

Highly polytypic taxon complex: interspecific and intraspecific integrative taxonomic assessment of the widespread pollinator *Bombus pascuorum* Scopoli 1763 (Hymenoptera: Apidae)

THOMAS LECOQC¹, NICOLAS BRASERO¹, BAPTISTE MARTINET¹, IRENA VALTEROVÁ² and PIERRE RASMONT¹

¹Laboratory of Zoology, Research Institute of Biosciences, University of Mons, Mons, Belgium and ²Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Abstract. The recent development of the integrative taxonomic approach in bumblebees has led to unexpected merging or splitting of several taxa. Here we investigate the taxonomic statuses of one of the most abundant, widespread and polytypic Palearctic bumblebees, *Bombus pascuorum*. The latest review of this species includes 24 subspecies. We used an integrative approach based on genetic markers and male chemical reproductive traits and compared our results with the former classifications. Our results show that all *B. pascuorum* taxa are conspecific and share the same male chemical reproductive traits. The genetic structure observed in one mitochondrial and two nuclear markers poorly reflects the current subspecific classification. Indeed, the concordance of population genetic differentiation, population geographic distribution, and population colour pattern similarity suggests a different meaningful prospective classification with four taxon complexes: (i) the *B. pascuorum dusmeti* group, including all taxa from the Iberian Peninsula and south-west France; (ii) *B. pascuorum rehbinderi*; (iii) *B. pascuorum siciliensis*; and (iv) *B. pascuorum floralis* groups (including all other taxa studied here).

Introduction

The species concept is central to biodiversity classification but remains debated (Mayr, 1942; De Queiroz, 2007). Indeed, the delimitation of species boundaries is a fundamental aspect in evolutionary biology (Lecocq *et al.*, 2013a,b) and conservation biology (Whittaker & Fernández-Palacios, 2007). However, establishing taxonomic boundaries between closely related species is often hindered by a lack of diagnostic morphological characters (Bickford *et al.*, 2007). Bumblebee (genus *Bombus* Latreille) taxonomy exemplifies this issue: different species can be morphologically similar (i.e. cryptic species) while conspecific specimens can be extraordinarily divergent, e.g. in their hair body's colour patterns (Michener, 1990; Williams, 1998; Hines & Williams, 2012). As in other taxonomically confusing

groups, there have been many attempts to clarify bumblebee taxonomy using alternative features to complement traditional morphological characters such as geometric morphometrics of wing shape, DNA or ecochemical traits (reviewed by Lecocq *et al.*, 2015c). Nevertheless, each approach has its own limitations (reviewed by Lecocq *et al.*, 2015a,c). Using a multisource approach to gather different lines of evidence of speciation in order to draw strongly supported species status hypotheses has been demonstrated to be a solution to overcome the limitations of each method (De Queiroz, 2007; Schlick-Steiner *et al.*, 2010). This integrative taxonomic approach has become widely used in bumblebee systematics (Bertsch, 1997; Bertsch *et al.*, 2005; Lecocq *et al.*, 2011, 2015a,c). Although previous studies have confirmed many expected taxonomic statuses, this integrative approach also has led to unexpected merging or splitting of several taxa (Bertsch *et al.*, 2005; Lecocq *et al.*, 2011, 2015a,c). These studies question the current bumblebee systematics mainly based on few criteria (i.e. genetic marker) or on traits (i.e. colour pattern or wing shape) that have been recently

Correspondence: Thomas Lecocq, Laboratory of Zoology, Research Institute of Biosciences, University of Mons, Place du Parc 20, 7000 Mons, Belgium. E-mail: thomas.lecocq@umons.ac.be



Fig. 1. Eight *Bombus pascuorum* subspecies: (A) *Bombus pascuorum floralis* (male); (B) *Bombus pascuorum gotlandicus* (male); (C) *Bombus pascuorum intermedius* (male); (D) *Bombus pascuorum melleofacies* (worker); (E) *Bombus pascuorum mniorum* (male); (F) *Bombus pascuorum rehbinderi* (male); (G) *Bombus pascuorum rufocitrinus* (worker); (H) *Bombus pascuorum septentrionalis* (male). All photographs are by P. Rasmont except for *B. p. rufocitrinus*, which is by D. Genoud.

showed as inefficient as taxonomic diagnostic characters (Carolan *et al.*, 2012; Lecocq *et al.*, 2015c). This places a premium on taxonomic status assessments of closely related taxa as well as of polytypic species within the bumblebee genus.

Here, we investigate the taxonomy of one of the most abundant, widespread and polytypic Palearctic bumblebee species (Rasmont *et al.*, 2015) known to be an important pollinator in Europe (Free, 1993): *Bombus pascuorum* Scopoli. The species displays a considerable coat colour variation (Fig. 1) (Krüger, 1928, 1931; Reinig, 1970; De Ruijter & Wiebes, 1974; Rasmont, 1983), a morphological differentiation (Løken, 1973; Pekkariinen, 1979) and a slight genetic differentiation (Pirounakis *et al.*, 1998; Widmer & Schmid-Hempel, 1999) within its geographic

range. Similar variation patterns have been observed in other species such as *B. lapidarius* or *B. terrestris* recently resolved as species complexes (Lecocq *et al.*, 2015a,c). Based on colour patterns, the last taxonomic revisions defined three allopatric subspecies and 21 parapatric subspecies (Fig. 2) (Rasmont, 1983; Rasmont & Adamski, 1995) (*B. p.*, *Bombus pascuorum*): *B. p. bofilli* (Vogt) (southern Iberian Peninsula), *B. p. dusmeti* (Vogt) (centre of the Iberian Peninsula), *B. p. flavobarbatus* (Morawitz) (east Siberia), *B. p. floralis* (Gmelin) (central and eastern Europe, Ireland and west Russia), *B. p. freygessneri* (Vogt) (central France), *B. p. gotlandicus* (Erlandsson) (Gotland), *B. p. intermedius* (Vogt) (south-west Alps), *B. p. maculatus* (Vogt) (western Pyrenees and south-west France), *B. p.*

Sampled taxa

<i>B. p. dusmeti</i>	<i>B. p. flavobarbatus</i>	<i>B. p. floralis</i>	<i>B. p. freygessneri</i>	<i>B. p. gotlandicus</i>	<i>B. p. intermedius</i>	<i>B. p. maculatus</i>	<i>B. p. melleofacies</i>	<i>B. p. michnoi</i>	<i>B. p. mniorum</i>
<i>B. p. moorseleensis</i>	<i>B. p. olympicus</i>	<i>B. p. pallidofacies</i>	<i>B. p. pascuorum</i>	<i>B. p. rehbinderi</i>	<i>B. p. rufocitrinus</i>	<i>B. p. septentrionalis</i>	<i>B. p. siciliensis</i>	<i>B. p. smithianus</i>	<i>B. p. sparreanus</i>
									<i>B. p. vulgo</i>

Nonsampled

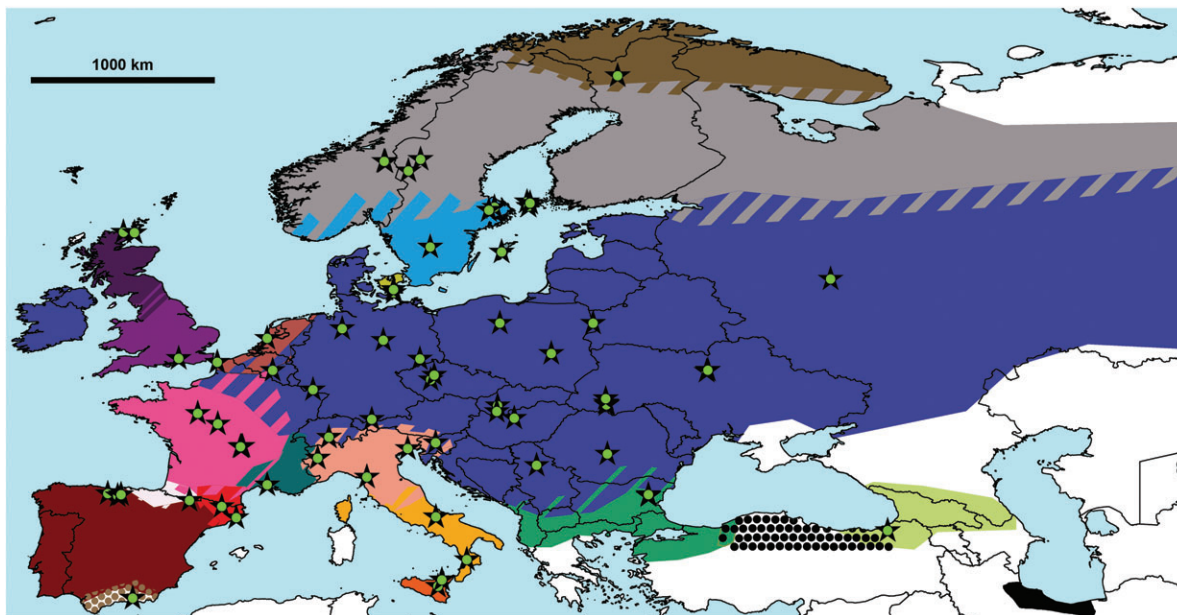
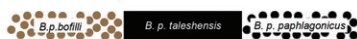


Fig. 2. Distribution and sampling map of *Bombus pascuorum* and its subspecies in Europe and surrounding regions based on traditional subspecies classification by Rasmont (1983). The approximate distribution of each subspecies and sympatric areas (hatched areas) between parapatric subspecies are shown according to the literature (Rasmont, 1983; Rasmont *et al.*, 2015). The green and black stars are the sampling sites.

melleofacies (Vogt) (centre and south of Italian Peninsula and Corsica), *B. p. michnoi* (Skorikov) (South Siberia), *B. p. mniorum* (Fabricius) (Sjaelland), *B. p. moorseleensis* (Ball) (northern Belgium and western Netherlands), *B. p. olympicus* (Vogt) (southern Balkan region), *B. p. pallidofacies* (Vogt) (southern Scandinavia), *B. p. paphlagonicus* (Reinig) (north-west Anatolia), *B. p. pascuorum* Scopoli (northern Italian Peninsula), *B. p. rehbinderi* (Vogt) (Crimea, north-east Anatolia, Georgia, and Caucasus), *B. p. rufocitrinus* (Krüger) (eastern Pyrenees), *B. p. septentrionalis* (Vogt) (northern part of Great Britain), *B. p. siciliensis* Tkalčú (Sicily), *B. p. smithianus* (White) (north of Fennoscandia), *B. p. sparreanus* (Löken) (central Fennoscandia), *B. p. taleshensis* Rasmont (Iran), and *B. p. vulgo* (Harris) (southern Great Britain). In this paper, we aim to assess the traditional *B. pascuorum* taxa classification with statuses and groups defined by an integrative taxonomy approach based on genetic markers and chemical reproductive traits (Lecocq *et al.*, 2015a).

Material and methods

Sampling

A total of 276 specimens were collected in the whole Palearctic region (Fig. 2; Table S1). We aimed to sample all

B. pascuorum taxa. However, we failed to collect *B. p. bofilli*, *B. p. paphlagonicus*, and *B. p. taleshensis* (Fig. 2). In southern Spain (i.e. Sierra Nevada) where *B. p. bofilli* should occur (Rasmont, 1983), we found only *B. p. dusmeti*. For subspecies with a large distribution (e.g. *B. p. floralis*), several populations were sampled. We used the phylogenetically closely related species *Bombus humilis*, *Bombus ruderarius*, and *Bombus sylvarum* as outgroups (Pedersen, 2002; Cameron *et al.*, 2007). We killed specimens by freezing at -20°C .

Genetic divergence

We sequenced three genes commonly used in bee phylogenetic studies (Pedersen, 2002; Cameron *et al.*, 2007; Hines, 2008; Lecocq *et al.*, 2013a,b; Dellicour *et al.*, 2014): mitochondrial cytochrome oxidase 1 (COI), nuclear protein-coding gene elongation factor-1 alpha, F2 copy (EF-1 α), and phosphoenolpyruvate carboxykinase (PEPCK). We followed the total DNA extraction protocol, polymerase chain reaction amplification reactions, sequencing procedures, and alignment method described in Lecocq *et al.* (2013a). We carried out haplotype network analyses and phylogenetic analyses to investigate the genetic differentiation as well as to define lineages

within *B. pascuorum* taxa. Sequences were deposited in GenBank (Table S1) and matrices were deposited on TreeBase (TB2:S17344).

Median-joining haplotype network analyses were carried out in NETWORK 4.6.1.0 (www.fluxus-engineering.com) for each gene individually. The median joining method uses a maximum parsimony approach to search for all the shortest phylogenetic trees for a given data set (Bandelt *et al.*, 1999). To reconstruct the network, we weighted transversions twice as high as transitions.

In phylogenetic analyses, we analysed each gene independently with maximum likelihood (ML) and Bayesian (MB) methods. For both methods, we partitioned each gene to explore the best substitution model: (i) EF-1 α into two exons and one intron; (ii) PEPCK into two exons and two introns; and (iii) COI and each nuclear exon by base positions (first, second and third). The best-fitting substitution models were chosen with JMODELTEST using the corrected Akaike information criterion (AICc) for each dataset. Secondly, we analysed all genes combined in ML and MB phylogenetic analyses in order to define the lineages within *B. pascuorum*. We assessed the level of incongruence in phylogenetic reconstructions among genes by comparing congruence and incongruence of well-supported clades across ML and MB trees of each gene. Overall, the gene trees were not strongly conflicting with each other and well supported clades were similar across all trees. In all phylogenetic analyses, trees were rooted with species from the outgroup. We conducted ML analyses with GARLI 2.0 (Zwickl, 2006). We performed 10 independent runs in GARLI for each gene; the topology and $-\ln L$ were identical among replicates. The run with the highest likelihood was retained. We evaluated statistical confidence in nodes with 10 000 nonparametric bootstrap replicates. Topologies with bootstrap values $\geq 70\%$ were considered well supported (Hillis & Bull, 1993). We performed MB analyses with MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). We carried out 10 independent analyses for each gene (100 million generations, four chains with mixed models, default priors, saving trees every 100 generations). Then we discarded the first 10 million generations 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).

Reproductive trait divergence

We analysed the male cephalic labial gland secretion (CLGS) involved in the bumblebee premating recognition (Ayasse & Jarau, 2014; Lecocq *et al.*, 2015b) and commonly used for species discrimination in bumblebees (Rasmont *et al.*, 2005; Bertsch & Schweer, 2012). CLGSs are species-specific secretions synthesized *de novo* by cephalic labial glands (Žáček *et al.*, 2013). CLGSs consist of a complex mixture of (mainly aliphatic) compounds, with several main components (Coppée *et al.*, 2008; Lecocq *et al.*, 2011). By main compounds, we mean compounds that have the highest relative proportion among all compounds of CLGSs at least in one individual of the taxa.

We extracted the CLGSs in 400 μ L n-hexane (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 on an quadrupol Finigan GCQ, with a DB-5 ms nonpolar capillary column [5% phenyl(methyl)polysiloxane stationary phase; column length 30 m; inner diameter 0.25 mm; film thickness 0.25 μ m]. We quantified the CLGS composition with a gas chromatograph Shimadzu GC-2010 with a SLB-5 ms nonpolar capillary column (same characteristic as the DB-5 ms nonpolar capillary column) and a flame ionization detector. For both, we injected 1 μ L, 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 $10^{\circ}\text{C}/\text{min}$ to 320°C . The temperature was then held at 320°C for 5 min. Compounds were identified using the retention times and mass spectra of each peak, in comparison to those at National Institute of Standards and Technology library (NIST, U.S.A.). Double bond positions were determined by dimethyl disulfide (DMDS) derivatization. We quantified the peak areas of compounds in GCsolution Postrun (Shimadzu Corporation) with automatic peak detection and noise measurement. We calculated relative amounts (RAs, 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 less than 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 (Table S2) on the alignment of each compound between all samples performed with GCaligner 1.0 (Dellicour & Lecocq 2013a,b).

We performed statistical comparative analyses of the CLGSs in R (R Development Core Team, 2013) to detect CLGS differentiations among *B. pascuorum*. We transformed data [$\log(x + 1)$] to reduce the great difference of abundance between compounds with highly and low concentration (De Meulemeester *et al.*, 2011). We compared the CLGS composition between individuals with a clustering analysis performed with the unweighted pair group method and the arithmetic mean based on Pearson r correlation distance matrices (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 taxa by performing a multiple response permutation procedure (MRPP) (R package VEGAN, Oksanen *et al.*, 2011).

Data integration and decision framework

Assuming that species diagnosis is more likely in multiple evidence detection (De Queiroz, 2007; Schlick-Steiner *et al.*, 2010), we based our species delimitation on genetic and ecochemical divergences considered as efficient diagnostic characters among bumblebees (Lecocq *et al.*, 2015c). Integration of different lines

of evidence in bumblebee taxonomy has recently been the focus of intensive research (reviewed by Lecocq *et al.*, 2015a,c). We considered that a taxon deserved a species status with a high degree of certainty if the taxon: (i) was genetically differentiated in all genetic markers; (ii) constituted a monophyletic group; (iii) is significantly differentiated in CLGS compositions; and (iv) is differentiated in the main CLGS compounds (see discussion in Lecocq *et al.*, 2015a).

Results

Totals of 1069 bp from COI, 789 bp from EF-1 α , and 928 bp from PEPCK were obtained (GenBank numbers are in Table S1). Our genetic analyses showed an expected divergence between outgroup and ingroup. Within *B. pascuorum* taxa, 50 unique haplotypes were observed for COI, 21 for EF-1 α and 21 for PEPCK among 250 Palearctic individuals (Fig. 3A–C). Only *Bombus pascuorum siciliensis* and *B. p. rehbinderi* displayed specific haplotypes in COI and PEPCK, while *B. p. rufocitrinus*, *B. p. gotlandicus*, and *B. p. smithianus* had specific COI haplotypes (Fig. 3A–C). Our genetic markers did not show that all other taxa had specific haplotypes, but some taxa from the same part of the *B. pascuorum* distribution shared specific haplotypes (Fig. 3A–C). Indeed, all taxa from Iberian Peninsula and southern/south-western France shared haplotypes in all genetic markers (Fig. 3A–C). Similarly, all taxa from the Italian Peninsula (*B. p. pascuorum* and *B. p. mellofacies*) displayed the same COI haplotypes (but shared one haplotype with other taxa; Fig. 3A–C) and all *B. p. olympicus* and south-eastern European *B. p. floralis* displayed the same COI haplotypes (Fig. 3A–C). For COI and PEPCK sequences, same haplotypes were found in *B. p. floralis* from Russia and *B. p. flavobarbatus* and *B. p. michnoi* from Siberia (Fig. 3A–C). All phylogenetic analyses (ML and MB) of each single gene and the combined dataset showed a partially similar topology with clades corresponding to haplotype groups found in the networks. Within *B. pascuorum* gene phylogenies, the relationships between taxa remained poorly resolved (see supplementary trees at TreeBase TB2:S17344). Nevertheless, phylogenetic analyses on COI, PEPCK and the three-gene combined dataset showed two main lineages within *B. pascuorum* (Fig. 3D and supplementary trees at TreeBase TB2:S17344): the south-western lineage (all subspecies from the Iberian Peninsula and south-west France) and the main lineage (all other subspecies). Within the main lineage, some geographic subgroups appeared (Fig. 3D): (i) the eastern group (*B. p. floralis* from Russia and *B. p. flavobarbatus* and *B. p. michnoi* from Siberia); (ii) the Sicily group (*B. p. siciliensis*); (iii) the eastern Turkey and Caucasus group (*B. p. rehbinderi*), (iv) the south-eastern Europe group (most of *B. p. floralis* from south-eastern Europe and most of *B. p. olympicus*).

In the chemical analyses, 56 compounds were detected in the CLGSs of *B. pascuorum* taxa (Table S2). The CLGS composition of *B. pascuorum* taxa were clearly different from those of outgroup species [comparison with Appelgren *et al.* (1991) and Terzo *et al.* (2005)]. In contrast, all *B. pascuorum* taxa shared all compounds. No discrimination between taxa was

found in the statistical analyses (Fig. 4; MRPP result, $T = 11.09$, $A = -0.29$, $P = 0.85$).

The concordance of the nuclear and mitochondrial divergence and the observed CLGS differentiation strongly confirmed the differentiation between *B. pascuorum* taxa and outgroup species (Figs 3, 4). The same decision framework strongly suggested that *B. pascuorum* taxa are conspecific, as they shared the same CLGS composition and only a few of them displayed a genetic differentiation (only private haplotypes) (Figs 3, 4).

Discussion

Species status

Our integrative taxonomic decision framework supported the specific status of *B. pascuorum* compared with its closely related consubgeneric species as commonly acknowledged in the literature (Williams, 1998; Rasmont *et al.*, 2000; Kuhlmann *et al.*, 2014); similar interspecific genetic and CLGS differentiations were previously recorded in the literature (Appelgren *et al.*, 1991; Pedersen, 2002; Terzo *et al.*, 2005; Cameron *et al.*, 2007; Hines, 2008). Within *B. pascuorum*, despite their conspicuous phenotypic differentiations, all studied taxa were regarded as conspecific based on diagnostic characters (Figs 3, 4). Therefore, contrary to some other polytypic groups of Palearctic taxa resolved as species complexes, such as *B. terrestris* and *B. lapidarius* (Lecocq *et al.*, 2013b, 2015a,c), *B. pascuorum* appears as a single species with a high geographic phenotypic differentiation. Nevertheless, our analyses revealed several differentiated groups of populations that lack of CLGS differentiation and with a low genetic differentiation (Figs 3, 4).

Intraspecific structure

The concordance of population genetic differentiation, population geographic distribution, and population colour pattern similarity suggests an intraspecific structure (Figs 1–3) where south-western European taxa (Iberian Peninsula and southern/south-western France) and other *B. pascuorum* taxa (main lineage) constitute two differentiated lineages (Fig. 3D). The main lineage includes four conspicuous subgroups: *B. p. siciliensis*, *B. p. rehbinderi*, eastern populations/taxa (*B. p. floralis* from Russia and *B. p. flavobarbatus* and *B. p. michnoi* from Siberia), and most of south-eastern European populations/taxa (Fig. 3D). The geographic distribution of these lineages and subgroups could suggest a phylogeographic structure within *B. pascuorum* fostered by allopatric differentiation between Ice Age refugia (i.e. Iberian Peninsula, Italian Peninsula, cryptic refugia within mainland; see Lecocq *et al.*, 2013a) or insular genetic drift (e.g. Lecocq *et al.*, 2013b); further phylogeographic studies are needed to assess this phylogeographic structure.

The observed lineages and subgroups poorly reflect the current *B. pascuorum* subspecific classification (Fig. 3D; Rasmont, 1983; Rasmont & Adamski, 1995). As the subspecies has been traditionally defined as a population group occurring

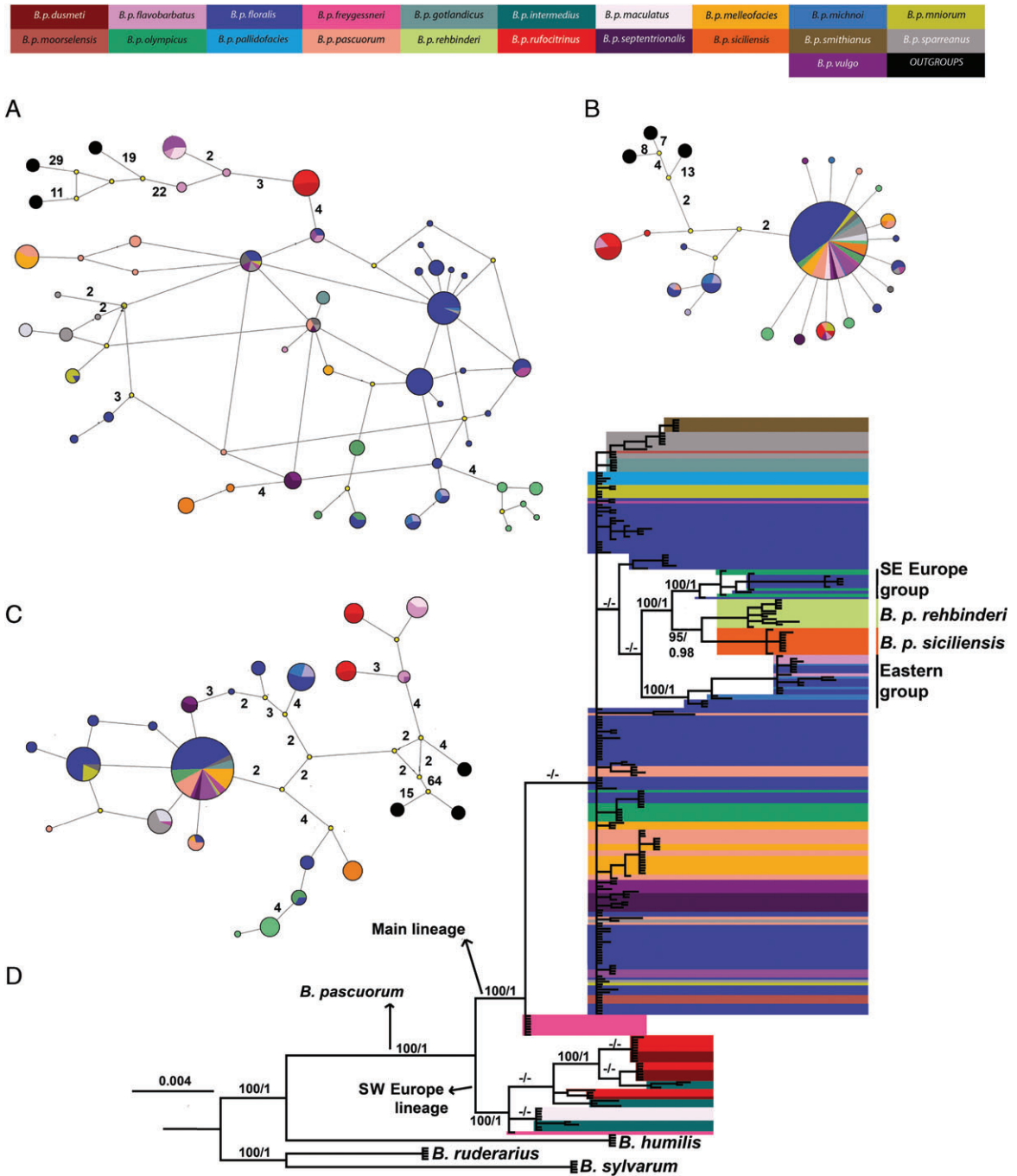


Fig. 3. Genetic differentiations within *Bombus pascuorum*. The colour chart given at the top edge of the figure is used in all subparts of the figure and refers to the traditional subspecies classification (see Rasmont, 1983). (A) Median-joining network of haplotypes based on cytochrome oxidase I (COI) sequences. (B) Median-joining network of haplotypes based on EF-1α sequences. (C) Median-joining network of haplotypes based on PEPCK sequences. For all median-joining networks: the sizes of the circles are proportional to the frequencies of haplotypes; the yellow points on the lines represent undetected/extinct intermediate haplotype states; the numbers on the lines represent the number of mutations between two close haplotypes (only numbers of mutation > 1 are shown). (D) Majority rule (50%) consensus tree based on Bayesian analyses of combined molecular data matrix (COI, EF-1α, and PEPCK). Values above tree branches are maximum likelihood bootstrap values/Bayesian posterior probabilities. Only maximum likelihood bootstrap values > 70% and posterior probabilities > 0.95 are shown. SE, south-eastern; SW, south-western.

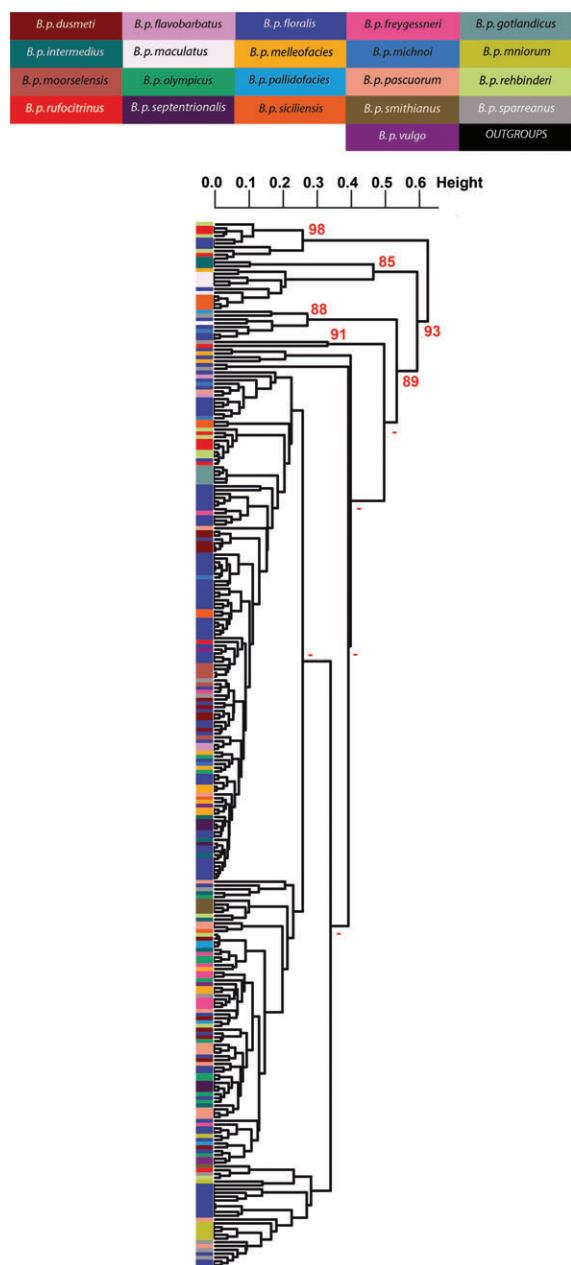


Fig. 4. Cephalic labial gland secretion differentiation within *Bombus pascuorum*. Colour chart given at the top edge of the figure refer to the traditional subspecies classification (see Rasmont, 1983). The cluster is an unweighted pair group method with arithmetic mean (UPGMA) cluster based on a correlation matrix calculated from the cephalic labial gland secretion matrix. The values near nodes are multiscale bootstrap resampling values (only values > 80 of main groups are shown).

in a distinct geographic area and phenotypically diagnosably distinct from other conspecific population groups (Mayr, 1942, 1963; Patten & Unitt, 2002), the observed discordances are presumably due to *a priori* phenotypically defined subspecies reflecting local adaptation to a particular environment rather than genetic differentiation in a neutral marker (Avice, 2000;

Frankham *et al.*, 2010; Braby *et al.*, 2012; Lecocq *et al.*, 2013a). Alternatively, the traditional bumblebee subspecies classification is based on an *a priori* classification reflecting colour pattern variation (e.g. for *B. pascuorum*: Krüger, 1928, 1931; Rasmont, 1983) but the definition of discrete diagnostic colour pattern that reflects the differentiation process requires a difficult to achieve extensive overview of the interindividual variability, making subspecies classifications dubious (Bertsch & Schweer, 2012). Taking these limitations into account, the usefulness of subspecies status in the bumblebee has been criticized during recent decades (Williams, 1991; Bertsch & Schweer, 2012), although other researchers have continued to consider the subspecies as a useful pragmatic taxonomical unit (Rasmont *et al.*, 2008; Lecocq *et al.*, 2015a), especially for evolutionary biology and biological conservation (see similar statements in other organisms: Phillimore & Owens, 2006; Allentoft & O'Brien, 2010; Crowhurst *et al.*, 2011; Hawlitschek *et al.*, 2012; Sackett *et al.*, 2014) because the subspecies can be seen as providing a handle for identifiable units of geographic variation and, therefore, frequently acting as an impetus for further investigations (Patten, 2009). This divergence of opinion repeats on a smaller scale the extensive long-standing debate on the subspecies' usefulness in the living world (Ebach & Williams, 2009; Patten, 2009). Assuming the subspecies' usefulness, some authors tried to develop efficient subspecies definitions that reconcile genetic structure/divergence and geographical distribution of phenotypic character, especially in butterflies, reptiles, birds, or even bacteria (Manier, 2004; Mulcahy, 2008; Tsao & Yeh, 2008; Hawlitschek *et al.*, 2012; Ennen *et al.*, 2014; Maia-Carvalho *et al.*, 2014). Under the unified species concept (De Queiroz, 2007) and Mayr's assumption that the subspecies reflect a step in the allopatric speciation process (Mayr, 1963), subspecies can be defined as a group of populations representing partially isolated lineages within a species that is allopatric and phenotypically diagnosably distinct from other conspecific population groups (Braby *et al.*, 2012; Hawlitschek *et al.*, 2012). In the context of integrative taxonomy, the subspecies' status has been assigned to phenotypically distinct allopatric groups of populations with divergences in some but not all criteria used in the integrative decision framework for species delimitation (i.e. disagreement in selected criteria in an integrative species delimitation framework that could suggest an incipient speciation; see discussion in Hawlitschek *et al.*, 2012; Lecocq *et al.*, 2015a,c). In *B. pascuorum*, as most of the lineages and subgroups display a parapatric distribution (Fig. 2) and a poorly fixed diagnosable phenotypic character state (i.e. there is a large colour pattern variation within lineages), such a subspecies delimitation could lead to merging of several differentiated population groups and therefore hide evolutionarily important and more complex intraspecific differentiation processes (a main risk when using a subspecies classification; e.g. Zink, 2004). The alternative subspecies concept, including parapatric subspecies (i.e. characterized by an abrupt change in phenotypic characters rather than a cline variation in contact zone with other differentiated populations; see the review in Braby *et al.*, 2012), remains doubtful and debated (review in Braby *et al.*, 2012), and fails to reflect the *B. pascuorum* lineages and subgroups (i.e. cline variations as well as

abrupt changes in colour pattern occur between lineages and subgroups; Rasmont, 1983). Therefore, subspecies classification seems unsuitable to reflect the intraspecific differentiation within *B. pascuorum*. In order to provide a prospective useful classification for further evolutionary biology or conservation biology studies, we regard the lineages and subgroups as different evolutionarily significant units (ESUs, *sensu* Conner & Hartl, 2004; see also Lecocq *et al.*, 2015a, and the review in Braby *et al.*, 2012) with a neutral divergence (i.e. genetic differentiation but without the necessary reciprocal monophyly; see James, 2010) in nuclear and mitochondrial markers and a divergence in characters (colour pattern) shaped by selective pressure (i.e. bumblebee colour pattern is shaped by Müllerian mimicry processes; Hines & Williams, 2012). We consider that these ESUs represent an important component in the evolutionary legacy of the species (Waples, 1995; Lecocq *et al.*, 2015a). The resulting classification is: (i) the *dusmeti* ESU including all taxa from Iberian Peninsula and southern/south-western France; (ii) *rehbinderi* ESU; (iii) *siciliensis* ESU; and (iv) *floralis* ESU (including all other here studied taxa). The nuclear and mitochondrial differentiation of the eastern group (*B. p. floralis* from Russia and *B. p. flavobarbatus* and *B. p. michnoi* from Siberia) could result from a low sampling effort in Russia that makes it impossible to estimate the haplotype diversity in this region and therefore to assess the genetic differentiation. Further studies based on larger sampling and other genetic markers are needed to assess the originality of this group.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/syen.12137

Table S1. Table of sampling. Sample code refers to the sample labels used in analyses and supplementary tree. Taxa is the name of taxa. COI, EF-1 α , and PEPCK are the GenBank accession numbers for each sample (when consubspecific samples display the same gene sequence, only one of them has been submitted to Genbank; the complete matrices are available on TreeBase TB2:S17344).

Table S2. Data matrix of cephalic labial gland secretions (relative amounts of each compound) and list of the identified compounds in the *Bombus pascuorum*. Unknown x are undetermined compounds.

Acknowledgements

We gratefully acknowledge K. Urbanová (Prague, Czech Republic) for her help with the chemical analyses. The authors acknowledge the *Fonds pour la recherche dans l'industrie et l'agriculture* (FRIA) for financial support. T.L. is post-doc in the context of the BELBEES project (BELSPO; BR/132/A1/BELBEES). The research leading to these results has received funding from the European Community's Seventh

Framework Programme (FP7/2007-2013) under grant agreement no. 244090, STEP Project (Status and Trends of European Pollinators, www.step-project.net). Partial financial support (I.V.) was provided by the Institute of Organic Chemistry and Biochemistry of the Academy of Sciences of the Czech Republic (subvention for development of research organization RVO: 61388963). This project was supported by the network *Bibliothèque du Vivant* funded by the CNRS, the *Muséum National d'Histoire Naturelle* (MNHN) and the *Institut National de la Recherche Agronomique* (INRA), and technically supported by *Genoscope*. Special thanks are also due to S. Lambert (Mons, Belgium) for helpful comments and discussions on earlier versions of this manuscript. We acknowledge David Genoud for providing photograph of *B. pascuorum*. We would like to thank the anonymous reviewers and the editor T. Simonsen for helpful comments and improvements. Computational resources have been provided by the *Consortium des Équipements de Calcul Intensif* (CÉCI), funded by the Belgian F.R.S.-FNRS.

References

- Allentoft, M.E. & O'Brien, J. (2010) Global amphibian declines, loss of genetic diversity and fitness: a review. *Diversity*, **2**, 47–71.
- Appelgren, M., Bergström, G., Svensson, B.G. & Cederberg, B. (1991) Marking pheromones of *Megabombus* bumble bee males. *Acta Chemica Scandinavica*, **45**, 972–974.
- Avice, J.C. (2000) *Phylogeography: The History and Formation of Species*. Harvard University Press, Cambridge, Massachusetts.
- Ayasse, M. & Jarau, S. (2014) Chemical ecology of bumble bees. *Annual Review of Entomology*, **59**, 299–319.
- Bandelt, H.-J., Forster, P. & Röhl, A. (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, **16**, 37–48.
- Bertsch, A. (1997) Discrimination of the bumblebee species *Bombus cryptarum* and *B. lucorum* by means of male labial gland secretions and morphological characters (Hymenoptera: Apidae). *Entomologia Generalis*, **22**, 129–145 (in German).
- Bertsch, A. & Schweer, H. (2012) Cephalic labial gland secretions of males as species recognition signals in bumblebees: are there really geographical variations in the secretions of the *Bombus terrestris* subspecies? *Beiträge zur Entomologie*, **62**, 103–124.
- Bertsch, A., Schweer, H., Titze, A. & Tanaka, H. (2005) Male labial gland secretions and mitochondrial DNA markers support species status of *Bombus cryptarum* and *B. magnus* (Hymenoptera, Apidae). *Insectes Sociaux*, **52**, 45–54.
- Bickford, D., Lohman, D.J., Sodhi, N.S. *et al.* (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution*, **22**, 148–155.
- Braby, M.F., Eastwood, R. & Murray, N. (2012) The subspecies concept in butterflies: has its application in taxonomy and conservation biology outlived its usefulness? *Biological Journal of the Linnean Society*, **106**, 699–716.
- Cameron, S.A., Hines, H.M. & Williams, P.H. (2007) A comprehensive phylogeny of the bumble bees (*Bombus*). *Biological Journal of the Linnean Society*, **91**, 161–188.
- Carolan, J.C., Murray, T.E., Fitzpatrick, Ú. *et al.* (2012) Colour patterns do not diagnose species: quantitative evaluation of a DNA barcoded cryptic bumblebee complex. *PLoS ONE*, **7**, e29251.
- Conner, J.K. & Hartl, D.L. (2004) *A Primer of Ecological Genetics*. Sinauer Associates, Sunderland, Massachusetts.

- Coppée, A., Terzo, M., Valterová, I. & Rasmont, P. (2008) Intraspecific variation of the cephalic labial gland secretions in *Bombus terrestris* (L.) (Hymenoptera: Apidae). *Chemistry & Biodiversity*, **5**, 2654–2661.
- Crowhurst, R.S., Faries, K.M., Collantes, J., Briggler, J.T., Koppelman, J.B. & Eggert, L.S. (2011) Genetic relationships of hellbenders in the Ozark highlands of Missouri and conservation implications for the Ozark subspecies (*Cryptobranchus alleganiensis bishopi*). *Conservation Genetics*, **12**, 637–646.
- Dellicour, S. & Lecocq, T. (2013a) *GALIGNER 1.0 and GCKOVATS 1.0 – Manual of a Software Suite to Compute a Multiple Sample Comparison Data Matrix from Eco-chemical Datasets Obtained by Gas Chromatography*. University of Mons, Mons.
- Dellicour, S. & Lecocq, T. (2013b) GALIGNER 1.0: an alignment program to compute a multiple sample comparison data matrix from large eco-chemical datasets obtained by GC. *Journal of Separation Science*, **36**, 3206–3209.
- Dellicour, S., Lecocq, T., Kuhlmann, M., Mardulyn, P. & Michez, D. (2014) Molecular phylogeny, biogeography, and host plant shifts in the bee genus *Melitta* (Hymenoptera: Anthophila). *Molecular Phylogenetics and Evolution*, **70**, 412–419.
- De Meulemeester, T., Gerbaux, P., Boulvin, M., Coppée, A. & Rasmont, P. (2011) A simplified protocol for bumble bee species identification by cephalic secretion analysis. *Insectes Sociaux*, **58**, 227–236.
- De Queiroz, K. (2007) Species concepts and species delimitation. *Systematic Biology*, **56**, 879–886.
- De Ruijter, A. & Wiebes, J.T. (1974) Colour variation of *Bombus pascuorum* in the Netherlands. *Netherlands Journal of Zoology*, **25**, 233–238.
- Ebach, M.C. & Williams, D.M. (2009) How objective is a definition in the subspecies debate? *Nature*, **457**, 785.
- Ennen, J.R., Kalis, M.E., Patterson, A.L., Kreiser, B.R., Lovich, J.E., Godwin, J. & Qualls, C.P. (2014) Clinal variation or validation of a subspecies? A case study of the *Graptemys nigrinoda* complex (Testudines: Emydidae). *Biological Journal of the Linnean Society*, **111**, 810–822.
- Frankham, R., Ballou, J.D. & Briscoe, D.A. (2010) *Introduction to Conservation Genetics*, 2nd edn. Cambridge University Press, Cambridge.
- Free, J.B. (1993) *Insect Pollination of Crops*, 2nd edn. Academic Press, London.
- Hawiltschek, O., Nagy, Z.T. & Glaw, F. (2012) Island evolution and systematic revision of Comoran snakes: why and when subspecies still make sense. *PLoS ONE*, **7**, e42970.
- Hillis, D.M. & Bull, J.J. (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology*, **42**, 182–192.
- Hines, H.M. (2008) Historical biogeography, divergence times, and diversification patterns of bumble bees (Hymenoptera: Apidae: *Bombus*). *Systematic Biology*, **57**, 58–75.
- Hines, H.M. & Williams, P.H. (2012) Mimetic colour pattern evolution in the highly polymorphic *Bombus trifasciatus* (Hymenoptera: Apidae) species complex and its comimics. *Zoological Journal of the Linnean Society*, **166**, 805–826.
- James, F. (2010) Avian subspecies. *Ornithological Monographs*, **67**, 1–5.
- Krüger, E. (1928) Über die farbenvariationen der hummelart *Bombus agrorum* fabr. *Zeitschrift für Morphologie und Ökologie der Tiere*, **11**, 361–494.
- Krüger, E. (1931) Über die farbenvariationen der hummelart *Bombus agrorum* fabr. II. Teil. *Zeitschrift für Morphologie und Ökologie der Tiere*, **24**, 148–237.
- Kuhlmann, M., Ascher, J.S., Dathe, H.H. *et al.* (2014) Checklist of the Western Palaearctic Bees (Hymenoptera: Apoidea: Anthophila) [WWW document]. URL <http://westpalbees.myspecies.info>. [accessed on 11 April 2015].
- Lecocq, T., Lhomme, P., Michez, D., Dellicour, S., Valterová, I. & Rasmont, P. (2011) Molecular and chemical characters to evaluate species status of two cuckoo bumblebees: *Bombus barbutellus* and *Bombus maxillosus* (Hymenoptera, Apidae, Bombini). *Systematic Entomology*, **36**, 453–469.
- Lecocq, T., Dellicour, S., Michez, D. *et al.* (2013a) Scent of a break-up: phylogeography and reproductive trait divergences in the red-tailed bumblebee (*Bombus lapidarius*). *BMC Evolutionary Biology*, **13**, 263.
- Lecocq, T., Vereecken, N.J., Michez, D. *et al.* (2013b) Patterns of genetic and reproductive traits differentiation in mainland vs. Corsican populations of bumblebees. *PLoS ONE*, **8**, e65642.
- Lecocq, T., Brasero, N., De Meulemeester, T. *et al.* (2015a) An integrative taxonomic approach to assess the status of Corsican bumblebees: implications for conservation. *Animal Conservation*, **18**, 236–248.
- Lecocq, T., Coppée, A., Mathy, T. *et al.* (2015b) Subspecific differentiation in male reproductive traits and virgin queen preferences, in *Bombus terrestris*. *Apidologie*. DOI: 10.1007/s13592-015-0349-y.
- Lecocq, T., Dellicour, S., Michez, D. *et al.* (2015c) Methods for species delimitation in bumblebees (Hymenoptera, Apidae, *Bombus*): towards an integrative approach. *Zoologica Scripta*, **44**, 281–297.
- Løken, A. (1973) Studies on Scandinavian bumble bees (Hymenoptera, Apidae). *Norsk Entomologisk Tidsskrift*, **20**, 1–218.
- Maia-Carvalho, B., Gonçalves, H., Martínez-Solano, I., Gutiérrez-Rodríguez, J., Lopes, S., Ferrand, N. & Sequeira, F. (2014) Intraspecific genetic variation in the common midwife toad (*Alytes obstetricans*): subspecies assignment using mitochondrial and microsatellite markers. *Journal of Zoological Systematics and Evolutionary Research*, **52**, 170–175.
- Manier, M.K. (2004) Geographic variation in the long-nosed snake, *Rhinocheilus lecontei* (Colubridae): beyond the subspecies debate. *Biological Journal of the Linnean Society*, **83**, 65–85.
- Mayr, E. (1942) *Systematics and the Origin of Species*. Columbia University Press, New York, New York.
- Mayr, E. (1963) *Animal Species and Evolution*. Harvard University Press, Cambridge, Massachusetts.
- Michener, C.D. (1990) Classification of the Apidae (Hymenoptera). *University of Kansas Natural History Museum Special Publication*, **54**, 75–164.
- Mulcahy, D.G. (2008) Phylogeography and species boundaries of the western North American Nightsnake (*Hypsiglena torquata*): revisiting the subspecies concept. *Molecular Phylogenetics and Evolution*, **46**, 1095–1115.
- Oksanen, F.J., Blanchet, G., Kindt, R. *et al.* (2011) *Tertiary Vegan: Community Ecology Package*.
- Paradis, E., Claude, J. & Strimmer, K. (2004) APE: analyses of phylogenetics and evolution in R language. *Bioinformatics*, **20**, 289–290.
- Patten, M.A. (2009) ‘Subspecies’ and ‘race’ should not be used as synonyms. *Nature*, **457**, 147.
- Patten, M.A. & Unitt, P. (2002) Diagnosability versus mean differences of Sage Sparrow subspecies. *Auk*, **119**, 26–35.
- Pedersen, B.V. (2002) European bumblebees (Hymenoptera: Bombini) – Phylogenetic relationships inferred from DNA sequences. *Insect Systematics and Evolution*, **33**, 361–386.
- Pekkarinen, A. (1979) Morphometric colour and enzyme variation in bumble bees (Hymenoptera, Apidae, *Bombus*) in Fennoscandia and Denmark. *Acta Zoologica Fennica*, **158**, 1–60.
- Phillimore, A.B. & Owens, I.P.F. (2006) Are subspecies useful in evolutionary and conservation biology? *Proceedings of the Royal Society of London Series B: Biological Sciences*, **273**, 1049–1053.
- Pirounakis, K., Koulianos, S. & Schmid-Hempel, P. (1998) Genetic variation among European populations of *Bombus pascuorum*

- (Hymenoptera: Apidae) from mitochondrial DNA sequence data. *European Journal of Entomology*, **95**, 27–33.
- Rasmont, P. (1983) La notion d'exerge appliquée à *Megabombus (Thoracobombus) pascorum* (Scopoli) (Hymenoptera, Apidae). *Bulletin Annales de la Société Royale Belge d'Entomologie*, **119**, 185–195.
- Rasmont, P. & Adamski, A. (1995) Les bourdons de la Corse (Hymenoptera, Apoidea, Bombinae). *Notes Fauniques de Gembloux*, **31**, 1–87.
- Rasmont, P., Verhaeghe, J.-C., Rasmont, R. & Terzo, M. (2000) West-palaearctic bumblebees. *Insect Pollination in Greenhouses: Proceedings of the Specialists' Meeting Held in Soesterberg, The Netherlands*, 30 September to 2 October 1999 (ed. by M.J. Sommeijer and A. Ruijter), pp. 93–97. Universiteit Utrecht, Utrecht.
- Rasmont, P., Terzo, M., Aytikin, A.M., Hines, H., Urbanova, K., Cahlikova, L. & Valterova, I. (2005) Cephalic secretions of the bumblebee subgenus *Sibiricobombus* Vogt suggest *Bombus niveatus* Kriechbaumer and *Bombus vorticosus* Gerstaecker are conspecific (Hymenoptera, Apidae, *Bombus*). *Apidologie*, **36**, 571–584.
- Rasmont, P., Coppée, A., Michez, D. & De Meulemeester, T. (2008) An overview of the *Bombus terrestris* (L. 1758) subspecies (Hymenoptera: Apidae). *Annales de la Société Entomologique de France (Nouvelle Série)*, **44**, 243–250.
- Rasmont, P., Franzen, M., Lecocq, T. *et al.* (2015) Climatic risk and distribution atlas of European bumblebees. *BioRisk*, **10**, 1–236.
- R Development Core Team (2013) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. [WWW document]. URL <http://www.R-project.org/> [accessed on 11 April 2015].
- Reinig, W.F. (1970) Bastardierungs-zonen und mischpopulationen bei hummeln (*Bombus*) und schmarotzerhummeln (*Psithyrus*) (Hymenopt., Apidae). *Mitteilungen der Münchener Entomologischen Gesellschaft*, **59**, 1–89.
- Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes 3: bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.
- Sackett, L.C., Seglund, A., Guralnick, R.P., Mazzella, M.N., Wagner, D.M., Busch, J.D. & Martin, A.P. (2014) Evidence for two subspecies of Gunnison's prairie dogs (*Cynomys gunnisoni*), and the general importance of the subspecies concept. *Biological Conservation*, **174**, 1–11.
- Schlick-Steiner, B.C., Steiner, F.M., Seifert, B., Stauffer, C., Christian, E. & Crozier, R.H. (2010) Integrative taxonomy: a multisource approach to exploring biodiversity. *Annual Review of Entomology*, **55**, 421–438.
- Suzuki, R. & Shimodaira, H. (2011) *Pvclust: Hierarchical Clustering with P-values via Multiscale Bootstrap Resampling*. Contributed package. Version 1-1.10. R Foundation for Statistical Computing, Vienna. [WWW document]. URL <http://www.R-project.org> [accessed on 11 April 2015].
- Terzo, M., Urbanová, K., Valterová, I. & Rasmont, P. (2005) Intra and interspecific variability of the cephalic labial glands' secretions in male bumblebees: the case of *Bombus (Thoracobombus) ruderarius* and *B. (Thoracobombus) sylvarum* Hymenoptera, Apidae. *Apidologie*, **36**, 85–96.
- Tsao, W.C. & Yeh, W.B. (2008) DNA-based discrimination of subspecies of swallowtail butterflies (Lepidoptera: Papilioninae) from Taiwan. *Zoological Studies*, **47**, 633–643.
- Waples, R. (1995) Evolutionary significant units and the conservation of biological diversity under the Endangered Species Act. *Evolution and the Aquatic Ecosystem* (ed. by J. Nielsen), pp. 8–27. American Fisheries Society, Bethesda, Maryland.
- Whittaker, R.J. & Fernández-Palacios, J.M. (2007) *Island Biogeography – Ecology, Evolution, and Conservation*, 2nd edn. Oxford University Press, Oxford.
- Widmer, A. & Schmid-Hempel, P. (1999) The population genetic structure of a large temperate pollinator species, *Bombus pascuorum* (Scopoli) (Hymenoptera: Apidae). *Molecular Ecology*, **8**, 387–398.
- Wilcox, T.P., Zwickl, D.J., Heath, T.A. & Hillis, D.M. (2002) Phylogenetic relationships of the dwarf boas and a comparison of Bayesian and bootstrap measures of phylogenetic support. *Molecular Phylogenetics and Evolution*, **25**, 361–371.
- Williams, P.H. (1991) The bumble bees of the Kashmir Himalaya (Hymenoptera: Apidae, Bombini). *Bulletin of the Natural History Museum (Entomology)*, **60**, 1–204.
- Williams, P.H. (1998) An annotated checklist of bumble bees with an analysis of patterns of description (Hymenoptera: Apidae, Bombini). *Bulletin of the Natural History Museum (Entomology)*, **67**, 79–152.
- Žáček, P., Prchalová-Horňáková, D., Tykva, R. *et al.* (2013) *De novo* biosynthesis of sexual pheromone in the labial gland of bumblebee males. *ChemBioChem*, **14**, 361–371.
- Zink, R.M. (2004) The role of subspecies in obscuring avian biological diversity and misleading conservation policy. *Proceedings of the Royal Society of London Series B: Biological Sciences*, **271**, 561–564.
- Zwickl, D.J. (2006) *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criteria*. PhD Dissertation, The University of Texas, Austin, Texas.

Accepted 12 June 2015

First published online 24 July 2015