ANIMAL GENETICS Immunogenetics, Molecular Genetics and Functional Genomics

doi: 10.1111/age.12874

Unveiling introgression in bumblebee (*Bombus terrestris*) populations through mitogenome-based markers

D. Cejas*, A. López-López*,†, I. Muñoz*, C. Ornosa‡ and P. De la Rúa*

*Área de Biología Animal, Departamento de Zoología y Antropología Física, Facultad de Veterinaria, Universidad de Murcia, Murcia 30100, Spain. †Área de Zoología, Departamento de Agroquímica y Medio Ambiente, Campus de Elche, Universidad Miguel Hernández, Elche 03202, Spain. ‡Departamento de Biodiversidad, Ecología y Evolución, Facultad de Ciencias Biológicas, Universidad Complutense, Madrid 28040, Spain.

Summary

The bumblebee, *Bombus terrestris*, is an important pollinator commercially used on a global scale. The exported subspecies *B. t. terrestris* has colonised diverse environments, in some cases displacing wild pollinators to the verge of local extinction. In this sense, the native Iberian subspecies *B. t. lusitanicus* may be threatened by the subspecies *B. t. terrestris*, naturally distributed from the Pyrenees to Central Europe but also observed in southern Spain due to escapes from commercial nests. Mitochondrial genomes have a low recombination rate and a small effective population size owing to their maternal inheritance, thus providing an accurate approach to study hybridisation events between populations. Therefore, we present the sequences of the mitogenomes of both subspecies as a molecular framework to select suitable markers to detect possible introgression events between them. We used metagenomics to obtain approximately 17 kbp of the mitogenome from both subspecies. Their mitogenomes differed in 358 bp (excluding the AT-rich region). Four mitogenomic fragments were selected to be tested as subspecific diagnostic markers. A RFLP detected in the gene *nad2* (NADH dehydrogenase subunit 2) has proven to be an efficient, quick and cost-effective tool to assess the dispersion of the non-endemic subspecies into Iberian native populations. Subspecific haplotypes were observed in both morphological subspecies, suggesting introgression events in the northern natural contact area and in the new human-mediated contact area in the south of the Iberian Peninsula.

Keywords conservation, introgression, mitogenome, molecular markers, pollinators

Introduction

The common bumblebee *Bombus terrestris* (Linnaeus, 1758) is an important pollinator distributed through the Palaearctic biogeographic region (Ornosa & Ortiz-Sánchez, 2004) in both natural and human-disturbed areas. Exportation has led to the species reaching new environments to the detriment of native pollinators. *B. terrestris* commercial breeds are able to easily colonise suitable environments (Kraus et al., 2011) and compete with native pollinators for food resources (Ings, Ward, & Chittka, 2006), thus becoming a threat to their conservation status as seen in endemic bumblebee species (Aizen et al., 2018).

Address for Correspondence

P. De la Rúa, Área de Biología Animal, Departamento de Zoología y Antropología Física, Facultad de Veterinaria, Universidad de Murcia, 30100 Murcia, Spain.
E-mail: pdelarua@um.es

Accepted for publication 04 October 2019

According to geographic distribution and morphological differences, *B. terrestris* can be classified into nine different subspecies (Rasmont, Coppée, Michez, & Meulemeester, 2008). Among them, *B. t. lusitanicus* Krüger, 1956, is native to the Iberian Peninsula. Its distribution range extends beyond the Pyrenees to southern France (Rasmont et al., 2008), possibly due to its ability to expand its altitude range (Ornosa, Torres, & De la Rúa, 2017). Another subspecies, *B. t. terrestris*, naturally occurs in Central Europe reaching the northeastern Pyrenees (Rasmont et al., 2008), and has been one of the most commonly used insects for greenhouse pollination since its domestication in the 1980s (Velthuis & Doorn, 2006). The distribution ranges of these two subspecies have changed during the last few decades. *B. t. terrestris* has been detected in the southern Iberian Peninsula (Ortiz-Sánchez, 1992), where bumblebee breeding companies are located, supplying pollinators to the many greenhouses. Currently, over 1 million colonies are produced commercially every year worldwide (Velthuis & van Doorn, 2006), and their use in Spain is growing (around 300 000 colonies per

year; Agrobío, personal communication) because they improve the quality of pollinated fruits (Klatt et al., 2014, but see Trillo, Herrera, & Vilà, 2018). These managed individuals can escape from agricultural facilities, as seen in Poland (Kraus et al., 2011) and Portugal (Seabra, Silva, & Nunes, 2018), and become naturalised (Cejas, Ornosa, Muñoz, & De la Rúa, 2018; Ornosa, 1996; Ortiz-Sánchez, 1992; Trillo et al., 2019).

The main diagnostic characteristic that discriminates *B. t. lusitanicus* and *B. t. terrestris* is a colour variation in the hair above the corbicula, which is ferruginous brown or black respectively (Ornosa & Ortiz-Sánchez, 2004). These subspecies can hybridise, creating a colour gradient in this feature across their natural contact area in northern Spain and southern France (Rasmont et al., 2008). Molecular approaches, such as the comparison of the pheromones produced by the cephalic labial glands of the male or the sequencing of nuclear markers, have not shown enough differences to distinguish between the two subspecies (Lecocq et al., 2016; Williams, Brown, & Carolan, 2012). However, recent studies have found molecular differences between *B. t. lusitanicus* and commercial breeds in the sequence of the *rrnL* (16S) and *cox*1 mitochondrial genes (Cejas et al., 2018; Seabra et al., 2018), although in both cases the differences were revealed after sequencing a small number of samples.

Although it is less variable than that in vertebrates, insect mitochondrial DNA has a higher mutational rate than nuclear DNA (Allio, Donega, Galtier, & Nabholz, 2017). Furthermore, mitochondrial genomes have a lower recombination rate and a smaller effective population size owing to their maternal inheritance, providing an accurate tool to study hybridisation events between populations and to analyse phylogenetic relationships between closely related taxa (Rubinoff & Holland 2005). However, mutation rates vary along the mitogenome: some of the most common regions used for phylogeny (e.g. *cox*1) might remain tightly preserved when analysing low-level phylogenetic relationships, whereas other regions might have a higher resolution in specific taxa (Cheng, Chen, Wang, Liang, & Lin, 2018). Scanning the mitochondrial genome in search of molecular markers for subspecies or population differences is a growing approach given the reduction in costs of high-throughput sequencing technologies. This perspective has already been used to find high-resolution markers at the population level in various organisms, in plants, mammals and insects (Brandt, Grigorev, & Afanador-Hernández, 2016; Crampton-Platt, Douglas, Zhou, & Vogler, 2016; Donnelly et al., 2016), including bees (Eimanifar, Kimball, Braun, & Ellis, 2018).

By sequencing the mitogenomes of the two *B. terrestris* subspecies present on the Iberian Peninsula, we aim to build a molecular framework to select potential subspecific markers to complement morphological differences between these two subspecies and their hybrids. Our final objectives are (i) to design a cost-effective, and quick test to differentiate the subspecies and (ii) to check possible introgression events between the two taxa. The results of this study will provide new knowledge on the spread of commercial breeds and tools for the conservation of the native Iberian subspecies.

Materials and methods

Sample collection and DNA extraction

Individuals used for sequencing the mitochondrial genome were sampled in the locations shown in Table S1 and Figure S1. The identification of the subspecies of each individual was morphologically performed by one of the co-authors (C. Ornosa). To ensure an adequate amount of DNA for sequencing, three groups of 10 individuals were made: TLSa comprising *B. t. lusitanicus* from central and northern Spain; TLSb comprising *B. t. lusitanicus* from southern Spain; and TTF comprising *B. t. terrestris* from northern France. DNA was extracted from muscular tissue following Quispe-Tintaya, White, Popov, Vijg, and Maslov (2013) using the *minipred* kit of Qiagen (Hilden, Germany), and pooled according to the defined groups. All of the samples were diluted to a final concentration of 15 ng/μl. One TLSa sample was discarded owing to its low DNA concentration.

Sequencing and quality controls

DNA was quantified by a fluorescence-based PicoGreen method using Victor 3 fluorometry (Invitrogen). Pools were shotgun sequenced on an Illumina HiSeq 2000 sequencer using an Illumina TruSeq Nano DNA Library Prep Kit for 350 bp (Macrogen, South Korea). Library read sizes were checked on an Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip. FastQC (Babraham Bioinformatics, 2012) was used to check the quality of the raw data. Reads were filtered before assembly (98.40% bp ≥ Q20).

Assembly, alignment and annotation

Filtered reads were assembled *de novo* using three independent algorithms: CELERA ASSEMBLER (Myers, Sutton, & Delcher, 2000), IDBA-UD (Peng, Leung, YiuS, & Chin, 2012) and NEWBLER (Margulies et al., 2005). The contigs were filtered using complete mitogenomes from GenBank (Table S2) as references to discard non-mitochondrial sequences. The consensus sequence of each group was aligned in Geneious (www.geneious.com) using MAFFT (Katoh & Standley, 2013) with the mitogenomes of *B. terrestris* (KT368150), *B. ignitus* (DQ870926) and *B. hypocrita sapporensis* (NC_011923), and cleaned manually. Consensus sequences were uploaded to MITOS (Bernt et al., 2013) to annotate protein-coding genes (PCGs) and tRNAs. The mitogenome map of *B. t. lusitanicus* was uploaded to CGView (Grant & Stothard, 2008) and then redrawn.

Subspecific variation, primer design and sequencing

The consensus sequences were exported to MEGA v.6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Variable positions, including both SNPs and indels (insertion and deletions), were detected using the mitogenome of *B. t. terrestris* as a reference. PCR primers were designed to amplify potential subspecific markers using Primer3 (Rozen & Skaletsky, 2000).

A dataset including 10 *B. t. lusitanicus* from Spain, five hybrids from the two contact areas at the north and south of the Iberian Peninsula, and 10 *B. t. terrestris* from northern France (Table S3), was used to prove the validity of the selected potential subspecific markers. Individuals were considered hybrids based on a mixed-colour pattern (morphological hybrids) or discrepancies between their morphology and the 16S haplotype (discrepant hybrids) (Cejas et al., 2018).

Total DNA was extracted from the hind leg of each individual to amplify the selected fragments following Ivanova, Dewaard, and Herbert (2006). PCR consisted of an initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing temperature at 52 °C for 1 min, elongation at 72 °C for 1 min and a final extension at 72 °C for 10 min. Amplicons were sequenced by Secugen (Madrid, Spain).

RFLP design and validation

To design a quick and economic test to distinguish the two subspecies, we searched for point mutations compatible with RFLP, comparing the sequences with the information of the restriction enzyme database REBASE (Roberts, Vincze, Posfai, & Macelis, 2007) in GENEIOUS.

To validate the RFLP test and explore the distribution of the *nad2* haplotypes, a third dataset was used consisting of 30 individuals: 10 *B. t. lusitanicus* and 10 *B. t. terrestris* collected in Spain, and 10 *B. t. terrestris* collected from Belgium (Table S4).

DNA extraction and subspecific fragment amplification were performed as previously mentioned. The enzyme digestion for the RFLP analysis was carried out with 0.4 μl of *Taq*I FastDigest enzyme (Thermo Scientific), 1 μl of 10X FastDygest Green Buffer and 10 μl of the amplicon for 30 min at 65 °C.

Results

Mitogenome structure and composition

After filtering, a total of 19765284 and 21211702 reads were obtained for the *B. t. lusitanicus* groups (TLSa and TLSb respectively), and 23009466 reads were obtained for the *B. t. terrestris* group (TTF) (Table S5). Mitogenome sequences of TLSa and TLSb groups were identical so both were combined, and one consensus sequence, named TLS, was used in further analyses. The complete consensus alignment of the control region for any of the two subspecies pools was not obtained. The assembled mitogenomes of *B. t. lusitanicus* and *B. t. terrestris* were 17049 bp and 17232 bp long respectively (Table S6), owing to the presence of intergenic regions of different sizes.

Mitogenome content and gene order were consistent with published data (Figure S2). All 13 PCGs, 22 tRNAs and two rRNAs (large and small rRNA) were obtained for each subspecies. The genes encoded on the heavy (H) and light (L) strands and the start and stop codons for the two subspecies were identical to previously published *Bombus* mitogenomes. The A+T content for both mitogenomes reached 86.2% in *B. t. lusitanicus* and 86.0% in *B. t. terrestris*. The total A+T content in all gene fragments was 84.5%, whereas in the intergenic regions it was 94.4% for *B. t. lusitanicus* and 93.6% for *B. t. terrestris* (Table S6).

Mitogenome variation

The alignment of the two mitogenomes showed variation in 358 positions: 55 corresponded to SNPs and 303 to indels. SNPs were found in 18 positions included in genes (PCGs, transfer genes and the two ribosomal RNAs) and in 37 positions in intergenic regions. Of the SNPs within genes, 83.3% were transitions (A⇔G, C⇔T), whereas in the intergenic regions, these mutations comprised 13.5%. Indels were located mainly in intergenic regions, consisting of fragments from 1 to 120 bp. This long insertion of 120 bp was located between the serine (*trnS*) and phenylalanine (*trnF*) transfer RNA genes in the sequence of *B. t. terrestris*.

Six of the 18 SNPs included in PCGs were non-synonymous in four proteins (NADH dehydrogenase subunits 1, 2, 5 and cytochrome c oxidase subunit I), but they did not change the polarity or the hydrophobic character of the protein. No indels were found within the PCGs, whereas one insertion of 2 bp was detected in the lysine transfer RNA gene (*trnK*), and one deletion of 2 bp and one insertion of 1 bp were detected in the large RNA (*rrnL*) of *B. t. lusitanicus*.

Subspecific diagnostic marker selection

Four fragments (named Bter_*rrnL-trnV*, Bter_*nad5-nad4*, Bter_*cox2-atp6* and Bter_*nad2*) fulfilled the parameters to be tested as diagnostic markers for the two *B. terrestris* subspecies, i.e. they had an appropriate amplicon length (from 330 to 804 bp) and included variable positions that differentiate *B. t. lusitanicus* from *B. t. terrestris* (Table 1).

The four fragments were successfully amplified using the designed primers in the set of 25 individuals (Table S3). After sequencing, some mutations showed no correlation with the subspecies determination of the individuals (Table 2). On the other hand, two SNPs and one insertion

 doi: 10.1111/age.12874

Table 1 Information about the mitogenome fragments tested as subspecific markers.

Fragment code	F-primer sequence	R-primer sequence	Amplicon length (bp)	Coding genes included	Mutations
Bter_*rrnLtrnV*	ACCCTGATACAAAAGGTACAAAAAT	GCCCGTCAGTTTCGTTTATGG	330	*rrnL*, *trnV*	1 SNP 1 indel
Bter_*nad5nad4*	CCCGAATTAAAAGCTAAATTCTTTTGA	TGTGTTTATAATTCAGGAGCTCCA	767	*nad5*, *trnH*, *nad4*	2 SNPs
Bter_*cox2atp6*	GGTCAATGTTCTGAAATTTGTGGA	TGGTGAATTTAATGGAACTAAATGTCT	804	*cox2*, *trnD*, *trnK*, *atp8*, *atp6*	2 SNPs 1 indel
Bter_*nad2*	AGCCTCTTCTAGAATTTATACCCAA	TGTTGTAAATAATGGAAATGAAGAA	676	*nad2*	1 SNP

PCR primer sequences are given in the 5′–3′direction.

Table 2 Sequencing results of the validation of the mitochondrial fragments used to differentiate *Bombus terrestris terrestris* and *B. t. lusitanicus*.

Consensus mitogenome		Bter_*rrnLtrnV*		Bter_*nad5nad4*		Bter_*cox2atp6*			Bter_*nad2*
B. t. terrestris		T–	A	A	A	T	– –	C	G
B. t. lusitanicus		– –	G	G	T	A	AT	T	A
Code	Subspecies								
TLS.71	*B. t. lusitanicus*	TT	G	G	T	A	AT	T	A
TLS.78	*B. t. lusitanicus*	– –	G	G	T	A	AT	T	A
TLS.79	*B. t. lusitanicus*	– –	G	G	T	A	AT	T	A
TLS.89	*B. t. lusitanicus*	TT	G	G	T	A	AT	T	A
TLS.90	*B. t. lusitanicus*	T–	G	G	T	A	AT	T	A
TLS.91	*B. t. lusitanicus*	– –	G	G	T	A	AT	T	A
TLS.94	*B. t. lusitanicus*	– –	G	G	T	A	AT	T	A
TLS.96	*B. t. lusitanicus*	T–	G	G	T	A	AT	T	A
TLS.97	*B. t. lusitanicus*	– –	G	G	T	A	AT	T	A
TLS.98	*B. t. lusitanicus*	T–	G	G	T	A	AT	T	A
THS.01	*B. terrestris**	– –	G	G	T	A	– –	T	A
THS.02	*B. terrestris**	– –	G	G	T	A	AT	T	A
TLS.62	*B. t. lusitanicus***	T–	G	A	T	T	– –	T	G
TLS.77	*B. t. lusitanicus***	– –	G	A	T	A	– –	C	G
TLS.95	*B. t. lusitanicus***	T–	G	A	T	A	– –	T	G
TTF.01	*B. t. terrestris*	– –	A	A	A	T	– –	C	G
TTF.02	*B. t. terrestris*	– –	A	A	A	T	– –	C	G
TTF.04	*B. t. terrestris*	– –	G	A	T	A	– –	T	G
TTF.06	*B. t. terrestris*	– –	A	A	A	T	– –	C	G
TTF.07	*B. t. terrestris*	– –	G	A	T	A	– –	T	G
TTF.09	*B. t. terrestris*	– –	G	A	T	A	– –	T	G
TTF.10	*B. t. terrestris*	– –	A	A	A	T	– –	C	G
TTF.11	*B. t. terrestris*	– –	G	A	T	A	– –	T	G
TTF.23	*B. t. terrestris***	TT	G	G	T	A	AT	T	A
TTF.24	*B. t. terrestris***	– –	G	G	T	A	AT	T	A

Subspecific diagnostic positions are labelled in grey.
Hybrid individuals are marked with asterisks: * morphological hybrids; ** discrepant hybrids whose morphological phenotype and mitochondrial haplotypes differed (Cejas et al., 2018).

within the fragments Bter_*nad5-nad4*, Bter_*cox2-atp6* and Bter_*nad2* did show a correlation with the subspecies identification and, therefore, were proposed as subspecific diagnostic markers.

Design and validation of a RFLP subspecific test

The sequences of the subspecific diagnostic markers were screened to identify potential discriminatory restriction sites. One of them (Bter_*nad2*) showed a unique restriction site for the enzyme *Taq*I that produced a pattern that was easily visible by agarose gel electrophoresis: two fragments in *B. t. terrestris* (cutting at T^CGA, *nad2*-G haplotype) and one fragment in *B. t. lusitanicus* (not cutting at TCAA, *nad2*-A haplotype) (Figure S3).

We validated this RFLP-based tool and explore the *nad2* haplotype distribution in a dataset with 30 individuals (Table S4); *B. t. lusitanicus* from Spain showed the *nad2*-A

haplotype, whereas *B. t. terrestris* from Belgium showed the *nad2*-G haplotype. Individuals morphologically classified as *B. t. terrestris* collected in southern Spain showed both haplotypes, and those with the *nad2*-A haplotype were described as discrepant hybrids.

Discussion

In this study, we have described the mitogenomes of two subspecies of the common buff-tailed bumblebee, *B. t. lusitanicus* and *B. t. terrestris*, to find subspecific markers and validate a cost-effective tool to differentiate both subspecies. By using it we have been able to infer introgression events on the genetic pool of wild native bumblebee populations in Spain owing to the introduction of commercial breeds.

The gene order of the consensus mitogenome was consistent with prior mitochondrial genomes sequenced in other *Bombus* species (Cha et al., 2007; Du et al., 2015; Takahashi, Sasaki, Nishimoto, Okuyama, & Nomura, 2018; Zhao, Wu, Huang, An, & Sun, 2017; Zhao, Wu, Huang, Liang, et al., 2017). The size of the consensus mitogenomes was larger than those in other *Bombus* species and varied in size between the two subspecies, mainly owing to the presence of intergenic regions that are typically observed in other insects (Rortais, Arnold, Alburaki, Legout, & Garnery, 2011). As expected, the coding regions and the rRNA genes did not show size variation. Genes *nad5* and *nad4* did not present stop codons, which is not uncommon in insects (Lin, Song, Li, Wu, & Wan, 2017). The A+T region was not completely sequenced, but we did not expect to find useful subspecific markers in the control region owing to its extreme variability.

We have found subspecific variation between the two mitogenomes, thus confirming the effectiveness of complete mitochondrial sequencing to discriminate close taxa in insects when other common molecular approaches fail (Coppée, Terzo, Valterova, & Rasmont, 2008; Lecocq, Rasmont, Harpke, & Schweiger, 2015; Moreira, Horgan, Murray, & Kakouli-Duarte, 2015). Our results on the mitochondrial variation between subspecies contrast with those of Eimanifar et al. (2018), who searched for molecular markers to differentiate South African *A. mellifera* subspecies. Nevertheless, their results might be influenced by overlapping distribution of subspecies, gene flow owing to beekeeping, a more recent divergence or the retention of ancestral genetic variation in recently diverged lineages.

Most of the differences found between the two mitogenomes were observed in intergenic regions, and the non-synonymous changes within coding regions did not modify the polarity or the hydrophobic character of the protein. The genes with the highest variability were *nad5* and *rrnL*, which were both included in the subspecific marker validation. In total, eight mutations (six SNPs and two indels) along four fragments were tested to validate their ability as subspecific markers. The variability found in Bter_*rrnLtrnV* showed no linkage with any of the two subspecies, in contrast to the pattern found in the genes *trnV*, *nad5*, *atp6* and *trnD*. Two SNPs and one indel within the fragments Bter_*nad5nad4*, Bter_*cox2atp6* and Bter_*nad2* defined a haplotype correlated to the Iberian subspecies *B. t. lusitanicus*; hence, they had enough discriminative power to be used as subspecific markers. Furthermore, the results of the *nad2*-RFLP validation were mainly conclusive, as we observed a correlation between the morphological identification and the presence of specific haplotypes in the individuals of each subspecies. Moreover, we found few contradictions between the subspecies morphological identification and the two haplotypes, what might be related to contemporary introgression events. These results suggest a complex situation of the European *B. terrestris* subspecies due to various events: a natural dispersion in France and an anthropogenic introduction in southern Spain. The fact that discrepant hybrids have been found in northern France means that the expansion of *B. t. lusitanicus* is advanced as described in Lecocq et al. (2015). If this expansion is driven by climate change, *B. t. lusitanicus* could continue to advance towards the rest of Europe (Lecocq et al., 2015). Therefore, studying the current situation in France and the effects of the northward expansion of *B. t. lusitanicus* may be an interesting starting point for investigating possible future scenarios. One consequence of introgression between commercial and native bumblebee populations is that the introgressed alleles from commercial individuals could be less suitable in the local habitat with warmer climate (Seabra et al., 2018), leading to potential negative fitness consequences in the native populations.

On the other hand, the presence of morphological and discrepant hybrids and individuals of *B. t. terrestris* in the south of Spain could become an alarming problem in the future, as the introduction of subspecies could cause problems in native populations (Facon et al., 2011) and have other unknown effects on the health of native pollinator populations. In this sense, introduced subspecies can produce the spillover of pathogens (Graystock et al., 2013), as observed in *Bombus* populations of Chile (Schmid-Hempel et al., 2014) and the USA (Cameron et al., 2011). Such an event has been evoked as one of the drivers of the present decline in pollinators (Goulson, Nicholls, Botías, & Rotheray, 2015) and might be taken into consideration for establishing conservation programmes, including periods of quarantine with appropriate pathogen testing.

To quantify the introduction of commercial breeds of *B. terrestris* in the Iberian Peninsula, we propose the RFLP assay with the restriction enzyme *Taq*I (*nad2* RFLP), which has proved to be a fast and economical tool for introgression studies that normally include a large number of individuals. In addition, given the sequence variability found in the *nad5* and *rrnL* regions and their usefulness for the study of the two *B. terrestris* subspecies present in the Iberian Peninsula, we propose their use in future phylogeographic and

population studies in globally economically important species such as *B. terrestris.*

Acknowledgements

This study was supported by the projects E-RTA2014-00003-C03 (INIA, Spain; European Regional Development Fund), AGL2015-64825-R (MINECO, Spanish Ministry of Economy and Competitivity) and 19908/GERM/2015 of Regional Excellence (Fundación Séneca, CARM). Sampling permissions were obtained from the corresponding authorities (Parques Nacionales de Sierra Nevada and de la Sierra de Guadarrama). We thank Dr. Carlos Ruiz for his technical support and comments on an earlier version, and two anonymous reviewers and the editor for comments on the manuscript. Irene Muñoz is supported by Fundación Séneca (Murcia, Spain) through the post-doctoral fellowship Saavedra Fajardo (20036/SF/16).

Conflict of interest

The authors declare that they have no conflict of interest.

Availability of data

Assembled sequences have been uploaded to Genbank under Accession nos. MK570128 (*Bombus terrestris lusitanicus*) and MK570129 (*Bombus terrestris terrestris*).

References

Aizen, M. A., Smith-Ramírez, C., Morales, C.L. *et al.* (2018). Coordinated species importation policies are needed to reduce serious invasions globally: The case of alien bumblebees in South America. *Journal of Applied Ecology*, **56**, 100–106.

Allio, R., Donega, S., Galtier, N., & Nabholz, B. (2017). Large variation in the ratio of mitochondrial to nuclear mutation rate across animals: Implications for genetic diversity and the use of mitochondrial DNA as a molecular marker. *Molecular Biology and Evolution*, **34**, 2762–72.

Bernt, M., Bleidorn, C., Braband, A. *et al.* (2013). A comprehensive analysis of bilaterian mitochondrial genomes and phylogeny. *Molecular Phylogenetics and Evolution*, **69**, 352–64. https://doi.org/10.1016/j.ympev.2013.05.002

Bioinformatics, B. (2012). *FastQC: A quality control tool for high throughput sequence data*. http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/ Accessed 26 Sep 2018.

Brandt, A.L., Grigorev, K., Afanador-Hernández, Y.M., *et al.* (2016). Mitogenomic sequences support a north-south subspecies subdivision within *Solenodon paradoxus*. *Mitochondrial DNA*, **28**, 662–70.

Cameron, S.A., Lozier, J.D., Strange, J.P., Koch, J.B., Cordes, N., Solter, L.F., & Griswold, T.L. (2011). Patterns of widespread decline in North American bumble bees. *Proceedings of the National Academy of Sciences*, **108**, 662–67.

Cejas, D., Ornosa, C., Muñoz, I., & De la Rúa, P. (2018). Searching for molecular markers to differentiate *Bombus terrestris* (Linnaeus) subspecies in the Iberian Peninsula. *Sociobiology*, **65**, 558–65. https://doi.org/10.13102/sociobiology.v65i4.3442

Cha, S.Y., Yoon, H.J., Lee, E.M. *et al.* (2007). The complete nucleotide sequence and gene organization of the mitochondrial genome of the bumblebee, *Bombus ignitus* (Hymenoptera: Apidae). *Gene*, **392**, 206–20.

Cheng, Y.-C., Chen, M.Y., Wang, J.F., Liang, A.P., & Lin, C.P. (2018). Some mitochondrial genes perform better for damselfly phylogenetics: Species- and population-level analyses of four complete mitogenomes of *Euphaea* sibling species. *Systematic Entomology*, **43**, 702–15.

Coppée, A., Terzo, M., Valterova, I., & Rasmont, P. (2008). Intraspecific variation of the cephalic labial gland secretions in *Bombus terrestris* (L.) (Hymenoptera: Apidae). *Chemistry & Biodiversity*, **5**, 2654–2661.

Crampton-Platt, A., Douglas, W.Y., Zhou, X., & Vogler, A.P. (2016). Mitochondrial metagenomics: Letting the genes out of the bottle. *GigaScience*, **5**, 15.

Donnelly, K., Cottrell, J., Ennos, R.A.,Vendramin, G.G., A'Hara, S., King, S., Perry, A., Wachowiak, W., & Cavers, S. (2016). Reconstructing the plant mitochondrial genome for marker discovery: A case study using *Pinus*. *Molecular Ecology Resources*, **17**, 943–54.

Du, Q., Bi, G., Zhao, E., Yang, J., Zhang, Z., & Liu, G. (2015). Complete mitochondrial genome of *Bombus terrestris* (Hymenoptera: Apidae). *Mitochondrial DNA*, **27**, 4455–56.

Eimanifar, A., Kimball, R.T., Braun, E.L., & Ellis, J.D. (2018). Mitochondrial genome diversity and population structure of two western honey bee subspecies in the Republic of South Africa. *Scientific Reports*, **8**, 1333.

Facon, B., Crespin, L., Loiseau, A., Lombaert, E., Magro, A., & Estoup, A. (2011). Can things get worse when an invasive species hybridizes? The harlequin ladybird *Harmonia axyridis* in France as a case study. *Evolutionary Applications*, **4**, 71–88.

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E.L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, **347**, 1255957.

Grant, J.R., & Stothard, P. (2008). The CGView server: A comparative genomics tool for circular genomes. *Nucleic Acids Research*, **36**, 181–184.

Graystock, P., Yates, K., Evison, S.E., Darvill, B., Goulson, D., & Hughes, W.O. (2013). The Trojan hives: Pollinator pathogens, imported and distributed in bumblebee colonies. *Journal of Applied Ecology*, **50**, 1207–15. https://doi.org/10.1111/1365-2664.12134

Ings, T.C., Ward, N.L., & Chittka, L. (2006). Can commercially imported bumble bees out-compete their native conspecifics? *Journal of Applied Ecology*, **43**, 940–48.

Ivanova, N., Dewaard, J., & Herbert, D. (2006). An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Molecular Ecology Notes*, **6**, 998–1002. https://doi.org/10.1111/j.1471-8286.2006.01428.x

Katoh, K., & Standley, D.M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, **30**, 772–80.

Klatt, B.K., Holzschuh, A., Westphal, C., Clough, Y., Smit, I., Pawelzik, E., & Tscharntke, T. (2014). Bee pollination improves crop quality, shelf life and commercial value. *Proceedings of the Royal Society B: Biological Sciences*, **281**, 2013244.

Kraus, F., Szentgyörgyi, H., Rozej, E., Rhode, M., Moron, D., Woyciechowski, M., & Moritz, R.F.A. (2011). Greenhouse bumblebees (*Bombus terrestris*) spread their genes into the wild. *Conservation Genetics*, **12**, 187–92.

Lecocq, T., Coppée, A., Michez, D., Brasero, N., Rasplus, J.Y., Valterová, I., & Rasmont, P. (2016). The alien's identity: consequences of taxonomic status for the international bumblebee trade regulations. *Biological Conservation* **195**, 169–76.

Lecocq, T., Rasmont, P., Harpke, A., & Schweiger, O. (2015). Improving international trade regulation by considering intraspecific variation for invasion risk assessment of commercially traded species: The *Bombus terrestris* case. *Conservation Letters*, **9**, 281–89.

Lin, Z.Q., Song, F., Li, T., Wu, Y.Y., & Wan, X. (2017). New mitogenomes of two Chinese stag beetles (Coleoptera, Lucanidae) and their implications for systematics. *Journal of Insect Science*, **17**, 63.

Margulies, M., Egholm, M., Altman, W.E. *et al.* (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature*, **437**, 376–80. https://doi.org/10.1038/nature03959

Moreira, A., Horgan, F., Murray, T., & Kakouli-Duarte, K. (2015). Population genetic structure of *Bombus terrestris* in Europe: Isolation and genetic differentiation of Irish and British populations. *Molecular Ecology*, **24**, 3257–68.

Myers, E.W., Sutton, G.G., Delcher, A.L., *et al.* (2000). A whole-genome assembly of *Drosophila*. *Science*, **287**, 2196–2204.

Ornosa, C. (1996). Una nota de atención sobre la introducción artificial de subespecies foráneas de abejorros polinizadores en la Península Ibérica (Hymenoptera, Apidae, Bombinae). *Boletín De La Asociación Española De Entomología*, **20**, 259–60.

Ornosa, C., & Ortiz-Sánchez, F. (2004). *Hymenoptera: Apoidea I, Fauna Ibérica (Vol. 23)*. Madrid: Museo de Ciencias Naturales, CSIC, 553.

Ornosa, C., Torres, F., & De la Rúa, P. (2017). Updated list of bumblebees (Hymenoptera: Apidae) from the Spanish Pyrenees with notes on their decline and conservation status. *Zootaxa*, **4237**, 41–77. https://doi.org/10.11646/zootaxa.4237.1.3

Ortiz-Sánchez, F. (1992). Introducción de *Bombus terrestris terrestris* (Linnaeus, 1758) en el Sur de España para la polinización de cultivos en invernadero (Hymenoptera, Apidae). *Boletín De La Asociación Española De Entomología*, **16**, 247–48.

Peng, Y., Leung, H.C.M., YiuS, M., & Chin, F.Y.L. (2012). IDBA-UD: A *de novo* assembler for single cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics*, **28**, 1420–28.

Quispe-Tintaya, W., White, R.R., Popov, V.N., Vijg, J., & Maslov, A.Y. (2013). Fast mitochondrial DNA isolation from mammalian cells for next-generation sequencing. *BioTechnique*, **55**, 133–6.

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*, **44**, 243–50.

Roberts, R.J., Vincze, T., Posfai, J., & Macelis, D. (2007). REBASE-enzymes and genes for DNA restriction and modification. *Nucleic Acids Research*, **35**, 269–70. https://doi.org/10.1093/nar/gkl891

Rortais, A., Arnold, G., Alburaki, M., Legout, H., & Garnery, L. (2011). Review of the *Dra*I COI-COII test for the conservation of the black honeybee (*Apis mellifera mellifera*). *Conservation Genetics Resources*, **3**, 383–91.

Rozen, S., & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. In S. Misener, & S. A. Krawetz (Eds.), *Bioinformatics Methods and Protocols. Methods in Molecular Biology™*, Vol. **132**. Totowa, NJ: Humana Press.

Rubinoff, D., & Holland, B.S. (2005). Between two extremes: Mitochondrial DNA is neither the panacea nor the nemesis of phylogenetic and taxonomic inference. *Systematics Biology*, **54**, 952–61. https://doi.org/10.1080/10635150500234674

Schmid-Hempel, R., Eckhardt, M., Goulson, D., *et al.* (2014). The invasion of southern South America by imported bumblebees and associated parasites. *Journal of Animal Ecology*, **83**, 823–37.

Seabra, S.G., Silva, S.E., Nunes, V.L., *et al.* (2018). Genomic signatures of introgression between commercial and native bumblebees, *Bombus terrestris*, in western Iberian Peninsula – implications for conservation and trade regulation. *Evolutionary Applications*, **00**, 1–13.

Takahashi, J., Sasaki, T., Nishimoto, M., Okuyama, H., & Nomura, T. (2018). Characterization of the complete sequence analysis of mitochondrial DNA of Japanese rare bumblebee species *Bombus cryptarum florilegus*. *Conservation Genetics Resources*, **10**, 387–91. https://doi.org/10.1007/s12686-017-0832-z

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis Version 6.0. *Molecular Biology and Evolution*, **30**, 2725–29. https://doi.org/10.1093/molbev/mst197

Trillo, A., Herrera, J.M., & Vilà, M. (2018). Managed bumble bees increase flower visitation but not fruit weight in polytunnel strawberry crops. *Basic and Applied Ecology*, **30**, 32–40. https://doi.org/10.1016/j.baae.2018.05.008

Trillo, A., Montero-Castaño, A., González-Varo, J.P., González-Moreno, P., Ortiz-Sánchez, F.J., & Vilà, M. (2019). Contrasting occurrence patterns of managed and native bumblebees in natural habitats across a greenhouse landscape gradient. *Agriculture, Ecosystems & Environment*, **272**, 230–36.

Velthuis, H.H.W., & Doorn, A. (2006). A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie*, **37**, 421–51. https://doi.org/10.1051/apido:2006019

Williams, P.H., Brown, M.J., Carolan, J.C., *et al.* (2012). Unveiling cryptic species of the bumblebee subgenus *Bombus* s. str. worldwide with COI barcodes (Hymenoptera: Apidae). *Systematics and Biodiversity*, **10**, 21–56.

Zhao, X., Huang, J., Sun, C., & An, J. (2017). Complete mitochondrial genome of *Bombus consobrinus* (Hymenoptera: Apidae). *Mitochondrial DNA Part B*, **2**, 770–2.

Zhao, X., Wu, Z., Huang, J., Liang, C.,An, J., & Sun, C. (2017). Complete mitochondrial genome of *Bombus breviceps* (Hymenoptera: Apidae). *Mitochondrial DNA Part B*, **2**, 604–6.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Sampling information of the individuals used to obtain the mitogenome sequences.

Table S2 Mitochondrial genome sequences used as references during the mitogenome assembly.

Table S3 Sampling information of the individuals used for the validation of the subspecific markers.

Table S4 Sampling information of the individuals used for the validation of the RFLP test.

Table S5 Results of the mitogenome sequencing.

Table S6 Mitogenome organisation of the studied subspecies of *Bombus terrestris*. *trn*, transfer RNA labelled by the one-letter amino acid code; *rrnL*, large subunit of ribosomal gene; *rrnS*, small subunit of ribosomal gene.

Figure S1 Map displaying location of origin for samples of *Bombus terrestris* analysed.

Figure S2 The mitochondrial genome map of *B. t. lusitanicus* in the absence of the complete control region.

Figure S3 Validation test of the Bter_*nad2* RFLP. Different RFLP patterns are due to the *Taq*I restriction enzyme that recognises a T^CGA site.