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

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Abstract. Cold-adapted species are expected to have reached their largest distribution range during a part of the Ice Ages whereas postglacial warming has led to their range contracting toward high-latitude and high-altitude areas. This has resulted in an extant allopatric distribution of populations and possibly to trait differentiations (selected or not) or even speciation. Assessing inter-refugium differentiation or speciation remains challenging for such organisms because of sampling difficulties (several allopatric populations) and disagreements on species concept. In the present study, we assessed postglacial 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 DNA/nuclear DNA markers and eco-chemical traits, we performed integrative taxonomic analysis to evaluate alternative species delimitation hypotheses and to assess geographical differentiation between interglacial refugia and speciation in arcto-alpine species. Our results show that trait differentiations occurred between most Southern European mountains (i.e. Alps, Balkan, Pyrenees, and Apennines) and Arctic regions. We suggest that the *monticola* complex actually includes three species: *B. konradini* **stat.n.** status distributed in Italy (Central Apennine mountains), *B. monticola* with five subspecies, including *B. monticola mathildis* **ssp.n.** distributed in the North Apennine mountains ; and *B. lapponicus*. Our results support the hypothesis that post-Ice Age periods can lead to speciation in cold-adapted species through distribution range contraction. We underline the importance of an integrative taxonomic approach for rigorous species delimitation, and for evolutionary study and conservation of taxonomically challenging taxa.

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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, 2004a,b; Thuiller, 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; Stewart *et al*., 2010; Hewitt, 2004a). These population dynamics have fostered intraspecific divergence processes leading to differentiation and possibly speciation (Avise, 2000; Hewitt, 2004a). 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 for better understanding and predicting the evolution of future biodiversity and to propose evidence-based mitigation strategies (Rasmont *et al*., 2015).

Although 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 little 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 postglacial warming and subsequent interglacial period is thought to have led to range contraction of such cold-adapted species toward the high-latitude and-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 postglacial warming (Reinig, 1937; Mardulyn *et al*., 2009; Dellicour *et al*., 2014a,b). 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 the arbitrary selection of variable traits whose accuracy continues to be debated (Mayr, 1942; De Queiroz, 2007; Lecocq *et al*., 2015a,d). Moreover, it is quite difficult to comprehensively sample specimens for phylogeographical 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 misunderstanding of 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 interpopulation differentiation processes and taxonomic statuses (Schlick-Steiner *et al*., 2010; Lecocq *et al*., 2015a,d). This reduces the likelihood of false taxonomic conclusions driven by single trait. Second, analysing multiple traits to investigate interpopulation differentiation facilitates an increase in 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 and (ii) have undergone diversification processes during the Pleistocene and Quaternary climatic cycles (Michener, 2007; Hines, 2008; Duennes *et al*., 2012; Martinet *et al*., 2015a; Rasmont *et al*., 2015; Dellicour *et al*., 2016). Their interspecific and interpopulation 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*., 2015d). This provides the opportunity to efficiently delimit 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,b,d). In bumblebees, subspecies definition is traditionally based on colour pattern variation, notwithstanding that this diagnostic character requires an extensive overview of the interindividual variability (Bertsch & Schweer, 2012a). However, colour 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,d).

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 of the allopatric regions where the species is known (infraspecific taxa). We analysed interpopulation 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,d) to assess the taxonomic status of major clades. In this approach, all taxonomic criteria used must be significantly differentiated to assign the species status.

Material & methods

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) (Fig. 1). *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 *B. monticola* diverged from its sister species *B. lapponicus* about 3 Ma. The species displays geographically differentiated colour patterns (Reinig, 1965) that have been used to define five phenotypically diagnosable allopatric subspecies (Table 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*.

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 Isles, *B. monticola rondoui* ($n = 9$) from the Pyrenees, *B. monticola alpestris* from the Alps ($n = 9$), Balkans ($n = 3$) and Mount Olympus ($n = 1$), 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 geographical 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* (Table 2). We further split *konradini* into *konradini*-N to indicate the Northern Apennines population and *konradini*-C to indicate the Central Apennines population.

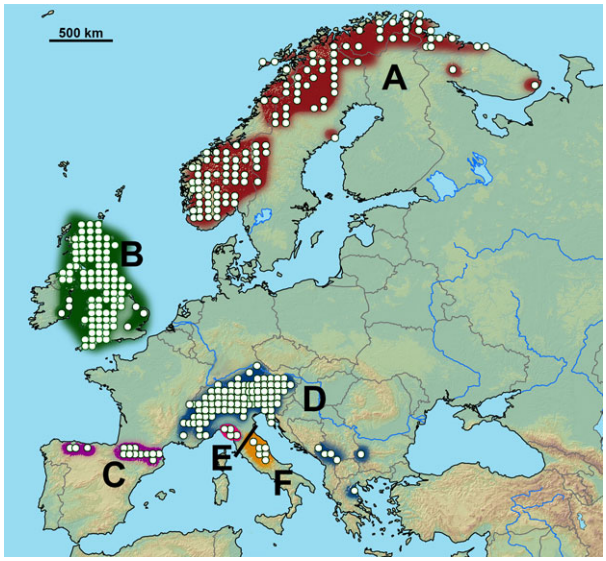

Fig. 1. Distribution map (Gall projection) of *Bombus monticola* (Rasmont & Iserbyt, 2014) and its traditional subspecies in Europe according to (Rasmont, 1983). (A) *Bombus monticola scandinavicus* queen, red area on the map; (B) *B. monticola monticola* queen, dark green area; (C) *B. monticola rondoui* queen, purple area; (D) *B. monticola alpestris* queen, blue area; (E) *B. monticola mathildis* **ssp.n.** Holotype male, pink area; (F) *B. konradini* **stat.n.** Lectotype queen, yellow area. White dots indicate the occurrence of the taxon in the region. [Colour figure can be viewed at wileyonlinelibrary.com].

Genetic differentiation analyses

In order 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, PCR (*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*. (2013a,b). 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*., 2015a,b).

In phylogenetic analyses, we analysed each gene with maximum parsimony (MP), maximum-likelihood (ML), and Bayesian (MB) methods. We carried out MP 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

Table 1. Range, conservation status, and main morphological and colour pattern differences (male and female) between *Bombus konradini* **stat.n.**, *monticola* subspecies including *mathildis* **ssp.n.** 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.n.**	*konradini* **stat.n.**	*lapponicus*
Range	Fennoscandia	British Isles	Pyrenees	Alps, Balkans, Mt. Olympus	North Apennines	Central Apennines	Fennoscandia
Conservation status	No regression was mentioned	In decline Evans & Potts (2013) and Fitzpatrick *et al.* (2006)	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 width of the metabasitarsus of this taxon is intermediate (Appendix S3).	Strong pubescence and the maximal width of the basitarsus are low (*sensu* Gjershaug *et al.*, 2013). The length of the metabasitarsus of this taxon is short (Appendix S3).
Coat colour 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)
	Colour pattern						
Face	Black	Black	Yellow	Black	Yellow or sometimes black (Figure S1)	Yellow (Figure 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

Table 1. Continued

	scandinavicus	*monticola*	*rondoui*	*alpestris*	*mathildis* **ssp.n.**	*konradini* **stat.n.**	*lapponicus*
Tergite 4	Dark red	Dark red	Red	Light red	Dark 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							
	Colour 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

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 (first position), TIM2 + I (second position), TrN + G (third position); (ii) for *PEPCK* intron 1: TPM1 uf + I; (iii) for *PEPCK* exon 1: HKY + I (first position), JC (second position), TrN + I (third position); (iv) for *PEPCK* intron 2: TrN + I; (v) for *PEPCK* exon 2: JC (first position), JC (second position), JC (third position). For ML analyses, we performed ten 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 to be 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 ten 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 to be statistically significant (Wilcox *et al*., 2002). We (re) rooted all trees with the taxon *B. bimaculatus*.

In order to recognize species threshold, we used a Bayesian implementation of the general mixed Yule-coalescent model (bGMYC) for species delimitation based on the *COI* tree (Reid & Carstens, 2012; see an example of the use of the approach in Lecocq *et al*., 2015d). 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, whereas a range of probabilities <0.05 suggested that taxa were heterospecific (Reid & Carstens, 2012). Because bGMYC required ultrametric trees, we performed a phylogenetic analysis with beast 1.7.2 (Drummond & Rambaut, 2007) using a phylogenetic clock model to generate a posterior distribution of trees [length of the Markov chain Monte Carlo (MCMC): 1 billion generations], with 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 was applied against the first sample tree to provide a 'heat' map'.

Molecular clock – estimating divergence time

Following the approach of Duennes *et al*. (2012) and Lecocq *et al*. (2013a), we analysed the *COI* dataset in beast v1.7.2

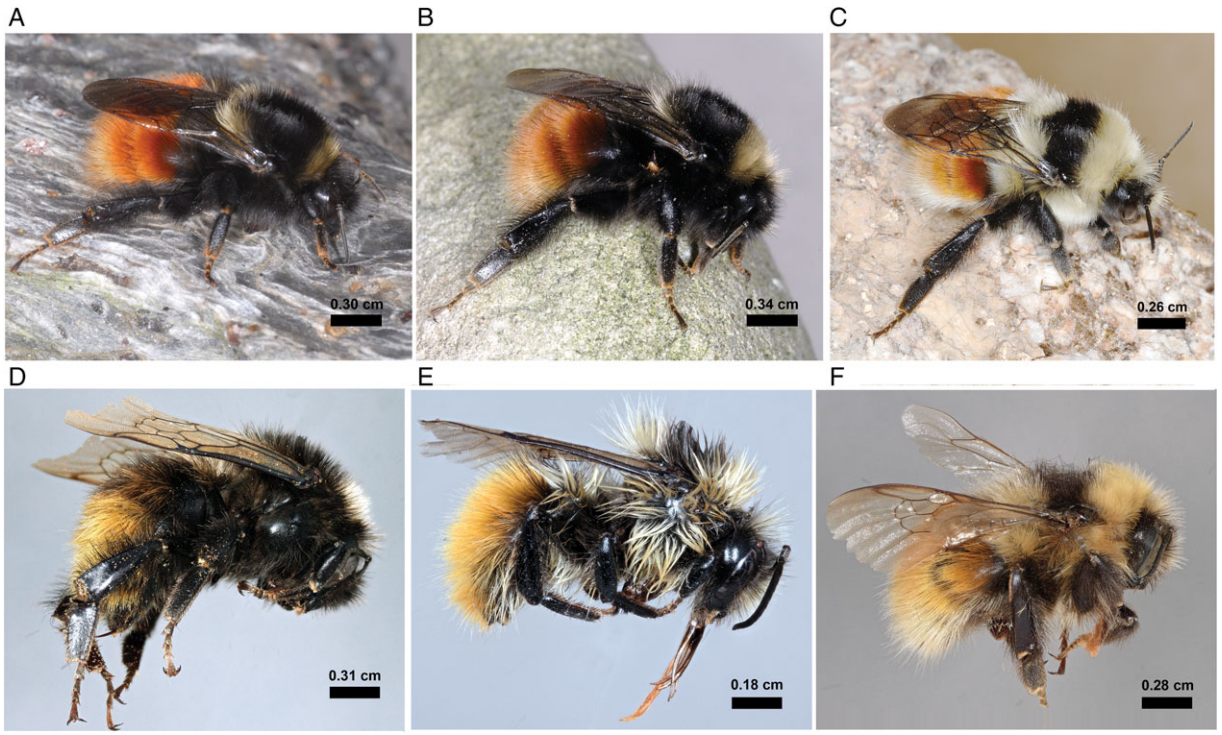

Fig. 2. Photos of the studied bumblebee taxa. (A) *Bombus monticola scandinavicus* queen; (B) *Bombus monticola monticola* queen; (C) *Bombus monticola rondoui* queen; (D) *Bombus monticola alpestris* queen; (E) *Bombus monticola mathildis* **ssp.n.** Holotype male; (F) *Bombus konradini* **stat.n.** Lectotype queen. All photographs are by P. Rasmont. [Colour figure can be viewed at wileyonlinelibrary.com].

(Drummond & Rambaut, 2007) to estimate the divergence time among different clades. Using the GTR + I model selected by jModeltest, we ran MCMC 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, 2013) 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, 2013) for final parameter estimates.

Eco-chemical trait differentiation

We focused on CLGS, the most studied eco-chemical trait involved in bumblebee pre-mating recognition (Baer, 2003; Ayasse & Jarau, 2014). 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; Žacek *et al*., 2013). We identified the main component as the

Table 2. Summary of sampling table with genetic and eco-chemical criteria for *Bombus* species and subspecies used in this study.

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

PEPCK, Phosphoenolpyruvate carboxykinase gene; *COI*: Cytochrome oxidase 1 gene; CLGS, cephalic labial gland secretions; M, male; F, female.

compound that had the highest relative area (RA) among all compounds of CLGSs at least in one specimen of the taxon. The CLGS are species-specific blends with some interpopulation variations and are, subsequently, commonly used for species discrimination and assessment of intraspecific variability in bumblebees (review in Lecocq *et al*., 2015a,d). 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 (GC/MS) using a Focus GC (Thermo Scientific) with a nonpolar 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 a DSQ II quadrupol mass analyser (Thermo Scientific, Waltham, MA, U.S.A.) 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 nonpolar 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 (Appendix S2) on the alignment of each relative proportion of compound between all samples performed with GCALIGNER 1.0 (Dellicour & Lecocq, 2013a,b).

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 50 cm/s). The oven temperature (of the column) was programmed isothermally, starting at 70°C for 2 min and then rising from 70 to 320°C at a rate of 10°C/min. The temperature was then held at 320°C for 5 min.

In order 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 (2013a,b).

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 (Dufrene & 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.

Morphological analyses

In order to investigate diagnostic morphological characters for species identification and new taxa description (not for species delimitation), a total of 60 worker bees were analysed morphologically to discriminate between *B. lapponicus*, *B. konradini*, and *B. monticola*. We included only workers to have a sufficient sampling (minimum 15 specimens) and because the differences in metabasitarsus measurements were more pronounced in females than males. We selected the maximum length and width of metabasitarsus following 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 such a way as to maximize focus on the metabasitarsus. The maximum metabasitarsus distance was measured on the picture with the software IMAGEJ 1.5 (Abràmoff *et al*., 2004) (Table 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.

Data integration and decision framework

Assuming that species diagnosis and interpopulation 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,d) 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 specific status. This strict approach can lead to underestimation of the species differentiation but reduces the taxonomic inflation

(Lecocq *et al*., 2015d) 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; and (iii) was significantly differentiated in CLGS composition (by indices 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 but not all traits, in order to highlight those populations displaying such differentiation (originality) and to reduce the 'underestimate's risk' of our strict approach to assigning species status by naming them as a subspecies (Zink, 2004; Hawlitschek *et al*., 2012; Ennen *et al*., 2014; Lecocq *et al*., 2016).

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).

Results

Intertaxa differentiation

Haplotype network analysis revealed six unique haplotypes for *COI* and two for *PEPCK* (Fig. 3) within the *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 geographical subgroups within '*monticola*' (Fig. 3): (i) the northern Apennine lineage of '*monticola*' (described hereafter as *mathildis* **ssp.n.**), (ii) a western group including taxa from Pyrenees (*rondoui*) and Scotland (*monticola*); and (iii) an eastern-northern group including specimens from Sweden (*scandinavicus*) and Alps + Balkans + Mt. Olympus (*alpestris*).

In comparison to the ML, MP, and MB analyses for *COI* data, the tree generated for bGMYC analysis displayed difference (not biologically significant) mainly in the branching of *mathildis* **ssp.n.** As discussed in the 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 (Barraclough *et al*., 2003; Lecocq *et al*., 2015d). 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 the 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* ($p > 0.95–1$), *alpestris* ($P > 0.95–1$), *scandinavicus* ($P > 0.98–1$), *monticola* ($P > 0.99–1$) and *mathildis* **ssp.n.** ($P > 0.99–1$) which are significantly conspecific. The pairwise matrix (Fig. 4) shows more structure within *B. monticola* ssp. where the group displays 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 $F = 122.52$, $P < 0.05$; Fig. 3). Except *konradini*, all other *B. monticola* taxa shared the same compounds with similar relative concentration (RA) (PERMANOVA $F = 6.00–13.20$, $P > 0.05$) (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* (Table 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% although 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 $F = 29.36$ $P < 0.05$, between *konradini* and other *B. monticola* taxa).

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) (Table 4), species status was assigned to *konradini* (detailed information is given

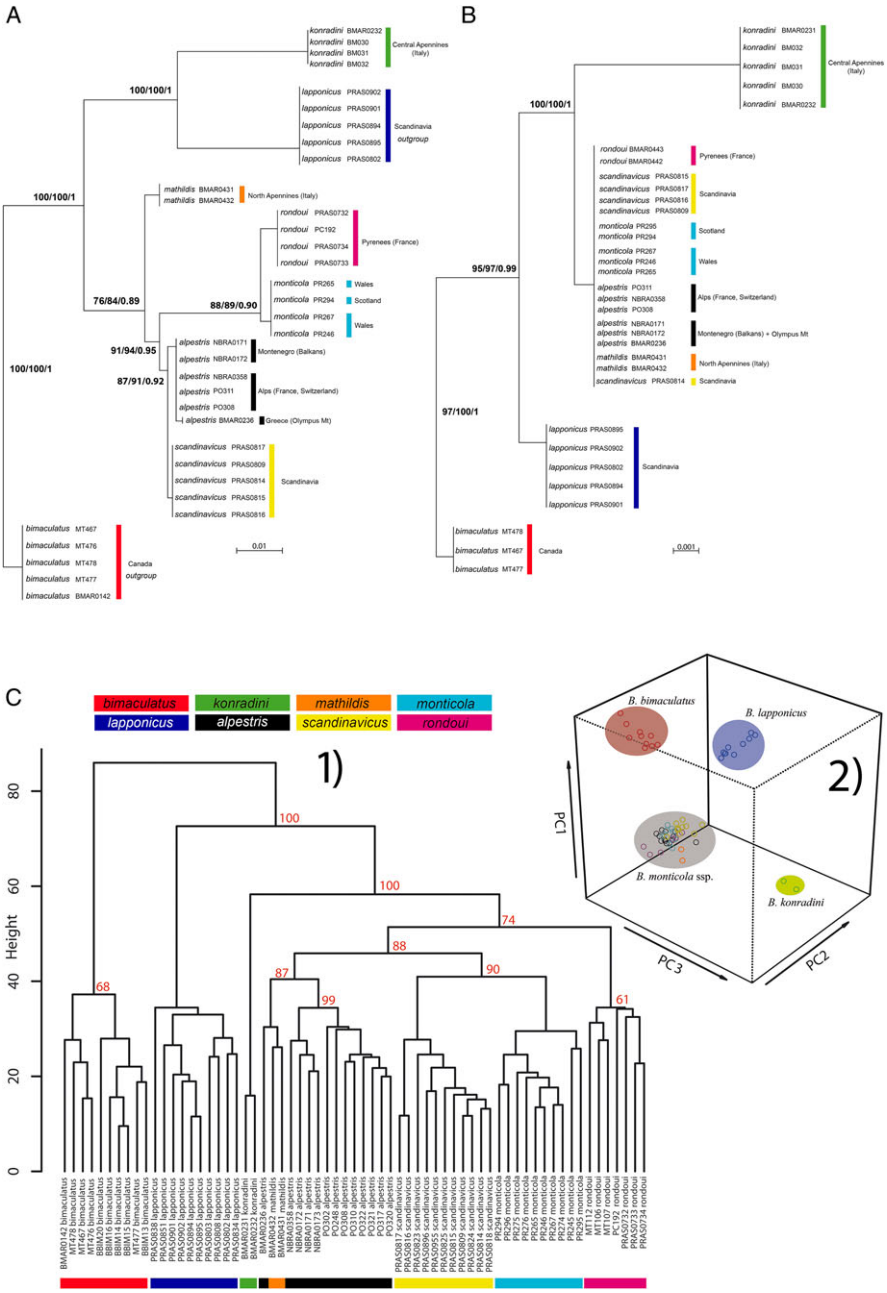

Fig. 3. Genetic and chemical analyses within the *monticola complex*. (A) Majority rule (50%) consensus tree based on maximum-likelihood (MB) analyses of *COI*. Values above tree branches are parsimony bootstrap values/ML bootstrap values/Bayesian posterior probabilities. Only ML and parsimony bootstrap values >70% and posterior probabilities >0.95 are shown. (B) Majority rule (50%) consensus tree based on ML analyses of *PEPCK* (MB). Values above tree branches are parsimony bootstrap values/maximum likelihood bootstrap values/Bayesian posterior probabilities. Only ML and parsimony bootstrap values >70% and posterior probabilities >0.95 are shown. (C) (1) Dendrogram of cephalic labial gland secretion (CLGS) differentiation within *monticola* complex and *Bombus 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 CLGS matrix of *B. bimaculatus* (red), *B. lapponicus* (dark blue), *B. konradini* **stat.n.** (green), *B. m. rondoui* (pink), *B. m. scandinavicus* (yellow), *B. m. monticola* (light blue), *B. m. alpestris* (black), *B. m. mathildis* **ssp.n.** (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 CLGS differentiation within *monticola* complex and *B. bimaculatus*: *B. bimaculatus* (red circles), *B. lapponicus* (dark blue circles), *B. konradini* **stat.n.** (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.n.** (orange circles). PC1, PC2 and PC3 are the first, the second and the third axes. [Colour figure can be viewed at wileyonlinelibrary.com].

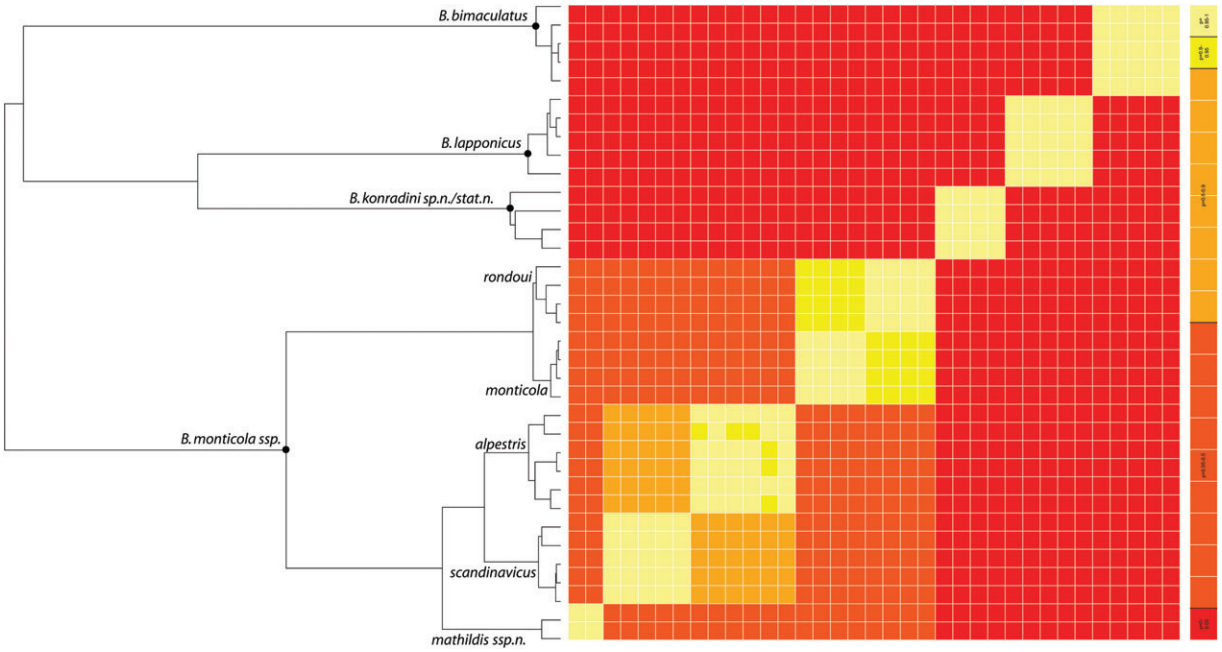

Fig. 4. Species recognition pairwise matrix. Species recognition pairwise matrix based on ultrametric tree of cytochrome oxidase 1 (*COI*) sequences with bGMYC pairwise probability of conspecificity plotted on a sample tree from BEAST. The coloured matrix corresponds to the pairwise probabilities of conspecificity returned by the Bayesian implementation of the general mixed Yule-coalescent model (bGMYC) method (colour scale on the right of the figure). Black spots show the coalescent node for each species. [Colour figure can be viewed at wileyonlinelibrary.com].

Table 3. List of indicator compounds (IndVal method, compounds >70%[a]) and main compounds[b] identified for *Bombus konradini* **stat.n.** within cephalic labial gland secretions.

Compounds	MW	*alpestris* ($n=13$) M	*monticola* ($n=10$) M	*rondoui* ($n=7$) M	*scandinavicus* ($n=11$) M	*mathildis ssp. nov* ($n=2$) M	*konradini nov status* ($n=2$) M	*lapponicus* ($n=10$) M	*bimaculatus* ($n=10$) M
Citronellol[a]	156	-	-	-	-	0.08	0.17	-	-
Ethyl tetradecenoate[a,c]	254	-	-	-	-	-	0.13	-	-
Ethyl tetradecanoate[a]	256	-	-	-	-	-	0.18	-	-
Hexadec-7-en-1-ol[a]	240	0.11	0.08	-	-	0.25	1.84	-	-
Ethyl hexadec-9-enoate[a]	282	-	-	1.00	-	-	1.41	0.03	-
Hexadec-9-enyl acetate[b]	282	52.34	55.15	57.05	53.96	35.27	51.53	0.08	32.95
Geranyl citronellol[c]	292	-	-	-	-	-	-	71.32	-
Ethyl octadecadienoate[a,b]	308	-	-	-	-	-	0.37	-	-
Ethyl octadec-9-enoate[a]	310	0.68	0.35	0.57	0.46	1.73	8.28	-	-
Geranyl geranyl acetate[d]	332	-	-	-	-	-	-	-	31.61
Dotriacontane[a]	451	-	-	-	-	-	0.09	-	-
Hexadecyl hexadecanoate[a,c]	480	0.04	0.07	0.09	0.22	0.25	0.94	0.19	0.09

[c]Main compound identified for *B. lapponicus*.
[d]Main compound identified for *B. bimaculatus*.
The full matrix is presented in Appendix S2.
MW, molecular weight; *n*, number of specimens; M, median of compound relative concentration (%); -, absent compounds.

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 colour pattern (Table 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.n.**) from the Central Apennines taxon (*B. konradini* **stat.n.**) 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 colour from *alpestris* (Table 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).

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 interglacial 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 7500 – max 548 000) yr ago.

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.n.** The ratio is also significantly different between *konradini* and *monticola* ssp. but not between *monticola alpestris* and *monticola mathildis* (Figure S2, Appendix S3). However, between *konradini* and *lapponicus*, although our results present a clear trend which highlights a larger ratio for *konradini*, there is no significant differentiation. According to these results, *konradini* appears as intermediate between *monticola* s.s. (large metabasitarsus ratio) and *lapponicus* (small metabasitarsus ratio). Diagnostic morphological characters are summarized in Table 1.

Impact of new taxa in zoological nomenclature

Bombus konradini ***stat.n.*** *(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, labelled: 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 labelled as paralectotypes (Table 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*.

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

Locus typicus: North Apennines, Emilia Romagna, Reggio Emilia, Villa Minozzo, Mt Cusna (Italy).
Holotype (present designation): 1 male, labelled: 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: Two males have been located, designated and labelled 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'.

Discussion

Interpopulation 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 the Alps, Balkans and Mt. Olympus) constitute, with the *COI* marker, five differentiated groups in three main lineages (Fig. 3) which diverged recently (about 40 000–18 000 year ago based on molecular clock estimates) during the Pleistocene/Quaternary. This could explain the weak divergence of the *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 a time of divergence matches with the start of the last postglacial warming. Thus, it appears that the geographical pattern is most likely a consequence of allopatric differentiation and genetic drift triggered by a range fragmentation subsequent to the last postglacial warming. We speculate that, at the beginning of the current interglacial period, taxa found refuge in southern mountainous areas (the Alps and

Table 4. Taxonomic decision table with all criteria used for *Bombus* species delimitation.

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*** **stat.n.**
B. bimaculatus	+	+/+	+	+/+	+	*B. bimaculatus*
B. lapponicus	+	+/+	+	+/+	+	*B. lapponicus*

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 maximum parsimony, maximum-likelihood and Bayesian 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.

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), in a similar way 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 similar to what has already been shown for insular populations of bumblebees (Lecocq *et al*., 2013b, Lecocq *et al*., 2015a).

Despite their relative geographical isolation, all other *B. monticola* allopatric taxa previously recognized by Reinig (1965) are considered as conspecific based on our diagnostic criteria with a low geographical genetic and phenotypic differentiation (decision framework Table 4; Fig. 3). Overall they shared the same CLGS composition (except for some low relative concentration differences) and are characterized by only 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 *B. monticola alpestris*, the three sampled populations (Alps, Balkans and Mt. Olympus) are clearly consubspecific.

Mountaintop speciation: Bombus konradini ***stat.n.***

Contrary to the situation within *B. monticola*, *B. konradini* **stat.n.** displays greater genetic and chemical trait differentiation (Fig. 3). Allopatry has most likely shaped the reproductive trait (CLGS) differentiation as observed in other species (Lecocq *et al*., 2013a,b; Lecocq *et al*., 2015c). 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 have led to a new shuffling of the genetic pool by re-contacting these populations without speciation (Coyne & Orr, 2004; Hewitt, 2004a,b). The modification of geographical range could trigger genetic and CLGS differentiation. Indeed, it has been shown that reproductive traits including CLGS can differentiate from both sides of the physical barriers that may exist between 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 phylogeographical 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*) (Cameron *et al*., 2007; Løken, 1973; Svensson, 1979; Gjershaug *et al*., 2013). Our results also supported the species status of *konradini* which is endemic at high altitudes (>1800 m a.s.l.) of the Central Apennines (Manino *et al*., 2007) (Fig. 3, Table 4). Concerning 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 for the *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 interglacial 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 (Martín-Bravo *et al.*, 2010). Several studies have shown the presence of endemic taxa in the Central Apennines (e.g. in amphibians, Canestrelli *et al.*, 2008; Canestrelli *et al*., 2012 and Mattoccia *et al*., 2011; in reptiles, Joger *et al*., 2007; in turtles, Fritz *et al*., 2005; in plants, Conti *et al*., 2005, Fuente *et al*., 2011 and Frattaroli *et al*., 2013; and in bumblebees, Lecocq *et al*., 2013a). For example, Lecocq *et al*. (2013a) provided evidence that the population of *B. lapidarius* (a Palaearctic 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-Labourdette *et al*., 2012).

The sympatry of two different species, defined by divergent taxonomic traits, reinforces the 'species' status because individuals co-inhabit the same area without hybridization. Our results suggest that *B. monticola* (s.s.) is absent in the Central Apennines unless this could be due to a sampling bias. Such an absence could have resulted in a lack of sympatry between *B. monticola* and *B. konradini*. Several hypotheses could explain the potential absence of *B. monticola* (s.s.) in the Central Apennines: (i) for eco-climatic constraints, historical or competition reasons, this taxon has never inhabited this region or has disappeared; and (ii) despite the significant observed differences (genetic, morphological and chemical traits), a limited hybridization between *monticola* and *konradini* still could be possible. Following this second hypothesis, along the contact zone between *monticola* and *konradini*, the subspecies *mathildis* could represent an intermediate population resulting from some introgressions of the population living in the Alps (*alpestris*). Our *COI* results suggest that the subspecies *mathildis* (low branch support) is closer to *B. konradini* than all other subspecies of *B. monticola* (s.s.) (Fig. 3). However, phenotypic and chemical trait results do not support this hypothesis (Figs 3C, Figure S2). Although distinct, *B. konradini* could be the 'replacement species' to *B. monticola* (s.s.) 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 our *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.

Conservation remarks on the B. monticola *complex and the practice of integrative taxonomy*

Considering all taxonomic criteria in our integrative approach (Fig. 3, Table 4), we propose conservation of the subspecies status for five *monticola* taxa (Hawlitschek *et al*., 2012; Lecocq *et al*., 2015a,d, 2016): *B. monticola rondoui* from the Pyrenees, *B. monticola monticola* from the British Isles, *B. monticola scandinavicus* from Fennoscandia, *B. monticola alpestris* from the Alps, Balkans and Mt. Olympus, and *B. monticola mathildis* **ssp.n.** from the 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 of 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, of defining the species or subspecies status of a population. Here the integrative taxonomy, considering several criteria independently, provide strong pieces of evidence to take decision concerning species status of taxa. We assigned 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,d; Lecocq *et al*., 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 the assignment of taxonomic status to any doubtful bumblebee taxa and marks 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 *B. patagiatus* and *B. magnus*, Bertsch & Schweer (2012b).

Conservation implication of the new taxonomic status of B. konradini ***stat.n.***

The new taxonomical status has implication for the red list assessments of the European bumblebees studied herein, according to the IUCN criteria (Nieto *et al*., 2014). Although 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 1800 m a.s.l. (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 back-up plans (mitigation measures).

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/syen.12268

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* ssp.n. and *Bombus konradini* **stat.n.**, designation of the holotype and lectotype and morphological differentiation.

Figure S1. Morphology and coloration variation of the face of *Bombus konradini* **stat.n.** (Lectotype female, A) and *Bombus monticola alpestris* (female, B). Photographs are by P. Rasmont.

Figure 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).

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BM, TL, NB, CU, IV and PR conceived and designed the experiments; BM, NB, PB, MC and PR carried out the sampling; BM analyzed the data; and BM, TL, NB, PB, MC, CU, IV, JOG, DM and PR wrote the paper.

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