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Methods for species delimitation in bumblebees (Hymenoptera, Apidae, *Bombus*): towards an integrative approach

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Delimitation of closely related species is often hindered by the lack of discrete diagnostic morphological characters. This is exemplified in bumblebees (genus *Bombus*). There have been many attempts to clarify bumblebee taxonomy by using alternative features to discrete morphological characters such as wing shape, DNA, or eco-chemical traits. Nevertheless each approach has its own limitations. Recent studies have used a multisource approach to gather different lines of speciation evidence in order to draw a strongly supported taxonomic hypothesis in bumblebees. Yet, the resulting taxonomic status is not independent of selected evidence and of consensus methodology (i.e. unanimous procedure, majority, different weighting of evidence). In this article, we compare taxonomic conclusions for a group of taxonomically doubtful species (the *Bombus lapidarius*-group) obtained from the four commonly used lines of evidence for species delimitation in bumblebees (geometric morphometric of wing shape, genetic differentiation assessment, sequence-based species delimitation methods and differentiation of cephalic labial gland secretions). We ultimately aim to assess the usefulness of these lines of evidence as components of an integrative decision framework to delimit bumblebee species. Our results show that analyses based on wing shape do not delineate any obvious cluster. In contrast, nuclear/mitochondrial, sequence-based species delimitation methods, and analyses based on cephalic labial gland secretions are congruent with each other. This allows setting up an integrative decision framework to establish strongly supported species and subspecies status within bumblebees.

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Introduction

The species is a fundamental unit, central to biodiversity classification (Mayr 1942; De Queiroz 2007). However, species delimitation between closely related species is often impractical with traditional discrete morphological characters (Bickford *et al.* 2007). Bumblebee (genus *Bombus*) taxonomy exemplifies this issue: different species can be morphologically similar (i.e. cryptic species) while conspecific specimens can be extraordinarily divergent in their hair body's colour patterns (Michener 1990; Williams 1998). As in other taxonomically confused groups, there have been many attempts to clarify bumblebee taxonomy by using alternative features to traditional morphological characters such as geometric morphometrics of wing shape (Aytekin *et al.* 2007), DNA (e.g. Ellis *et al.* 2006) or eco-chemical traits (e.g. Rasmont *et al.* 2005).

The geometric morphometric approach based on wing shapes remains poorly used in bumblebee taxonomy despite promising pioneer studies (e.g. Aytekin *et al.* 2007). Further knowledge on the evolution of this trait between closely related species is needed to assess its usefulness for bumblebee species delimitation. In contrast, the genetic approach (e.g. Brower 1994; Hebert *et al.* 2003) has been widely used on several bumblebee species groups (e.g. Williams *et al.* 2011, 2012). Nevertheless, species delimitation based on solely genetic evidence remains controversial because (i) DNA sequences analysed are chosen arbitrarily, (ii) speciation processes are not always characterized by accumulation of many genetic differences while conspecific populations can display high genetic divergence (e.g. Ferguson 2002; Salvato *et al.* 2002; Kuhlmann *et al.* 2007) and (iii) mating isolation can happen faster than the differentiation of genetic markers (e.g. Trewick 2008; Symonds *et al.* 2009; Bauer *et al.* 2011). A solution to this last issue is to base the species delimitation on species-specific reproductive traits involved in the species mating recognition (Paterson 1993). In bumblebees, the male cephalic labial gland secretions (CLGS), a key species-specific chemical reproductive trait for mate attraction (Calam 1969; Bergström *et al.* 1981; Baer 2003; Bertsch *et al.* 2005), have been widely used as chemical markers in resolving species status (e.g. Svensson 1979; Bertsch *et al.* 2005; Lecocq *et al.* 2011). However, it is difficult to determine a threshold of species-level differentiation because the consequence of reproductive trait differentiation can vary from low regional variation with minor behavioural consequences (e.g. Vereecken *et al.* 2007) to the rise of reproductive isolation barrier (e.g. Martens 1996). These consequences are not predictable without field observations or ethological tests that are most of the time unavailable (Lecocq *et al.* 2013b).

Few recent studies have used a multisource approach to gather different lines of evidence of speciation in order to draw strongly supported taxonomic hypotheses for bumblebees (e.g. Bertsch *et al.* 2005; Lecocq *et al.* 2011). This type of approach combines taxonomic tools from different areas, such as geometric morphometric, genetics and chemistry to obtain to a more informed consensus. The development of integrative taxonomy based on the unified species concept (USC) provides a methodological framework for this taxonomic evaluation (De Queiroz 2007; Schlick-Steiner *et al.* 2010). The USC argues that all species concepts agree on the fact that species exist as separately evolving metapopulation lineages but diverge in criteria for delimiting species (De Queiroz 2007). The USC proposes that the numerous delimiting species criteria are maintained as operational criteria (De Queiroz 2007). Therefore, separation of metapopulation lineages could be inferred from evidence for reproductive isolation, phylogenetic divergence or ecological differentiation. Integrative taxonomy considers these to be separate line of evidence when assigning species status (e.g. Burns *et al.* 2008; Fisher & Smith 2008), although species diagnose is more likely in multiple evidence detection. Therefore, integrative taxonomy may provide an efficient approach to species delimitation. Moreover, by considering subspecies as a step in the process of allopatric speciation (Mayr 1942), assigning subspecies rank to lineages in ambiguous allopatric cases (i.e. differentiation in only one character) has been proposed as a solution (see argumentation in Hawlitschek *et al.* 2012). Nevertheless, the resulting taxonomic status is not independent of the kind of evidence chosen and of consensus methodology (i.e. unanimous procedure, majority, different weighting of evidence).

In this article, we compare the taxonomic conclusions obtained by a 'discovery-like approach' (Schlick-Steiner *et al.* 2010) on the four commonly used lines of evidence for species delimitation in bumblebees (geometric morphometric approach, genetic differentiation assessment, sequence-based species delimitation methods and CLGS differentiation) in a group of taxonomically doubtful bumblebee species (the *Bombus lapidarius*-group; subgenus *Melanobombus*; Cameron *et al.* 2007; Hines 2008; Williams 1998). We ultimately aim to assess the usefulness of these lines of evidence as components of an integrative decision framework for bumblebee species delimitation.

Material and methods

Studied species group and sampling

The *Bombus lapidarius*-group includes seven species (Williams 1998). Here, we focused on a group of closely related taxa that includes the West-Palearctic taxa (Cameron *et al.* 2007; Williams *et al.* 2008): *B. erzurumensis* Özbek, 1990,

B. incertus Morawitz 1881, *B. lapidarius* (L.) and *B. sichelii* Radoszkowski 1860 (Fig. 1A).

Bombus erzurumensis and *B. sichelii* are two closely related taxa considered as conspecific (Williams 1998) or as distinct species (Rasmont *et al.* 2000). *Bombus erzurumensis* is endemic to North East Anatolia and North Iran while *B. sichelii* is a widespread Palearctic species (Rasmont & Iserbyt 2012). *Bombus sichelii* currently includes five recognized subspecies (Fig. 1A): *B. sichelii alticola* Kriechbaumer, 1873 (central and eastern Alps), *B. sichelii cazurroi* Vogt, 1911 (North-East Turkey, Caucasus, and North Iran), *B. sichelii drenowskii* Vogt 1911 (Balkans), *B. sichelii flavissimus* Tkalců, 1974 (Pyrenees and western Alps) and *B. sichelii sichelii* Radoszkowski, 1860 (Russia and Siberia).

Bombus incertus is restricted to Anatolia, Transcaucasia and North Iran (Rasmont & Iserbyt 2012) (Fig. 1A).

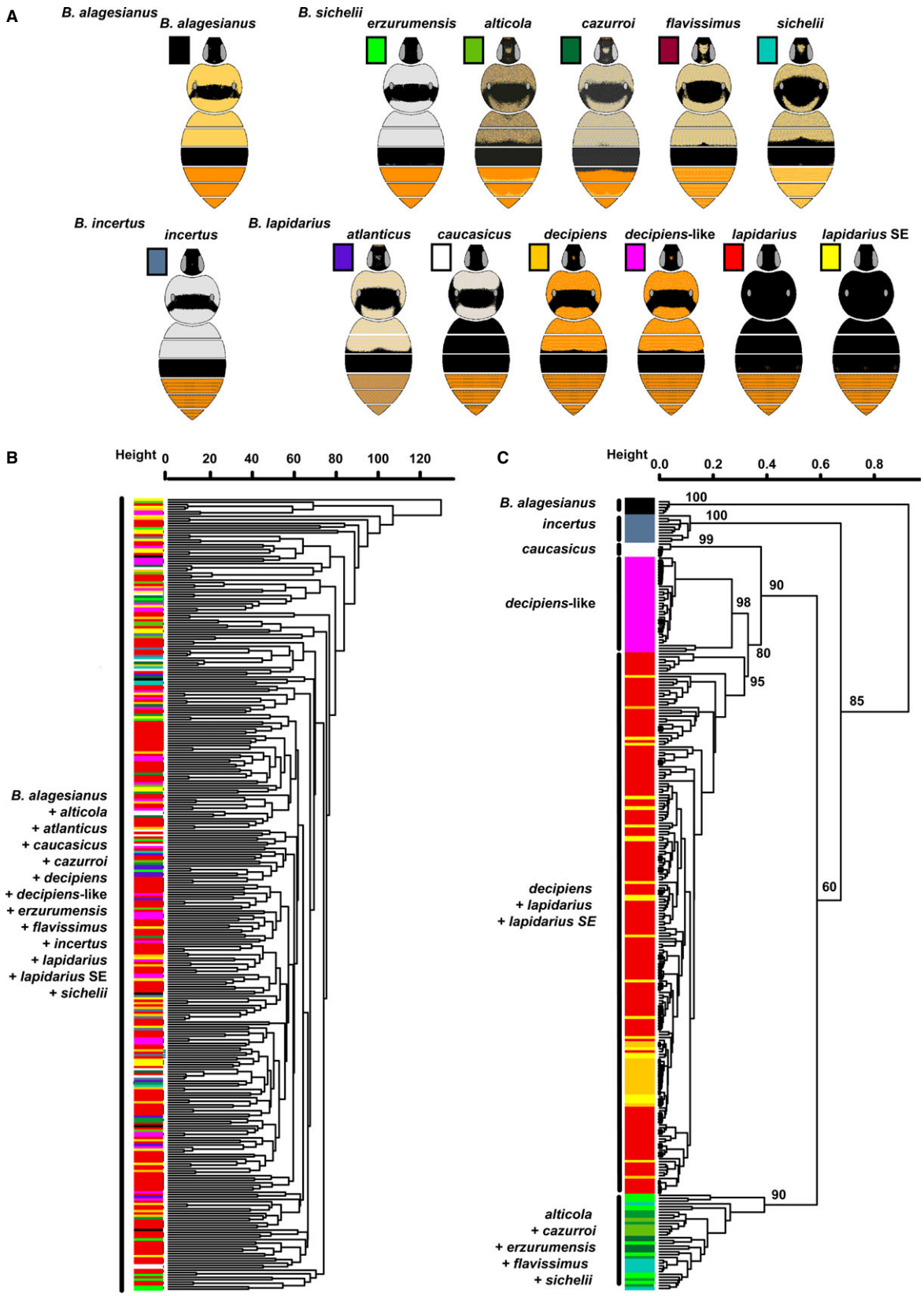
Bombus lapidarius is a common and widespread species in temperate West-Palearctic except in Southern Europe where it is relatively rare (Reinig 1935; Rasmont & Iserbyt 2012). *Bombus lapidarius* currently includes five subspecies based on colour patterns (Fig. 1A) (Reinig 1935, 1970; Tkalců 1960; Rasmont 1983) despite their poor reliability as diagnostic characters in bumblebees (Bertsch & Schweer 2012a; Carolan *et al.* 2012): (i) *B. lapidarius lapidarius* (L.) in the European plains, Balkans and West Anatolia, (ii) *B. lapidarius decipiens* Pérez 1890 in the Iberian Peninsula and in Southern Italy, (iii) *B. lapidarius caucasicus* Radoszkowski 1859 in the North East Anatolia and Caucasus, (iv) *B. lapidarius eriophorus* Klug 1807 in Caucasus and (v) *B. lapidarius atlanticus* Benoist 1928 in the Moroccan Atlas. A recent genetic and eco-chemical study does not support this classification and points out that *B. lapidarius* could be a species complex (Lecocq *et al.* 2013a). First, the large genetic divergence of *B. lapidarius caucasicus* makes its conspecificity with other *B. lapidarius* taxa doubtful. Second, the European populations of *B. lapidarius* are clustered in three monophyletic groups (Lecocq *et al.* 2013a) which do not reflect the current intraspecific taxonomy: (i) the Italian *B. lapidarius decipiens* group, (ii) the South Eastern European *B. lapidarius lapidarius* group, (iii) the main group that includes all other European *B. lapidarius lapidarius* and the Iberian *B. lapidarius decipiens*. Moreover, the South Italian *B. lapidarius decipiens* displays diagnostic CLGS (Lecocq *et al.* 2013a).

We sampled 327 specimens (Table S1): *B. erzurumensis* [genetic data (GD) = 7, CLGS data (CD) = 10, morphological data (MD) = 10], *B. sichelii alticola* (GD = 4, CD = 5, MD = 5), *B. sichelii cazurroi* (GD = 5, CD = 5, MD = 5), *B. sichelii flavissimus* (GD = 5, CD = 7, MD = 7), *B. sichelii sichelii* (GD = 7, CD = 7, MD = 7), *B. incertus* (GD = 6, CD = 10, MD = 10), *B. lapidarius lapidarius* (GD = 196, CD = 174, MD = 196), Iberian *B. lapidarius decipiens* (GD = 20, CD = 17, MD = 23), Italian *B. lapidarius decipiens* (GD = 20, CD = 35, MD = 35), *B. lapidarius atlanticus* (GD = 5, CD = 0, MD = 10) and *B. lapidarius caucasicus* (GD = 10, CD = 5, MD = 13). We failed to collect *B. sichelii drenowskii* and *B. lapidarius eriophorus*. All samples used in CLGS and wing shape analyses were males while females/workers were included in the genetic analyses; male samples were analysed in all kind of analyses (Table S1). We also sampled *B. alagesianus* Reinig, 1930 as outgroup (GD = 5, CD = 5, MD = 5). The dataset included new data and data from Lecocq *et al.* (2013a) (see Table S1). Specimens were killed by freezing at −20 °C (Table S1).

In the following analyses, we considered both taxa defined in the literature (Reinig 1935, 1970; Tkalců 1960; Rasmont 1983) and genetic groups defined by Lecocq *et al.* (2013a). We referred to taxa as *erzurumensis* (*B. erzurumensis*), *alticola* (*B. sichelii alticola*), *cazurroi* (*B. sichelii cazurroi*), *flavissimus* (*B. sichelii flavissimus*), *sichelii* (*B. sichelii sichelii*), *incertus* (*B. incertus*), *caucasicus* (*B. lapidarius caucasicus*), *decipiens*-like (Italian *B. lapidarius decipiens*), *decipiens* (Iberian *B. lapidarius decipiens*), *atlanticus* (*B. lapidarius atlanticus*), *lapidarius* SE Europe (*B. lapidarius lapidarius* from the SE European group; see Lecocq *et al.* 2013a) and *lapidarius* (all other *B. lapidarius lapidarius*) (Fig. 1A).

Geometric morphometric approach

Wing venation is a traditional discrete character for insect taxonomy (e.g. Grimaldi & Engel 2005). Wing shape variation has been increasingly studied by geometric morphometric methods to discriminate taxa at intra- and supraspecific levels (e.g. Aytekin *et al.* 2007; Tofilski 2008; Wappler *et al.* 2012; Dehon *et al.* 2014). These methods compare the shapes themselves (see Adams *et al.* 2004) and produce informative data for separating groups (Monteiro & Coelho 2002). We used the landmark based geometric morphometrics on the *B. lapidarius*-group. We used only males to avoid sexual dimorphism (Pretorius 2005; Jeratthitikul *et al.* 2014). We photographed the right forewings of all specimens ($n = 321$) using a D70 Nikon coupled to an Olympus SZ010 binocular. Photographs were gathered in one file using tps-UTIL 1.58 and then one author (MD) digitized two-dimensional Cartesian coordinates of 18 landmarks placed on the wing veins with tps-DIG 2.17 (Rohlf 2010a,b) (Fig. S1, Table S2). First, the landmark configurations were scaled, translated and rotated against the consensus configuration by the generalized least square (GLS) Procrustes superimposition method in R (R-package shapes, Dryden 2012). The GLS Procrustes superimposition removed all the non-shape differences and separated the size and shape components of the structure. Further statistical analyses were performed on



landmark configurations projected in the Euclidean tangent space approximate to Kendall's shape space. This approximation is allowed if the amplitude of shape variation in the dataset is small enough. To check this assumption, we calculated with tps-SMALL (Rohlf 2013) the least-squares regression slope and the correlation coefficient between the two distances (Euclidean and Procrustes distances between pairs of specimens) computed by tps-SMALL. We then performed a clustering analysis performed with unweighted pair group method with arithmetic mean (UPGMA) clustering method on Procrustes distance matrix (R-package ape, Paradis *et al.* 2004). We did not use discriminate approaches commonly used in geometric morphometric analyses on bees (e.g. Aytekin *et al.* 2007) since we developed a 'discovery-like approach' (without *a priori*; Schlick-Steiner *et al.* 2010).

Genetic differentiation assessment

Genetic differentiation has been previously used for species delimitation in bumblebees (e.g. Bertsch 2010). Here, we assessed the genetic differentiation in two gene fragments commonly used to analyse interspecific and intraspecific relationships in bumblebees (Pedersen 2002; Cameron *et al.* 2007): mitochondrial cytochrome oxidase 1 (COI) and phosphoenolpyruvate carboxykinase (PEPCK) following the phylogenetic approach of Lecocq *et al.* (2011). We extracted total DNA using a QIAGEN DNeasy® Tissue Kit (Qiagen Inc., Valencia, CA, USA). Legs were removed, crushed using liquid nitrogen and digested (4 h in proteinase K at 56 °C). We carried out polymerase chain reaction (PCR) amplifications with primer pair Apl2013/ApH2931 (Pedersen 1996) for COI and FHv4/RHv4 (Cameron *et al.* 2007) for PEPCK. We carried out PCR amplifications by initial denaturing for 3 min at 94 °C, 35 (COI) or 40 (PEPCK) cycles of 1 min denaturing at 94 °C, 1 min annealing at 51 °C (COI) or 48.5 °C (PEPCK), 2 min elongation at 72 °C and a final extension for 10 min at 72 °C. Genes were sequenced with an ABI 3730 DNA analyser or by GENOSCOPE (*Centre National de Séquençage*; Evry, France). We sequenced both strands of each PCR product. We performed the consensus of both strands with CodonCode Aligner 3.0.1. We checked the bumblebee origin of each sequence with BLAST 2.2.20 (Zhang *et al.* 2000). We performed the alignment with MAFFT ver.6. (FFT-NS-2 algorithms, default parameters; Katoh *et al.* 2002) and edited the data matrix in Mesquite 2.75 (Maddison & Maddison 2007). We performed translation to proteins (*Drosophila* mitochondrial DNA genetic code or Universal genetic code) with Mesquite. Sequences were deposited in GenBank (Table S1). The final molecular dataset spanned 2757 aligned nucleotides: 1056 bp from COI [185 parsimony informative sites (PIS)] and 910 bp from PEPCK (18 PIS). Sequences are available on GenBank (Table S1) and genetic data matrices are deposited on TreeBase (TB2:S15458).

We analysed each gene independently using maximum likelihood (ML) and Bayesian methods (MB). Trees were rooted with outgroup species. We partitioned each gene to explore the best substitution model: (i) PEPCK into two exons and two introns, (ii) COI and each PEPCK exon by base position (1st, 2nd and 3rd). We used the Akaike information criteria corrected for small sample sizes (Hurvich & Tsai 1989) to choose the best fitting substitution models with jModeltest (Posada 2008) for each dataset: (i) for COI: TIM1 + G (1st), F81 (2nd) and TPM1uf + G (3rd); (ii) for PEPCK introns 1 and 2: HKY; (iii) for PEPCK exon 1: TrN (1st), JC (2nd) and TrNef (3rd); (iv) for PEPCK exon 2: F81 + I (1st), K80 (2nd) and JC (3rd). We conducted ML analyses with GARLI 2.0 (Zwickl 2006). We used a random starting tree and the automated stopping criterion (stop when the ln score remained constant for 20 000 consecutive generations). We performed 10 independent runs in GARLI for each of the genes; the topology and –ln L were identical among replicates. We retained the highest likelihood of one of those runs. We evaluated statistical confidence in nodes with 10 000 non-parametric bootstrap replicates (Felsenstein 1985) using the automated stopping criteria set at 10 000 generations. More bootstrap replicates could not be performed because it would have required unpractical computing times. Topologies with bootstrap values ≥70% were considered well supported (Hillis & Bull 1993). We performed Bayesian analyses (MB) with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). The model selection process was the same as that for ML analysis. We substituted selected models which are not implemented in MrBayes by the closest overparameterized model (Huelsenbeck & Rannala 2004). The TIM1, TIM1uf, and TrN were replaced by the GTR

Fig. 1 Colour pattern, wing shape, and cephalic labial gland secretion (CLGS) differentiations. —A. Colour pattern of taxa studied. The name in the top left-hand corner of each colour pattern group corresponds to the species that included these taxa according to Williams (1998). —B. Unweighted pair group method with arithmetic mean (UPGMA) cluster based on Procrustes distance matrix based on the 18 landmarks coordinates of wing. Colours refer to the colour chart of taxa on the Fig. 1A. —C. UPGMA cluster based on a correlation matrix calculated from the CLGSs matrix. The values near nodes are multiscale bootstrap resampling (only values >80 of main groups are shown). Colours refer to the colour chart of taxa on the Fig. 1A.

model and the TPM3 and TrNef were replaced by the SYM model. We conserved the proportion of invariable sites and gamma distributed rates defined in jModeltest in all models. We carried out five independent analyses for each gene and for the combined data (500 million generations, four chains with mixed-models, default priors, saving trees every 100 generations). We stopped the analyses after checking convergence between runs using the average standard deviation of split frequencies and by plotting likelihood values across generations with Tracer 1.4 (Rambaut & Drummond 2007). We discarded the first 20 million generations (200 000 first trees saved) as burn-in. The phylogeny and posterior probabilities were then estimated from the remaining trees and a majority-rule 50% consensus tree was constructed. Topologies with posterior probabilities ≥0.95 were considered as well-supported (Wilcox *et al.* 2002).

Given the substantially greater coalescence time of nuclear gene sequences compared to mitochondrial genes, we also calculated PEPCK phylogenetic networks (neighbourNet method) using SplitsTree V.4 (for a review of application see Huson & Bryant 2006) with heterozygous characters treated as averaged. Phylogenetic networks allow a more efficient representation when an incomplete lineage sorting occurs.

Sequence-based species delimitation for the DNA taxonomy

Several recent bumblebee taxonomic studies (Williams *et al.* 2012; Lecocq *et al.* 2014) have used sequence-based methods for species delimitation such as the general mixed Yule-coalescent (GMYC) model (Pons *et al.* 2006) or its Bayesian implementation (bGMYC; Reid & Carstens 2012). The GMYC approach delineates species by searching for the transition between a coalescent-type intraspecific genealogy and a Yule-type inter-specific diversification pattern (Yule 1925). While the single-threshold (Pons *et al.* 2006) and multiple-threshold (Monaghan *et al.* 2009) variants of GMYC return species delimitation *per se* and are based on only one ultrametric tree, the bGMYC method returns a pairwise matrix of posterior probabilities that specimens are conspecific and can be based on several distinct ultrametric trees. When these trees are sampled from the same posterior distribution, this latter characteristic allows taking the phylogenetic uncertainty into account. For bGMYC results, the probability that a lineage was conspecific with other lineages was here estimated by reporting ranges of posterior probabilities among sequences from different lineages. These approaches rely on the prediction that independent evolution leads to the appearance of distinct genetic clusters (i.e. monophyly), separated by longer internal branches (Barraclough *et al.* 2003). We applied the single-threshold GMYC, the multiple-threshold GMYC as well as the bGMYC methods to the *B. lapidarius*-group. For bGMYC, a range of probabilities >0.95 was considered as strong evidence that the groups compared were conspecific while a range of probabilities <0.05 strongly suggested that the groups compared was not conspecific (Reid & Carstens 2012). Other probabilities were interpreted as indicating non-significance (i.e. the method was not able to confirm if the specimens were conspecific or not) (Reid & Carstens 2012). GMYC methods all require ultrametric trees (i.e. trees whose tips are all equidistant from the root). We then used BEAST 1.7.4 (Drummond *et al.* 2012) with a phylogenetic clock model to generate a posterior distribution of trees (length of the MCMC chain: 1 billion generations). GMYC and bGMYC analyses were, respectively, conducted with the 'splits' (Ezard *et al.* 2013) and 'bGMYC' (Reid & Carstens 2012) R packages. Single and multiple-threshold GMYC analyses were both based on the mitochondrial consensus tree build with TreeAnnotator v1.8.0 (Drummond *et al.* 2012), discarding 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.

Eco-chemical trait comparative approach

Courtship signals of male bumblebees include both behavioural and chemical features (Baer 2003). Here, we focus on the most studied trait, the CLGS involved in the premating recognition (Ayasse *et al.* 2001; Baer 2003; Ayasse & Jarau 2014). Most bumblebee males patrol along paths (i.e. patrolling behaviour) where they scent-mark objects with their CLGS. Several authors have used the CLGS as chemical markers for resolving species status (e.g. Svensson 1979; Bertsch *et al.* 2005; Lecocq *et al.* 2011). CLGS are species-specific secretions synthesized *de novo* by cephalic labial glands (Žáček *et al.* 2013). CLGS consist of a complex mixture of (mainly aliphatic) compounds, with several main components (Coppée *et al.* 2008; Lecocq *et al.* 2011). By main compounds of a taxon, we mean every compound that has the highest relative amount (RA) within the CLGS at least in one individual.

We extracted the CLGS in 400 μL *n*-hexane following De Meulemeester *et al.* (2011). All samples were stored at −40 °C prior to the analyses. We determined the CLGS composition by gas chromatography-mass spectrometry (GC/MS) and quantified the CLGS composition with gas chromatograph-flame ionization detector (GC/FID). We used a GC/MS Finnigan Focus GC Thermo (Waltham, MA, USA) with a DB-5 ms non-polar capillary column (5%

phenyl (methyl) polysiloxane stationary phase; 30 m × 0.25 mm × 0.25 μm) coupled to Fisons MD 800 quadrupol mass analyser Fisons (Ipswich, UK) with 70 eV electron ionization. We used a GC/FID gas chromatograph Shimadzu GC-2010 with a SLB-5 ms non-polar capillary column (5% diphenyl/95% dimethyl siloxane; 30 m × 0.25 mm × 0.25 μm) and a FID. For both, we used a splitless injection mode (220 °C) and helium as carrier gas (1 mL/min). The temperature program of the column was set to 70 °C for 2 min and then increased at a rate of 10 °C/min to 320 °C. The temperature was then held at 320 °C for 5 min. We identified compounds in Xcalibur™ with their mass spectra compared to those at National Institute of Standards and Technology library (NIST, U.S.A) with NIST MS Search 2.0. We determined the double bond positions (i) from mass spectra of dimethyl disulphide adducts of unsaturated components (Francis 1981) (reaction time: 4 h) and (ii) by chemical ionization with acetonitrile as a reaction gas (Oldham & Svatoš 1999). An ion trap GC/MS instrument Varian (Palo Alto, CA, USA) was used for chemical ionization. We quantified the peak areas of compounds in GCsolution Postrun Shimadzu (Kyoto, Japan) with automatic peak detection and noise measurement. We calculated RA (in %) of compounds in each sample by dividing the peak areas of compounds by the total area of compounds in each sample. We did not use any correction factor to calculate the RA of individual compounds. We discarded all compounds for which RA were recorded as <0.1% for all specimens (De Meulemeester *et al.* 2011). We elaborated the data matrix for each species with the relative proportion of each compound for each individual. We based the data matrix on the alignment of each compound between all samples performed with GCAligner 1.0 (Dellicour & Lecocq 2013a,b). The data matrix is available as supporting materials (Table S3).

We performed statistical comparative analyses of the CLGS of each taxa in R (R Development Core Team 2013) to detect CLGS differentiation among *B. lapidarius* group. We transformed data (log (*x*-1)) to reduce the great difference of abundance between compounds (De Meulemeester *et al.* 2011). We explored the CLGS composition inside the studied group with a clustering analysis performed with UPGMA clustering method on Pearson r Correlation distances matrix (R-package ape, Paradis *et al.* 2004). We assessed the uncertainty in hierarchical cluster analysis with *P*-values calculated via multiscale bootstrap resampling with a bootstrap sample size of 100 000 (R-package pvclust, Suzuki & Shimodaira 2011). We assessed CLGS differentiations between cluster groups by performing a multiple response permutation procedure (MRPP) (R-package vegan, Oksanen *et al.* 2011). The MRPP is a non-parametric, multivariate procedure that tests the null hypothesis of no difference between groups. MRPP has the advantage of not requiring distributional assumptions (such as multivariate normality and homogeneity of variances). To determine compounds specific and regular to each *B. lapidarius* taxa (indicator compounds), we used the indicator value (IndVal) method (Dufrêne & Legendre 1997; Claudet *et al.* 2006; De Meulemeester *et al.* 2011). The value given is the product of relative abundance and relative frequency of occurrence of a compound within a group. We evaluated the statistical significance of a compound as an indicator at the 0.01 level with a randomization procedure.

Results

Wing shape differentiations

The test of correlation between Euclidean and Procrustes distances revealed a regression coefficient higher than 0.99, meaning that the linear tangent space closely approximates the shape space. This allowed us to be confident in the variation amplitude of the dataset. The cluster analysis showed no separation between the different taxa analysed based on inter-individual Procrustes distances (Fig. 1B).

Genetic differentiations

All phylogenetic analyses (ML and MB) performed on the same dataset led to similar tree topologies and to identical relationships between taxa (supplementary tree available at TreeBase: TB2:S16318). Mitochondrial and nuclear datasets produced different topologies (Figs 2 and S2). COI phylogenetic analyses detected nine strongly supported monophyletic groups (Fig. 2A): (i) *alticola* + *cazurroi* + *flavissimus* + *sichelii*, (ii) *erzurumensis*, (iii) *incertus*, (iv) *caucasicus* part 1, (v) *caucasicus* part 2, (vi) *decipiens*-like, (vii) *lapidarius* SE Europe, (viii) *lapidarius* + *decipiens* + *atlanticus* and (ix) the outgroup *B. alagesianus*. PEPCK phylogenetic analyses failed to resolve all taxa in distinct monophyletic groups (Fig. S2). Nevertheless, several taxa display only private haplotypes (haplotypes not shared with other taxa) (Figs 2B and S2): (i) *alticola* + *flavissimus*, (ii) *cazurroi* + *sichelii*, (iii) *erzurumensis*, (iv) *incertus*, (v) *atlanticus*, (vi) *caucasicus*, (vii) *decipiens*, (viii) *decipiens*-like, (ix) *lapidarius* + *lapidarius* SE Europe and (x) the outgroup *B. alagesianus*. The PEPCK phylogenetic network led to a similar pattern with the same groups (Fig. 2B).

Sequence-based species delimitation

Single-threshold GMYC, multiple-threshold GMYC and bGMYC analyses led to different species delimitation. The single-threshold GMYC analysis splits off the consensus tree in several entities that partially recovered the taxa (Fig. 2A): (i) three groups that all included several *alticola*, *cazurroi*, *flavissimus* and *sichelii*, (ii) one group with all

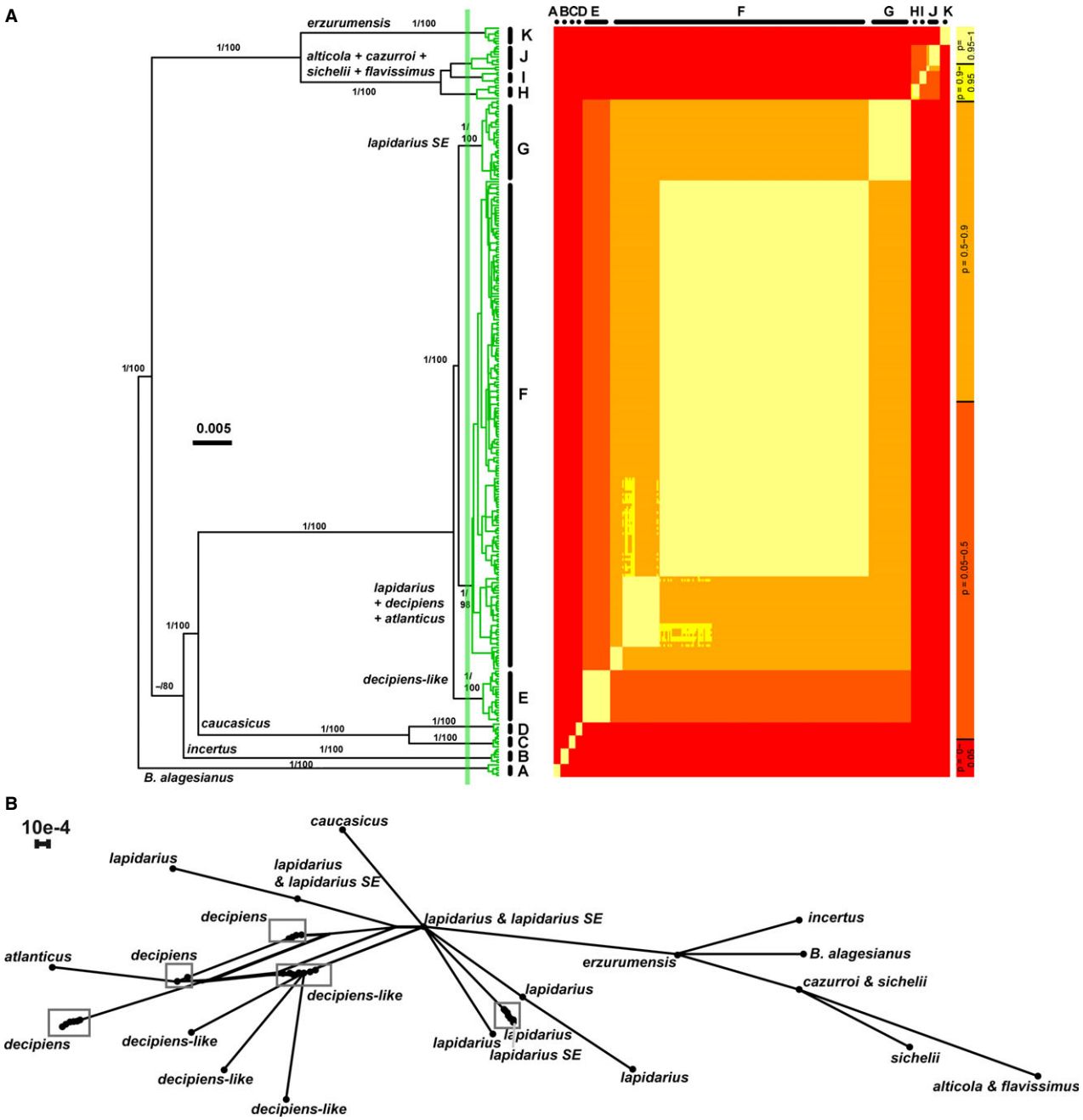


Fig. 2 Phylogenetic, general mixed Yule-coalescent (GMYC) and bGMYC results. —A. Bayesian ultrametric tree based on cytochrome oxidase 1 (COI) sequences with single threshold GMYC model applied and bGMYC pairwise probability of conspecificity. Values above tree branches are Bayesian posterior probabilities/maximum likelihood (ML) bootstrap values. Only posterior probabilities >0.95 and ML bootstrap values >70% are shown. The green branches are entities detected with the single-threshold GMYC method. The adjusted single threshold from the GMYC model is shown by the vertical green bare. The coloured matrix corresponds to the pairwise probabilities of conspecificity returned by the bGMYC method (see also the related colour scale on the right). —B. Phylogenetic network based on phosphoenolpyruvate carboxykinase (PEPCK) data matrix. The scale bar represents the split support for the edges. Dots are haplotypes. Names summarize all individuals from the same taxon included in one haplotype. Grey frames include several closely related haplotypes from the same taxon.

erzurumensis, (iii) one group with all *incertus*, (iv) two groups that all included only *caucasicus*, (v) one group with all *decipiens*-like, (vi) one group with all *lapidarius* from SE Europe, (vii) one group with all *lapidarius*, *decipiens* and *atlanticus* and (viii) the one group for the outgroup *B. alagesianus*. In contrast, the multiple-threshold GMYC analysis identified a very high number of specific entities. Finally, the bGMYC analysis showed fewer entities with low probabilities (<0.05–0) to be conspecific with the other ones (Fig. 2A, Table S4): (i) one group that included all *alticola*, *cazurroi*, *flavissimus* and *sichelii* [bGMYC conspecificity probabilities between individuals included in the group (intragroup probabilities, IP >0.18–1)], (ii) one group with all *erzurumensis* (IP >0.99–1), (iii) one group with all *incertus* (IP >0.99–1), (iv) two groups that all included only *caucasicus* (for each group, IP >0.99–1), (v) one group that included *decipiens*-like, *lapidarius* SE Europe, *lapidarius*, *decipiens*, *atlanticus* (intragroup probabilities: >0.39–1) and (vi) one group for the outgroup *B. alagesianus* (IP >0.99–1).

CLGS differentiation

Seventy-three compounds were detected in the CLGS of studied taxa (Table S3). The cluster analysis of the CLGS revealed six strongly supported (bootstrap >90) groups (Fig. 2C): (i) *alticola* + *cazurroi* + *flavissimus* + *sichelii* + *erzurumensis*, (ii) *incertus* (iii) *caucasicus*, (iv) *decipiens*-like, (v) *lapidarius* + *lapidarius* SE Europe + *decipiens* and (vi) the outgroup *B. alagesianus*. Global MRPP tests confirmed these divergences ($T = 0.23$, A = 0.36, P-value <0.01). Pairwise MRPP confirmed divergences between these groups (Table S5). For each CLGS group, the IndVal method revealed several significantly indicator compounds including main compounds (Table S3).

Discussion

The species delimitation analyses based on wing shape, COI, PEPCK and CLGS lead to quite divergent results (Figs 1–2). All taxa have similar wing shape while most of them display diagnostic COI haplotypes and CLGS. These discrepancies are most probably a consequence of the specific evolution rates/type of each operational criterion (wing shape, COI, PEPCK and CLGS) (see below).

Wing shape as evidence for species delimitation

Clustering based on Procrustes distances between wing shape do not detect an inter-taxa differentiation, even between the outgroup and the ingroup despite that wing shape is recognized as a diagnostic character to discriminate morphologically similar taxa in many insect groups (Hill *et al.* 2012; Schutze *et al.* 2012) including other bumblebee groups (Aytekin *et al.* 2007; Kozmus *et al.* 2011). Nevertheless we used a 'discovery-like approach' (Schlick-Steiner *et al.* 2010) while previous wing shape bee studies used a 'hypothesis-driven approach' (Schlick-Steiner *et al.* 2010) with an *a priori* based on other evidence such as genetic or putative species status (e.g. previous taxonomic revision; Aytekin *et al.* 2007). In this context, the wing shape usefulness as a diagnostic character between closely related bumblebee species should be explored further with a 'hypothesis-driven approach' such as discriminate analyses of wing shape based on *a priori* species status defined by genetics and chemical reproductive traits. Nevertheless, the lack of inter-taxa differentiation observed in our analysis could be explained by the fact that the main variation in wing shape is not always related to species differences. Hypotheses on stronger stabilizing selection on wing shape in particular species groups that minimize the interspecific variations (but see Dockx 2007) should not be avoided. Further studies on the evolution of wing shape using the bumblebee phylogeny are needed to explain this observation. Moreover shape variation in alternative characters could be explored such as those of head or other structure (e.g. Gurgel-Gonçalves *et al.* 2011).

Genetic divergences and DNA sequence-based species delimitations

Monophyly based on molecular data or at least original haplotypes can provide evidence of speciation between taxa (Avise 2000, 2004). These pieces of speciation evidence can be reinforced if there is a concordance of genetic divergence in tree topologies derived from mitochondrial and nuclear genes and if there is a persistence of the genetic differentiation through time despite sympatric distribution (Avise 2004). Nevertheless, the detection of genetic differentiation depends on the variability of the targeted markers that could lead to different tree topologies and thus to conflicting results. Nuclear gene sequences do not resolve closely related species in a distinct monophyletic clade as mitochondrial markers do (also observed in the present study see Figs 2 and S2), presumably due to the substantially greater coalescence time of nuclear genes (Boursot & Bonhomme 1986). In bumblebees, all phylogenetic analyses based on widely used nuclear markers (i.e. PEPCK, nuclear protein-coding genes long-wavelength rhodoposin copy 1, elongation factor-1 alpha F2 copy) have failed to resolve relationships among some groups of closely related taxa commonly recognized as species (Lecocq *et al.* 2013a, 2014). This is most probably a consequence of the recent radiation of bumblebees (near the Miocene–Pliocene boundary; Hines 2008 but see Dehon *et al.* 2014). However, further studies on the variation rate of nuclear markers in the context of species delimitation are needed.

Beside the tree topology incongruence between genes, the determination of objective markers for species

delimitation is difficult because several factors can cause the genealogy from a particular locus to be discordant with the true history of speciation (Maddison 1997; Reid & Carstens 2012). Developing a multilocus approach such as restriction-site-associated DNA sequencing (RAD) to avoid taxonomic conclusions based on few loci whose power to discriminate species may be limited (Cruaud *et al.* 2014). However since such approaches are not yet within an easy reach for all taxonomists, delimiting species approaches based on one single locus such as GMYC and bGMYC remain useful. The GMYC and bGMYC methods allow taking into account the evolutionary theory, the variation in typical levels of intraspecific and interspecific variation among clades, and the substitution rate variation among lineages (Barraclough *et al.* 2009). These methods assume that species are distinct genetic clusters (i.e. monophyly) separated by longer internal branches (Barraclough *et al.* 2009) even if this can be not observed between closely related species (Esselstyn *et al.*, 2012; Zhang *et al.*, 2013; see also an example in bumblebees in Lecocq *et al.* 2014). In the present study, the GMYC and bGMYC analyses lead to different species delimitation (Fig. 2A). This could be at least partially explained by (i) the intrinsic difference between the single and multiple threshold methods (the multiple version allowing independent transition times on different branches of the phylogeny) and also by (ii) the difference between GMYC and bGMYC outputs (i.e. delimitation *per se* vs. a pairwise matrix of posterior probabilities). Regarding the differences between the single and multiple-threshold models, Esselstyn *et al.* (2012) showed that the latter one often overestimates the number of species. For the bGMYC method, we here based our conclusions on two selected significance levels (0.05 and 0.95) but a change of these values will obviously have an impact on the conclusions.

CLGS differentiation

In contrast with genetic markers and wing shape, reproductive traits such as CLGS are under a strong selective pressure to promote a species-specific signal (Andersson 1994; Symonds & Elgar 2007). This explains why the CLGS differentiation partially corroborates the genetic groups ('prospective species' defined by GMYC and bGMYC) observed in our results and in other bumblebee species groups (Lecocq *et al.* 2013a,b, 2014). Genetic differentiated allopatric groups can display similar reproductive traits because (i) they are isolated populations (limited or null gene flow) from the same species (Lecocq *et al.* 2011, 2014) or (ii) they are allopatric species where the very low rate of interspecific miss-mating has not fostered the premating isolation through reproductive trait differentiation (Paterson 1993; Symonds & Elgar 2007; Lecocq *et al.* 2013b). In contrast, group of genetically undifferentiated (in targeted markers) individuals can display local reproductive trait variations (e.g. Clearwater *et al.* 1991; Förschler & Kalko 2007). Indeed, local reproductive trait variations, promoted by selection for specific optimized reproductive traits (Löfstedt 1993; Symonds *et al.* 2009), can appear due to changes in factors that affect communication systems: (i) mutation of genes involved in reproductive traits (Löfstedt 1993), (ii) intraspecific interactions like local preferences of the receivers (Roelofs *et al.* 2002), (iii) the presence/abundance of sympatric species with a similar courtship signal which would result in selection for releasers with the most distinct, optimized reproductive traits (Symonds *et al.* 2009).

The use of CLGS differentiation as species delimitation evidence remains difficult since few ethological studies have showed the consequences of the CLGS differentiation on the species premating recognition (Coppée 2010; Ayasse & Jarau 2014). Nevertheless, the comparison of this semiochemical between closely related bumblebee taxa with a recognized species status suggests that the interspecific differentiation involves its main compounds (e.g. Calam 1969; Rasmont *et al.* 2005; Bertsch & Schweer 2012b). Therefore, these main compound differentiations could be considered as a strong indicator of potential ethological consequences for premating recognition. Further bioassays are needed to allow defining a threshold of species-level differentiation in *Bombus* CLGS, but this requires species-specific year-round rearing methods (Lhomme *et al.* 2012, 2013) that are not available for all species (Hasselrot 1960). Moreover, the usefulness of the CLGS for species delimitation remains definitely limited since (i) few bumblebee species use alternative premating behaviour (i.e. nest waiting behaviour; Bergman & Bergström 1997) and (ii) the CLGS are sex-specific chemical features. Therefore, alternative chemical species-specific signals should be explored such as cuticular hydrocarbons produced by both sexes as a cue involved in the nest mate recognition and used as taxonomical tools in other organisms (Bagnères & Wicker-Thomas 2010).

Integrative decision framework and method limitations

The development of an integrative approach in taxonomy aims to overcome the specific limitations of each single criterion in order to draw a strongly supported taxonomic hypothesis (Schlick-Steiner *et al.* 2010). Our independent analyses on the operational criteria tested here seem to rule out the wing shape as efficient evidence for species delimitation in the *B. lapidarius*-group (see Discussion before). Therefore, an effective integrative decision framework for bumblebee taxa should be based on mitochondrial, nuclear and CLGS evidence according to currently available

criteria. However, since species delimitation approaches based on traditional discrete morphological characters (e.g. colour pattern) are the earliest and the widest method used in most of previous studies (e.g. Løken 1973), because the wing shape geometric morphometric analyses have led to conclusive results in other species groups using more complex exploratory analyses or a 'hypothesis-driven approach' (e.g. Aytekin *et al.* 2007), and because natural selection acts on phenotype (Schlick-Steiner *et al.* 2010), an integrative decision framework should not put aside this evidence (Schlick-Steiner *et al.* 2010).

For each criterion, a threshold of divergence where two taxa can be presumably considered as distinct species must be defined. Establishing a morphological differentiation threshold (e.g. colour pattern or wing shape) to define species remains doubtful since the difficulty to determine objective morphological characters that accurately reflect species (Bickford *et al.* 2007; e.g. colour pattern Carolan *et al.* 2012 but the same issue concerns any other characters, Schlick-Steiner *et al.* 2010). Therefore, any morphological differentiation should be considered as differentiation evidence but not as enough to define species without concordance with other evidence. Similarly, the lack of morphological differentiation should not invalidate a species status (i.e. cryptic species). In genetic traits, the concordance of mitochondrial and nuclear differentiation can be considered as a first piece of evidence for a species status (Avise 2000, 2004). Indeed, taxonomic conclusion only based on mitochondrial marker can lead to false taxonomic status as mitochondrial differentiation may result from sex-specific characteristics (e.g. lower dispersion for females; Kraus *et al.* 2009; Lepais *et al.* 2010 or mtDNA introgression or incomplete lineage sorting). However, the observed different mutation rates between nuclear and mitochondrial markers imply a differential threshold between the types of marker. The empirical observation of interspecific differentiation between commonly recognized bumblebee species suggests that the speciation lead most probably to distinct haplotypes rather than to distinct monophyletic lineage between closely related species (Pedersen 2002; Lecocq *et al.* 2013b, 2014). In contrast, distinct monophyletic mitochondrial groups can reflect speciation processes as well as interpopulational differentiation (Lecocq *et al.* 2011, 2013b; Williams *et al.* 2012); these mitochondrial divergences should be interpreted in the light of objective DNA-based species delimitation methods such as bGMYC and GMYC despite their own limitation (see before). In CLGS, similar composition between taxa can be interpreted as strong evidence of the lack of premating isolation (at least through this reproductive trait) (Lecocq *et al.* 2011; Bertsch & Schweer 2012b). In the opposite, the CLGS differentiation should not be interpreted as evidence of a speciation process without conclusive bioassays (Coppée 2010), except if it is consistent with genetic evidence (Bertsch *et al.* 2005; Lecocq *et al.* 2013a,b).

As species diagnosis is more likely in multiple evidence detection, the species status should be assigned to taxa with a nuclear and mitochondrial differentiation, a status of 'prospect species' according to GMYC and/or bGMYC, and a CLGS differentiation (including main compound divergence). This restrictive approach recognizes only the strongly supported species. This avoids overestimating the species diversity that leads to a taxonomic inflation which is problematic for several fields in biology (e.g. taxonomic inflation making it increasingly difficult to provide funding for conservation; Isaac *et al.* 2004). Moreover, by considering subspecies as a first step in the process of allopatric speciation (Mayr 1942; Patten 2010), assigning subspecies rank to lineages in allopatric ambiguous cases (i.e. where only there are divergence in some operational criteria) can be proposed as a solution (see argumentation in Hawlitschek *et al.* 2012). This procedure allows assigning a taxonomic status to any doubtful bumblebee taxa and points these taxa out for further taxonomic studies.

The accuracy of the integrative approach is not depending on selected features only (see Discussion before) but also on sampling. All modern taxonomic methods based on intra- and interspecific variability comparisons are expected to consider monophyletic groups. Not considering all members of a monophyletic group is especially likely to affect the GMYC and bGMYC results because the method compares branching patterns within and among subgroups (Fujisawa & Barraclough 2013). However obtaining a comprehensive sampling of several individuals of all bumblebee taxa included in one targeted monophyletic group remains most of the time difficult, especially in the context of the worldwide *Bombus* decline (Williams & Osborne 2009). Similarly, limited sampling of a group of taxa makes it difficult to estimate the morphological and CLGS diversity among the group. This places a premium to sample as many individuals as possible. However, since the sampling of common species is more likely than uncommon ones, this leads to a significant oversampling of some taxa that could blur statistical analyses (e.g. principal component analysis). This issue can be solved by subtracting a part of oversampled taxa but means losing information. Therefore, all sampling effects should be taken into account in taxonomic conclusion.

Taxonomic implications

The concordance of the nuclear and mitochondrial divergence, the species status according to GMYC and bGMYC, and the observed CLGS differentiation strongly suggest that our dataset included five species: *B. alagesianus*

Reinig, 1930 (the outgroup), *B. caucasicus* Radoszkowski, 1859, *B. incertus* Morawitz 1881, *B. lapidarius* (L.) and *B. sichelii* Radoszkowski 1860. With the exception of *B. caucasicus*, these species delimitations are congruent with current bumblebee taxonomy (Williams 1998 update at NHM: www.nhm.ac.uk/research-curation/research/projects/bombus/). We have sampled most of the taxa included in *B. lapidarius* group but we have failed to collect *B. ladakhensis*, *B. semenovianus* and *B. tanguticus* (Williams 1998 update at NHN). However, we speculate that limited sampling did not significantly affect our results as these species are considered as very morphologically distinct from our ingroup (Williams 1998 update at NHN).

Our results strongly suggest that *erzurumensis*, *alticola*, *cazurroi*, *flavissimus* and *sichelii* are conspecific and included in *B. sichelii* Radoszkowski 1860 (oldest available name). The *erzurumensis* and *B. sichelii* have been previously considered as conspecificity by Williams (1998) despite the large phenotypic differentiation in colour pattern (Fig. 1A) between *erzurumensis* and *cazurroi* (i.e. previously considered as the Turkish subspecies of *B. sichelii*). According to the conspecificity suggested by our analyses, this colour pattern differentiation could be regarded as a local intraspecific dimorphism as observed in other bumblebee species (Rasmont *et al.* 2005; De Meulemeester *et al.* 2011; Williams *et al.* 2013). Nevertheless, the *erzurumensis* displays specific COI haplotypes while *cazurroi* shares its haplotypes with other *B. sichelii* taxa. Therefore, an alternative hypothesis could be that *erzurumensis* would be an old lineage previously isolated in Anatolia prior to colonization of the region by *cazurroi* (i.e. the two taxa are barely sympatric; Rasmont & Flagothier 1996). For other *B. sichelii* taxa, our integrative decision framework does not support that *alticola*, *flavissimus* and *sichelii* deserve a subspecies status (lack of genetic and CLGS differentiation. However, most of these allopatric taxa display obvious specific phenotype (i.e. specific colour pattern used in traditional morphology) that suggests differentiation and specific characters. Therefore the subspecies status of these taxa should be maintained awaiting further studies based on a larger set of characters (i.e. whole genome, alternative ecological character, Fig. 1A). In order to be conservative in taxonomic status, we currently consider that *B. sichelii* included five subspecies: *B. sichelii alticola*, *B. sichelii cazurroi*, *B. sichelii erzurumensis*, *B. sichelii flavissimus* and *B. sichelii sichelii*.

The results of our integrative decision framework suggest that *B. lapidarius* and *B. caucasicus* Radoszkowski 1859 (resurrected species status) should be considered as distinct species. This is conflicting with most previous morphological studies (Reinig 1935; Tkalců 1960) but agrees with the species status assigned by the taxon descriptor (Radoszkowski 1960). *Bombus lapidarius eriophorus* (not sampled here) and *B. caucasicus* have been considered as two forms of the same taxon by Reinig (1935) while Rasmont (1983) regarded them as two different taxa. If *B. lapidarius eriophorus* and *B. caucasicus* are to be considered conspecific, *B. eriophorus* (Klug, 1807) would be the oldest available name for the species. Further analyses on *B. lapidarius eriophorus* and *B. caucasicus* are needed to assess their conspecificity.

According to the differentiation in mitochondrial and nuclear markers, the subspecies status was assigned to *atlanticus*, *decipiens* and *lapidarius*, respectively, *B. lapidarius atlanticus* Benoist, 1928, *B. lapidarius decipiens* Pérez, 1890 and *B. lapidarius lapidarius* (L.) (Table 1). The *lapidarius* SE Europe was regarded as consubspecific with *B. lapidarius lapidarius* according to the lack of nuclear and phenotypic differentiation (Table 1). The mitochondrial and nuclear differentiation, the species status according the GMYC methods, and the CLGS differentiation of *decipiens*-like suggest that this taxon deserved a species status while bGMYC analysis suggest a subspecies status within *B. lapidarius*. The potential species status of *decipiens*-like (from S. Italy and Sicily) still needs to be explored in further analyses since there is an obvious lack of gene flow with *B. lapidarius* in sympatry (Lecocq *et al.* 2013a).

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Table 1 Comparison of alternative criteria used for species delimitation on bumblebees

			Genetic analyses						
			Private haplotypes		Phylogenetic analyses		Sequence-based species delimitations		
Taxa	Colour pattern	Wing shape	COI	PEPCK	COI	PEPCK	GMYC	bGMYC	CLGS
erzurumensis	+	– [1]	+	+	+	– [1]	+	+	– [1]
alticola	+	– [1]	– [1]	– [1]	– [1]	– [1]	– [1]	– [1]	– [1]
cazurroi	+	– [1]	– [1]	– [2]	– [1]	– [1]	– [1]	– [1]	– [1]
flavissimus	+	– [1]	– [1]	– [1]	– [1]	– [1]	– [1]	– [1]	– [1]
sichelii	+	– [1]	– [1]	– [2]	– [1]	– [1]	– [1]	– [1]	– [1]
incertus	+	– [1]	+	+	+	– [1]	+	+	+
caucasicus	+	– [1]	+	+	+	– [1]	+	+	+
decipiens-like	– [1]	– [1]	+	+	+	– [1]	+	– [2]	+
decipiens	– [1]	– [1]	+	+	– [1]	– [1]	– [2]	– [2]	– [2]
atlanticus	+	– [1]	+	+	– [1]	– [1]	– [2]	– [2]	?
lapidarius SE Europe	– [2]	– [1]	+	– [3]	+	– [1]	+	– [2]	– [2]
lapidarius	– [2]	– [1]	+	– [3]	– [1]	– [1]	– [2]	– [2]	– [2]
B. alagesianus	+	– [1]	+	+	+	– [1]	+	+	+

PEPCK, phosphoenolpyruvate carboxykinase; GMYC, general mixed Yule-coalescent; COI, cytochrome oxidase 1; CLGS, cephalic labial gland secretion; bGMYC, Bayesian implementation.

Wing Shape indicates if a taxon has a diagnostic wing shape (+/- means that wing shape is/is not diagnostic. When the wing shape is not diagnostic, the numbers group together taxa that share a similar wing shape). Private haplotypes indicates 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 numbers group together taxa that share haplotypes). Phylogenetic analyses indicates if a taxon forms a monophyletic group strongly supported and distinct from other taxa (+/- means that the taxon is/is not a monophyletic group. When the taxon is not a distinct monophyletic group, the numbers group together taxa included in the same monophyletic group). Sequence-based species delimitations indicates if a taxon is a prospective species according to GMYC or bGMYC analyses (+/- means that the taxon is/is not a prospective species. When the taxon is not regarded as a prospective species, the numbers group together taxa recognized as conspecific according to GMYC or bGMYC methods). CLGS indicates if the taxon has/has not specific composition of CLGSs (+/- means that the taxon has/has not a specific CLGS composition. When the taxon shares CLGS composition with other ones, the numbers group together taxa that share similar CLGS. ? means that the CLGS composition is unknown).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Right forewing of *B. lapidarius lapidarius* male with 18 landmarks.

Fig. S2. Majority rule (50%) consensus tree based on Bayesian analyses based on PEPCK data matrix.

Table S1. Table of sampling.

Table S2. Wing shape data matrix.

Table S3. Data matrix of cephalic labial gland secretions (relative amounts of each compound) and list of the identified compounds in the *B. lapidarius* complex.

Table S4. Results of the bGMYC analysis (pairwise table).

Table S5. The results of multiple response permutation procedure test between each group.