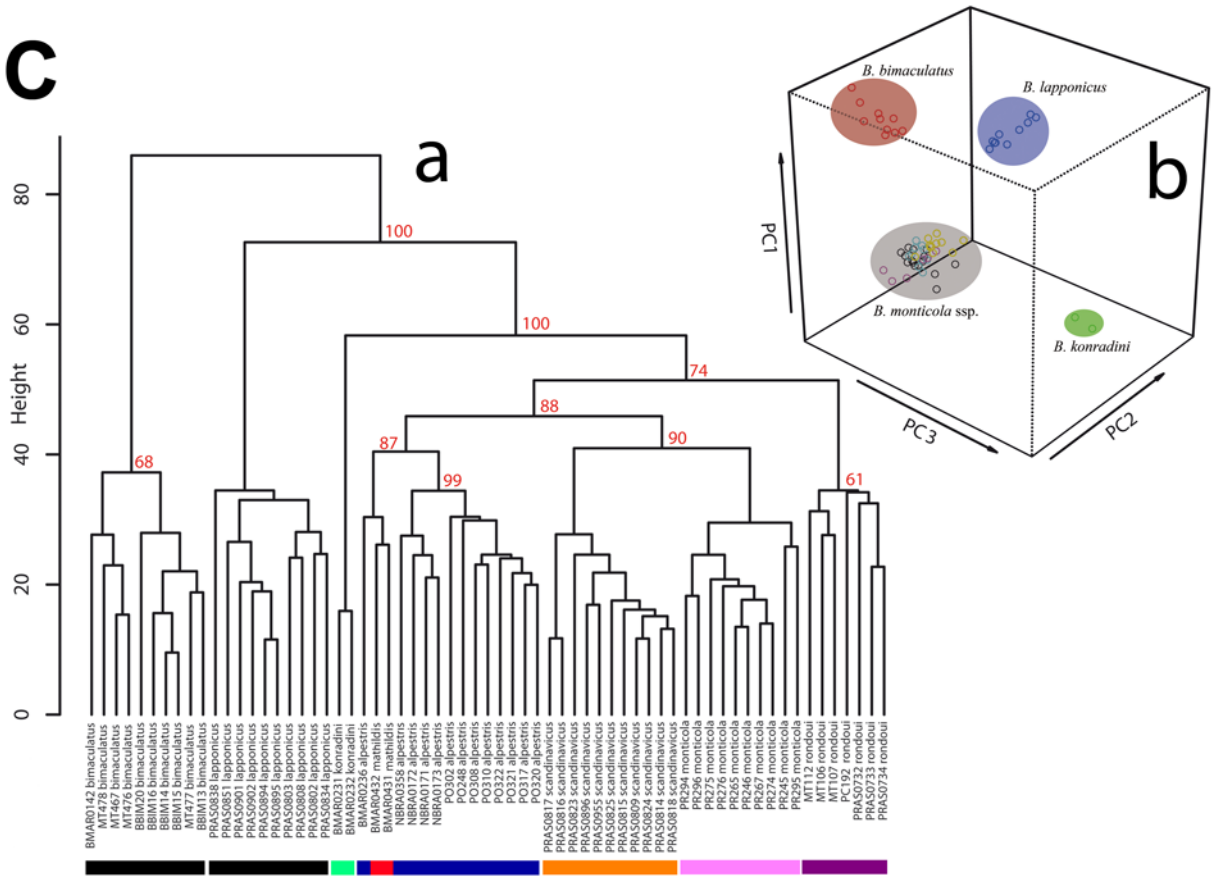

500 km
A
B
C
D
E
F

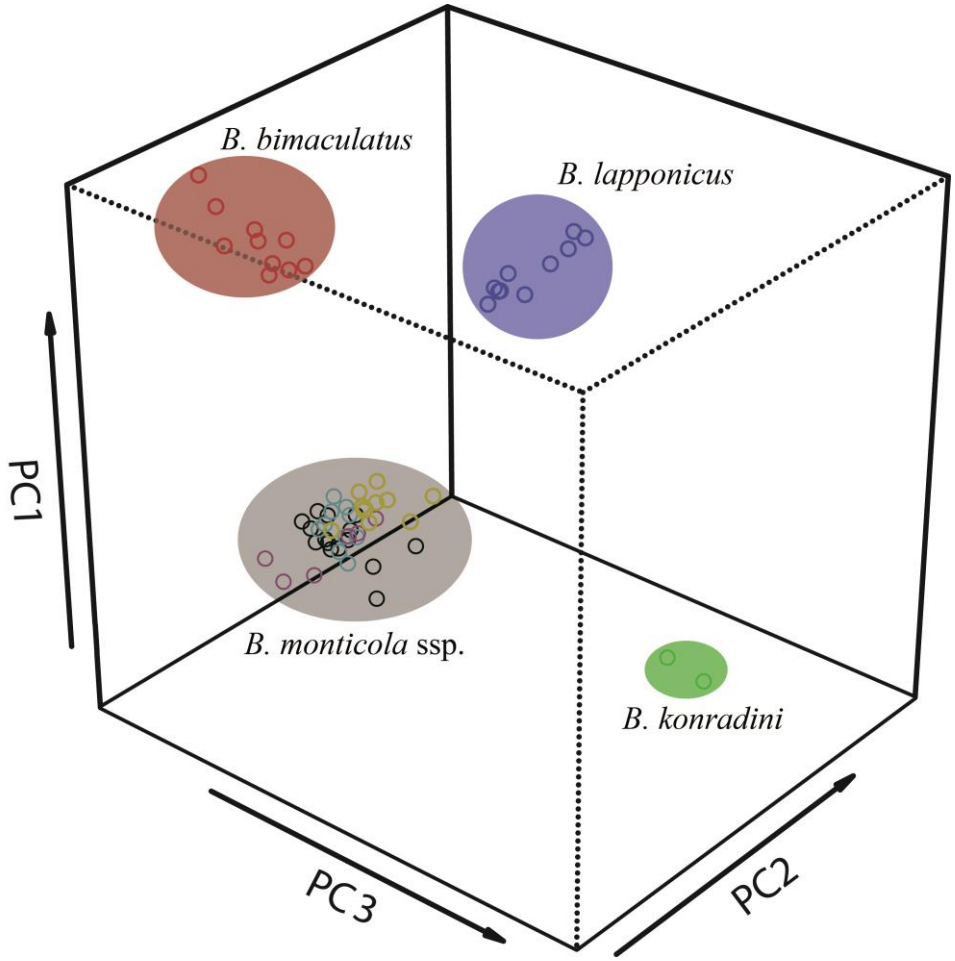
1155



1156


A
konradini BMAR0232
konradini BM030
konradini BM031
konradini BM032
Central Apennines (Italy)
100/100/1
lapponicus PRAS0902
lapponicus PRAS0901
lapponicus PRAS0894
lapponicus PRAS0895
lapponicus PRAS0802
Scandinavia
outgroup
100/100/1
mathildis BMAR0431
mathildis BMAR0432
North Apennines (Italy)
rondoui PRAS0732
rondoui PC192
rondoui PRAS0734
rondoui PRAS0733
Pyrenees (France)
76/84/0.89
monticola PR265
monticola PR294
monticola PR267
monticola PR246
Wales
Scotland
Wales
91/94/0.95
alpestris NBRA0171
alpestris NBRA0172
Montenegro (Balkans)
alpestris NBRA0358
alpestris PO311
Alps (France, Switzerland)
alpestris PO308
alpestris BMAR0236
Greece (Olympus Mt)
100/100/1
scandinavicus PRAS0817
scandinavicus PRAS0809
scandinavicus PRAS0814
scandinavicus PRAS0815
scandinavicus PRAS0816
Scandinavia
bimaculatus MT467
bimaculatus MT476
bimaculatus MT478
bimaculatus MT477
bimaculatus BMAR0142
Canada
outgroup
0.01
B
konradini BMAR0231
konradini BM032
konradini BM031
konradini BM030
konradini BMAR0232
Central Apennines (Italy)
100/100/1
rondoui BMAR0443
rondoui BMAR0442
Pyrenees (France)
scandinavicus PRAS0815
scandinavicus PRAS0817
scandinavicus PRAS0816
scandinavicus PRAS0809
Scandinavia
monticola PR295
monticola PR294
Scotland
monticola PR267
monticola PR246
monticola PR265
Wales
95/97/0.99
alpestris PO311
alpestris NBRA0358
alpestris PO308
Alps (France, Switzerland)
alpestris NBRA0171
alpestris NBRA0172
alpestris BMAR0236
Montenegro (Balkans) + Olympus Mt
mathildis BMAR0431
mathildis BMAR0432
North Apennines (Italy)
scandinavicus PRAS0814
Scandinavia
97/100/1
lapponicus PRAS0895
lapponicus PRAS0902
lapponicus PRAS0802
lapponicus PRAS0894
lapponicus PRAS0901
Scandinavia
bimaculatus MT478
bimaculatus MT467
bimaculatus MT477
Canada
0.001


C
a
Height
0
20
40
60
80
100
100
74
88
90
87
99
68
61
BMAR0142 bimaculatus
MT478 bimaculatus
MT467 bimaculatus
MT476 bimaculatus
BBIM20 bimaculatus
BBIM16 bimaculatus
BBIM14 bimaculatus
BBIM15 bimaculatus
MT477 bimaculatus
BBIM13 bimaculatus
PRAS0838 lapponicus
PRAS0851 lapponicus
PRAS0901 lapponicus
PRAS0902 lapponicus
PRAS0894 lapponicus
PRAS0895 lapponicus
PRAS0803 lapponicus
PRAS0808 lapponicus
PRAS0802 lapponicus
PRAS0834 lapponicus
BMAR0231 konradini
BMAR0232 konradini
BMAR0236 alpestris
BMAR0432 mathildis
BMAR0431 mathildis
NBRA0358 alpestris
NBRA0172 alpestris
NBRA0171 alpestris
NBRA0173 alpestris
PO302 alpestris
PO248 alpestris
PO308 alpestris
PO310 alpestris
PO322 alpestris
PO321 alpestris
PO317 alpestris
PO320 alpestris
PRAS0817 scandinavicus
PRAS0816 scandinavicus
PRAS0823 scandinavicus
PRAS0896 scandinavicus
PRAS0835 scandinavicus
PRAS0825 scandinavicus
PRAS0815 scandinavicus
PRAS0809 scandinavicus
PRAS0824 scandinavicus
PRAS0814 scandinavicus
PRAS0818 scandinavicus
PR294 monticola
PR295 monticola
PR276 monticola
PR265 monticola
PR246 monticola
PR267 monticola
PR274 monticola
PR245 monticola
PR293 monticola
MT113 rondoui
MT106 rondoui
MT107 rondoui
PC192 rondoui
PRAS0732 rondoui
PRAS0733 rondoui
PRAS0734 rondoui
B. bimaculatus
B. lapponicus
b
PC1
B. monticola ssp.
B. konradini
PC3
PC2


B. bimaculatus
B. lapponicus
B. monticola ssp.
B. konradini
PC1
PC3
PC2

1158