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ORIGINAL PAPER

Contrasting patterns of genetic and morphological diversity in the bumblebee *Bombus lucorum* (Hymenoptera: Apidae: *Bombus*) along a European gradient

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Abstract
The Iberian Peninsula is known to have acted as a glacial refugium for many species during the Pleistocene in Europe. Several phylogeographical studies have been carried out within the genus *Bombus* which indicate a genetic differentiation of some of its species in the southern European peninsulas. *Bombus lucorum* (Linnaeus, 1761) is one of the three cryptic species belonging to the *B. lucorum* complex. In recent years, this complex has been widely studied; however, there is a lack of information about the genetic diversity of this species and its possible postglacial recolonization events. To overcome this knowledge gap, in this study several populations from the centre of the Iberian Peninsula to Belgium have been characterized using mitochondrial and nuclear markers (*cox1* barcoding and 11 microsatellite loci) and the geometric morphometrics of the wings. Results from *cox1* indicate a genetic differentiation of the population of Sierra de Guadarrama at the centre of the Iberian Peninsula, while microsatellite loci and geometric morphometrics analyses do not show any population structure. These results point to a past event of genetic differentiation of *B. lucorum* in the Iberian Peninsula although they also suggest a current gene flow with populations from mainland Europe.

Keywords *Bombus lucorum* · Gene flow · Genetic differentiation · Population structure · Glacial refugium · Iberian Peninsula

Introduction

The Iberian Peninsula is considered one of the most important glacial refugia of the Pleistocene in Europe. In addition, mountainous areas as well as the influence of both the Mediterranean Sea and the Atlantic Ocean create different climatic zones that are thought to have functioned as "microrefugia" in different parts of the Peninsula during the last maximum glacial (LMG). In this way, a wide variety of organisms is known to either have differentiated genetically in the Peninsula or have colonized the north and east of Europe from this region (Gómez and Lunt 2007).

Recently, several phylogeographic studies of the genus *Bombus* Latreille, 1802 have been carried out due to the increasing interest about the conservation of this group given their status as important pollinators of wild flora and crops (Widmer et al. 1998; Widmer and Schmid-Hempel 1999; Duennes et al. 2012; Lecocq et al. 2013a, b, 2015; Françoso et al. 2016). *Bombus lucorum* (Linnaeus, 1761) (subgenus *Bombus*) is a Palearctic species which extends from southern Europe north to the Barents Sea coast, reaching Iceland to the west and the Pacific coast to the east (Rasmont et al. 2015). It is abundant all over Western, Central and Northern Europe but is restricted to the mountainous regions in Southern Europe (Bertsch et al. 2004). Recent studies (Bertsch et al. 2005; Bertsch 2009; McKendrick et al. 2017) have provided evidence that *B. lucorum* is a complex of three cryptic species: *B.*

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lucorum, *B. magnus* (Vogt 1911) and *B. cryptarum* (Fabricius 1775). Analyses of male labial gland secretions and sequence variation of the mitochondrial cytochrome oxidase I (*cox1*) consistently distinguish the three species in Europe (Bertsch et al. 2004, 2005; Bertsch 2009; Carolan et al. 2012), but there is a lack of morphological characters to easily discriminate among them (Carolan et al. 2012). The form of the first collar in the queens may be the unique character to discriminate them in the field (Rasmont 1984; Bertsch 1997; Bertsch et al. 2004). In *B. lucorum* the lateral border of the collar is higher than in *B. magnus* and *B. cryptarum* and is almost exclusively restricted to the pronotal lobes (Bertsch et al. 2004). Regarding their ecology and based on the most recent studies that used genetics-supported identification, *B. lucorum* is generally the most abundant of the three species in the complex at the majority of European sites (Murray et al. 2008; Vesterlund et al. 2014; Scriven et al. 2015; Bossert et al. 2016), with the exception of Waters et al. (2011) who found *B. cryptarum* as the most frequent species in north-western Scotland. *B. lucorum* feeds on a wide range of flowers and seems to be most closely associated with urbanized areas than other members of the species complex (Murray et al. 2008; Waters et al. 2011; Scriven et al. 2015). However, the fact that the three species of the complex can be found living simpatrically in various habitats and the insufficient comparative studies across large geographic areas make it difficult to establish the factors determining the particular niche of *B. lucorum* (Bossert 2015).

Many *B. lucorum* populations have been characterized using *cox1* gene sequence variation throughout Europe with the aim of differentiating the three species of the *lucorum* complex (Bertsch et al. 2005; Murray et al. 2008; Bertsch 2009; Carolan et al. 2012; Williams et al. 2012). However, only one individual from the Iberian Peninsula was included in one of those studies (Bertsch 2009). The Iberian Peninsula is the southwest limit of the species distribution and it becomes less abundant here, being present only in the mountains of the northern half (Ornosa and Ortiz-Sánchez 2004). As stated in Penado et al. (2016), the lack of knowledge of bumblebee distribution in the Iberian Peninsula combined with the susceptibility to a rapid decline of these marginal populations, urge studies in this area. Indeed, *B. lucorum* is considered a species of Least Concern in the IUCN Red List of European Bees, but it has already become less abundant in Belgium and western France and a projected climatic scenario for 2050 has shown the reduction and even loss of the Iberian populations (Rasmont et al. 2015). Taking this into account, and the possibility that the peninsula could have acted as a glacial refugium for bumblebee species (Widmer and Schmid-Hempel 1999; Lecocq et al. 2013a, 2015), it may be particularly informative to sample Iberian populations and perform molecular analyses to infer the genetic diversity of the species and to clarify possible postglacial recolonization events.

In this work, several populations of *B. lucorum* ranging from the Iberian Peninsula to Belgium have been characterized by *cox*1 sequencing adding published sequences to the data set. A median-Joining haplotype network was constructed to infer intraspecific relationships (Bandelt et al. 1999). Furthermore, individuals were genotyped with nine nuclear microsatellite loci to infer the intraspecific population structure, and finally the geometric morphometry of the wings has been analyzed to resolve fine-scale variation (Aytekin et al. 2007; Kozmus et al. 2011; Schutze et al. 2012). As has been observed in other insect species (Cooper and Hewitt 1993; Schmitt and Seitz 2004; Habel et al. 2005; Lecocq et al. 2013a, 2015), a differentiation is expected of the Iberian individuals with respect to those from central Europe.

Materials and methods

Sampling

Sampling was conducted along a latitudinal gradient from central Iberian Peninsula to Belgium between the summers of 2013 and 2017. Ninety-nine individuals were collected from the Spanish National Parks Sierra de Guadarrama (central Spain), and Aigüestortes and Ordesa (Pyrenees), and the Cantabrian mountain range (Spain) and preserved in absolute ethanol (Fig. 1). Forty-five individuals from the Pyrenees and Monts d'Ardèche (southern France), Namur and the High Fens (Belgium) sampled between 1986 and 2001 (Fig. 1) were provided from the collection of Dr. Denis Michez (University of Mons, Belgium) (Table S1).

DNA extraction

DNA was extracted from a hind leg removed from every individual using the Chelex method (Walsh et al. 1991). In the case of the bumblebees from the Belgian collection, total DNA was extracted using the Canadian Centre for DNA Barcoding Glass Fiber Plate DNA Extraction Protocol (Ivanova et al. 2006).

Cox1 amplification and sequencing

PCR amplification of a fragment of the *cox1* gene was carried out using the primers pair LCO1490/HCO2198 (Folmer et al. 1994) with an initial denaturation at 94 °C for 3 min; followed by 40 cycles of 94 °C for 30 s, 48/50 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. As some individuals had degraded DNA, the *cox1* amplification was carried out for those individuals using the

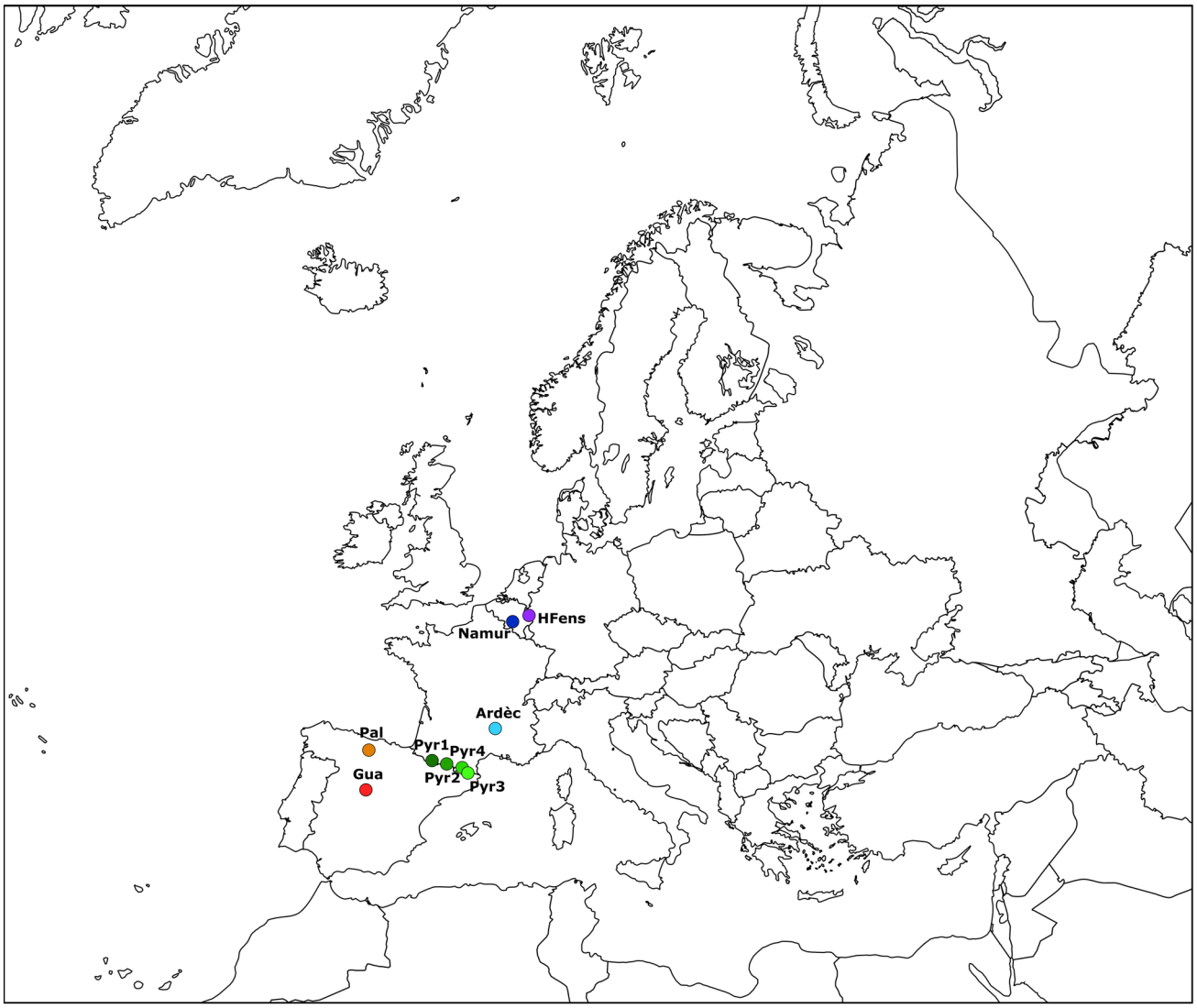

Fig. 1 Map showing the sampling locations of *Bombus lucorum*: *Hfens* high fens, *Ardèc* Monts d'Ardèche, *Pyr1* Pyrenees1, *Pyr2* Pyrenees2, *Pyr3* Pyrenees3, *Pyr4* Pyrenees4, *Pal* Palencia, *Gua* Sierra de Guadarrama

mini barcode approach (Françoso and Arias 2013). PCR conditions included in this case an initial denaturation at 94 °C for 5 min; followed by 35 cycles of 94 °C for 1 min, 46 °C for 1 min 20 s, 64 °C for 2 min, and a final extension step at 64 °C for 10 min. Amplicons of the *cox1* fragments were Sanger sequenced (Secugen SL, Madrid, Spain).

Species identification and haplotype analysis

Bombus lucorum individuals were initially identified through morphological characters and lately confirmed by *cox1* sequencing and BLAST comparisons (https://blast.ncbi.nlm.nih.gov/Blast.cgi). *B. lucorum* sequences available for the same mitochondrial fragment were downloaded from GenBank (accession numbers in Table S2). Sequences were aligned using Geneious 7.1 and the final matrix comprised 216 sequences. Sequences were translated to check for aminoacid changes and to discard possible sequencing mistakes and the presence of NUMTs (nuclear mitochondrial DNA segments).

To study haplotype relationships, a haplotype network was constructed with PopArt 1.7 (Leigh and Bryant 2015) using the median-Joining distance method. Nucleotide divergence and differentiation coefficients (Φ_{ST}) were calculated using the package StrataG (Archer et al. 2017) in R software only in those sampling locations consisting in more than ten individuals (including both females and males). Bonferroni correction was applied to *p*-values.

Microsatellite amplification and genotyping

Eleven microsatellite loci (B10, B11, B96, B100, B118, B119, 121, B124, B126, B131, B132, (Estoup et al. 1995, 1996) were amplified with two multiplex reactions (Cejas et al. 2019). PCR consisted in an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 92 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 30 min. Allele discrimination was carried out on an AB3700 capillary genotyping system (Servei Central de Suport a la Investigació Experimental,University of Valencia, Spain). Alleles were scored using GeneMapper 4.8 (Applied Biosystems Inc.).

Microsatellite analysis

Only females from sampling locations with more than nine individuals were used in the microsatellite analysis. Putative full siblings were identified with the software Colony 2.0 (Jones and Wang 2010). Individuals with probabilities of sibship above 0.95 were discarded from further molecular and geometric morphometrics analyses.

The presence of null alleles, scoring error due to stuttering, and allele dropout were analyzed with the software Micro-Checker 2.2 (Van Oosterhout et al. 2004). Genetic structure was estimated using two methods. Firstly, a Bayesian clustering algorithm was performed using the program STRUCTURE 2.3.4 (Pritchard et al. 2000). The admixture correlated frequency model was used and K (number of clusters) was tested from 1 to 10. Five independent runs were performed with a burn-in period of 250,000 and a run length of 750,000 iterations. The optimal number of clusters (K) was selected using Structure Harvester (Earl 2012) according to the ad hoc statistic deltaK. In a first step temporal stability of populations was tested performing an analysis only with Pyrenean samples collected in 2001 and 2013–2017. Then a second analysis was done with all the samples independently of their geographical location. Additionally, a discriminant analysis of principal components (DAPC) (Jombart et al. 2010) was also performed with the package adegenet in R. Number of clusters was identified following the Bayesian Information Criterion (BIC) and an optimal number of principal components that explained more than 90% of the variance was selected.

Mean allele richness (A_R), number of private alleles (Npa), observed (H_O) and unbiased expected heterozigosity (uH_E), F_{IS} values, departure from Hardy–Weinberg equilibrium, linkage disequilibrium, migration rate, and differentiation coefficients (pairwise F_{ST}) were calculated using the software GenAlEx v6.5 (Smouse and Peakall 2012) and the packages GenePop (Rousset 2008), adegenet (Jombart 2008), StrataG (Archer et al. 2017) and diveRsity (Keenan et al. 2013) in R. Due to the different number of individuals of each group A_R values were corrected after rarefaction with diveRsity (Keenan et al. 2013). Bonferroni correction was applied to *p*-values when testing for linkage disequilibrium, Hardy–Weinberg equilibrium and F_{ST} pairwise comparison.

Wing morphometrics

Wing shape was considered to characterize the phenotype of the individuals. Wings are two dimensional structures where crossing veins and maximum curve can be considered as homologous (e.g. Dehon et al. 2017). The left forewings were removed and mounted with water in glass slides with glass slipcovers. Only females were considered to avoid differences based on sexual dimorphism (Gerard et al. 2015). Pyrenean samples were grouped in this analysis given their geographical proximity. All slide-mounted wings were photographed with a Spot Insight Firewire camera and the application SPOT Advanced (SPOT Imaging). Images were converted to tps format with tpsUtil 1.74 (Rohlf 2017a). Eighteen landmarks were selected (Fig. S1) and manually plotted on the forewings images using tpsDig2 2.32 (Rohlf 2016). Correlation between the Procrustes distances in the shape space with the Euclidean distances in the tangent space was checked using the software tpsSmall 1.34 (Rohlf 2017b).

Geometric morphometrics analysis

The program MorphoJ 1.06d (Klingenberg 2011) was used for geometric morphometrics analyses. A Procrustres fit was performed to eliminate all the variations due to non-shape differences and outliers were checked. Individuals were classified by population clusters. A principal component analysis (PCA) was performed to visualize shape variation among groups. A canonical variance analysis (CVA) was performed and the Mahalanobis and Procrustres distances were computed for each group and tested by a permutation test applying Bonferroni correction. The separation of the groups was also tested in a discriminant function analysis (DFA) with a leave-one-out cross-validation test. ANOVA tests were performed to see if the origin of the individuals had any effect on the size and the shape of the wings.

Results

Although southern locations suitable for Iberian bumblebees were sampled, including the mountain ranges Sierra Nevada and Sierra Espuña, and the natural park Calares del Mundo y de la Sima (southeastern Spain), no *B. lucorum* individuals were found supporting its limited distribution

to the northern half of the Iberian Peninsula (Ornosa and Ortiz-Sánchez 2004).

Cox1 analyses

The resulting matrix of the partial *cox1* gene included 216 sequences of 564 base pairs (bp) with a total of 28 segregating variable sites, 11 parsimony-informative sites and a nucleotide diversity (π) of 0.0028. Sequences obtained in this study were submitted to GenBank (accession numbers MK279535–MK279653). All variable nucleotides corresponded to synonym mutations, thus discarding the presence of NUMTs. Eleven haplotypes were found in the locations analyzed with seven being private and only two of them (IV and V) being predominant in the individuals of the Iberian Peninsula. The private haplotype of Palencia was obtained from one sample of this location (Fig. 2). Individuals from Sierra de Guadarrama showed a differentiation from the rest of the populations with two main private haplotypes, V and IV, observed in 92.7% of the samples of this location. These haplotypes, V and IV, were 3 and 4 mutation steps apart from the most common haplotype in Europe and Asia, respectively. Individuals from the locations of High Fens and the Pyrenees shared haplotypes I and VII with the rest of Europe with 83.3% and 91.9% of the samples, respectively. One sample of Monts d'Ardèche had the haplotype I and the two individuals sampled of Namur had a different haplotype, XIII. Remarkably, one sample with haplotype V was found in the Pyrenees and one sample with haplotype I was found in Sierra de Guadarrama. The haplotype IV found in Palencia occupied an intermediate position in the network between the haplotype VII found mainly in the Pyrenees and the haplotype V found mainly in Sierra de Guadarrama (Fig. 2).

Only a single individual from Monts d'Ardèche yielded DNA of enough quality for sequencing, therefore this population was excluded from further analyses. Intra-population nucleotide divergence for the populations of this study was low, with a maximum value of 0.0014. Inter-population nucleotide divergence values were within the same range as intra-population values, except for the population of Sierra de Guadarrama, which had values of divergence ranging from 0.0056 to 0.0065 in relation to the rest of populations (Table S3). The pairwise Φ_{ST} indicated a significant differentiation only in the population of Sierra de Guadarrama (Φ_{st} = 0.768–0.806) after applying the Bonferroni correction (Table 1).

Microsatellite analysis

Three putative full sibling individuals were identified with a probability > 0.95 and discarded from the analyses. Locus B131 was monomorphic and loci B121 and B132 gave unclear peak patterns in the electropherograms, consequently they were discarded from further analyses. MicroChecker did not detect the presence of null alleles, nor found evidence for scoring error or allele dropout.

The lack of structure in the temporal analysis of the old (2001) and new (2013–2017) samples from the Pyrenees allowed us to gather all the samples independently of the sampling year in the following analyses (data not shown). Although STRUCTURE and Structure Harvester indicated an optimal number of groups with K = 2, none of the populations showed a higher proportion of their individuals belonging to one of the two genetic groups, i.e. all individuals had a similar probability of belonging to both groups (Fig. S2).

The DAPC showed that the BIC reached its minimum value and an elbow in the curve at K = 3. The first 35

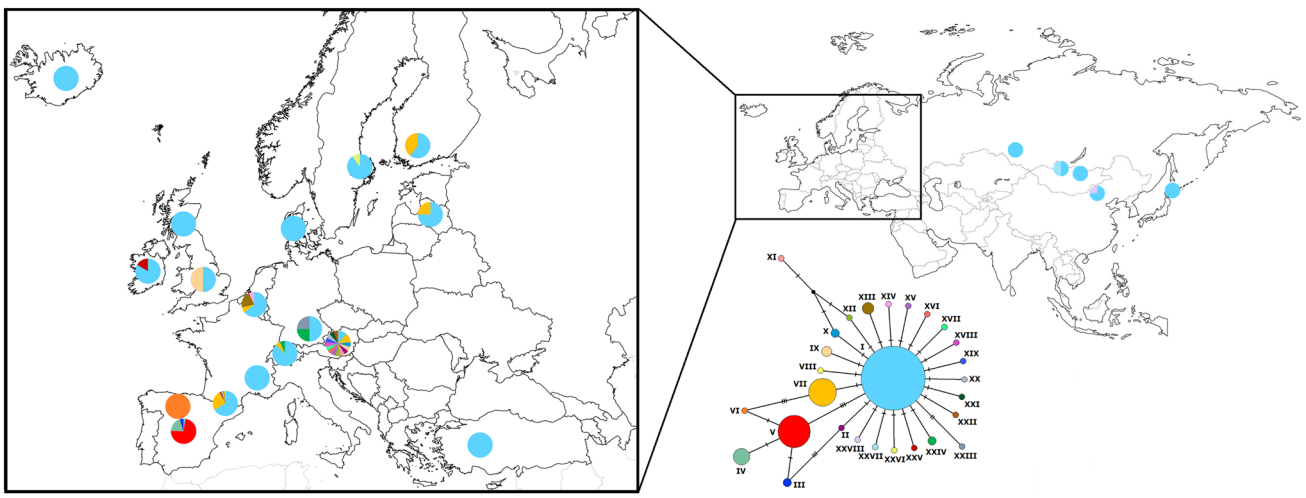

Fig. 2 *Bombus lucorum* haplotype frequencies and haplotype network based on *cox1* gene. Analysis included all *B. lucorum* sequences available in GenBank for the same *cox1* gene fragment

Table 1 Differentiation test values (Φ_{ST}) between *B. lucorum* populations based on mitochondrial variation

	Sierra de Guadarrama	Pyrenees1	Pyrenees2	Pyrenees3	Pyrenees4	High Fens
Sierra de Guadarrama	NA	0.0009*	0.0009*	0.0009*	0.0009*	0.0009*
Pyrenees1	0.7682*	NA	0.6263	0.0819	0.6483	0.1488
Pyrenees2	0.7896*	−0.0284	NA	0.0349	0.2807	0.0129
Pyrenees3	0.8058*	0.0960	0.2747	NA	0.2467	0.9500
Pyrenees4	0.7907*	−0.0247	0.0010	0.0720	NA	0.4625
High Fens	0.7890*	0.0374	0.1215	−0.0166	−0.0109	NA

Φ_{ST} coefficients are shown in the lower part of the matrix with their corresponding *p*-values for 1000 permutations in the upper part of the matrix. The asterisks indicate the significance at the 5% level after applying Bonferroni correction (*p*-value < 0.0033)

principal components explaining 92.4% of the variation and two discriminant functions were retained. Microsatellite loci B119 and B96, contributed more to define the discriminant function 1 and 2, respectively. DAPC classification of individuals in clusters was consistent with a membership probability > 91.9% in all cases. However, the clustering showed no correspondence between genetic clusters and spatial distribution of the samples (Fig. 3a), with individuals from distant locations included in the three genetic clusters (Fig. 3b).

Given the lack of structure, individuals were finally grouped according to their geographical proximity to calculate population genetics parameters. Allelic richness ranged from 3.8 in Monts d'Ardèche to 4.0 in High Fens. Populations of Sierra de Guadarrama and Pyrenees showed the same value of expected heterozigosity ($H_E = 0.624$) meanwhile the highest inbreeding coefficient value ($F_{IS} = 0.466$) was observed in Sierra de Guadarrama (Table 2).

Significant linkage disequilibrium was not observed for any loci after applying the Bonferroni correction. Only the population of Sierra de Guadarrama showed a significant departure from Hardy–Weinberg equilibrium due to a significant heterozygosity deficiency in the loci B96, B100 and B124 (Table 2). Pairwise differentiation coefficients F_{ST} ranged from 0.0015 to 0.1277 and were not significant in any case after applying the Bonferroni correction (*p*-value < 0.0024) (Table 3). An overall migration rate of Nm = 3.40 was obtained using private alleles.

Geometric morphometrics

The same populations as above were considered in this analysis. When the PCA was performed, the first two principal components explained an accumulated variation of 34.7% (PC1 = 21.4%, PC2 = 13.3%). The scatterplot of variation

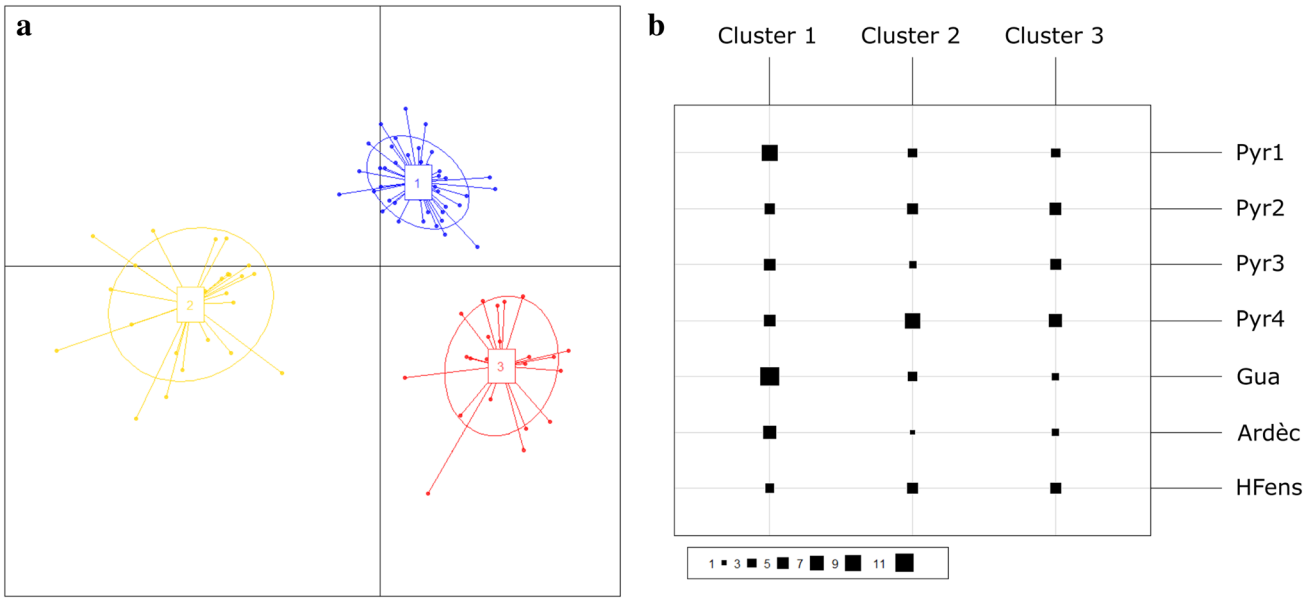

Fig. 3 Results of a nested discriminant analysis of principal components (DAPC). **a** Scatterplot of the first two principal components based on microsatellite genotypes. **b** Distribution of *B. lucorum* populations per cluster

with the two principal components did not show any clear differentiation between the individuals of different populations (Fig. 4a).

The differences between the Mahalanobis distances were significant in all the pairwise comparisons except between the populations from Monts d'Ardèche and High Fens (Table S4). The differences between the Procrustres distances were not significant in any case after applying the Bonferroni correction (Table S5). The total accumulated variation explained by the first two canonical variates was 81.9% (CV1 = 49.1%, CV2 = 32.8%). The scatterplot of variation with the first two canonical variates showed an overlap in the shape of the wings for all the groups although the population of Sierra de Guadarrama seemed more differentiated with a higher within group variance (Fig. 4b). The discrimination between the groups was assessed with a DFA by calculating the percentage of individuals correctly classified to one population of each pairwise comparison with a leave-one-out cross-validation

Table 2 Population genetic parameters for every *B. lucorum* population based on microsatellite variation

	N	A_R	Npa	H_O	uH_E	F_{IS}	H–W
Sierra de Guadarrama	16	3.9	4	0.358	0.624	0.466	B96, B100, B124
Pyrenees	57	3.9	18	0.605	0.624	0.043	–
Monts d'Ardèche	9	3.8	1	0.500	0.535	−0.111	–
High Fens	11	4.0	2	0.577	0.612	0.014	–

Number of individuals (N), allelic richness (A_R) after rarefaction, number of private alleles (Npa), observed (H_O) and unbiased expected heterozigosity (uH_E), inbreeding coefficient (F_{IS}) and loci showing a significant departure from Hardy–Weinberg equilibrium (H–W)

Table 3 Differentiation test values (pairwise F_{ST}) between *B. lucorum* populations based on microsatellite data

	Sierra de Guadarrama	Pyrenees	Monts d'Ardèche	High Fens
Sierra de Guadarrama	NA	0.1878	0.4482	0.0710
Pyrenees	0.0136	NA	0.0370	0.0200
Monts d'Ardèche	0.0015	0.0483	NA	0.0221
High Fens	0.0576	0.0433	0.1277	NA

F_{ST} coefficients are shown in the lower part of the matrix with their corresponding *p*-values for 1000 permutations in the upper part of the matrix

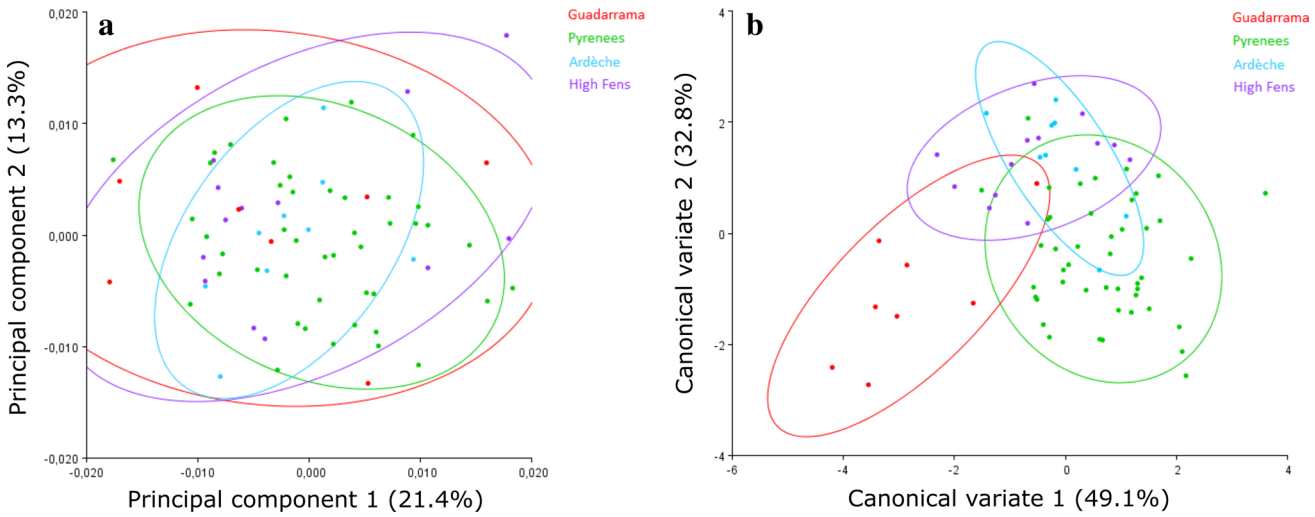

Fig. 4 Results from geometric morphometrics analysis of the wings of *B. lucorum* individuals. **a** Scatterplot showing the first two principal components from the principal components analysis (PCA). **b** Scatterplot showing the first two canonical variates from the canonical variance analysis (CVA)

test. The percentage of correct classifications ranged from 54.5 to 76.2% in all the pairwise comparisons (Table S6).

The results from the ANOVA analyses indicated that there was not a significant effect of the population origin of the individuals on the size of the wings (p value = 0.8822) (Table S7) but significant on the shape of the wings (p-value = 0.0022) that explained a 5.9% of total sum of squares (Table S8).

Discussion

Mitochondrial and nuclear markers were analyzed in *B. lucorum* individuals collected along a European gradient from the centre of the Iberian Peninsula to Belgium, with the aim of increasing our understanding of the genetic diversity and phylogeography of this bumblebee species. The results from the *cox1* gene sequence analysis resolved a divergence of *B. lucorum* from the central Iberian Peninsula from those of the Pyrenees and central Europe. However, microsatellite and geometric morphometrics analyses failed to provide any additional evidence of differentiation.

The haplotype network based in the *cox1* gene showed a clear differentiation between the *B. lucorum* population from Sierra de Guadarrama and those from the rest of Europe, with evidence for an intermediate haplotype in the Cantabrian mountain range. Differentiation tests also supported this distinction with a highly significant Φ_{ST} value obtained between Sierra de Guadarrama and the other populations analyzed. In contrast, the analysis of population structure using microsatellite data, supported no clear genetic differentiation. Although structure analysis identified a similar composition of the two genetic clusters for all the individuals, DAPC analysis did not display a correlation between the geographical location of the populations and the genetic clustering. The contrast between the degree of differentiation obtained with the *cox1* gene and the microsatellite loci could be explained by differences in coalescence times (Boursot and Bonhomme 1986). While the number of microsatellite loci and their high variability make them useful to study local gene flow and inbreeding (Queller et al. 1993), the mitochondrial marker *cox1* has a shorter coalescence time and is sex-biased because of the maternal inheritance of the mitochondrial molecule. Differentiation mirrored in *cox1* gene diversity could reflect a low dispersion of the queens. However, queens are known to migrate long distances (Mikkola 1984; Lepais et al. 2010) so the differentiation observed probably results from the short coalescence time and may reflect a differentiation event that took place in the past. Previous studies within the genus *Bombus* have already observed a contrast between the divergence of mitochondrial genes and the homogeneity with nuclear markers that could be explained due to a current gene flow between populations (Lecocq et al. 2013a, b; Moreira et al. 2015: Maebe et al. 2019).

In the case of the morphometric analysis, a slight differentiation could be seen in the CVA, but this analysis is known to maximize the differences between groups established a priori (Campbell and Atchley 1981). The DFA with a cross validation test and the PCA are more reliable and in both cases did not indicate any population differentiation. PCA failed to resolve the different populations and DFA was unable to classify most individuals according to their population in the leave-one-out cross-validation. Furthermore, insufficient individuals from Sierra de Guadarrama were included in these analyses as most of them were males and only females were used to avoid inter-caste bias (Gerard et al. 2015). Although some studies have discriminated taxa at an intraspecific level with this method (Schachter-Broide et al. 2004; Tofilski 2008; Francoy et al. 2011), there could be a convergent or stabilizing selection on wing shape (Dockx 2007). Nevertheless, given the lack of isolation between the populations as indicated by the microsatellite data, a differentiation in wing morphology would not be expected.

Several studies in other *Bombus* species have found a differentiation in the southern European Peninsulas that support the hypothesis that they could have acted as glacial refugia during the Pleistocene (Widmer and Schmid-Hempel 1999; Lecocq et al. 2013a, 2015). In the case of *B. lucorum*, Bossert et al. (2016) already pointed out that the low genetic diversity of the species through Europe could be indicative of a rapid expansion from a glacial refugium. The divergence observed in the population of Sierra de Guadarrama with respect to European populations supports the hypothesis that the Iberian Peninsula could have acted as a glacial refugium during the Pleistocene glacial cycles. A high haplotype diversity would be expected if the peninsula acted as a refugium, therefore it would be necessary to sample more populations to finally confirm our hypothesis. Alternatively, an expansion from central Europe after the last glacial period should be also tested. Nevertheless, the differentiation found in the gene *cox1* in Sierra de Guadarrama coupled with the high inbreeding value obtained, highlight that populations of central Spain are important when evaluating the genetic diversity of *B. lucorum* as these population parameters are important factors to be measured in conservation strategies (Boettcher et al. 2010).

The lack of population structure observed with microsatellite data and geometric morphometrics is suggestive of ongoing gene flow between the populations of the Iberian Peninsula and those from central Europe. The presence of two samples from Sierra de Guadarrama and the Pyrenees with shared haplotypes supports this view. Microsatellite data obtained for *B. lucorum* and other *Bombus* species have previously indicated that in general, there is gene flow

between bumblebee populations on the mainland of Europe (Estoup et al. 1996; Moreira et al. 2015; Potapov et al. 2018; Maebe et al. 2019) resulting in low levels of genetic differentiation despite of the geographic distance. Only significant geographical barriers, such as seas or mountain ranges seem to result in the reduction or interruption of gene flow what could be mirrored in the microsatellite loci variation (Estoup et al. 1996; Widmer and Schmid-Hempel 1999; Moreira et al. 2015). However, our data suggests that Pyrenees fail to act as a geographical barrier that interrupts gene flow between the northern and southern populations. It would be possible that a rapid expansion of *B. lucorum* from the Iberian Peninsula had caused a genetic homogeneity in microsatellite loci but given the high mutation rate of these markers it is more plausible that a minimum gene flow exists. Nevertheless, the population of Sierra de Guadarrama is the only one where a high inbreeding coefficient and a departure from Hardy–Weinberg equilibrium are observed. These results may reflect a higher degree of isolation or selective pressure in this population with respect to the northern populations analysed, probably affected by the distribution of this species: while in Europe *B. lucorum* is a common and abundant species, in the Iberian Peninsula is restricted to northern mountains (Ornosa and Ortiz-Sánchez 2004).

Although our work provides a first step in understanding the genetic structure and diversity of *B. lucorum* in the Iberian Peninsula, there is a need to sample other Iberian populations to better characterize the genetic and morphology of their populations. This will help to determine the intraspecific variability of this widespread and common species through Europe. Nonetheless, this study highlights the importance of including Iberian populations of bumblebees to determine and characterise their genetic diversity. Given the current situation of decline of insects in general (Sánchez-Bayo and Wyckhuys 2019) and pollinators in particular (Potts et al. 2010; Powney et al. 2019), our results must be taken into account when managing the conservation of bumblebee species. The observed absence of genetic structure suggests that neither geographical barriers such as mountain ranges nor the European agricultural landscape are a barrier to gene flow. Although observed levels of genetic diversity are not as low as those observed in other species of the genus *Bombus* (Maebe et al. 2019), conservation and management plans should be developed to maintain or even increase current levels of genetic diversity. The connectivity of the current populations may be favoured to avoid the impact that changes in the landscape due to human, environmental or climate change pressure could produce limiting gene flow between populations of this species.

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References

Archer FI, Adams PE, Schneiders BB (2017) Stratag: an R package for manipulating, summarizing and analysing population genetic data. Mol Ecol Resour 17:5–11

Aytekin MA, Terzo M, Rasmont P, Çağatay N (2007) Landmark based geometric morphometric analysis of wing shape in Sibiricobombus Vogt (Hymenoptera: Apidae: Bombus Latreille). Ann Soc Entomol Fr 43:95–102

Bandelt HJ, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37–48

Bertsch A (1997) Abgrenzung der Hummel-Arten *Bombus cryptarum* und *B. lucorum* mittels männlicher Labialdrüsen-Sekrete und morphologischer Merkmale (Hymenoptera, Apidae). Entomol Gen 22:129–145

Bertsch A (2009) Barcoding cryptic bumblebee taxa: *B. lucorum*, *B. crytarum* and *B. magnus*, a case study. Beitr Entomol 59:287–310

Bertsch A, Schweer H, Titze A (2004) Discrimination of the bumblebee *species Bombus lucorum*, *B. cryptarum* and *B. magnus* by morphological characters and male labial gland secretions. Beitr Entomol 54:365–386

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). Insect Soc 52:45–54

Boettcher PJ, Tixier-Boichard M, Toro MA, Simianer H, Eding H, Gandini G et al (2010) Objectives, criteria and methods for using molecular genetic data in priority setting for conservation of animal genetic resources. An Genet 41:64–77

Bossert S (2015) Recognition and identification of bumblebee species in the *Bombus lucorum*-complex (Hymenoptera, Apidae)—a review and outlook. Deut Entomol Z 62:19

Bossert S, Gereben-Krenn BA, Neumayer J, Schneller B, Krenn HW (2016) The cryptic *Bombus lucorum* complex (Hymenoptera: Apidae) in Austria: phylogeny, distribution, habitat usage and a climatic characterization based on COI sequence data. Zool Stud 55:2016–2055

Boursot P, Bonhomme F (1986) Génétique et évolution du génome mitochondrial des Métazoaires. Genet Sel Evol 18:73–98

Campbell NA, Atchley WR (1981) The geometry of canonical variate analysis. Syst Biol 30:268–280

Carolan JC, Murray TE, Fitzpatrick Ú, Crossley J, Schmidt H, Cederberg B et al (2012) Colour patterns do not diagnose species: quantitative evaluation of a DNA barcoded cryptic bumblebee complex. PLoS ONE 7:e29251

Cejas D, Ornosa C, Muñoz I, De la Rúa P (2019) Preliminary report on cross-species microsatellite amplification for bumblebee biodiversity and conservation studies. Archivos de Zootecnia 68:422–426

Cooper SJB, Hewitt GM (1993) Nuclear DNA sequence divergence between parapatric subspecies of the grasshopper *Chorthippus parallelus*. Insect Mol Biol 2:185–194

Dehon M, Perrard A, Engel MS, Nel A, Michez D (2017) Antiquity of cleptoparasitism among bees revealed by morphometric and

phylogenetic analysis of a Paleocene fossil nomadine (Hymenoptera: Apidae). Syst Entomol 42:543–554
Dockx C (2007) Directional and stabilizing selection on wing size and shape in migrant and resident monarch butterflies, *Danaus plexippus* (L.), in Cuba. Biol J Linn Soc 92:605–616
Duennes MA, Lozier JD, Hines HM, Cameron SA (2012) Geographical patterns of genetic divergence in the widespread Mesoamerican bumble bee *Bombus ephippiatus* (Hymenoptera: Apidae). Mol Phylogenet Evol 64:219–231
Earl DA (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4:359–361
Estoup A, Scholl A, Pouvreau A, Solignac M (1995) Monoandry and polyandry in bumble bees (Hymenoptera; Bombinae) as evidenced by high variable microsatellites. Mol Ecol 4:89–93
Estoup A, Solignac M, Cornuet J, Goudet J, Scholl A (1996) Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in Europe. Mol Ecol 5:19–31
Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3:294–299
Françoso E, Arias MC (2013) Cytochrome c oxidase I primers for corbiculate bees: DNA barcode and mini-barcode. Mol Ecol Resour 13:844–850
Françoso E, de Oliveira FF, Arias MC (2016) An integrative approach identifies a new species of bumblebee (Hymenoptera: Apidae: Bombini) from northeastern Brazil. Apidologie 47:171–185
Francoy TM, Grassi ML, Imperatriz-Fonseca VL, de Jesús May-Itzá W, Quezada-Euán JJG (2011) Geometric morphometrics of the wing as a tool for assigning genetic lineages and geographic origin to *Melipona beecheii* (Hymenoptera: Meliponini). Apidologie 42:499–507
Gerard M, Michez D, Fournier D, Maebe K, Smagghe G, Biesmeijer JC et al (2015) Discrimination of haploid and diploid males of *Bombus terrestris* (Hymenoptera; Apidae) based on wing shape. Apidologie 46:644–653
Gómez A, Lunt DH (2007) Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: Weiss S, Ferrand N (eds) Phylogeography of southern European refugia. Springer, Netherlands, Dordrecht, pp 155–188
Habel JC, Schmitt T, Müller P (2005) The fourth paradigm pattern of post-glacial range expansion of European terrestrial species: the phylogeography of the Marbled White butterfly (Satyrinae, Lepidoptera). J Biogeogr 32:1489–1497
Ivanova NV, Dewaard JR, Hebert PD (2006) An inexpensive, automation-friendly protocol for recovering high-quality DNA. Mol Ecol Notes 6:998–1002
Jombart T (2008) adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24:1403–1405
Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genet 11:94
Jones OR, Wang J (2010) COLONY: a program for parentage and sibship inference from multilocus genotype data. Mol Ecol Resour 10:551–555
Keenan K, McGinnity P, Cross TF, Crozier WW, Prodöhl PA (2013) diveRsity: an R package for the estimation and exploration of population genetics parameters and their associated errors. Methods Ecol Evol 4(8):782–788
Klingenberg CP (2011) MorphoJ: an integrated software package for geometric morphometrics. Mol Ecol Resour 11:353–357
Kozmus P, Virant-Doberlet M, Meglič V, Dovč P (2011) Identification of *Bombus* species based on wing venation structure. Apidologie 42:472–480
Lecocq T, Dellicour S, Michez D, Lhomme P, Vanderplanck M, Valterová I et al (2013a) Scent of a break-up: phylogeography and reproductive trait divergences in the red-tailed bumblebee (*Bombus lapidarius*). BMC Evol Biol 13:263
Lecocq T, Vereecken NJ, Michez D, Dellicour S, Lhomme P, Valterova I 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, Martinet B, Valterova I, Rasmont P (2015) Highly polytypic taxon complex: interspecific and intraspecific integrative taxonomic assessment of the widespread pollinator *Bombus pascuorum* Scopoli 1763 (Hymenoptera: Apidae). Syst Entomol 40:881–890
Leigh JW, Bryant D (2015) popart: full-feature software for haplotype network construction. Methods Ecol Evol 6:1110–1116
Lepais O, Darvill B, O'Connor S, Osborne JL, Sanderson RA, Cussans J et al (2010) Estimation of bumblebee queen dispersal distances using sibship reconstruction method. Mol Ecol 19:819–831
Maebe K, Karise R, Meeus I, Mänd M, Smagghe G (2019) Pattern of population structuring between Belgian and Estonian bumblebees. Sci Rep 9(1):9651
McKendrick L, Provan J, Fitzpatrick Ú, Brown MJ, Murray TE, Stolle E et al (2017) Microsatellite analysis supports the existence of three cryptic species within the bumble bee *Bombus lucorum* sensu lato. Conserv Genet 183:573–584
Mikkola K (1984) Spring migrations of wasp and bumblebee queens across the Gulf of Finland (Hymenoptera: Vespidae and Apidae). Not Entomol 64:125–128
Moreira AS, Horgan FG, Murray TE, Kakouli-Duarte T (2015) Population genetic structure of *Bombus terrestris* in Europe: isolation and genetic differentiation of Irish and British populations. Mol Ecol 24:3257–3268
Murray TE, Fitzpatrick Ú, Brown MJ, Paxton RJ (2008) Cryptic species diversity in a widespread bumble bee complex revealed using mitochondrial DNA RFLPs. Conserv Genet 9:653–666
Ornosa C, Ortiz-Sánchez FJ (2004) Hymenoptera: Apoidea I. CSIC Press, Madrid
Penado A, Rebelo H, Goulson D (2016) Spatial distribution modelling reveals climatically suitable areas for bumblebees in undersampled parts of the Iberian Peninsula. Insect Conserv Diver 9(5):391–401
Potapov GS, Kondakov AV, Kolosova YS, Tomilova AA, Filippov BY, Gofarov MY et al (2018) Widespread continental mtDNA lineages prevail in the bumblebee fauna of Iceland. ZooKeys 774:141
Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. Trends Ecol Evol 25:345–353
Powney GD, Carvell C, Edwards M, Morris RK, Roy HE, Woodcock BA, Isaac NJ (2019) Widespread losses of pollinating insects in Britain. Nat Commun 10:1018
Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959
Queller DC, Strassmann JE, Hughes CR (1993) Microsatellites and kinship. Trends Ecol Evol 8:285–288
Rasmont P (1984) Les bourdons du genre *Bombus* Latreille sensu stricto en Europe Occidentale et Centrale (Hymenoptera, Apidae). Spixiana 7:135–160
Rasmont P, Franzén M, Lecocq T, Harpke A, Roberts SP, Biesmeijer JC et al (2015) Climatic risk and distribution atlas of European bumblebees. BioRisk 10:1–236
Rohlf FJ (2016) tpsDig2 ver 2.32. Department of Ecology & Evolution, State University of New York, Stony Brook
Rohlf FJ (2017a) tpsSmall ver 1.34. Department of Ecology & Evolution, State University of New York, Stony Brook
Rohlf FJ (2017b) tpsUtil ver 1.74. Department of Ecology & Evolution, State University of New York, Stony Brook

Rousset F (2008) genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol Ecol Resour 8:103–106

Sánchez-Bayo F, Wyckhuys KA (2019) Worldwide decline of the entomofauna: a review of its drivers. Biol Conserv 232:8–27

Schachter-Broide J, Dujardin JP, Kitron U, Gürtler RE (2004) Spatial structuring of *Triatoma infestans* (Hemiptera, Reduviidae) populations from northwestern Argentina using wing geometric morphometry. J Med Entomol 41:643–649

Schmitt T, Seitz A (2004) Low diversity but high differentiation: the population genetics of *Aglaope infausta* (Zygaenidae: Lepidoptera). J Biogeogr 31:137–144

Schutze MK, Jessup A, Clarke AR (2012) Wing shape as a potential discriminator of morphologically similar pest taxa within the *Bactrocera dorsalis* species complex (Diptera: Tephritidae). Bull Entomol Res 102:103–111

Scriven JJ, Woodall LC, Tinsley MC, Knight ME, Williams PH, Carolan JC et al (2015) Revealing the hidden niches of cryptic bumblebees in Great Britain: implications for conservation. Biol Conserv 182:126–133

Smouse RPP, Peakall R (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28(19):2537–2539

Tofilski A (2008) Using geometric morphometrics and standard morphometry to discriminate three honeybee subspecies. Apidologie 39:558–563

Van Oosterhout C, Hutchinson WF, Wills DP, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538

Vesterlund SR, Sorvari J, Vasemägi A (2014) Molecular identification of cryptic bumblebee species from degraded samples using PCR–RFLP approach. Mol Ecol Resour 14:122–126

Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10:506–513

Waters J, Darvill B, Lye GC, Goulson D (2011) Niche differentiation of a cryptic bumblebee complex in the Western Isles of Scotland. Insect Conserv Diver 4:46–52

Widmer A, Schmid-Hempel P (1999) The population genetic structure of a large temperate pollinator species, *Bombus pascuorum* (Scopoli) (Hymenoptera: Apidae). Mol Ecol 8:387–398

Widmer A, Schmid-Hempel P, Estoup A, Scholl A (1998) Population genetic structure and colonization history of *Bombus terrestris* sl (Hymenoptera: Apidae) from the Canary Islands and Madeira. Heredity 81:563–572

Williams PH, Brown MJF, Carolan JC, An J, Goulson D, Aytekin AM et al (2012) Unveiling cryptic species of the bumblebee subgenus *Bombus* s. str. worldwide with COI barcodes (Hymenoptera: Apidae). Syst Biodivers 10:21–56