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.
**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 (intraspecific taxa). We analysed interpopulation differentiation through multiple diagnostic traits: (i) a mtDNA marker (cytochrome oxidase I, COI), (ii) a nuDNA marker (phospho-enolpyruvate 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* (= *hypophilus*, 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 (Sibillini 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.

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 Ap12001/Aph2931, Pedersen, 2002; PEPCK primers FHv4/RFh4, Cameron et al., 2007), sequencing procedures and DNA sequence alignment using the method described in Lecoq 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 (Lecoq 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 replicates. 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>
<thead>
<tr>
<th>Range</th>
<th>Scandinavian</th>
<th>Monticola</th>
<th>Rondoui</th>
<th>Alpestris</th>
<th>Mathildis ssp.n.</th>
<th>Konradini stat.n.</th>
<th>Lapponicus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conservation status</td>
<td>No regression was mentioned</td>
<td>In decline Evans &amp; Potts (2013) and Fitzpatrick et al. (2006)</td>
<td>In decline Iserbyt &amp; Rasmont (2012)</td>
<td>Few data show a decline in Italy Manino et al. (2007)</td>
<td>No regression was mentioned</td>
<td>Rare and localized Ricciardelli &amp; Piatti (2003)</td>
<td>Stable Nieto et al. (2014)</td>
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<td>Female</td>
<td></td>
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<tr>
<td>Morphology</td>
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<tr>
<td>Furrow of gena</td>
<td>The surface between the punctures on vertex is shiny, and there is a slight depression with some punctures near the compound eye</td>
<td>Similar to Monticola</td>
<td>The surface between the punctures on vertex is rugose and dull and the furrow is distinct, nearly reaching the compound eye</td>
<td>Strong pubescence and the maximal width of the basitarus is high (sensu Gjershaug et al., 2013). The length of the metabasitarus of these taxa is large (Appendix S3)</td>
<td>Strong pubescence and the maximal width of the basitarus is low (sensu Gjershaug et al., 2013) as in lapponicus. The ratio maximum length/maximum width of the metabasitarus of this taxon is intermediate (Appendix S3).</td>
<td>Strong pubescence and the maximal width of the basitarus are low (sensu Gjershaug et al., 2013). The length of the metabasitarus of this taxon is short (Appendix S3).</td>
<td></td>
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<tr>
<td>Coat colour variation</td>
<td>Dark</td>
<td>Dark</td>
<td>Light</td>
<td>Relatively dark</td>
<td>Light and colorful</td>
<td>Large and light Reinig (1965)</td>
<td>Varies from very light and colorful in Northern Fennoscandia, to rather dark in Southern Fennoscandia (Southern Norway)</td>
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<tr>
<td>Colour pattern</td>
<td>Black</td>
<td>Black</td>
<td>Yellow</td>
<td>Black</td>
<td>Yellow or sometimes black (Figure S1)</td>
<td>Yellow (Figure S1)</td>
<td>Black</td>
</tr>
<tr>
<td>Face</td>
<td>Black</td>
<td>Black</td>
<td>Yellow</td>
<td>Black</td>
<td>Yellow or sometimes black (Figure S1)</td>
<td>Yellow (Figure S1)</td>
<td>Black</td>
</tr>
<tr>
<td>Collare and scutellare</td>
<td>Small dark yellow and black</td>
<td>Light yellow/yellow</td>
<td>Small dark yellow/dark yellow</td>
<td>Wide light yellow with a black line near the tegulae/yellow</td>
<td>Wide yellow band to the tegulae/yellow</td>
<td>Yellow/red/black</td>
<td>Yellow/red/black</td>
</tr>
<tr>
<td>Tergite 1</td>
<td>Black/Red</td>
<td>Black</td>
<td>Yellow</td>
<td>Yellow/black</td>
<td>Yellow/black (center of tergite)</td>
<td>Yellow/red/black</td>
<td>Yellow/red/black</td>
</tr>
</tbody>
</table>
Table 1. Continued

<table>
<thead>
<tr>
<th>Tergite 4</th>
<th>Tergite 5</th>
<th>Colour pattern</th>
<th>Face</th>
<th>Collar/scutellum</th>
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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: TPM1uf + 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 10000 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
(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 $1 \times 10^{-9}$ to $1 \times 10^{-7}$ 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.

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

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 CLGSs 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 CLGSs 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 GC Kovats 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 (Dufrène & 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 (Abrámoff et al., 2004) (Table 1, Appendix S3). Kruskal–Wallis analyses (Kruskal-Wallis test and multiple comparison test after Kruskal-Wallis; ‘pigrimess’ 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.
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 PECK (Fig. 3) within the B. monticola taxon. Konradini-C was the only taxon displaying unique COI (6.8% sequence difference from monticola alpestris and 5.3% from lapponicus) and PECK (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 PECK 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 (rondou) 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 (Barraculous 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 probability between individuals included in the group, P > 0.98–1), (ii) another group with all konradini from the Central Apennines (P < 0.99–1), (iii) one group with all bimaculatus (P > 0.98–1), (iv) one group with all monticola subspecies (P > 0.13–0.95) including rondou (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, octadecadienoate, and 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].
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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%) and main compounds identified for Bombus konradini stat.n. within cephalic labial gland secretions.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>alpestris (n = 13)</th>
<th>monticola (n = 10)</th>
<th>rondoui (n = 7)</th>
<th>scandinavicus (n = 11)</th>
<th>mathildis ssp. nov (n = 2)</th>
<th>konradini nov status (n = 2)</th>
<th>lapponicus (n = 10)</th>
<th>bimaculatus (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citronellol(^a)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.08</td>
<td>0.17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl tetradecenoate(^a)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl tetradecanoate(^b)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.18</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hexadec-7-en-1-ol(^a)</td>
<td>240 0.11</td>
<td>0.08</td>
<td>-</td>
<td>-</td>
<td>0.25</td>
<td>1.84</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl hexadec-9-enoate(^b)</td>
<td>282 -</td>
<td>-</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
<td>1.41</td>
<td>0.03</td>
<td>-</td>
</tr>
<tr>
<td>Hexadec-9-enyl acetate(^b)</td>
<td>282 52.34</td>
<td>55.15</td>
<td>57.05</td>
<td>53.96</td>
<td>35.27</td>
<td>51.53</td>
<td>0.08</td>
<td>32.95</td>
</tr>
<tr>
<td>Geranyl citronellol(^c)</td>
<td>292 -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>71.32</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl octadecadienoate(^a)</td>
<td>308 -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>93.77</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl octadec-9-enoate(^b)</td>
<td>310 0.68</td>
<td>0.35</td>
<td>0.57</td>
<td>0.46</td>
<td>1.73</td>
<td>8.28</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Geranyl geranyl acetate(^d)</td>
<td>332 -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>31.61</td>
<td>-</td>
</tr>
<tr>
<td>Dotriacontane(^a)</td>
<td>451 -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hexadecyl hexadecanoate(^a)</td>
<td>480 0.04</td>
<td>0.07</td>
<td>0.09</td>
<td>0.22</td>
<td>0.25</td>
<td>0.94</td>
<td>0.19</td>
<td>0.09</td>
</tr>
</tbody>
</table>

\(^a\)Main compound identified for B. lapponicus.
\(^b\)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*. 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).


*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 pararlectotypes: ‘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 carpini-folius, 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 within the subspecies of *monticola* (Fig. 3). The western subspecies (*B. monticola rondou* 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 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.

<table>
<thead>
<tr>
<th>Former taxonomic status</th>
<th>Morphology (Gjershaug et al., 2013)</th>
<th>Private haplotypes (COI/PEPCK)</th>
<th>COI gene/ BGMYC</th>
<th>PEPCK gene</th>
<th>Proposed taxonomic status</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. monticola scandanavicus</td>
<td>−</td>
<td>++/−</td>
<td>− (B)</td>
<td>− LS (B)/−</td>
<td>−</td>
</tr>
<tr>
<td>B. monticola monticola</td>
<td>−</td>
<td>++/−</td>
<td>− (B)</td>
<td>− LS (A)/−</td>
<td>−</td>
</tr>
<tr>
<td>B. monticola rondoni</td>
<td>−</td>
<td>++/−</td>
<td>− (A)</td>
<td>− LS (A)/−</td>
<td>−</td>
</tr>
<tr>
<td>B. monticola alpestris</td>
<td>−</td>
<td>++/−</td>
<td>− (C)</td>
<td>− LS (B)/−</td>
<td>−</td>
</tr>
<tr>
<td>B. monticola konradini (North Apennines)</td>
<td>−</td>
<td>++/−</td>
<td>− (C)</td>
<td>− LS (C)/−</td>
<td>−</td>
</tr>
<tr>
<td>B. monticola konradini (Central Apennines)</td>
<td>+</td>
<td>++/−</td>
<td>+/++</td>
<td>+/++</td>
<td>+/++</td>
</tr>
<tr>
<td>B. bimaculatus</td>
<td>+</td>
<td>++/−</td>
<td>+/++</td>
<td>+/++</td>
<td>+/++</td>
</tr>
<tr>
<td>B. lapponicus</td>
<td>+</td>
<td>++/−</td>
<td>+/++</td>
<td>+/++</td>
<td>+/++</td>
</tr>
</tbody>
</table>

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 CLGSs. 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.

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 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 mountains 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 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-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 sympathy 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 rondoni 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 & Unit, 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 conspecific 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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