UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Conservation Genetics of Pollinators: Situation of the Bumblebee Species *Bombus terrestris* in the Iberian Peninsula

Genética de la Conservación de Polinizadores: Situación de la Especie de Abejorro *Bombus terrestris* en la Península Ibérica

D. Diego Manuel Cejas Acuña

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2020

Directoras: Mª Pilar de la Rúa Tarín e Irene Muñoz Gabaldón

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No one spoke.

Then Snufkin said slowly: “It would be awful if the world exploded. It is so wonderfully splendid.”

Comet in Moominland, Tove Jansson. 1946.

Contribution Statement

Chapter 1

This chapter was published in Archivos de Zootecnia. I share coauthorship with Concepción Ornosa, who provided samples and subspecific determination; and Irene Muñoz and Pilar de la Rúa, who contributed with manuscript writing and revision. Ana Asensio and Laura Jara helped with technical support. I contributed with experimental work, data analysis and manuscript writing.

Chapter 2

This chapter was published in Sociobiology. I share coauthorship with Concepcion Ornosa, who organized the sampling and determined the subspecies of the individuals; Irene Muñoz, who contributed with data analysis and result interpretation; and Pilar de la Rúa, who contributed to the design of the experiments and manuscript writing. All coauthors contributed to the revision of the article. I carried out the experimental work, data analysis, result interpretation and writing of the manuscript. Ana Asensio and Vicente López helped with technical support.

Chapter 3

This chapter was published in Animal Genetics. I share coauthorship with Alejandro López-López, who contributed to the methodology revision and bioinformatic analyses; Concepción Ornosa, who contributed with the subspecific determination; and Irene Muñoz and Pilar de la Rúa, who contributed to the experiment design, result interpretation and manuscript writing. All coauthors contributed to the revision of the article. I carried out the laboratory work and bioinformatic analyses, result interpretation and writing of the manuscript. Dr. Carlos Ruiz and Ana Asensio provided advice and technical support.

Chapter 4

The manuscript corresponding to this chapter is in preparation for publication. I share coauthorship with Concepción Ornosa and Denis Michez, who contributed with samples and subspecific determination of the individuals; and Irene Muñoz and Pilar de la Rúa, who contributed with the experiment design, result interpretation and manuscript writing. All coauthors contributed to the revision of the manuscript. I carried out the experimental work, data analysis, result interpretation and writing of the manuscript. Ana Asensio and Nuria Blasco provided technical support.

This thesis was revised by Tamara Gómez, Baptiste Martinet & Laura Bortolotti

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Introducción

(Spanish summary)

La sexta extinción masiva

En la historia geológica del planeta, sabemos de la existencia de cinco acontecimientos en los que las condiciones ambientales se volvieron tan devastadoras que la vida en la Tierra fue erradicada casi por completo: las extinciones masivas. Aunque es difícil hacer estimaciones sobre la diversidad perdida ya que no todas las especies dejan registros fósiles (Plotnick et al., 2016), una extinción en masa se describe como la pérdida de más del 75% de las especies en un período de menos de dos millones de años. Según esta definición, las tasas actuales de extinción de la mayoría de los vertebrados y de reducción de biomasa sugieren que nos encontramos ante la sexta extinción en masa (Barnosky et al., 2011).

El impacto de la vida humana en la naturaleza está llevando al planeta a una crisis de diversidad. Los cambios en el uso del suelo, la contaminación, el aceleramiento del cambio climático o la introducción de especies exóticas son algunos de los principales factores de extinción, todos ellos influidos directamente por el aumento de la población mundial y los métodos de obtención y consumo de recursos (Diaz et al., 2019). En este contexto, la Biología de la Conservación surge con el fin de proteger la diversidad de especies y ecosistemas de estas amenazas, mediante el estudio de su impacto y la propuesta de medidas y acciones que puedan mitigar nuestra huella en el medio ambiente (Soulé, 1985).

El declive de las abejas

La acción humana en el medio ambiente afecta severamente a la entomofauna (Habel et al., 2019). En la década de los 80 se detectaron las primeras evidencias de un declive poblacional de abejas silvestres (Williams, 1982). Dado lo estrechamente ligado que se encuentra el bienestar de los polinizadores al humano y los riesgos asociados a una reducción de sus servicios ecosistémicos,

la vulnerabilidad de las poblaciones de abejas manejadas y silvestres se ha estudiado más a fondo en las últimas décadas. Hoy en día, aunque todavía existen muchas lagunas sobre la distribución y la abundancia de especies en muchas partes del mundo (Potts et al, 2016), se supone que el declive de las abejas es mundial, tal y como se observa en los datos sobre disminución de la abundancia de las especies, el aumento del número de especies amenazadas en las listas rojas de todo el mundo y los eventos de extinción (Williams & Osborne, 2009; Cameron et al., 2011; Schmid-Hempel et al., 2014; Goulson et al., 2015; Potts et al., 2016, Drossart et al., 2019). Los factores de declive identificados son varios, y es importante señalar que sus efectos pueden ser sinérgicos (Brook et al., 2008; Goulson et al., 2015), por lo que las propuestas y medidas de gestión y conservación deben presentarse desde un enfoque holístico.

Pérdida de hábitat

La pérdida de hábitat debida a los cambios en el uso del suelo y a la agricultura intensiva es el principal factor del declive de los insectos (Habel et al., 2019). Esto es debido a que la destrucción del hábitat conlleva la pérdida de recursos y sitios de nidificación, e indirectamente, a la fragmentación y el aislamiento de las poblaciones (Brown & Paxton, 2009; Goulson et al., 2010a). El aislamiento y la reducción del tamaño de las poblaciones, a su vez, darán lugar a un aumento de la endogamia y, por lo tanto, a la pérdida de diversidad genética (Ellis et al., 2006). Estas circunstancias pueden hacer que la población alcance la extinción local, ya que conforme se reduce el número de individuos se reducen las posibilidades de encuentro entre sus miembros (efecto Allee).

Cambio climático

Aunque es difícil evaluar todos los efectos del cambio climático, las pruebas demuestran que la fenología de los himenópteros está cambiando con las variaciones climáticas (Duchenne et al., 2020); sin embargo, hay que tener en

cuenta que los efectos sobre las interacciones planta-polinizador actuales siguen sin estar claros (Hegland et al., 2009). El cambio climático está asociado no sólo con una regresión de los rangos de distribución de las abejas silvestres hacia altitudes más altas y gradientes septentrionales (Kerr et al., 2015; Ornosa et al., 2017), sino también con eventos de extinción local y cambios drásticos en la riqueza de especies de los ecosistemas (Soroye et al., 2020).

Plaguicidas

En la última década se ha debatido extensamente sobre el impacto del uso de plaguicidas en la entomofauna. Se ha demostrado que incluso el uso de herbicidas es un problema para los polinizadores, ya que reduce la densidad de las flores en el cultivo (Goulson et al., 2010b). Sin embargo, sacar conclusiones del efecto directo de los plaguicidas ha sido más difícil, dada su potencial capacidad sinérgica con el resto de los vectores de declive (Collison et al., 2016; Woodcock et al., 2017).

Los neonicotinoides son un grupo de insecticidas que actúan sobre el sistema nervioso central de los insectos. Los efectos de las dosis subletales en los polinizadores han sido ampliamente estudiados, algunos de los cuales son parálisis, reducción en la capacidad de búsqueda de alimento y geolocalización, o una reducción en el crecimiento de las colonias (Whitehorn et al., 2012; Goulson et al., 2015). Tras una moratoria en 2013, hay indicios que apoyan su implicación en el declive de las abejas, razón por la cual el uso de algunos insecticidas neonicotinoides se prohibió en la Unión Europea a partir de 2018 (EFSA, 2018). Aunque queda mucho por descubrir sobre la implicación de los plaguicidas en el declive de las abejas, y en particular en el declive de las abejas silvestres (Potts et al., 2016), la legislación debería ser más rigurosa y sólida para evitar el uso de productos insuficientemente probados (Sgolastra et al., 2020).

Dispersión de patógenos

Los patógenos desempeñan un papel importante en la dinámica de las poblaciones ya que la relación coevolutiva entre patógeno y huésped moldea el sistema inmunológico y las poblaciones del huésped (Evans et al., 2007). Sin embargo, la introducción de nuevos patógenos y la expansión de las enfermedades que éstos causan pueden ser devastadoras. La exportación internacional de colonias comerciales, con mayor sensibilidad al contagio de enfermedades al ser criadas en gran densidad (Goulson et al., 2010c; Graystock et al., 2016) o la trashumancia de las abejas de la miel facilita la propagación de nuevas enfermedades, ya que los individuos foráneos actúan como vectores del patógeno para las poblaciones locales (Muñoz et al., 2014a; Potts et al., 2016; Chandler et al., 2019). Uno de los casos más conocidos es la dispersión del ácaro *Varroa destructor* debido al contacto de la abeja europea con la abeja asiática *Apis cerana* (Rosenkranz et al., 2010). *V. destructor* es además vector del virus de las alas deformadas (DWV) y el efecto combinado de ambos patógenos ha causado estragos en las poblaciones de la abeja de la miel en Europa y América del Norte (Nazzi et al., 2012). En el caso de otro patógenos también se ha descrito la transmitisión a otras especies de abejas silvestres (Fürst et al., 2014).

Especies exóticas invasoras

Para mejorar la productividad de los cultivos, dependemos del uso de polinizadores comerciales (Lautenbach et al., 2012; Kleijn et al., 2015). La exportación de especies a lugares donde son exóticas puede llevar a su desarrollo como especies invasoras (Westphal et al., 2008) ya que, si la población foránea coloniza el medio y se expande, se convertirá en un competidor del resto de los polinizadores locales (Inoue et al., 2008), y afectará a las interacciones dentro de los ecosistemas. Además, puede dar lugar a eventos de introgresión en caso de que las poblaciones de las especies locales se reproduzcan con las introducidas (Muñoz et al., 2014b; Ellis et al., 2018). En esta tesis, se investiga con mayor

profundidad este último factor de declive y su impacto en la diversidad genética de las poblaciones locales de abejorros *B. terrestris* en la península Ibérica.

La especie modelo *Bombus terrestris*

El género *Bombus*

El género *Bombus* (Orden Hymenoptera, Familia Apidae) está compuesto por 250 especies agrupadas en 15 subgéneros. Cada género presenta una gran variabilidad en cuanto a las necesidades alimentarias, los patrones de color, las preferencias de hábitat y la fenología, aunque conservan una estructura morfológica similar (Williams et al., 2008).

Distribución

El abejorro común *Bombus terrestris* (Linnaeus, 1758) muestra un tamaño relativamente grande entre los miembros de la Familia Apidae y está cubierto por un denso pelaje. Son polinizadores con una dieta muy generalista que utilizan su fuerte zumbido para agitar las anteras de las flores de las angiospermas y así recoger polen en su cesta o corbícula (Buchmann & Hurley, 1978). Esta especie de abejorro es una de las más extendidas, con una gran abundancia dentro de su área de distribución, principalmente situada en Europa, desde el norte de África hasta Suecia, y alrededor de la cuenca del Mediterráneo (Rasmont et al., 2008). Investigaciones recientes han registrado la expansión de su área de distribución hacia el norte, alcanzando el Círculo Polar Ártico al norte de Noruega (Martinet et al., 2015), y hacia el este alcanzando Mongolia (Williams et al., 2012). Según la UICN (Unión Internacional para la Conservación de la Naturaleza) no se considera una especie amenazada (Least Concern, LC), aunque los modelos de distribución según diferentes escenarios de cambio climático predicen que su área de distribución se reducirá en gran medida en el sur de Europa y desaparecerá de África en los próximos 100 años (Rasmont et al., 2015).

La especie presenta nueve subespecies a lo largo de su distribución geográfica, con diferencias en su patrón de color.

Fenología

B. terrestris es un insecto eusocial con castas de individuos que cohabitan en colonias subterráneas, incluyendo una reina y su progenie de obreras. Las dos castas realizan diferentes tareas: mientras que la reina pone huevos, las obreras protegen y mantienen la colonia. Algunas de las principales funciones de las obreras son: criar y alimentar a las larvas, mantener una temperatura basal en la colonia (Heinrich, 2004), retirar los cadáveres y los individuos enfermos para evitar la propagación de enfermedades (Jandt & Dornhaus, 2004) y forrajear o pecorear néctar y polen. Se ha registrado que su área de pecoreo se extiende a más de 700 metros de sus colonias (Knight et al., 2005; Nagamitsu et al., 2009), y que utilizan la comunicación por feromonas para reclutar más pecoreadoras cuando se han encontrado nuevos recursos (Dornhaus & Chittka, 2001).

En los abejorros, la principal diferencia fenotípica entre las castas es el tamaño y, en algunos casos, los patrones de color. En el género *Bombus* las reinas no han perdido sus corbículas, lo que les permite pasar parte de su ciclo de vida forrajeando solas. En las obreras, la variación de tamaño es alta y aunque no hay una adscripción estricta de las funciones, las obreras de mayor tamaño tienden a convertirse en recolectoras (para lo cual son más productivas), mientras que las obreras más pequeñas tienden a realizar tareas dentro de la colonia (Spaethe et al., 2002). Otros comportamientos, como la alimentación de la cría o la termorregulación de la colonia mediante aleteo, se ven menos afectados por el tamaño y más condicionados por otros factores, como la genética, la mediación hormonal o las experiencias individuales, lo que pone de relieve la complejidad del comportamiento social y la importancia de la diversidad intracolonial (Jandt & Dornhaus, 2014).

El sexo de los individuos está regulado por un sistema de determinación sexual complementaria (sl-CSD). La heterocigosidad en este *locus* da lugar al fenotipo femenino, y la hemicigosidad (cuando sólo hay un conjunto de cromosomas) o la homocigosidad da lugar al fenotipo masculino (Van Wilgenburg et al., 2006). Dada la gran diversidad de alelos en el *locus* sl-CSD, lo más probable es que los individuos diploides sean heterocigotos y se desarrollen como hembras. Por otra parte, cuando un individuo es haploide, sólo hay un alelo y por lo tanto es hemizoide y se desarrolla como macho.

Las obreras son capaces de poner huevos, aunque el desarrollo ovárico y la oviposición son inhibidos en su mayor parte por las feromonas y el comportamiento de la reina y otras obreras (Alaux et al., 2004; Zanette et al., 2012). Sin el apareamiento, las obreras sólo pueden poner huevos haploides, ya que son huevos no fecundados, mientras que las reinas ponen tanto huevos no fecundados (haploides, se desarrollaran como machos), como fecundados (diploides, se desarrollaran como hembras).

Sin embargo, al no haber comportamientos que impidan el apareamiento entre individuos estrechamente relacionados, o en caso de escasa diversidad genética en la población, es posible que un individuo diploide presente el mismo alelo en ambos cromosomas (homocigótico) desarrollándose como macho diploide (Bogo et al., 2018). Los machos diploides son canibalizados por las obreras de *A. mellifera* en su etapa larvaria temprana, pero en las colonias de abejorros se desarrollan normalmente, lo que afecta a la tasa de crecimiento de la colonia y hace que la población de obreras descienda al 50% (Duchateau et al., 1994), ya que la mitad de la cría sería diploide en el *locus* sl-CSD. La aparición de machos diploides puede ser perjudicial, ya que presentan una vida más corta, tienen una respuesta inmunológica más débil que los machos haploides y producen espermatozoides diploides (Gosterit et al., 2016a). Si una reina se aparea con un macho diploide,

la descendencia será triploide e infértil, la mayoría de las obreras serán inviables, dando lugar a una colonia pequeña y débil (Ayabe et al., 2004).

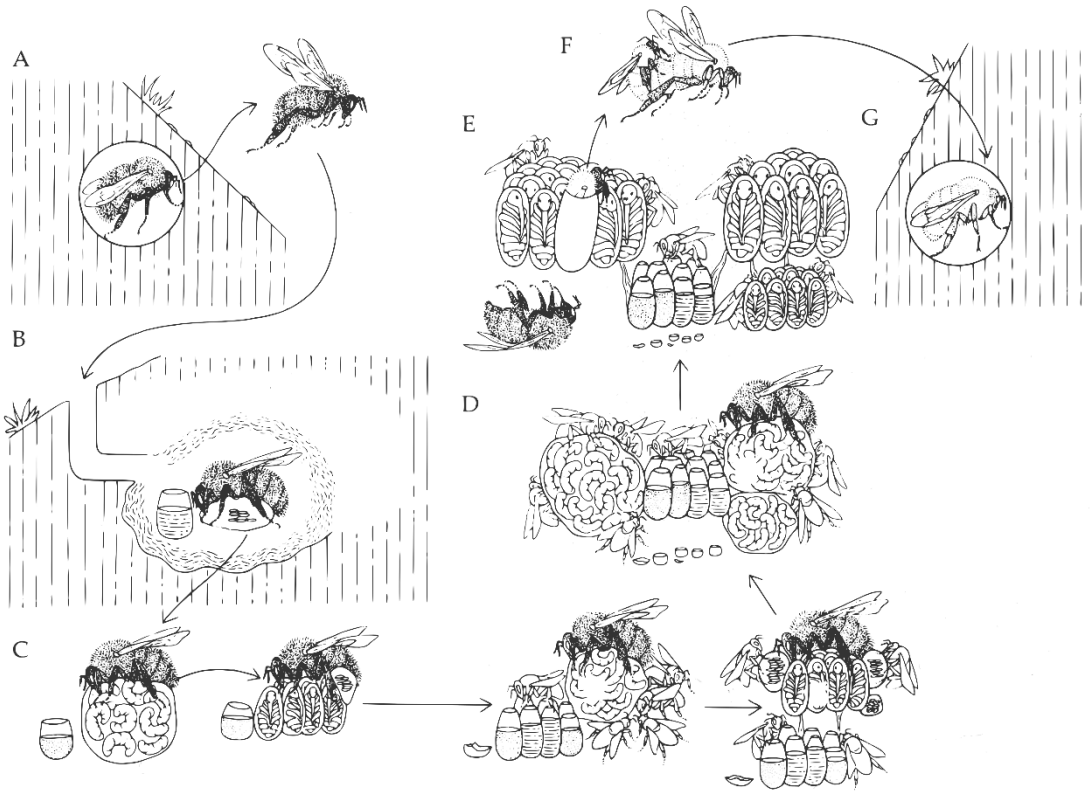

Figura 1. Diagrama del ciclo de una colonia de abejorros. A. La reina emerge de la diapausa. B. La reina encuentra una ubicación para hacer la puesta. C. La reina cuida de la primera generación de obreras que se encargará de forrajear y cuidar a las crías. D. Una colonia completamente operativa, la reina pone huevos mientras que las obreras cuidan de la colonia. E Pasado el punto de competición, la reina madre muere y las nuevas reinas son alimentadas hasta la madurez. F. Una reina y un macho copulando. G. La reina que ya se ha apareado encuentra un lugar en el que comenzar la diapausa. Modificado de Bernard Heinrich (2004).

B. terrestris tiene un ciclo anual, aunque es el único abejorro europeo que puede convertirse en multivoltínico (más de dos ciclos anuales, Rasmont et al., 2015), tal y como se ha observado en el sur de Europa (De Jonghe, 1986). Las reinas emergen de su diapausa (Fig 1.A) normalmente a principios de la primavera en la mayor parte de Europa o en otoño en el Mediterráneo, aunque su despertar puede sincronizarse con la floración de un recurso importante (Gurel et al., 2008).

Las reinas se alimentan por sí solas hasta que encuentran agujeros en el suelo o madrigueras vacías de pequeños roedores para establecer sus colonias y comenzar a incubar su primera puesta (Fig 1.B) (Goulson et al., 2010b). Los nidos de *B. terrestris* no siguen la arquitectura hexagonal de los panales de la abeja de la miel, sino que presentan celdas esféricas donde almacenan el polen o crían larvas. Cuatro o cinco semanas más tarde, la primera generación eclosiona (Fig 1.C), y las nuevas obreras reemplazan a la reina en sus tareas de búsqueda de alimento, almacenamiento de polen y cuidado de la cría (Fig 1.D) (Goulson et al., 2010b).

Al final del ciclo (normalmente al final del verano), la reina cambia su comportamiento y deja de poner nuevos huevos de obrera y comienza a poner huevos haploides de macho, en lo que se llama el punto de cambio de la colonia. La reina pierde el dominio sobre la colonia y cesa la inhibición por feromonas, de forma que los últimos lotes de huevos diploides se convierten en las nuevas reinas después de ser alimentadas por las obreras (Fig 1.E). Aunque no está claro cómo las colonias alcanzan el punto de cambio, ya que las larvas de los machos y las reinas necesitan más tiempo y nutrientes para desarrollarse plenamente, se cree que los factores desencadenantes de la maduración de la colonia podrían ser la relación obrera/larva, el estrés de la reina debido a la densidad de obreras (Alaux et al., 2004; Goulson et al., 2010b) o incluso un control total del proceso por parte de la reina (Duchateau & Velthuis, 1988; Holland et al., 2013). Cuando las obreras cambian su comportamiento para empezar a poner huevos no fecundados, la colonia alcanza el punto de competición (Duchateau & Velthuis, 1988). La ingesta de huevos y la dinámica de eliminación de huevos por parte de las obreras (*worker policing*) se hacen más frecuentes, afectando sobre todo a los huevos puestos por las obreras (Zanette et al., 2012). En este punto, la reina madre ha perdido el dominio sobre la colonia. Las agresiones entre las obreras y de las

obreras a la reina llevan a la colonia a un punto de no retorno y la colonia colapsa (Duchateau & Velthuis, 1988).

Una vez que han alcanzado la madurez completa, los machos abandonan la colonia, mientras que las nuevas reinas se quedan hasta el momento del apareamiento. Los machos vuelan a mayor distancia que las obreras, lo que es una actividad útil para mantener el flujo de genes y reducir la endogamia dentro de las poblaciones (Kraus et al., 2009). Los machos atraen a reinas compatibles con las feromonas de sus glándulas labiales cefálicas para aparearse (Fig 5.F) (Valterová et al., 2019). A diferencia de la abeja de la miel, las reinas de *B. terrestris* son monándricas, ya que se aparean con un único macho para fecundar sus huevos (Estoup et al., 1996), mientras que los machos pueden aparearse con varias reinas. Cuando las reinas se han apareado, inician la diapausa y el ciclo comienza de nuevo (Fig 5.G) (Goulson et al., 2010b).

Subespecies

Las nueve subespecies de *B. terrestris* actualmente reconocidas tienen una distribución geográfica particular. Mientras que las subespecies insulares están aisladas del continente, las subespecies continentales presentan, en algunos casos, un gradiente fenotípico, de modo que el fenotipo de una subespecie cambia gradualmente conforme se introduce en el rango de distribución de una subespecie colindante. Dadas las sutiles diferencias entre algunas de ellas y su capacidad de hibridación, a veces puede ser muy difícil clasificarlas en base a los rasgos morfológicos (Figura 2).

Los principales marcadores moleculares utilizados para estudiar la genética poblacional en *B. terrestris* han sido hasta ahora un fragmento del gen mitocondrial de la citocromo-oxidasa I (*cox1*), las secreciones de la glándula labial cefálica masculina (CLG) y las repeticiones en tándem (STR) o microsatélites. En la mayoría de los casos, las diferencias entre las subespecies son inexistentes o

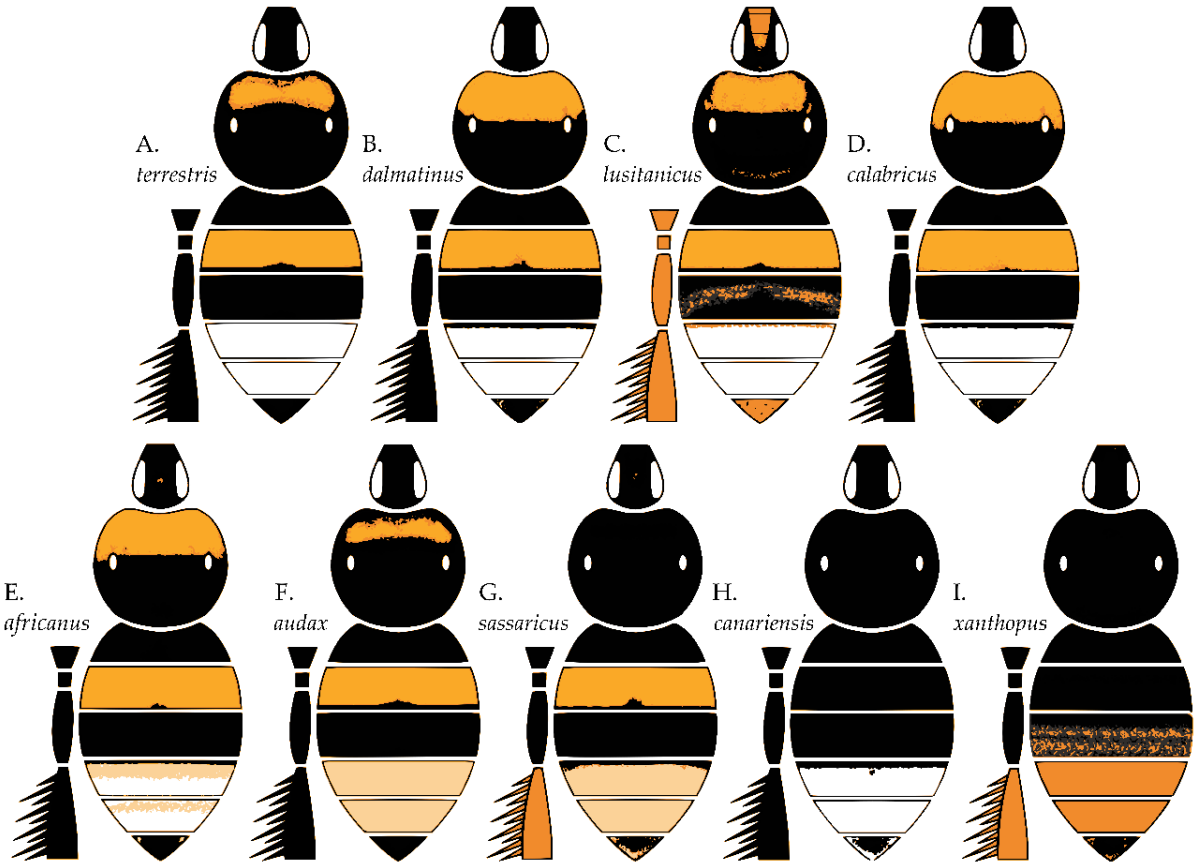

Figura 2. Esquema de la coloración de reinas de diferentes subespecies de *Bombus terrestris*. De Rasmont et al. (2008).

escasas, lo que se ha atribuido al elevado flujo génico entre las poblaciones y a la capacidad para hibridar de los abejorros (De Jonghe, 1986; Estoup et al., 1996; Ings et al., 2005; Moreira et al., 2015; Lecocq et al., 2016a). En cualquier caso, en un estudio reciente restringido a la península Ibérica se han encontrado haplotipos mitocondriales y cierta estructura poblacional debido al aislamiento geográfico (Silva et al., 2020). A pesar de estas circunstancias, la naturaleza endémica de algunas de estas subespecies y su distribución geográfica han llevado a que algunos países adopten medidas para conservar y proteger sus poblaciones locales de abejorros (Chandler et al., 2019). Aquí se presenta su rango de distribución natural y las diferencias morfológicas descritas en Rasmont et al. (2008), con anotaciones sobre su uso comercial, conservación y diferenciación molecular:

- *B. t. terrestris* ocupa Europa occidental y central, desde los Pirineos hasta el norte del continente. Su patrón de color está definido por un predominio del negro y un collar amarillo en el tórax (menos evidente que en otras subespecies), un segundo collar amarillo en el abdomen y una coloración blanca en el extremo del abdomen (Fig 6.A). Es la subespecie nominativa y es muy afín molecularmente a la otra subespecie continental europea, *B. t. dalmatinus*. En Noruega, sólo se permite el uso comercial para la polinización de esta subespecie (Velthuis & Doorn, 2006).
- La otra subespecie de distribución más amplia es *B. t. dalmatinus*, que ocupa la parte meridional del área de distribución de la especie, desde el sudeste de Francia hasta Irán. Su área de distribución se superpone a la de *B. t. terrestris*, y su fenotipo es muy similar, siendo las principales diferencias la anchura y los límites del primer collar (Fig. 2.B). Junto con *B. t. terrestris*, son las dos principales subespecies utilizadas para la polinización en invernadero al nivel mundial (Velthuis & Doorn, 2006).
- *B. t. lusitanicus* es autóctona de la península Ibérica, con un área de intergradación con *B. t. terrestris* en los Pirineos y el sur de Francia. Se puede diferenciar de otras subespecies por su color cobrizo herrumbroso en los segmentos superiores de las patas y a veces en cuerpo y cabeza (Ornosa & Ortiz Sanchez, 2004) (Fig. 2C). La composición de las secreciones de la glándula labial cefálica es prácticamente idéntica a la de las subespecies citadas antes.
- *B. t. calabricus* se distribuye naturalmente sólo en Calabria y en Sicilia. Su patrón de color es diferente al de otras subespecies en su amplio primer collar, que se extiende por debajo de la tégula (Fig. 2D).
- *B. t. africanus* se distribuye actualmente en el norte de África por separado del resto de la especie, aunque en el pasado su área de distribución podría haberse extendido al sur de la península Ibérica (Rasmont, comunicación

personal). Su patrón de color está definido por un primer collar ancho y una coloración amarilla clara en el extremo del abdomen (Fig. 2E).

- *B. t. audax* se encuentra en las Islas Británicas y presenta un primer collar delgado y a veces incompleto. Las reinas además presentan tonos de amarillo claro en el extremo del abdomen (Fig. 2F). Se crían comercialmente para su uso en la polinización en el Reino Unido, Nueva Zelanda y Tasmania (Venthuis & Doorn, 2006). En el Reino Unido, una legislación estricta protege a la subespecie contra la introducción de otras subespecies en el país para su uso comercial (Graystock et al., 2016).
- *B. t. sassaricus* habita en la isla de Cerdeña. Normalmente carece de collar torácico y presenta un collar abdominal amarillo y una coloración amarilla clara en el extremo del abdomen. Puede presentar una coloración rojiza (Fig. 2G). Se utiliza principalmente para la polinización en Cerdeña, aunque también se ha utilizado en Europa, donde las poblaciones comerciales no lograron colonizar el medio ambiente (Ings et al., 2010).
- *B. t. canariensis* habita en las Islas Canarias. Presenta una coloración negra en todo el cuerpo, con un segmento blanco en el extremo del abdomen. Junto con *B. t. xanthopus*, es la subespecie con mayor diferenciación genética y morfológica de la especie (Fig. 2H). Su clasificación taxonómica como especie o subespecie ha sido discutida en el pasado (Erlandsson, 1979) y actualmente es aceptada como subespecie (Estoup et al., 1996; Lecocq et al., 2016a). Dada su condición endémica, las Islas Canarias presentan una estricta legislación en lo referente al uso de otras subespecies comerciales en el territorio (Velthuis & Doorn, 2006).
- *B. t. xanthopus* se distribuye en las islas de Córcega, Capraia y Elba. Tiene un patrón principal de color negro con tonos rojos en el abdomen y las extremidades (Fig. 2I). Su clasificación como subespecie o especie se ha discutido recientemente debido a la variabilidad encontrada en los análisis moleculares (Lecocq et al., 2016a).

Box 1: El problema de las subespecies

Para clasificar y comprender la diversidad de las formas de vida, los niveles taxonómicos más básicos propuestos son los de las especies y subespecies (De Queiroz, 2011). Dependiendo del organismo de estudio, se pueden utilizar diferentes criterios para definir un taxón. En el caso de los insectos, los criterios más usados son: morfológicos, basados en rasgos fenotípicos; moleculares, basados en diferencias genéticas (ampliamente utilizados cuando se estudian especies crípticas); o reproductivos, según los cuales dos organismos pertenecen a especies diferentes cuando muestran barreras reproductivas que impiden la existencia de descendencia híbrida fértil. En algunos casos estos criterios se contradicen entre sí, por lo que es aconsejable utilizar varios para establecer hipótesis taxonómicas sólidas (Taxonomía integrativa) (Lecocq et al., 2015; Galtier et al., 2019). Qué criterios utilizar, cómo denominar a un taxón específico y qué es exactamente lo que hace que un taxón sea diferente, es todavía un debate abierto en Taxonomía que se conoce como el problema de las subespecies, estudiado por la microtaxonomía.

Lograr que esta clasificación sea precisa es un desafío cuando se trabaja en la conservación de subespecies (Braby et al., 2012). Para esta tesis, hemos decidido seguir nominalmente la taxonomía morfológica de la subespecie *B. terrestris* al referirnos a las poblaciones o grupos locales. Sin embargo, independientemente del concepto y la hipótesis taxonómica, desde un punto de vista biológico, hemos trabajado con diferentes poblaciones u OTUs (unidades taxonómicas operativas) dentro del gradiente de la especie, y no en grupos herméticos. Se han utilizado términos como hibridación y endogamia de acuerdo con las definiciones de Facon et al. (2011) sobre la interacción entre individuos, es decir, "los cruces entre individuos de poblaciones genéticamente diferenciadas de la misma especie también se consideran hibridación". Además, según estos autores, para que los cruces intraespecíficos desempeñen un papel en las invasiones biológicas deben cumplirse tres criterios: en primer lugar, las poblaciones que intervienen en el proceso de mezcla deben estar genéticamente diferenciadas; en segundo lugar, deben ser posibles los cruces entre individuos de poblaciones diferentes y, por último, los individuos mezclados deben diferir de los progenitores en algunos de sus rasgos para poder influir en el proceso de introgresión.

Servicios ecosistémicos e importancia económica

Los abejorros y otros insectos polinizan tanto las plantas silvestres como los cultivos. Esta contribución directa e indirecta de los ecosistemas al bienestar humano es lo que conocemos como servicios ecosistémicos. Gallai et al. (2009) estimaron que el valor económico total de la polinización en todo el mundo en 2005 era de unos 153 000 millones de euros, lo que equivalía al 9,5% de la producción agrícola mundial utilizada para la alimentación humana. Lautenbach et al. (2012) estimaron posteriormente que el valor de la polinización mundial en 2009, basado en la dependencia media de los cultivos de la polinización (Klein et al., 2007) era de alrededor de 300 000 millones de euros. Sin embargo, no todos los polinizadores contribuyen de la misma manera. El valor de la producción de cultivos dependientes de las abejas silvestres se estimó en un promedio de 3.000 € por hectárea (Kleijn et al., 2015), aunque el resultado fue más dependiente de las especies polinizadoras no amenazadas más comunes, como *B. terrestris*, cuyos servicios de polinización de cultivos se valoraron en un promedio de 360 € por hectárea.

La importancia económica de *B. terrestris* ha aumentado desde que la especie fue domesticada en la década de los años 80 para su utilización en invernaderos en todo el mundo (Velthuis & Doorn, 2006). *B. terrestris* es mejor polinizador que la abeja de la miel para algunos cultivos como las solanáceas (tomate, pimiento) o las curcubitáceas (melón, calabaza) entre otros (Palacios Estay, 2007; Kraus et al., 2011) debido a su capacidad de vibración. El uso de *B. terrestris* en estos cultivos proporciona un mayor tamaño de los frutos, lo que aumenta la productividad de los cultivos al reducir las pérdidas debidas a las normas de calidad del mercado (Goulson et al., 2010b). El valor económico global de este servicio, sólo para el tomate, se ha medido en 12 000 millones de euros anuales (Velthuis & Doorn, 2006).

En los últimos cuarenta años, su cría y exportación se han convertido en una industria mundial, aunque es difícil obtener datos sobre los beneficios que genera su comercio. La estimación más reciente disponible establece unas ventas mundiales de alrededor de dos millones de colonias en 2016 (Lecocq et al., 2016b).

La expansión invasiva de *B. terrestris* a nivel mundial

Al principio, se creía que el uso de *B. terrestris* en invernaderos no entrañaba riesgos como la colonización del medio ambiente o la introgresión con poblaciones locales. Las principales razones que apoyaban esta hipótesis eran que parecía poco probable que las reinas y machos pudieran escapar de los invernaderos y que, aunque lo hicieran, se creía que los individuos manejados no estaban sincronizados con los individuos silvestres y no podrían prosperar en el medio ambiente. Sin embargo, se ha demostrado que estas hipótesis eran erróneas (Kraus et al., 2011) y que *B. terrestris* tiene la capacidad de sobrevivir y colonizar nuevos entornos en todo el mundo hasta el punto de que se considera actualmente una importante amenaza para la conservación de las poblaciones silvestres (Sutherland et al., 2017).

B. terrestris fuera de su rango de distribución

B. terrestris muestra muchas cualidades que la convierten en una especie altamente invasiva. Tiene una alta tolerancia al estrés térmico, una gran distancia máxima de vuelo, una dieta generalista, una emergencia temprana y una alta producción de reinas (Dafni et al., 2010; Vanderplanck et al., 2019; Zambra et al., 2020). Debido a la intensificación de su exportación a escala mundial, la especie ha ampliado su área de distribución más allá de la región Paleártica llegando a todos los continentes habitados (Dafni et al., 2010) y se considera una amenaza si se exportara descuidadamente a nuevos entornos (Acosta et al., 2016; Lecocq et

al., 2016b). Según las características del ecosistema, el daño causado por la introducción de una nueva especie invasora puede ser diferente (Moore et al., 2013; Aizen et al., 2018; Tsuchida et al., 2019). A continuación, se exponen algunos de los casos más estudiados.

Tasmania y Nueva Zelanda

Algunas de las primeras introducciones de *B. terrestris* ocurrieron en Nueva Zelanda en 1985 y en Tasmania en 1992. Su expansión no se vio limitada por los depredadores existentes, las variables climáticas o estacionales, o los requisitos alimentarios, ya que puede encontrarse todo el año y es altamente compatible con la mayoría de la flora local. En 2002, se podían encontrar colonias de *B. terrestris* en Tasmania no sólo en jardines urbanos sino también en entornos naturales (Hingston et al., 2002), y ahora están totalmente naturalizadas en el medio ambiente. Su exportación no está permitida en Australia (Moore et al., 2013).

Japón

Las primeras colonias de abejorros se exportaron a Japón con fines experimentales en 1991. En 1996 su uso para la polinización en invernaderos ya estaba establecido en el país y se detectaron por primera vez colonias naturalizadas en el medio ambiente. En 2004 el comercio alcanzó las 70.000 colonias vendidas en el país (Inari et al., 2005; Tsuchida et al., 2019). La expansión de *B. terrestris* en Japón ha afectado a las poblaciones de especies endémicas como *B. hypocrita* y *B. ardens* hasta el punto de la extinción local en algunas zonas por la competencia por recursos florales y usurpación de sitios de nidificación (Matsumura et al., 2004; Inoue et al., 2008; Tsuchida et al., 2019), y por la hibridación interespecífica, ya que especies como *B. hypocrita* o *B. ignitus* pueden aparearse con *B. terrestris* aunque los huevos son inviables debido a la interferencia reproductiva (Tsuchida et al., 2010, 2019), lo que conduce a una

menor densidad de población y al deterioro genético. Además, la competencia está dando lugar a una división de los recursos y a una polinización ineficaz, debido a la tendencia de *B. terrestris* al aprovechamiento exclusivo de néctar, lo que está afectando negativamente a la producción de semillas de algunas especies de plantas nativas (Dohzono et al., 2008, Nishikawa et al., 2015).

Las pruebas científicas llevaron a la categorización de *B. terrestris* como especie exótica invasora en 2006, y su uso está ahora restringido en el país bajo protocolos estrictos (Tsuchida et al., 2010). El uso de especies nativas como *B. hypocrita* para complementar esta función de polinización se ha probado en el país y se supone menos perjudicial para el medio ambiente (Takeuchi et al., 2018). Aunque *B. terrestris* todavía no ha llegado al continente asiático, según las simulaciones de Naeem et al. (2018), si el abejorro comercial llegase a colonizar el territorio, se convertiría en una amenaza para las poblaciones locales de abejorros en China, Corea o Rusia.

Chile y Argentina

La especie europea *B. ruderatus* fue introducida con éxito en Chile en 1982. Seis años después, se probó la utilidad de las colonias de *B. terrestris* en cultivos de invernadero tanto abiertos como cerrados en este país (Schmid-Hempel et al., 2014) y después de los experimentos, comenzó su importación para uso comercial.

Dada la capacidad invasora de ambas especies, Argentina prohibió su exportación para proteger a los polinizadores locales (Aizen et al., 2018). No obstante, ambas especies lograron cruzar los Andes hacia Argentina; *B. ruderatus* en 1994 (Roig Alsina & Aizen, 1996) y *B. terrestris* en 2006 (Torretta et al., 2006). *B. terrestris* se ha expandido por el continente a un ritmo de 200 km al año, llegando a la costa atlántica de Argentina en 2011 (Schmid-Hempel et al., 2014). El abejorro comercial compite con los polinizadores locales y, paralelamente, ha

aumentado la polinización de las plantas invasoras no nativas (Morales et al., 2017; Medel et al., 2018). Su capacidad como vector de transmisión de parásitos o *spillover* no está clara en la actualidad (Revainera et al., 2020), aunque la expansión de la especie está relacionada con la extinción local de las poblaciones de la especie argentina *B. dahlbomii* (Schmid-Hempel et al., 2014). Este caso pone de relieve la necesidad de alcanzar respuestas consensuadas a través de las fronteras de los países en lo que respecta a la conservación y protección de la biodiversidad (Aizen et al., 2018).

B. terrestris dentro de su rango de distribución

Dentro del rango de distribución de la especie, la existencia de poblaciones de origen comercial en el medio también puede suponer una amenaza para las poblaciones locales. Las colonias de *B. terrestris* presentan muchas características que les dan una mejor aptitud para vivir tanto en entornos alterados como en entornos naturales frente a las poblaciones locales. Las razas comerciales de abejorros han demostrado tener el cuerpo más grande, mayor capacidad de forrajeo y una mayor producción de reinas que las colonias silvestres (Ings et al., 2006), lo que las hace más exitosas para la colonización y la expansión, y posibles competidores frente a sus conespecíficos. Los efectos de la hibridación entre los individuos comerciales y silvestres de *B. terrestris* fueron estudiados por Gosterit et al. (2016b), quienes confirmaron que las colonias de las razas comerciales producen larvas mucho más rápidamente y en mayor número, lo cual está determinado por el genotipo materno, señalando la importancia de la invasión de reinas comerciales.

Reino Unido

En el Reino Unido la exportación de colonias comerciales comenzó en 1989 (Velthuis & Doorn, 2006) pero no fue hasta décadas más tarde cuando se estudió su efecto sobre las poblaciones de la subespecie endémica *B. t. audax*, bajo la

creencia de que las poblaciones comerciales no podrían sobrevivir en el medio ambiente. No obstante, ahora se sabe que la subespecie comercial *B. t. dalmatinus* presenta la misma tolerancia al frío que la subespecie británica, pudiendo sobrevivir a las condiciones climáticas del Reino Unido y competir con las poblaciones locales (Owen et al., 2016). Dada la singular diversidad genética de la subespecie insular (Lecocq et al., 2016a), las poblaciones silvestres inglesas e irlandesas corren riesgo de introgresión y pérdida de alelos raros bajo la presencia de razas comerciales en el medio (Moreira et al., 2015). Además, se ha demostrado que los invernaderos son fuentes de transmisión de patógenos como *Crithidia bombi* y *Nosema bombi*, posiblemente debido a que los individuos criados en alta densidad, como en las instalaciones de cría, tienen más probabilidades de infectarse (Murray et al., 2013). Por esas razones, desde 2015 el comercio de subespecies no autóctonas está estrictamente regulado, aunque se ha debatido la falta de legislación para realizar pruebas más exhaustivas para detectar enfermedades (Graystock et al., 2016).

Box 2: La península Ibérica como hotspot de biodiversidad

Durante la última glaciación del Cuaternario, debido a sus diferentes sistemas montañosos, la península Ibérica se convirtió en un intrincado refugio glacial a largo plazo para diferentes especies que se diversificaron y especializaron, dando lugar al actual alto número de endemismos y linajes locales (Hewitt, 2011), por lo que hoy en día se considera un *hotspot* o punto caliente de diversidad. Además, la ubicación de la península, entre África y Europa, promueve la convergencia y la migración de especies y poblaciones, fomentando también su posición como *hotspot*.

Este nivel de biodiversidad ha sido ampliamente estudiado en numerosos organismos (Gómez & Lunt, 2007), entre ellos la abeja de la miel ibérica *A. m. iberiensis*. En relación con este importante polinizador, Pinto et al. (2012) observaron que la península Ibérica es una de las regiones europeas con mayor diversidad de haplotipos mitocondriales en la especie, lo cual podría ser debido al contacto que se produjo entre la subespecie *A. m. mellifera* del norte de Europa y *A. m. intermissa* del norte de África durante la última glaciación.

En relación con el género *Bombus*, en el territorio ibérico habitan 39 especies y 56 subespecies de abejorros (además de las formas híbridas en las zonas superpuestas, Ornosa y Ortiz-Sánchez, 2004). La fauna ibérico-balear está compuesta por especies paleárticas, euroasiáticas, europeas, mediterráneas y endémicas, estas últimas sobre todo a nivel de subespecie (Ornosa y Ortiz-Sánchez, 2004). Según el Atlas y la Lista Roja de Invertebrados Amenazados de España (Verdú et al., 2011) hay seis especies con algún grado de amenaza de extinción en la península Ibérica: 13 VU (Vulnerable) y 3 EN (En Peligro). Sin embargo, según la Lista Roja Europea de Abejas (Nieto et al., 2014) hay nueve especies de abejorros con algún grado de amenaza de extinción: 2 NT (Casi Amenazado), 3 VU, 3 EN y 1 CR (En Peligro Crítico).

Hipótesis y objetivos

El interés sobre los efectos de la introducción de razas comerciales de *B. terrestris* en poblaciones de la subespecie endémica ibérica (*Bombus terrestris lusitanicus*) ha aumentado en los últimos años. Sin embargo, no existe ninguna legislación que regule la importación de colonias comerciales foráneas, ni que apoye el uso de subespecies autóctonas para la polinización en el territorio español (con la excepción de las Islas Canarias y la subespecie *B. t. canariensis*). Las hipótesis de esta tesis son que el acervo genético de la subespecie endémica *B. t. lusitanicus* permite su diferenciación del resto de subespecies de *B. terrestris* debido a su distribución geográfica en lo que es considerado un *hotspot* de biodiversidad, y, en segundo lugar, que la diversidad genética de las poblaciones actuales está siendo afectada por la introducción de poblaciones comerciales que pueden naturalizarse en el medio ambiente.

Con el fin de verificar estas hipótesis y proporcionar una base científica para impulsar la legislación sobre la importación y utilización de abejorros comerciales, en esta tesis se han planteado los siguientes objetivos:

- Buscar marcadores subespecíficos para caracterizar la diversidad genética de las poblaciones locales de *B. terrestris* en la península Ibérica.
- Cuantificar la posible introgresión de las razas comerciales en las poblaciones de la subespecie *B. t. lusitanicus* en un gradiente de distribución peninsular y en zonas de contacto entre subespecies: natural (Pirineos) y artificial (próximas a invernaderos).
- Describir la diversidad genética de la subespecie *B. t. lusitanicus* y evaluar el impacto de la introducción de subespecies comerciales.

Estructura de la Tesis Doctoral

Esta tesis está dividida en cuatro capítulos: los tres primeros están orientados a la búsqueda y validación de marcadores moleculares fiables para realizar ensayos económicos y rápidos que ayuden a diferenciar las poblaciones ibéricas y centroeuropeas de abejorros mientras que el cuarto capítulo tiene como objetivo describir la diversidad genética de la subespecie *B. t. lusitanicus* y evaluar si la introducción de los abejorros comerciales está teniendo un impacto en su estructura genética. De este modo, los resultados y consideraciones alcanzados en cada capítulo sirvieron de base para los experimentos siguientes.

Capítulo 1: Informe preliminar sobre la amplificación de microsatélites para estudios de biodiversidad y conservación de abejorros

La península Ibérica alberga una gran diversidad de abejorros, pero existe en general una falta de información sobre su biodiversidad en el territorio. Para solventar esto y facilitar estudios de conservación, presentamos dos nuevos análisis múltiplex de marcadores microsatélites para la amplificación de seis y cinco loci respectivamente. Ambas PCRs multiplex, se amplificaron con éxito en la mayoría de las especies estudiadas de las poblaciones ibéricas. El parentesco y los parámetros genéticos de las poblaciones en la especie gestionada *B. terrestris* y en las especies silvestres *B. monticola* y *B. mesomelas* fueron analizados, demostrando la utilidad de estos análisis para estudios de biodiversidad de especies de abejorros tanto gestionadas como silvestres.

Capítulo 2: Búsqueda de marcadores moleculares para diferenciar subespecies de *Bombus terrestris* (Linneo) en la península Ibérica

Los abejorros (género *Bombus* Latreille) son insectos polinizadores de gran importancia ecológica y económica, cuyo uso comercial para la polinización ha aumentado desde la década de 1980. Sin embargo, la introducción de la especie

foránea *Bombus terrestris* (Linneo) ha dado lugar a una disminución de las poblaciones locales de abejorros en Japón, Chile y Argentina, entre otros países. Para estudiar la posible introgresión de individuos comerciales de *B. terrestris* en la subespecie endémica ibérica *Bombus terrestris lusitanicus* Krüger es necesario encontrar un marcador molecular preciso que diferencie a ambas subespecies. Con este fin, se realizaron análisis comparativos entre *B. t. lusitanicus* y *Bombus terrestris terrestris* (Linneo) de España y Bélgica mediante la secuenciación de los genes nucleares factor de elongación 1-α y arginina quinasa, el gen mitocondrial *16S* ARN ribosomal, y el genotipado de once loci de microsatélites. No se observó ninguna diferenciación al nivel nuclear, pero los haplotipos encontrados en la secuencia del gen mitocondrial *16S* se correlacionaron con la caracterización morfológica de la subespecie. En un caso de estudio que incluía individuos muestreados antes del establecimiento de empresas de cría de abejorros y otros de muestreos recientes, se detectaron en la actualidad individuos híbridos (aquellos con subespecie morfológica no coincidente con el haplotipo *16S*) con mayor frecuencia en el sur, lo que confirma la naturalización de *B. t. terrestris* comerciales y los eventos de introgresión entre ambas subespecies. Este marcador debería utilizarse en las poblaciones ibéricas con el fin de apoyar las acciones de gestión y conservación de las poblaciones endémicas de *B. t. lusitanicus*.

Capítulo 3: Descubriendo introgresión en poblaciones de abejorro (*Bombus terrestris*) mediante marcadores basados en el mitogenoma

El abejorro, *Bombus terrestris*, es un importante polinizador utilizado comercialmente a escala global. La subespecie exportada *B. t. terrestris* ha colonizado diversos ambientes, en algunos casos llevando a polinizadores silvestres al borde de la extinción local. En este sentido, la subespecie ibérica *B. t. lusitanicus* podría estar amenazada por la subespecie *B. t. terrestris*, distribuida naturalmente desde los Pirineos hasta Europa Central, pero también observada

en el sur de España debido a fugas de los nidos comerciales. Los genomas mitocondriales tienen una baja tasa de recombinación y un pequeño tamaño efectivo poblacional debido a su herencia materna, proporcionando por tanto un enfoque preciso para estudiar eventos de hibridación entre poblaciones. Por ello, presentamos las secuencias de los mitogenomas de ambas subespecies como un marco molecular para seleccionar marcadores adecuados con los que detectar posibles eventos de introgresión en el territorio ibérico. Obtuvimos aproximadamente 17 kbp del mitogenoma de ambas subespecies. Sus mitogenomas difieren en 358 pb (excluyendo la región control, rica en AT). Se seleccionaron cuatro fragmentos mitogenómicos para ser probados como marcadores de diagnóstico subespecíficos. El RFLP detectado en el gen *nad2* (subunidad 2 de la NADH deshidrogenasa) ha demostrado ser una herramienta eficiente, rápida y rentable para evaluar la dispersión de la subespecie no endémica en las poblaciones nativas ibéricas. Se observaron ambos haplotipos en las dos subespecies morfológicas, lo que sugiere eventos de introgresión tanto en el área de contacto natural del norte como en la nueva zona de contacto mediada por el hombre en el sur de la península Ibérica.

Capítulo 4: Patrones espaciales y temporales de la diversidad genética de poblaciones endémicas de *Bombus terrestris* en la península Ibérica y sus implicaciones para la conservación.

El abejorro *Bombus terrestris* es utilizado actualmente en todo el mundo para la polinización de los cultivos. A pesar de tener un efecto positivo en la agricultura, se ha convertido en una amenaza para la biodiversidad en diferentes regiones debido a sus interacciones con las poblaciones locales. Las subespecies comerciales introducidas en la península Ibérica desde la década de los 90, sin ninguna regulación, han colonizado el medio ambiente según las evidencias documentadas de eventos de naturalización y posterior hibridación con la subespecie endémica *Bombus terrestris lusitanicus*. En este trabajo se han utilizado

marcadores genéticos mitocondriales y nucleares con el objetivo de describir la diversidad genética de poblaciones de *B. t. lusitanicus* y estimar el posible efecto de las variedades comerciales sobre su acervo genético. Se han utilizado muestras de Europa Central (área de distribución natural de la subespecie comercial), de los Pirineos (área de intergradación natural entre las dos subespecies) y de colecciones antiguas de la península Ibérica (antes de la introducción de la subespecie comercial) para la comparación. Nuestros resultados indican que el haplotipo mitocondrial más frecuente en la subespecie comercial se ha encontrado en diferentes lugares de la península Ibérica, lo que evidencia que se están produciendo eventos de hibridación e introgresión que podría tener efectos inesperados a largo plazo. Sin embargo, no se ha detectado un impacto claro en la diversidad genética nuclear de las poblaciones ibéricas locales. Aun así, estos resultados sugieren que es necesario mejorar la legislación actual sobre la gestión y la exportación de abejorros comerciales para así preservar las poblaciones endémicas de abejorros ibéricos.

Conclusiones

En esta tesis se han llegado a las siguientes conclusiones:

- Las secuencias de *loci* de microsatélites están conservadas al nivel del género *Bombus* y por tanto se pueden usar reacciones múltiplex para obtener parámetros de diversidad genética, tanto en poblaciones silvestres de especies del género *Bombus* como *B. mesomelas*, como para poblaciones manejadas de *B. terrestris*.
- La diversidad haplotípica encontrada en las poblaciones ibéricas de *B. t. lusitanicus* al secuenciar el fragmento del gen mitocondrial *rnnL* (*16S*) aporta la suficiente precisión para diferenciar las poblaciones endémicas de la península Ibérica de las poblaciones centro europeas de *B. t. terrestris* seleccionadas como referencia.
- Una segunda zona de intergradación entre *B. t. lusitanicus* y las razas comerciales de *B. terrestris* está emergiendo en el sur de la península Ibérica, debido a la hibridación e introgresión entre los individuos de las poblaciones nativas y aquellos procedentes de invernaderos y empresas de cría de la zona.
- La información obtenida de la secuencia del mitogenoma es útil para la discriminación de taxones y la gestión de la conservación de los abejorros, ya que ha aportado la información necesaria para el diseño de marcadores subespecíficos.
- Los individuos de *B. terrestris* que habitan la península Ibérica se pueden identificar mediante un test rápido y económico basado en la técnica PCR-RFLP.
- Los rangos de distribución de las subespecies están cambiando como consecuencia tanto del cambio climático como de acciones humanas. En ese sentido el haplotipo más frecuente de las poblaciones de *B. terrestris* de Europa Central está presente en el sur de la Península mientras que el

haplotipo más frecuente en *B. t. lusitanicus* ha sido encontrado en Normandía (Francia), lo que sugiere que la subespecie ibérica está expandiendo su rango de distribución hacia el norte.

- El haplotipo característico de *B. t. terrestris* de Europa central se ha expandido sobre el territorio peninsular en las últimas décadas, ya que la comparación con el grupo de referencia temporal muestra que en el pasado su presencia se encontraba acotada a la zona natural de intergradación (Pirineos).
- Los efectos de la introgresión en la diversidad genética nuclear de las poblacionales actuales aún no son relevantes, pero a largo plazo podrían suponer la pérdida de adaptaciones locales de las poblaciones, por lo tanto, aconsejamos legislar la exportación y el uso de razas comerciales en el territorio, y aumentar la divulgación sobre el correcto uso de las colonias comerciales y los problemas potenciales que pueden causar.

Introduction

The sixth mass extinction

There are five events in the geological history of the planet in which environmental conditions became so catastrophically harsh that life on Earth was almost completely eradicated, these are mass extinctions. Although it is difficult to assess the diversity that was lost during these events, since not all species left fossil records (Plotnick et al., 2016), a mass extinction is described as the loss of more than the 75% of species over a period of less than two million years.

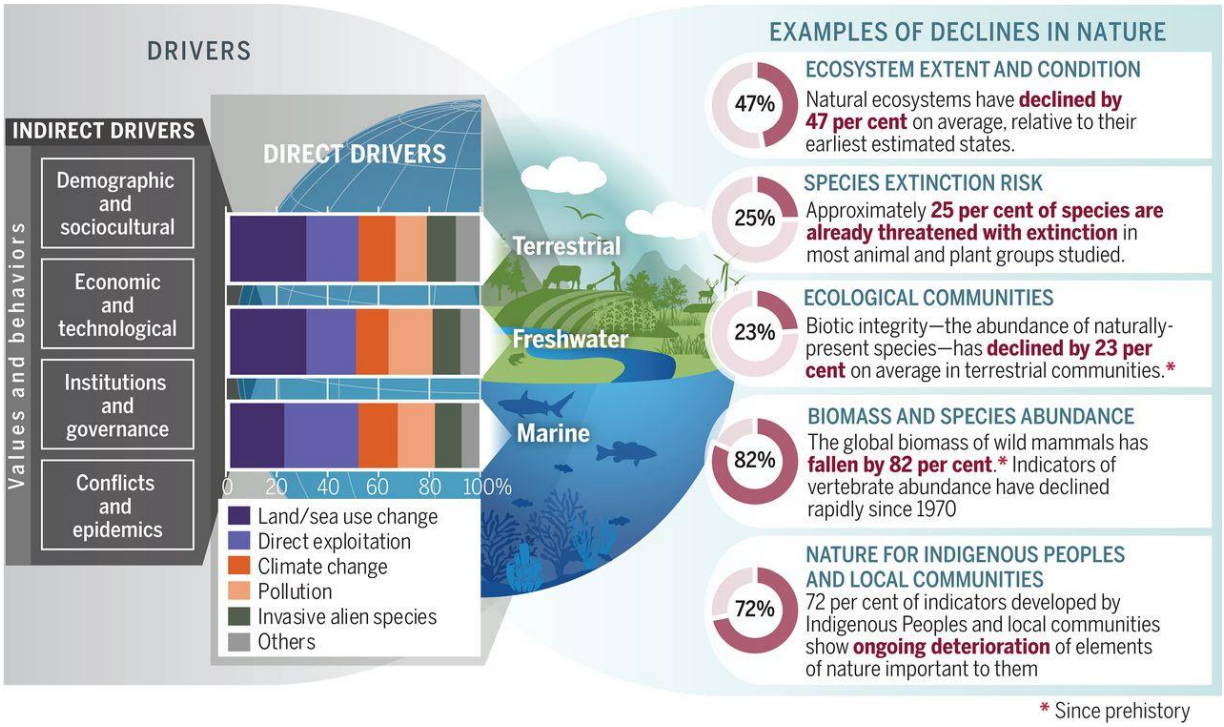

Figure 1. Drivers of extinction and some of their effects on nature. Extracted from Diaz et al. (2019).

According to this definition, the current extinction rates of most vertebrates and the reduction of biomass suggest that we are experiencing a sixth mass extinction (Barnosky et al., 2011). The impact of human life on nature is leading the planet into a diversity crisis (Figure 1). Changes in land use, pollution, accelerated climate change or the introduction of exotic species are some of the main drivers

of extinction (Figure 1), all of which are directly influenced by the increase in world population and the way we obtain and consume resources (Diaz et al., 2019). In this context, Conservation Biology has emerged to focus on the study of these threats to nature in order to the protect the diversity of species and ecosystems, while seeking ways to mitigate our impact on the environment (Soulé, 1985).

Bee decline

Human interference with the environment affects harshly the entomofauna (Habel et al., 2019). In the 80s, some of the first evidences of population decline were detected in wild bees (Williams, 1982). Given how intertwined is the welfare of pollinators to ours and the risks associated with the reduction on their ecosystem services (Figure 2), the vulnerability of bee populations has been more deeply studied in the last decades. Nowadays, even if there are still many gaps of knowledge referring to the distribution and abundance of bee species in many parts of the world (Potts et al., 2016), it can be assumed that the declining trend is global, mirrored in the declining abundance of species, and the increasing number of both threatened species on red lists worldwide and extinction events (Williams & Osborne, 2009; Cameron et al., 2011; Schmid-Hempel et al., 2014; Goulson et al., 2015; Potts et al., 2016, Drossart et al., 2019). The drivers of decline identified are several, and it is important to note that their negative effects are synergistic (Brook et al., 2008; Goulson et al., 2015), so management and conservation proposals and measures must be presented from a holistic approach.

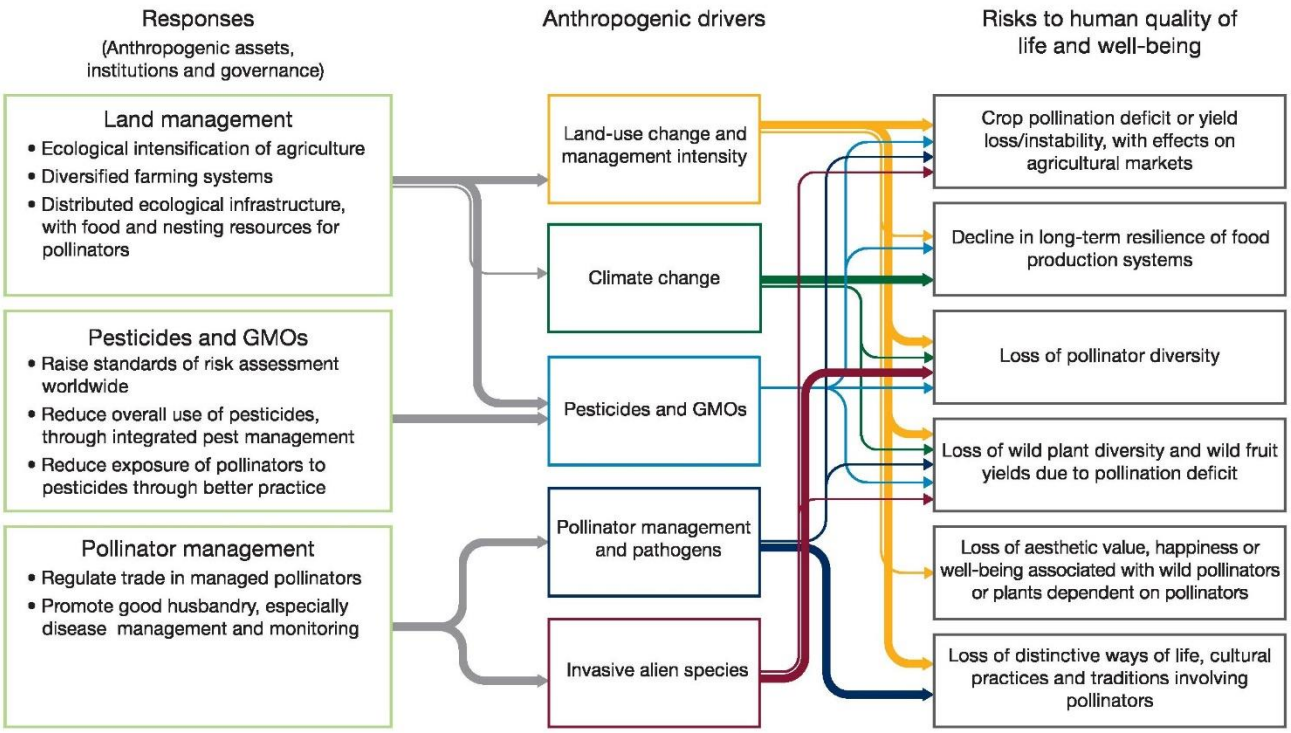

Figure 2. Drivers, risks and responses to pollinator decline. Extracted from Potts et al. (2016).

Habitat loss

Habitat loss due to changes in land use and agricultural intensification is the main driver of insect decline (Habel et al., 2019), directly by habitat destruction leading to loss of resources and nesting sites, and indirectly by population fragmentation and isolation (Brown & Paxton, 2009; Goulson et al., 2010a). Fragmentation, if unresolved, leads to inbreeding and thus loss of genetic diversity (Ellis et al., 2006), reduction of populations that can no longer be maintained due to low numbers of individuals (Allee effect) or even local extinction.

Climate change

Although the full effects of climate change are difficult to assess, the evidence shows that the phenology of Hymenoptera species is shifting with the new climate variables (Duchenne et al., 2020), although the effects on current plant-pollinator interaction matrices remain unclear (Hegland et al., 2009). Climate

change is not only associated with a regression of distribution ranges towards higher altitudes and northern gradients (Kerr et al., 2015; Ornosa et al., 2017), but also with local extinction events and drastic changes in the species of ecosystems (Soroye et al., 2020).

Pesticides

The impact of pesticide use on agriculture has been largely discussed in the last decade. It has been shown that even the use of herbicides is a problem for pollinators, as it reduces the density of flowers in the crops (Goulson et al., 2010b). However, drawing conclusions from the study of the direct effect of pesticides has been more difficult, given their potential synergistic capacity with respect to other drivers (Collison et al., 2016; Woodcock et al., 2017).

Neonicotinoids are a group of insecticides that target the central nervous system. The effects of sublethal doses on pollinators have been extensively studied, some of which are paralysis, reduced foraging and homing capabilities, and reduced colony growth (Whitehorn et al. 2012; Goulson et al., 2015). Following a moratorium in 2013, the evidence supported their involvement in bee decline, which is why the use of some neonicotinoids-based insecticides was banned in the European Union from 2018 (EFSA, 2018). Although much remains to be revealed about the involvement of pesticides in bee decline, particularly in wild bees (Potts et al., 2016), regulation should be more rigorous and robust to avoid future uses of inadequately tested products (Sgolastra et al., 2020).

Pathogens

Pathogens play an important role in population dynamics because the co-evolutionary relationship between pathogen and host shapes the immune system of the host species (Evans et al., 2007). However, the introduction of new pathogens and the development of the diseases they cause, can be catastrophic. International export of commercial colonies, more sensitive to the spread of

pathogens as are reared in high densities (Goulson et al., 2010c; Graystock et al., 2016), or transhumance of honey bees facilitates the spread of pathogens, as foreign individuals act as vectors to native populations (Muñoz et al. 2014a; Potts et al., 2016; Chandler et al., 2019). One of the best-known cases is the dispersion of the mite *Varroa destructor* due to the contact of the European honeybee with the Asian *Apis cerana* (Rosenkranz et al., 2010). *V. destructor* is vector of the Deformed Wing Virus (DWV) and the combined effect of both pathogens has devastated European and North American honeybee populations (Nazzi et al., 2012). In other cases, pathogens can also spill over other species of wild bees (Fürst et al., 2014).

Invasive alien species

In order to improve productivity of crops, we rely on the use of managed pollinators (Lautenbach et al., 2012; Kleijn et al., 2015). The export of species to locations where they are non-native can lead to them considered as invasive species (Westphal et al., 2008), because if the foreign population colonizes and expands into the wild, it will become a competitor to the rest of the native pollinators (Inoue et al., 2008), and will impact interactions within ecosystems. In addition, it can give rise to introgression in cases that local populations hybrize with introduced ones (Muñoz et al., 2014b; Ellis et al., 2018).

In this thesis, we will further investigate the latter driver, and its impact on the genetic diversity of local bumblebee *B. terrestris* populations in the Iberian Peninsula.

The model species *Bombus terrestris*

The genus *Bombus*

The genus *Bombus* (Order Hymenoptera, Family Apidae) is composed by 250 species grouped in 15 subgenera. The genus presents a high variability in diet necessities, colour patterns, habitat preferences and phenology, while conserving a similar morphological structure (Williams et al., 2008).

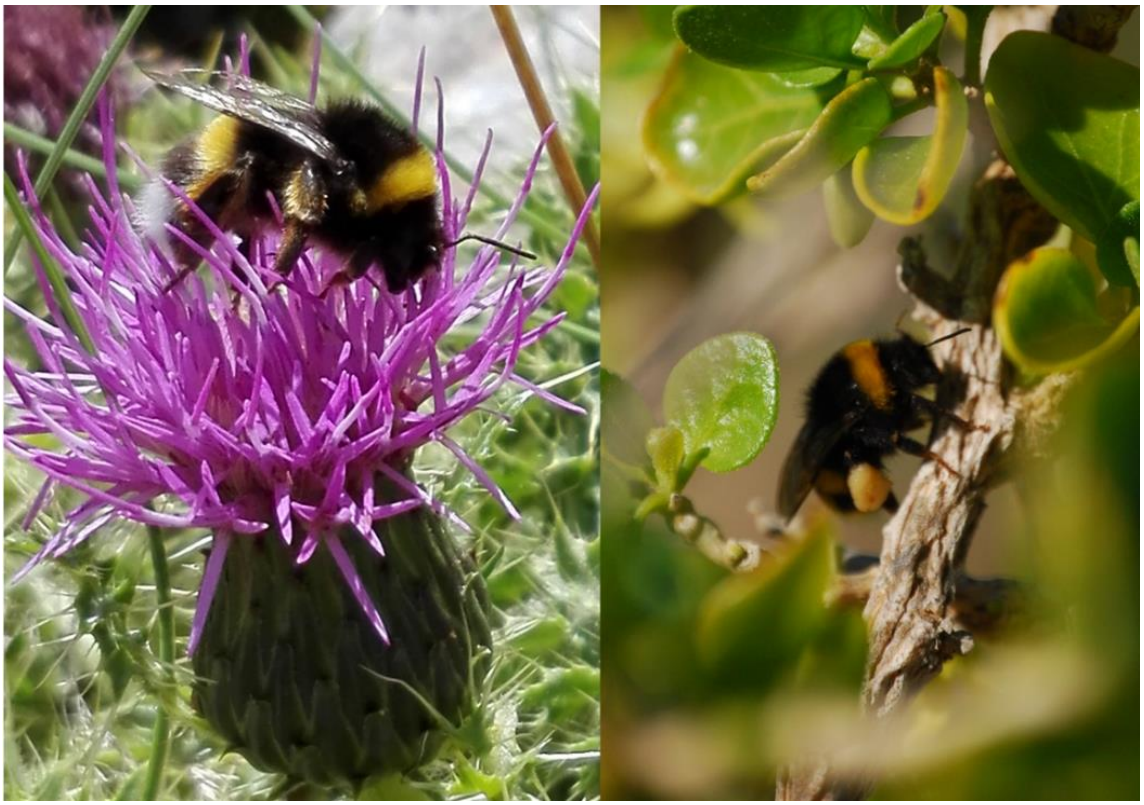

Figure 3. Pictures of *Bombus terrestris* individuals. On the left, a queen posed on *Centaurea sp.* On the left, a worker carrying pollen on her corbiculae. Pictures by Alba Gambín, used with permission.

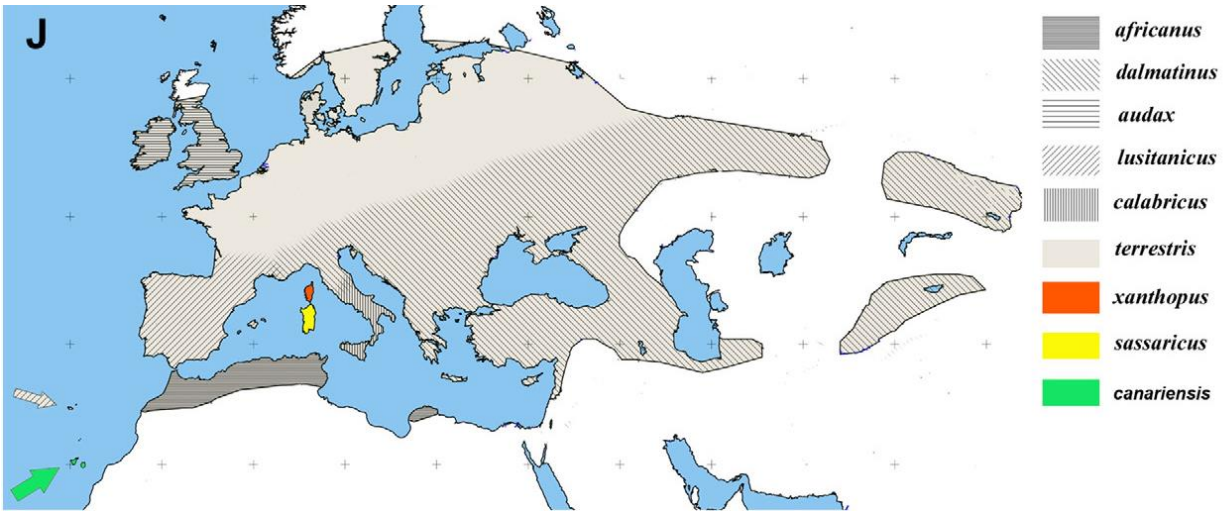

Figure 4. Geographic distribution of *Bombus terrestris* and its subspecies. From Lecocq et al. (2016a).

Distribution

The buff-tailed bumblebee *Bombus terrestris* (Linnaeus, 1758) shows a relatively large size among Apidae and is covered by a dense fur. It is a pollinator with a very generalist diet who uses its strong buzzing to shake the anthers of angiosperm flowers to collect pollen in their pollen basket or corbiculae (Buchmann & Hurley, 1978) (Figure 3). This species is one of the most widespread and abundant within the range of distribution of the genus *Bombus*, which has been set to occupy mainly Europe, from North of Africa to Sweden, and around the Mediterranean Basin (Rasmont et al., 2008) (Figure 4). Recent research has recorded its expanding range northwards, reaching the Arctic Circle north of Norway (Martinet et al., 2015), and eastwards, reaching Mongolia (Williams et al., 2012). According to the IUCN it is not considered a threatened species (Least Concern, LC), but models of different climate change scenarios predict that its range will be greatly reduced in southern Europe and disappear from Africa in the next 100 years (Rasmont et al., 2015). The species presents nine subspecies along its geographical distribution, with differences in their body colour pattern.

Phenology

B. terrestris is a eusocial insect with castes of individuals cohabiting in underground colonies including a queen and her progeny of workers. The two castes perform different tasks: while the queens lay eggs, the workers protect and maintain the colony. Some of the main functions of the workers are rearing and feeding the larvae, maintaining a basal temperature in the colony (Heinrich, 2004), removing dead corpses and sick individuals to prevent the spread of diseases (Jandt & Dornhaus, 2004) and foraging nectar and pollen. It has been recorded that their foraging area extends over 700 meters from their colonies (Knight et al., 2005; Nagamitsu et al., 2009), and that they use pheromone communication to recruit more foragers after resources have been found (Dornhaus & Chittka, 2001).

Bumblebees are commonly considered a primitive eusociality. Queens of *Apis mellifera* Linnaeus, 1758, a highly social insect, present a very different morphotype than workers without foraging structures. They depend on their colony to survive and new colonies are made by swarming: the division of part of the colony to follow the old queen to a new location (Michener, 1969). In bumblebees, the main phenotypic difference between castes is the size and, in some cases, the colour patternsof the body. Queens have not lost their corbiculae, allowing them to spend part of their life cycle foraging. Moreover, size variation is high among bumblebee workers. Although there is not strict acquisition of functions by individuals, larger workers tend to become foragers (for which they are more productive), while smaller workers tend to perform tasks within the colony (Spaethe et al., 2002). Some other behaviours, such as feeding the brood or thermoregulation of the colony, are less affected by size and more conditioned by other factors, such as genetics, hormonal mediation or individual experience, highlighting the complexity of social behaviour and the importance of intracolony diversity (Jandt & Dornhaus, 2014).

The sex of individuals is regulated by a single-*locus* complementary sex determination system (sl-CSD). Heterozygosity at this locus gives rise to the female phenotype, and hemizigosity (when only one set of chromosomes is present) or homozygosity gives rise to the male phenotype (haplodiploidy) (Van Wilgenburg et al., 2006). Given the high diversity of alleles in the sl-CSD *locus*, it is very likely that diploid individuals are heterozygous and develop as females. On the other hand, when an individual is haploid, there is only one allele and therefore it is hemizygote, so is becomes a male.

Bumblebee workers are able to lay eggs, although the ovarian development and oviposition are mostly inhibited by the pheromones and behaviour of the queen and other workers (Alaux et al., 2004; Zanette et al., 2012). Without mating, workers can only lay male eggs as they are unfertilized (haploid), while queens can lay both unfertilized (haploid, will develop as males) and fertilized eggs (diploid, will develop as females).

However, as there is no behaviour that prevents mating between closely related individuals, or in case of low genetic diversity in the population, it is possible that some diploid individuals carry the same allele on both chromosomes (homozygous) and developing as males being diploid (Bogo et al., 2018).

Diploid males are cannibalized by *A. mellifera* workers in their early larval stage, but they develop normally in bumblebee colonies, affecting growth rate and dropping the worker population of a colony down to the 50% (Duchateau et al., 1994), as half of the brood would be diploid for the sl-CSD gene. The appearance of diploid males can be detrimental, as they will not support the colony, present a shorter life spam, have a weaker immune response than haploid males and will produce diploid spermatozoids, (Gosterit et al., 2016a). If a queen mates with a diploid male, the offspring will be triploid and infertile, most of them unviable, resulting on a small and weak colony (Ayabe et al., 2004).

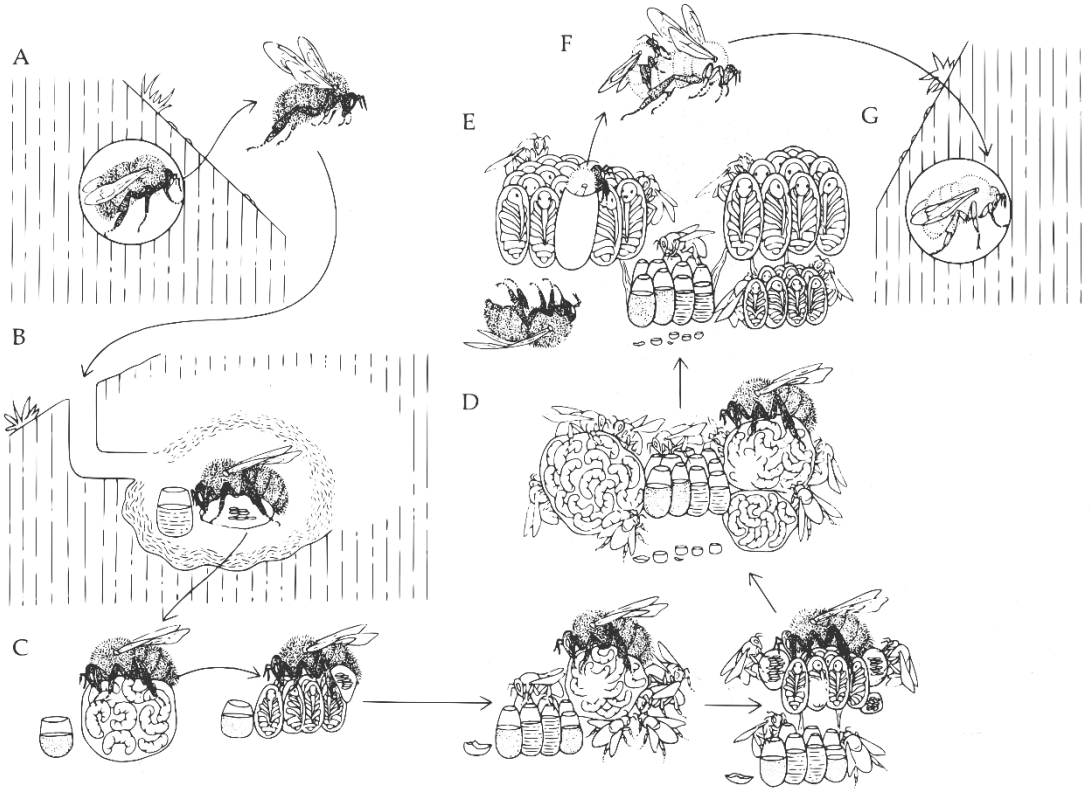

Figure 5. Diagram of a bumblebee colony cycle. A. Queen wakes from diapause. B. Queen finds a place to nest and lay eggs. C. Queen takes care of her first brood, which when hatched will take charge of foraging and rearing the larvae. D. Fully operational colony, the queen lays eggs while the workers take care of the colony. E. Past the competition point, the queen dies and the gynes (new queens) are feed to maturity. F. A male and a queen mating. G. The new fertilized queen finds a place to start her diapause. Modified from Bernard Heinrich (2004).

B. terrestris has an annual cycle, although is the only European bumblebee known to be able to become multivoltine (more than two cycles a year, Rasmont et al., 2015), as observed in the south of Europe (De Jonghe, 1986). Queens emerge from their diapause (Fig 5.A) usually in early spring in most of Europe or in autumn in the Mediterranean region, albeit their awakening can be synchronized with the blooming of an important resource (Gurel et al., 2008). Queens forage on their own until they find holes in the ground or empty dens of little rodents to stablish their colonies and start to hatch their first brood (Fig 5.B) (Goulson et

al., 2010b). *B. terrestris* are pollen-storers, their colonies do not follow the hexagonal architecture of the honeybee frames, they create spherical cells where they storage pollen or rear larvae.

Four to five weeks later, the first brood hatchs (Fig 5.C), and the new workers replace the queen on foraging duties, storing pollen and rearing the brood (Fig 5.D) (Goulson et al., 2010b). At the end of the cycle (usually at the end of the summer), the queen changes her behaviour, stops laying new worker eggs and starts laying male haploid eggs, what it is called the *switch point*. Pheromone inhibition in workers ceases, which is believed to be due to the queen losing dominance over the colony, and the last batches of diploid eggs become the new queens after being fed by the workers (Fig 5.E). Albeit it is not clear how colonies reach the *switch point*, as male and queen larva need more time and nutrients to fully develop, it is believed that some of the triggering factors for the colony maturation could be worker/larva ratio, as well as queen stress due to worker density (Alaux et al., 2004; Goulson et al., 2010b) or even a full control of the process by the queen (Duchateau & Velthuis, 1988; Holland et al., 2013). When workers change their behaviour to start laying male eggs, the colony reaches the *competition point* (Duchateau & Velthuis, 1988). Egg eating and worker policing dynamics become more frequent, affecting above all the eggs laid by the workers (Zanette et al., 2012). At this point, the mother queen has lost the dominance over the colony. The aggressions between workers and from workers to the queen bring the colony to a point of no return and the colony collapses (Duchateau & Velthuis, 1988).

Once they have reached full maturity, males leave the colony, while the gynes (new queens) stay until mating. Male flight longer distance than workers, which is a useful trait to maintain gene flow and lower endogamy within populations (Kraus et al., 2009). Males attract conspecific queens with the pheromones of their cephalic labial glands (CLG) to mate (Fig 5.F) (Valterová et al., 2019). Contrary to

honeybees, *B. terrestris* queens are monandrous as they mate with one male (Estoup et al., 1996), while males can mate with several queens. When queens have been mated, they will start the diapause and the cycle starts again (Fig 5.G) (Goulson et al., 2010b).

Subspecies

The nine subspecies of *B. terrestris* currently recognized have a particular geographic distribution (Figure 4). While the island subspecies are isolated from the mainland, the continental subspecies present in some cases, a phenotypic gradient so that the phenotype of one subspecies gradually changes with respect to the phenotype of the adjacent subspecies. The genetic differentiation in the continental continuum of the species is low as observed in Figure 6. Given the subtle differences among some of them and their ability to hybridize, it can sometimes be very difficult to classify them according to morphological traits (Figure 6).

The main molecular markers used to study population genetics in *B. terrestris* have so far been the mitochondrial cytochrome oxidase I (*cox1*) fragment, the secretions from the male cephalic labial gland of and short tandem repeats (STR) or microsatellites. In most cases, the differences among subspecies are non-existent or sparse, which has been attributed to the high gene flow of these flying insects and to their capacity to hybridize (De Jonghe, 1986; Estoup et al., 1996; Ings et al., 2005; Moreira et al., 2015; Lecocq et al., 2016a). In any case, in a recent study restricted to the Iberian Peninsula, mitochondrial haplotypes and some population structure due to geographical isolation have been found (Silva et al., 2020). Despite these circumstances, the endemic nature of some of the subspecies and their geographical distribution have led some Governments to adopt measures to manage and protect local populations of bumblebees (Chandler et al., 2019).

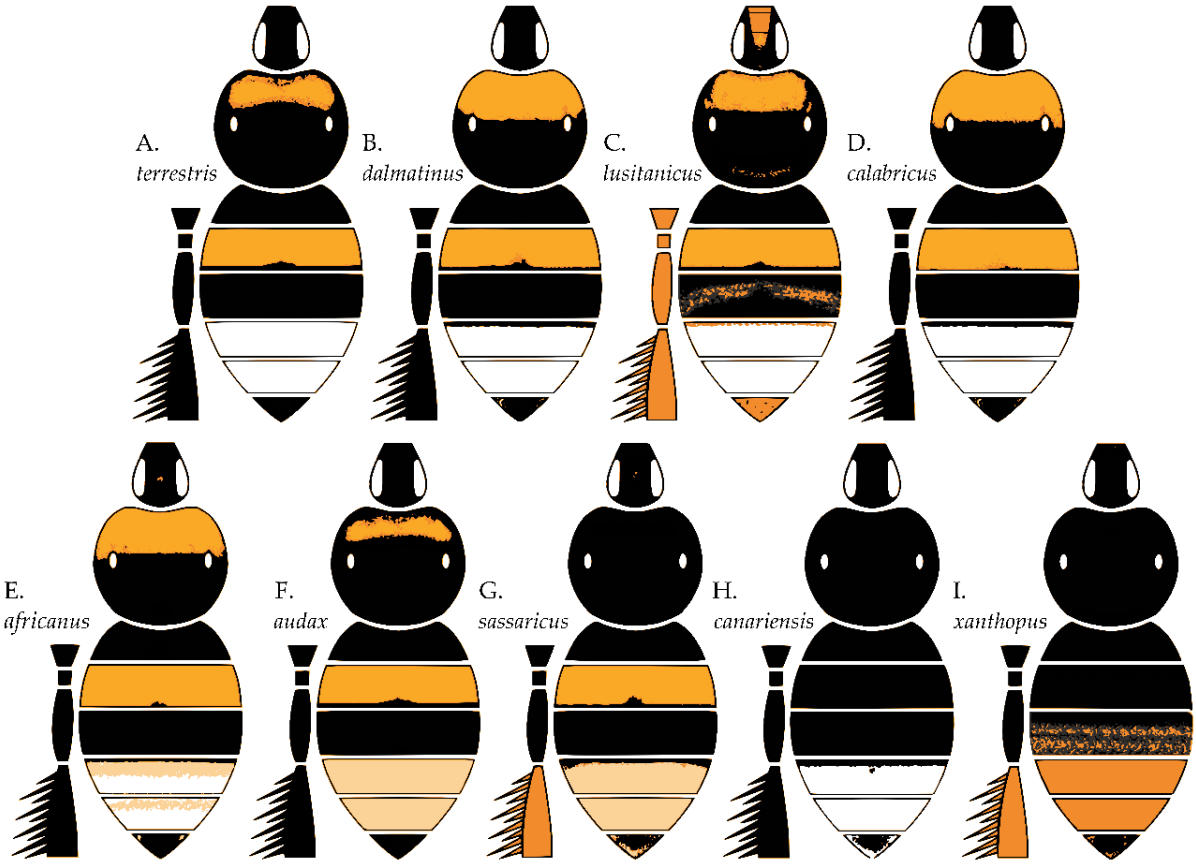

Figure 6. Schematic coloration of queens from the different subspecies of *Bombus terrestris*. From Rasmont et al. (2008).

Here is presented their natural distribution range and morphological differences as described in Rasmont et al. (2008), with annotations on their commercial use, conservation and molecular differentiation:

- *B. t. terrestris* occupies Western and Central Europe, from the Pyrenees to the north of the continent. Its colour pattern is defined by a predominance of black and yellow stripes on the thorax collar (less evident than in other subspecies), a second yellow band on the abdomen and a white coloration at the end of the abdomen (Fig 6.A). It is the nominative subspecies and forms a molecular group together with the other European continental subspecies. In Norway, only the local subspecies is allowed to be managed for pollination (Velthuis & Doorn, 2006).

- The other subspecies with a wider distribution is *B. t. dalmatinus*, which occupies the southern part of the species distribution area from south-east France to Iran. (Fig. 4). Its range overlaps with *B. t. terrestris*, and its phenotype is very similar, the main differences being the width and limits of the first collar (Fig. 6.B). Together with *B. t. terrestris*, these are the two main subspecies used for greenhouse pollination globally (Velthuis & Doorn, 2006).
- *B. t. lusitanicus* is native to the Iberian Peninsula, with an area of intergradation with *B. t. terrestris* at the Pyrenees and southern France (Fig. 4). It can be differentiated from other subspecies by its rusty brown colour on the legs and sometimes on the main body and head (Ornosa & Ortiz Sanchez, 2004) (Fig. 6C). The composition of its cephalic labial gland secretions is almost identical to the rest of the continental European subspecies.
- *B. t. calabricus* is naturally distributed only on Calabria and Sicily islands. Its colour pattern is different from that of other subspecies in its wide first collar, which extends below the tegula (Fig. 6D).
- *B. t. africanus* is currently distributed in the north of Africa separately from the rest of the species, although in the past its range might have extended to the south of the Iberian Peninsula (Rasmont, personal information). Its colour pattern is defined by a first wide collar and a light-yellow colouring at the end of the abdomen (Fig. 6E).
- *B. t. audax* occupies the British Islands and presents a thin and sometimes incomplete first collar. Queens also present a light-yellow coloration at the end of the abdomen (Fig. 6F). They are commercially reared for pollination use in the United Kingdom, New Zealand and Tasmania (Venthuis & Doorn, 2006). In the United Kingdom, strict legislation protects the subspecies against the introduction of other subspecies into the country for commercial use (Graystock et al., 2016).

- *B. t. sassaricus* inhabits the island of Sardinia. It normally lacks the yellow collar on the thorax and has a yellow segment on the abdomen and a light-yellow colouring on the end of the abdomen. It might present a reddish colour (Fig. 6G). It is mainly used for pollination in Sardinia, although it has also been used in Europe where the commercial populations failed to colonize the environment (Ings et al., 2010) (Fig. 7B).
- *B. t. canariensis* inhabits the Canary Islands. It presents a black coloration in the whole body, with a white segment at the end of the abdomen. Along with *B. t. xanthopus,* it is the subspecies with the most genetic and morphologic differentiation within the clade (Fig. 6H, 7). Its taxonomic classification as species or subspecies has been discussed in the past (Erlandsson, 1979) and it is currently accepted as a subspecies (Estoup et al., 1996; Lecocq et al., 2016a). Given the endemic status of the subspecies, the Canary Islands present a strict legislation concerning the use of other commercial subspecies in the territory (Velthuis & Doorn, 2006).
- *B. t. xanthopus* is distributed on the islands of Corsica, Capraia and Elba. It has a main pattern of black colour with red shades on the abdomen and legs. It may also present few yellow hairs on the thorax and variable reddish areas on the abdomen (Fig. 6I). Its classification as subspecies or species has been recently discussed due to the variability found in molecular analyses (Fig. 7) (Lecocq et al., 2016a).

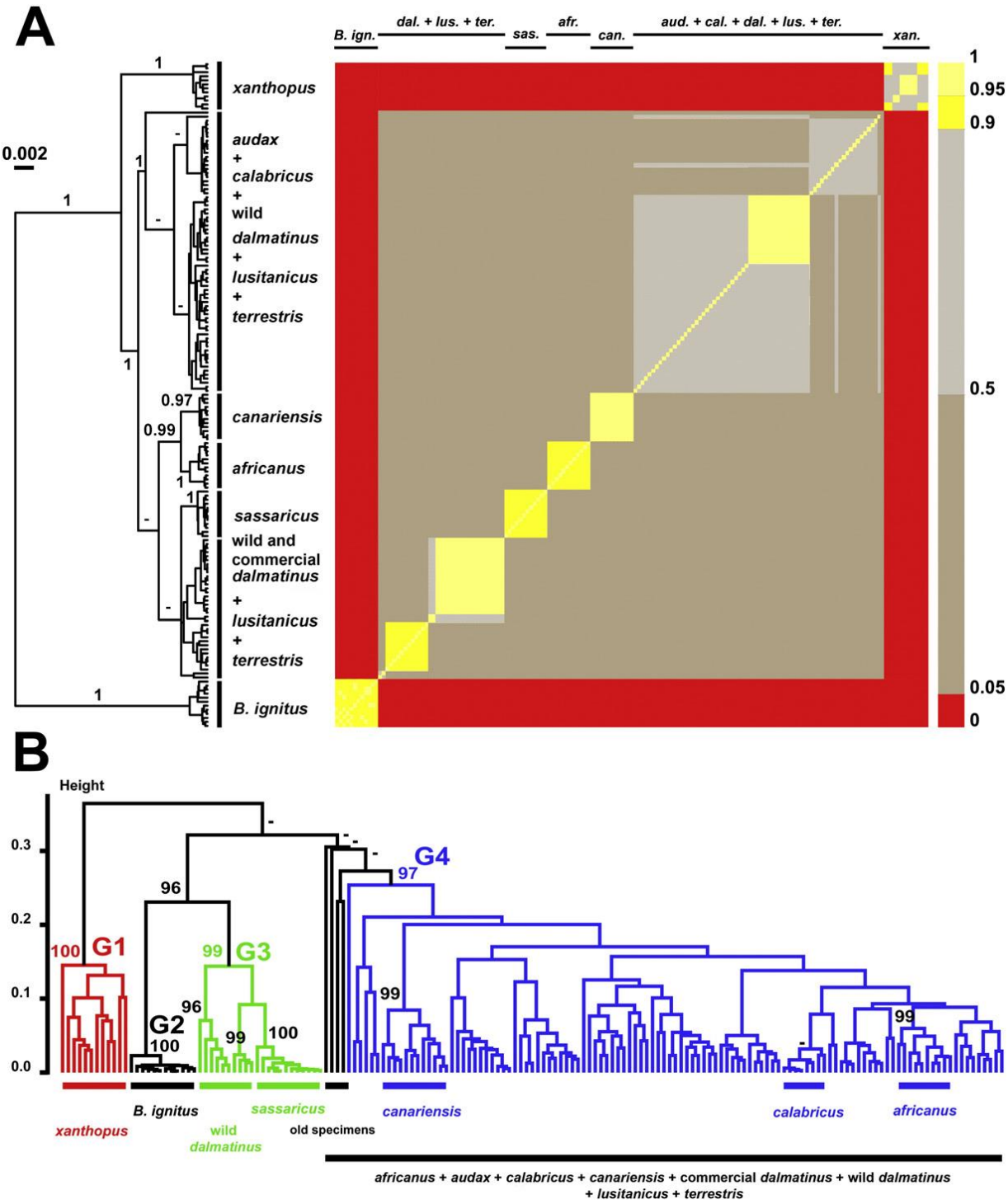

Figure 7. 7A. Bayesian ultrametric tree of *Bombus terrestris* subspecies based on a fragment of the cytochrome oxidase I *(cox1)* gene. Colors in the matrix correspond to the pairwise probability of conspecificity obtained from the Bayesian implementation of the general mixed Yule-coalescent (bGMYC). 7B. Unweighted pair group method with arithmetic mean (UPGMA) based on a correlation matrix calculated from the cephalic labial gland secretions. *B. ignitus* was used as outgroup (From Lecocq et al. 2016a).

Box 1: The subspecies problem

To classify and understand the diversity of life forms, the most basic taxonomic levels proposed are those of species and subspecies (De Queiroz, 2011). Depending on the study organism, different concepts can be used to define a taxon. In the case of insects, the most commonly used criteria are: morphological based on phenotypic traits, molecular based on differences between their genomes, widely used when studying cryptic species, or reproductive according to which two organisms belong to different species when they show reproductive barriers that prevent the existence of fertile hybrid offspring. In some cases, these criteria contradict each other, so it is advisable to use several to establish robust taxonomic hypotheses (integrative taxonomy) (Lecocq et al., 2015; Galtier et al., 2019). What criteria to use, how to label a specific taxon, and what exactly makes a taxon different is still an open debate in Taxonomy that is known as the species problem, which is studied by microtaxonomy.

Making this classification concrete is a challenge when working on subspecies conservation (Braby et al., 2012). In this thesis, we have decided to follow the morphological taxonomy of *B. terrestris* subspecies when referring to local populations or groups. However, regardless of the concept and taxonomic hypothesis, from a biological point of view, we have worked with different populations or OTUs (operational taxonomic units) within the species gradient, and not in hermetic groups. Terms such as hybridization and inbreeding have been used according to the definitions of Facon et al (2011) on the interaction between individuals, i.e. "crosses between individuals from genetically differentiated populations of the same species are also considered hybridization". Furthermore, according to these authors, three criteria must be met for intraspecific crosses to play a role in biological invasions: firstly, the populations involved in the mixing process must be genetically differentiated, secondly, crosses between individuals from different populations must be possible and finally, the mixed individuals must differ from the parents in some of their traits in order to influence the invasion process.

Ecosystem services and economic importance

Bumblebees and other insects pollinate both wild plants and crops. This direct and indirect contribution of ecosystems to human well-being is what we know as an ecosystem service. Gallai et al. (2009) estimated that the total economic value of pollination worldwide in 2005 was about 153 000 million €, which was equivalent to the 9.5% of the global agricultural production used for human food. Lautenbach et al. (2012) later estimated the global value of pollination in 2009, based on the average dependence of crops on pollination (Klein et al. 2007) as around 300 000 million €(converted to € from Figure 8).

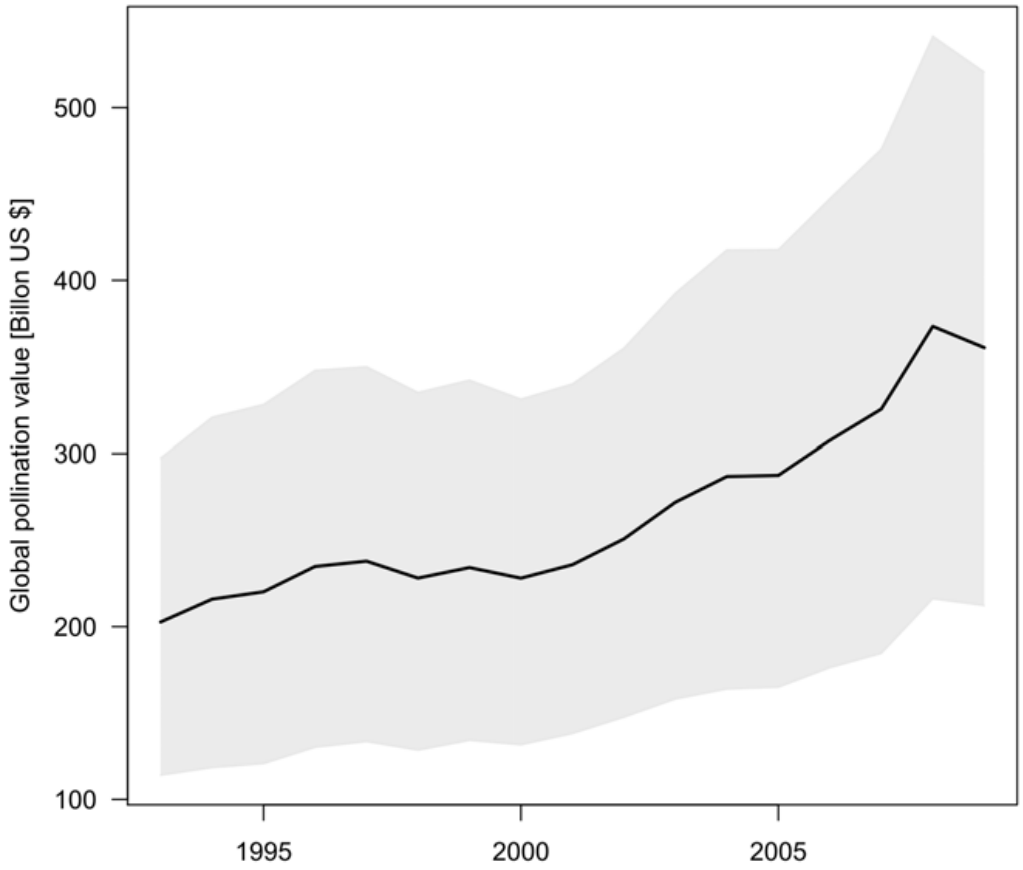

Figure 8. Temporal trend of global pollination benefit (1990-2009) based on data from Klein et al. (2007). Extracted from Lautenbach et al. (2012).

However, not all pollinators contribute in the same way. The value of crop production dependent on wild bees was estimated at an average of 3 000 € per

hectare (Kleijn et al. 2015), although the result was more dependent on the most common non-threatened pollinator species, as *B. terrestris*, whose crop-pollination services were valued at an average of 360 € per hectare.

The economic importance of *B. terrestris* has increased since the species was domesticated in the 1980s for use in greenhouses worldwide (Velthuis & Doorn, 2006). *B. terrestris* is a better pollinator than the honeybee for some crops such as berries and *Solanaceae* (tomato, pepper) or *Curcibitaceae* (melons, pumpkin) among others (Palacios Estay, 2007; Kraus et al., 2011) due to its buzzing capacity. The use of *B. terrestris* in these crops ensures larger fruit size, which increases crop productivity by reducing losses due to market quality standards (Goulson et al., 2010b). The global economic value of this service, for tomato alone, has been measured in 12 000 million € per year (Velthuis & Doorn, 2006).

In the last forty years, its breeding and export have become a worldwide industry, although it is difficult to obtain data on its profit. The most recent estimation we are aware of sets the trend for worldwide sales at around two million colonies for 2016 (Lecocq et al., 2016b).

The invasive expansion of *B. terrestris* worldwide

At the beginning of the domestication of *B. terrestris* it was thought that its use in greenhouses did not entail risks of potential colonization of the environment or introgression into local populations. The main reasons were that it seemed unlikely that gynes and males would be able to escape outdoors and, even if they did, it was assumed that commercial individuals were out of synchronization with wild ones and unable to thrive in the environment. However, these hypotheses were wrong (Kraus et al., 2011) and it has been demonstrated that *B. terrestris* has the capacity to survive and colonize new environments around the world (Figure 9) to the extent that it is considered a major threat to wild bees conservation (Sutherland et al., 2017).

B. terrestris outside its distribution range

B. terrestris shows many qualities that make it a highly invasive species. It has, among other characteristics, a high tolerance to heat stress, a long flight distance, a generalist diet, early emergence, and a high gynes production (Dafni et al., 2010). Due to its intensified export on a global scale, the species has expanded its distribution range beyond the Palearctic region, reaching all inhabited continents (Vanderplanck et al., 2019; Zambra et al., 2020; Dafni et al., 2010) and will become a threat if it is carelessly exported to new environments (Acosta et al., 2016; Lecocq et al., 2016b). Depending on the characteristics of the ecosystem, the damage caused by the introduction of a new invasive species may be different (Moore et al., 2013; Aizen et al., 2018; Tsuchida et al., 2019). Here, we will address some of the most studied cases.

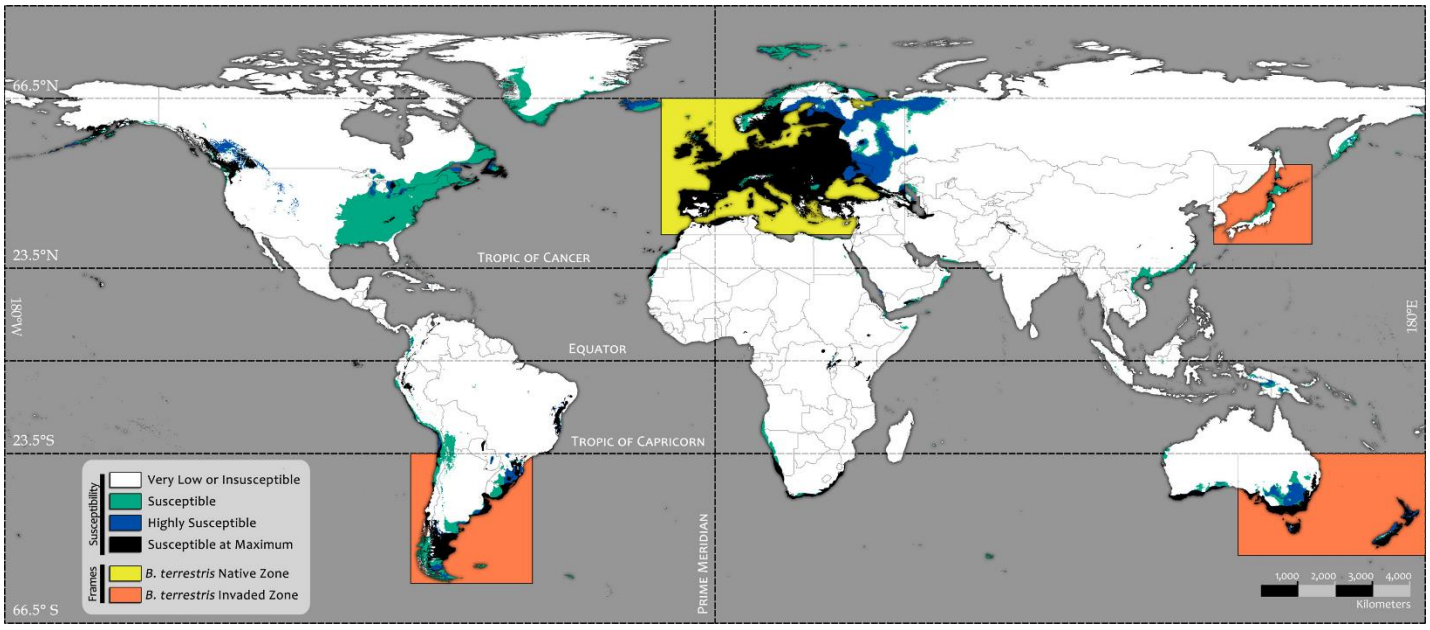

Figure 9. Map of current and potential distribution of *B. terrestris* worldwide. In yellow it is presented the native distribution range of the species, while in orange are the colonized territories. Susceptibility to new invasions has been modelled and graded by a colour scale from white to green and blue to black. From Acosta et al. (2016).

Tasmania and New Zealand

Some of the first introductions of *B. terrestris* occurred in New Zeeland in 1985 and in Tasmania in 1992. Its expansion was not limited by existing predators, climatic and seasonal variables, or diet requirements, so it can be found year-round and is highly compatible with most local flora. In 2002, *B. terrestris* colonies were found in Tasmania not only in urban gardens but also in natural environments (Hingston et al., 2002), and are now fully naturalized in the environment. Its exportation is not permitted to Australia (Moore et al., 2013).

Japan

The first bumblebee colonies were exported to Japan for experimental purposes in 1991. By 1996 their use for greenhouse pollination was already established in the country and the first naturalized colonies were discovered in the environment. The export was not regulated by 2004, when the trading reached 70 000 colonies sold in Japan (Inari et al., 2005; Tsuchida et al., 2019). The expansion of *B. terrestris* in Japan has affected the populations of endemic species as *B. hypocrita* or *B. ardens* to the point of local extinction, by competition for floral resources and nesting sites (usurpation) (Matsumura et al., 2004; Inoue et al., 2008; Tsuchida et al., 2019), and by interspecific hybridization. Species as *B. hypocrita* or *B. ignitus* can mate with *B. terrestris* but the eggs are unviable due to reproductive interference (Tsuchida et al., 2010; 2019), leading to lower populations density and genetic deterioration. In addition, competition is leading to a division of resources and inefficient pollination, due to the tendency of *B. terrestris* to use only nectar, which is negatively affecting the seed production of some native plants species (Dohzono et al., 2008, Nishikawa et al., 2015).

Scientific evidences led to the categorisation of *B. terrestris* as an invasive species in 2006, and its use is now restricted in the country under strict protocols (Tsuchida et al., 2010). The use of native bees as *B. hypocrita* to complement the

pollination function has been tested in the country and would be less environmentally disadvantageous (Takeuchi et al., 2018). Although *B. terrestris* has not yet reached the Asian mainland, according to simulations by Naeem et al. (2018), if the commercial bumblebee becomes naturalized there, it would become a threat to local populations of bumblebees in China, Korea or Russia.

Chile and Argentina

The European bumblebee *B. ruderatus* was successfully introduced to Chile in 1982. Six years later, the usefulness of *B. terrestris* colonies was tested in both open and closed greenhouse crops in this country (Schmid-Hempel et al., 2014) and after the experiments, its importation for commercial use began.

Lately, given the invasive capacity of both species, Argentina banned their export to protect local pollinators (Aizen et al., 2018). Nevertheless, both species managed to cross the Andes to Argentina; *B. ruderatus* in 1994 (Roig Alsina & Aizen, 1996) and *B. terrestris* in 2006 (Torretta et al., 2006). *B. terrestris* has expanded across the continent at a rate of 200 km per year, reaching the Atlantic coast of Argentina in 2011 (Schmid-Hempel et al., 2014). Commercial bumblebees compete with the native pollinators and in parallel has increased pollination of non-native invasive plants (Morales et al., 2017; Medel et al., 2018). Its capacity as a vector of spillover is nowadays unclear (Revainera et al., 2020), although the expansion of the species is ligated to the local extinction of the Argentinean populations of the species *B. dahlbomii* (Schmid-Hempel et al., 2014). This case highlights the need to achieve consensual responses across country borders when it comes to biodiversity conservation and protection (Aizen et al., 2018).

B. terrestris within its distribution range

Within the distribution range of the species, the dispersal of commercial individuals into the environment can also pose a threat to local populations. *B. terrestris* colonies have many characteristics that make them better suited to living

in both disturbed and natural environments comparedto local populations of the species. Commercial bumblebee breeds have shown to have larger bodies, greater nectar foraging activity and a higher production of gynes than wild colonies (Ings et al., 2006), making them more successful for colonization and expansion, and therefore, potential competitors against their conspecifics. The effects of hybridization between commercial and wild individuals of *B. terrestris* were studied by Gosterit et al. (2016b), who confirmed that commercial colonies produce larvae much more rapidly and in greater numbers, which is determined by the maternal genotype, placing an extra concern in the invasion of commercial queens.

United Kingdom

In the United Kingdom (UK), the export of commercial colonies began in 1989 (Velthuis & Doorn, 2006) but it was not until decades later that its effect on the populations of the endemic subspecies *B. t. audax* was studied, in the belief that commercial populations would not be able to survive in the environment. Nevertheless, the commercial subspecies *B. t. dalmatinus* presents the same cold tolerance as the endemic subspecies and can survive to the climatic conditions of the UK and compete with local populations (Owen et al., 2016). Given the genetic diversity of the insular subspecies (Lecocq et al., 2016a), the wild British and Irish populations are at risk of introgression and loss of rare alleles under the presence of commercial breeds in the wild (Moreira et al., 2015). In addition, greenhouses have been shown to be sources of spillover of pathogens such as *C. bombi* and *N. bombi*, possibly because individuals reared in high density, as in breeding facilities, are highly likely to become infected (Murray et al., 2013). For these reasons, from 2015 the exportation of non-native subspecies is regulated, although the lack of legislation on more comprehensive testing for pathogens remains a matter of debate (Graystock et al., 2016).

Box 2: The Iberian Peninsula as a diversity hotspot

During the last Quaternary glaciation, due to its different mountain systems, the Iberian Peninsula became an intricate long-term glacial refugia for different species that diversified and speciated, giving rise to the current high number of endemisms and local lineages (Hewitt, 2011). For this reason, it is nowadays considered a hotspot of diversity. In addition, the location of the peninsula between Africa and Europe promotes the convergence and migration of species and populations also enhacing its position as a hotspot.

This level of biodiversity has been widely studied in numerous organisms (Gómez & Lunt, 2007), among them the Iberian honeybee *A. m. iberiensis*. Regarding this important pollinator, Pinto et al. (2012) observed that the Iberian Peninsula is one of the European regions with the highest diversity of mitochondrial haplotypes, which they argued is due to past contact between the subspecies *A. m. mellifera* from Northern Europe and *A. m. intermissa* from Northern Africa during the last glacial maximum.

There are 39 *Bombus* species and 56 subspecies in the Iberian Peninsula (in addition to hybrid forms in the overlapping areas, Ornosa and Ortiz-Sanchez, 2004). The Iberian-Balearic fauna is composed of Palearctic, Eurasian, European, Mediterranean and endemic species, the latter mostly at the subspecies level (Ornosa and Ortiz-Sánchez, 2004). According to the Atlas and Red List of Threatened Invertebrates of Spain (Verdú et al., 2011) there are seven species with some degree of extinction threat in the Iberian Peninsula: 1 LC (Least Concern), 3 VU (Vulnerable) and 3 EN (Endangered). However, according to the European Red List of Bees (Nieto et al., 2014) there are 38 threatened bumblebee species: 2 NT (Near Threatened), 3 VU, 3 EN and 1 CR (Critically Endangered).

Objectives and hypotheses

Interest in the effects of the introduction of commercial breeds of *B. terrestris* on populations of the endemic Iberian subspecies (*Bombus terrestris lusitanicus*) has increased in recent years. However, there is no legislation regulating the importation of foreign commercial colonies, nor supporting the use of native subspecies for pollination in Spain (with the exception of the Canary Islands and the subspecies *B. t. canariensis)*. The hypotheses of this thesis are that the genetic pool of the endemic subspecies *B. t. lusitanicus* allows its differentiation from other *B. terrestris* subspecies, due to its geographic distribution in what is considered a biodiversity hotspot , and, secondly, that the genetic diversity of current populations is being affected by the introduction of commercial populations that can be naturalized in the environment. In order to verify these hypotheses and provide a scientific basis for further legislation on the import and use of commercial bumblebees, the following objectives have been set out in this thesis:

- To search for subspecific markers to characterize the genetic diversity of the local populations of *B. terrestris* in the Iberian Peninsula.
- To quantify the possible introgression of commercial breeds into populations of the subspecies *B. t. lusitanicus* in a gradient of peninsular distribution and in areas of contact between the two subspecies: natural (Pyrenees) and artificial (near greenhouses).
- To describe the genetic diversity of the subspecies *B. t. lusitanicus* and evaluate the impact of the introduction of commercial subspecies.

Structure of this thesis

This thesis is divided into four chapters: the first three are oriented towards the search for and validation of reliable molecular markers to perform inexpensive

and rapid assays to help differentiate Iberian and Central European bumblebee populations, while the forth aim is to describe the genetic diversity of the subspecies *B. t. lusitanicus* and assess whether the introduction of the commercial subspecies has an impact on its genetic structure. Therefore, the results and considerations reached in each chapter served as a basis for the following experiments.

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Chapter 1.

Preliminary report on cross-species microsatellite amplification for bumblebee biodiversity and conservation studies

Abstract

The Iberian Peninsula holds a high diversity of bumblebees but there is a general lack of information about their biodiversity in this area. To overcome this and facilitate conservation studies, we present two novel multiplex assays for the amplification of six and five microsatellite loci respectively. Both assays successfully amplified for most of the studied species in the Iberian populations. Sibling workers and population genetic parameters were analysed in the managed species *B. terrestris* and in the wild species *B. monticola* and *B. mesomelas*, demonstrating the capability of these multiplex assays for biodiversity studies of both managed and wild bumblebee species.

Introduction

The need to conserve genetic diversity in wild bee populations and understanding its structure and function to design successful breeding and conservation programs has been highlighted recently (López-Uribe et al., 2017). Because of several potential causes, populations of both managed and wild bees have been reported to be declining during the last decade in different parts of the world (Biesmeijer et al., 2006). In this context, despite the large bumblebee species richness present in the Iberian Peninsula with 39 out of the 79 West-Palearctic species distributed in this area (Ornosa & Ortiz-Sánchez, 2004), few molecular studies have been undertaken to know the genetic diversity of Iberian bumblebee taxa. Such studies are becoming crucial to the conservation of these important pollinators given the reduction of the altitudinal distribution range towards better-preserved high areas observed in Pyrenean populations of several bumblebee species (Ornosa et al., 2017). On the other hand, the distribution ranges of managed species as *B. terrestris* has changed due to escapes from agricultural facilities, especially in southern Spain, where bumblebee breeding companies are located to supply pollinators to the many greenhouses (Ortiz-Sánchez, 1992; Ornosa, 1996; Cejas et al., 2018; Trillo et al., 2019). An efficient approach for population genetic studies is to analyse the variation of microsatellite markers which allows genotyping different individuals with many loci, and thus elucidation of their genetic diversity and conservation status. Such markers are widely used in studies on the honeybee (Evans et al., 2013) and have been also implemented in stingless bees (see Hurtado-Burillo et al., 2014 as an example). Given the importance of the pollinating function of the bumblebees, and the economic benefits derived from its trade (Velthuis & Doorn, 2006), the validation of tools to explore the genetic diversity of *B. terrestris* are necessary to maintain the genetic diversity of commercial breeds. In this work, we selected 11 microsatellite loci developed by Estoup et al. (1995, 1996) from *B. terrestris* and

designed two novel multiplex assays of six and five microsatellites based on Wolf et al. (2010). Multiplex assays were cross-amplified in ten wild *Bombus* species and one reference species (*B. terrestris*) to test their efficacy. The chosen species were *B. lucorum*, *B. hortorum*, *B. lapidarius*, *B. humilis*, *B. mesomelas*, *B. ruderarius*, *B. vestalis*, *B. pratorum*, *B. soroeensis* and *B. monticola*. These species have a conservation status of Less Concern (Nieto et al., 2014); however, some of them, like *B. mesomelas*, are in regression in its distribution range in Europe (Rasmont et al., 2015) and the Pyrenees (Ornosa et al., 2017) while the managed *B. terrestris* is expanding its distribution range. To our knowledge *B. mesomelas* and *B. monticola* are here microsatellite-genotyped for the first time.

Material and Methods

Sampling and DNA extraction

Individuals were collected during sampling campaigns in 2013-2015. DNA was extracted from one leg of each individual following Walsh et al. (1991) or Ivanova et al. (2006).

Genotyping and amplification effectivity

Each amplification reaction contained 1X reaction buffer, 1.2 mM of MgCl2, 0.3 mM of dNTPs, 0.2 μM of each primer (multiplex RB1: B10, B100, B11, B124, B126, B96 and multiplex RB2: B118, B119, B121, B131 and B132 with forward primers fluorescent-labelled, Table 1), 1.2 mg/ml of BSA, 1.5 U of Kapa Biosystems Taq and ~40 ng of DNA. The same PCR conditions were used for both assays: initial denaturation at 95 ºC for 5 min, followed by 30 cycles of 30 s at 92 ºC, 30 s at 54 ºC and 30 s at 72 ºC, with a final elongation of 30 min at 72 ºC. Alleles were separated using capillary electrophoresis on an ABI Prism 3700 (Applied Biosystems) and scored with Genemapper 4.8 software (Applied Biosystems). The fluorescence intensity (measured in relative fluorescent units, RFU) was ranked as low (L, fluorescence intensity <900 RFUs and high failure percentage),

medium (M, fluorescence intensity ≈1000 RFUs) and high (H, fluorescence intensity >1900 RFUs) (Appendix 1) and used as a measure of amplification effectivity. GenAlEx 6.5 (Peakall & Smouse, 2012) was used to estimate allele size range (SR) and number of alleles (Na) per locus.

Population genetic analysis

Population parameters were only analysed in the managed species *B. terrestris* (reference species) and in the wild species *B. monticola* and *B. mesomelas* (tested species) to study the efficacy of our multiplex assays to obtain genetic diversity parameters. Sibling workers from the same colony inferred with Colony 2.0.6.2 (Wang, 2012) were excluded from further analyses. Micro-Checker 2.2.3 (Van Oosterhout et al., 2004) was used to calculate the frequency of null alleles. Observed (Ho) and expected heterozygosity (He) values were obtained with GenAlEx 6.5. Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were estimated with Genepop on the wb (Rousset, 2008). Bonferroni correction was applied to LD results to avoid type I errors.

Results and Discussion

Of the 121 species-locus combinations, 91.7% amplified correctly (based on the relative fluorescence intensity observed), showing B118 the lowest amplification success (Table 2). In total 82% of combinations were polymorphic, although this rate could be bias by the number of individuals analysed per species (i. e. only three *B. vestalis* individuals could be sampled). The obtained results show that both multiplexes presented here are suitable for population studies of the analysed species since even datasets with only eight microsatellite loci (as in Maebe et al., 2015) provide appropriate genetic information for conservation purposes.

Table 1 Summary information for microsatellite multiplex reactions. Observed (H_O) and expected (H_E) heterozygosity and null alleles frequency (F_{NULL}) are given for *B. terrestris* (reference species) and *B. mesomelas* and *B. monticola* (tested species).

Multiplex/Locus	Primer sequence 5′–3′	Dye	*B. terrestris* (N=12)			*B. mesomelas* (N=13)			*B. monticola* (N=10)		
RB1			H_O	H_E	F_{NULL}	H_O	H_E	F_{NULL}	H_O	H_E	F_{NULL}
B10	5'- GTGTAACTTTCTCTCGACAG-3' 5'-GGGAGATGGATATAGATGAG-3'	PET	0.875	0.900	0.0365	-	-	-	0.846	0.722	0
B100	5'-CGTCCTCCTATCGGGCTAAC-3' 5'-CCTCGAAACCTCGTGACG-3'	VIC	0.750	0.771	0.0625	0.308	0.260	0	0.462	0.355	0
B11	5'-GCAACGAAACTCGAAATCG-3' 5'-GTTCATCCAAGTTTCATCCG-3'	FAM	0.750	0.805	0.0308	0.923	0.867	0	1	0.822	0
B124	5'-GCAACAGGCGGGTTAGAG-3' 5'-CAGGATAGGGTAGGTAACAG-3'	NED	0.875	0.846	0	0.692	0.675	0	0.385	0.556	0.110
B126	5'-GCTTGCTGGTGAATTGTGC-3' 5'-CGATTCTCTCGTGTACTCC-3'	NED	0.938	0.840	0	0.769	0.749	0	0.615	0.530	0
B96	5'-GGGAGAGAAAGACCAAC-3' 5'-GATCGTAATGACTCGATATG-3'	VIC	0.563	0.611	0.022	0.846	0.781	0	0.385	0.746	0.207
RB2											
B118	5'-CCTAACTCCCTATATClTCG-3' 5'-GAAACACCTATCTACATCTACAG-3'	FAM	0.750	0.781	0.061	-	-	-	-	-	-
B119	5'-CATCGTGCTAGAAAAGGAAG-3' 5'-CCACACTGCAAAGlTTCTG-3'	NED	0.563	0.461	0	0	0	0	0	0	0
B121	5'-GAACATGTGGAACGACGG-3' 5'-GAACAATCGATATGTCACCC-3'	NED	0.250	0.361	0.1089	0.846	0.858	0.0069	0.308	0.260	0
B131	5'-GATCGCCTATCTCITCTCGG-3' 5'-GAGGCGCTCTCGACCTC-3'	FAM	0	0	0	0.462	0.604	0.1333	0.923	0.793	0
B132	5'-GAAATCGTGCCGAGGG-3' 5'-CAGAGAACTACCTAGTGCTACGC-3'	VIC	0.667	0.840	0.0615	0.153	0.145	0	0.923	0.754	0

Table 2. Scoring efficiency of the cross-species amplification of 11 microsatellite loci in *Bombus* species. For each species is given the number of individuals (N), percentage of amplified individuals for each locus (%), locus size range (SR) and number of alleles (Na) and relative fluorescence intensity (RFI) of the amplification, ranked as low (L, fluorescence intensity <900 RFUs and high failure percentage), medium (M, fluorescence intensity ≈1000 RFUs) and high (H, fluorescence intensity >1900 RFUs).

Species		B10	B100	B11	B124	B126	B96	B118	B119	B121	B131	B132
B. lucorum	%	100	100	100	90	100	100	100	100	70	100	80
N= 10	SR (Na)	186-230 (13)	132-182 (10)	157-171 (5)	238-252 (5)	166-202 (11)	233-235 (2)	210-218 (5)	128-130 (2)	157 (1)	121 (1)	145-185 (9)
	RFI	H	H	H	H	H	H	H	H	H	H	H
B. terrestris	%	100	100	100	100	100	100	100	100	100	100	94
N= 16	SR (Na)	186-222 (14)	152-170 (7)	154-172 (8)	232-260 (10)	116-202 (12)	234-11246 (5)	208-222(7)	128-132 (3)	158-166 (3)	121 (1)	157-175 (9)
	RFI	H	H	H	H	H	H	H	H	H	H	H
B. hortorum	%	40	83	100	100	100	100	0	80	100	100	60
N=5	SR (Na)	178-180 (2)	146-162 (4)	140-158 (5)	260-280 (7)	148-204 (7)	238-248 (5)	-	124 (1)	155-181 (6)	122-136 (5)	149-175 (6)
	RFI	L	M	M	M	M	M	-	L	H	H	L
B. lapidarius	%	100	100	100	100	100	100	100	100	100	100	100
N=10	SR (Na)	208-240 (10)	156-174 (5)	160-186 (6)	266-278 (5)	139-159 (8)	244-256 (6)	207-223 (7)	124 (1)	157-165 (2)	136-152 (6)	153-161 (5)
	RFI	H	H	H	H	H	H	H	H	H	H	H
B. humilis	%	100	100	100	100	100	100	100	100	100	100	100
N=8	SR (Na)	178-186 (4)	136 (1)	130-132 (2)	248-252 (2)	134-144 (2)	224-236 (4)	212-216 (2)	124-128 (2)	158-164 (2)	121-125 (3)	143-155 (5)
	RFI	M	H	H	H	H	H	H	H	H	H	H
B. ruderarius	%	100	100	100	100	100	100	100	0	100	100	100
N=6	SR (Na)	174-194 (8)	136 (1)	136-144 (4)	246-258 (4)	136-142 (3)	241-251 (6)	212-216 (3)	-	158-164 (2)	121 (1)	145-151 (4)
	RFI	H	H	H	H	H	H	H	-	H	H	H
B. mesomelas	%	0	100	100	100	100	100	0	94	100	100	94
N=16	SR (Na)	-	148-154 (2)	156-202 (11)	256-264 (5)	162-182 (8)	238-250 (5)	-	124 (1)	140-174 (12)	121-125 (3)	143-155 (5)
	RFI	-	H	M	H	H	M	-	M	H	H	L
B. vestalis	%	100	100	100	100	100	100	100	100	0	100	100
N=3	SR (Na)	196 (1)	144-154 (1)	138 (1)	234 (1)	147-161 (3)	254-256 (2)	230-248 (2)	136 (1)	-	126-128 (2)	153-163 (3)
	RFI	L	L	M	M	L	L	L	H	-	M	M
B. pratorum	%	100	86	100	100	100	100	0	86	100	100	86
N=7	SR (Na)	194-196 (2)	148-154 (2)	132-138 (2)	232-234 (2)	136 (1)	237 (1)	-	124 (1)	149-165 (3)	117-127 (3)	176-180 (3)
	RFI	M	M	H	H	H	M	-	H	M	H	L
B. soroeensis	%	100	86	86	100	100	100	0	0	0	100	100
N=7	SR (Na)	200-212 (4)	150-168 (6)	160-176 (6)	250-258 (4)	194-224 (8)	251-265 (7)	-	-	-	137-153 (6)	169-179 (5)
RFI	-	M	L	M	M	L	M	-	-	-	M	L
B. monticola	%	100	100	100	100	100	100	0	100	100	100	100
N=13	SR (Na)	212-226 (5)	154-156 (2)	137-157 (8)	232-236 (3)	160-164 (3)	245-253 (4)	-	124 (1)	151-153 (2)	129-143 (7)	164-182 (8)
	RFI	H	H	H	H	H	H	-	H	H	M	M

In the reference species (*B. terrestris*), the number of alleles ranged from 1 (B121) to 14 (B10) (mean: 7.18) (Table 2). Four sibling workers were excluded for population analysis (final N=12). Heterozygosity and null allele frequency results were similar to those obtained by Moreira et al. (2015) at the European level, confirming the efficacy of the multiplex assays in the Iberian population. Neither significant deviation from HWE nor LD were found. In the tested species *B. mesomelas*, two (B10 and B118) of the 11 loci did not amplify (Table 2). Number of alleles varied from 1 (B119) to 11 (B11) (mean: 5.7). Three sibling workers were removed from population analysis (final N=13). No significant deviations from HWE, LD or null alleles were found. In *B. monticola*, one marker (B118) did not amplify, while in the other ten loci, the percentage of amplification achieved 100% and the number of alleles ranged from 1 (B119) to 8 (B11) (mean: 4.3). Three sibling workers were removed from population analysis (final N=10). Significant deviation from HWE was found in B96, which might be due to the existence of null alleles, observed at a frequency of 0.207. LD test revealed no significantly linked loci after applying Bonferroni correction.

Conclusions

In conclusion, the obtained results showed that the amplification of these 11 microsatellite *loci* optimized in two multiplex assays might be useful to genotype both wild Iberian bumblebee populations of many of the studied species and the commercial breeds of *B. terrestris*. Moreover, they could potentially be used in populations with other origin. As demonstrated by the results obtained in the population analysis of *B. mesomelas* and *B. monticola*, the use of these loci might be helpful to depict the population structure and genetic differentiation of bumblebee populations and for future assessments of their conservation status. This is especially urgent in mountain habitats as the Pyrenees since an upward trend towards better-preserved high areas has been observed in the bumblebee

populations (Ornosa et al., 2017). On the other hand, multiplex assays have shown a good resolution to infer the genetic parameters of the managed species *B. terrestris*. This result provides a suitable tool to value the genetic diversity of the bumblebee breeds in companies producing nests for pollination of crops, as well as to determine the gene flow between managed and wild populations of this important species (Kraus et al., 2011).

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Chapter 2.

Searching for Molecular Markers to Differentiate *Bombus terrestris* (Linnaeus) Subspecies in the Iberian Peninsula

Abstract

Bumblebees (genus *Bombus* Latreille) are pollinator insects of great ecological and economic importance, which commercial use for pollination has increased since the 80s. However, the introduction of foreign *Bombus terrestris* (Linnaeus) has resulted in a decline of native bumblebee populations in Japan, Chile and Argentina among others. To study the potential introgression of commercial *B. terrestris* into the Iberian endemic subspecies *Bombus terrestris lusitanicus* Krüger it is necessary to find a precise molecular marker that differentiates both subspecies. For this purpose, comparative analyses were carried out between B. *t. lusitanicus* and *B. t. terrestris* (Linnaeus) from Spain and from Belgium by sequencing the nuclear genes elongation factor 1-α and arginine kinase and the mitochondrial gene *16S* ribosomal RNA, and genotyping with eleven microsatellite loci. No differentiation was observed at the nuclear level, but haplotypes found within the *16S* sequence correlated with the morphological characterization of the subspecies. In a case study including individuals sampled before the establishment of bumblebee rearing companies and others from recent samplings, we detected hybrid individuals (those with non-matching morphological subspecies and *16S* haplotype) more frequently in the south supporting the naturalization of commercial *B. t. terrestris* and introgression events between both subspecies. This marker should be used in Iberian populations with the aim to support management and conservation actions in endemic populations of *B. t. lusitanicus*.

Introduction

The average pollination dependency of crops was estimated to around 300 000 million of euros in 2009 (Lautenbach et al., 2012). Among the insect pollinators, the bumblebee *Bombus terrestris* (Linnaeus) is used in greenhouses all over the world because they are better pollinators than honeybees for crops as strawberry, tomato or melon, due to their large body and buzzing capacity. The economic value of this service, just for tomato, has been measured in 12 000 million of euros per year (Velthuis & Doorn, 2006). Nevertheless, in the current scenario of bee decline, the introduction of non-native *Bombus* species and subspecies without a proper legislation may affect local bumblebee biodiversity (Goulson et al., 2015). Such introductions suppose a risk for the conservation of endemic species and subspecies in many countries (Lecocq et al., 2016a) to the extent that invasions of commercial nonnative bumblebees have been reported as one of the 15 emerging issues for global conservation in 2017 (Sutherland et al., 2016). In this sense, *Bombus terrestris* (Linnaeus) can leave greenhouses and colonize the environment (Kraus et al., 2010), presenting some negative effects on native bees, for instance, displacing wild species while competing for resources such as pollen or nesting places (Matsumura et al., 2004; Aizen et al., 2018). This colonization might affect native species by spreading exotic diseases and parasites (Whitehorn et al., 2013) and changing plant-pollinator interactions in non-native environments, impacting crops, native plants and pollinators (Morales et al., 2013; Aizen et al., 2014). *B. terrestris* have also better competitive capacities such as large foraging ranges and a broad diet, and they emerge early in the season making them adaptable to different environments (Matsumura et al., 2004). Currently, the invasive distribution of *B. terrestris* is increasing due to direct human intervention. Bumblebee introduction has impacted local bee populations in countries as Chile, China, Israel, Japan, Mexico, South Africa, South Korea, Taiwan, and New Zealand, where they are displacing native bee species to the

edge of local extinction (Inoue et al., 2008; Schmid-Hempel et al., 2014; Acosta et al., 2016). The effect of reared *B. terrestris* in areas with consubspecific populations has been less investigated (Lecocq et al., 2016b). *B. terrestris* presents nine subspecies classified by their body hair color pattern and distribution range (Rasmont et al., 2008). Among them, *Bombus terrestris terrestris* (Linnaeus) and *Bombus terrestris dalmatinus* Dalla Torre are the two most widely used in artificial rearing (Velthuis & Doorn, 2006). The endemic subspecies *Bombus terrestris lusitanicus* Krüger inhabits the Iberian Peninsula and reaches southern France where there is a natural contact zone with *B. t. terrestris* (Ornosa & Ortiz-Sánchez, 2004). During the last years, *B. t. terrestris* has been also detected at the south of the Peninsula (Ornosa, 1996; Vargas et al., 2013), mainly in Almeria where more than 30 000 hectares of greenhouses are located. We hypothesize that the contact between the endemic and the introduced subspecies in this region leads to introgression events that may yield to a loss of genetic diversity, or even displacement of the local endemic subspecies by individuals of the commercial subspecies. The taxonomy of these two *B. terrestris* subspecies has been discussed before, but neither the barcoding approach (based on mitochondrial *cox1* gene fragment variation) nor the cephalic labial gland secretions have been able to differentiate them (Coppée et al., 2008; Williams et al., 2012; Lecocq et al., 2016b). In this work, we aim to evaluate the sequence variation of two nuclear genes and one mitochondrial gene as potential subspecific markers to differentiate *B. t. terrestris* and *B. t. lusitanicus*. Nuclear arginine kinase (*ArgK*) and elongation factor-1α (*EF-1α*) genes include introns that have been proven useful to differentiate *Bombus* species (Kawakita et al., 2003) and to characterize geographic variations (Hines et al., 2006). The mitochondrial *16S* ribosomal RNA (*16S*) gene has a higher substitution rate than barcoding fragment *cox1*, what results useful to lower-level (genera and species) analysis on the Hymenoptera (Rokas et al., 2002; Hines et al., 2006; Cameron et al., 2007). Furthermore, we have also used microsatellite loci developed by Estoup et al. (1995, 1996) adequate to

analyze the genetic structure of bumblebee populations (Kraus et al., 2010, Dreier et al., 2014, Moreira et al., 2015).

Material and Methods

Sampling

- Preliminary sampling

For the first experiment searching for molecular markers to differentiate *B. terrestris* subspecies in the Iberian Peninsula, we included 10 female individuals of *B. t. lusitanicus* and 20 *B. t. terrestris* sampled in 2011-2015. *B. t. lusitanicus* individuals were sampled along the bank of Duero River (Soria, Spain), National Park (N. P.) of Guadarrama (Madrid, Spain) and the Sierra Nevada N. P. (Granada, Spain). *B. t. terrestris* individuals were sampled also in Sierra Nevada N. P. and in Belgium (Table S1). The species of every individual was confirmed through barcoding (Murray et al., 2008), whereas the subspecies was determined through morphological characters. Individuals were classified in three groups according to their subspecies and geographic origin: TLS for *B. t. lusitanicus* from Spain, TTS for *B. t. terrestris* from Spain and TTB for *B. t. terrestris* from Belgium (Table S1). Samples were maintained in absolute ethanol and stored at -20 ºC in the laboratory until processed.

- Case study

In order to confirm the discriminative subspecific power of the *16S* haplotypes, we designed a case study by adding 123 individuals to the prior sampling: 48 *B. t. lusitanicus* individuals collected in Spain between 1977 and 1985 before the settlement of bumblebee rearing companies plus 40 *B. t. lusitanicus* from different Spanish locations, 29 *B. t. terrestris* (21 from the north of France and eight from Sierra Nevada N. P., Spain) and six individuals considered

morphologically as hybrids from the north of Spain (Huesca) and surroundings of Sierra Nevada N. P. (Spain). These 75 individuals were collected between 2013 and 2015 (Table 1 and Table S2 in Supplementary Material) and their species status was also confirmed through barcoding (Murray et al., 2008).

Table 1. Subspecies, number of *Bombus terrestris* individuals (N), country and years of sampling in the case study. Distribution of *16S* haplotypes detected is also shown.

Subspecies	N	Country	Years	*16S* haplotype	
				A	G
B. t. lusitanicus	48	Spain	1977/1985	1	47
	50	Spain	2013/2015	7	43
Total	98			8	90
B. t. terrestris	18	Spain	2013/2014	7	11
	10	Belgium	2013	10	-
	21	France	2015	21	-
Total	49			38	11
Hybrids	6	Spain	2014	2	4

DNA extraction, amplification and sequencing

Total DNA was extracted from a hind leg of every individual following the protocol of Ivanova et al. (2006). As a first step, the tissue was digested with proteinase K during six hours at 56 ºC in continuous shaking. PureTaqTM Ready-To-Go PCR beads (GE Healthcare) were used for DNA amplification. PCR profile consisted on initial denaturation at 94 ºC for 3 min followed by 36 cycles of denaturation at 94 ºC for 1 min, annealing temperature at 48 ºC (*ArgK*), 53 ºC (*EF-1α*) and 49 ºC (*16S*) for 1 min, elongation at 68 ºC (72 ºC for *EF-1α*) for 1 min and a final extension at 72 ºC for 10 min (Hines et al., 2006). Efficacy of PCR reactions was checked in 1.5% agarose gel stained with Redsafe (Ecogen). Amplicons were

sequenced using forward primers for *EF-1α* and *ArgK* fragments, and with both forward and reverse primers for the *16S* fragment (Secugen, Madrid, Spain).

Genotyping

Microsatellites loci were amplified in two PCR multiplex reactions: RB1 (B10, B11, B100, B96, B124 and B126), and RB2 (B118, B119, B121, B131 and B132), with 0.2 μM concentration for each primer. Reactions also included $MgCl_2$ (1.2 mM), dNTPs (0.3 mM), BSA (1.2 mg/ ml) and Kappa DNA polymerase (1.5 U, Kapa Biosystems®). PCR profiles for both multiplex were set following Cejas et al. (personal information) as follows: initial denaturation at 95 ºC for 5 min followed by 30 cycles of denaturation at 92 ºC for 30 s, annealing temperature at 54 ºC for 30 s, elongation at 72 ºC for 30 s and a final extension at 72 ºC for 30 min. PCR products were sent to the Servei Central de Suport a la Investigació Experimental (University of Valencia, Spain). Allele scoring was performed using GENEMAPPER 4.8 (Applied Biosystems Inc.) by comparing alleles with an internal size standard (GeneScan-500 Liz, Applied Biosystems Inc.). Binsets were built to correct genetic analyzer errors. Quality control was made manually.

Data analysis

DNA sequences were edited and aligned using Geneious 7.1.3 (http://www.geneious.com, Kearse et al., 2012). *B. terrestris* sequences of *ArgK* (AF492888.1), *EF-1α* (AF492955.1) (Kawakita et al., 2003) and *16S* (AY737386.1) (Hines et al., 2006) genes were downloaded from Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and included for comparison purposes. MEGA6 (Tamura et al., 2013) was used for sequence similarity analysis. Individuals with less than nine microsatellites amplified were discarded. Sibling workers from the same location inferred with Colony 2.0.6.4 (Wang, 2012) were excluded from further analyses. Colony parameters were selected as default for a haplodiploid species. Genetic parameters were calculated

taken the three groups as three independent populations. GenAlex 6.5 (Peakall & Smouse, 2012) was used to obtain the number of alleles (Na), effective number of alleles (Ne), private number of alleles (Np) and observed (Ho) and expected (He) heterozygosity values. R program (R Core Team, 2013), with the adegenet package (Jombart, 2008; Jombart & Ahmed, 2011) was used to perform a discriminant analysis of principal components (DAPC), that is a multivariate method which allows probabilistic assignment of individuals to different clusters. Information about the subspecies (*B. t. lusitanicus* or *B. t. terrestris*), country, year of sampling and *16S* haplotype of each individual in both samplings (preliminary and case study) was used to calculate (1) Kendall´s tau to estimate the correlation between subspecies and haplotype (hybrids were not taken into account, N = 147) and (2) Pearson´s chi-squared test with Yates continuity correction to analyze whether differences of *16S* haplotype frequency in past and present samplings of *B. t. lusitanicus* were significant (N = 98). Statistical analyses were carried in R with package stats version 3.4.3 (R Core Team, 2013).

Results

Sequence analyses

In total 23 *ArgK*, 27 *EF-1α* and 30 *16S* sequences were obtained in the preliminary study. After trimming the sequences, the size of the *ArgK* fragment was 755 (base pairs) bp long. Seven point-mutations were detected (Table 2): five transitions (4 A↔G and 1 C→T) and two transversions (1 C→G and 1 C→A), but none of them were subspecies or geographic specific. The fragment amplified of the *EF-1α* gene was 522 bp long. Sequence alignment did not show any point mutations. The *16S* trimmed fragment was 504 bp long. Two point-mutations were detected (Table 2): a transversion (A→T) and a transition (A→G). The transversion was only

found in one *B. t. terrestris* individual (TTB.02), whereas the transition yielded two haplotypes that discriminate between the subspecies: individuals from the TLS group (Iberian *B. t. lusitanicus*) presented a guanine (haplotype-G) and those from TTB (Belgian *B. t. terrestris*) presented an adenine (haplotype-A). In the TTS group (*B. t. terrestris* from Spain), both haplotypes were found in five individuals each.

Case study

The final sequence set included 153 individuals (Table 1, Table S2 in Supplementary Material). In relation to *B. t. lusitanicus* 47 out of the 48 (97.92%) sampled in the 80s and 43 out of the 50 (86%) from the recent samplings presented the G-haplotype. The individual from the old sampling with G-haplotype was located at the north of Spain near the Pyrenees, whereas the individuals from the most recent surveys were distributed at the north (2), center (2) and south (3) of Spain (Fig S1 in Supplementary Material). All the French and Belgian *B. t. terrestris* individuals (100%) presented the A-haplotype; however, seven of the 18 *B. t. terrestris* individuals (38.89%) recently sampled in Spain showed the A-haplotype, all of them from southern Spain (Sierra Nevada N. P.). Individuals considered morphologically as hybrids presented both haplotypes: two bear A-haplotype and four G-haplotype. Overall, 91.84% *B. t. lusitanicus* individuals showed G-haplotype, while 77.56% *B. t. terrestris* individuals showed A-haplotype. Kendall´s tau coefficient showed a significant correlation between subspecies and haplotype (tau = 0.705, $p < 2.2e-16$). However, when comparing French and Belgian *B. t. terrestris* populations with Spanish *B. t. lusitanicus* from the 80s, the correlation between both variables is almost one (tau = 0.973, $p < 2.2e-16$). Pearson´s chi-squared test for Iberian *B. t. lusitanicus* populations showed no significant differences ($p = 0.074$) between old and current individuals in haplotype segregation.

Table 2. Point mutations and indels found in the alignments of the two genes (*ArgK* and *16S*). GenBank sequences from *Bombus terrestris* were concatenated and taken as reference (*ArgK*: AF492888.1, *16S*: AY737386.1). The codon is included in the case of indels (between brackets is shown the nucleotide inserted) (bp = position of the mutation in base pairs; . = same nucleotide as in the reference sequence; — = missing data).

	ArgK							*16S*	
bp	69	269	393	558	705	706	749	161	263
reference	G	C	G	C	A	G	C	A	A
TLS.01	.	.	.	.	.	.	.	G	.
TLS.02	A	.	A	.	.	.	.	G	.
TLS.03	.	.	A	.	.	.	.	G	.
TLS.04	—	—	—	—	—	—	—	G	.
TLS.05	A	.	.	.	.	.	.	G	.
TLS.06	.	.	.	.	.	.	.	G	.
TLS.07	.	.	.	.	.	.	.	G	.
TLS.08	.	.	.	.	.	.	.	G	.
TLS.09	A	.	A	.	.	.	.	G	.
TLS.10	A	.	A	.	.	.	.	G	.
TTS.01	.	.	.	.	.	.	.	G	.
TTS.02	A	.	A	.	.	.	.	.	.
TTS.03	A	.	A	.	.	.	.	.	.
TTS.04	A	.	.	.	.	.	.	G	.
TTS.05	—	—	—	—	—	—	—	.	.
TTS.06	A	T	A	.	.	.	.	G	.
TTS.07	A	.	.	.	.	.	.	.	.
TTS.08	.	.	.	.	.	.	.	.	.
TTS.09	A	.	A	.	.	.	.	G	.
TTS.10	A	.	A	.	.	.	.	G	.
TTB.01	A	.	.	.	.	.	.	.	.
TTB.02	—	—	—	—	—	—	—	.	.
TTB.03	A	.	.	.	.	.	G	.	.
TTB.04	—	—	—	—	—	—	—	.	.
TTB.05	—	—	—	—	—	—	—	.	.
TTB.06	—	—	—	—	—	—	—	.	.
TTB.07	.	.	.	A	G	A	.	.	G
TTB.09	A	.	.	.	.	.	.	—	.
TTB.10	A	.	A	.	.	.	.	.	.

Genotyping and clustering results

One individual (TTB.05) was removed due to low amplification quality. Two possible siblings (TTS.01 and TTS.03, p = 1) were detected, therefore one sample (TTS.01) was randomly chosen and removed from posterior analyses (N = 28). Values of the number of alleles and heterozygosity (Table 3) were similar between TLS and TTB and slightly higher in TTS. The number of private alleles was different in the three groups, again higher in TTS. Bayesian Information Criterion (BIC) calculated prior to the DAPC yielded one as the most probable number of clusters. Even though, DAPC models with K = 2 and K = 3 were studied, but no correlation was found between the formed clusters with TLS, TTS and TTB groups (data not shown).

Discussion

Sequences of the two nuclear genes *EF-1α* and *ArgK* did not discriminate between the subspecies *B. t. terrestris* and *B. t. lusitanicus* in congruence with previous studies based on DNA barcoding (Williams et al., 2012) and the cephalic labial gland secretions (Coppée et al., 2008; Lecocq et al., 2016b). Though some diversity was found along the *ArgK* sequence, it did not allow grouping the individuals into the two morphologically described subspecies. These genes were selected because they were previously used by Kawakita et al. (2003) and Hines et al. (2006) for analyzing *Bombus* intraspecificity. In fact, Hines et al. (2006) were able to distinguish between *Bombus lucorum* (Linnaeus) and *Bombus hypnorum* (Linnaeus) populations from Europe and China. In the same sense, the DAPC analysis based on microsatellite did not show a clustering agreement with the previously morphological defined groups. This low genetic differentiation between *Bombus* subspecies based on microsatellite variation has

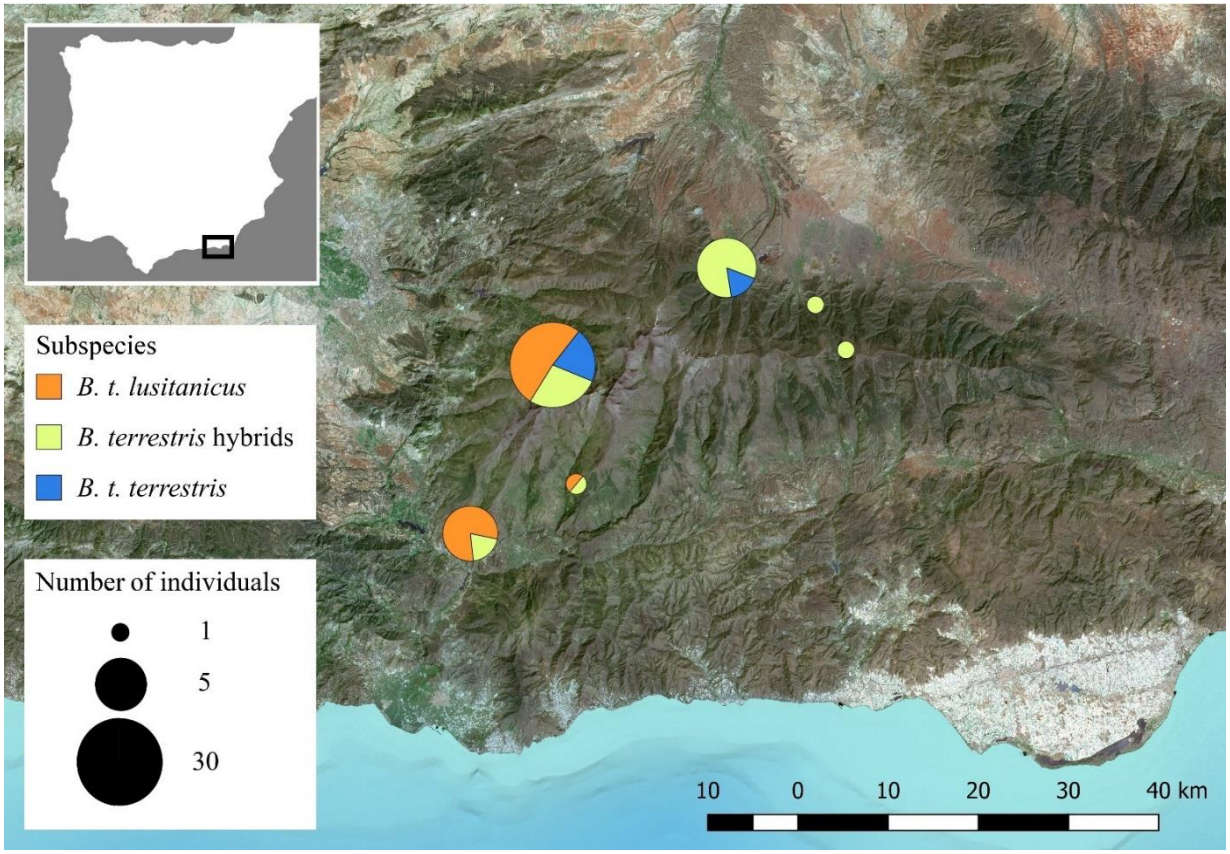

Fig 1. Frequency of *Bombus terrestris lusitanicus*, *Bombus terrestris terrestris* and hybrids (those with morphological subspecies identification and *16S* haplotype that did not match i. e. *B. t. lusitanicus* with A-haplotype and *B. t. terrestris* with G-haplotype) in the National Park of Sierra Nevada (southern Spain). Size of the circles is proportional to the total number of individuals sampled at each location. The high density of greenhouses can be seen in the lower right corner of the figure. Ortophotograph obtained from http://www.juntadeandalucia.es

been reported before in mainland Europe (Estoup et al., 1996; Moreira et al., 2015), as a consequence of the high gene flow in *B. terrestris* populations, probably intensified due to contact and hybridization between commercial and wild *B. terrestris* populations. Interestingly, the mitochondrial gene sequenced here showed a variation which allows discriminating two haplotypes. The unique haplotype of the mitochondrial gene *16S* retrieved in the Iberian individuals was not found in the Belgian and French *B. t. terrestris* individuals used as a reference. Furthermore, a significant correlation was found between haplotypes and subspecies in the case study, so it might be considered that G-haplotype

identified *B. t. lusitanicus* and A-haplotype to *B. t. terrestris*. This is supported by the fact that all except one of the individuals collected between 1977 and 1985 before the establishment of the rearing companies and the start of the commercial use of *B. terrestris* (Velthuis & Doorn, 2006), showed the G-haplotype. The presence of individuals of *B. t. terrestris* in Spain may be due to two situations: on one hand, a natural contact area at the north of the Iberian Peninsula may have been established given the overlapping distribution range of both subspecies in the Pyrenees (Ornosa & Ortiz-Sánchez, 2004; Rasmont et al., 2008; Ornosa et al., 2017); on the other hand the presence of *B. t. terrestris* in the south of the Iberian Peninsula may be due to individuals that have escaped from greenhouses where they are widely used for pollination of crops such as tomatoes (Fig 1). The detection of hybrids and individuals with non-matching morphological subspecies and *16S* haplotype is an evidence of subspecific introgression. The existence of individuals with *B. t. terrestris* phenotype and *B. t. lusitanicus* haplotype suggests that hybridization between commercially reared bumblebees and wild *B. t. lusitanicus* populations has occurred. The opposite case has also been observed: individuals with *B. t. lusitanicus* phenotype and *B. t. terrestris* haplotypes. Given the maternal inheritance of the mitochondrial molecule we can deduce that both commercial queens and males have colonized the environment, which is still a discussed topic (Velthuis & Doorn, 2006; Kraus et al., 2010, Lecocq et al., 2016a), meaning that *B. t. terrestris* is naturalized, for now, in the south of Spain. *B. terrestris* subspecies usually do not interbreed due to the specificity of the hormones secreted by the male cephalic labial glands (Coppée et al., 2008). Yet, in the case of *B. t. terrestris* and *B. t. lusitanicus*, the secretions are chemically very similar (Coppée et al., 2008) allowing more usual copulation between them than with other *B. terrestris* subspecies. In other attempts of colonizing new lands by *B. terrestris* (Ings et al., 2010), the subspecies *B. t. sassaricus* stopped to be seen in the south of France two years after the end of its importation, and no hybrids were found although no molecular analyses were performed. In the case study,

naturalized individuals of *B. t. terrestris* on the Iberian Peninsula may have colonized the environment in the south where the breeding companies are based and, given their ability to breed with *B. t. lusitanicus*, the number of hybrid individuals in this area may increase in the future. The effects that this hybridization is having on wild populations are unknown (Facon et al., 2011), as are the effects that these introduced populations may have on native flora (Aizen et al., 2018). However, according to the expansion models of Lecocq et al. (2016a), *B. t. terrestris* should not be able to continue its colonization on the Iberian Peninsula, as it is not suitable for its climatic conditions. Future studies including more localities within the Iberian Peninsula and using preferably the *16S* marker and other highly variable nuclear markers such as SNPs (Single Nucleotide Polymorphisms) will show the degree of introduction of commercial *B. terrestris* over the territory, what is of conservation concern due to a possible impact on the genetic diversity of Iberian populations or even to the displacement of the native wild populations.

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Supplementary Material

Table S1. Sampling sites and geographical information of the *Bombus terrestris* individuals included in the preliminary study (TLS = *B. t. lusitanicus* from Spain; TTS = *B. t. terrestris* from Spain; and TTB = *B. t. terrestris* from Belgium; N= number of sampled individuals).

Group	N	Sampling site	Province	Country	Geographical coordinates		Altitude
TLS	4	Duero river bank	Soria	Spain	41°46'6.96" N	2°27'17.20" W	1010m
TLS	3	Fuenfría	Madrid	Spain	40º47´10" N	4º3´11" W	1750m
TLS/TTS	3/10	Sierra Nevada1	Granada	Spain	37° 5'6.82" N	3°22'54.46" W	2646m
TTB	1	Malchamps	Liège	Belgium	50º27'54" N	5º55'22.8" E	579m
TTB	9	Saint-Vaast	Hainaut	Belgium	50º26'52.8" N	4º9'39.599" E	68m

Table S2. Sampling information of the *Bombus terrestris* individuals used in the preliminary and case studies. The morphological subspecies identification is given for each individual. GenBank sequence codes for *ArgK, EF-1α* and *16S* fragments of the sequenced individuals are given. (* = Individual determined as hybrid due to incongruency between its morphological subspecies identification and the *16S* haplotype.)

Code	Subspecies	Sampling Site	Country	Year	*ArgK*	*EF-1α*	*16S*
TLS.01	*B. t. lusitanicus*	Duero river bank	Spain	2013	MH464193	MH464166	MH558385
TLS.02	*B. t. lusitanicus*	Duero river bank	Spain	2013	MH464194	MH464167	MH558386
TLS.03	*B. t. lusitanicus*	Duero river bank	Spain	2013	MH464195	MH464168	MH558387
TLS.04	*B. t. lusitanicus*	Duero river bank	Spain	2013		MH464169	MH558388
TLS.05	*B. t. lusitanicus*	Fuenfría	Spain	2014	MH464196	MH464170	MH558389
TLS.06	*B. t. lusitanicus*	Fuenfría	Spain	2014	MH464197	MH464171	MH558390
TLS.07	*B. t. lusitanicus*	Fuenfría	Spain	2014	MH464198	MH464172	MH558391
TLS.08	*B. t. lusitanicus*	SierraNevada1	Spain	2013	MH464199	MH464173	MH558392
TLS.09	*B. t. lusitanicus*	SierraNevada1	Spain	2013	MH464200	MH464174	MH558393
TLS.10	*B. t. lusitanicus*	SierraNevada1	Spain	2013	MH464201	MH464175	MH558394
TTS.01	*B. t. terrestris**	SierraNevada1	Spain	2013	MH464202	MH464176	MH558489
TTS.02	*B. t. terrestris*	SierraNevada1	Spain	2013	MH464203	MH464177	MH558490
TTS.03	*B. t. terrestris*	SierraNevada1	Spain	2013	MH464204	MH464178	MH558491
TTS.04	*B. t. terrestris**	SierraNevada1	Spain	2013	MH464205	MH464179	MH558492
TTS.05	*B. t. terrestris*	SierraNevada1	Spain	2013		MH464180	MH558493
TTS.06	*B. t. terrestris**	SierraNevada1	Spain	2013	MH464206	MH464181	MH558494
TTS.07	*B. t. terrestris*	SierraNevada1	Spain	2013	MH464207	MH464182	MH558495
TTS.08	*B. t. terrestris*	SierraNevada1	Spain	2013	MH464208	MH464183	MH558496
TTS.09	*B. t. terrestris**	SierraNevada1	Spain	2013	MH464209		MH558497
TTS.10	*B. t. terrestris**	SierraNevada1	Spain	2013	MH464210	MH464184	MH558498
TTB.01	*B. t. terrestris*	Malchamps	Belgium	2014	MH464211	MH464185	MH558507
TTB.02	*B. t. terrestris*	Saint-Vaast	Belgium	2014		MH464186	MH558508

Code	Subspecies	Sampling Site	Country	Year	*ArgK*	*EF-1α*	*16S*
TTB.03	*B. t. terrestris*	Saint-Vaast	Belgium	2014	MH464212	MH464187	MH558509
TTB.04	*B. t. terrestris*	Saint-Vaast	Belgium	2014			MH558510
TTB.05	*B. t. terrestris*	Saint-Vaast	Belgium	2014		MH464188	MH558511
TTB.06	*B. t. terrestris*	Saint-Vaast	Belgium	2014		MH464189	MH558512
TTB.07	*B. t. terrestris*	Saint-Vaast	Belgium	2014	MH464213	MH464190	MH558513
TTB.08	*B. t. terrestris*	Saint-Vaast	Belgium	2014		MH464191	MH558514
TTB.09	*B. t. terrestris*	Saint-Vaast	Belgium	2014	MH464214		MH558515
TTB.10	*B. t. terrestris*	Saint-Vaast	Belgium	2014	MH464215	MH464192	MH558516
TLS.11	*B. t. lusitanicus*	Salnes	Spain	1986			MH558395
TLS.12	*B. t. lusitanicus*	Llanes	Spain	1986			MH558396
TLS.13	*B. t. lusitanicus**	Alsasua	Spain	1984			MH558397
TLS.14	*B. t. lusitanicus*	Peguerinos	Spain	1980			MH558398
TLS.15	*B. t. lusitanicus*	El Tiemblo	Spain	1980			MH558399
TLS.16	*B. t. lusitanicus*	Comarruga	Spain	1983			MH558400
TLS.17	*B. t. lusitanicus*	Barcelona	Spain	1985			MH558401
TLS.18	*B. t. lusitanicus*	Saelices del Río	Spain	1977			MH558402
TLS.19	*B. t. lusitanicus*	Budia	Spain	1986			MH558403
TLS.20	*B. t. lusitanicus*	Budia	Spain	1977			MH558404
TLS.21	*B. t. lusitanicus*	El Molar	Spain	1978			MH558405
TLS.22	*B. t. lusitanicus*	Cerceda	Spain	1983			MH558406
TLS.23	*B. t. lusitanicus*	Cerceda	Spain	1986			MH558407
TLS.24	*B. t. lusitanicus*	Algete	Spain	1986			MH558408
TLS.25	*B. t. lusitanicus*	Madrid	Spain	1978			MH558409
TLS.26	*B. t. lusitanicus*	Madrid	Spain	1978			MH558410
TLS.27	*B. t. lusitanicus*	Madrid	Spain	1979			MH558411
TLS.28	*B. t. lusitanicus*	Madrid	Spain	1979			MH558412
TLS.29	*B. t. lusitanicus*	Madrid	Spain	1980			MH558413
TLS.30	*B. t. lusitanicus*	Madrid	Spain	1980			MH558414
TLS.31	*B. t. lusitanicus*	Madrid	Spain	1980			MH558415
TLS.32	*B. t. lusitanicus*	Madrid	Spain	1980			MH558416
TLS.33	*B. t. lusitanicus*	Madrid	Spain	1980			MH558417
TLS.34	*B. t. lusitanicus*	Madrid	Spain	1981			MH558418
TLS.35	*B. t. lusitanicus*	Madrid	Spain	1981			MH558419
TLS.36	*B. t. lusitanicus*	Madrid	Spain	1981			MH558420
TLS.37	*B. t. lusitanicus*	Madrid	Spain	1981			MH558421
TLS.38	*B. t. lusitanicus*	Madrid	Spain	1981			MH558422
TLS.39	*B. t. lusitanicus*	Madrid	Spain	1981			MH558423
TLS.40	*B. t. lusitanicus*	Madrid	Spain	1982			MH558424
TLS.41	*B. t. lusitanicus*	Madrid	Spain	1982			MH558425
TLS.42	*B. t. lusitanicus*	Madrid	Spain	1982			MH558426
TLS.43	*B. t. lusitanicus*	Madrid	Spain	1982			MH558427
TLS.44	*B. t. lusitanicus*	Madrid	Spain	1977			MH558428
TLS.45	*B. t. lusitanicus*	Madrid	Spain	1983			MH558429
TLS.46	*B. t. lusitanicus*	Madrid	Spain	1983			MH558430
TLS.47	*B. t. lusitanicus*	Madrid	Spain	1983			MH558431
TLS.48	*B. t. lusitanicus*	Madrid	Spain	1985			MH558432

TLS.49	*B. t. lusitanicus*	Madrid	Spain	1985			MH558433
TLS.50	*B. t. lusitanicus*	Madrid	Spain	1977			MH558434
Code	Subspecies	Sampling Site	Country	Year	*ArgK*	*EF-1α*	*16S*
TLS.51	*B. t. lusitanicus*	Madrid	Spain	1978			MH558435
TLS.52	*B. t. lusitanicus*	Madrid	Spain	1986			MH558436
TLS.53	*B. t. lusitanicus*	Madrid	Spain	1986			MH558437
TLS.54	*B. t. lusitanicus*	Madrid	Spain	1986			MH558438
TLS.55	*B. t. lusitanicus*	Villares del Saz	Spain	1986			MH558439
TLS.56	*B. t. lusitanicus*	Mallorca	Spain	1977			MH558440
TLS.57	*B. t. lusitanicus*	Cazorla	Spain	1986			MH558441
TLS.58	*B. t. lusitanicus*	Marbella	Spain	1986			MH558442
TLS.59	*B. t. lusitanicus*	Vizcaya	Spain	2013			MH558443
TLS.60	*B. t. lusitanicus**	Guipuzcoa	Spain	2013			MH558444
TLS.61	*B. t. lusitanicus*	Logroño	Spain	2013			MH558445
TLS.62	*B. t. lusitanicus**	Jaca	Spain	2014			MH558446
TLS.63	*B. t. lusitanicus*	Jaca	Spain	2014			MH558447
TLS.64	*B. t. lusitanicus*	Duero river bank	Spain	2013			MH558448
TLS.65	*B. t. lusitanicus*	Duero river bank	Spain	2013			MH558449
TLS.66	*B. t. lusitanicus*	Duero river bank	Spain	2013			MH558450
TLS.67	*B. t. lusitanicus*	Duero river bank	Spain	2013			MH558451
TLS.68	*B. t. lusitanicus**	Burgos	Spain	2013			MH558452
TLS.69	*B. t. lusitanicus*	Fuenfría	Spain	2015			MH558453
TLS.70	*B. t. lusitanicus*	Fuenfría	Spain	2015			MH558454
TLS.71	*B. t. lusitanicus*	Fuenfría	Spain	2015			MH558455
TLS.72	*B. t. lusitanicus*	Fuenfría	Spain	2015			MH558456
TLS.73	*B. t. lusitanicus*	Fuenfría	Spain	2015			MH558457
TLS.74	*B. t. lusitanicus**	Fuenfría	Spain	2015			MH558458
TLS.75	*B. t. lusitanicus*	Fuenfría	Spain	2015			MH558459
TLS.76	*B. t. lusitanicus*	Fuenfría	Spain	2015			MH558460
TLS.77	*B. t. lusitanicus**	Huelma	Spain	2015			MH558461
TLS.78	*B. t. lusitanicus*	Huelma	Spain	2015			MH558462
TLS.79	*B. t. lusitanicus*	Carrascoy	Spain	2015			MH558463
TLS.80	*B. t. lusitanicus**	SierraNevada1	Spain	2013			MH558464
TLS.81	*B. t. lusitanicus*	SierraNevada1	Spain	2013			MH558465
TLS.82	*B. t. lusitanicus*	SierraNevada1	Spain	2013			MH558466
TLS.83	*B. t. lusitanicus*	SierraNevada1	Spain	2013			MH558467
TLS.84	*B. t. lusitanicus*	SierraNevada1	Spain	2013			MH558468
TLS.85	*B. t. lusitanicus*	SierraNevada1	Spain	2013			MH558469
TLS.86	*B. t. lusitanicus*	SierraNevada1	Spain	2013			MH558470
TLS.87	*B. t. lusitanicus*	SierraNevada1	Spain	2013			MH558471
TLS.88	*B. t. lusitanicus*	SierraNevada1	Spain	2013			MH558472
TLS.89	*B. t. lusitanicus*	SierraNevada1	Spain	2015			MH558473
TLS.90	*B. t. lusitanicus*	SierraNevada1	Spain	2015			MH558474
TLS.91	*B. t. lusitanicus*	SierraNevada1	Spain	2015			MH558475
TLS.92	*B. t. lusitanicus*	SierraNevada1	Spain	2013			MH558476
TLS.93	*B. t. lusitanicus*	SierraNevada5	Spain	2013			MH558477
TLS.94	*B. t. lusitanicus*	SierraNevada6	Spain	2015			MH558478

TLS.95	*B. t. lusitanicus**	SierraNevada6	Spain	2015			MH558479
TLS.96	*B. t. lusitanicus*	SierraNevada6	Spain	2015			MH558480
TLS.97	*B. t. lusitanicus*	SierraNevada6	Spain	2015			MH558481
TLS.98	*B. t. lusitanicus*	SierraNevada6	Spain	2015			MH558482
Code	Subspecies	Sampling Site	Country	Year	*ArgK*	*EF-1α*	*16S*
TTS.11	*B. t. terrestris**	SierraNevada1	Spain	2013			MH558499
TTS.12	*B. t. terrestris**	SierraNevada1	Spain	2013			MH558500
TTS.13	*B. t. terrestris*	SierraNevada1	Spain	2013			MH558501
TTS.14	*B. t. terrestris**	SierraNevada2	Spain	2014			MH558502
TTS.15	*B. t. terrestris*	SierraNevada2	Spain	2014			MH558503
TTS.16	*B. t. terrestris**	SierraNevada2	Spain	2014			MH558504
TTS.17	*B. t. terrestris**	SierraNevada4	Spain	2014			MH558505
TTS.18	*B. t. terrestris**	SierraNevada5	Spain	2013			MH558506
TTF.01	*B. t. terrestris*	Chartres	France	2015			MH558517
TTF.02	*B. t. terrestris*	Chartres	France	2015			MH558518
TTF.03	*B. t. terrestris*	Chartres	France	2015			MH558519
TTF.04	*B. t. terrestris*	Chartres	France	2015			MH558520
TTF.05	*B. t. terrestris*	Chartres	France	2015			MH558521
TTF.06	*B. t. terrestris*	Chartres	France	2015			MH558522
TTF.07	*B. t. terrestris*	Chartres	France	2015			MH558523
TTF.09	*B. t. terrestris*	Grandcamp-Maisy	France	2015			MH558524
TTF.10	*B. t. terrestris*	Grandcamp-Maisy	France	2015			MH558525
TTF.11	*B. t. terrestris*	Grandcamp-Maisy	France	2015			MH558526
TTF.12	*B. t. terrestris*	Colleville-sur-Mer	France	2015			MH558527
TTF.13	*B. t. terrestris*	Beaumont-en-Auge	France	2015			MH558528
TTF.14	*B. t. terrestris*	Beaumont-en-Auge	France	2015			MH558529
TTF.15	*B. t. terrestris*	Beaumont-en-Auge	France	2015			MH558530
TTF.16	*B. t. terrestris*	Beaumont-en-Auge	France	2015			MH558531
TTF.17	*B. t. terrestris*	Beuvron-en-Auge	France	2015			MH558532
TTF.18	*B. t. terrestris*	Beuvron-en-Auge	France	2015			MH558533
TTF.19	*B. t. terrestris*	Beuvron-en-Auge	France	2015			MH558534
TTF.20	*B. t. terrestris*	Beuvron-en-Auge	France	2015			MH558535
TTF.21	*B. t. terrestris*	Beuvron-en-Auge	France	2015			MH558536
TTF.22	*B. t. terrestris*	Beuvron-en-Auge	France	2015			MH558537
THS.01	*B. terrestris* hybrid	Biescas	Spain	2014			MH558483
THS.02	*B. terrestris* hybrid	Jaca	Spain	2014			MH558484
THS.03	*B. terrestris* hybrid	SierraNevada2	Spain	2014			MH558485
THS.04	*B. terrestris* hybrid	SierraNevada2	Spain	2014			MH558486
THS.05	*B. terrestris* hybrid	SierraNevada2	Spain	2014			MH558487
THS.06	*B. terrestris* hybrid	SierraNevada3	Spain	2014			MH558488

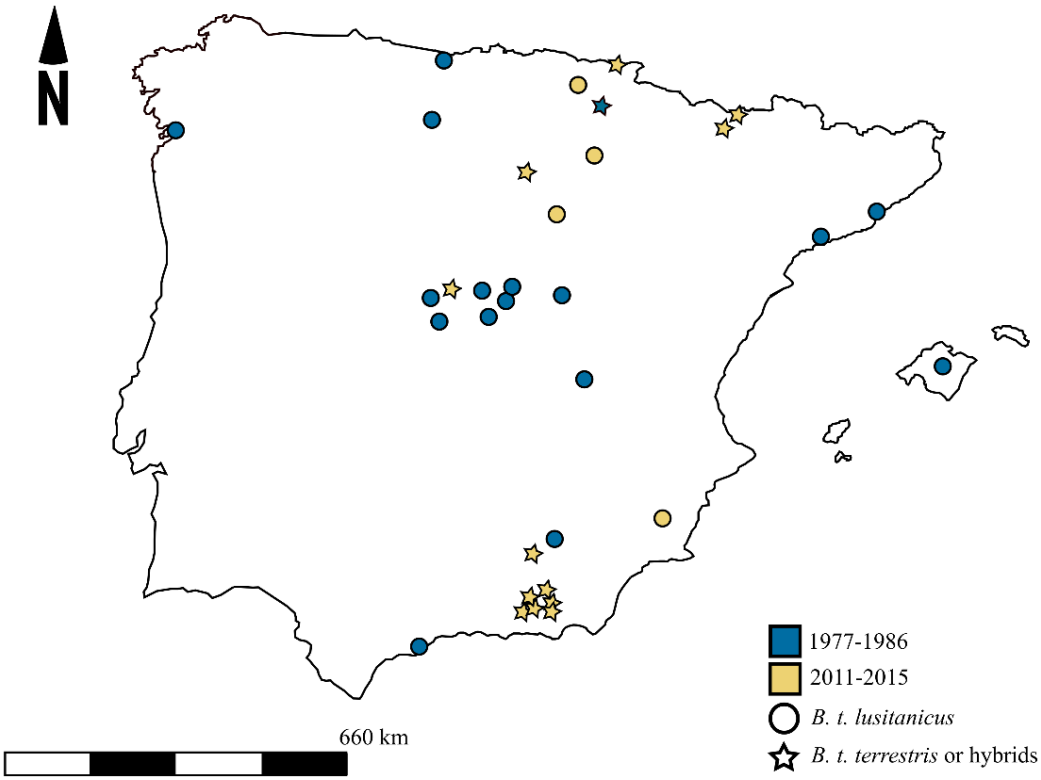

Figure S1. Sampling localities of *Bombus terrestris* subspecies in the Iberian Peninsula. Blue colour indicates those localities from the sampling between 1977 and 1986, and yellow those sampled between 2011 and 2015. Shape of the sampling point indicates the subspecies identification of the bumblebees sampled in each locality: a circle referred to *B. t. lusitanicus* and a star to *B. t. terrestris* and/or hybrids (individuals with non-matching morphological subspecies and *16S* haplotype).

Chapter 3.

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

Abstract

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.

Introduction

The common bumblebee *Bombus terrestris* (Linnaeus, 1758) is an important pollinator distributed through the Palaearctic biogeographic region (Ornosa & Ortiz-Sanchez, 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 et al., 2006), thus becoming a threat to their conservation status as seen in endemic bumblebee species (Aizen et al., 2018). According to geographic distribution and morphological differences, *B. terrestris* can be classified into nine different subspecies (Rasmont et al., 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 et al., 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- Sanchez, 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; Agrobio, personal communication) because they improve the quality of pollinated fruits (Klatt et al., 2014, but see Trillo et al., 2018). These managed individuals can escape from agricultural facilities, as seen in Poland (Kraus et al., 2011) and Portugal (Seabra et al., 2018), and become naturalised (Cejas et al., 2019; Ornosa, 1996; Ortiz-Sanchez, 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-Sanchez, 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., 2016a; Williams et al., 2012). However, recent studies have found molecular differences between *B. t. lusitanicus* and commercial breeds in the sequence of the *rrnL* (*16S*) and *cox1* 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 et al., 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. *cox1*) might remain tightly preserved when analysing low-level phylogenetic relationships, whereas other regions might have a higher resolution in specific taxa (Cheng et al., 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 et al., 2016; Crampton-Platt et al., 2016; Donnelly

et al., 2016), including bees (Eimanifar et al., 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 coauthors (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 et al. (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 et al., 2000), IDBA-UD (Peng et al., 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.

Consensus sequences were exported to MEGA V.6 (Tamura et al., 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., 2019). Total DNA was extracted from the hind leg of each individual to amplify the selected fragments following Ivanova, Dewaard &

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 et al., 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 ll of *TaqI* FastDigest enzyme (Thermo Scientific), 1 ll of 10X FastDygest Green Buffer and 10 ll 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* (MK570128) and *B. t. terrestris* (MK570129) were 17 049 bp and 17 232 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 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.

Table 1. Information about the mitogenome fragments tested as subspecific markers. PCR primer sequences are given in the 5′-3′ direction.

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

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 *TaqI* 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 expore 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.

Table 2. Sequencing results of the validation of the mitochondrial fragments used to differentiate *B. t. terrestris* and *B. t. lusitanicus*. 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., 2019).

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

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 et al., 2018; Zhao et al., 2017). The size of the consensus mitogenomes varied in size between the two subspecies, mainly owing to the presence of intergenic regions that are typically observed in other insects (Rortais et al., 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 et al., 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 et al., 2008; Lecocq et al., 2016a; Moreira et al., 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 nonsynonymous 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* has advanced as described in Lecocq et al. (2016b). If this expansion is driven by climate change, *B. t. lusitanicus* could continue to advance towards the rest of Europe (Lecocq et al., 2016b). 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 et al., 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 *TaqI* (*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*.

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Supplementary Material

Table S1 Sampling information of the individuals used to obtain the mitogenome sequences. The same number (in parentheses) has been given to locations very close to each other (see map in Figure S1). Geographical coordinates are given for each individual.

ID	Group	Subspecies	Location	Country	Latitude	Longitude
TLS.059	TLSa	*B. t. lusitanicus*	Vizcaya (6)	Spain	43°16'1.75"N	2°29'45.45"W
TLS.001	TLSa	*B. t. lusitanicus*	Soria (7)	Spain	41°46'6.96"N	2°27'17.20"W
TLS.004	TLSa	*B. t. lusitanicus*	Soria (7)	Spain	41°46'6.96"N	2°27'17.20"W
TLS.067	TLSa	*B. t. lusitanicus*	Soria (7)	Spain	41°46'6.96"N	2°27'17.20"W
TLS.068	TLSa	*B. t. lusitanicus*	Burgos1 (8)	Spain	41°57'7.01"N	3°26'38.47"W
TLS.099	TLSa	*B. t. lusitanicus*	Burgos2 (8)	Spain	42°20'57.25"N	3°42'15.82"W
TLS.100	TLSa	*B. t. lusitanicus*	Burgos2 (8)	Spain	42°20'57.25"N	3°42'15.82"W
TLS.101	TLSa	*B. t. lusitanicus*	Burgos2 (8)	Spain	42°20'57.25"N	3°42'15.82"W
TLS.102	TLSa	*B. t. lusitanicus*	Burgos2 (8)	Spain	42°20'57.25"N	3°42'15.82"W
TLS.103	TLSa	*B. t. lusitanicus*	Burgos2 (8)	Spain	42°20'57.25"N	3°42'15.82"W
TLS.104	TLSb	*B. t. lusitanicus*	SierraNevada10 (12)	Spain	37°10'59.96"N	3°3'54.05"W
TLS.105	TLSb	*B. t. lusitanicus*	SierraNevada2 (12)	Spain	37°11'48.50"N	3°10'3.31"W
TLS.106	TLSb	*B. t. lusitanicus*	SierraNevada2 (12)	Spain	37°11'48.50"N	3°10'3.31"W
TLS.107	TLSb	*B. t. lusitanicus*	SierraNevada2 (12)	Spain	37°11'48.50"N	3°10'3.31"W
TLS.108	TLSb	*B. t. lusitanicus*	SierraNevada2 (12)	Spain	37°11'48.50"N	3°10'3.31"W
TLS.109	TLSb	*B. t. lusitanicus*	SierraNevada2 (12)	Spain	37°11'48.50"N	3°10'3.31"W
TLS.110	TLSb	*B. t. lusitanicus*	SierraNevada2 (12)	Spain	37°11'48.50"N	3°10'3.31"W
TLS.111	TLSb	*B. t. lusitanicus*	SierraNevada9 (12)	Spain	37°7'22.26"N	3°13'4.70"W
TLS.112	TLSb	*B. t. lusitanicus*	SierraNevada9 (12)	Spain	37°7'22.26"N	3°13'4.70"W
TLS.113	TLSb	*B. t. lusitanicus*	SierraNevada9 (12)	Spain	37°7'22.26"N	3°13'4.70"W
TTF.012	TTF	*B. t. terrestris*	Coleville-sur-Mer (3)	France	49°21'37.86"N	0°51'27.11"W
TTF.013	TTF	*B. t. terrestris*	Beaumont-en-Auge (3)	France	49°16'39.58"N	0°6'33.28"E
TTF.014	TTF	*B. t. terrestris*	Beaumont-en-Auge (3)	France	49°16'39.58"N	0°6'33.28"E
TTF.015	TTF	*B. t. terrestris*	Beaumont-en-Auge (3)	France	49°16'39.58"N	0°6'33.28"E
TTF.016	TTF	*B. t. terrestris*	Beaumont-en-Auge (3)	France	49°16'39.58"N	0°6'33.28"E
TTF.017	TTF	*B. t. terrestris*	Beuvron-en-Auge (3)	France	49°11'23.1"N	0°02'44.2"W
TTF.018	TTF	*B. t. terrestris*	Beuvron-en-Auge (3)	France	49°11'23.1"N	0°02'44.2"W
TTF.019	TTF	*B. t. terrestris*	Beuvron-en-Auge (3)	France	49°11'23.1"N	0°02'44.2"W
TTF.020	TTF	*B. t. terrestris*	Beuvron-en-Auge (3)	France	49°11'23.1"N	0°02'44.2"W
TTF.021	TTF	*B. t. terrestris*	Beuvron-en-Auge (3)	France	49°11'23.1"N	0°02'44.2"W

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

Species	Genbank code	Authors
Apis mellifera	KJ396191	Fuller et al. unpublished research
Bombus terrestris	KM268150	Du et al. (2015)
Bombus hypocrita sapporensis	NC_011923	Hong et al. (2008)
Melipona bicolor	NC_004529	Silvestre & Arias (2004)

Du, Q., Bi, G., Zhao, E., Yang, J., Zhang, Z. & Liu, G. (2015). Complete mitochondrial genome of *Bombus terrestris* (Hymenoptera: Apidae). Mitochondrial DNA, 27. doi: 10.3109/19401736.2015.1089568

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Table S3 Sampling information of the individuals used for the validation of the subspecific markers. Hybrid individuals are marked with asterisks: *: morphological hybrids, **: discrepant hybrids, in which the phenotype and the mitochondrial haplotype dissent. The same number (in parentheses) has been given to locations very close to each other (see map in Figure S1). Geographical coordinates are given for each individual.

ID	Subspecies	Location	Country	Latitude	Longitude
TLS.71	*B. t. lusitanicus*	Pto. Fuenfria (9)	Spain	40°47'31"N	4°3'35"W
TLS.78	*B. t. lusitanicus*	Huelma (11)	Spain	37°43'8.75"N	3°27'5.36"W
TLS.79	*B. t. lusitanicus*	Murcia (10)	Spain	37°55'13.40"N	1°6'53.44"W
TLS.89	*B. t. lusitanicus*	SierraNevada1 (12)	Spain	37°5'6.82"N	3°22'54.46"W
TLS.90	*B. t. lusitanicus*	SierraNevada1 (12)	Spain	37°5'6.82"N	3°22'54.46"W
TLS.91	*B. t. lusitanicus*	SierraNevada1 (12)	Spain	37°5'6.82"N	3°22'54.46"W
TLS.94	*B. t. lusitanicus*	SierraNevada6 (12)	Spain	36°54'58.54"N	3°28'20.57"W
TLS.96	*B. t. lusitanicus*	SierraNevada6 (12)	Spain	36°54'58.54"N	3°28'20.57"W
TLS.97	*B. t. lusitanicus*	SierraNevada6 (12)	Spain	36°54'58.54"N	3°28'20.57"W
TLS.98	*B. t. lusitanicus*	SierraNevada6 (12)	Spain	36°54'58.54"N	3°28'20.57"W
THS.01	*B. terrestris**	Biescas (5)	Spain	42°37'49.19"N	0°18'58.17"W
THS.02	*B. terrestris* *	Jaca (5)	Spain	42°33'52.50"N	0°34'1.39"W
TLS.62	*B. t. lusitanicus***	Jaca (5)	Spain	42°33'52.50"N	0°34'1.39"W
TLS.77	*B. t. lusitanicus***	Huelma (11)	Spain	37°43'8.75"N	3°27'5.36"W
TLS.95	*B. t. lusitanicus***	SierraNevada6 (12)	Spain	36°54'58.54"N	3°28'20.57"W
TTF.01	*B. t. terrestris*	Chartres (4)	France	48°29'17.84"N	1°30'47.31"E
TTF.02	*B. t. terrestris*	Chartres (4)	France	48°29'17.84"N	1°30'47.31"E
TTF.04	*B. t. terrestris*	Chartres (4)	France	48°29'17.84"N	1°30'47.31"E
TTF.06	*B. t. terrestris*	Chartres (4)	France	48°29'17.84"N	1°30'47.31"E
TTF.07	*B. t. terrestris*	Chartres (4)	France	48°29'17.84"N	1°30'47.31"E
TTF.09	*B. t. terrestris*	Grandcamp-Maisy (3)	France	49°23'13.53"N	1°3'8.17"E
TTF.10	*B. t. terrestris*	Grandcamp-Maisy (3)	France	49°23'13.53"N	1°3'8.17"E
TTF.11	*B. t. terrestris*	Grandcamp-Maisy (3)	France	49°23'13.53"N	1°3'8.17"E
TTF.23	*B. t. terrestris*	Grandcamp-Maisy (3)	France	49°23'13.53"N	1°3'8.17"E
TTF.24	*B. t. terrestris*	Grandcamp-Maisy (3)	France	49°23'13.53"N	1°3'8.17"E

Table S4 Sampling information of the individuals used for the validation of the RFLP test. **: discrepant hybrids, in which the phenotype and the mitochondrial haplotype dissent. The same number (in parentheses) has been given to locations very close to each other (see map in Figure S1). Geographical coordinates are given for each individual.

ID	Subspecies	Location	Country	Latitude	Longitude
TLS.01	*B. t. lusitanicus*	Soria (7)	Spain	41°46'6.96"N	2°27'17.20"W
TLS.02	*B. t. lusitanicus*	Soria (7)	Spain	41°46'6.96"N	2°27'17.20"W
TLS.03	*B. t. lusitanicus*	Soria (7)	Spain	41°46'6.96"N	2°27'17.20"W
TLS.04	*B. t. lusitanicus*	Soria (7)	Spain	41°46'6.96"N	2°27'17.20"W
TLS.05	*B. t. lusitanicus*	SierraGuadarrama1 (9)	Spain	40º47´10"N	4º3´11"W
TLS.06	*B. t. lusitanicus*	SierraGuadarrama2 (9)	Spain	40º49´57"N	3º57´23"W
TLS.07	*B. t. lusitanicus*	SierraGuadarrama2 (9)	Spain	40º49´57"N	3º57´23"W
TLS.08	*B. t. lusitanicus*	SierraNevada1 (12)	Spain	37° 5'6.82"N	3°22'54.46"W
TLS.09	*B. t. lusitanicus*	SierraNevada1 (12)	Spain	37° 5'6.82"N	3°22'54.46"W
TLS.10	*B. t. lusitanicus*	SierraNevada1 (12)	Spain	37° 5'6.82"N	3°22'54.46"W
TTS.01**	*B. t. terrestris*	SierraNevada8 (12)	Spain	37° 7'40.20"N	3°21'39.20"W
TTS.02	*B. t. terrestris*	SierraNevada1 (12)	Spain	37° 5'6.82"N	3°22'54.46"W
TTS.03	*B. t. terrestris*	SierraNevada1 (12)	Spain	37° 5'6.82"N	3°22'54.46"W
TTS.04**	*B. t. terrestris*	SierraNevada1 (12)	Spain	37°5'6.82"N	3°22'54.46"W
TTS.05	*B. t. terrestris*	SierraNevada1 (12)	Spain	37°5'6.82"N	3°22'54.46"W
TTS.06**	*B. t. terrestris*	SierraNevada1 (12)	Spain	37°5'6.82"N	3°22'54.46"W
TTS.07	*B. t. terrestris*	SierraNevada1 (12)	Spain	37°5'6.82"N	3°22'54.46"W
TTS.08	*B. t. terrestris*	SierraNevada1 (12)	Spain	37°5'6.82"N	3°22'54.46"W
TTS.09**	*B. t. terrestris*	SierraNevada1 (12)	Spain	37°5'6.82"N	3°22'54.46"W
TTS.10**	*B. t. terrestris*	SierraNevada1 (12)	Spain	37°5'6.82"N	3°22'54.46"W
TTB.01	*B. t. terrestris*	Malchamps (1)	Belgium	50°27'54''N	5°55'22.8''E
TTB.02	*B. t. terrestris*	Saint-Vaast (2)	Belgium	50°27'10.80"N	4°7'26.40"E
TTB.03	*B. t. terrestris*	Saint-Vaast (2)	Belgium	50°27'10.80"N	4°7'26.40"E
TTB.04	*B. t. terrestris*	Saint-Vaast (2)	Belgium	50°27'10.80"N	4°7'26.40"E
TTB.05	*B. t. terrestris*	Saint-Vaast (2)	Belgium	50°27'10.80"N	4°7'26.40"E
TTB.06	*B. t. terrestris*	Saint-Vaast (2)	Belgium	50°27'10.80"N	4°7'26.40"E
TTB.07	*B. t. terrestris*	Saint-Vaast (2)	Belgium	50°27'10.80"N	4°7'26.40"E
TTB.08	*B. t. terrestris*	Saint-Vaast (2)	Belgium	50°27'10.80"N	4°7'26.40"E
TTB.09	*B. t. terrestris*	Saint-Vaast (2)	Belgium	50°27'10.80"N	4°7'26.40"E
TTB.10	*B. t. terrestris*	Saint-Vaast (2)	Belgium	50°27'10.80"N	4°7'26.40"E

Table S5 Results of the mitogenome sequencing. TLSa, TLSb and TTF are acronyms of *B. t. lusitanicus* from the north of the Iberian Peninsula, *B. t. lusitanicus* from the south of the Iberian Peninsula and *B. t. terrestris*, respectively.

		Number of contigs		
Pool	Reads	Celera	IDBA-UD	Newbler
TLSa	19,765,284	38,564	10,315	641,323
TLSb	21,211,702	46,513	10,635	631,031
TTF	23,009,466	38,117	11,532	645,611

Table S6. Mitogenome organization of the studied subspecies *B. t. terrestris* (TTF) and *B. t. lusitanicus* (TLS). *trn*: transfer RNA labelled by the one-letter amino acid code; *rrnL*: large subunit of ribosomal gene; *rrnS*: small subunit of ribosomal gene. Strand refers to the strand (H: heavy strand, L: light strand) where the open reading frame of each gene is located. The percentage of A+T is the average content of both subspecies. Mutations (SNPs and indels: ins for insertion, del for deletion) are described for *B. t. lusitanicus* using *B. t. terrestris* as a reference. Selected genes for subspecific differentiation are marked in bold.

	Strand	Start codon	Stop codon	A+T (%)	Length		Mutations		
					TTF	TLS	SNPs	Indels	Variable aa
trnM	H	-	-	82.4	68	68			
trnA	H	-	-	89.9	69	69			
trnI	H	-	-	85.9	64	64			
nad2	H	ATA	TAA	88.1	987	987	2		1
trnC	L	-	-	92.4	66	66			
trnY	L	-	-	86.6	67	67			
trnW	H	-	-	91.7	72	72			
cox1	H	ATT	TAA	76.7	1560	1560	1		1
trnL	H	-	-	82.4	68	68			
cox2	H	ATT	TAA	81.4	693	693	1		
trnD	H	-	-	85.7	70	70	1		
trnK	H	-	-	81.7	69	71		1 ins	
atp8	H	ATA	TAA	91.3	150	150			
atp6	H	ATG	TAA	84.7	678	678	1		
cox3	H	ATG	TAA	82.8	783	783			
trnG	H	-	-	90.6	64	64			
nad3	H	ATA	TAA	88.1	360	360	1		
trnR	L	-	-	87	69	69			
trnN	H	-	-	83.6	67	67			
trnE	H	-	-	95.9	74	74			
trnS	H	-	-	82.1	57	57			
trnF	L	-	-	84.8	66	66			
nad5	L	ATT	-	86.1	1653	1653	3		2
trnH	L	-	-	87.1	62	62			
nad4	L	ATG	-	85.1	1299	1299	2		
nad4L	L	ATT	TAA	87.6	267	267	1		
trnP	L	-	-	86.4	66	66			
trnT	H	-	-	91.2	68	68			
nad6	H	ATG	TAA	92.6	540	540			
cob	H	ATG	TAG	81.6	1143	1143			
trnS	H	-	-	86	67	67			
nad1	L	ATA	TAA	84.1	933	933	2		2
trnL	L	-	-	89.1	64	64			
rrnL	L	-	-	84.6	1363	1362	2	1 ins 1 del	
trnV	L	-	-	91	67	67	1		
rrnS	L	-	-	85.7	747	747			
trnQ	L	-	-	88.2	68	68			
Total genes				84.5			18	2 ins 1 del	
Intergenic regions				94	2673	2489	37	7 ins 9 del	
Sequenced genome	-	-	-	86.1	17232	17049	55	9 ins 10 del	

Figure S1. Map displaying location of origin for samples of *Bombus terrestris* analysed. A circle referred to *B. t. lusitanicus* and a star to *B. t. terrestris* and/or morphological hybrids.

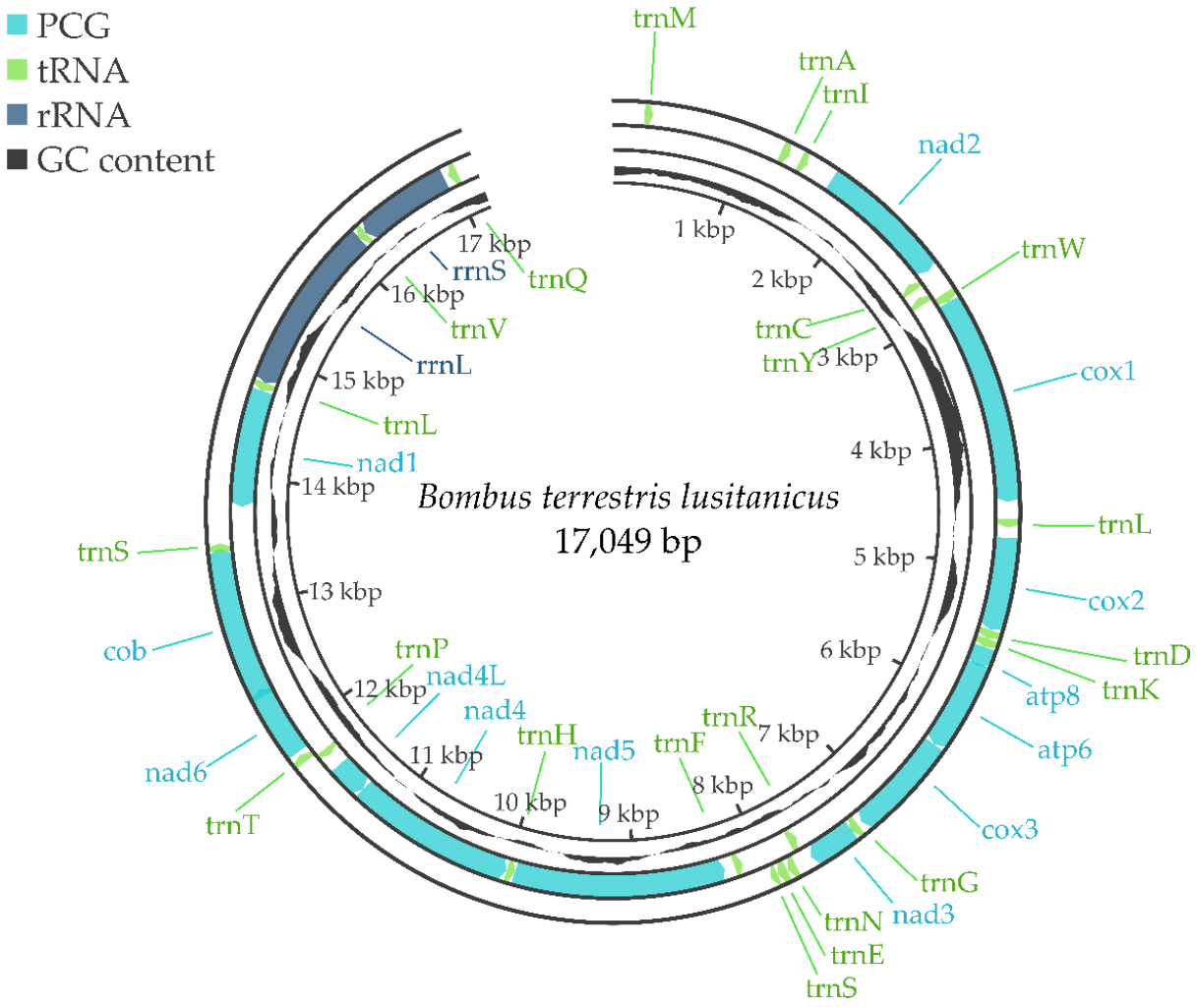

Figure S2. The mitochondrial genome map of *B. t. lusitanicus* in the absence of the complete control region. External circles show the position and orientation of protein coding genes (PCG) and transfer and ribosomal RNAs (tRNA and rRNA, respectively). The circle in dark grey shows the proportion of GC content in each region.

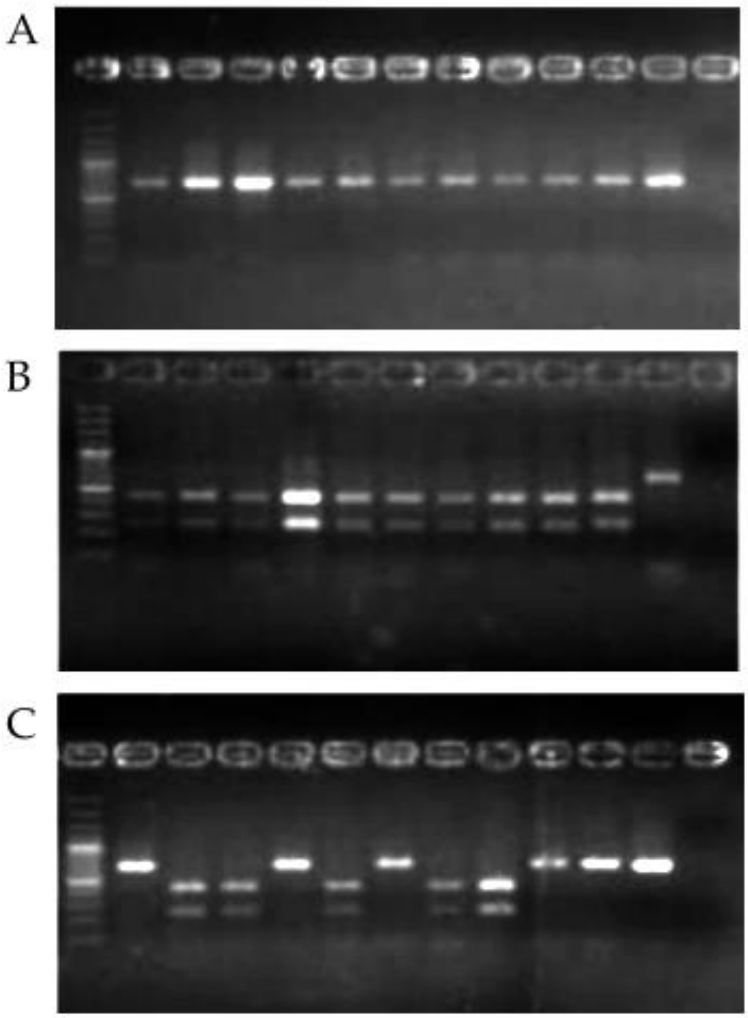

Figure S3. Validation test of the Bter_nad2 RFLP. Different RFLP patterns are due to the *TaqI* restriction enzyme that recognizes a T^CGA site. A) *B. t. lusitanicus* individuals from the Iberian Peninsula showing the banding pattern of the *nad2*-A haplotype. B) *B. t. terrestris* individuals from Belgium showing the banding pattern of the *nad2*-G haplotype. C) *B. t. terrestris* individuals sampled in the south of the Iberian Peninsula showing either the *nad2*-A or the *nad2*-G haplotypes. On the left side a 100 bp ladder was loaded showing bands of 500 and 1000 bp with more intensity. Controls for restriction (uncut amplicon) and PCR (negative) reactions were loaded in the penultimate and last positions on the right side of the gel.

Chapter 4.

Spatial and temporal patterns of genetic diversity in *Bombus terrestris* endemic populations of the Iberian Peninsula and their conservation implications.

Abstract

The bumblebee *Bombus terrestris* is currently used worldwide for crop pollination. Despite having a positive impact on crop yield, it has become a threat to biodiversity in different regions due to its interactions with local populations. Commercial subspecies introduced in the Iberian Peninsula since the 80s without any regulation have colonized the environment, with registered evidences of naturalization, hybridization and introgression with the endemic subspecies *Bombus terrestris lusitanicus*. Here, we have used mitochondrial and nuclear genetic markers to describe the current genetic diversity of *B. t. lusitanicus* populations and to estimate the expansion of the commercial bumblebees in the Iberian Peninsula. Samples from Central Europe (natural distribution range of the commercial subspecies), the Pyrenees (natural intergradation area between subspecies), and a temporal collection from the Iberian Peninsula (before the introduction of commercial subspecies) have been used for comparison. Our results indicate that the presence of the commercial breeds is affecting the Iberian populations, as we have observed not only that the most frequent mitochondrial haplotype of the commercial subspecies is present throughout the peninsula but also subtle changes in their population nuclear diversity. These outcomes evidence that hybridization and consequent introgression are taking place in most of the peninsula, which could have unexpected long-term effects, such as the loss of local adaptation. It is therefore necessary to improve the current legislation about management and exportation of commercial bumblebees to conserve endemic Iberian bumblebee populations.

Introduction

In the current context of human-induced biodiversity crisis (Barnosky et al., 2011), scientific knowledge about the drivers of decline and how they affect organisms is extremely important to propose adequate conservation measures (Sutherland et al., 2018; Habel et al., 2019). In the last decades, given their economic and environmental importance (Lautenbach et al., 2012), the interest on the welfare of pollinators continues to rise. Many studies have explored the main potential causes of their decline (Potts et al., 2016). Beyond some of those identified as major causes (e.g., pesticides and loss of habitat, see Goulson et al., 2015), anthropogenic translocations of domesticated taxa like honey bees or bumblebees within their natural range are receiving more attention lately (De la Rúa et al. 2009; Lecocq et al., 2016). Such human-mediated movements may result in detrimental effects on other pollinators due to competition for resources and the spread of parasites (e.g. microsporidia and trypanosomatids) from commercial to wild populations (Ings et al., 2006; Murray et al, 2013; Graystock et al., 2015; Trillo et al. 2019a). In this context, more research is needed to provide scientific evidence to the regulatory authorities in order to implement conservation measures (Chandler et al., 2019).

The bumblebee species *Bombus terrestris* (Linnaeus, 1758) began to be used commercially in greenhouses for crop pollination in the decade of the 80s in Belgium, and nowadays the subspecies *B. t. terrestris* and *B. t. dalmatinus* Dalla Torre, 1882 are widely used in artificial rearing (Velthuis & Doorn, 2006). Since then, its breeding and exportation have increased, reaching 300,000 colonies sold per year only in Spain (Agrobio pers. comm. in Cejas et al., 2020) with annual estimations above the two million colonies shipped globally (Lecocq et al., 2016). Commercial breeds can escape from greenhouses to natural habitats looking for floral resources (Trillo et al., 2019b) or nesting places, being able to colonize suitable environments (Kraus et al., 2011) as *B. terrestris* is a species with great

invasive capacity thanks to a high thermic resistance, a generalist diet and a wide climatic tolerance (Lecocq et al., 2015). Its exportation outside its Palearctic distribution range has turned this bumblebee into an invasive species worldwide (Inoue et al., 2008; Schmid-Hempel et al., 2014; Montalva et al., 2017; Aizen et al., 2018) yielding some native species to local extinction due to competition, pathogen spillover and/or reproductive interference (Whitehorn et al., 2013; Tsuchida et al., 2019).

Many countries have already taken legislative actions to manage this situation. In Japan, *B. terrestris* was classified as an invasive species in 2006 after studying the effects of its introduction on the wild populations of endemic bumblebees and its exportation is at present severely restricted (Tsuchida et al., 2019). In Argentina, the importation of *B. terrestris* and *B. impatiens* Cresson, 1863 is also strongly regulated to protect native South American species as *B. dahlbomii* Guérin-Méneville, 1835 (Aizen et al., 2018), although commercial species can expand from Chile (Schmid-Hempel et al., 2014), where exportation regulations are still under discussion (Smith-Ramírez et al., 2018). Within the natural distribution range of the species, specific regulations to protect endemic subspecies of *B. terrestris* have also been approved. Norway and the Canary Islands have a strict policy on the importation of foreign bumblebees (Velthuis & Doorn, 2006), while in the United Kingdom the trade of non-native subspecies is restricted, favouring the use of the endemic subspecies *B. t. audax* (Harris, 1776) (Graystock et al., 2016). However, no regulation has been proclaimed yet to protect the endemic subspecies of the Iberian Peninsula, *B. t. lusitanicus* Krüger, 1956. This subspecies differs from others because of the rusty brown colour in the setae and sometimes in the main body (Ornosa & Ortiz-Sánchez, 2004; Rasmont et al., 2008). It is broadly accepted that some subspecies of *B. terrestris* are able to mate (Ings et al., 2005; Ayasse & Jarau, 2014). Despite of their morphological differences, the composition of the cephalic labial gland secretions of *B. t.*

lusitanicus and *B. t. terrestris* is very similar (Bertsch & Schweer, 2012; Lecocq et al., 2015), which is an important requirement for gyne selection (Bergström et al., 1981; Bergman et al., 1997; Coppée et al., 2011). Natural events of hybridization between the two subspecies occur in the intergradation area where their distribution ranges overlap in the Pyrenees and southern France (Rasmont et al., 2008). However, there are records of escapes and naturalization of managed commercial bumblebees in the Iberian Peninsula soon after the establishment of rearing companies in the 1990s in the region of Andalusia (southern Spain) (Ortiz-Sánchez, 1992; Ornosa, 1996). Nowadays, events of hybridization and introgression with wild populations have been confirmed with genetic evidences (Seabra et al., 2019; Bartomeus et al., 2020; Cejas et al., 2020).

In order to preserve endemic taxa, it is necessary to first investigate the genetic diversity and structure of local populations (Coates et al., 2018). Conservation genetics provides a theoretical framework to apply appropriate methodologies to examine bumblebee populations, revealing differences between taxa (Williams et al., 2015), populations (Ellis et al., 2006; Gosterit, 2016) and temporal samplings (Cameron et al., 2011; Maebe et al., 2016). The use of mitochondrial and nuclear (microsatellites) markers in population genetics is advisable due to the different inheritance mechanisms of both genomes (Moritz et al., 1987; Allio et al., 2017), especially in haplodiploid species that can show a strong mitochondrial bias in introgression rates (Patten et al., 2015). The use of mitochondrial DNA has certain implications because of its maternal inheritance. In bumblebees, its analysis has confirmed the presence of foreign queens in the environment (Cejas et al., 2020) and even identified drifter workers that can lay unfertilized eggs in foreign colonies resulting in males (Chandler et al., 2019). Furthermore, it has been considered based on empirical data, that the maternal genotype is decisive for the inheritance of the colony traits (Gosterit & Baskar, 2016). In the present study both markers were analysed to estimate genetic diversity parameters and

clustering patterns across *B. t. lusitanicus* populations. In order to further understand the status of populations, the determination of male diploidy (2n♂) was also implemented as has been considered a proxy of the inbreeding rate in bumblebee populations. In haplodiploid species such as *B. terrestris*, male diploidy is caused by homozygosis at the sex determination *locus* (sl-CSD) in diploid individuals, which otherwise would have to develop in females if they were heterozygous (Van Wilgenburg et al., 2006). The presence of diploid males implies a reduction in the number of workers which can negatively impact the fitness of the colony (Gosterit, 2016). Although male diploidy occurs naturally (Bogo et al., 2018), high rates have been linked with declines in effective population size and low genetic diversity (Kent et al., 2018).

The aim of this research is to study the genetic diversity and structure of the Iberian *B. terrestris* populations from a broader perspective on both, spatial and temporal scales, taking into account the known presence of commercial breeds (i.e. central European subspecies), and possible introgression events between wild and commercial populations. We hypothesize that after the dispersion of the subspecies used commercially, hybridization with the wild populations of the endemic subspecies is not limited to the south and east of the Iberian Peninsula (Cejas et al., 2018, 2020; Seabra et al., 2019; Bartomeus et al., 2020), and that introgression events are occurring in all the peninsular territory. These events would have a more pronounced impact on the genetic diversity of the populations in the south of the peninsula, where many bumblebee breeding companies are located to meet the pollination needs of the agricultural crops that depend on these pollinators (Ministerio de Agricultura, Pesca y Alimentación de España, 2018).

Material and methods

Study areas

Individuals were collected along the Iberian Peninsula, the Pyrenees, north of France, and Belgium. The sampling was focused on the National Parks of Sierra de Guadarrama and Sierra Nevada (central and southern regions of the Iberian Peninsula, respectively), which are immersed in very different landscape contexts. In this sense, agriculture in the surroundings of Sierra de Guadarrama is more dependent on irrigated and rain-fed crops (greenhouse crops in the surrounding provinces only cover up to 350 hectares), while in the vicinity of Sierra Nevada, greenhouse crops have expanded over the last fifty years to more than 30,000 hectares, some of them less than 20 kilometres from the boundaries of the National Park (Ministerio de Agricultura, Pesca y Alimentación de España, 2018). To determine the impact of the introduction of commercial bumblebees, a set of individuals collected in Spain around the 1980s before the use of bumblebee colonies started in the peninsula in the 1990s, was included in the analyses. Populations were delimited based on the maximum flight distance recorded for *B. terrestris* (10 km, Kraus et al., 2009) and geographic barriers were also taken into consideration.

Sample collection

A total of 594 individuals were initially selected for this work. From them, 437 individuals were sampled (256 females and 181 males) in a north-south gradient in 17 locations of the Iberian Peninsula between 2013 and 2017 (Table 1 in Supplementary Material). Two sets of reference populations were additionally considered for spatial comparisons: 78 individuals from the Pyrenees (sampled along the period 1998-2002) as it is the natural intergradation area between the two subspecies, 23 individuals from France (Normandy, 2015) and 12 from Belgium (2013-2014) as they are within the natural distribution range of *B. t. terrestris*. As a temporal reference, 44 individuals collected in Spain before the

introduction of commercial *B. terrestris* (Cejas et al., 2018) were included (Tables 1 and 2, Figure 1 in Supplementary Material). A first subspecies identification of individuals was performed through their morphological characters: reddish pilosity for *B. t. lusitanicus*, black pilosity for the non-Iberian *B. terrestris*, intermediate colours for hybrid individuals (Ornosa & Ortiz-Sánchez, 2004; Rasmont et al., 2008).

Individuals from the Iberian sampling and Belgian and French reference populations were caught with clean nets and maintained individually in absolute ethanol at -20 ºC until processed, while individuals from the Pyrenean and temporal reference populations were obtained from pinned collections.

DNA extraction

Total DNA was extracted from a hind leg of each individual following the protocol of Ivanova et al. (2006) or the NucleoSpin DNA Tissue Kit (Hudlow et al., 2011). Concentration and purity of DNA extractions from pinned individuals were measured with Nanodrop, as DNA obtained from antique individuals has normally a lesser quality (Wandeler et al., 2007; Maebe et al., 2016). Samples with less than 10 ng/uL of DNA concentration and unfitting quality were discarded.

Mitochondrial haplotype screening

Fragments of either the cytochrome oxidase I (*cox1*) (Murray et al., 2008) or the *16S RNA* gene (*rrnL)* (Hines et al., 2006) were sequenced to confirm the species through BLAST tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Haplotype information on *B. terrestris* individuals was obtained with the RFLP approach on the NADH dehydrogenase subunit 2 gene (*nad2*) following Cejas et al. (2020). In this case, 1.2 mg/mL of Bovine Serum Albumin (BSA) were added to the PCR reaction, to improve the success rate of the amplification.

For dry-stored samples from the Pyrenees collection, as the DNA is often highly fragmented in pinned individuals, it is difficult to amplify larger sequences (Wandeler et al., 2007). Therefore, a new set of primers (F: 5'-

TCAACATCGAGGTCGCAATCA-3′ and R: 5′-TGGCTGCGGTATAATTGACTGT-3′) was designed with Primer3 (Rozen & Skaletsky, 2000) to amplify a sorter *rrnL* fragment (388 bp) under the same PCR conditions as for the *rrnL* fragment in Hines et al., (2006) as genes *rrnL* and *nad2* provide the same haplotype information (Cejas et al., 2020). In this case, amplicons were sequenced in Secugen (Madrid, Spain).

Microsatellite amplification and sample genotyping

Ten microsatellite *loci* designed for *B. terrestris* (Estoup et al., 1995; 1996) were amplified in all individuals with two multiplex sets following Cejas et al. (2019): RB1 (B10, B11, B100, B96, B124 and B126), and RB2 (B118, B119, B121 and B132). PCR products were sent to the Servei Central de Suport a la Investigació Experimental (University of Valencia, Spain) for capillary electrophoresis on an ABI Prism 3700 (Applied Biosystems). Allele scoring was performed using GENEMAPPER 4.8 (Applied Biosystems Inc.) by comparing alleles with an internal size standard (GeneScan-500 Liz, Applied Biosystems Inc.). Quality control was made manually. Individuals with less than seven amplified microsatellites were discarded. Siblings were inferred with Colony 2.0.6.4 (Wang, 2012) with a Full-Likelihood analysis under default parameters for haplodiploid species. When the probability of two or more individuals belonging to the same sibship was greater than 70%, one individual was selected from each sibship and the rest were excluded for further genetic analyses.

Genetic diversity and effect of hybridization

To evaluate the potential effect of the hybridization with introduced *B. terrestris* on the genetic pool of the endemic subspecies, genetic diversity parameters were calculated for each Iberian sampling location (resembling one population) with sufficient number of samples (≥14 individuals, 7 populations), for the Iberian Peninsula as one population (SP+PT), and for the four reference populations (dataset *hIN*). The same genetic diversity parameters were estimated again after

removing all detected hybrids (morphologically identified hybrids and those individuals with discrepant mitochondrial haplotype) and naturalized *B. t. terrestris* individuals (dataset *hOUT*). In the French reference population, *B. terrestris* individuals with an Iberian haplotype were tagged as hybrids (for more information on the subject, check Table 2 in Supplementary Material). Finally, to avoid type-I error the analyses were conducted again after removing a random set of the same size (dataset *rOUT*).

To avoid bias due to the haplodiploidy of the species, only genotypes of female individuals were considered. Micro-Checker 2.2.3 (Van Oosterhout et al., 2004) was used to examine genotyping errors such as stutter bands and the frequency of null alleles. Departure of Hardy-Weinberg equilibrium (HWE) and estimates of linkage disequilibrium (LD) were calculated for the Iberian and reference populations using Genepop on the web (Rousset, 2008). The Markov chain for the HWE analysis had 10,000 steps and 1,000 dememorization steps. Level of significance for the analyses was adjusted with Bonferroni corrections (p-value = 0.005). Pairwise tests of linkage disequilibrium were conducted over 10,000 repetitions and the significance level was also corrected under Bonferroni (p-value = 0.0002). Observed and expected heterozygosity (Ho and He) were estimated with GenAlEx 6.5 (Peakall & Smouse, 2006). Allele richness (Ar) and private allelic richness (pAr) were calculated with Hp-Rare 1.1 (Kalinowski, 2005), and rarefacted to a sample size of 10 gene copies. The inbreeding coefficient Fis was obtained with FSTAT 2.9.4 (Goudet, 1995).

Obtained parameters from datasets *hIN* and *hOUT* were compared with the non-parametric Wilcoxon signed-rank test for related samples using package *stats* 3.6.0 (function *wilcox.test*, paired = TRUE) in R (R Core Team, 2017). For this test, data was paired according to their label and ranked by their absolute differences. Level of significance for the analysis was adjusted under Bonferroni corrections (p-value = 0.0083). Wilcoxon signed-rank test was repeated against

the random removal of individuals (datasets *hIN* and *rOUT*), in order to investigate whether the significance of the results was due to the removal of individuals itself or to the identity of those excluded. The Iberian Peninsula as a population (SP+PT) was not included in this analysis to avoid oversampling.

The percentage of hybrid individuals found in each location was compared with the values of expected heterozygosity (He) and with the change in heterozygosity (ΔHe), after removing the hybrids (*hOUT − hIN),* to estimate the effect of introgression on the genetic diversity of the populations through linear regressions in R with package *stats* v3.6.2 (R Core Team, 2017).

Genetic structure

A clustering analysis was carried out on Structure (Pritchard et al., 2000) with a burn-in period of 100,000 steps and 1,000,000 MCMC repetitions. Potential clustering values (K) 1 to 10 were tested under 10 iterations each. Structure Harvester (Earl & von Holdt, 2012) was used to select the best fitting K by calculating ΔK (Evanno et al., 2005). Software Clumpak (Kopelman et al., 2015) was used to reach a consensus between the different iterations of each selected K, following the Markov clustering algorithm (Jakobsson & Rosenberg, 2007). Furthermore, Distruct 1.1 software (Rosenberg, 2004), also implemented in Clumpak, was used to obtain a graphical representation of the population structure.

Discriminant analyses of principal components (DAPC) was performed using R (R Core Team, 2013) package *adegenet* 2.1.1 (Jombart, 2008; Jombart & Ahmed, 2011). Function *find.clusters* was used to identify the number of genetic clusters according to the Bayesian Inference Criterion (BIC). Nevertheless, from a biogeographic perspective, other K numbers were also analysed. Finally, populations were selected as clusters to investigate their identity and relationship. The number of principal components for the analysis was selected

after α-score estimations to explain the data while avoiding overfitting. Results were graphically represented in a scatter plot.

Diploid male monitoring

The proportion (ϕ) of diploid males (2n ♂) was assessed for each population. Males with two or more diploid *loci* were considered as diploids. Diploidy proportion was calculated following Zayed et al. (2009) by dividing the number of diploid males between the total number of sampled male individuals in the population.

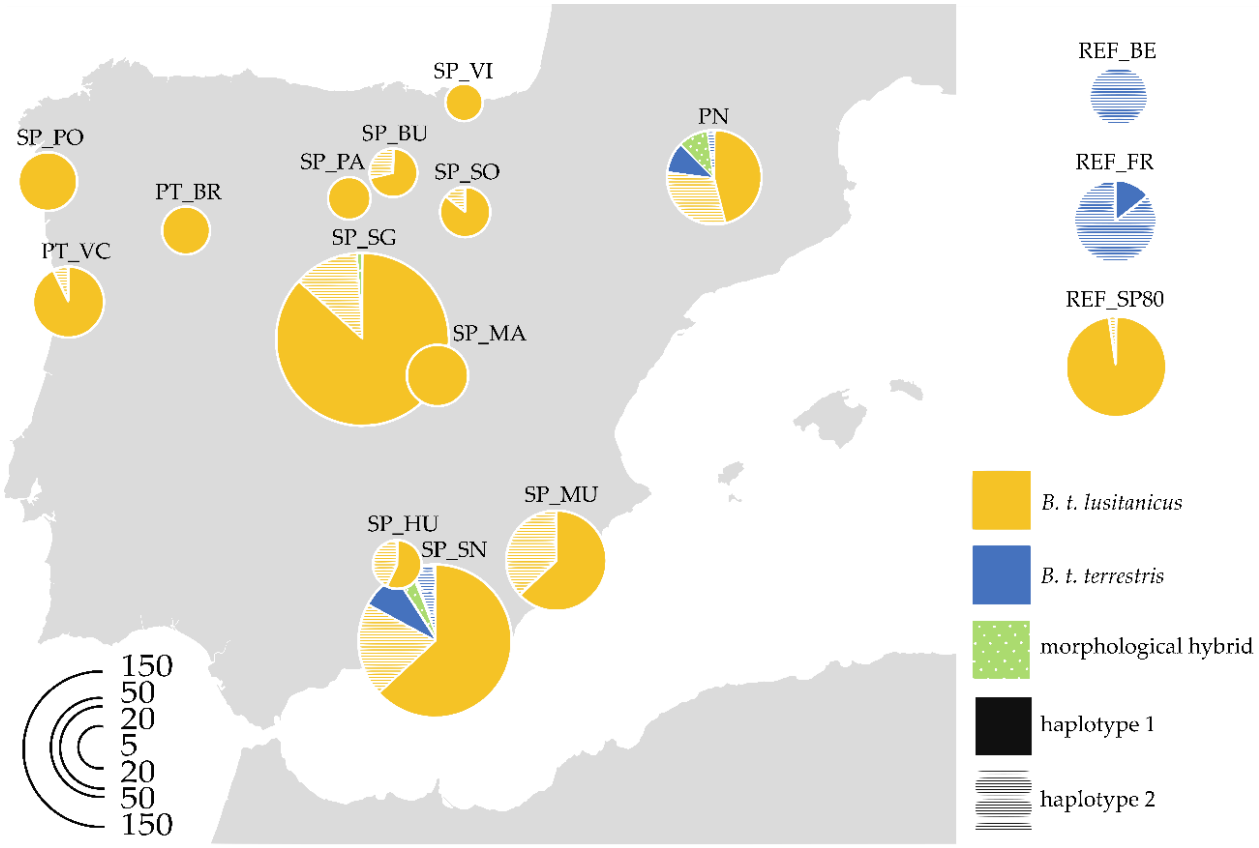

Figure 1. *Bombus terrestris* subspecies and haplotype distribution in the Iberian Peninsula based on their morphologic traits and the genetic variation within the mitochondrial genes nad2 and rrnL. Subspecies information is shown by color: yellow for *B. t. lusitanicus* and blue for *B. t. terrestris*; haplotype information is shown by pattern: plain for hapotype 1 (Iberian) and stripped for haplotype 2 (central European). Morphological hybrids are represented with green color and spot pattern. The data of the reference populations are shown on the top right. The radius of the circles is proportional to the number of individuals analyzed in each location. The data from nearby locations have been merged for visual reasons.

Results

Subspecies and hybrid distribution based on morphology

From the 594 individuals, 39 were discarded as eight corresponded to a different species after BLAST analyses (seven *B. lucorum* [Linnaeus, 1761] and one *B. soroeensis* [Fabricius, 1777]), and 31 from pinned collections had low quality DNA extractions. From the remaining 555 individuals (365 females and 190 males), 487 were morphologically identified as *B. t. lusitanicus* (87.75%), 58 as *B. t. terrestris* (10.45%) and 10 as hybrids (1.8%). Within the Iberian sampling, *B. t. terrestris* individuals and most hybrids were found in the south of the peninsula and in the Pyrenees (Table 1), where the two main current areas of intergradation have been previously detected. However, two hybrids were found in Sierra de Guadarrama (SP_SG3, central peninsula), where supposedly the two subspecies are not in contact.

Subspecies and hybrid distribution based on mitochondrial haplotypes

Mitochondrial haplotype information was obtained for 555 morphologically identified individuals. The mitochondrial haplotype was determined in 433 individuals (78.01%) with the *nad2*- RFLP approach and in 33 individuals through sequencing of the *Bter_rrnL* fragment (5.95%), while haplotype information of 89 individuals was retrieved from other studies (16.04%) (Cejas et al., 2018, 2020). ´

Two haplotypes have been detected in the *B. terrestris* individuals: a haplotype more frequent in the Spanish and Portuguese locations (from now on, Iberian haplotype, SP+PT: 82.37%) and another more frequent in the Belgian and French reference populations (from now on, central-European haplotype, REF_BL: 100%; REF_FR: 86.9%) (Figure 1).

Table 1. Subespecies identification of *B. terrestris* individuals and hybrids detected per location (see Figure 1 for code location). Hybrids (labeled with * in the column subspecies) have been identified either morphologically or because of discrepancies between morphologic identification and mitochondrial haplotype. Sex is also indicated and for males, the number of diploid males (2n) is also shown in parenthesis. Percentage of hybrid individuals is shown by sampling site.

Location	subspecies	N	♀	hybr.	♂(2n)	hybr.	% hybr.
SP_VI	*lusitanicus*	3			3(1)		0
SP_PO	*lusitanicus*	11	7		4		0
SP_BU	*lusitanicus*	7	7	2			28.57
SP_PA	*lusitanicus*	5	5				0
SP_SO	*lusitanicus*	11	9	2	2		18.18
PT_BR	*lusitanicus*	7	3		4		0
PT_VC	*lusitanicus*	14	14	1			7.14
SP_SG1	*lusitanicus*	10	10	1			10
SP_SG2	*lusitanicus*	138	67	6	71(5)	9	10.87
SP_SG3	*lusitanicus*	33	23	2	10	2	18.18
	hybrids*	2	1		1		
SP_MA	*lusitanicus*	12	9		3(2)		0
SP_MU1	*lusitanicus*	23	16	4	7(2)	3	30.43
SP_MU2	*lusitanicus*	25	18	7	7(2)	4	44
SP_HU	*lusitanicus*	7	2	1	5	2	42.86
SP_SN1	*lusitanicus*	16	8	1	8	1	17.65
	terrestris	1			1	1	
SP_SN2	*lusitanicus*	40	15	3	25	4	29.17
	hybrids*	4			4		
	terrestris	4	4	3			
SP_SN3	*lusitanicus*	51	27	6	24(1)	10	45.31
	terrestris	13	11	5	2	2	
SP+PT	*lusitanicus*	413	240	36	173(13)	35	21.74
	hybrids*	6	1		5		
	terrestris	18	15	8	3	3	
REF_BL	*terrestris*	12	12				0
REF_FR	*terrestris*	23	18	3	5		13.04
PN_AM	*lusitanicus*	14	14	5			40
	terrestris	1	1	1			
PN_EY	*lusitanicus*	15	15	6			47.37
	terrestris	4	4	3			
PN_JA	*lusitanicus*	1	1	1			100
	hybrids*	4	4	4			
PN	*lusitanicus*	30	30	12			53.85
	hybrids*	4	4	4			
	terrestris	5	5	4			
REF_SP80	*lusitanicus*	44	40	1	4	0	2.27

A different haplotype from the one expected based on their morphology (discrepant haplotype) was detected in 20.18% of the analysed individuals. In this sense, *B. t. lusitanicus* individuals with the central-European haplotype (discrepant hybrids) were found in 12 of the 17 Iberian locations sampled (range 0-44%) and in the Pyrenees (18.74%). In the same way, 61.1% of the naturalized *B. t. terrestris* found in the south of the peninsula bear the Iberian haplotype. The higher percentages of discrepant hybrids were found in the two intergradation areas: the Pyrenees (PN_JA: 100%; PN_EY: 40%; PN_AM: 35.7%) and southern peninsula (SP_MU2: 44%; SP_SN3: 31.40%), while three individuals from France presented the Iberian haplotype (REF_FR: 13.04%) and no hybrids were found in the Belgian reference group. In the Iberian temporal reference population, the Iberian haplotype was more frequent than in the current sampling (REF_SP80: 97.73%).

Microsatellite data validation

Of the ten microsatellite *loci* amplified, *locus* B10 was excluded due to low amplification rate and difficult scoring. Twenty-seven individuals with missing data in more than three of the remaining *loci* were not taken into account in further analyses. In addition, 18 female individuals were also removed after sibship inference analysis, leaving a total of 320 female individuals to carry out population analyses. Null alleles due to homozygote excess were only found in the Iberian populations (SP+PT) and the temporal sampling (REF_SP80) at low frequencies (Oosterhout > 0.25%). In fact, most *loci* presented a statistically significant deviation from HWE expectations due to heterozygote deficit. None of the microsatellite *loci* analysed showed significant linkage disequilibrium after 10,000 repetitions.

Genetic diversity and effect of hybridization

Allelic richness ranged from 4.27 to 4.63 across the Iberian locations (Table 2). The lowest allelic richness was found in the Pyrenean population (PN_EY: 4.16)

whereas the highest value was found in Normandy (Ref_FR: 4.70). The higher number of private alleles was found in the overall Iberian population. Genetic diversity values estimated from He values varied from 0.630 to 0.716 (observed in the Pyrenean PN_EY and French Ref_FR populations respectively). The fixation index Fis showed signs of inbreeding in both the Iberian and the temporal reference populations.

Table 2. Allelic richness (Ar), private allelic richness (pAr), observed heterozygosity (Ho), expected heterozygosity (He) and inbreeding index (Fis) calculated independently for each population based on the genotypes of female individuals. N= population size. hIN include all the individuals while in hOUT hybrid individuals have been excluded.

Location	N		Ar		pAr		Ho		He		Fis	
	hIN	*hOUT*	*hIN*	*hOUT*	*hIN*	*hOUT*	*hIN*	*hOUT*	*hIN*	*hOUT*	*hIN*	*hOUT*
PT_VC	14	13	4.45	4.43	0.19	0.16	0.586	0.572	0.659	0.660	0.148	0.174
SP_SG2	54	49	4.29	4.32	0.11	0.10	0.532	0.524	0.671	0.672	0.217	0.229
SP_SG3	22	19	4.27	4.21	0.13	0.10	0.590	0.576	0.680	0.665	0.156	0.162
SP_MU1	15	11	4.63	4.57	0.30	0.31	0.708	0.733	0.700	0.687	0.033	-0.01
SP_MU2	17	11	4.44	4.60	0.12	0.09	0.570	0.619	0.698	0.690	0.215	0.157
SP_SN2	19	12	4.45	4.42	0.11	0.13	0.570	0.624	0.687	0.662	0.197	0.101
SP_SN3	36	20	4.50	4.37	0.16	0.16	0.629	0.611	0.701	0.683	0.117	0.131
SP+PT	177	135	4.46	4.43	0.39	0.40	0.587	0.584	0.701	0.696	0.166	0.165
REF_BL	11	11	4.48	4.48	0.13	0.18	0.717	0.717	0.661	0.661	-0.036	-0.036
REF_FR	17	14	4.70	4.62	0.23	0.15	0.739	0.778	0.716	0.707	-0.001	-0.064
PN_EY	15	8	4.16	3.85	0.11	0.13	0.588	0.644	0.630	0.589	0.107	0.07
REF_SP80	34	33	4.69	4.66	0.36	0.37	0.498	0.488	0.710	0.706	0.314	0.326

In order to study introgression at the nuclear level on the Iberian populations, population parameters were compared with those obtained after the removal of hybrid individuals in each location (*hIN*/*hOUT*) (Table 2). Wilcoxon signed-rank test showed a strong variation in the expected heterozygosity values after the removal of the hybrids, although not significant after Bonferroni correction (p-value = 0.014, V = 52). In any case, the results after random removal (*hIN*/*rOUT*) (p-value = 0.082, V = 45) pointed out that the decrease of He in *hOUT* might not be due to the removal of individuals *per se* (Table 3), but to the identity of the

removed individuals as hybrids. Linear regressions showed that genetic diversity values do not depend directly on the percentage of hybrid individuals in each location ($r^2 = 0.012$; $p = 0.741$), probably being affected by other variables. However, the variation in He due to the presence of hybrid individuals (ΔHe = He *hIN* — He *hOUT*) does present a significant dependence on the percentage of hybrids present in each population ($r^2 = 0.593$; $p = 0.005$) (Figure 2).

Table 3. Wilcoxon signed-rank test for related samples. hIN/hOUT: population parameters from the whole dataset (hIN) were compared with those obtained without hybrids (hOUT). To avoid type I error, the Wilcoxon test was repeated comparing the results with those obtained after the random removal of individuals (hIN/rOUT). N= population size, Ar= allelic richness, pAr= private allelic richness, Ho= observed heterozygosity, He= excepted heterozygosity, Fis= inbreeding index, * = p-value<0.008.

hIN/hOUT						
	N	Ar	pAr	Ho	He	Fis
V Wilcoxon	55	43	32	15	52	40
p-value	0.006*	0.125	0.682	0.221	0.014	0.221
hIN/rOUT						
	N	Ar	pAr	Ho	He	Fis
V Wilcoxon	55	32	32	17	45	30.5
p-value	0.006*	0.684	0.679	0.944	0.082	0.374

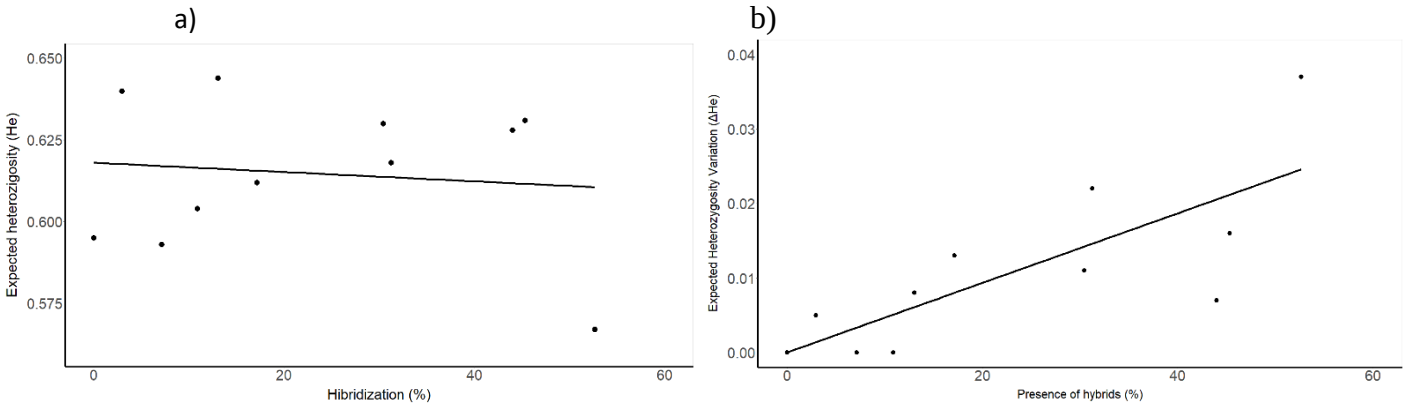

Figure 2. Linear regressions between (a) genetic diversity (He) / (b) variation of genetic diversity (ΔHe) and percentage of hybrids observed in each population. Genetic diversity does not decrease significantly as the percentage of hybrids rises (r2 = 0.012, p= 0.741) whereas when the percentage of hybrids increases, the variation of genetic diversity increases as well (r2 = 0.593, p= 0.005).

Genetic structure

Structure Harvester showed K=4 as the most likely number of clusters (Figure 2 in Supplementary Material), although this result is probably a consequence of the limitations of the program, which cannot detect values of K=1 (Earl and von Holdt 2012). However, K=2 was also studied to investigate possible structuration due to the subspecies (*lusitanicus* and *terrestris*) identification (Figure 3 in Supplementary Material). Visualisation of Structure results indicates the approximately equal contribution to each population (K), implying that the true value of K is indeed 1, with no clear structuring due to the subspecies determination.

DAPC analysis with the function *find.clusters* showed that BIC reached its minimum value at K=4 with K=2 presenting the largest increase in BIC (Figure 4 in Supplementary Material). Clusters observed in K=2 to K=4 were not related either to the subspecies identification or population distributions (Figure 5 in Supplementary Material). In a final analysis in which populations were selected as clusters (Figure 3), they showed an overlapping distribution in the axis, indicating a low degree of genetic differentiation and corroborating the results of Structure. However, the Iberian populations from the temporal reference group REF_SP80 presented a higher degree of clustering than the other populations, (Figure 3).

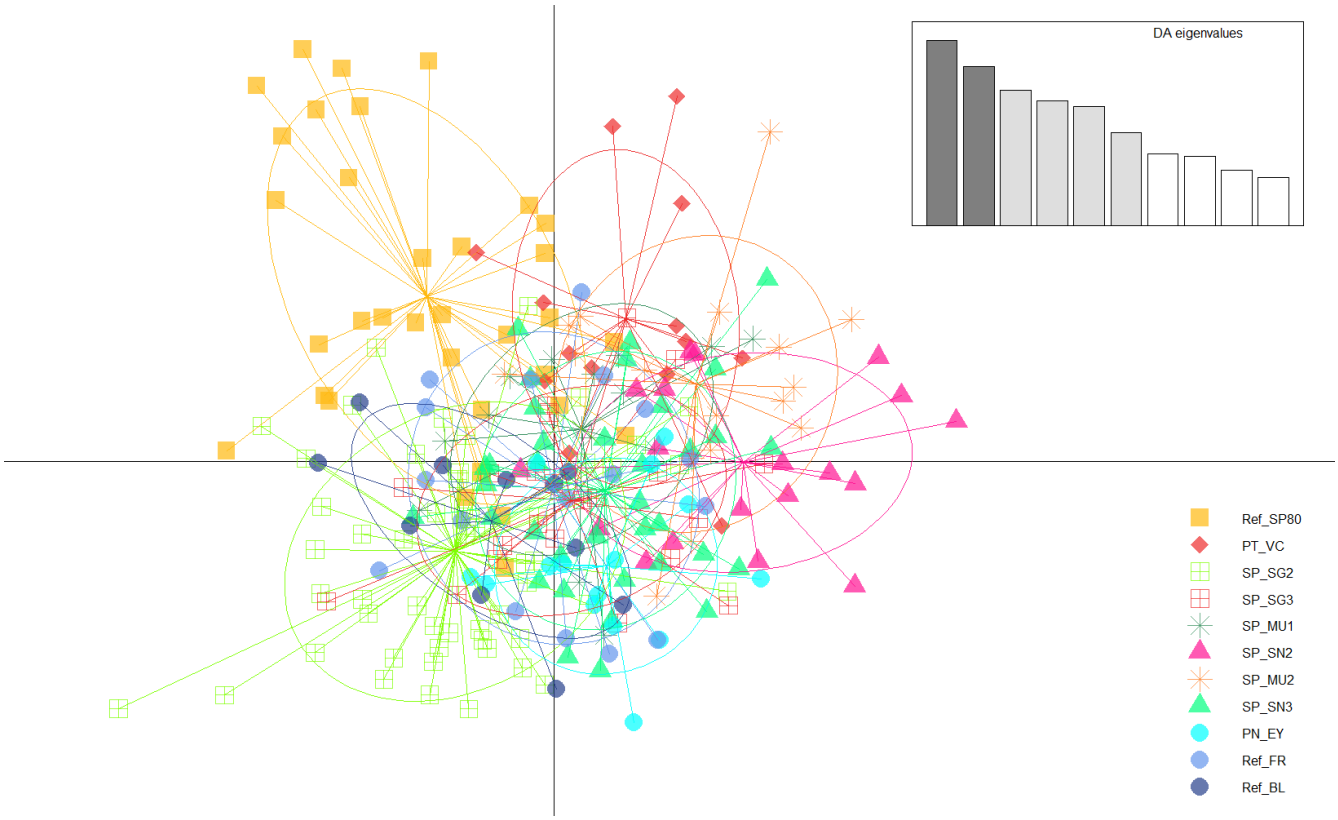

Figure 3. Scatter plot from discriminant analysis of principal components. 80 Principal Components (PC) were selected after α-score estimations to avoid overfitting. Chosen discriminant analysis eigenvalues are depicted in the top right of the plot. Ellipse indicate 95% confidence interval of assignment, yellow squares identify bumblebee individuals collected before the 90's, orange rhombuses represent Portuguese samples, green colours represent individuals from Sierra de Guadarrama, pink colours represent individuals from Sierra Nevada and blue colours represent samples outside the Iberian Peninsula, as displayed in the figure legend in the bottom right.

Diploid male detection

A total of 13 diploid males were found out of the 181 male individuals sampled in the Iberian Peninsula (Table 1): one at the north (SP_VI: 1, ϕ=0.3), seven in the central area (SP_SG2: 5, ϕ=0.07; SP_MA: 2, ϕ=0.66) and five at the south (SP_MU1: 2, ϕ=0.29; SP_MU2: 2, ϕ=0.29; SP_SN3: 1, ϕ=0.04), with a ϕ value for the Iberian Peninsula of 0.07. A male from the locality SP_SG2 was identified as a triploid (3n) after amplifying each microsatellite *locus* individually.

Discussion

The aim of this research was to study the genetic diversity at both spatial and temporal scales of the Iberian *B. terrestris* populations under threat of introgression from commercial populations. Our results based on an extensive sampling throughout the Iberian Peninsula (Spain and Portugal) indicate that the mitochondrial haplotype linked to the central European populations of *B. terrestris* has expanded beyond the natural contact area and near greenhouses, reaching most of the north-south gradient with some exceptions at the northwest. These results contrast with the haplotype distribution found in the reference Iberian population used for temporal comparison (i.e. REF_SP80, specimens collected in Spain forty years ago) in which, except for one individual spatially close to the natural intergradation area in the Pyrenees, all individuals presented the Iberian haplotype. This result supports that the endemic Iberian haplotype was predominant in the territory and that, due to hybridization and introgression events with naturalized commercial breeds, the central European haplotype has recently spread in the environment. This expansion has modified the distribution of haplotypes what may cause losses of local adaptation in the not too distant future (Ellis et al., 2018).

Our results expand the current knowledge about the genetic integrity of *B. t. lusitanicus* in the Iberian Peninsula. Previous records of *B. t. terrestris* (Ortiz-Sánchez, 1992; Ornosa, 1996; Vargas et al., 2013 Cejas et al., 2018, 2020) and hybrid individuals have been found in the south (morphological and putative genetic hybrids; Cejas et al., 2018, 2020; Bartomeus et al., 2020) and west (putative genetic hybrids; Seabra et al., 2019) of the Iberian Peninsula, whereas in the present study we have found evidence of introgression in most of the territory (see Table 1), including the area of natural introgression in the Pyrenees. The presence of the central European haplotype in the central area of the peninsula where the subspecies *B. t. terrestris* has not been detected, could be due to the dispersion of

individuals from areas where commercial breeds are being used. On the other hand, in the most north western localities sampled (SP_PO, SP_BR and SP_PA), the central European haplotype has not been found, which suggests that these populations of *B. t. lusitanicus* are less affected by introgression probably due to a lower density of greenhouses in the area (Ministry of Agriculture, Fisheries and Food of Spain, 2018) which implies the absence of the foreign subspecies.

This study confirms that the inclusion of samples from old collections is crucial to obtain information on the evolution of the genetic diversity of local populations as has been done before in other *Bombus* species (Maebe et al., 2016). Furthermore, it is important to continue studying the natural expansion trend of *B. terrestris* subspecies, not only within the Iberian Peninsula, but also from there, as it seems that *B. t. lusitanicus* is expanding into northern Europe from the peninsula (Rasmont et al., 2008; Lecocq et al., 2015; Ornosa et al., 2017). In any case, although we must assume that hybridization can play a key role in the evolution of many plant and animal taxa (Allendorf et al., 2001; Arnold & Kunte, 2017), there is a general consensus that human-induced introgression of non-indigenous organism has negative effects into native gene pools (Mallet, 2005; Allendorf & Luikart, 2009; De la Rúa et al. 2013).

In line with previous studies on the genetic diversity of the species, heterozygosity values obtained in this analysis and the clusters inferred (Structure and DAPC) suggest the existence of an intense gene flow among *B. terrestris* populations, which leads to a reduced population structure in a gradient not only continental (Estoup et al., 1996; Moreira et al., 2015) but also peninsular. When comparing the Iberian populations with the temporal reference, only slight variations can be observed in the allelic frequencies, although the data shows an overall decrease of allelic richness and expected heterozygosity values. On the other side, the Wilcoxon test suggest that the hybridization and introgression events are affecting the native populations (Bartomeus et al. 2020, Facon et al.,

2011; Hamilton & Miller, 2016) by rising the genetic diversity in the presence of hybrid individuals, which is expected from the admixture of different genetic pools (Facon et al., 2011). Given the low structure of bumblebee populations, the effect of this change is difficult to measure, but it can be assumed that if this dynamic is followed, the loss of endemicity towards a deeper homogenization of the European populations will be a recurrent concern in the future.

The analysis of the genetic diversity in the Iberian Peninsula yielded the lowest values in the central peninsular populations (Sierra de Guadarrama, SP_SG2, SP_SG3), where the highest number of diploid males and a triploid male were found. The excess of homozygosis observed in the Iberian populations could be affecting the proportion of diploid males. *B. terrestris* does not present mating preferences among related or unrelated individuals (Bogo et al., 2018), which means that there are no barriers to prevent diploidy or even triploidy from occurring in males (Ayabe et al., 2004). Although the ploidy values obtained in this work have been higher than those of the populations of threatened species such as *B. florilegus* Panfilov, 1956 (2.7%) (Takahashi et al., 2008) or *B. muscorum* Linnaeus, 1758 (5%) (Darvill et al., 2006), it has been previously discussed that the species *B. terrestris* could have some capacity to support inbreeding in their populations (Gerloff & Schmid-Hempel, 2005). However, given the lack of population structure observed in the *B. terrestris* populations studied in this work, it is difficult to say whether the data obtained could represent a sign of potential threat of inbreeding depression in the Iberian populations (Schenau & Jha, 2017). In any case, these results should be taken into account in future studies on the species.

It has been recorded that *B. terrestris* can escape from greenhouses (Kraus et al., 2011; Seabra et al., 2019; Trillo et al., 2019b). In addition, this bumblebee species has a great capacity to expand as seen in South America where individuals escaping from the greenhouses in Chile were able to cross the Andes

to Argentina (Torreta et al., 2006), reaching expansion rates higher than expected from their recorded maximum flight distances (Kraus et al., 2009; Schmid-Hempel et al., 2014). Also, Moreira et al. (2015) found traces of migration across the English Channel. All this leads us to argue that the main driver of the population alterations we have inferred in this research is the propagation of the commercial *B. terrestris* subspecies from greenhouses across the Iberian Peninsula. According to natural dispersal models of *B. terrestris* (Lecocq et al., 2015) the European subspecies should not be able to adapt to the environmental conditions of the Iberian Peninsula, but the situation observed may not be compatible with such model, since commercial breeds have a greater feeding capacity and larger colony sizes than local ones, in addition to having the capacity to hybridise with locally adapted populations (Ings et al., 2006).

Moreover, there is another factor contributing to the emergence and dispersion of commercial breeds (inferred by the presence of the central European haplotype) in the environment: inadequate management of the colonies by breeding companies and growers after colony use due to lack of information on the consequences of their dispersal in the environment. To our concern, there is still no legislation about the management of bumblebee colonies for agricultural use in the Iberian Peninsula beyond the instructions of the seller. There are cases of outdoor use of colonies, incomplete removal or simply abandonment of colonies (Seabra et al., 2019), as happens in UK (Chandler et al., 2019), and even attempts to manage the nesting of commercial queens in the environment (personal information). In order to avoid escapes, bumblebee nests should be placed only inside greenhouses and removed at the end of the crop pollination campaign. The arrival of commercial breeds of *B. terrestris* into new environments and their subsequent colonization is guaranteed unless stricter regulations are adopted on their transport and use (Aizen et al., 2018; Naeem et al., 2018), both outside and within the species natural distribution range (Ings et

al., 2006; Moreira et al., 2015). In the case of the Iberian Peninsula, as a large producer and exporter of fruits and vegetables and therefore an intensive user of commercial pollination services, we suggest that a stricter legislation must be applied to the import of foreign subspecies and that companies breeding local subspecies (Velthuis & Doorn, 2006) should be supported, as the breeding of *B. t. lusitanicus* does not involve additional costs compared to other subspecies of *B. terrestris* (de Jonghe; Rasmont, personal information). Such regulations are already being used in other regions within the natural distribution range of the species such as United Kingdom, Norway or the Canary Islands. Authorisation of the importation of foreign taxa should be discussed after studying the survival rate, expansion capacity and environmental impact of the managed populations on the environment. It is important to coordinate an update of existing legislation with more active dissemination to first-hand users on the correct management of commercial breeds and the benefits of using local subspecies.

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Supplementary Material

Table S1. Sampling and geographical information of the Iberian and reference *B. terrestris* populations. Location codes correspond to country and locations (SP=Spain; PT=Portugal). N= number of individuals with the number of females between brackets.

Code	Location	Country	Latitude (N)	Longitude (W)	N (♀)
Iberian (SP+PT)					437 (256)
SP_VI	Vitoria	Spain	42°50'59.96"	2°40'18.91"	3 (0)
SP_PO	Pontevedra	Spain	42°19'12.76"	8°30'19.27"	11 (7)
SP_BU	Burgos	Spain	42º26´32"	4º15´50"	7 (7)
SP_PA	Palencia	Spain	4302´29"	4º27310"	5 (5)
SP_SO	Soria	Spain	41° 45' 58"	2°27'17.20"	11 (9)
PT_BR	Braganza	Portugal	41°47'56.15"	6°45'55.72"	7 (3)
PT_VC	Vila do Conde	Portugal	41°20'38.35"	8°44'45.85"	14 (14)
SP_SG1	Sierra Guadarrama	Spain	40°39'38"	4°0'41"	10 (10)
SP_SG2	Sierra Guadarrama	Spain	40°45'14"	4°3'50"	138 (67)
SP_SG3	Sierra Guadarrama	Spain	40°49'53"	3°57'34"	35 (24)
SP_MA	Madrid	Spain	40°26'52.9"	3°43'34.1"	12 (9)
SP_MU1	Murcia	Spain	38° 9'38.72"	2°13'18.85"	23 (16)
SP_MU2	Murcia	Spain	37°52'23.94"	1°33'42.64"	25 (18)
SP_HU	Huelma	Spain	37°43'8.75"	3°27'5.36"	7 (2)
SP_SN1	Sierra Nevada	Spain	36°57'54.70"	3°20'27.13"	17 (8)
SP_SN2	Sierra Nevada	Spain	37°10'50.18"	3° 9'47.24"	48 (19)
SP_SN3	Sierra Nevada	Spain	37° 5'36.98"	3°23'13.13"	64 (38)
REF_BL					12(12)
	Moorsel	Belgium	50°57'10.8"	4°06'21.6"E	1(1)
	Malchamps	Belgium	50°27'54"	5°55'22.8"E	1(1)
	Saint-Vaast	Belgium	50°27'10.80"	4°7'26.40"E	9(9)
	Torgny	Belgium	49°31'01.2"	5°27'54.0"E	1(1)
REF_FR					23(18)
	Colleville-sur-Mer	France	49°21'37.86”	0°51'27.11”W	1(1)
	Beaumont en Auge	France	49°16'39.58”	0° 6'33.28”E	3(3)
	Beuvron-en-Auge	France	49°11'23.1"	0°02'44.2"W	6(5)
	Chartres	France	48°29'17.84”	1°30'47.31”E	8(4)
	Grandcamp-Maisy	France	49°23'13.53”	1° 3'8.17”E	5(5)
PN					78(78)
PN_AM	Argelès-sur-Mer	French Pyrenees	42°28'27"	3°00'33"E	27 (27)
PN_EY	Eyne	French Pyrenees	42°29'12.8"	02°05'06.4"E	46 (46)
PN_JA	Jaca	Spanish Pyrenees	42°33'52.50"	0°34'1.39"W	5 (5)
REF_SP80					44(40)
	Salnes	Spain			1(1)
	Alsasua	Spain			1(1)
	Llanes	Spain			1(1)
	Barcelona	Spain			1(1)
	Comarruga	Spain			1(1)
	Saelices del Río	Spain			1(1)
	El Espinar	Spain			1(1)
	Ávila	Spain			2(2)
	Guadalajara	Spain			1(1)
	Budia	Spain			1(1)
	Cerceda	Spain			1(1)
	Algete	Spain			1(1)
	Madrid	Spain			27(24)
	Villares del Saz	Spain			1(1)
	Mallorca	Spain			1(1)
	Cazorla	Spain			1(1)
	Marbella	Spain			1(1)
TOTAL	(Iberian + Reference)				594 (404)

Table S2. Information on selected individuals for the genetic diversity and structure analyses. For the Iberian populations, only those with 14 or more individuals were selected. Haplotype 1 corresponded to the most frequent in *B. t. lusitanicus* and 2 to the most frequently observed in *B. t. terrestris*. Individuals were marked as potential hybrids in the *analysis_use* column and removed for the *hOUT* analysis when their morphological characters were intermediate between the two subspecies, when their mitochondrial haplotype did not correspond to their subspecies, or when given the geographical distribution of the subspecies, it was suspected that the individual was naturalized.

Location	ID	Subspecies	Haplotype	*Analysis_use*	Sex
PT_VC	TLPT.010	lusitanicus	2	hybrid	worker
PT_VC	TLPT.009	lusitanicus	1		worker
PT_VC	TLPT.008	lusitanicus	1		worker
PT_VC	TLPT.011	lusitanicus	1		worker
PT_VC	TLPT.012	lusitanicus	1		worker
PT_VC	TLPT.013	lusitanicus	1		worker
PT_VC	TLPT.014	lusitanicus	1		worker
PT_VC	TLPT.015	lusitanicus	1		worker
PT_VC	TLPT.016	lusitanicus	1		worker
PT_VC	TLPT.017	lusitanicus	1		worker
PT_VC	TLPT.018	lusitanicus	1		worker
PT_VC	TLPT.019	lusitanicus	1		worker
PT_VC	TLPT.020	lusitanicus	1		worker
PT_VC	TLPT.021	lusitanicus	1		worker
SP_SG2	TLS.074	lusitanicus	2	hybrid	worker
SP_SG2	TLS.252	lusitanicus	2	hybrid	worker
SP_SG2	TLS.262	lusitanicus	2	hybrid	worker
SP_SG2	TLS.264	lusitanicus	2	hybrid	worker
SP_SG2	TLS.276	lusitanicus	2	hybrid	worker
SP_SG2	TLS.190	lusitanicus	1		worker
SP_SG2	TLS.253	lusitanicus	1		worker
SP_SG2	TLS.183	lusitanicus	1		worker
SP_SG2	TLS.005	lusitanicus	1		worker
SP_SG2	TLS.070	lusitanicus	1		queen
SP_SG2	TLS.071	lusitanicus	1		worker
SP_SG2	TLS.072	lusitanicus	1		worker
SP_SG2	TLS.073	lusitanicus	1		worker
SP_SG2	TLS.075	lusitanicus	1		worker
SP_SG2	TLS.076	lusitanicus	1		worker
SP_SG2	TLS.153	lusitanicus	1		worker
SP_SG2	TLS.154	lusitanicus	1		worker
SP_SG2	TLS.156	lusitanicus	1		worker
SP_SG2	TLS.157	lusitanicus	1		worker
SP_SG2	TLS.161	lusitanicus	1		worker
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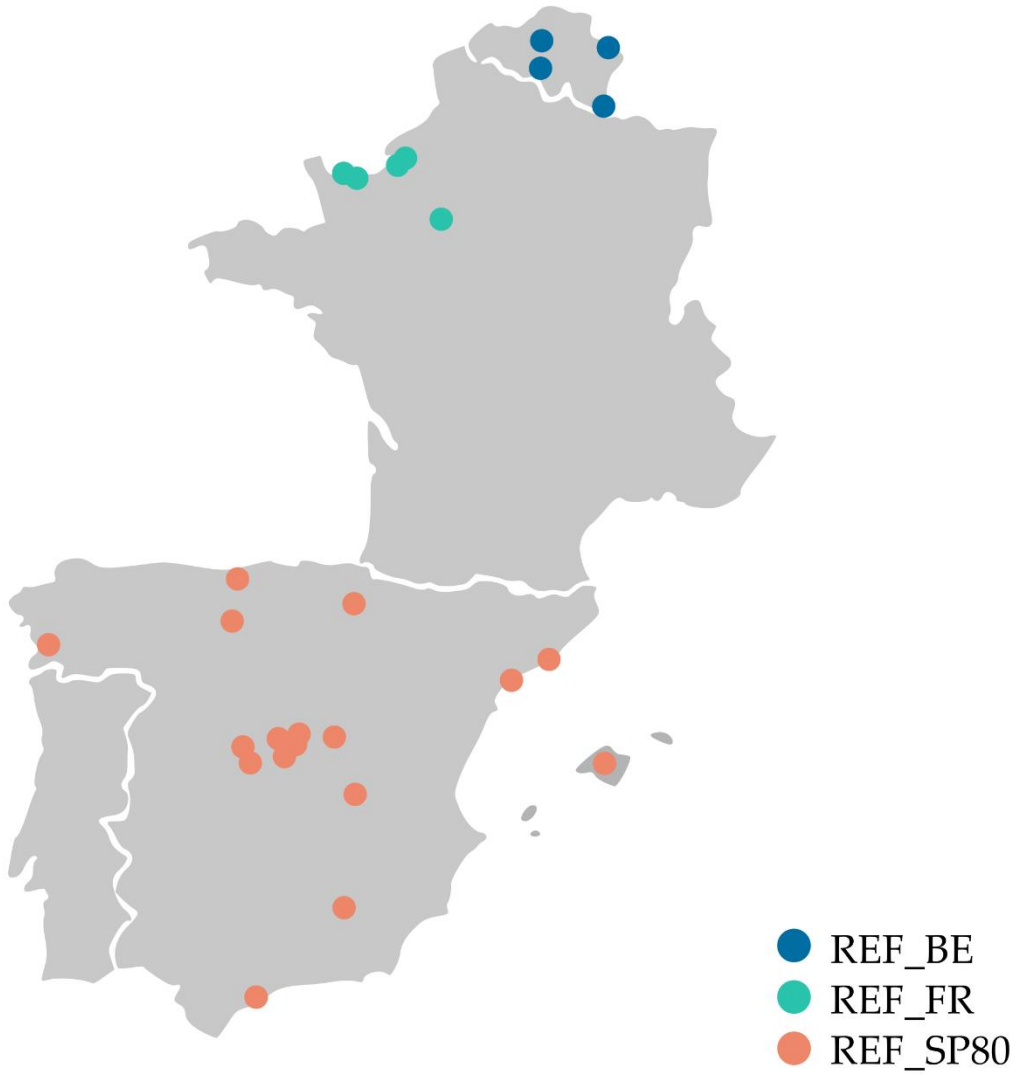

Figure S1. Locations of the reference data extracted from Cejas et al. (2018), as displayed in Table S1. REF_BE in blue colour, REF_FR in green and REF_SP80 in orange.

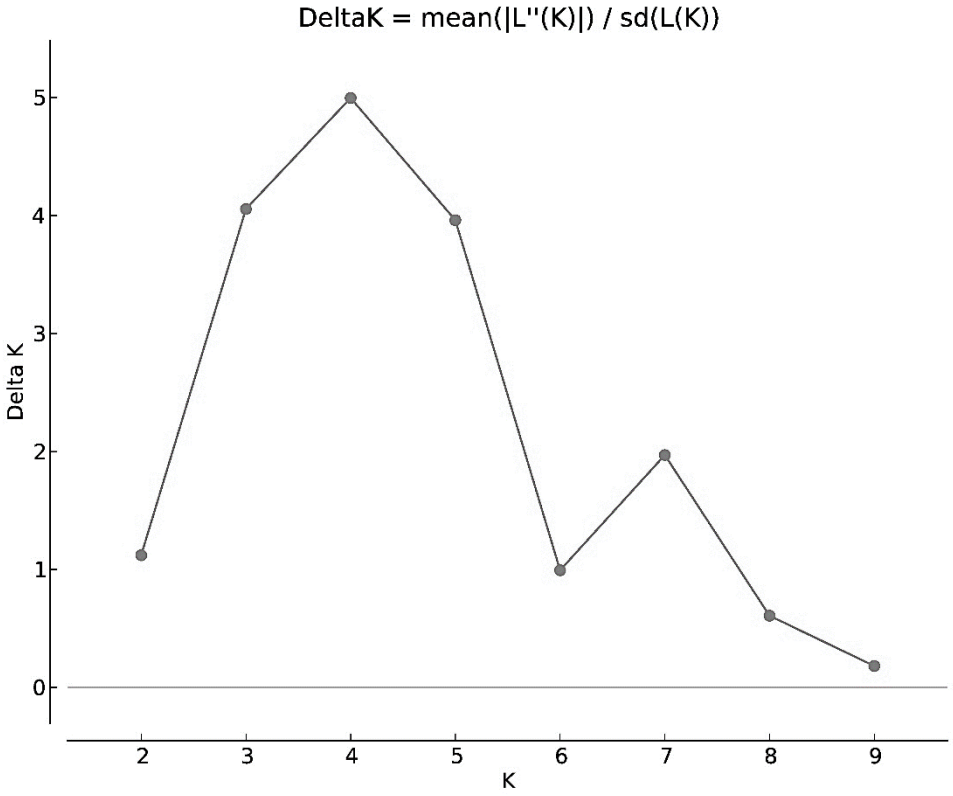

Figure S2. ΔK calculated by the Evanno method for models K=1 to K=10.

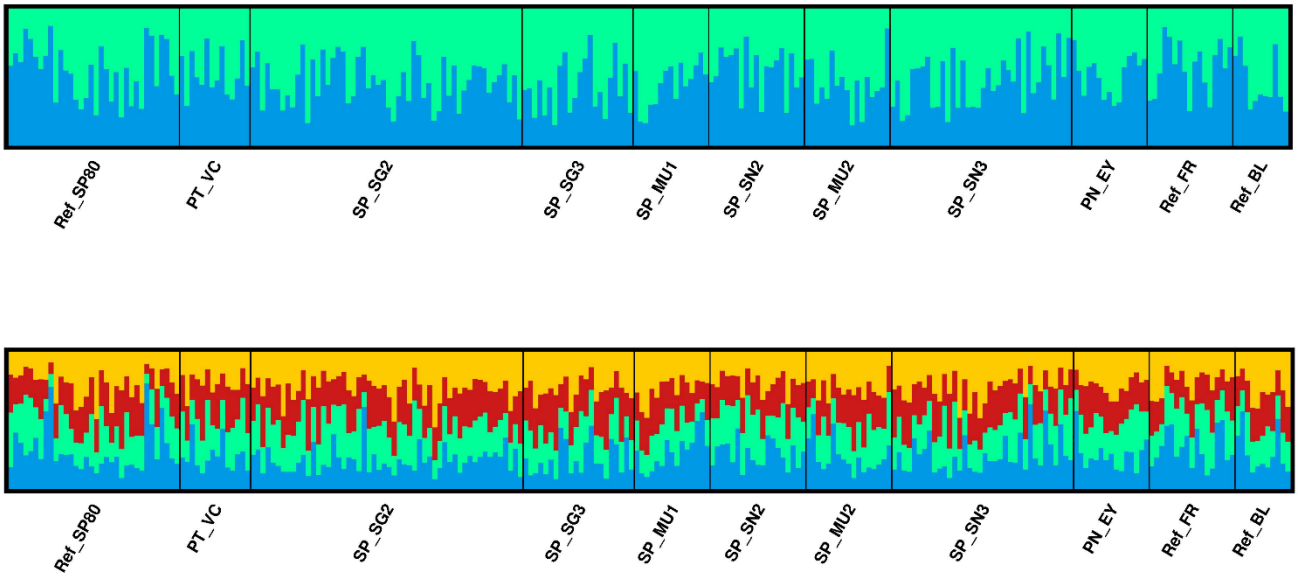

Figure S3. Results from STRUCTURE clustering analysis on the microsatellite genotype of 254 females. The consensus of the iterations for K=2 and K=4 after Markov clustering algorithm are shown.

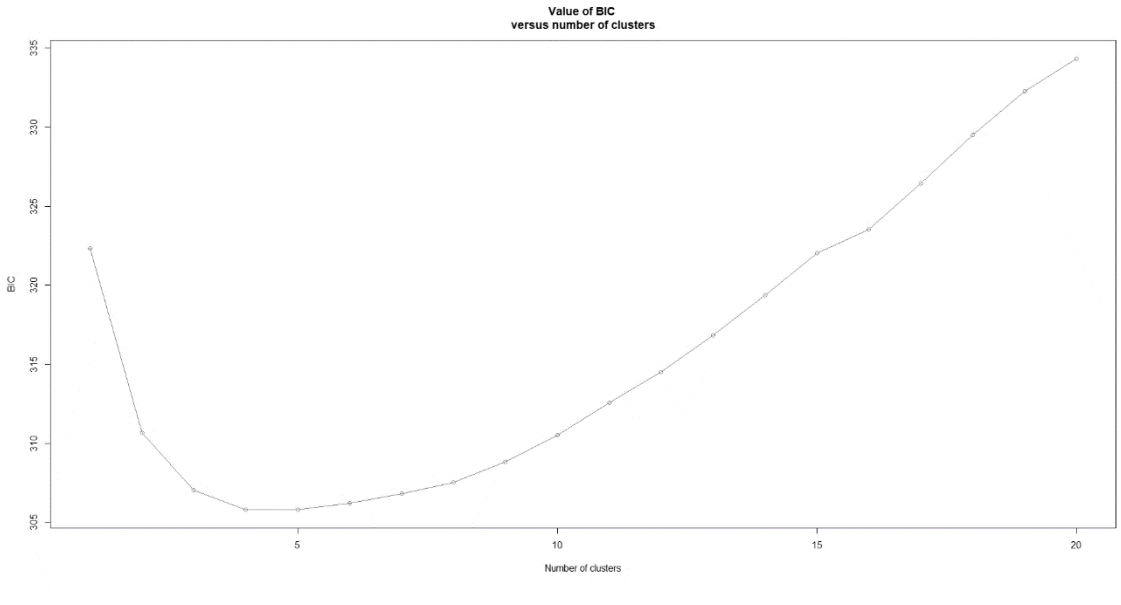

Figure S4. Inference of the number of clusters with function *find.clusters* in package *adegenet 2.1.1*, prior to the discriminant analysis of principal components. Bayesian information criterion (BIC) is provided for every possible number of clusters.

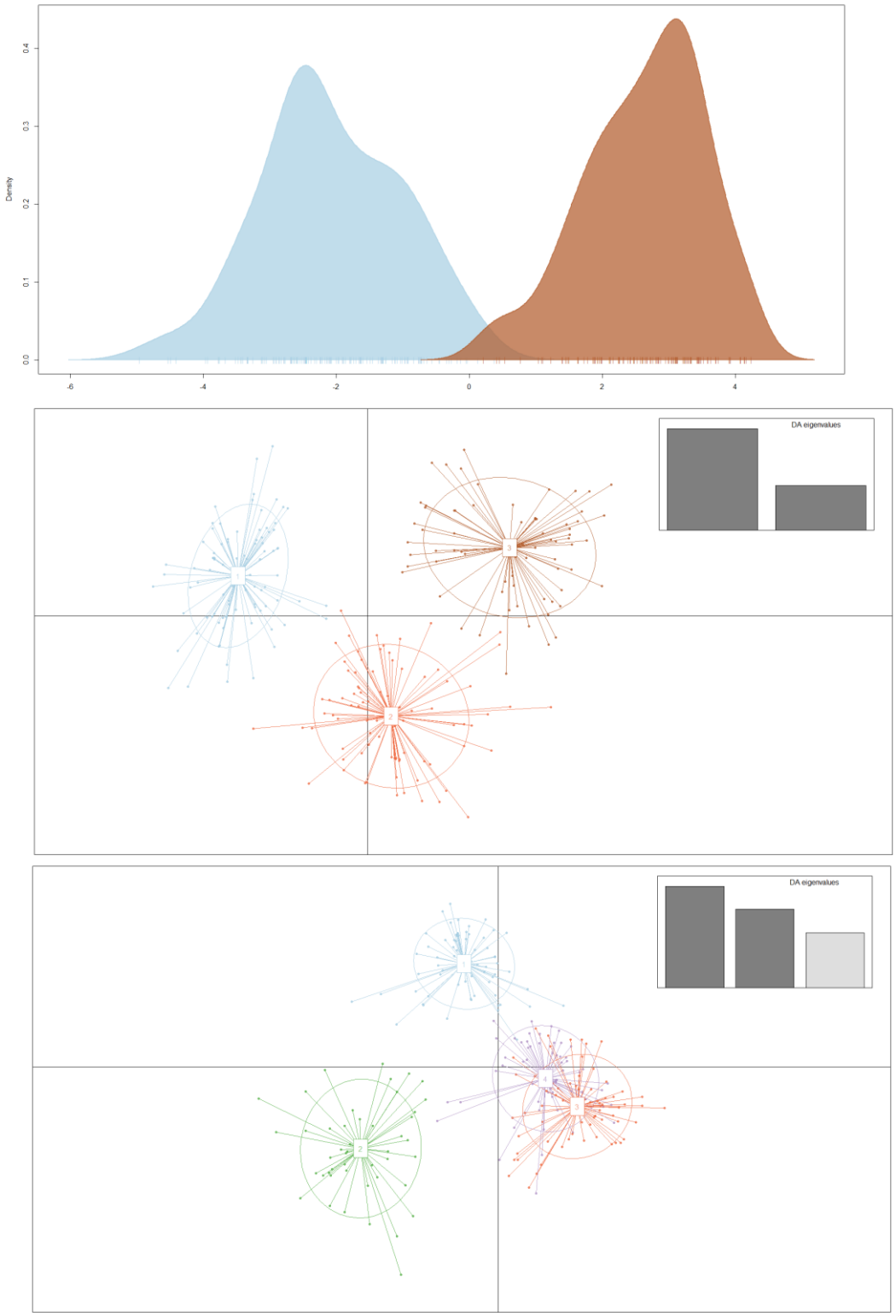

Figure S5. Scatter plot from discriminant analysis of principal components based on *find.clusters* results from K=2 to K=4. Chosen discriminant analysis eigenvalues are depicted in the top right of the plot. Ellipse indicate 95% confidence interval of assignment.

Conclusions

Conclusions

The following conclusions can be drawn from this thesis:

- Microsatellite loci sequences are conserved at the level of the genus *Bombus* and therefore multiplex reactions can be used to obtain genetic diversity parameters, both in wild populations of *Bombus* species such as *B. mesomelas*, and for managed populations of *B. terrestris*.
- The haplotypic diversity found in Iberian populations of *B. t. lusitanicus* by sequencing the fragment of the mitochondrial gene *rnnL* (16S) provides sufficient precision to differentiate the endemic populations of the Iberian Peninsula from the central European populations of *B. t. terrestris* selected as reference.
- A second area of intergradation between *B. t. lusitanicus* and commercial breeds of *B. terrestris* is emerging in the south of the Iberian Peninsula, due to hybridisation and introgression between individuals from native populations and those from greenhouses and breeding companies in the area.
- The information obtained from the mitogenome sequence is useful for taxon discrimination and conservation management of bumblebees, as it has provided the necessary information for the design of sub-specific markers.
- Individuals of *B. terrestris* inhabiting the Iberian Peninsula can be identified by means of a quick and inexpensive test based on the PCR-RFLP technique.
- The distribution ranges of the subspecies are changing as a consequence of both climate change and human actions. In this sense, the most frequent haplotype of the Central European populations of *B. terrestris* is

present in the south of the Peninsula while the most frequent haplotype of B. t. lusitanicus has been found in Normandy (France), which suggests that the Iberian subspecies is expanding its range towards the north.

- The characteristic haplotype of *B. t. terrestris* from central Europe has expanded over the peninsula in the last decades, since the comparison with the temporal reference group shows that in the past its presence was limited to the natural area of intergradation (Pyrenees).
- The effects of introgression on the nuclear genetic diversity of the current populations are not yet relevant, but in the long term they could mean the loss of local adaptations of the populations. Therefore, we advise to legislate the export and use of commercial breeds in the territory, and to increase the dissemination about the correct use of commercial colonies and the potential problems they can cause.