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Interactions between the Genetic Diversity and the Prevalence of Pathogens in Populations of *Apis mellifera iberiensis*

Interacciones entre la Diversidad Genética y la Prevalencia de Patógenos en Poblaciones de *Apis mellifera iberiensis*

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Tesis Doctoral

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Facultad de Biología
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>> CON EL APOYO DE (SUPPORT):

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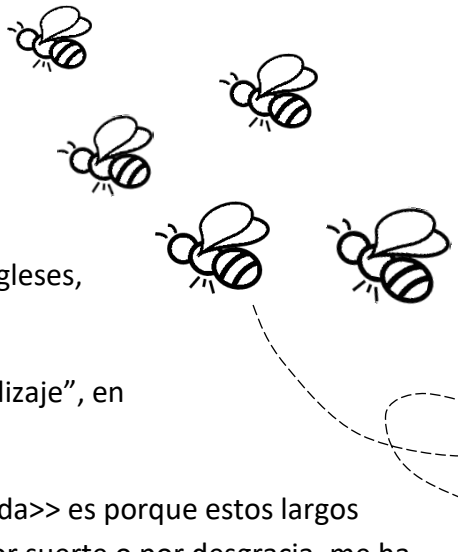
Porque a veces…
<<…lo esencial es invisible a los ojos>>

Le Petit Prince

<<A mi familia,

por haber sido siempre mi apoyo >>



>> Un camino con parada: "La tesis"

"La tesis", todo un ensayo de vida,
un simulacro de lo que aún queda por venir después,
un período de prueba, un *"training"* que dirían los ingleses,
la mili de la ciencia.

Si tuviera que definirla con una palabra sería "aprendizaje", en todos los sentidos de la palabra ¡Vaya si se aprende!

Cuando me refiero a la tesis como <<un ensayo de vida>> es porque estos largos cinco años de tesis han dado para mucho, y ya sea por suerte o por desgracia, me ha tocado vivir muchas cosas; y como en la vida, unas han sido muy buenas y otras, no tanto.

En estos siete años desde que acabé la carrera, cinco de tesis, más dos previos de Másters (en plural, que hay que diversificar las salidas laborales), hemos pasado por una "desaceleración económica", que luego resultó ser una "señora crisis", unas elecciones europeas, un rescate a la banca, la crisis griega, dos mundiales de futbol, dos candidaturas fallidas a los juegos olímpicos, el movimiento 15M, tres elecciones generales, otras tantas elecciones catalanas, cuatro presidentes regionales, dos Papas, la teórica "recuperación económica", la fuga masiva de científicos y jóvenes cualificados de España, el 20% de paro (50% entre los jóvenes), la crisis de los refugiados, la elección de Trump como presidente de EEUU, el *Brexit*, … ¡Y casi mejor no seguir!

En definitiva, un contexto algo convulso en el que decidir hacer una tesis.

En este tiempo hay mucha gente a la que dar las gracias, por todo lo que me llevo, por las enseñanzas, por los buenos ratos y por el apoyo en los malos, por mil y una cosas que han hecho que a pesar del complejo contexto laboral, social y económico, y de las muchas piedras del camino y dificultades, al final "el viaje" haya merecido, y mucho, la pena.

>> Agradecimientos (Acknowledgements)

Como toda buena historia, ésta de la tesis también tiene su inicio, su nudo y su desenlace: el enamoramiento, relación y crisis, y la reconciliación.

"El enamoramiento"

En primer lugar, y como no podía ser de otra manera, me gustaría dar las gracias a Pilar De la Rúa y a Pepe Galián por acogerme en el grupo de investigación y por introducirme y guiarme en mis primeros pasos en el mundo de la ciencia en general, y de la genética en particular. Fue en el último año de carrera cuando, al elegir como optativa, la asignatura de "Técnicas de Ecología Molecular", conocí a Pilar De la Rúa y me volví a

encontrar con Pepe Galián y con sus siempre llamativas historias sobre viajes de muestreo *around the world* (Australia, México, Madagascar, …) en busca de escarabajos. La genética siempre había sido una de mis asignaturas favoritas dentro de la biología y me llamaba la atención todo lo relacionado con ella, así que ya estaba la mitad del trabajo hecho. Las historias sobre los viajes y el buen *feeling* personal con Pilar y Pepe hicieron el resto. "Genética, buen ambiente de trabajo, muestreos de campo, ciencia y viajes"… ¡¿Qué más se podía pedir?! ¡Ya me tenían ganada! Así que decidí hacer mi proyecto fin de carrera (PFC) con ellos, y con éste inicié, sin darme mucha cuenta, el camino hacia la tesis ¡Gracias a los dos por esos inicios! Además gracias también a Pilar por ofrecerme seguir trabajando juntas y por ser primero mi tutora de tesina y luego también de tesis, además de compañera de viajes, amiga y a veces, hasta una madre.

Con el PFC me inicié en el mundo de la ciencia y en mi caso concreto también, en el fascinante mundo de "las abejas". Aprendí sobre su organización en castas, su peculiar comportamiento de "superorganismo" y sus amenazas. Conocí a *Varroa destructor*, a *Nosema*, al pollo escayolado (así como suena), a los virus y tantos otros patógenos que atacan a las abejas…; también visité por primera vez un colmenar, descubrí lo que eran las PCRs a tiempo real o qPCRs, los endógenos, las curvas de *melting*, el *RNA later*, el *SYBRGreen*, y como no, también los resultados negativos, los no significativos, las PCRs que no salen y sobre todo, que en la palabra "paciencia" está incluida la palabra "ciencia", y no se puede trabajar en la segunda si no se trabaja antes la primera.

En esta etapa me gustaría dar las gracias a Juan José Quereda, a Guillermo Ramís y a Cesar Flores por su inestimable ayuda con las qPCRs, que no salían; pero sobre todo a Obdulia por acompañarme en ese camino y porque con ella aprendí casi todo lo que sé de laboratorio hoy en día y otras tantas cosas sobre la vida. Gracias por su paciencia con los principiantes y con los pequeños desastres de laboratorio. Gracias por su cercanía, su humor, los viajes de muestreo y los de placer, por los mil contactos a los que acudir cuando estás perdido en algún tema y en definitiva, por su amistad durante todo este tiempo y por esa fuerza que transmite y que tanto inspira a los que la conocemos y estamos cerca.

Gracias también a Anita por esa empatía infinita y esa calidez, por su ayuda en el laboratorio en momentos de estrés y por todo el cariño, el apoyo y la comprensión en los momentos malos, y por las alegrías compartidas en los buenos. Gracias en definitiva a todos los compañeros del Área de Biología Animal por ser "una gran familia" y por hacerme parte de ella.

"Relación y crisis"

Tras el PFC, vino la tesina, los dos Másters y un camino algo más cuesta arriba debido a la situación económica y laboral en España y la precariedad en el mundo de la ciencia. En esta etapa aparecieron en escena las promesas de un contrato que nunca llegaba,

primero por el cambio de gobierno en 2012 y la ya si “CRISIS” con todas las letras, luego por la deuda regional, y más tarde por la demora, de casi dos años, del pago del proyecto por parte de la empresa colaboradora. También vinieron los recortes en investigación, las becas que nunca se convocaron, las que se convocaban un año tarde y se resolvían con casi otro año de retraso, y las becas, que después de concedidas, resultaron tener que ser para otras personas.

En definitiva, un tramo del camino pedregoso y de mucha pendiente, que como tantos otros en la vida, me puso a prueba y que en alguna que otra ocasión me hizo plantearme si no habría errado la senda. Ésta fue la etapa de la tesina por las mañanas, los Másters por la tarde, las prácticas y estudio en las horas libres, la época de depender económicamente de mis padres y también de los fines de semana y veranos de echar *currículums* y de reinventarse en días sueltos como niñera, profesora de clases particulares, monitora infantil, camarera, captadora de socios, etc., etc., etc.

Como en toda etapa dura, se pasan momentos difíciles, de crisis laboral, personal y casi existencial, pero la parte positiva es que de esos malos tragos se aprende muchísimo (gracias a Silvia Congost, Pequi Jordà y Marina Díaz por sus lecciones de vida a este respecto), y se sacan también grandes amistades. En este punto no puedo más que dar las gracias a toda esa gente que tanto me ha animado y apoyado en estos años y a las fuerzas que sea que rigen este mundo por cruzar en mi camino tantísima gente estupenda y tan valiosa para mí. Gracias a los que estuvieron en los inicios, a los que aparecieron para quedarse, y a los que siempre han estado y que siguen estando ahí.

De este último grupo me gustaría dar las gracias a toda mi familia y muy en especial a mis padres por su enorme apoyo en los momentos más complicados y decisivos y por todo el cariño recibido. También a mi hermano por su sentido del humor y por meterme caña cuando más falta me ha hecho. Gracias a mis abuelos por ser un ejemplo a seguir, a Fran y Caro por sus consejos y su ayuda con la estadística y a toda la familia por el cariño, por los ánimos y por su apoyo incondicional.

Por supuesto no puedo dejar de dar las gracias también a mis amigos por tantos años de amistad, por los buenos ratos que hacen olvidar los malos, por haberme aguantado mis historias sobre abejas, mis penurias con los contratos, mis crisis existenciales… Gracias a mis niñas (Dori, Mª José, Paqui, Ana Cristina, Marina y Mari Cruz) por estar ahí, en algunos casos, a pesar de la distancia. Gracias por las “conejadas” compartidas, por los viajes, y los buenos ratos. Gracias a Juanfran por ser amigo y confidente desde casi que nos salieran los dientes, a Martínez por sus bromas, a Luis por las batallas compartidas y la confianza (que ya sabes lo que dicen de ella xD), a Salva por la estadística y los buenos y malos ratos compartidos en estos años de tesis.

De mi grupo de Calabardina, gracias a mi Anita por ser como una hermana, por esa energía positiva que derrocha, por las lecciones de vida a pesar de ser la pequeña de las

dos y por acordarse de mí cada vez que ve algo sobre abejas o que le toca estudiarse las leyes que regulan la propiedad de los enjambres ;P. Gracias también a Miriam, por tantos años de hacer de consejera espiritual y a veces psicóloga, y por su risa contagiosa, que tantos buenos ratos da. Gracias a todo el grupito de "Cala" (Jay, Deby, Seta, Carlos, Vicente, Vicky, …) por tantos grandes veranos.

"La reconciliación"

Como dicen que después de la tormenta siempre llega la calma, en este caso por fin llegó, en forma de contrato para los últimos años de tesis. Con el contrato vino una etapa mucho más amable, en la que por fin podía centrarme casi en exclusiva en la tesis, sin tener que estar buscándome la vida con otras mil cosas a la vez. Digo "casi" porque aún estaban los abejorros y su alimento alternativo.

Aquí quiero dar las gracias a mucha gente cuya ayuda ha sido básica para que al final, yo pudiera llevar a buen fin esta tesis. Entre ellos, gracias a Gheri por aparecer en el momento en el que más falta me hacía y por su inestimable ayuda en mi lucha a oscuras con los abejorros ¡No sé qué hubiera hecho sin ti! Mil gracias también a Fuensanta Hernández y Josefa Madrid del departamento de Producción Animal de la Universidad de Murcia, por toda su ayuda con los análisis de alimentos y por su cercanía. En mi buceo por el genoma de la abeja quiero dar las gracias a Dora Henriques, Julio Chávez y Alice Pinto del Instituto Politécnico de Bragança (Portugal), por ayudarme a resolver mis dudas en la búsqueda de genes bajo selección ¡Obrigada! También a Antonio Franco y a su hijo por la ayuda con las colmenas y las muestras, y a tantos otros que quizá me deje en el tintero y que también debieran estar en estas líneas. Gracias a Meli del Centro de Investigación Apícola de Marchamalo (Guadalajara) por los análisis del polen y el tiempo dedicado; y por supuesto gracias a Raquel Martín y a Mariano Higes del Departamento de Patología Apícola del mismo centro, por ser cómplices directos de la mayor parte de los artículos de esta tesis; también a las técnicos: Tere, Virginia, María y Mª Carmen por estar siempre dispuestas a ayudar; a Luis por echarme una mano en mi última semana intensiva allí; a Almu Cepero por ser una amiga y mi guía turística oficial en Guadalajara; y a todo el resto del equipo (Javi, Cris, Almudena, María, Sol,...) por su cálida acogida en mis recurrentes estancias en el centro apícola.

Además de estas estancias en Marchamalo, a lo largo de la tesis también he tenido la oportunidad de realizar una estancia de tres meses en la Universidad de Gante (Bélgica). *I want to thank Professor Guy Smagghe and the University of Ghent for inviting me, as well as to the people from the laboratory of Agrozoology for the nice welcoming. In especial, I would like to thank Niels Piot and Ivan Meeus for their help in the laboratory. I also want to thank the wonderful group of friend that I met in Ghent. Thanks to Marta Hernández, Katty Prentice, Dora Quispe, Ruben Verbeek, Clauvis Taning, Kleber Pereira, Cintia Mosquera, Haidong, Wimbledon, Gosia, Gisel, Henok, … and many others for making my stay in Ghent much more fun, enjoyable and enriching. Thank you all!*

Ya estamos llegando al final de este camino con última parada “La tesis”, un viaje que aunque complicado a veces, al final ha resultado ser muy gratificante.

En esta última etapa de la tesis han aparecido algunos nuevos personajes en escena, pero no por ello menos importantes. Gracias a Mª José y Caty por los meses de convivencia juntas, y a mis dos soletes Lore y Palo por haberse convertido en muy poco tiempo en dos personas súper importantes para mí. Gracias por los buenos ratos y por ser como sois. Estoy encantada de haberme cruzado con vosotras en el camino y espero que sigáis estando en él por mucho tiempo. También quiero dar las gracias a alguien, que aunque ya estaba, ha tomado un protagonismo especial en esta última etapa de tesis. Gracias Alberto por ser como eres, por quererme como soy y por ser el mejor compañero de viaje que se puede pedir. Gracias por los ánimos, por el apoyo, por las maratones de trabajo hasta las tantas y por tu empeño en hacerme este camino más ameno consiguiendo hacerme reír cada día con ese humor tan tuyo ¡Gracias compañero por querer ser el 50% de este equipo!

Y ahora ya sí, gracias a esa “gran familia” que forma el grupo de investigación de Filogenia y Evolución Animal, que ha sido mi segunda casa (aunque por el número de horas podría ser la primera) y que tantísimo me ha dado en estos 7 años desde que acabé la carrera. Gracias al cabeza de familia, Pepe Serrano, por ser un ejemplo a seguir, por haber iniciado esta gran familia y sobre todo por su humanidad y calidad como persona. Gracias también por aceptar ser mi co-tutor de tesis, con todo lo que eso conlleva, y por ayudar a dar forma a esta tesis. A Pilar y a Pepe (“los papis” del grupo), por transmitirnos a todos la pasión por la ciencia y por tratar con la “persona” antes que con “el doctorando”. A Ana y Odu porque sin ellas aún estaría con la tesis. A Carlitos e Irene por su papel de “hermanos mayores”, por sus consejos, sus ánimos y por toda su ayuda y colaboración en esta tesis. A Javi y Alejandro por abrir el camino para los que venimos detrás. Cómo no, a mis chotonis, porque qué habría sido de este viaje sin vosotras Gracias Juli y Clau por vuestra amistad y por tantas risas y penas compartidas ¡Me llevo dos joyitas! Gracias también a los que vienen detrás pisando fuerte. Alberto (y sus chistes malos), Andrés (gran filósofo y pensador), Vicente (y sus maldades), Diego (mi pequeño aprendiz de conejo)…; a los grandes fichajes extranjeros (William, Nora, Vilmar, Gheri, Matteo, …) y a todo el resto de compañeros que han formado o forman parte de este gran equipo (Raquel, Miguel, Loli, Carmelo, Fermín, Jose Manuel, Víctor, Nuria, José,…) por el buen ambiente de trabajo, el apoyo, el compañerismo, las ganas siempre de ayudar y los buenos ratos y las risas, que han hecho mucho más ameno y llevadero todo este camino.

¡Gracias a todos por haber formado parte de esta aventura!

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Resumen

(Spanish summary)

Resumen (español)

La abeja de la miel *Apis mellifera* Linneo 1758 es uno de los insectos más beneficiosos del mundo, no solo por la producción de miel, sino también por su papel como principal polinizador de cultivos y especies silvestres entomófilas a nivel mundial (Klein et al., 2007; Aizen et al., 2009). La historia evolutiva de esta especie presenta una notable complejidad debido a la intervención tanto de factores naturales como de otros antrópicos, ya que se trata de una especie manejada desde hace siglos. En la actualidad, se conocen alrededor de 31 subespecies de *A. mellifera* y numerosos ecotipos (Ruttner, 1988; Engel, 1999; Sheppard y Meixner, 2003; Chen et al., 2016), que se han agrupado en base a datos morfométricos, genéticos, ecológicos, fisiológicos y de comportamiento (revisión en De la Rúa et al., 2009b) en cuatro linajes evolutivos principales: Africano (A), Europeo Occidental (M) y Oriental (C), y de Oriente Medio (O) (Ruttner et al., 1978; Ruttner, 1988; Garnery et al., 1992; Arias y Sheppard, 1996; Franck et al., 2000; Miguel et al., 2011). El origen de estos linajes se ha situado en Asia, donde se cree que se habría originado *A. mellifera* (Ruttner et al., 1988; Han et al., 2012; Wallberg et al., 2014), expandiéndose más tarde a Europa y África; aunque aún existe controversia en torno al origen de esta especie (Ruttner, 1988; Cornuet y Garnery, 1991; Whitfield et al., 2006; Han et al., 2012; Wallberg et al., 2014; Cridland et al., 2017).

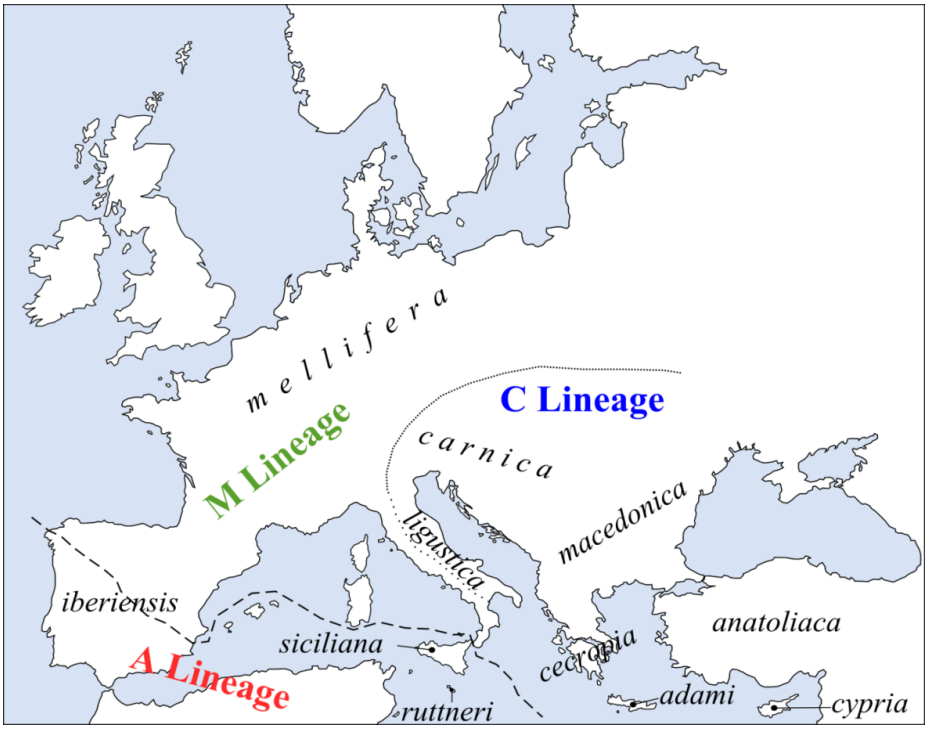

Fig.1. Distribución de los linajes evolutivos y subespecies de* Apis mellifera *en Europa (tomado de De la Rúa et al., 2009b).

En la península Ibérica se dispersan de forma natural los linajes evolutivos A y M. Diferentes estudios basados en enzimas, feromonas, ADN mitocondrial, microsatélites y SNPs (De la Rúa et al., 2009a; Chávez-Galarza et al., 2015, 2017) apoyan la hipótesis planteada previamente mediante estudios de morfometría y comportamiento (Engel, 1999), de que las poblaciones de abeja ibérica de la miel, *A. m. iberiensis* Engel 1999, son el resultado de una amplia integración entre abejas del linaje M que sobrevivieron al último máximo glacial y abejas norteafricanas del linaje A que recolonizaron el sureste europeo (Serrano et al., 2011) (Fig. 1.). La hibridación entre abejas de ambos linajes habría dado lugar a una distribución clinal de las poblaciones, con una disminución de la frecuencia del linaje A desde el SO al NE peninsular, mientras que la frecuencia del M aumenta (Cánovas et al., 2008; De la Rúa et al., 2009b).

La estructura poblacional de la abeja de la miel refleja, no solo su historia evolutiva sino también las adaptaciones concretas a las distintas áreas biogeográficas que ocupa. Esta diversidad genética es fundamental para mantener en buen estado las poblaciones, ya que les permite resistir a corto plazo perturbaciones debidas a cambios ambientales y/o presencia de patógenos, y evolucionar en respuesta a estos cambios a mayor escala (Frankham et al., 2010). En este sentido, la distribución natural de *A. mellifera* en la península Ibérica ha estado probablemente influida en las últimas décadas por las técnicas apícolas. La práctica intensiva de la trashumancia (que afecta ya al 80% de las colonias), la compraventa de colonias a escala trans-regional y sobre todo, la pérdida masiva de colonias por la expansión de enfermedades como la varroosis, la nosemosis y otras patologías, están cambiando de forma acelerada el citado patrón clinal (Cánovas et al., 2011).

Durante las últimas décadas, la pérdida de colonias en todo el mundo ha aumentado la preocupación y la sensibilización pública sobre el futuro de las abejas, siendo muchos los esfuerzos que se han dedicado a cuantificar la incidencia y discutir las causas de esta pérdida (Cox-Foster et al., 2007; Coloss, 2009; Giray et al., 2010; EFSA, 2008). Un caso paradigmático ha sido el del conocido como Síndrome del Colapso de las Colonias (SCC), una pérdida inexplicable de colonias de abejas de la miel, que se ajusta a unos criterios definidos (Cox-Foster et al., 2007; vanEngelsdorp et al., 2009). Hasta la fecha, una de las variables más significativa que se ha relacionado con la pérdida masiva de abejas en Europa y con el SCC en EEUU, es precisamente el incremento en la incidencia de parásitos y patógenos de la abeja de la miel (vanEngelsdorp et al., 2009). Entre los agentes patógenos actuales más perjudiciales para las abejas de la miel se encuentran el ácaro *Varroa destructor* y las enfermedades víricas asociadas a este ácaro, así como los microsporidios del género *Nosema* (Higes et al., 2006; Cox-Foster et al., 2007; Rosenkranz et al., 2010; de Miranda y Genersch, 2010; Johnson et al., 2009). Sin embargo, el conocimiento que se tiene de estos organismos patógenos y de sus interacciones con el hospedador *A. mellifera* es aún insuficiente (Martinson et al., 2011).

V. destructor es un ectoparásito obligado de *A. mellifera* que se alimenta de la hemolinfa tanto de abejas inmaduras como de adultas y se reproduce en las celdillas del panal que contiene a la cría (Sammataro et al., 2000). Tiene su origen en Asia, donde parasita a su hospedador original, la abeja asiática *Apis cerana* Fabricius 1793. Las colonias infectadas sufren tanto daños directos por el parasitismo, como indirectos por los patógenos víricos y microbianos de los que el ácaro es vector (Johnson et al., 2009; Highfield et al., 2009). Además *V. destructor* induce una inmunosupresión en las abejas, lo cual da lugar a una amplificación de las infecciones pre-existentes (Yang y Cox-Foster, 2007), agravando aún más su impacto. El salto de hospedador de *A. cerana* a *A. mellifera* se produjo hace unos 50 años tras la introducción de la abeja occidental en Asia (Rosenkranz et al., 2010). Debido al comercio de abejas y productos apícolas al nivel global, en la actualidad el ácaro se ha expandido por colonias de todo el mundo. En España fue detectado por primera vez en la frontera con Francia en diciembre de 1985 y desde entonces se ha expandido por el país, causando la muerte de aproximadamente el 40% de las colonias españolas es sus primeros años de incidencia (Llorente, 2003).

***Fig.2.* V. destructor *sobre una pupa de abeja (izqda.) y una abeja obrera adulta (dcha.). Fuentes:* https://i0.wp.com/www.blythewoodbeecompany.com/wp-content/uploads/Varroa-destructor-reproductive.jpg *y* http://wildlife.org/wp-content/uploads/2016/10/bayer.png**

Los efectos letales de *V. destructor* sobre las colonias se atribuyen en buena parte a la propiedad del ácaro para activar y transmitir un buen número de enfermedades víricas (Ball y Allen, 1988; Bailey y Ball, 1991; Bowen-Walker et al., 1999; Sumpter y Martin, 2004; Chen y Siede, 2007; Tentcheva et al., 2006; Todd et al., 2007). El virus de las alas deformadas (DWV) es uno de los virus más importantes que infectan a las abejas y uno de los más estudiados en relación con el colapso de las colonias inducido por *V. destructor* (Ball, 1983; Ball y Allen, 1988; Bowen-Walker et al., 1999; Nordström et al., 1999; Ribière et al., 2008; Sumpter y Martin, 2004; Tentcheva et al., 2004; de Miranda y Genersch, 2010). Este virus fue aislado por primera vez en abejas adultas enfermas de Japón (Bailey y Ball, 1991), y debido a su asociación con el ácaro *V. destructor*, hoy día aparece distribuido al nivel global (Allen y Ball, 1996; Ellis y Munn, 2005; Ribière et al., 2008).

En ausencia de *V. destructor*, DWV aparece en niveles bajos en las colonias y no causa impactos negativos evidentes sobre ellas. Por el contrario, la inyección directa de partículas del virus en el hemocele de las pupas de abeja a través del parasitismo por *Varroa*, permite que se desarrolle la infección por DWV, apareciendo en las colmenas los síntomas típicos de la enfermedad, como la emergencia de abejas con alas deformadas, abdomen acortado, un tamaño disminuido, y en ocasiones también decoloración y parálisis (Genersch y Aubert, 2010) (Fig. 3). Estas malformaciones provocan la muerte prematura de las abejas (Yang y Cox-Foster, 2007) y un menor rendimiento de éstas en la colonia, lo que afecta finalmente al desarrollo normal de la misma si la infección se extiende.

Fig.3. Abeja con alas deformadas.
Fuente: ***https://www.flickr.com/photos/klaasdegelder/5137860060***

En la actualidad, la estructura poblacional del ácaro *Varroa* en relación con su hospedador original *A. cerana*, ha sido estudiada mediante marcadores moleculares como el ADN mitocondrial (Warrit et al., 2006) y los marcadores nucleares microsatélites (Ruepell et al., 2011). Los resultados sugieren que la distribución actual de los linajes genéticos de *Varroa* y de *A. cerana* (determinados mediante el análisis de la variación de regiones específicas del cromosoma mitocondrial) es una consecuencia de la historia biogeográfica y de patrones actuales de migración más que de una posible co-evolución parásito-hospedador. En el caso de que esta última hipótesis hubiera sido confirmada, se habría observado una correspondencia tanto de los haplotipos mitocondriales como del genoma nuclear (microsatélites) entre los linajes del ácaro y los de la abeja hospedadora.

En cuanto a la relación del ácaro con su nuevo hospedador *A. mellifera*, no se ha realizado hasta la actualidad ningún estudio extenso. Tan sólo en la península Ibérica se

ha determinado mediante caracterización del cromosoma mitocondrial, el perfil genético del ácaro presente en 575 colonias (Muñoz et al., 2008), observándose la presencia de un haplotipo denominado Corea (más virulento) en todas las colonias analizadas menos una. Se determinaron también los haplotipos mitocondriales de las abejas del apiario donde se detectó un único haplotipo denominado Japón (menos virulento), no observándose ninguna relación significativa en la distribución de linajes del parásito y del hospedador.

El contacto entre las dos especies de abejas, *A. cerana* y *A. mellifera* pudo haber sido también la causa de la expansión de los microsporidios. Estos parásitos han sido reclasificados en 2005 como hongos (Sina et al., 2005).y se transmiten por esporas; son parásitos intracelulares obligados que infectan el epitelio del ventrículo de las abejas. El transporte de *A. mellifera* a lo largo de todo el mundo se asocia con la dispersión de *Nosema apis* Zander 1909, el microsporidio que históricamente ha infectado a la abeja de la miel. Sin embargo a partir de 1996, una nueva especie denominada *N. ceranae* fue descrita en su hospedador asiático *A. cerana* (Fries et al., 1996). Inicialmente se creía que esta especie estaba restringía a la abeja oriental y geográficamente limitada a Asia, pero recientemente se ha detectado infectando a *A. mellifera* tanto en España como en Europa y el resto del mundo (Higes et al., 2010). Se trata por tanto, de un nuevo agente en un nuevo hospedador, el cual se ha planteado si podría estar desplazando al parásito original, ya que los últimos estudios realizados muestran actualmente una mayor prevalencia de *N. ceranae* que de *N. apis* en *A. mellifera,* aunque ambas se encuentran ampliamente distribuidas (Higes et al., 2006; Klee et al., 2007; Paxton et al., 2007).

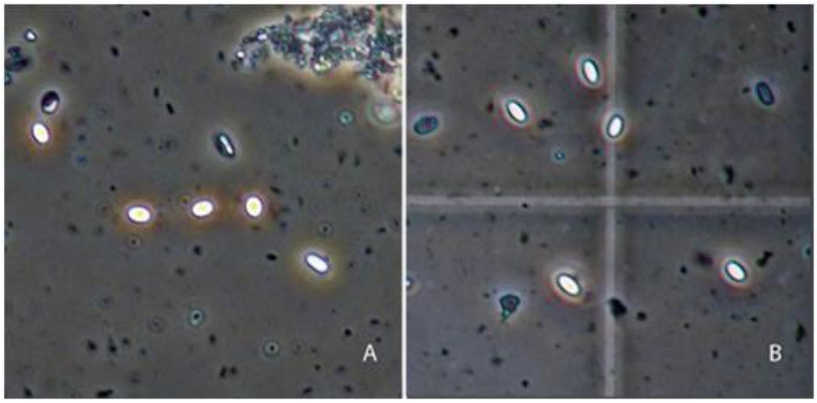

Fig.4.* Nosema apis *(A) y* Nosema ceranae *(B) vistas al microscopio óptico. (Foto: W. F. Huang).

La prevalencia y la abundancia de las especies de *Nosema* (Fig. 4) tanto en las colonias como en las abejas individuales, pueden estar influidas por múltiples factores incluyendo la variación genética del hospedador, el clima, los niveles de exposición y los tratamientos realizados (Runckle et al., 2011). En ese sentido los estudios sobre las

relaciones parásito-hospedador son escasos al igual que en el caso de la varroosis descrito anteriormente. Únicamente se ha hecho mención a la caracterización molecular (mediante el análisis del ADN mitocondrial, Solorzano et al., 2009) de las poblaciones de abejas hospedadoras, en dos trabajos recientes sobre la detección molecular de *N. ceranae* y *N. apis* en abejas de Turquía (Whitaker et al., 2011) y de los países balcánicos (Stevanovic et al., 2011), pero en ninguno de ellos se precisó con detalle la existencia de algún tipo de relación significativa entre la infestación por las dos especies de *Nosema* y los linajes genéticos de las abejas.

La extensa dispersión de este nuevo parásito, no solo por España sino también al nivel mundial (Fries, 2010), sugiere un exitoso proceso de colonización y una reducida adaptación del nuevo hospedador *A. mellifera*. *V. destructor* y los microsporidios del género *Nosema*, principalmente *N. ceranae,* se encuentran entre las especies invasoras actuales más perjudiciales para las abejas de la miel; y sin embargo, el conocimiento que se tiene sobre estos organismos patógenos y de sus interacciones con el hospedador *A. mellifera*, es aún escaso e insuficiente (Martinson et al., 2011).

En ese sentido la península Ibérica proporciona un interesante escenario para analizar las relaciones de los parásitos con las poblaciones de abejas hospedadoras, debido entre otros factores, a la presencia de dos linajes evolutivos de *A. m. iberiensis* y a la notable diversidad genética de sus poblaciones (Cánovas et al., 2008; De la Rúa et al., 2009b).

De todo lo anterior, se deriva el **objetivo principal de esta tesis**, que es estudiar las interacciones entre la diversidad genética de *A. m. iberiensis* en España y la prevalencia de los parásitos y patógenos más dañinos que afectan a las colonias de abejas melíferas.

Con el fin de lograr este objetivo, la presente tesis se ha dividido dos partes, las cuales constan de dos capítulos cada una:

<u>Parte 1</u>: Relación entre la diversidad genética y la prevalencia de patógenos en colonias de *A. m. iberiensis*

- ***Capítulo I: Relacionando el linaje evolutivo con la prevalencia de parásitos y patógenos en la abeja ibérica***

  La reciente disminución de colonias de abejas de miel, observada en los países europeos y en todo el mundo, es de gran interés y preocupación, aunque las causas subyacentes siguen siendo poco conocidas. En los últimos años, ha sido cada vez más evidente la implicación de parásitos y patógenos en esta disminución de la vitalidad y el número de colonias de abejas. La península Ibérica ofrece un entorno interesante en el que estudiar la distribución de patógenos y parásitos en las poblaciones de abejas debido a la presencia de los

dos linajes evolutivos de *A. m. iberiensis* (europeo occidental [M] y africano [A]). En este capítulo inicial de la tesis se presenta la primera evidencia que vincula la estructura poblacional de la abeja ibérica con la prevalencia de algunos de sus parásitos y patógenos más importantes: el ácaro *Varroa destructor* y los microsporidios *Nosema apis* y *Nosema ceranae*. A partir de abejas procedentes de dos muestreos realizados en 2006 y 2010 en 41 provincias españolas, se detectó el linaje evolutivo y la presencia de los tres organismos patógenos citados anteriormente en un total de 228 colonias. En 2006, *N. apis* fue detectada en una proporción significativamente mayor en abejas del linaje M que en abejas del linaje A, discutiéndose en el presente trabajo las posibles causas de esta tendencia. No obstante, en 2010 esta situación cambió significativamente debido a una mayor prevalencia de *N. ceranae*. No se observaron relaciones significativas en ninguno de los dos años muestreados entre las distribuciones de *V. destructor* o *N. ceranae* y el linaje evolutivo presente en las colonias de *A. m. iberiensis*. Se desconocen los posibles efectos de estos organismos sobre la diversidad genética de las poblaciones melíferas, lo cual requiere de estudios específicos.

- ***Capítulo II: Diversidad genética estable a pesar de la propagación de parásitos y patógenos en colonias de abejas melíferas***

  En las últimas décadas, la rápida propagación de enfermedades como la varroosis y la nosemosis, asociadas con la mortalidad masiva de colonias en todo el mundo ha reducido significativamente el número y el tamaño de las poblaciones de abeja melífera, y posiblemente su diversidad genética. Aquí se compara la diversidad genética de las colonias de abejas ibéricas en dos muestreos realizados en 2006 y 2010 en relación a la presencia de los agentes patógenos *Varroa destructor, Nosema apis* y *Nosema ceranae* para determinar si la propagación de parásitos y patógenos en colonias de abejas melíferas refleja cambios en la diversidad genética de éstas. En este segundo capítulo, se determinó que la diversidad genética se mantuvo similar, mientras que la incidencia de *N. ceranae* aumentó y la incidencia de *N. apis* y *V. destructor* disminuyó ligeramente. Estos resultados indican que la diversidad genética de las poblaciones de abeja melífera en España, no se ha visto afectada por la presencia de estos agentes patógenos en el período analizado. Sin embargo, los dos grupos de colonias, con y sin los patógenos *Varroa* y *Nosema,* mostraron diferencias genéticas significativas (prueba G). El análisis pormenorizado de la segregación alélica de *loci* de microsatélites en colonias negativas y positivas a la parasitación por *Varroa* y *Nosema* reveló dos *outlier loci* relacionados con genes implicados en la respuesta inmune de las abejas.

<u>Parte 2</u>: Efecto de las prácticas apícolas sobre la diversidad genética y la dispersión de patógenos en las colonias de abeja ibérica

- ***Capítulo III: Efecto de los movimientos migratorios a pequeña escala en la dispersión del hongo* Ascosphaera apis *en colonias de* Apis mellifera iberiensis**

  Ascosphaera apis es el agente causal de la enfermedad de la cría encalada o pollo escayolado, una patología que, aunque menos grave que otras en las colmenas, afecta a las larvas de abejas y que perjudica significativamente el crecimiento de la población y la productividad de las colonias. En este estudio, se detectó la presencia de *A. apis* en abejas obreras adultas a partir de la amplificación por PCR del espaciador interno transcrito (ITS1) del gen ribosomal (rDNA). La extracción de ADN se optimizó a partir del testado de diferentes protocolos de extracción en abejas adultas individuales y en *pool* (a nivel de colonia). Posteriormente, se evaluó la presencia del hongo *A. apis* en colonias estacionarias y migratorias (sometidas a movimientos a pequeña escala al nivel regional) para determinar el efecto de las prácticas migratorias sobre la dispersión de este patógeno. Los resultados confirmaron una correlación positiva entre la apicultura migratoria y una mayor prevalencia de *A. apis*, lo que indica que las colonias migratorias son más propensas a desarrollar la enfermedad de la cría encalada. Teniendo en cuenta estos resultados, sugerimos que los apicultores deben ser conscientes de los riesgos de propagación de patógenos durante los movimientos de colmenas, incluso cuando éstos se realizan a una escala geográfica reducida.

- ***Capítulo IV: El efecto de la apicultura migratoria en la prevalencia de patógenos en las colonias de abejas melíferas y en su composición genética***

  El manejo apícola migratorio es una práctica ampliamente extendida destinada a aumentar el rendimiento de los productos de la colmena y de los servicios de polinización. Sin embargo, esta práctica representa un factor de estrés para las colonias, lo que afecta al funcionamiento de las mismas y favorece la propagación y proliferación de enfermedades. En España además, esta práctica supone también un factor de homogeneización genética para las poblaciones de abejas ibéricas. Para analizar el alcance de estos efectos, se realizó un seguimiento del estado de salud y la composición genética de las colonias en un experimento de campo comparando colonias estacionarias y migratorias pertenecientes a apicultores aficionados y profesionales, compartiendo las mismas condiciones ambientales pero diferenciándose en el tipo de manejo (estacionario vs. migratorio), y con un origen o base genética diferente. El diseño experimental de este cuarto capítulo de tesis comprende el estudio de la prevalencia de importantes patógenos de las abejas (*Varroa destructor*, *Nosema*

spp. y el DWV) en cuatro momentos diferentes: previo a la operación apícola migratoria, dos semanas después del transporte de las colmenas, al final del período migratorio, y dos semanas después del regreso de las colmenas migratorias a su lugar de origen. En este trabajo se detectó una incidencia creciente de *V. destructor* y *N. ceranae*, aunque no de DWV, en las colonias migratorias. También se detectaron cambios temporales en la diversidad genética, aunque independientes del grupo de colmenas (migratorias vs. estacionarias), lo que sugiere un posible efecto de otros factores de estrés distintos al tipo de manejo sobre la diversidad genética individual de las colonias.

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Introduction

(Introducción)

1. The species *Apis mellifera* Linnaeus 1758

The European honey bee, *Apis mellifera* L. belongs to the order Hymenoptera, family Apidae. This family comprises about 40,000 species of bees (Michener and Charles, 2000), including the bees from the genus *Apis*, or honey bees. *Apis* bees have attracted wide interest because of the products they provide (honey, wax, pollen, propolis), their importance for pollination of crops and their social organization. The genus *Apis* is constituted by a total of ten recognized species: *A. florea, A. andreniformis, A. dorsata, A. laboriosa, A. binghami, A. nigrocincta, A. cerana, A. koschevnikovi, A. nulensis* and *A. mellifera* (Engel, 1999; Arias and Sheppard, 2005) (Fig 1). Among these species, the European honey bee *A. mellifera* is the most commonly managed species and therefore, it is worldwide distributed.

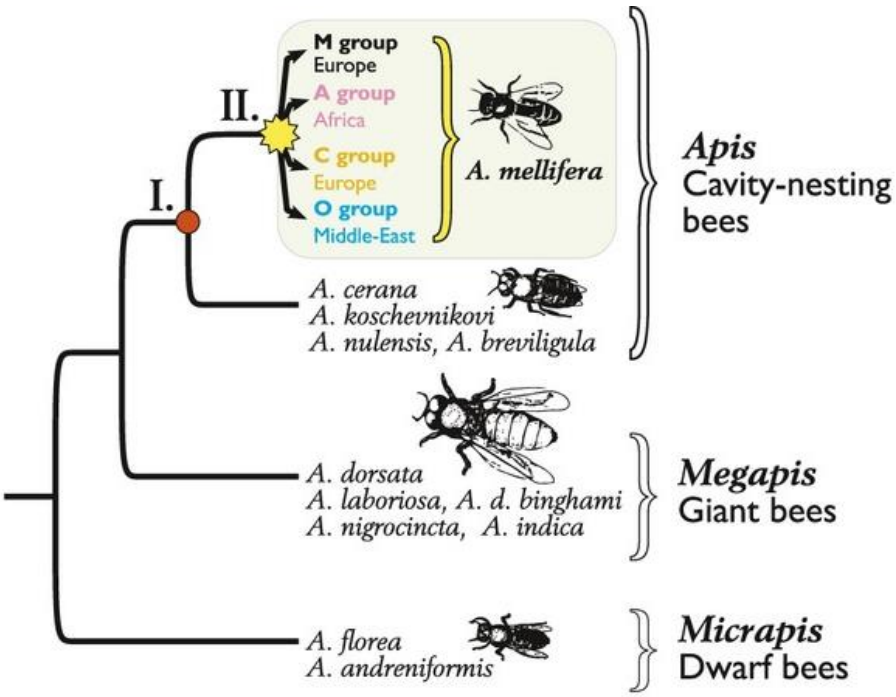

Fig. 1. Evolutionary relationships of* Apis bees *(from Han et al., 2012). Node I represents the split between* A. mellifera *and other cavity-nesting bees. Node II represents the most recent common ancestor of extant subspecies of* A. mellifera*.

The adaptability and the high variability of this species has allowed it to occupy an extensive geographic area and to show plasticity in its interaction with humans. To date, at least 31 subspecies of *A. mellifera* (Ruttner, 1988; Engel, 1999; Sheppard and Meixner, 2003; Meixner et al., 2011; Chen et al., 2016) have been described, with a natural distribution area that spans from the Southern parts of Scandinavia to Central Asia and Africa (Ruttner, 1988; Sheppard and Meixner, 2003; Meixner et al., 2011). This area extends to almost every corner of the globe today as a result of the transportation of beehives by man throughout history (Crane, 1999).

The evolutionary history of *A. mellifera* presents a remarkable complexity due to the intervention of both natural and anthropic factors. Morphometric, genetic, ecological, physiological and behavioral studies (review in De la Rúa et al., 2009b) group the described subspecies of *A. mellifera* into four main evolutionary lineages: lineage A, which includes subspecies throughout Africa; lineage M, including subspecies of Western and Northern Europe; lineage C, which includes subspecies of Eastern Europe; and the O lineage, which includes species from Turkey and the Middle East (Ruttner et al., 1978; Ruttner, 1988; Garnery et al., 1992; Arias and Sheppard, 1996; Franck et al., 2000; Miguel et al., 2011). A sub-division of the African lineage into three sub-lineages (AI, AII, AIII, Franck et al., 2001) and a new lineage named Z have been later proposed (Alburaki et al., 2011). The origin of these lineages has been commonly found in Asia, where it is believed that *A. mellifera* originated (Ruttner et al., 1988; Han et al., 2012; Wallberg et al., 2014; Cridland et al., 2017), later expanding to Europe and Africa. However, there is a controversy in this respect with different hypotheses regarding the origin of the species (Fig 2.).

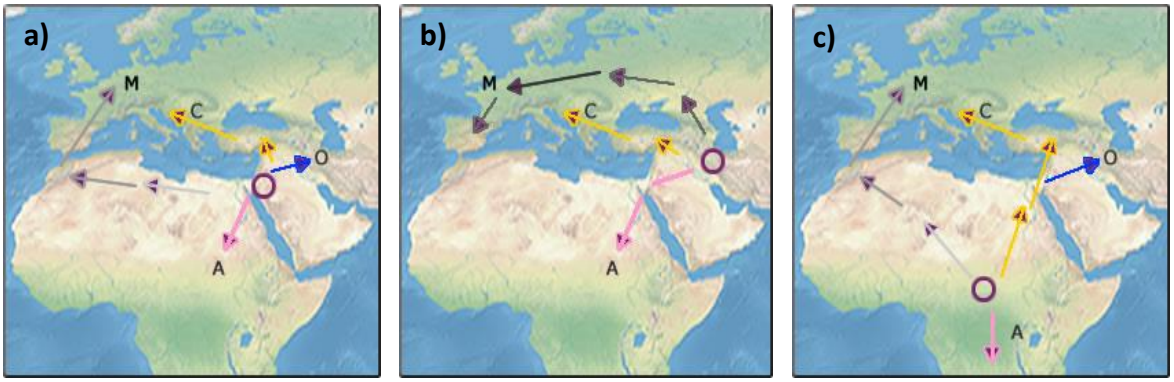

***Fig. 2. Three hypotheses proposed for the origin and expansion of* A. mellifera: *Source:* http://www.killowen.com/genetics14.html**

- **a)** *Based on morphological data, Ruttner (1988) proposed the origin of* A. mellifera *in the Middle East. Ruttner's hypothesis has been reaffirmed by two recent studies (Han* et al.*, 2012; Wallberg* et al.*, 2014)*
- **b)** *Based on mtDNA results, Cornuet and Garnery (1991) proposed the Middle East as the origin of* A. mellifera *but following a different expansion route.*
- **c)** *Based on results from WGS Whitfield* et al. *(2006) proposed the origin of* A. mellifera *in Africa, from where the different lienages would have expanded.*

2. *Apis mellifera* in the Iberian Peninsula

According to molecular data (Whitfield et al., 2006), African (A) and Western European (M) evolutionary lineages are naturally dispersed in the Iberian Peninsula. Different studies based on allozymes, pheromones, mitochondrial DNA (mtDNA) and

microsatellites (STRs) (De la Rúa et al., 2009a) support the hypothesis previously established through morphometry and behavioral studies (summarized in Ruttner, 1988). This hypothesis postulated that populations of the Iberian honey bee, *A. m. iberiensis* (Engel, 1999), are the result of a wide integration between bees of the M lineage, that survived the last glacial maximum harbored in the Iberian Peninsula, and the bees of the A lineage from North Africa that recolonized the Southeast of Europe (Serrano et al., 2011) (Fig. 3).

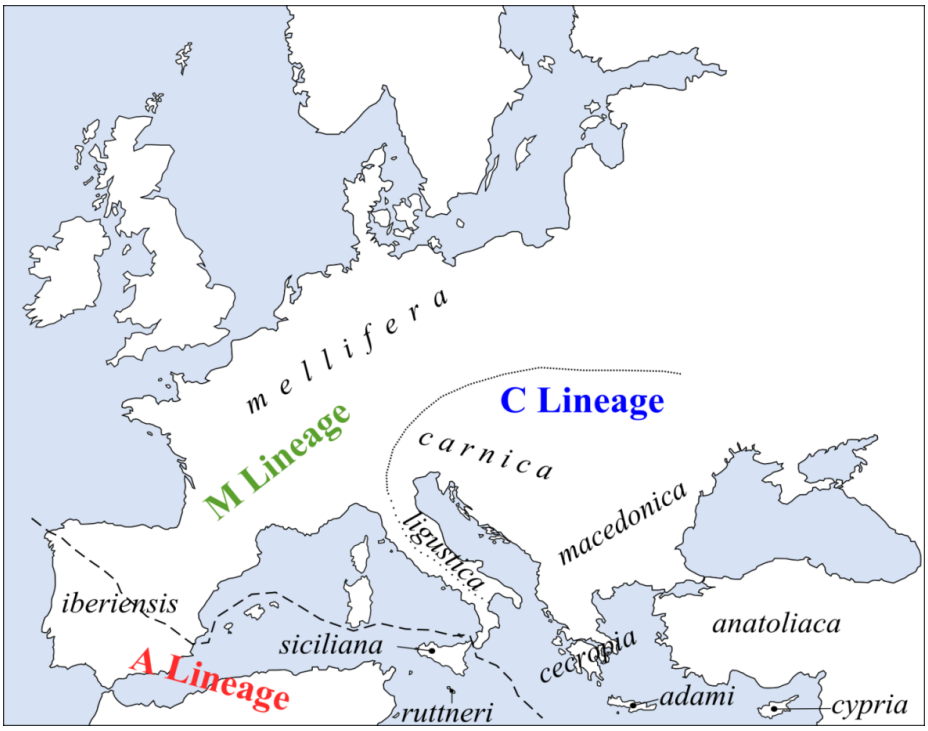

Fig.3. Distribution of the evolutionary lineages and subspecies of* Apis mellifera *in Europe (from De la Rúa et al., 2009b).

Mitochondrial evolutionary history of A. m. iberiensis

The mitochondrial DNA is one of the most widely used marker in studies on the biogeography of the populations and subspecies of *A. mellifera*, as it allows to reconstruct the evolutionary history of the populations through a maternal route. This molecule is inherited through the ovum, since the spermatozoid usually does not provide mitochondria to the zygote (Meusel and Moritz, 1993). Therefore, all the individuals from one colony (workers and drones) share the same mtDNA, thus allowing to characterize the queen and the whole colony through the analysis of a single specimen.

The analysis of variation of the mtDNA sequence supports the hybrid origin of the subspecies *A. m. iberiensis*, as haplotypes (or mitochondrial variants) belonging to both evolutionary lineages A and M, show a gradient of distribution in the Iberian Peninsula

(Miguel et al., 2007; Cánovas et al., 2008; Pinto et al., 2012, 2013; Chávez-Galarza et al., 2017).

A secondary contact between bees of the African (A) and Western European (M) evolutionary lineages would have resulted in a clinal distribution of populations, with a decrease of the frequency of haplotypes of the A lineage from the SW to the NE of the peninsula, while the frequency of M lineage haplotypes increases (Cánovas et al., 2008; De la Rúa et al., 2009b; Chávez-Galarza et al., 2017). This population structure of the Iberian honey bee reflects not only their evolutionary history but also different adaptations to local conditions (Miguel et al., 2007; Cánovas et al., 2008).

Microsatellites studies in the Iberian honey bee

Microsatellites (short tandem repeats; STRs) are polymorphic loci present in the nuclear DNA. These are non-coding repeating DNA segments from 1-6 bp, arranged in tandem and dispersed throughout the eukaryotic genome (Goldstein and Schlötterer, 1999; Scribner and Pearse, 2000; Fontdevilla and Moya, 2003). These regions present high rates of mutation and genetic variability (Tautz et al., 1986; Tautz, 1989; Goldstein and Schlöterter, 1999; Scribner and Pearse, 2000), and are used as molecular markers in a wide variety of applications in the field of population genetics. At present, about 550 microsatellite loci have been described in *A. mellifera* (Solignac et al., 2003, 2007).

Numerous studies have demonstrated the usefulness of microsatellites unravelling the signatures of historical and contemporary human-driven events in the honey bee populations (Franck et al., 1998, 2001; Garnery et al., 1998; De la Rúa et al., 2001, 2003, 2006; Pinto et al., 2005; Muñoz et al., 2009; Miguel et al., 2011; Cánovas et al., 2011; Galindo-Cardona et al., 2013; Uzunov et al., 2014; Rangel et al., 2016). Also, these markers have resulted useful for identifying introgression of C lineage-derived genes into gene pools of native honey bees, and monitoring conservation programs of different subspecies of *A. mellifera* (Jensen et al., 2005; Soland-Reckeweg et al., 2009; Muñoz et al., 2014), including *A. m. iberiensis* on the Canary Islands (Muñoz et al., 2012a).

The clinal variation found in the Iberian Peninsula for mtDNA haplotypes is not observed with nuclear markers such as microsatellites. Furthermore, the results derived from the analysis of these markers show a lower variability of the microsatellite loci in the Iberian Peninsula and France with respect to Africa, as a result of a hypothetical bottleneck suffered by the Iberian populations during the last glaciation (Franck et al., 1998; Garnery et al., 1998). These also show a clear rupture between Africa and Europe, while genetic profiles are rather homogeneous from Spain to Scandinavia (Garnery et al., 1998). According to the hypothesis proposed by Miguel et al. (2007), this similarity is due to the higher mutation rates of microsatellites with respect to mtDNA, which would lead to an accelerated increase in the initial diversity of refugee populations after the last glacial maximum, masking in a relatively short time, the effects of the recolonization

process from Africa. However, studies carried out in Spain, found intermediate genetic diversity values between those of the African and Western European populations, in populations of bees of Murcia (De la Rúa et al., 2002), Balearic Islands (De la Rúa et al., 2003) and Andalusia (De la Rúa et al., 2004).

As compared to mtDNA, microsatellites provide more information about population events such as introgressive hybridization through mating between drones and queens. Due to this mating behavior, the geographical distribution of microsatellite markers in Iberian populations may have been modified by beekeeping practices of the last decades. In Spain, around 80% of the hives are transported seasonally resulting in potential bi-directional gene flow between stationary and migratory colonies (Perrier et al., 2003). Similarly, the massive loss of colonies by the spread of diseases such as varroosis and nosemosis, and the replacement of these colonies by beekeepers may have accelerated the changes in the population structure and the genetic diversity of the Iberian bee (Cánovas et al., 2011).

***Single-nucleotide polymorphisms (SNPs) assays in* A. m. iberiensis**

Single-nucleotide polymorphisms (SNPs) represent the most recent addition to the molecular toolkit available for honey bee genetic analysis. As a usually biallelic marker, the per locus information content of a SNP is lower than that of a multiallelic microsatellite. This drawback can be offset by employing large numbers of SNPs, whose identification is greatly facilitated in the genomics era (Consortium HGS, 2006; Muñoz et al., 2016).

To date, SNPs have been used to assess the evolutionary history of the honey bee (Whitfield et al., 2006; Wallberg et al., 2014; Chen et al., 2016), to search for footprints of selection (Zayed and Whitfield 2008; Spotter et al., 2012; Chávez-Galarza et al., 2013; Wallberg et al., 2014; Chen et al., 2016), to examine the evolutionary complexities of the Iberian honey bee hybrid zone (Chávez-Galarza et al., 2015, 2017), to inspect genome-wide recombination patterns (Wallberg et al., 2015), and to investigate introgression of C lineage-derived genes into gene pools of native honey bees for commercial and conservation purposes (Pinto et al., 2014; Muñoz et al., 2015).

In the Iberian Peninsula, a genome-wide scan using SNPs has been conducted in searching for the footprints of selection in the environmentally heterogeneous Iberian honey bee range (Chávez-Galarza et al., 2013). The results from this study, showed signatures of selection in the Iberian honey bee genome, suggesting that this evolutionary force has had an important role in structuring Iberian honey bee diversity. Furthermore, a more recent study (Chávez-Galarza et al., 2015) using a genome-wide dataset of SNPs, confirmed the clinal pattern of populations structure in the peninsula, previously observed with mtDNA, but not with other nuclear markers such as microsatellites. Together, these results highlight the complexity of the Iberian honey bee patterns, and strength the hypothesis of an ancestral secondary contact between

divergent honey bee populations previously isolated in glacial refuges (Smith et al., 1991).

3. Importance and genetic diversity of *Apis mellifera*

The International Union for the Conservation of Nature (IUCN) recognizes genetic diversity as one of the three forms of biodiversity that deserves to be conserved (McNeely et al., 1990). The importance of preserving the genetic variability of populations is based on two arguments: (1) the need of this variability for evolution to occur, and (2) the observed relationship between heterozygosity and the biological efficacy (or fitness) of the populations (Reed and Frankham, 2003).

Greater genetic diversity in a species means greater ability of individuals to adapt. In this way, genetic variability at the species level becomes a necessary element for populations to withstand short-term environmental disturbances, such as the appearance of new diseases. It is also crucial to enable populations to evolve and adapt to long-term environmental changes, such as climate change (Frankham et al., 2010). In this sense, the honey bee has adapted to a wide variety of ecosystems in its natural range (Africa, Europe and West and Central Asia). At present, about 31 subspecies of *A. mellifera* and numerous ecotypes are known on the basis of behavioral, morphological and molecular evidences (Ruttner, 1988; Engel, 1999; Sheppard and Meixner, 2003; Meixner et al., 2011; Chen et al., 2016). This process of local adaptation can occur naturally (natural selection), but it may also be influenced by the management performed by the beekeepers.

European honey bees are among the most important insects for humans and terrestrial ecosystems due to their fundamental role as pollinators of wild plants and crops worldwide (Klein et al., 2007; Aizen et al. 2009). Many studies have highlighted the great value of this pollinating service for agriculture, estimating its economic benefits in millions of euros per year (Morse and Calderone, 2000; Klein et al., 2007; Gallai et al., 2008), and being incalculable in the case of wild flora. To this, it must be added the value and utility of the products derived from beekeeping such as honey, pollen, propolis, wax, royal jelly or bee venom (apitoxin). In this sense, *A. mellifera* has been managed by man for centuries, both to produce honey and wax, and for the pollination of crops. The human management of a species often brings profound changes in the genetic variation levels. The reduction of the effective size of the populations and the diminution of their genetic diversity, are habitual consequences of the domestication process. This phenomenon is known as the "bottleneck of domestication" (Wright et al., 2005; Zeder et al., 2006), and it is sometimes followed by secondary bottlenecks as a result of successive colonization events associated with the dispersion induced by man (Zeder et al., 2006). However, it should be mentioned that the honey bee, in contrast to other

livestock species, it is not completely domesticated (Oxley and Oldroyd, 2010), since mating of the honey bee queen occurs naturally. Although artificial insemination of queens has been practiced since the 1940s (Laidlaw, 1944), and there is a large industry of bee rearing for sale in the American continent and in some European countries (Delaney et al., 2009, Laidlaw and Page, 1997), the beekeeper cannot fully control the reproduction process, as it does with other domestic animals such as poultry, pigs or sheep. During the mating season, usually in spring, queen bees can successfully mate with unselected drones. Because of this, it would be more correct to consider the honey bee as a wild animal, which we can maintain and use as long as we provide it of access to its resources (Moritz, 2005).

Even though honey bees are only semi-domestic, their populations in Europe have been seriously affected by human activities (Jaffé et al., 2010). In recent decades, there has been an overall reduction in the number of colonies in different regions of the world. This fact is presumably affecting the genetic diversity and the population structure of the honey bee, especially if it is taken into account that in their attempts to replace lost colonies, beekeepers import colonies from different regions (Muñoz et al., 2012b).

Genetic diversity at the intra-colony level has been shown to be important (Crozier and Page, 1985; Palmer and Oldroyd, 2000; Crozier and Fjerdingstad, 2001). Several studies show that multiple mating of the bee queens results in an increase of the genetic diversity within the colony, which positively impacts its biological fitness and productivity (Oldroyd et al., 1992; Mattila and Seeley, 2007). In addition, colonies with higher patriline diversity appear to be more capable of maintaining homeostasis (Oldroyd and Fewell, 2007), thermoregulation (Jones et al., 2004; Graham et al., 2006), and are more resistant to diseases (Tarpy, 2003).

4. Current threats for honey bees and beekeeping

The studies of the last decades indicate a growing tendency to the disappearance of honey bee colonies world-wide. Between 1970 and 2007, the number of colonies in Europe gradually decreased from over 21 million to about 15.5 million (FAO, 2009, Aizen and Harder, 2009), and in recent years, beekeepers in North America and in Europe have been repeatedly affected by high, and sometimes unexplained, winter losses (Higes et al., 2006, Oldroyd, 2007, vanEngelsdorp and Meixner, 2010).

The first alarm occurred in the fall of 2006 when commercial migratory beekeepers on the East Coast of the United States began to register high colony losses in their apiaries. The winter of 2006-2007 witnessed the disappearance of bee colonies throughout the United States, with more than half of the states affected and losses to beekeepers of between 30% and 90% of their colonies (vanEngelsdorp et al., 2007). This

phenomenon seems to have spread also to Europe where, to a greater or lesser extent, similar episodes have been recorded every year since then (vanEngelsdorp et al., 2009; vanEngelsdorp and Meixner, 2010). In the US, total colonies losses were of 32% during the winter of 2006-2007, increasing to 36% in 2007-2008, and apparently 'stabilizing' at 29% in 2008-2009.

These losses are not new in the beekeeping activity. Since the late XIX century there are references of at least 18 episodes of unusual and high mortality rates in bee colonies (Underwood and vanEngelsdorp, 2007). Many efforts have been devoted in recent years to quantify the incidence and discuss the possible causes and factors involved in these colony losses (Cox-Foster et al., 2007; EFSA, 2008; COLOSS, 2009; Giray et al., 2010). Several national programs, such as 'The German bee monitoring project' (Genersch et al., 2010) or the American research team on the Colony Collapse Disorder (CCD) of the US Department of Agriculture (USDA), have been launched to collect data on bee mortality and to conduct an overall assessment of potential risk factors for colony survival. Similarly, the European Union has funded a number of programs such as COLOSS (Preventing COlony LOSSes, Action COST FA0803, www.coloss.org) with the aim of creating a network of scientists from different countries to collaborate on the study of various aspects of bee health: monitoring and diagnosis, pests and pathogens, environment and beekeeping, and diversity and fitness.

Despite substantial efforts, there are still many unknown aspects remaining. Numerous factors have been analyzed and discussed in the recent literature for their possible influence on the weakening and eventual disappearance of the honey bee colonies; however, to date none of them has been identified as the ultimate cause of this phenomenon, but rather takes strength the idea of a multi-causal phenomenon (Higes et al., 2006; Cox-Foster et al., 2007; Desneux et al., 2007; Johnson et al., 2009; Nguyen et al., 2009; de Miranda et al., 2010; de Miranda and Genersch, 2010; Rosenkranz et al., 2010; vanEngelsdorp and Meixner, 2010).

5. Factors related to the global massive loss of colonies

As increases our dependence on honey bees for world-wide pollination of crops, the decline is more evident in both man-managed (Potts et al., 2010) and wild colonies (Kraus and Page, 1995; Moritz et al., 2007; Jaffé et al., 2010). This decrease appears to be mainly caused by anthropic action, with several factors including beekeepers education, playing important causal roles (Jacques et al., 2017).

5.1. Changes in land use

The loss of natural habitats due to the incessant urban activity and the continuous transformation from forest to agricultural land, causes the diminution or the dispersion of important resources for the bees, such as food (Hines and Hendrix, 2005; Potts et al., 2005), seriously affecting *A. mellifera* populations (Rathcke and Jules, 1994; Kremen et al., 2002, 2004; Steffan-Dewenter et al., 2002, 2006; Larsen et al., 2005; Cane et al., 2006). Likewise, habitat fragmentation, a direct result of habitat loss, may have a negative impact on the populations, either through genetic isolation and the subsequent inbreeding (Zayed, 2009), or simply because of the inability of the resulting habitats to maintain viable populations of bees (Ellis et al., 2006). In Europe, the commonly practiced intense land use (extensive fields of a single annual crop or monoculture timber forests) is likely to reduce the availability of floral resources for honey bees (Biesmeijer et al., 2006; Murray et al., 2009).

5.2. The massive use of pesticides and herbicides in agriculture

Intoxications due to the inappropriate use of pesticides and herbicides in agriculture are another problem of the human activity associated with honey bees (Ingram et al., 1996). In modern crop systems routinely treated against insect pests, honey bees are readily exposed to pesticides (Mullin et al., 2010). Some of them, particularly systemic pesticides, diffuse through all tissues of growing plants and eventually contaminate nectar and pollen (Rortais et al., 2007). Forager bees are therefore directly exposed, and so is the rest of the colony, since when returning from foraging, they store or exchange the contaminated material with their congeners of the hive (Rortais et al., 2007; Krupke et al., 2012).

The legal commercialization of these products requires studies to ensure that doses in the field are kept below lethal levels to honey bees. However, several studies have indicated that sublethal doses of these insecticides (levels equal to or lower than those applied in the field) also lead to profound alterations in various biological aspects of honey bees, such as the division of labor, the foraging behavior (dysfunction in learning, olfactory memory, and field orientation), the development of the colony, and the recognition of 'nest mate' (Thompson, 2003; Bortolotti et al., 2003; Decourtye et al., 2003, 2004a, 2004b; Colin et al., 2004; Desneux et al., 2007; Yang et al., 2008; Schneider et al., 2012). Some scientists and beekeepers suspect that pesticides play a central role in the processes of weakening colonies (Oldroyd, 2007) or at least in the interaction with other stressors (Alaux et al., 2010; Vidau et al., 2011).

5.3. Intensive beekeeping practices

Some apicultural practices like the purchase of honey bee queens and the intensive migratory movements also affect genetic and health conditions of the honey bee populations (Simone-Finstrom et al., 2016). An excessive gene flow among populations result in the homogenization of the genetic pool, which can lead to the loss of certain genetic combinations resulted from the adaptation to their local conditions (ecotypes) (Cánovas et al., 2011). On the other hand, the introduction of honey bees from different origins into local populations can lead to the introduction of pathogens as observed in island honey bee populations (Muñoz et al., 2014).

5.4. Increased incidence of the parasites and pathogens of the honey bee

To date, one of the most significant variables associated to the massive loss of honey bees in Europe and in the US is the increase of the incidence of parasites and pathogens in the honey bee (vanEngelsdorp et al., 2009). Among the current most damaging agents are the mite *Varroa destructor* and viral diseases associated to it, as well as the microsporidia of the genus *Nosema* (Higes et al., 2006, Cox-Foster et al., 2007; Johnson et al., 2009; Rosenkranz et al., 2010; de Miranda and Genersch, 2010;).

It seems unlikely that a single factor would be the cause of all the losses recorded in recent years worldwide. The more recent hypotheses point to the possibility that several of the factors mentioned here may be occurring simultaneously or interacting with each other. Combinations particularly virulent of parasites and pathogens seem to be the most likely explanation (Chen and Evans, 2007; Johnson et al., 2009). On the other hand, chronic exposure to pesticides, which does not cause serious damage in healthy colonies, is suspected to interact with pathogens and parasites, causing lethal consequences in colonies already weakened by diseases (Thompson, 2003).

5.4.1. The mite *Varroa destructor*

Varroa destructor Anderson and Trueman 2000 is an obligate ectoparasite of the bee species *A. mellifera* and *A. cerana* Fabricius 1793. It is the causative agent of the varroosis, a parasitic disease that affects bees in all their stages of development and one of the current most serious diseases of the honey bees (Sammataro et al., 2000; Llorente, 2003). This mite has killed hundreds of thousands of colonies around the world and is to date, one of the most important agents associated with major winter losses recorded in recent years in Europe.

Origin and distribution

The mite initially named *Varroa jacobsoni* was described by A. C. Oudemans in 1904 from specimens found on the island of Java (Indonesia) on the Asian honey bee, *A. cerana.* On its original host, the mite does not cause serious damage, mainly because it only reproduces in drone brood cells. However, on the new host *A. mellifera*, this species can reproduce in both drone and worker cells, so that the effects are much more severe and may end up causing the death of colonies.

The host hop from *A. cerana* to *A. mellifera* occurred about 50 years after the introduction of the Western honey bee in Asia (Rosenkranz et al., 2010). Due to the global trade of bees and bees' products, the mite has now expanded to colonies of all continents, except Australia (Llorente, 2003). In Spain, *V. destructor* was first detected on the French border in December 1985, and since then it has stretched throughout the country, causing the death of approximately 40% of the Spanish colonies in its first years of incidence (Llorente, 2003).

Morphology

V. destructor shows a marked sexual dimorphism (Ifantidis, 1983) with morphological adaptations to its host. The females are diploid, with an elliptical shape and a size of 0.8-1.5 mm in length and 1.3-1.9 mm in width. In contrast, males are haploid, with a triangular shape and a size clearly inferior to that of the female (0.75 x 0.8 mm) (Fig. 4).

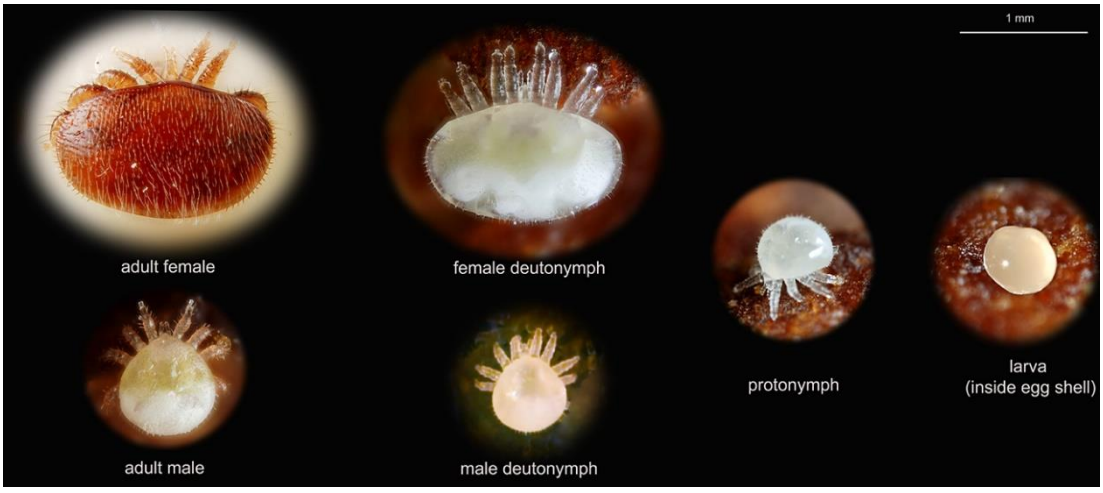

Fig.4. Detail of the body of* Varroa destructor *showing the morphological differentiation between the male and the female of the species in the adult stages of development. Source: *http://idtools.org/id/mites/beemites/bmites_lifestages.php*

Females are those that parasitize bees. As an adaptation to this parasitism, the female body is dorso-ventrally flattened, and it is covered by a strong chitin reddish membrane, on which hundreds of hairs are inserted. The chelicerae of the oral apparatus perforate the chitin of the bee and its small excrescences allow a better

fixation in the body of the bee (Llorente, 2003). In the case of males, less consistent and yellowish-white, these adaptations to parasitism do not appear. The body of the male is almost spherical and its chelicerae are not knife-shaped as in females, but are tube-shaped and adapted to transfer sperm into females

***Biology of* V. destructor**

The life cycle of *Varroa* presents two clearly differentiated phases, a phoretic phase and a reproductive phase. The phoretic phase is only carried out by the adult females, who adhere to the worker bees and drones to colonize new hives. During this stage, the mite feeds on the hemolymph of the adult bee until there is bee brood in the hive. At that moment, *V. destructor* is dropped on a cell containing a bee larva at the end of its open stage. Once the cell is capped, the parasite begins the laying and its descendants develop at the expense of that larva. Mating of adult mites takes place inside the capped cell, and once mature, the gravid females of *Varroa* leave the cell together with the young bee until they find another brood cell about to occlude. Each female can complete about 2-3 of these cycles, so it is assumed that the population of *Varroa* doubles approximately every month, provided there is brood in the colony (Calatayud and Verdú, 1997) (Fig. 5).

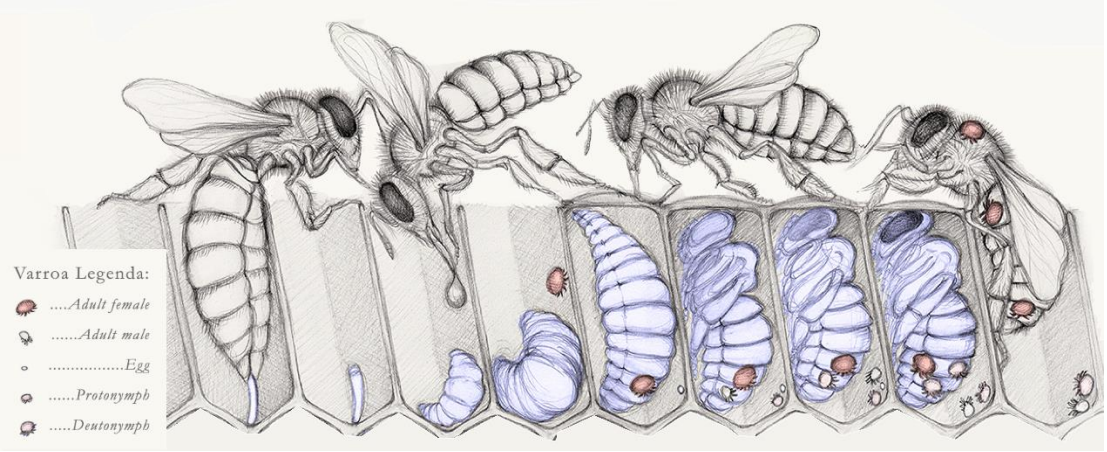

***Fig. 5.* Varroa destructor *life cycle. Source:* https://es.pinterest.com/drmck03/bees/. *See also animation of* V. destructor *life cycle in:* https://www.youtube.com/watch?v=h-wDqd5yURo**

Pathology

The colonies infected by *Varroa* suffer both, direct damage by the parasitism of the mite, and indirect damage by viral and microbial pathogens vectored by *Varroa* (Johnson et al., 2009, Highfield et al., 2009). In addition, *V. destructor* induces immunosuppression in the honey bees, which leads to an amplification of pre-existing infections (Yang and Cox-Foster, 2007), further exacerbating their impact.

Individual level damage. At the individual level, loss of haemolymph, especially in developing larvae, causes a significant reduction in the weight of the bee that emerges, a delay in its development and eclosion, and even its death before emerging when the degree of parasitism is high (De Jong et al., 1982; Schatton-Gadelmayer and Engels, 1988; Duay et al., 2002, 2003).

The worker bees parasitized during development, start foraging earlier and have a significantly reduced life (Amdam et al., 2004; De Jong et al., 1982; Schneider and Drescher, 1987). In addition, the parasitized foragers show a decrease in non-associative learning capacity, as well as prolonged absence and a lower rate of return to the colony (Kralj and Fuchs, 2006; Kralj et al., 2007), which may be caused by a decrease in their flight capacity and orientation (Ruano et al., 1991).

Colony level damage. The fitness of the honey bee colony is noticeably reduced, even if the infestation is moderate. On the one hand, drones that have been parasitized during their development are significantly less likely to mate (Duay et al., 2002), and on the other, infected colonies tend to swarm less (Fries et al., 2003; Villa et al., 2008). Furthermore, a moderate rate of infestation can reduce the growth of the bee population, and therefore, the honey production due to the lack of vitality and the premature death of parasitized bees. When the infestation is moderate, the clinical symptoms are not visible, so the varroosis usually goes unnoticed. This lack of detection and premature treatment, usually leads to irreversible damages in the colony. Once high rates of parasitism are reached in the colony, the visible symptoms of varroosis are:

i. Sealed brood combs degraded (similar to those observed in hives attacked by American foulbrood): scattered brood pattern, sunken and chewed cappings, and even a typical stench due to rotting processes of dead larvae.
ii. Young bees of small size and appearance of malformations (deformed wings and legs, shortened abdomen, etc.) typical of the infection by deformed wing virus or other pathogens linked to *Varroa* infestation.
iii. A more intense activity of the bees, which are restless in their eagerness to get rid of their guests, and consequently, an increased consumption of food.

All this, combined with possible secondary infections (either induced by the parasite or that appear opportunistically when the colony is more vulnerable), cause the generalized weakening of the colony and ultimately, its collapse.

5.4.2. Viruses associated with *V. destructor* infestation

The lethal effects of *V. destructor* on honey bee colonies are largely due to the capability of this mite to activate and transmit several viral diseases (Ball and Allen, 1988; Bailey and Ball, 1991; Bowen-Walker et al., 1999; Sumpter and Martin, 2004; Chen and Siede, 2007; Tentcheva et al., 2006; Todd et al., 2007). So far, about 23 different

viruses have been isolated from honey bees (Chen and Siede, 2007; McMenamin and Genersch, 2015) and many of them can be transmitted by *Varroa*. This has been demonstrated for the Kashmir bee virus (KBV), sacbrood bee virus (SBV), acute bee paralysis virus (ABPV), Israeli acute bee paralysis virus (IAPV), and the deformed wing virus (DWV) (Boecking and Genersch, 2008). Before the appearance of *Varroa*, the bee viruses had been considered a minor problem for the health of the colonies (Allen et al., 1986; Bailey and Ball, 1991; Bowen-Walker et al., 1999; Yue and Genersch, 2005). At present, however, many of the pathological alterations and the mortality detected in colonies highly parasitized by *V. destructor* are considered to be related to the synergistic action of the parasite with the viruses it transmits.

In the absence of the *Varroa* mite, it has been observed that many of the honey bee pathogenic viruses only cause covert infections, which show no clinical signs and have no detectable impact on infected bees or colonies (de Miranda and Genersch, 2010). However, the mite has very quickly become established as a mechanical and biological vector of many honey bee viruses, so that, the virus transmission to the pupae through the parasitic mite is the prerequisite for overt viral infections with the manifestation of clear disease symptoms. This augmented prevalence and virulence due to mite transmission is marked for members of the DWV/VDV-1 (deformed wing virus/*V. destructor* virus-1) and ABPV/KBV/IAPV (acute bee paralysis virus/Kashmir bee virus/Israeli acute bee paralysis virus) clades (de Miranda and Genersch, 2010; de Miranda et al. 2010, Genersch and Aubert, 2010).

5.4.3. The Deformed Wing Virus (DWV)

The deformed wing virus (DWV) is one of the major viruses infecting honey bees, and one of the most studied in relation to the colony collapse induced by *V. destructor* (Ball, 1983; Ball and Allen, 1988; Bowen-Walker et al., 1999; Nordström et al., 1999; Ribière et al., 2008; Sumpter and Martin, 2004; Tentcheva et al., 2004; de Miranda and Genersch, 2010). In the absence of *V. destructor*, however, DWV appears at low levels in the colonies and does not cause obvious negative impacts on them.

Origin and distribution

The virus was first isolated in symptomatic bees from Japan in 1982. Initially it was related to the Egyptian bee virus (EBV), being briefly named as "Japanese variant of EBV", but the existence of serological differences between both viruses led to rename it as deformed wing virus (DWV) in reference to the symptoms with which it appears associated in the colonies (Bailey and Ball, 1991; Ribière et al., 2008). The mite *V. destructor* is the main transmission vector of the DWV, so that the virus is now distributed in colonies of *A. mellifera* worldwide (Allen and Ball, 1996; Ellis and Munn, 2005; Ribière et al., 2008). The close association with the mite has raised the question of

whether the original host of this virus is *A. mellifera*, or whether the virus originates, as *Varroa*, in the Asian honey bee *A. cerana*, on which it has also been detected. As well as it has been detected on different species of wild and domestic bumblebees showing deformed wings (Genersch et al., 2006), on the bee *Apis florea*, the mites *V. destructor* and *Tropilaelaps mercedesae* (Dainat et al., 2009) and the small hive beetle *Aethina tumida* (Eyer et al., 2009).

Structure and genetics of DWV

The virion is a 30-nm icosahedral particle consisting of a single, positive strand RNA genome and three major structural proteins (de Miranda and Genersch 2010; Škubník et al., 2017) (Fig.6).

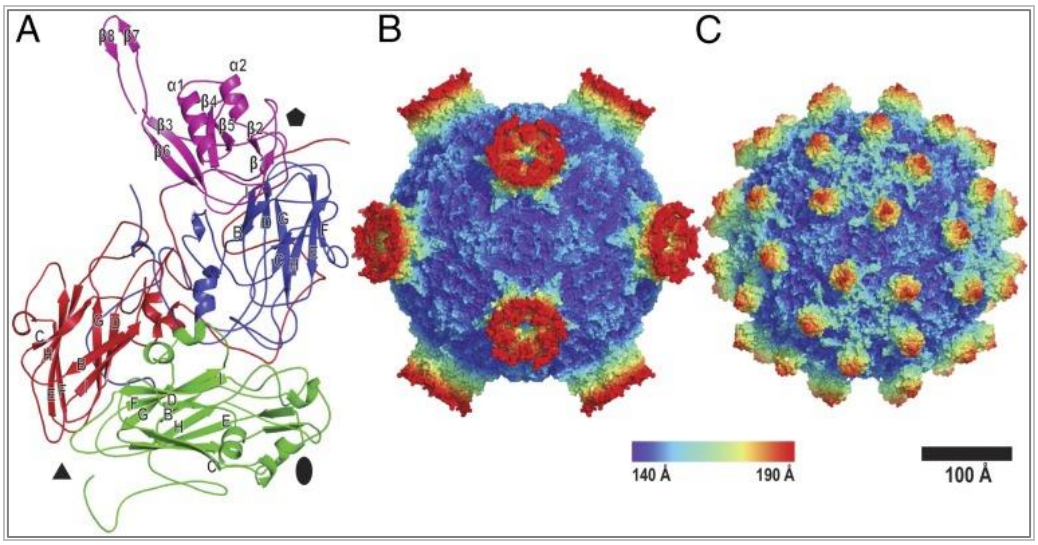

Fig. 6. Structures of the icosahedral asymmetric unit of DWV and its virions in alternative conformations (from Škubník et al., 2017). *Icosahedral asymmetric unit of DWV in schematic representation* ***(A)*** *with major capsid protein VP1 colored in blue, VP2 in green, and VP3 in red. The P domain, which is part of VP3, is highlighted in magenta. Selected secondary structure elements are labeled. The locations of the fivefold, threefold, and twofold symmetry axes are denoted by a pentagon, triangle, and oval, respectively.* ***(B)*** *Molecular surface of DWV virions determined by cryo-EM and* ***(C)*** *X-ray crystallography. The virion surfaces are rainbow-colored according to their distance from the particle center (Scale bar: 100 Å.).*
Source: ***https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5373406/figure/fig01/***

The DWV genome was published in 2006 (Lanzi et al., 2006). The viral genome is 10140 nucleotides in length excluding the poly-A tail. Its organization (Fig. 7) is the typical of the iflaviruses genus, and consists of a single open reading frame (ORF) flanked by a long 50 untranslated region (50 UTR), a short, highly conserved 30 UTR, and ending in a 30 poly-A tail. Both untranslated regions are involved in regulating the replication and translation of the genome (Belsham, 2009; Gromeier et al., 1999; Nakashima and

Uchiumi, 2009; Roberts and Groppelli, 2009). Within the 50 UTR lies an Internal Ribosome Entry Site (IRES) which is active in vivo in several mammalian, insect and plant cell-free translation systems (Roberts and Groppelli, 2009).

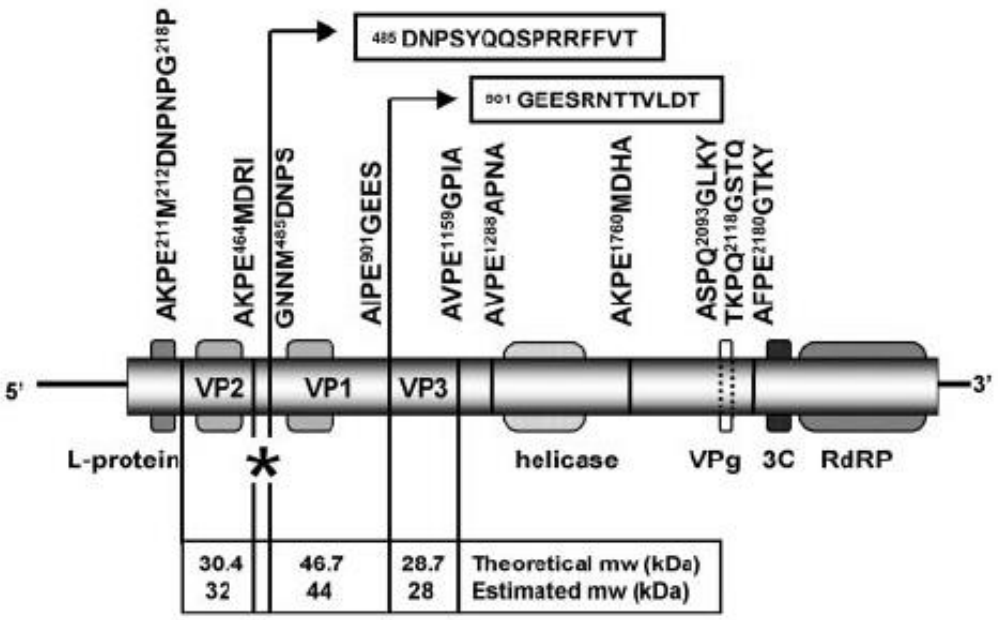

Fig. 7. Map of the DWV genome (from Lanzi et al., 2006). *The long shaded box represents the single open reading frame, with various recognizable protein domains shown. The short vertical lines (dashed and solid) represent predicted protease cleavage sites: their positions and surrounding amino acid sequences are also shown. The long vertical lines lead to the experimentally determined N-terminal amino acid sequences of VP1 and VP3 (boxed). The figure also shows, for each of the main structural proteins, the size estimated by PAGE and the theoretical calculations based on putative protease cleavage sites. The asterisk marks the position of the putative VP4 capsid protein (2.3 kDa).*
Source: ***http://jvi.asm.org/content/80/10/4998/F3.expansion.html***

Biology of DWV

The predominant role of *Varroa* in the epidemiology of DWV, causes the seasonal distribution of DWV to be closely associated to that of the mite, growing in prevalence and viral load as the bee season progresses (Gauthier et al., 2007; Tentcheva et al., 2004). When brood is available in the colony, DWV is injected into developing bee pupae by the feeding activities of *Varroa.* These indirect horizontal transmissions trough *Varroa* parasitism lead to an overt acute infection, recognizable by the characteristic symptoms of deformed wing disease (Boecking and Genersch, 2008; de Miranda and Genersch, 2010) (Fig. 9). The ability of the mite to act both as a mechanical vector of DWV but also as a biological vector supporting DWV replication prior to transmission, have been linked to overt outbreaks of DWV disease (Yue and Genersch, 2005; Gisder et al., 2009). Indeed, high virus levels in mites are generally associated with high virus levels in the

corresponding pupae (Bowen-Walker et al., 1999; Nordström, 2000). Furthermore, *V. destructor* parasitism is known to cause immunosuppression in parasitized bees and bee pupae, which will also activate covert infections by the virus (Yang and Cox-Foster, 2007).

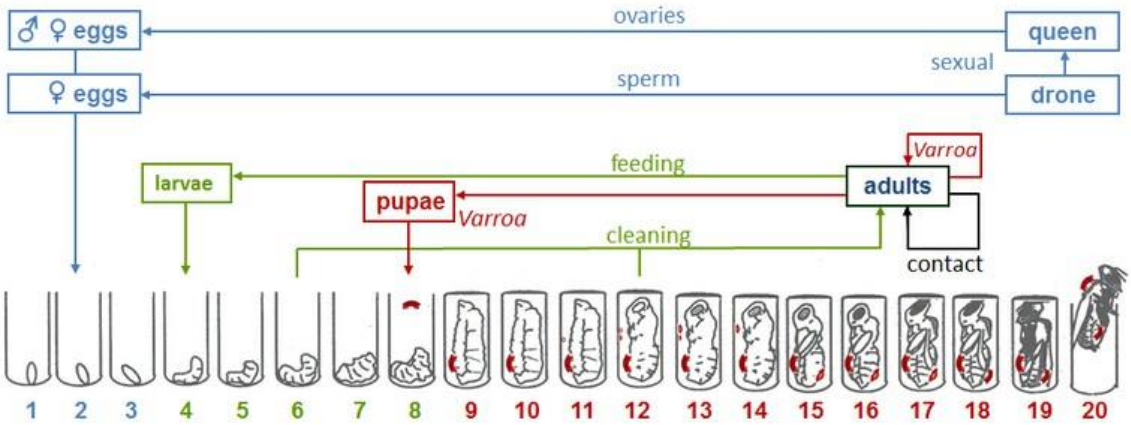

Fig. 8. Diagram describing the different possible transmission routes for honey bee viruses. Source: *http://www.coloss.org/beebook/II/virus/1/1* ***(Adapted from de Miranda et al. 2011).***

Vertical transmission of the virus is also detected at the individual-insect level through drones and queens as well as at the colony level by reproductive swarming of infected colonies (Chen et al., 2006; de Miranda and Fries, 2008; Yue et al., 2006, 2007). Another alternative route of infection is through larval contact with nurse bees. The virus has been detected in hypopharyngeal secretions (royal jelly), brood-food, eggs, and early larval stages not parasitized by mites (Chen et al., 2004, 2005; Yue and Genersch, 2005) and in uninfested pupae fed in infected colonies (Nordström, 2000, 2003). These findings suggest the horizontal transmission of the virus through feeding and trophallaxis (Yue and Genersch, 2005) (Fig. 8). However, none of these transmission routes result in any detectable or measurable negative impact on the fitness of bees and colonies. So that, while horizontal vectorial transmission of DWV by *V. destructor* leads to overt acute and lethal infections, transmission of DWV in the absence of *V. destructor* causes asymptomatic, covert infections. In addition, there is evidence that horizontal vectorial transmission of DWV to adult bees also affects host fitness through an overt chronic infection, although the associated neurological symptoms (learning deficits) constitute a less obvious, sub-lethal pathology than that on pupae (Iqbal and Mueller, 2007).

Pathology

Individual level damage. The typical symptoms associated to DWV disease in individual bees are: death in the pupal stage or adult bees dying shortly (<67 h) after

emergence with vestigial and crumpled wings and sometimes associated with shortened and bloated abdomens, miscolouring and paralysis (Genersch and Aubert, 2010) (Fig. 9).

Colony level damage. Scattered brood nest, crippled bees, eventual loss of coordinated social behavior like hygienic behavior and queen attendance, as well as rapid loss of bee population, constitute the typical ‘‘damage symptoms” caused by DWV infection (Rosenkranz et al., 2010).

Bees emerging with deformed wings succumb soon to the infection (Yang and Cox-Foster, 2007). The premature loss of worker bees and their contribution to colony performance, as well as the energy expended by raising these bees result in a clear negative impact on colony fitness when too many of these non-viable bees are produced. Also, chronic infections due to the vectorial DWV transmission leading to learning deficits will affect colony fitness (de Miranda and Genersch, 2010). Additionally, the combination of mites and viruses causing immunosuppression in the bees, will increase their susceptibility to other opportunistic pathogens (Yang and Cox-Foster, 2005), leading to a progressive reduction in colony performance and a complex disease profile at colony level that often includes other pathogens as well (Ball and Allen, 1988; Shimanuki et al., 1994; Nordström et al., 1999; Tentcheva et al., 2004).

Fig.9. Honey bee with typical symptoms of deformed wing disease. Source: *https://www.flickr.com/photos/klaasdegelder/5137860060*

Symptomatic adult bees typically appear in the final stages of colony collapse, usually late summer-autumn of the second or third year of uncontrolled *Varroa* mite infestation. Occasionally, symptomatic bees can also appear in early spring and recover during the summer, only for symptoms to reappear at the end of the year (Lanzi et al.,

2006). When a colony is building up (i.e. in spring), bee losses due to overt DWV infections of individuals can be compensated, but when brood rearing slows down and mite levels peak (i.e. in autumn), the virus epidemic accelerates and the excessive loss of working bees causes the colony to decline rapidly, and ultimately to collapse (Schroeder and Martin, 2012).

DWV, which is one of the less virulent viruses usually associated with *V. destructor,* induces colony collapse, while more virulent viruses cause less damages into the colonies (Berthoud et al., 2010). It is precisely this low virulence that allows the bee brood to develop through the pupal stage to adulthood (Bailey and Ball, 1991; Ball and Bailey, 1997); while the more virulent viruses kill the pupae (and hence the mite) before the mite can complete its reproduction on the bee pupae, and transmit the virus to new hosts (Martin, 2001; Sumpter and Martin, 2004).

5.4.4. Micrisporidia of the genus *Nosema*

Microsporidia of the genus *Nosema* are intestinal parasites of adult bees, recently reclassified as fungi (Sina et al., 2005) and causing nosemosis in honey bee colonies around the world. They are transmitted by spores and are responsible of significant economic losses in the beekeeping industry worldwide, either by their individual action (Higes et al., 2008, 2009; Hornitzky, 2008; Heintz et al., 2011) or in combination with other factors (Alaux et al., 2010; Bromenshenk et al., 2010; Bacandritsos et al., 2010; Vidau et al., 2011). There are currently two species of *Nosema* that affect the viability of hives, *Nosema apis* Zander 1909, and *Nosema ceranae* Fries, Feng, da Silva, Slemenda and Pieniazek 1996. In spite of their morphological and genetic similarity, they present a different symptomatology and are therefore treated as two independent pathologies (COLOSS workshop, 2009): nosemosis type A produced by *N. apis*, and nosemosis type C produced by *N. ceranae*.

Origin and distribution

N. apis was first described more than 100 years ago and its effects on individual bees and colonies have been extensively documented (reviewed by Neveu-Lemaire, 1938; Bailey and Ball, 1991; OIE, 2008). *N. apis* is the species of microsporidium that historically infected the honey bee and its dispersion is associated with transport of *A. mellifera* throughout the world. From 1996, a new species named *N. ceranae* was described on the Asian bee, *A. cerana*. Initially, it was believed that this species was restricted to the eastern bee and geographically limited to Asia, but recently it has been detected to infect *A. mellifera* in Spain, other European countries, and in the rest of the world (Fries et al., 2006; Fries, 2010; Higes et al., 2006, 2010; Chen and Huang, 2010). It is therefore a new agent in a new host, and it is discussed whether it could be displacing the original parasite (Martín-Hernández et al., 2012; Milbrath et al., 2015; Natsopoulou

et al., 2016), since the latest studies show a higher prevalence of *N. ceranae* than of *N. apis* in *A. mellifera*, despite both are widely distributed (Higes et al., 2006; Klee et al., 2007; Paxton et al., 2007).

Morphology

Microsporidia of the genus *Nosema* have vegetative and resistance phases in the form of spores, which act as elements of resistance and diffusion of the disease. Morphologically, the spores of both species are similar, although those of *N. ceranae* present a slightly smaller size (3.3-5.5 x 2.3-3 µm) than those of *N. apis* (5-7 x 3-4 µm) (Fries et al., 1996; Chen et al., 2009). These are also binucleate (Fries et al., 1996; Fries, 2006; Higes et al., 2007), but the polar filament is considerably shorter than in *N. apis* (Fries et al., 1996; Higes et al., 2007; Chen et al., 2009). The two species are, however, difficult to differentiate under an optical microscope (Fig. 10). To date, molecular techniques based on the polymerase chain reaction (PCR) are the most reliable for the diagnosis and identification of the two *Nosema* species (Martín-Hernández et al., 2007).

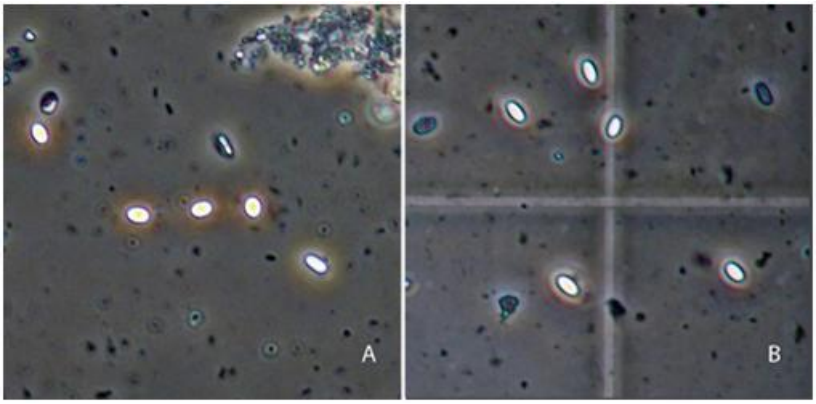

Fig.10. **Nosema apis** ***(A) and*** **Nosema ceranae** ***(B) under the microscope. (Photo: W. F. Huang).***

Biology of **Nosema** ***spp.***

All microsporidia are obligate intracellular parasites that disperse among their hosts in the form of spores and have exclusive organs for cell invasion. Infection occurs when adult bees ingest viable spores during feeding (Bailey, 1981; Webster, 1993), trophallaxis (Webster, 1993), while performing beekeeping tasks (Fries 1989; Webster, 1993), or through water (L'Arrivee, 1965). The spores enter the bee through the alimentary canal and germinate in the middle intestine, where they infect the epithelial cells. The mechanism of infection is based on the mechanical injection of a polar filament that protrudes when the spore germinates, acquiring the shape of a hollow tubule. The filament crosses the membrane of the host cell by introducing itself into it and injecting the infectious sporoplasm into it, where replication and subsequent spore production of

the parasite begins (Larsson, 1986). Once the parasitized cells are full of mature spores, they can rupture releasing them into the intestinal lumen (Fig. 11).

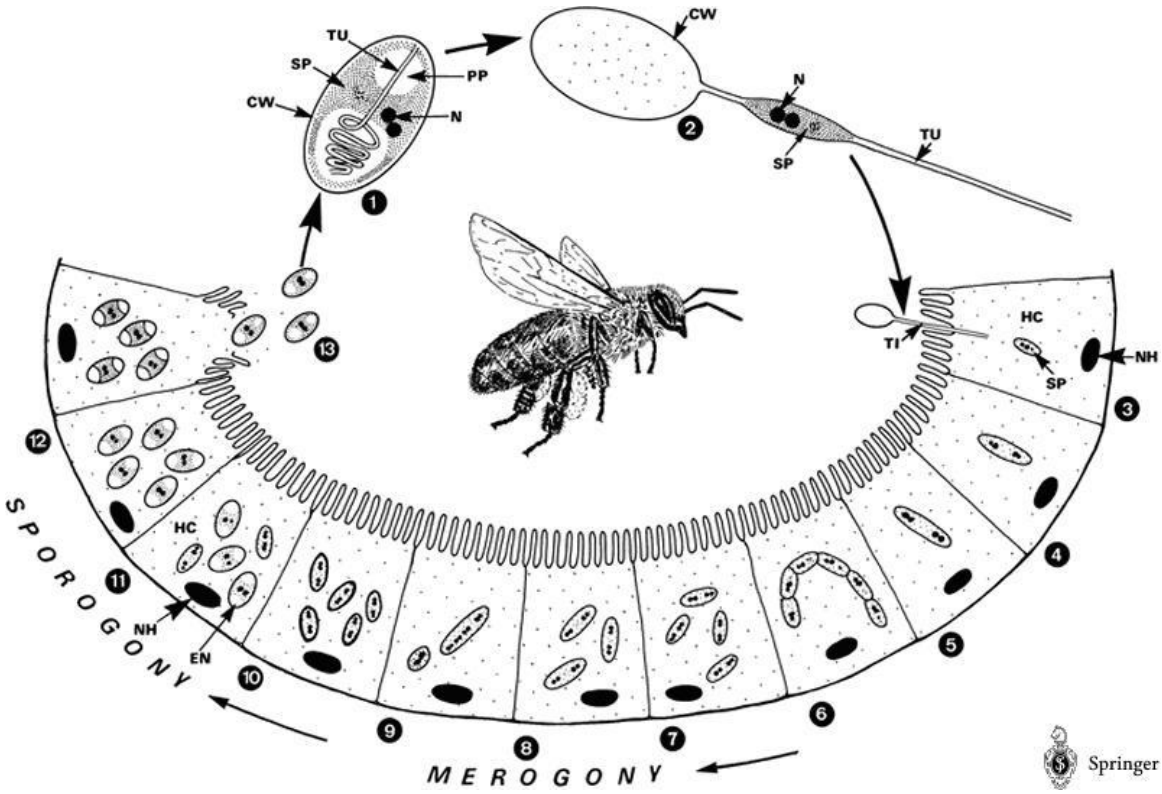

Fig. 11. Life cycle of* Nosema apis*. The spore injects its contents into a gut epithelial cell, multiplies, and eventually causes the cell to burst and release the new spores back into the gut.* Nosema *can also reproduce "vegetatively", cell to cell. © Springer Life Sciences Source: http://scientificbeekeeping.com/images/stories/nosema/nosema1-1.jpg

The intracellular development of *N. ceranae* in ventricular cells seems to be similar to that of *N. apis* (Fries et al., 1996; Higes et al., 2007; Chen et al., 2009). However, epidemiological differences between these two microsporidia may explain the increased prevalence of *N. ceranae* over that of *N. apis* in colonies of *A. mellifera*. For example, *N. ceranae* appears to have less host specificity than *N. apis*. In addition, differences have been found in relation to the season in which the infection can be detected. In this sense, infections by *N. apis* have been associated with temperate climates and with seasonal patterns, being difficult to detect during the summer, presenting a slight increase in autumn and high detection in spring (Bailey, 1955; Hornitzky, 2008). On the contrary, *N. ceranae* does not appear to have this seasonality, but rather appears to infect bees throughout the year in some latitudes (Martín-Hernández et al., 2007; Giersch et al., 2009; Tapaszti et al., 2009). This could be related to the higher biotic potential of *N. ceranae* at different temperatures when compared to *N. apis* (Martín-

Hernández et al., 2007, 2009), though a recent study suggests the presence of certain seasonality of *N. ceranae* infections (Traver et al., 2012).

Pathology

The nosemosis can affect individuals of the three bee castes (queen, workers and drones) (Bailey and Ball, 1991; Fries, 1989, 1993), with a notable influence of age, as it affects more to adult bees (Wang and Moeller, 1970). On the Asian honey bee, the parasitosis caused by *N. apis* is self-limiting, which determines a lower pathogenicity. However, in European honey bees it is not, and parasitization by *N. apis* progresses rapidly in the epithelial cells of the ventricle, mainly in adult bees (foragers). In the last stages of the disease, the queen is also affected, which compromises the viability of the bee colony.

Individual level damage. The effects of *Nosema* spp. in queens are particularly important for beekeeping. A decreased development of the ovaries leads to infertility (Fyg, 1964; Liu, 1992) and consequently, to the substitution of the queen (Farrar, 1947; Furgala, 1962; Czekonska, 2000). The pathogenic action of *Nosema* species on honey bees focuses almost exclusively on the epithelium of the ventricle (Bailey, 1981; Liu, 1984). Macroscopically it appears inflamed and of whitish appearance, and it breaks easily when removed from the abdomen. The bees infected by *N. apis* accumulate excrement and thus present the abdomen dilated and distended. As a final consequence, diarrhea appears (Bailey, 1981). This is the most characteristic sign of nosemosis type A, which does not appear in infections caused by *N. ceranae*. Other visible symptoms in the bees are a bright appearance, the inability to fly as a result of the compression of the abdominal aerial sacs, and a generalized weakness resulting from malnutrition. As a consequence of the generalized weakness of parasitized bees, other symptoms appear, such as tremors and paralysis. In fact, it is common to find groups of dying or dead bees in front of the hive in colonies with high levels of infection. All these damages may be aggravated by the possible pathogenic action of various viruses associated with *Nosema* spp., such as the filamentous virus (VF) or the blackback virus (VRN) (Bailey, 1982).

Colony level damage. As in the individual bees, in the colonies affected by nosemosis no obvious signs of disease are observed. The latent form of the disease is more common during the winter, and though the symptoms are not apparent, the physiology of the bees is affected. The only perceptible symptoms in the hives are: a decrease in the number of individuals in the colony, a greater food consumption and a lack of dynamism in the spring, which leads to lower production of honey in the hives affected. Premature aging of bees has also been described, which results in reduced longevity of the bees (Furgala and Mussen, 1990). These effects are also accompanied by changes in the division of tasks and precocity in the foraging (Wang and Moller, 1970), which affects

the behavior of the bees. Woyciechowski and Kozlowski (1998) also found an increased foraging activity in workers affected by *N. apis* during adverse weather conditions.

The global weakening of the colony is probably due to the fact that heavily infected bees (mainly foragers) often do not return to the hive, which is evidenced with the presence of dead and infected bees far from the colony (Higes et al., 2008, 2009; Krajl and Fuchs, 2010). As other microsporidia, *N. ceranae* partially suppresses the humoral and cellular defense mechanisms of the bees, which does not occur with *N. apis* (Antúnez et al., 2009). The nosemosis type-C is considered a health problem of great impact both in the individual bee (Paxton et al., 2007; Martín-Hernández et al., 2009; Antúnez et al., 2009) and in the colony as a whole (Higes et al., 2008). Indeed, this type of nosemosis has been associated with the sudden collapse of *A. mellifera* colonies (Higes et al., 2008, 2009), and with an increased risk of colony death if it is not actively controlled (Martín-Hernández et al., 2007)

8. Relationship between pathogens prevalence and host genetic diversity

In the case of the *Varroa* mite, its population structure has been studied by molecular markers such as mitochondrial DNA (mtDNA, Warrit et al., 2006) and microsatellites (Rueppell et al., 2011) in relation to its original host *A. cerana*. However, the interaction of this mite with its new host *A. mellifera*, is scarcely known from the molecular point of view. In a study carried out in the Iberian Peninsula (Muñoz et al., 2008), the genetic profile of the mites present in 575 colonies was determined by mtDNA characterization, identifying the presence of a haplotype called Korea (more virulent) in all the colonies analyzed except one. In this study, any significant relationship between the genetic diversity of the parasite and that of the host was detected.

Regarding the microsporidia of the genus *Nosema*, the first studies that refer to the molecular characterization of the host bee populations (through mtDNA, Solorzano et al., 2009), and to the relationship with the presence of *N. ceranae* and/or *N. apis*, have been performed on honey bees from Turkey (Whitaker et al., 2011) and the Balkan countries (Stevanovic et al., 2011). None of them detailed the existence of any significant relationship between the presence of *Nosema* and the genetic lineages of the bees. Indeed, in the case of *N. ceranae,* the rapid dispersion of these new pathogen, not only in Spain but also worldwide (Fries, 2010), suggests a successful colonization process and a reduced adaptation of the new host *A. mellifera*.

Presently, the invasion of an ecosystem by exotic species is regarded as one of the most important sources of biodiversity loss, which may even lead to the eradication of the local species in the ecosystem (Deredec and Courchamp, 2003). *V. destructor* and the microsporidia of the genus *Nosema*, mainly *N. ceranae*, are among the current invasive species most damaging to the honey bees. However, the current knowledge on

these pathogenic organisms and their interactions with the host *A. mellifera*, is still scarce and insufficient (Martinson et al., 2011).

To make decisions on bee management and conservation priorities, it is essential to document the extent and the infestation rates of the parasites, as well as to elucidate the factors that contribute to the parasitism and its potential effects on the structure and genetic variability bee populations. In this sense, the Iberian Peninsula provides an interesting scenario to analyze the relationships of the parasites with the populations of host bees, due, among other factors, to the presence of two evolutionary lineages of *A. m. iberiensis* and the remarkable genetic diversity of its populations (Cánovas et al., 2008, De la Rúa et al., 2009b).

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Thesis outline, objectives and hypotheses

(Estructura de la tesis, objetivos e hipótesis)

>> Main objective and outline of the thesis

The main objective of this thesis is to study the interactions between the genetic diversity of *A. m. iberiensis* populations in Spain and the incidence of the current most harmful parasites and pathogens affecting honey bee colonies.

To achieve this objective, the thesis has been divided in two parts:

In Part I, the genetic diversity and the pathogens prevalence was temporally analyzed at a Iberian scale in colonies sampled randomly in two surveys carried out in 2006 (113 colonies) and 2010 (115 colonies). First, we examined the relation between the two mitochondrial evolutionary lineages (African and Western European) of the Iberian honey bees, and the distribution of three major pathogens (*V. destructor, N. ceranae* and *N. apis*) on colonies sampled in 2006 and 2010 (Chapter I). Subsequently, microsatellite markers were used to assess the potential impact of the rapid spread of these pathogens on the genetic diversity of Iberian honey bees collected over the two sampling periods (Chapter II).

In Part II, experimental designs were focused on the study of colony managing practices in Spain, in relation to the genetic diversity of the Iberian honey bee, and the dispersion of parasites and pathogens across the national territory. A first study was designed to study the effect of migratory practices on the dispersal of the fungus *Ascosphaera apis* in colonies subjected to small-scale regional level movements (Chapter III). Finally, a wider assay was designed to evaluate the effects of long-scale migratory beekeeping on the prevalence of important pathogens (*V. destructor*, *Nosema* spp. and DWV), as well as on the genetic diversity of honey bee colonies in Spain (Chapter IV).

>> Objectives and hypotheses of the four chapters:

<u>Chapter I:</u> Linking evolutionary lineage with parasite and pathogen prevalence in the Iberian honey bee

The main goal of this study was to examine whether the prevalence of three major honey bee pathogens (*V. destructor, N. ceranae* and *N. apis*) differed between the two evolutionary lineages (African and Western European) of the honey bees in the Iberian Peninsula.

The particular objectives in this chapter are:

(i) to determine the distribution of the evolutionary lineages and the mitochondrial haplotypes of the honey bee colonies sampled in 2006 and 2010

(ii) to detect the presence in the colonies of the mite *V. destructor,* by visual inspection, and the microsporidia *N. apis and N. ceranae*, by molecular identification

(iii) to analyze the relations between the distribution of the evolutionary lineages and mitochondrial haplotypes of the colonies and the prevalence of the three pathogens detected in 2006 and 2010

The initial hypothesis to test in this work is that the prevalence of parasites and pathogens differs between honey bees belonging to the two evolutionary lineages (African and Western European) present in the Iberian honey bee populations.

Chapter II: Stable genetic diversity despite parasite and pathogen spread in honey bee colonies

The main goal of this study was to evaluate the effect of the incidence of *V. destructor*, *N. ceranae* and *N. apis*, on the genetic diversity of the populations of *A. m. iberiensis* in 2006 and 2010 in Spain.

The particular objectives in this chapter are:

(i) to analyze the genetic diversity of *A. m. iberiensis* in two different samples obtained in 2006 and 2010 using microsatellite markers

(ii) to assess the relationship between the prevalence of *V. destructor* and *Nosema* spp. with the genetic diversity in *A. m. Iberiensis* populations, in the two years analyzed (2006 and 2010)

(iii) to evaluate whether any of the microsatellite alleles analyzed was associated to genes possibly involved in the adaptive response of honey bees to these harmful agents

The initial hypothesis here is that the rapid spread of diseases, such as varroosis and nosemosis, associated with massive honey bee colonies mortality in Spain and around the World, has affected the genetic diversity of the Iberian honey bee populations, leading to a reduction in the global genetic diversity level and a directional selection of certain genotypes.

Chapter III: Effect of small-scale migratory movements on the dispersion of *Ascosphaera apis* in *Apis mellifera iberiensis* colonies

The main objective of this study is to investigate the possible correlation between the prevalence of *Ascosphaera apis* in honey bee colonies and the practice of small-scale migratory beekeeping.

The particular objectives in this chapter are:

(i) to implement the molecular characterisation of the causal agent of chalkbrood in adult worker honey bees of *A. m. iberiensis*.

(ii) to search for *A. apis* in symptomatic and asymptomatic colonies of both stationary and migratory apiaries from Murcia (south-eastern Spanish province)

The hypothesis in this chapter is that small-scale transportation of beehives affects colonies health in migratory apiaries, leading to higher incidence of pathogens.

<u>Chapter IV:</u> The effect of migratory beekeeping on the prevalence of pathogens in honey bee colonies and on their genetic composition

The main goal of this study is to determine the effects of long-scale migratory beekeeping on the prevalence of important pathogens (*Varroa destructor*, *Nosema* spp. and DWV) in honey bee colonies in Spain, and on their genetic composition.

The particular objectives in this chapter are:

(i) to determine the pathogen prevalence (*V. destructor*, *Nosema* spp.) and viral load (DWV) in stationary and migratory colonies sampled in four different moments of a migratory period (June - October 2015)

(ii) to assess the effects of migratory beekeeping on the prevalence of pathogens by comparing groups of migratory and stationary hives, that: (a) share the same genetic background but different management strategies; (b) differ in genetic backgrounds but are treated with the same management; and (c) are settled in the same location and subjected to the same environmental conditions, but have different genetic background and are treated with different management strategies

(iii) to assess whether migratory beekeeping has an effect on the genetic diversity and patrilines composition (through microsatellites genotyping) of colonies, by comparing the changes in genetic diversity between migratory and stationary colonies from the same apiary in two different moments of a migratory period (June - October 2015)

The first hypothesis to be tested is that stress factors associated to migratory management affect colony health and disease transmission, resulting in a higher prevalence of pathogens and viruses in the migratory colonies. Secondly, it is also hypothesized that factors associated to migratory beekeeping affect genetic diversity within the colonies, and favor particular patrilines and alleles, depending on their selective value against the external stressors prevailing in the migratory areas. Additionally, changes in the genetic composition of colonies due to queen replacement and its subsequent matting in the region of migration are also expected.

PART I

Relationship between genetic diversity and pathogens prevalence in *A. m. iberiensis* colonies

Chapter I

(Capítulo I)

Linking evolutionary lineage with parasite and pathogen prevalence in the Iberian honey bee

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Linking evolutionary lineage with parasite and pathogen prevalence in the Iberian honey bee

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Abstract

The recent decline in honey bee colonies observed in both European countries and worldwide is of great interest and concern, although the underlying causes remain poorly understood. In recent years, growing evidence has implicated parasites and pathogens in this decline of both the vitality and number of honey bee colonies. The Iberian Peninsula provides an interesting environment in which to study the occurrence of pathogens and parasites in the host honey bee populations due to the presence of two evolutionary lineages in *A. m. iberiensis* (Western European [M] or African [A]). Here, we provide the first evidence linking the population structure of the Iberian honey bee with the prevalence of some of its most important parasites and pathogens: the *Varroa destructor* mite and the microsporidia *Nosema apis* and *Nosema ceranae*. Using data collected in two surveys conducted in 2006 and 2010 in 41 Spanish provinces, the evolutionary lineage and the presence of the three parasitic organisms cited above were analyzed in a total of 228 colonies. In 2006 *N. apis* was found in a significantly higher proportion of M lineage honey bees than in the A lineage. However, in 2010 this situation had changed significantly due to a higher prevalence of *N. ceranae*. We observed no significant relationships in either year between the distributions of *V. destructor* or *N. ceranae* and the evolutionary lineage present in *A. m. iberiensis* colonies, but the effects of these organisms on the genetic diversity of the honey bee populations need further research.

Keywords: *Apis mellifera iberiensis*, *Varroa destructor*, *Nosema apis*, *Nosema ceranae*, Evolutionary lineages, Parasitic prevalence.

1. Introduction

The honey bee *Apis mellifera* Linnaeus 1758 is one of the world's most beneficial insects, given its crucial role both in honey production and as the main pollinator of crops and entomophilous wild species around the globe. The value of the crop pollination service performed by honey bees is estimated at millions of euros per year (Morse and Calderone, 2000; Klein et al., 2007; Gallai et al., 2008) and it is incalculable in the case of wild flora. The evolutionary his-tory of *A. mellifera* is quite complex due to the contribution of both natural and anthropic factors (Moritz et al., 2005). Indeed, this species has been managed by man for centuries. According to recent molecular data (Whitfield et al., 2006) this species appeared in Africa about two million years ago, and from there it spread throughout Europe and the Middle East, resulting in the generation of five distinct evolutionary lineages through successive colonization: African (A), Western European (M), Eastern European (C), Middle East (O) and Ethiopian (Y).

The A and M evolutionary lineages are dispersed naturally throughout the Iberian Peninsula. Through morphometric and behavioral studies it was hypothesized that the Iberian population of honey bees, *A. m. iberiensis* (Engel, 1999), is the result of an extensive integration among honey bees of the M lineage that survived the last glacial maximum, and of North African honey bees (A linage) that recolonized south-eastern Europe (Serrano et al., 2011). Multiple studies based on the analysis of enzymes, pheromones, and microsatellite and mitochondrial DNA (De la Rúa et al., 2009a) support this proposition. Hybridization between honey bees of both lineages have resulted in a clinal distribution of populations, characterized by a decrease in the frequency of the A lineage along the SW to NE axis of the peninsula, accompanied by a parallel increase in the frequency of the M lineage (Cánovas et al., 2008; De la Rúa et al., 2009b).

The structure of the honey bee population reflects both its evolutionary history and its specific adaptations to the different bio-geographic areas it occupies. This genetic diversity is essential to maintain population fitness, allowing the population to withstand short-term disruption due to environmental changes or the presence of parasites and/or pathogens, and to evolve in response to these changes (Frankham et al., 2010). The natural distribution of *A. mellifera* in the Iberian Peninsula has probably been influenced in recent decades by beekeeping techniques. The intensive practice of transhumance (which currently affects 80% of the colonies), the sale of colonies from one region to another, and particularly, the massive loss of colonies due to the spread of parasites and pathogens, nosemosis and other diseases, is rapidly altering the previously described clinal pattern (Cánovas et al., 2011).

In recent decades, the loss of honey bee colonies worldwide has increased public awareness and concern about the future of honey bees, and numerous efforts have been devoted to quantifying this phenomenon and identifying the causes (Cox-Foster et al., 2007; Coloss, 2009; Giray et al., 2010; EFSA, 2008; Higes et al., 2010 a, b; Bromenshenk et al., 2010; Bacandritsos et al., 2010; Hatjina et al., 2011; Soroker et al., 2011). A typical case is the phenomenon of Colony Collapse Disorder (CCD), an unexplained loss of honey bee colonies that meets specific defined criteria (Cox-Foster et al., 2007; van Engelsdorp et al., 2009). To date, the most significant variable related to the CCD is the increased incidence of pathogens in honey bees (van Engelsdorp et al., 2009; Bromenshenk et al., 2010). However, our understanding of the pathogenic organisms and their interactions with the host *A. mellifera* remains limited (Martinson et al., 2011).

The mite *Varroa destructor* Anderson and Trueman (2000) and the microsporidium *Nosema ceranae* Fries et al. (1996) are among these parasitic organisms. *V. destructor* is an obligate ectoparasite of *A. mellifera* that feeds on the haemolymph of both adult and immature honey bees and reproduces in cells of the comb containing the brood (Sammataro et al., 2000; Rosenkranz et al., 2010). It originated in Asia, where it parasitizes the Asian honey bee *Apis cerana* Fabricius 1793. Infected colonies suffer both

direct damage by parasitism and indirect damage from microbial and viral pathogens of which the mite is a vector (Johnson et al., 2009; Highfield et al., 2009). Moreover, *V. destructor* suppresses the immune response in honey bees, resulting in the amplification of pre-existing infections (Yang and Cox-Foster, 2007), which further accentuates their impact. The jump from the host *A. cerana* to *A. mellifera* occurred about 50 years ago when the Western honey bee was introduced into Asia (Rosenkranz et al., 2010). Currently, the mite is found in colonies worldwide due to the global trade of honey bees and honey bee products. In Spain it was first detected on the French border in December 1985 and it has since spread through-out the country, killing approximately 40% of the Spanish colonies within the first year of parisitization (Llorente, 2003).

The population structure of this parasite in relation to its original host *A. cerana* has been studied using molecular markers, including mitochondrial DNA (Warrit et al., 2006) and nuclear microsatellite markers (Rueppell et al., 2011). The results suggest that the current distribution of genetic lineages of *Varroa* and *A. cerana* (determined by analyzing the variation of specific regions of the mitochondrial chromosome) is a consequence of the biogeographic history and current patterns of migration, rather than host-parasite co-evolution. Such co-evolution would require a correspondence of both the mitochondrial haplotypes and the nuclear genome (microsatellites) between the lineages of the mite and those of the host honey bee, and to date, no such analysis of the mite and its new host (*A. mellifera*) has been performed. In the Iberian Peninsula, the genetic profile of the mites present in 575 colonies was determined by characterizing the mitochondrial chromosome (Muñoz et al., 2008). This analysis identified the more virulent haplotype, termed Korea, in all but one of the colonies analyzed. However, an analysis of the mitochondrial haplotypes of the honey bees from the apiary in which the less virulent Japan haplotype was found revealed no significant relationship between the lineage of the parasite and that of its host.

Contact between the two sister honey bee species, *A. cerana* and *A. mellifera*, may have also contributed to the spread of microsporidia (Fries, 2010). Microsporidia such as *Nosema apis* and *Nosema ceranae* are obligate intracellular pathogens that infect the ventricular epithelium of honey bees and that are transmitted by spores. The transport of *A. mellifera* throughout the world has been associated with the spread of *N. apis* Zander 1909, the microsporidium that historically has infected these honey bees (Mutinelli, 2011). However in 1996, a new species called *N. ceranae* was described in the Asian host *A. cerana* (Fries et al., 1996). Initially this species was thought to be restricted to the eastern honey bee and geographically limited to Asia; however it was recently reported in *A. mellifera* in Spain, Europe and throughout the rest of the world (Higes et al., 2006, 2010a). In fact, recent studies have described *N. ceranae* to have a higher prevalence than *N. apis* in *A. mellifera*, although both are widely distributed (Higes et al., 2006; Klee et al., 2007; Paxton et al., 2007; Martín-Hernández et al., 2007, 2011).

The variable prevalence and abundance of *Nosema* species, both at the colony and individual honey bee level, may be influenced by many factors, including host genetic variation, climate, levels of exposure and treatments administered or beekeeping practices (Runckel et al., 2011; Botías et al., 2011a). However, studies of *Nosema* prevalence in different hosts are scarce. Molecular characterization of the host honey bee populations (analyzing mitochondrial DNA: De la Rúa et al., 2009a) was mentioned in two recent studies in which the prevalence of *N. ceranae* and *N. apis* in honey bees in Turkey (Whitaker et al., 2011) and Balkan countries (Stevanovic et al., 2011) was analyzed. However, neither study reported any significant relationship between infestation by the two species of *Nosema* and the genetic lineage of honey bees.

The Iberian Peninsula provides an interesting environment in which to analyze the relationships between parasites and host honey bee populations due to the presence of two evolutionary distinct lineages of *A. m. iberiensis*, and the wide genetic variability of the populations (Cánovas et al., 2008; De la Rúa et al., 2009b). Given the existence of a distribution gradient of two lineages (African and Western European) in the Iberian honey bee population, we examined whether parasite prevalence differed between these two lineages. We determined the distribution of the evolutionary lineages and mitochondrial haplotypes of honey bees in Spain collected over two sampling periods in 2006 and 2010. The presence of the *V. destructor* mite had been previously determined in these samples by visual inspection and that of the microsporidian *Nosema* species (*N. apis* or *N. ceranae*) by molecular identification.

2. Materials and methods

2.1. Sampling

The present study analyzed bees from a total of 228 colonies in 41 different Spanish provinces, sampled randomly in two surveys carried out in 2006 (113 colonies) and 2010 (115 colonies: Fig. 1). This sampling process was part of two wider surveys de-signed to study the honey bee colony loss phenomenon in Spain. Each sample was composed of about 150 adult inner worker honey bees from each colony. One adult worker honey bee from each colony was placed directly into 100% ethanol and stored at 20 ºC until processing in the laboratory of Molecular Ecology (Department of Zoology and Anthropology, Faculty of Veterinary Medicine, University of Murcia).

2.2. Genetic determination of evolutionary honey bee lineages

Two left hind legs were removed from one worker honey bee per colony and the total DNA was extracted using a 5% Chelex solution according to a modified version of the protocol described previously (Walsh et al., 1991).

Determination of the evolutionary lineages was performed by RFLP analysis (restriction fragment length polymorphism) of the cytochrome oxidase gene I (COI) (Hall and Smith, 1991) using the primers known as Jerry and Pat (C1-J-2183 and TL2-N-3014: Simon et al., 1994). The amplification program consisted of: an initial denaturation of 5 min at 94 ºC; 35 cycles of 45 s at 92 ºC, 45 s at 48 ºC, 2 min at 62 ºC; and a final extension over 10 min at 65 ºC. The PCR products were digested with the Hinc-II restriction enzyme at 37 ºC for 10h in a thermocycler PTC 100 (MJ Research) and after digestion the resulting fragments were electrophoretically separated on 1.5% agarose gels.

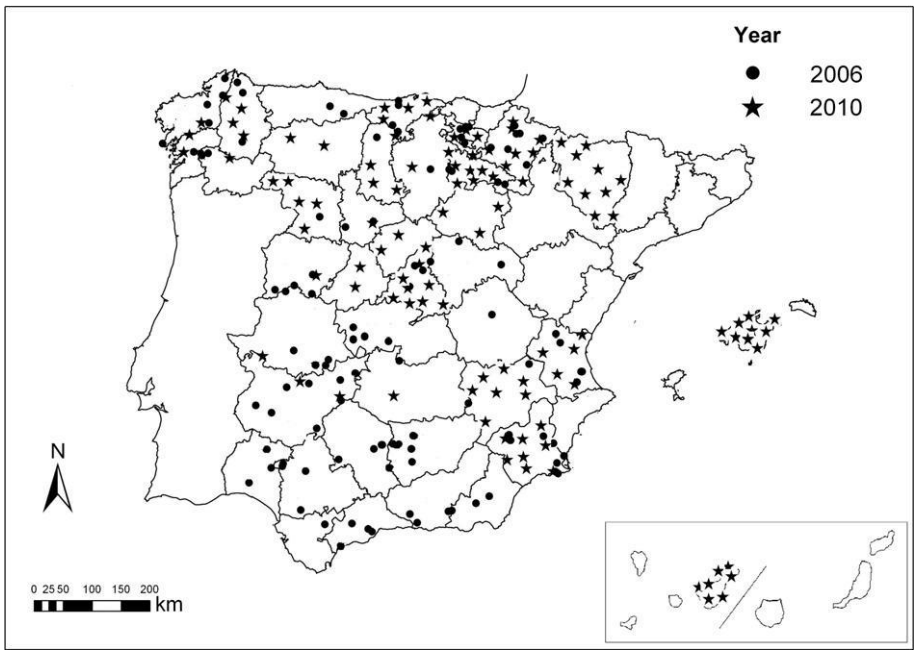

Fig. 1. Map showing the location of the colonies sampled in 2006 and 2010.

Analysis of the intergenic region between the $tRNA^{leu}$ and the cytochrome oxidase gene II (cox2) was carried out as described previously (Garnery et al., 1992) using the E2 and H2 primers. The PCR program used was the same as that described above and the size of the fragments amplified was determined by agarose gel electrophoresis (1.5%). The remaining volume was digested at 37 ºC with the enzyme Dra-I for 10h in a PTC 100 thermocycler (MJ Research) and the resulting digestion fragments were electrophoresed on 4% NuSieve agarose gels. Haplotypes were determined by comparison with the patterns described previously (Garnery et al., 1993; Franck et al., 2001; Rortais et al., 2011).

2.3. Detection of the *Varroa* and *Nosema spp* in worker bee samples

About 100 inner worker honey bees from each colony were analyzed to detect the presence of *Nosema* spp. spores and *V. destructor* according to OIE recommendations (OIE, 2008).

To determine *Nosema* species, the abdomens of 100 honey bees from each colony were pooled and macerated in 10 ml of ultrapure PCR-grade water (Milli-Q system). DNA extraction and PCR was performed as described previously, using an internal PCR control to determine the reliability of analysis (Martín-Hernández et al., 2007; Botías et al., 2011a). PCR reactions (50 µL) were carried out in a Mastercycler® ep gradient S apparatus (Eppendorf), each containing 25 µL of Fast Start PCR Master (No. 04710452001 Roche Diagnostic), 0.4 µM of each primer for *Nosema* spp. (321-APIS-F/R and 218-MITOC-F/R), 0.03 µM of COI-F/R primers (Martín-Hernández et al., 2007), 0.2 mg/ml BSA, 0.1% Triton X-100 and 5 µL of DNA template. The thermocycler program used was as follows: 95 ºC (10 min); 35 cycles of a 30 s denaturation at 95 ºC, a 30 s elongation at 61.8 ºC, a 45 s extension at 72 ºC; and a final extension step at 72 ºC for 7 min. Negative controls (for DNA extraction and PCR) were included in all PCR experiments and all PCR products were analyzed in a QIAxcel System (Qiagen) using a QIAxcelDNA High Resolution Kit (Qiagen, No. 929002).

2.4. Statistical analysis

The relationship between the categorical variables ‘‘evolutionary lineage of Iberian honey bees’’ and ‘‘presence of *V. destructor*, *N. apis* and *N. ceranae*’’ was analyzed using the Pearson Chi-square test and Fisher exact test for contingency tables. The raw data (Ta-bles 1 and 2 in Supplementary Material) were analyzed at the Section of Scientific Computing and Statistical Support of the University of Murcia. The SPSS 15 program for Windows was used for all analyzes.

3. Results

3.1. Genetic characterization of honey bee colonies

The apiaries sampled belonged to three evolutionary lineages: A, M and C. In 2006, 63% of the 113 colonies sampled corresponded to the A lineage, while the remaining 37% were of the M lineage. No colonies belonging to the evolutionary lineage C were detected. By contrast, in 2010 the C lineage was detected in 2 of the 115 colonies sampled, while A and M lineages were detected in 48% and 50% of the colonies, respectively.

The lineage distribution observed for the different provinces demonstrated the existence of a clinal pattern similar to that de-scribed previously (De la Rúa et al., 2009b), whereby the frequency of M haplotypes decreased in the NE-SW direction, while there was an increase in the frequency of the A haplotypes.

A total of 21 mitochondrial haplotypes were identified after the RFLP-PCR analysis of the tRNAleu-cox2 intergenic region: 12 belonged to African haplotypes, of which A2 was the most common in both 2006 and 2010, 8 belonged to the M lineage, with M4 and M7 the most frequent, and the C1 haplotype corresponding to the Eastern Europe lineage, which was only detected in 2% of the 2010-collected colonies (Tables 1 and 2 in Supplementary Material).

Genetic diversity was not significantly different between 2006 and 2010 (Student´s t-test; t = 0.442, p = 0.681). In the sample taken in 2006, 8 A and 6 M haplotypes were detected, while 11 A, 7 M and 1 C haplotype appeared in 2010. The high number of haplotypes found and the low frequency of several of these haplotypes were associated with a large degree of statistical error. Thus, statistical analyzes were conducted using only the variables ‘‘evolutionary lineage’’ and ‘‘presence of *Varroa*, *N. apis* and *N. ceranae*’’.

3.2. Presence of *Varroa* in the colonies sampled

A total of 31% of the colonies from the 2006 study tested positive for *Varroa*, as opposed to 19% of those sampled in 2010. Moreover, in 2006 *Varroa* parasitization was more common in colonies of the M than of the A lineage (Fig. 2), while this trend was reversed in 2010. Of the two C lineage colonies detected in 2010, one tested positive for *Varroa.*

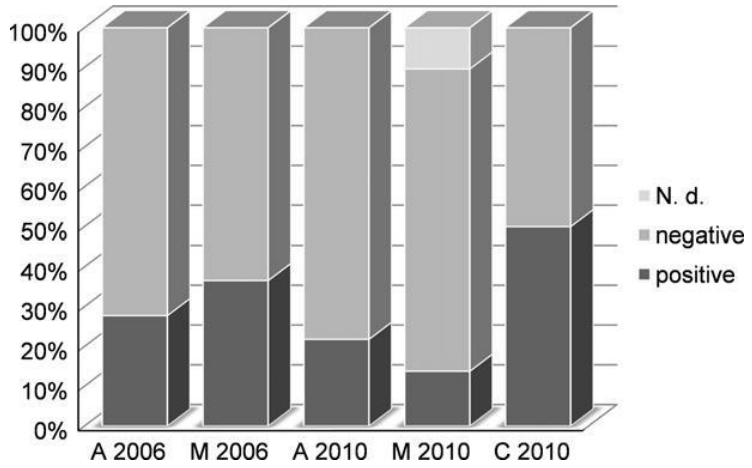

Fig. 2. **Varroa destructor** ***occurrence per evolutionary lineage and sampling year. N.d., not determined.***

Statistical analysis revealed no significant relationship between the variables ''evolutionary lineage'' and ''presence of *Varroa*'' in samples collected in 2006 (X^2 = 0.948, d.f. = 1, p = 0.330) or those collected in 2010 (X^2 = 0.728, d.f. = 1, p = 0.394), and the Chi-square tests resulted in P values >0.05 for both samples. Together these findings indicated that the two variables studied are independent.

3.3. Presence of *Nosema* in the colonies sampled

In 2006, *Nosema* spp were detected in 62.5% of the honey bee colonies sampled. Of the infected colonies 20.8% tested positive for *N. apis*, 77.8% for *N. ceranae*, and 1.4% were infected by both species simultaneously (Fig. 3). The analysis of the relationship be-tween the parasitic species and the host lineage showed that 88.9% of the infected lineage A colonies were parasitized by *N. ceranae*, whereas only 9.8% were infected by *N. apis* and in 2.2% were both species identified simultaneously. By contrast, 59.3% of infected lineage M colonies were infected with *N. ceranae*, and the remaining 40.7% with *N. apis*.

In 2010, the percentage of microsporidia-infected colonies had increased to 72.2% and while *N. ceranae* was detected in 81.9% of infected colonies, only 3 (3.6%) of the 113 colonies examined were infected by *N. apis* alone (Fig. 3). Simultaneous infection by *N. apis* and *N. ceranae* was observed in 12 colonies (14.5% *vs.* 1.4% in 2006). There were no significant differences in the pattern of infection by the two microsporidia species between the two host lineages. *N. ceranae* was present in 81.1% of infected lineage A colonies, *N. apis* in 5.4% and both microsporidia in 13.5%. A similar distribution was observed in the M lineage in which 82.6% of infected colonies were parasitized by *N. ceranae*, 2.2% by *N. apis* and 15.2% by both species. Both C lineage colonies tested negative for both *N. ceranae* and *N. apis*.

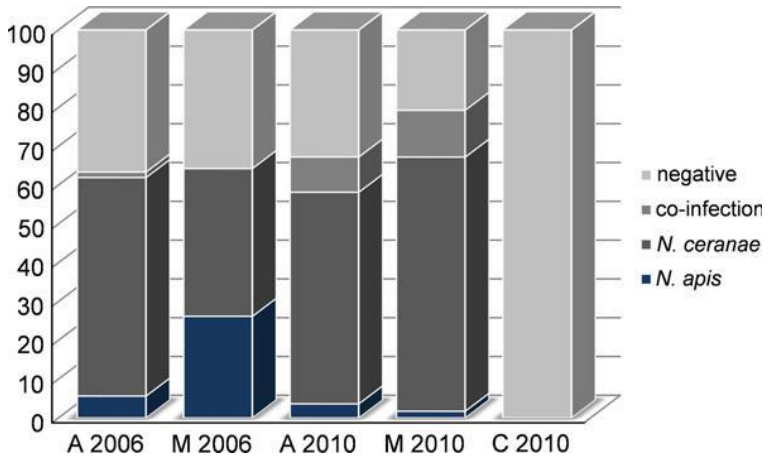

Fig. 3. **N. apis *and* N. ceranae *occurrence in each evolutionary lineage and sampling year.***

Analysis of the 2006 sampling using the Chi-square test revealed a significant relationship between the honey bee evolutionary lineage and the presence of *N. apis*. Comparing the variables two by two (*N. apis* evolutionary lineage and *N. ceranae* evolutionary lineage) and correcting with the Bonferroni post hoc method, we observed significantly higher *N. apis* infection of M lineage colonies than in those of the A lineage (X^2 = 10.384, d.f. = 2, p = 0.006). When colonies with no microsporidia infestation were excluded from the analysis, this difference was even greater (X^2 = 10.059, d.f. = 1, p = 0.002). The X^2 and P values obtained indicate that these differences are unlikely if we assume both variables to be independent and thus, we reject the null hypothesis and assume that these two variables are dependent.

The relationship between microsporidian species and honey bee evolutionary lineage found in the 2006 sampling was not observed in those collected in 2010, when *N. ceranae* was present in almost all the colonies of both the A and M lineages. All comparisons of variables resulted in P values >0.05 and thus, the null hypothesis of independence could not be rejected.

4. Discussion

The present study provides the first description of the prevalence of some of the most important honey bee parasites (*V. destructor*) and pathogens (*N. apis* and *N. ceranae*) in individual evolutionary lineages of the Iberian honey bee *A. m. iberiensis*. These three parasitic organisms (especially *N. ceranae*) have been associated with depopulation of honey bee colonies and increased colony losses in temperate areas (Martín-Hernández et al., 2007; Higes et al., 2008, 2009, 2010b; Borneck et al., 2010; Bacandritsos et al., 2010; Soroker et al., 2011; Hatjina et al., 2011).

The distribution of African (A) and West-European (M) lineages observed was in agreement with that described elsewhere (De la Rúa et al., 2009b), whereby A lineage haplotypes are substituted by M lineage haplotypes along a SW-NE gradient across the peninsula. The mitochondrial diversity of the colonies analyzed corroborated the large variation among Iberian honey bees (Cánovas et al., 2008; De la Rúa et al., 2009b). We detected 21 different haplotypes in the 228 colonies analyzed: 12 belonging to the A lineage, 8 to the M lineage and 1 to the Eastern (C) lineage. This distribution represents a 5-fold greater variability than that observed in honey bee populations in France, where 85 different haplotypes were detected in 4552 colonies analyzed (Rortais et al., 2011).

The presence of the evolutionary lineage C is evidence of the introduction of foreign queens to the peninsula by professional beekeepers. The practice of buying queens of different origins may contribute to the spread of pathogens (Mutinelli, 2011), as there are often inadequate sanitary control measures or quarantine of the queens and their cohort of worker honey bees. The effective-ness of these control measures was

demonstrated in Portugal in 2004, when the Portuguese National Veterinary Service prevented the introduction of larvae from the small hive beetle *Aethina tumida* Murray 1867, which was detected during quarantine of 122 queens legally imported from the United States (Murilhas, 2005).

The distribution of honey bee lineages and the incidence of the parasite mite did not differ depending on the year of sampling. No significant relationship was observed between parasitization by the *V. destructor* mite and the colony evolutionary lineages in either 2006 or 2010. Any type of adaptation by the honey bee to this parasite would depend on the evolutionary lineage of the host, as the Korea (K) haplotype is almost uniquely found in Spain (Muñoz et al., 2008). However, statistical analysis suggests that the distribution pattern of *V. destructor* is independent of that of the evolutionary lineages of *A. m. iberiensis* in Spain. These results are consistent with those reported for the original host species *A. cerana* (Warrit et al., 2006), when it was proposed that the population structure of *V. destructor* and *A. cerana* are not related but rather they are a consequence of the biogeographic history and current migration patterns of both species.

The relationship between microsporidia and the population structure of *A. mellifera* has not been analyzed previously. We only observed more frequent infection by *N. apis* in samples from 2006 whereas infection by this microsporidium was significantly reduced in samples collected in 2010. Moreover, in the 2006 sampling we observed a significant relationship between honey bee evolutionary lineage and infection by specific microsporidia species. In particular, *N. apis* infected colonies of M lineage to a greater degree the than those of the A lineage. This may be due to factors such as adverse weather conditions with low temperatures and high rainfall, which are more frequent in the northern peninsula where the M lineage is predominantly distributed. These conditions favor the spread of *N. apis* in the colony due to the decreased activity of forager honey bees (Bailey 1955; OIE 2008). Taken together, these findings suggest that the variation in the prevalence of *N. apis* between the two evolutionary lineages of honey bees could be due to adaptation by both the parasite and the host to the different climates of the Iberian Peninsula, rather than to host-parasite co-evolution.

The pattern of infection described for 2006 had significantly shifted by 2010, as the number of infected colonies by *N. ceranae* (alone or mixed with *N. apis*) markedly increase from 79.2% to 96.4%, whereas those exclusively infected by *N. apis* decreased from 20.8% to 3.6%. Given that the sampling was limited to these two years, it is necessary to test again these levels of infection to check whether the increase in *N. ceranae* prevalence is somehow related to the decrease of *N. apis*. The recent dispersion of *N. ceranae*, both in Spain and worldwide (Fries, 2010; Botías et al., 2011b), is indicative of the successful colonization by the parasite and the limited adaptation of the new host, *A. mellifera*. Indeed, we found no relationship between *N. ceranae* infection and the evolutionary lineage of the infected colonies in either 2006 or 2010. Thus, in the

case of *N. ceranae*, these results contradict the hypothesis initially proposed regarding a relationship between the presence of the pathogens and the evolutionary lineage of the host.

Another potential effect of the rapid expansion of nosemosis type C due to *N. ceranae* (Higes et al., 2010a) in Iberian honey bee populations is the reduction of genetic diversity due to the high risk of collapse in colonies infected by *Nosema* (including asymptomatic colonies during the incubation period: Martín-Hernández et al., 2007; Higes et al., 2008). Therefore, this study should be complemented by future analyzes of nuclear markers such as microsatellites (Solignac et al., 2003), which are particularly suitable for analyzing the genetic structure of populations (De la Rúa et al., 2009a).

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Supplementary Material

Table 1: Sample data 2006

SAMPLE NAME	REGION	PROVINCE	*NOSEMA*	*VARROA*	LINEAGE	HAPLOTIPE
PA 1004	Andalucía	Almería	Negativo	Negativo	A	A2
PA 1128	Andalucía	Almería	Cer	Negativo	A	A4
PA 1072	Andalucía	Cádiz	Negativo	Negativo	A	A8
PA 1001	Andalucía	Córdoba	Cer	Varroa	A	A2
PA 1212	Andalucía	Córdoba	Cer	Negativo	A	
PA 1229	Andalucía	Córdoba	Cer	Negativo	A	A2
PA 1230	Andalucía	Córdoba	Cer	Negativo	A	A2
PA 1102	Andalucía	Granada	Negativo	Negativo	A	A1
PA 1103	Andalucía	Granada	Cer	Negativo	A	A4
PA 1144	Andalucía	Granada	Cer	Varroa	A	A2
PA 1145	Andalucía	Granada	Cer	Negativo	A	A2
PA 1011	Andalucía	Huelva	Cer	Negativo	A	A2
PA 1213	Andalucía	Huelva	Cer	Negativo	A	A1
PA 1214	Andalucía	Huelva	Cer	Negativo	A	A1
PA 1217	Andalucía	Huelva	Cer	Varroa	A	A2
PA 1090	Andalucía	Jaén	Cer	Negativo	A	A2
PA 1223	Andalucía	Jaén	Negativo	Negativo	A	A14
PA 1231	Andalucía	Jaén	Cer	Varroa	A	
PA 1235	Andalucía	Jaén	Cer	Varroa	A	A2
PA 1236	Andalucía	Jaén	Negativo	Negativo	A	A1
PA 1237	Andalucía	Jaén	Cer	Varroa	A	A2
PA 1066	Andalucía	Málaga	Negativo	Varroa	A	A2
PA 1123	Andalucía	Málaga	Cer	Negativo	A	A2
PA 1215	Andalucía	Málaga	Cer	Negativo	A	A2
PA 1241	Andalucía	Málaga	Cer	Varroa	A	A2
PA 1069	Andalucía	Sevilla	Cer	Varroa	A	A9
PA 1163	Andalucía	Sevilla	Cer	Varroa	A	A9
PA 1218	Andalucía	Sevilla	Cer	Varroa	M	
PA 1224	Andalucía	Sevilla	Cer	Negativo	A	A2
PA 0750	Asturias	Asturias	Cer	Negativo	M	M7
PA 0751	Asturias	Asturias	Negativo	Negativo	A	A2
PA 0216	Cantabria	Cantabria	Cer	Varroa	M	M4
PA 0219	Cantabria	Cantabria	Apis	Negativo	M	M4
PA 0532	Cantabria	Cantabria	Cer	Varroa	M	M7
PA 0535	Cantabria	Cantabria	Apis	Negativo	M	M12
PA 0294	Castilla La Mancha	Albacete	Negativo	Varroa	M	M4
PA 0715	Castilla La Mancha	Ciudad Real	Negativo	Negativo	A	A1
PA 1015	Castilla La Mancha	Ciudad Real	Cer	Negativo	M	M7
PA 0566	Castilla La Mancha	Cuenca	Negativo	Negativo	M	M7

PA 0293	Castilla La Mancha	Guadalajara	Negativo	Varroa	M	M4
PA 0910	Castilla La Mancha	Guadalajara	Negativo	Varroa	M	M7
PA 0790	Castilla La Mancha	Toledo	Cer	Varroa	A	A8
PA 0791	Castilla La Mancha	Toledo	Negativo	Negativo	A	A8
PA 1269	Castilla La Mancha	Toledo	Negativo	Varroa	A	A8
PA 0573	Castilla y León	Burgos	Cer	Negativo	M	M7
PA 0693	Castilla y León	Burgos	Apis	Negativo	M	M4
PA 0671	Castilla y León	Palencia	Apis	Negativo	M	M12
PA 1044	Castilla y León	Salamanca	Cer	Negativo	M	M7
PA 1051	Castilla y León	Salamanca	Cer	Negativo	A	A3
PA 1142	Castilla y León	Salamanca	Cer	Negativo	A	A9
PA 0730	Castilla y León	Valladolid	Negativo	Negativo	M	M12
PA 0752	Castilla y León	Valladolid	Apis	Negativo	M	M7
PA 0755	Castilla y León	Zamora	Apis	Negativo	A	A10
PA 1196	Extremadura	Badajoz	Cer	Negativo	A	A8
PA 1198	Extremadura	Badajoz	Cer	Negativo	A	A3
PA 1199	Extremadura	Badajoz	Cer	Negativo	A	A8
PA 1200	Extremadura	Badajoz	Cer	Negativo	M	
PA 1204	Extremadura	Badajoz	Cer	Negativo	A	A2
PA 1206	Extremadura	Badajoz	Cer	Negativo	A	A2
PA 1207	Extremadura	Badajoz	Negativo	Negativo	A	A2
PA 1208	Extremadura	Badajoz	Cer	Negativo	M	M4
PA 1209	Extremadura	Badajoz	Negativo	Negativo	A	A2
PA 1210	Extremadura	Badajoz	Cer	Varroa	M	M8
PA 1040	Extremadura	Cáceres	Cer	Negativo	A	A2
PA 1096	Extremadura	Cáceres	Cer	Negativo	A	A3
PA 1201	Extremadura	Cáceres	Negativo	Negativo	A	A8
PA 1202	Extremadura	Cáceres	Cer	Negativo	A	A2
PA 1203	Extremadura	Cáceres	Negativo	Negativo	A	A3
PA 1205	Extremadura	Cáceres	Cer	Varroa	A	A3
PA 1182	Galicia	La Coruña	Apis	Negativo	A	A2
PA 1185	Galicia	La Coruña	Negativo	Varroa	M	M7
PA 1194	Galicia	La Coruña	Negativo	Varroa	M	M7
PA 1251	Galicia	La Coruña	Negativo	Varroa	A	A2
PA 1179	Galicia	Lugo	Cer	Varroa	M	M8
PA 1187	Galicia	Lugo	Negativo	Negativo	M	M4
PA 1192	Galicia	Lugo	Negativo	Negativo	M	M4
PA 1195	Galicia	Lugo	Negativo	Negativo	M	M4'
PA 1244	Galicia	Pontevedra	Negativo	Negativo	A	A2
PA 1246	Galicia	Pontevedra	Cer	Negativo	A	A8
PA 1253	Galicia	Pontevedra	Negativo	Negativo	A	
PA 0644	La Rioja	La Rioja	Cer	Varroa	A	A2
PA 0657	La Rioja	La Rioja	Negativo	Negativo	M	M7
PA 0840	La Rioja	La Rioja	Cer	Negativo	M	M7
PA 0845	La Rioja	La Rioja	Apis	Negativo	M	M8

PA 0358	Madrid	Madrid	Cer	Negativo	A	A4
PA 1020	Madrid	Madrid	Negativo	Varroa	A	A2
PA 1092	Madrid	Madrid	Cer	Varroa	M	M4'
PA 1093	Madrid	Madrid	Negativo	Varroa	A	A4
PA 0295	Murcia	Murcia	Apis	Varroa	A	A2
PA 0296	Murcia	Murcia	Cer	Negativo	M	M4
PA 0299	Murcia	Murcia	Negativo	Negativo	A	A2
PA 0353	Murcia	Murcia	Negativo	Negativo	A	A2
PA 0392	Murcia	Murcia	Negativo	Negativo	A	A2
PA 0393	Murcia	Murcia	Cer	Negativo	A	A2
PA 0558	Murcia	Murcia	Cer	Negativo	A	A2
PA 0579	Murcia	Murcia	Negativo	Varroa	A	A2
PA 0713	Navarra	Navarra	Negativo	Negativo	M	M7
PA 0773	Navarra	Navarra	Negativo	Negativo	M	M7
PA 0780	Navarra	Navarra	Apis	Varroa	M	M4
PA 0865	Navarra	Navarra	Cer	Negativo	M	M4
PA 0921	Navarra	Navarra	Negativo	Varroa	M	M4
PA 1119	Navarra	Navarra	Apis	Negativo	M	M7
PA 1284	Navarra	Navarra	Cer	Varroa	M	M8
PA 1313	Navarra	Navarra	Apis	Varroa	M	M8'
PA 0166	País Vasco	Álava	Apis	Negativo	M	M4
PA 0167	País Vasco	Álava	Apis	Varroa	A	A2
PA 0170	País Vasco	Álava	Apis	Negativo	M	M7
PA 0363	País Vasco	Álava	Negativo	Negativo	M	M4
PA 0583	Valencia	Valencia	Apis+Cer	Negativo	A	A2
PA 0585	Valencia	Valencia	Cer	Negativo	A	A2
PA 0586	Valencia	Valencia	Negativo	Negativo	A	A2
PA 0587	Valencia	Valencia	Negativo	Negativo	A	A1
PA 0588	Valencia	Valencia	Negativo	Negativo	A	A2

Table 2. Sample data 2010

SAMPLES NAME	REGION	PROVINCE	*NOSEMA*	*VARROA*	LINEAGE	HAPLOTIPE
PA10-1014	Aragón	Huesca	Negativo	Negativo	M	M8
PA10-1021	Aragón	Huesca	Cer	Negativo	M	M4
PA10-1024	Aragón	Huesca	Negativo	Negativo	M	M4'
PA10-1025	Aragón	Huesca	Cer	Negativo	M	M4
PA10-1026	Aragón	Huesca	Cer	Varroa	M	M4
PA10-1029	Aragón	Huesca	Cer	Negativo	M	M4
PA10-1030	Aragón	Huesca	Negativo	Negativo	M	M4
PA10-1343	Aragón	Huesca	Cer	Negativo	M	M4
PA10-1346	Aragón	Huesca	Cer	Negativo	M	M4
PA10-1349	Aragón	Huesca	Cer	Varroa	M	M7'
PA10-0760	Cantabria	Cantabria	Cer	Negativo	M	M6
PA10-0763	Cantabria	Cantabria	Cer	Negativo	M	M6
PA10-0881	Cantabria	Cantabria	Apis+Cer	Negativo	M	M4
PA10-0882	Cantabria	Cantabria	Apis+Cer	Negativo	M	M6
PA10-0884	Cantabria	Cantabria	Cer	Negativo	A	A1
PA10-0767	Castilla La Mancha	Albacete	Negativo	Negativo	A	M6
PA10-0791	Castilla La Mancha	Albacete	Cer	Negativo	A	A4
PA10-0900	Castilla La Mancha	Albacete	Apis	Varroa	A	A2
PA10-2050	Castilla La Mancha	Albacete	Cer	Negativo	A	A2
PA10-2051	Castilla La Mancha	Albacete	Apis+Cer	Varroa	A	A2
PA10-2053	Castilla La Mancha	Albacete	Cer	Negativo	A	M6
PA10-2054	Castilla La Mancha	Albacete	Apis	Negativo	A	A2
PA10-0682	Castilla La Mancha	Ciudad Real	Negativo	Negativo	A	A2
PA10-1013	Castilla y León	Ávila	Cer	Negativo	A	A2
PA10-1051	Castilla yLeón	Ávila	Cer	Negativo	A	A8
PA10-0834	Castilla y León	Burgos	Cer	Negativo	M	M4
PA10-0846	Castilla y León	León	Apis+Cer	Negativo	A	A16
PA10-1054	Castilla y León	León	Apis+Cer	Negativo	M	M4'
PA10-1017	Castilla y León	Palencia	Negativo	Negativo	M	M7
PA10-1019	Castilla y León	Palencia	Apis+Cer	Negativo	A	A8
PA10-2024	Castilla y León	Palencia	Negativo	Negativo	M	M4
PA10-1059	Castilla y León	Salamanca	Apis+Cer	Negativo	A	A3
PA10-0831	Castilla y León	Segovia	Cer	Negativo	A	A16
PA10-1031	Castilla y León	Segovia	Apis+Cer	Negativo	A	A2
PA10-0807	Castilla y León	Soria	Negativo	Varroa	A	A3
PA10-0845	Castilla y León	Soria	Apis+Cer	Negativo	M	M7
PA10-1005	Castilla y León	Soria	Cer	Negativo	M	M7
PA10-0798	Castilla y León	Valladolid	Apis+Cer	Negativo	M	M12
PA10-0662	Castilla y León	Zamora	Cer	Negativo	A	A16
PA10-0663	Castilla y León	Zamora	Cer	Negativo	A	A11
PA10-0991	Castilla y León	Zamora	Cer	Negativo	A	A2
PA10-1009	Castilla y León	Zamora	Cer	Negativo	A	A16
PA10-1050	Castilla y León	Zamora	Cer	Negativo	A	A16
PA10-1499	Extremadura	Badajoz	Cer	Negativo	A	A3
PA10-1566	Extremadura	Badajoz	Cer	Negativo	M	M4'
PA10-1567	Extremadura	Badajoz	Cer	Negativo	A	A9
PA10-0432	Galicia	La Coruña	Negativo	Negativo	A	A3
PA10-0433	Galicia	Lugo	Negativo	Negativo	M	M4
PA10-0770	Galicia	Lugo	Cer	Negativo	M	M4

PA10-0841	Galicia	Lugo	Negativo	Varroa	M	M6
PA10-1724	Galicia	Lugo	Cer	Negativo	M	M7'
PA10-0661	Galicia	Orense	Cer	Negativo	A	A16
PA10-0839	Galicia	Pontevedra	Negativo	Negativo	A	A16
PA10-1580	Islas Baleares	Mallorca	Negativo	Varroa	A	A1
PA10-1582	Islas Baleares	Mallorca	Cer	Negativo	A	A1
PA10-1584	Islas Baleares	Mallorca	Cer	Varroa	A	A1
PA10-1585	Islas Baleares	Mallorca	Negativo	Varroa	A	A1
PA10-1588	Islas Baleares	Mallorca	Negativo	Varroa	A	A1
PA10-1717	Islas Baleares	Mallorca	Cer	Negativo	M	M7
PA10-1722	Islas Baleares	Mallorca	Negativo	Negativo	A	A1
PA10-2003	Islas Baleares	Mallorca	Negativo	Varroa	M	M8
PA10-2006	Islas Baleares	Mallorca	Negativo	Varroa	A	A1
PA10-1356	Islas Canarias	Santa Cruz de Tenerife	Negativo	Varroa	A	A14
PA10-1359	Islas Canarias	Santa Cruz de Tenerife	Cer	Varroa	A	A1
PA10-1360	Islas Canarias	Santa Cruz de Tenerife	Cer	Negativo	A	A15
PA10-1361	Islas Canarias	Santa Cruz de Tenerife	Cer	Varroa	A	A14
PA10-1362	Islas Canarias	Santa Cruz de Tenerife	Negativo	Varroa	A	A4
PA10-1366	Islas Canarias	Santa Cruz de Tenerife	Negativo	Varroa	C	C
PA10-1367	Islas Canarias	Santa Cruz de Tenerife	Negativo	Negativo	C	C
PA10-1986	La Rioja	La Rioja	Cer		M	M7
PA10-1988	La Rioja	La Rioja	Cer	Varroa	M	M7
PA10-1989	La Rioja	La Rioja	Apis	Varroa	M	M4
PA10-1991	La Rioja	La Rioja	Cer	Negativo	M	M7
PA10-1997	La Rioja	La Rioja	Cer		M	M7
PA10-1999	La Rioja	La Rioja	Cer		M	M7
PA10-2000	La Rioja	La Rioja	Cer		M	M7
PA10-2001	La Rioja	La Rioja	Cer		M	M4
PA10-2002	La Rioja	La Rioja	Cer		M	M7
PA10-0670	Madrid	Madrid	Apis+Cer	Negativo	M	M12
PA10-0672	Madrid	Madrid	Cer	Negativo	M	M12
PA10-0673	Madrid	Madrid	Cer	Negativo	A	A9
PA10-0674	Madrid	Madrid	Cer	Negativo	M	M4'
PA10-0675	Madrid	Madrid	Negativo	Negativo	A	A2
PA10-0757	Madrid	Madrid	Cer	Negativo	A	A4'
PA10-0800	Madrid	Madrid	Cer	Negativo	A	A2
PA10-0805	Madrid	Madrid	Cer	Negativo	A	A2
PA10-0835	Madrid	Madrid	Cer	Negativo	A	A2
PA10-1039	Madrid	Madrid	Cer	Negativo	M	M7'
PA10-0783	Murcia	Murcia	Negativo	Negativo	A	A2
PA10-0784	Murcia	Murcia	Negativo	Negativo	A	A2
PA10-0785	Murcia	Murcia	Cer	Negativo	A	A1
PA10-0786	Murcia	Murcia	Cer	Negativo	A	A16
PA10-0787	Murcia	Murcia	Negativo	Negativo	A	A1
PA10-0788	Murcia	Murcia	Cer	Negativo	A	A2
PA10-0789	Murcia	Murcia	Cer	Negativo	A	A8
PA10-1707	Murcia	Murcia	Negativo	Negativo	A	A2
PA10-0773	Navarra	Navarra	Negativo	Varroa	M	M4'
PA10-0774	Navarra	Navarra	Cer	Negativo	A	A2
PA10-0775	Navarra	Navarra	Negativo	Negativo	M	M7
PA10-1046	Navarra	Navarra	Cer	Negativo	M	M7
PA10-1048	Navarra	Navarra	Negativo	Varroa	M	M7
PA10-1979	Navarra	Navarra	Cer	Negativo	M	M4
PA10-1980	Navarra	Navarra	Cer	Negativo	M	M4

PA10-2043	Navarra	Navarra	Negativo	Negativo	M	M8
PA10-2044	Navarra	Navarra	Cer	Negativo	M	M4
PA10-0856	País Vasco	Álava	Cer	Negativo	M	M4
PA10-0858	País Vasco	Álava	Cer	Negativo	M	M4
PA10-0859	País Vasco	Álava	Cer	Negativo	M	M4
PA10-1370	País Vasco	Álava	Apis+Cer	Negativo	M	M4
PA10-1372	País Vasco	Álava	Cer	Negativo	M	M4'
PA10-1378	País Vasco	Álava	Cer	Negativo	M	M4
PA10-1379	País Vasco	Álava	Cer	Negativo	M	M8
PA10-1382	País Vasco	Álava	Cer	Negativo	M	M7
PA10-1385	País Vasco	Álava	Cer	Negativo	M	M4
PA10-1387	País Vasco	Álava	Negativo	Negativo	A	A1

Chapter II

(Capítulo II)

Stable genetic diversity despite parasite and pathogens spread in honey bee colonies

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ORIGINAL PAPER

Stable genetic diversity despite parasite and pathogen spread in honey bee colonies

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Abstract

In the last decades, the rapid spread of diseases, such as varroosis and nosemosis, associated with massive honey bee colonies mortality around the world has significantly decreased the number and size of honey bee populations and possibly their genetic diversity. Here, we compare the genetic diversity of Iberian honey bee colonies in two samplings performed in 2006 and 2010 in relation to the presence of the pathogenic agents *Nosema apis*, *Nosema ceranae*, and *Varroa destructor* in order to determine whether parasite and pathogen spread in honey bee colonies reflects changes in genetic diversity. We found that the genetic diversity remained similar, while the incidence of *N. ceranae* increased and the incidence of *N. apis* and *V. destructor* decreased slightly. These results indicate that the genetic diversity was not affected by the presence of these pathogenic agents in the analyzed.period. However, the two groups of colonies with and without *Nosema*/*Varroa* detected showed significant genetic differentiation (G test). A detailed analysis of the allelic segregation of microsatellite loci in *Nosema*/*Varroa*-negative colonies and parasitized ones revealed two outlier loci related to genes involved in immune response.

Keywords: Heterozygosity, Disease, Selection, Genotyping, Colony losses.

1. Introduction

The International Union for Conservation of Nature (IUCN) recognizes genetic diversity as one of the three forms of bio-diversity worthy of conservation (McNeely et al. 1990). Indeed, the need to conserve genetic diversity within populations is based on two arguments: the importance of genetic variability for evolution to occur and the tight relationship between heterozygosity and population fitness (Reed and Frankham 2003). In the case of honey bees, within-colony genetic diversity has proven to reduce the negative impacts of pathogens and parasites (Crozier and Page 1985; Palmer and Oldroyd 2000; Crozier and Fjerdingstad 2001; Oldroyd and Fewell 2007). Meanwhile, the increased adaptive capacity associated with wider genetic diversity at the population level allows short-term environmental perturbations to be with-stood, such as the emergence of new diseases, enabling bee populations to evolve and adapt to long-term environmental changes (Frankham et al. 2010).

Human management often brings profound changes in the genetic variation of species, and accordingly, the effect of domestication on the genetic diversity of honey bee populations has recently been addressed (Harpur et al. 2012; De la Rúa et al. 2013). In contrast to other livestock species, honey bees are not fully domesticated (Oxley and Oldroyd 2010) given the lack of control that beekeepers have on the natural mating of honey bee queens with unselected drones during the breeding season. Nevertheless,

honey bee populations have been severely affected by human activities, and in recent decades, there has been an overall reduction in the number of colonies from different regions (Biesmeijer et al. 2006; vanEngelsdorp and Meixner 2010), potentially affecting the diversity of honey bee populations. The importation of foreign queens in an attempt to replace lost colonies has become an important driver of these changes (Muñoz et al. 2013).

Different factors have been analyzed and discussed in the literature for their potential influence on the decline and eventual disappearance of honey bee colonies. The most significant of them is possibly the increased incidence of parasites and pathogens (vanEngelsdorp et al. 2009). Particularly, virulent combinations of different pathogenic agents, instead of a single pathogenic factor, are thought to be the most likely explanation for this decline (Chen and Evans 2007; Johnson et al. 2009). Pathogens like the *Varroa destructor* mite (Anderson and Trueman 2000) and its associated viruses, as well as the microsporidia of the genus *Nosema*, are currently considered to be the most harmful pathogens of honey bees (Higes et al. 2006; Cox-Foster et al. 2007; Johnson et al. 2009; Rosenkranz et al. 2010; de Miranda and Genersch 2010; de Miranda et al. 2010), while other agents that have been around since the 1950s (American foulbrood; chalkbrood) are reported much less often nowadays.

There is accumulating evidence that genetic variation can influence host susceptibility to pathogens (Reed and Frankham 2003; Spielman et al. 2004; Whitehorn et al. 2011). However, we still have a limited and insufficient understanding of the interaction of these pathogenic organisms with their host, *Apis mellifera* (Martinson et al. 2011). In a recent study (Jara et al. 2012), the distribution of the *V. destructor* mite was not significantly related to that of the two evolutionary lineages making up the Iberian honey bee population, *Apis mellifera iberiensis* (M, Western European lineage at the North-West, and A, African lineage at the South-East; Cánovas et al. 2008). This data coincided with that obtained in the original host species of this mite, *Apis cerana* (Rueppell et al. 2011). However, a significantly higher incidence of the microsporidian *Nosema apis* was detected in northern Iberian honey bee populations belonging to the M lineage, a result that changed due to the rapid expansion of *Nosema ceranae* that now affects more than 50 % of Iberian colonies (Jara et al. 2012; Martín-Hernández et al. 2012; Botías et al. 2012).

The wide dispersion of *N. ceranae*, both in Spain and worldwide (Fries 2010; Higes et al. 2010a, b; Muñoz et al. 2014), is indicative of the successful colonization of this new pathogen and the limited adaptation of its new host, *A. mellifera*. A potential effect of this rapid expansion of *N. ceranae* is a reduction in genetic diversity, since invasive species may affect certain genotypes more than others, and the high risk of collapse in infected colonies (including asymptomatic colonies during the incubation period: Martín-Hernández et al. 2007; Higes et al. 2008). To assess the relationship between the spread of three major pathogens (*N. ceranae*, *N. apis*, and *V. destructor*), and the genetic

diversity in Iberian honey bee colonies (*A. mellifera iberiensis*), we used microsatellite markers to analyze the genetic diversity of *A. m. iberiensis* in two different samplings obtained in 2006 and 2010. The null hypothesis tested was that changes in the frequency of pathogens and parasites are unrelated to the changes in genetic diversity of honey bee colonies. In addition, because transmissible diseases or pests are an important and universal selective evolutionary force (Seal 1991), we assessed whether any of the microsatellite alleles analyzed was associated to genes possibly involved in the adaptive response of honey bees to these harmful agents.

2. Materials and methods

2.1. Sampling

The samplings of this study were designed to be representative of Spanish honey bees at a country scale including samples from the peninsula and from islands such as Majorca and Tenerife. We analyzed adult worker honey bees from 228 colonies sampled in 41 Spanish provinces, corresponding to two surveys carried out in spring 2006 (113 colonies) and 2010 (115 colonies: Fig. 1). Each sample contained about 150 adult inner worker honey bees from each colony. An etiological analysis was performed for some of the major pathogenic agents affecting honey bees, and subsequently, one adult worker honey bee from each colony was placed directly into 100 % ethanol and stored at −20 °C for later DNA analysis (Department of Zoology and Anthropology, Faculty of Veterinary Medicine, University of Murcia). The evolutionary lineage of each colony was determined previously by analyzing the mitochondrial DNA variation (Jara et al. 2012).

2.2. DNA extraction and microsatellite analysis

The two left legs were removed from one worker honey bee per colony, and the total DNA was extracted in a 5 % Chelex solution according to a modified version of the protocol pub-lished by Walsh et al. (1991). The final solution of DNA (2 µl) was then used for PCR amplification, performing two multiplex PCR reactions in order to study a total of 12 microsatellite loci in the genotype of the worker honey bees (Chahbar et al. 2013; Evans et al. 2013). PCR products were visualized by capillary electrophoresis (ABI-3730, Applied Biosystems) and sized with an internal standard (Servei Central de Suport a la Investigació Experimental, University of Valencia, Spain). The individual alleles were subsequently scored using GeneMapper v3.7 software (Applied Biosystems).

2.3. Detection of pathogenic agents

All the samples came from colonies treated against *Varroa* (mandatory in the Spanish beekeeping regulation); therefore, the finding of *Varroa*-positive samples indicates that the treatment was not successful or incorrectly applied (>50 % of the treatments are applied improperly in Spain: see Garrido Bailón 2012). Conversely, *Varroa*-negative colonies indicate either the result of a recent treatment (reducing infestation to levels below the threshold of detection) or the development of natural tolerance to *Varroa* as that reported by other authors (Rinderer et al. 2001; Büchler et al. 2002, 2008; Le Conte et al. 2007; Seeley 2007; Rosenkranz et al. 2010; Locke et al. 2012).

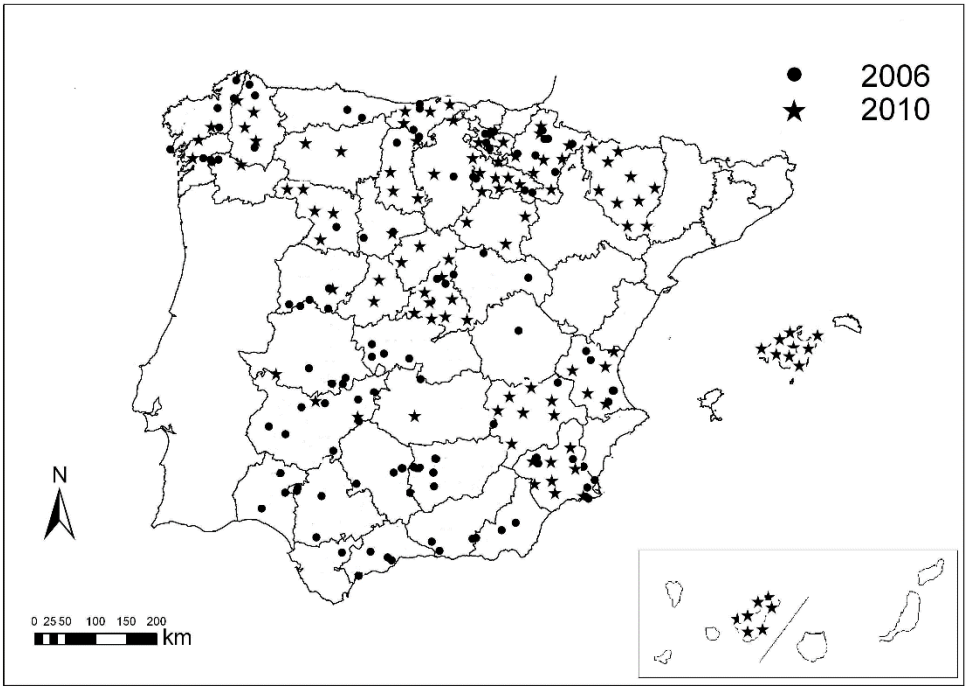

Fig. 1. Map showing the location of the* Apis mellifera iberiensis *colonies sampled in 2006 and 2010 (from Jara et al. 2012).

Nosema spp. and *V. destructor* presence had already been analyzed in these colonies (Jara et al. 2012). Briefly, about 150 inner worker honey bees from each colony were pooled and analyzed to detect the presence of *Nosema* spp. and *V. destructor* according to OIE recommendations (2008). To assay *Nosema* species, DNA extraction and PCR reactions were performed as described previously, using an internal PCR control to determine the reliability of the analysis (Martín-Hernández et al. 2007; Botías et al. 2011). Therefore, *Nosema* spp. and *V. destructor*-negative colonies are defined here as colonies in which none of these parasites were detected in a sample of 150 bees.

2.4. Genetic and statistical analysis

Population genetic parameters were calculated for the samples in the two samplings (2006 and 2010) using GenAlex (Peakall and Smouse 2006). Given the notable genetic homogeneity found in the microsatellite analyses of Iberian honey bees across the Peninsula (Cánovas et al. 2011), we considered that all samples collected in the same year make up a single population. Genetic diversity was evaluated by calculating allele frequencies, comparing the observed *(Ho)* and expected *(He)* heterozygosity values. Population genetic differentiation was tested using Genepop software (Raymond and Rousset 1995; Rousset 2008), and the relationship between genetic diversity in the population and the prevalence of the pathogenic elements (*V. destructor* and *Nosema* spp.) was analyzed with the Pearson's chi-squared and Fisher exact tests, as implemented in SPSS 19 for Windows.

2.5. Detecting loci under selection

To investigate whether selection affected any of the loci analyzed, we used the Lositan software (Antao et al. 2008). Adaptive differentiation has traditionally been identified from the differences in allele frequencies among different populations, summarized by an estimate of the F_{ST} (Beaumont 2005). Low-frequency outliers relative to an appropriate neutral population genetic model indicate loci subject to balancing selection, whereas high-frequency outliers suggest adaptive (directional) selection. Lositan is based on a method that describes the expected distribution of allele frequencies (F_{ST}) *vs* He under an island model of migration with neutral markers (Wright 1931). This distribution is used to identify outlier loci that have excessively high or low F_{ST} scores com-pared to neutral expectations. Such outlier loci are therefore candidates to be subject to selection.

To minimize the bias on the estimation of the mean neutral F_{ST}, Lositan was run using all loci through 50,000 simulations. After the first run, those loci that appeared outside the desired confidence intervals (95 % CIs) were removed and the mean neutral F_{ST} was computed again. In this way, a more accurate estimate of the neutral F_{ST} value was obtained using only neutral loci. This refined estimate was used for a final set of 50,000 simulations over all loci, and the estimated selection status of each of them was reported.

The outlier loci were submitted to a sequence similarity search using Blast (www.ncbi.nlm.nih.gov) and BeeBase (http://hymenopteragenome.org/beebase/), and likewise, they were screened for annotation of genes using the Map Viewer tool (NCBI; Amel 4.5). As the functional annotation of the honey bee genome is incomplete, putative Gene Ontology classifications were ascribed to genes based on homology to

Drosophila melanogaster FlyBase (www.flybase.org), complemented by NCBI annotation.

3. Results

Of the 12 microsatellite loci analyzed, one (AC011) was removed from the analysis due to the poor efficiency of amplification and posterior fragment detection. For the 11 remaining microsatellite loci, we found a total of 122 different alleles within the 228 samples studied (Table 1). All the loci were polymorphic in both the 2006 and 2010 samplings, and the allele frequencies for all of them are listed in Supplementary material, including the observed *(Ho)* and expected *(He)* heterozygosity values.

There was allelic loss at the Ap43 and Ap55 loci in 2010 compared to 2006, and whereas five loci exhibited an in-crease (A8, Ap274, A79, A88, and Ap249), the remaining four maintained the same number of alleles in both samplings (A113, A7, B124, and Ap224). Of the 87 alleles identified in 2006, only 63 were found in samples collected in 2010, a loss of 24 alleles all of which had a low frequency (below 0.015). Moreover, of the 98 alleles recorded in 2010, 35 corresponded to alleles not detected in 2006, and again, these were alleles with a low frequency (between 0.005 and 0.018).

[a] Average values across loci

Locus	**Na**		H_o		H_e	
	2006	**2010**	**2006**	**2010**	**2006**	**2010**
A113	13	13	0.604	0.588	0.592	0.619
A7	7	7	0.459	0.330	0.478	0.416
AP43	16	14	0.735	0.489	0.719	0.669
AP55	12	11	0.673	0.689	0.744	0.755
B124	11	11	0.815	0.806	0.834	0.821
A79	9	11	0.146	0.194	0.294	0.301
A8	3	7	0.062	0.172	0.145	0.278
A88	3	7	0.081	0.400	0.132	0.363
Ap224	6	6	0.545	0.578	0.614	0.631
Ap249	2	5	0.033	0.058	0.464	0.122
Ap274	5	6	0.042	0.051	0.081	0.108
All [a]	**7.9**	**8.9**	**0.381**	**0.396**	**0.463**	**0.462**

Table 1. Microsatellite descriptive statistics for 2006 and 2010 samples of* Apis mellifera iberiensis*. Columns indicate the total number of alleles observed (Na), observed heterozygosity (H_o) and expected heterozygosity (H_e) estimates per locus.

Highly significant genetic differentiation was found be-tween 2006 and 2010, as evident with the G test implemented in the Genepop software (p<0.001). However, genetic diversity levels *(He)* were similar in both surveys (He in 2006= 0.463±0.080 and 0.462±0.075 in 2010). These values are within the expected range of genetic diversity for *A. m. iberiensis* (Cánovas et al. 2011).

Of the 228 colonies randomly sampled in 2006 and 2010, at least one of the pathogenic agents analyzed was detected in almost 80 % of them (*V. destructor*, *N. apis*, and *N. ceranae*), although they were less prevalent in 2006 (74 %) than in 2010 (83 %). About 31 % of the colonies analyzed in 2006 were parasitized by *V. destructor*, which decreased to 19 % in 2010 (Table 2), while *Nosema* microsporidia were more prevalent than the *V. destructor* mite in 2006 (64 %) and 2010 (72 %). In 2006, *N. ceranae* was detected in 50 % of the colonies while *N. apis* was less frequent (13 %). However, by 2010, the presence of *N. ceranae* reached to 59 %, whereas in only three of the colonies sampled in 2010, *N. apis* was detected alone (3 %). Indeed, while *N. apis* and *N. ceranae* were detected together in only 1 % of the colonies in 2006, this had risen to 10 % in 2010 (Table 2).

[a] *Varroa* presence was not analyzed in six of the 115 colonies from the sampling in 2010; furthermore, the percentage is calculated in this case based on 109 colonies analyzed

	% Positive colonies (number of positive colonies)	
	2006 (*N*=113)	**2010** (*N*=115)
Prevalence of *Varroa* mite	**31%** (35)	**19%** [a] (68)
N. ceranae	**50%** (56)	**59%** (68)
N.apis	**13%** (15)	**3%** (3)
Coinfection by *N.apis* + *N.ceranae*	**1%** (1)	**10%** (12)
Total prevalence of *Nosema spp.*	**64%** (72)	**72%** (83)
Presence of at least one of the analysed agents	**74%** (84)	**83%** (95)

Table 2. Presence of* Nosema *spp. and* Varroa destructor *by sampling year in* Apis mellifera iberiensis *colonies.

Since the genetic diversity of the *A. m. iberiensis* population was similar in 2006 and 2010, no temporal correlation could be established between this parameter and the prevalence of pathogens and parasites in the Iberian honey bee. Nevertheless, we analyzed the allelic patterns in the colonies parasitized by *Varroa* or *Nosema* separately

vs Nosema/*Varroa*-negative colonies (i.e., without any of these pathogenic agents). Greater genetic diversity was found in these *Nosema*/*Varroa*-negative colonies (He =0.480±0.075) than in the parasitized colonies (He =0.464±0.075), and although these differences did not appear to be statistically significant (Student t=−0.149, p=0.441), the G test revealed highly significant genetic differentiation between these two groups of colonies (p<0.001).

[a] Locus Ap249 shows a high probability of being subjected to selective pressures

Locus	H_e	F_{ST}	P (Simul F_{ST} < sample F_{ST})
A113	0.606836	-0.003254	0.33287
A7	0.450309	0.002717	0.475918
AP43	0.703883	0.008342	0.464157
AP55	0.789031	0.045271	0.824397
B124	0.829635	-0.003618	0.142272
A79	0.29875	-0.002601	0.450995
A8	0.216294	0.017493	0.593753
A88	0.267323	0.069342	0.869986
Ap224	0.623494	-0.00542	0.30051
Ap249 [a]	**0.39438**	**0.250268**	**0.999406**
Ap274	0.094999	-0.001697	0.386087

Table 3. Results of the outlier analysis of* Apis mellifera iberiensis *populations when comparing 2006 and 2010 samples.

[a] Locus B124 shows a high probability of being subjected to selective pressures

Locus	H_e	F_{ST}	P (Simul F_{ST} < sample F_{ST})
A113	0.62406	-0.000357	0.613779
A7	0.478716	2.8e-05	0.69447
AP43	0.681419	-0.007087	0.436818
AP55	0.791319	0.001775	0.562544
B124 [a]	**0.866319**	**0.047409**	**0.985782**
A79	0.401316	0.021712	0.798066
A8	0.336097	0.029368	0.85626
A88	0.201974	-0.008837	0.470685
Ap224	0.648352	-0.00876	0.437697
Ap249	0.357919	0.025789	0.829389
Ap274	0.077703	-0.000715	0.595662

***Table 4. Results of the outlier analysis when comparing* Apis mellifea iberiensis *populations (2006 plus 2010 samples) negative for* Varroa/Nosema *infestation vs colonies parasitized by* V. destructor.**

To further analyze these genetic differences, we studied the allele frequency patterns of each locus in colonies sampled in 2006 *vs* colonies sampled in 2010. Coalescent

simulations performed with Lositan software detected an outlier locus (Ap249) as a candidate of being subjected to positive selection (Table 3). The comparison between *Nosema*/*Varroa*-negative colonies *vs* colonies parasitized by *Varroa* revealed another outlier locus, B124, as a candidate for positive directional selection (Table 4). No outliers were detected when comparing *Nosema*/*Varroa*-negative colonies *vs* positive colonies to *Nosema* spp., irrespective of the sampling year. Screening for annotated genes (Map Viewer tool NCBI; Amel 4.5) showed that the Ap249 locus is located on chromosome LG2 of *A. mellifera* and that it is linked to the protein-coding gene Aos1 related to protein degradation, whereas B124 locus is located on chromosome LG13 and it is associated with the dpr7 gene that encodes immuno-globulin proteins.

4. Discussion

This study indicates that the genetic diversity level among honey bee colonies in Spain has remained stable over the last decade, despite the colony losses recorded in this and other European countries (Higes et al. 2010a, b; vanEngelsdorp and Meixner 2010). Therefore, the initial hypothesis that the in-creased incidence of parasites (*V. destructor*) and pathogens (*N. apis* and *N. ceranae*) in recent years was associated with a decrease in genetic diversity of the Iberian honey bee population was not corroborated here. This result does not rule out the possibility of such effects at a local or intra-colony scale, an issue that deserves more specific research. However, the genetic diversity levels at the peninsular scale *(He)* remained similar in both years surveyed, 2006 (He =0.463±0.080) and 2010 (He =0.462±0.075). In fact, the genetic diversity recorded in this study is concordant with that obtained previously in the Iberian Peninsula studying microsatellite loci (Cánovas et al. 2011: He 0.401 to 0.518; Miguel et al. 2007: He 0.442 to 0.516). As discussed by these authors, honey bee populations in Spain might be considered to make up a single large population for certain purposes, given the large population interchange and gene flow derived from the extensive beekeeping mobility around the Peninsula (Cánovas et al. 2011; 2014). In fact, around 80 % of the estimated number of Spanish colonies move in a yearly cycle, which leads to a potential bi-directional genetic flow between migratory and stationary colonies (Perrier et al. 2003; Hernández-García 2010), an effect that is enhanced by the lack of control that beekeepers have on natural honey bee mating (Baudry et al. 1998; Koeniger and Koeniger 2000; Hernández-García et al. 2009; Jaffé et al. 2009). Additional factors promoting the genetic homogeneity in the Iberian Peninsula are the capture of swarms and the purchase of colonies or mated queens, often originating from distant locations (Cánovas et al. 2008; Serrano et al. 2011). All these factors would account for the maintenance of genetic diversity in Iberian honey bee populations reported in this study, despite of the colony losses occurred in recent decades. However, there is an evident risk of disease spreading as a result of the intense colony movement between most

regions in the Iberian Peninsula. In this sense, the increased prevalence of pathogens in Spain during the study period is not surprising, consistent with data found in other studies (Higes et al. 2010a, b; Botías et al. 2012; Martín-Hernández et al. 2012; Muñoz et al. 2014) and in other regions (Fries 2010; Traver and Fell 2011; Martin et al. 2013; Bekele et al. 2015). It is noteworthy that around 74 % (2006) or 86 % (2010) of the colonies presented at least one of the searched pathogenic agents (*V. destructor*, *N. apis*, and *N. ceranae*) showing *N. apis* a lower prevalence even though the samples were obtained in Spring, the peak season for *N. apis* infestation (Fries 2010). By contrast, *N. ceranae* was the pathogen that showed the greatest expansion with an increase in prevalence of 10 % in the 4 years of our study. This high prevalence of parasites and pathogens, and the fast expansion of *N. ceranae*, are of particular concern given the potential of *V. destructor* (Rosenkranz et al. 2010) and *N. ceranae* (Martín-Hernández et al. 2007; Higes et al. 2008) to produce colony collapse.

A relationship between honey bee genetic diversity and the prevalence of parasites and pathogens could not be established in this study due to the genetic stability observed. However, we did detect significant genetic differences in the surveys performed in 2006 and 2010 (G test, $p<0.001$), as well as *Varroa*/*Nosema*-negative colonies and parasitized colonies (G test, $p< 0.001$), with tentatively higher He values in the *Varroa*/*Nosema*-negative colonies compared to parasitized ones. This is consistent with studies showing that colonies of genetically diverse social insects have a selective advantage because of increased resistance to pests (Sherman et al. 1988; Liersch and Schmid-Hempel 1998; Tarpy 2003; vanBaalen and Beekman 2006; Mattila and Seeley 2007; Oldroyd and Fewell 2007). It is thought that different host genotypes have distinct susceptibilities to diverse parasitic strains; thus, parasitic infections are not likely to spread in genetically heterogeneous colonies as rapidly as in more homogeneous ones (Sherman et al. 1988; Schmid-Hempel 1998). Studies on different Hymenoptera species also support this hypothesis, including those on bumblebees like *Bombus terrestris* (Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 2001) and *Bombus pascuorum* (Whitehorn et al. 2011) and on ants (Hughes and Boomsma 2004).

To further investigate the genetic differences between the groups of colonies, we studied whether any of the loci analyzed was subject to selective pressure. A comparison of allele frequencies between the samples collected in 2006 and 2010 showed that the Ap249 locus was subjected to positive selection during that period. Searches of the complete *A. mellifera* genome (Consortium HGS 2006) showed that this locus is located on the chromosome LG2 and that it is linked to the protein coding gene Aos1. Experimental data in *D. melanogaster* indicate that Aos1 (FlyBase: http://flybase.org/reports/FBgn0029512.html, 16/01/2015) is involved in the biological processes of protein sumoylation, a protein modification implicated in various cellular processes that include the stress response (Hay 2005; Paddibhatla et al. 2010), and the positive regulation of the NF-kappaB transcription factor, a modulator of the immune response

to infection and of nervous system plasticity (Ghosh et al. 1998; Albensi and Mattson 2000; Li and Verma 2002; Meffert et al. 2003).

When comparing *Varroa*/*Nosema*-negative colonies *vs* colonies parasitized by *V. destructor*, we found a second outlier locus, B124. As noted before, *Varroa*-negative colonies might be false negatives because of a recent treatment, but also the result of the development of natural tolerance to the mite. It is interesting that the screening of B124 in the annotated genome of *A. mellifera* (Map Viewer tool, NCBI; Amel 4.5) suggested the linkage of this locus with the dpr7 gene that encodes immunoglobulin proteins (FlyBase: http://flybase.org/ reports/FBgn0053481.html, 16/01/2015).

In conclusion, in this study, we show that the genetic diversity in Spanish honey bee populations remained stable between 2006 and 2010, despite the increasing levels of pathogens and parasites. This result possibly reflects the multi-drone mating system of the queens and the high gene flow between colonies over most regions of the country. We identify two outlier loci related to genes that respond to stress and that could potentially reflect selective processes a hypothesis that should be assessed in more specific analyses.

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Supplementary Material

Allele frequencies,* He *and* Ho *per locus in* Apis mellifera iberiensis *populations surveyed in 2006 and 2010

Locus	Allele/n	2006	2010
A113		*N*=96	*N*=80
	202	0.036	0.050
	204	0.005	-
	208	0.005	0.006
	210	0.005	0.013
	212	-	0.013
	214	-	0.006
	216	0.005	0.025
	218	0.609	0.588
	220	0.104	0.075
	222	0.151	0.156
	224	0.031	0.050
	226	0.016	0.006
	228	0.010	-
	230	0.016	-
	232	0.005	0.006
	234	-	0.006
	He	0.592	0.619
	Ho	0.604	0.588

		2006	2010
AP55		*N*=98	*N*=90
	103	-	0.006
	105	0.005	-
	169	-	0.006
	171	-	0.017
	173	0.015	0.039
	175	0.071	0.189
	177	0.168	0.317
	179	0.378	0.317
	181	0.276	0.083
	183	0.061	0.017
	185	0.005	-
	187	0.005	-
	189	0.005	0.006
	191	0.005	-
	193	0.005	-
	195	-	0.006
	He	0.744	0.755
	Ho	0.673	0.689

		2006	2010
A7		*N*=98	*N*=94
	99	0.005	0.005
	101	0.158	0.160
	103	0.082	0.016
	105	0.699	0.745
	106	0.010	-
	107	0.041	0.064
	109	-	0.005
	111	0.005	-
	128	-	0.005
	He	0.478	0.416
	Ho	0.459	0.330

		2006	2010
B124		*N*=92	*N*=72
	218	0.049	0.049
	220	0.310	0.326
	222	0.147	0.188
	224	0.098	0.104
	226	0.136	0.104
	228	0.092	0.090
	230	0.087	0.056
	232	0.022	0.028
	234	0.027	0.035
	236	0.022	0.007
	238	0.011	0.014
	He	0.834	0.821
	Ho	0.815	0.806

A8		2006 *N*=65	2010 *N*=93
	159	-	0.005
	163	0.046	0.038
	165	0.923	0.844
	166	-	0.005
	169	-	0.011
	171	0.031	0.091
	175	-	0.005
	He	0.145	0.278
	Ho	0.062	0.172

Ap274		2006 *N*=96	2010 *N*=98
	104	-	0.010
	106	0.005	-
	110	-	0.010
	112	0.010	0.020
	120	0.021	0.010
	122	0.958	0.944
	124	0.005	0.005
	He	0.081	0.108
	Ho	0.042	0.051

A79		2006 *N*=89	2010 *N*=93
	83	0.011	-
	91	0.837	0.833
	93	-	0.016
	101	-	0.016
	103	-	0.005
	105	-	0.005
	107	-	0.005
	109	-	0.011
	111	0.034	0.016
	113	0.028	0.038
	115	0.051	0.038
	117	0.011	-
	119	0.011	0.016
	123	0.011	-
	141	0.006	-
	He	0.294	0.301
	Ho	0.146	0.194

AP43		2006 *N*=98	2010 *N*=90
	123	-	0.006
	125	-	0.011
	129	0.005	-
	131	0.423	0.378
	133	0.306	0.428
	135	0.026	0.022
	149	0.010	-
	151	0.010	0.011
	153	0.026	0.044
	155	0.026	0.011
	157	0.056	0.039
	159	0.031	0.017
	161	0.015	-
	163	0.026	0.006
	165	0.015	0.017
	167	0.010	-
	169	-	0.006
	171	0.010	-
	173	-	0.006
	181	0.005	-
	He	0.719	0.669
	Ho	0.735	0.489

Ap249		2006 *N*=60	2010 *N*=86
	201	-	0.012
	209	-	0.012
	215	0.633	0.936
	217	0.367	0.035
	219	-	0.006
	He	0.464	0.122
	Ho	0.033	0.058

Ap224		2006 *N*=66	2010 *N*=83
	277	0.015	-
	279	0.144	0.169
	281	0.523	0.506
	283	0.303	0.289
	285	-	0.018
	287	0.008	-
	289	-	0.006
	291	0.008	0.012
	He	0.614	0.613
	Ho	0.545	0.578

A88		2006 *N*=99	2010 *N*=100
	105	-	0.005
	137	-	0.010
	139	0.066	0.190
	141	0.929	0.775
	145	-	0.010
	147	-	0.005
	149	-	0.005
	157	0.005	-
	He	0.132	0.363
	Ho	0.081	0.400

PART II

Effects of beekeeping practices on the genetic diversity and the dispersion of pathogens in Iberan honey bee colonies

Chapter III

(Capítulo III)

Effect of small-scale migratory movements on the dispersion of *Ascosphaera apis* in *Apis mellifera iberiensis* colonies

Effect of small-scale migratory movements on the dispersion of *Ascosphaera apis* in *Apis mellifera iberiensis* colonies

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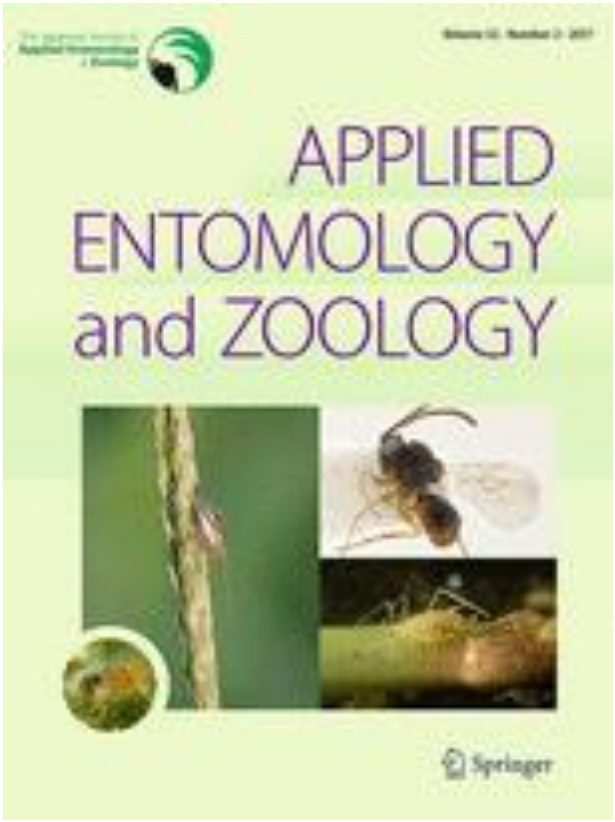

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Abstract

Ascosphaera apis is the causative agent of the chalkbrood disease, a pathology that though less severe than others in the hive, affects bees larvae and significantly impairs population growth and colony productivity. In this study, we detected the presence of *A. apis* in adult worker honey bees by PCR-amplification of the intergenic transcribed spacer (ITS1) of the ribosomal gene (rDNA). DNA extraction was optimised by testing different protocols in individual and pooled (colony level) adult honey bee samples. Subsequently, the presence of the fungus *A. apis* was assessed in both stationary and migratory colonies (subjected to small-scale regional level movements) to determine the effect of migratory practices on the dispersal of this pathogen. Results confirmed a positive correlation between migratory beekeeping and a higher prevalence of the *A. apis*, indicating that migratory colonies are more likely to develop chalkbrood. Given these results, we suggest that beekeepers should be aware of the risks of pathogens spreading while moving beehives, even within a reduced geographic range.

Keywords: migratory management, honey bee health, *Ascosphaera apis*, *Apis mellifera iberiensis*, Spain

1. Introduction

The benefits of safeguarding pollinators, especially the honey bee *Apis mellifera* as the most economically important pollinator insect, have been recently articulated (Potts et al., 2016). Sustainable hive management is among the strategies to avoid pollinator decline, as it has been shown that techniques such as migratory beekeeping affect oxidative stress levels in honey bees (Simone-Finstrom et al., 2016) and favour the spread of pathogens (Cavigli et al., 2016). To date, little attention has been paid to the impact of transporting beehives on spreading disease (Ahn et al., 2012; Zhu et al., 2014; Simone-Finstrom et al., 2016; Traynor et al., 2016).

While in North America honey bee hives are moved yearly for pollination of monocultures, in Spain beehives are mainly moved to take advantage of consecutive flowering events (to get as many harvests of honey as possible) and to find favourable sites for food sources and summer temperatures. Migratory beekeeping is an extended practice in Spain. There are 2.7 million beehives (data from 2015; REGA 2016), of which 80% are moved by professional beekeepers (a beekeeper is considered to be professional when he/she manages more than 150 beehives; mean is 406 beehives per beekeeper). Although this practice usually leads to increased honey production, it can imply higher costs due to transportation and colony losses that may counteract the actual economic benefits.

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Pathogen dissemination has been cited as one of the main causes of the severe decline of honey bee populations (Neumann and Carrek, 2010). Among pathogens, fungi might play an important role since spores can resist up to 15 years (Gilliam, 1986). *Ascosphaera apis*, the causative agent of chalkbrood disease in honey bee larvae, can survive one year in honey and two in pollen (Flores et al., 2005a, b). This disease is found worldwide, is typically common during spring (Aronstein and Murray, 2010), and causes significant harm to population growth and colony productivity. Honey bee colonies contaminated with *A. apis* often show no symptoms of the disease, therefore, diagnostic techniques, such as molecular assays, are needed to detect the pathogen. *A. apis* has been shown to be difficult to identify due to the lack of distinctive morphological features and the requirement of a special medium and growth conditions (Jensen et al., 2013). The first biochemical method for identifying this fungus was based on isozyme analysis (Gilliam and Lorenz, 1993; Chorbinski, 2003). Later, internal spacers (ITS1, ITS2) of ribosomal rDNA have been shown to be particularly useful in elucidating the relationships between closely related species of *Ascosphaera* (Anderson et al., 1998; Chorbinski, 2004; Borum and Ulgen, 2008). These analyses facilitated the design of primers specific for the ITS1-5.8S-ITS2 rDNA region for each *Ascosphaera* species (Murray et al., 2005; James and Skinner, 2005; Yoshiyama and Kimura 2011). More recent diagnostic methods rely upon information from the *Ascosphaera* genome (Qin et al., 2006) depicting polymorphic loci that can be used to differentiate haplotypes in *A. apis* (Jensen et al., 2012).

As Aronstein and Murray noted (2010), 'the migratory nature of commercial beekeeping in North America and Australia is probably the most important factor contributing to the rapid spread of chalkbrood disease within these two continents'. It can be then hypothesised that transporting beehives, which increases the stress in the honey bees (Simone-Finstrom et al., 2016), affects the immune response of colonies in migratory apiaries, leading to higher incidence of the pathogens. To test this hypothesis, we first implemented the molecular characterisation of the causal agent of chalkbrood in adult worker honey bees. Then we searched for *A. apis* in symptomatic and asymptomatic colonies of both stationary and migratory apiaries from Murcia, a south-eastern Mediterranean Spanish province. Murcia has 93,954 beehives grouped in 485 apiaries, of which 420 usually practice migratory beekeeping, and transportation occurs at different scales, nationally (around 700 km) to regionally (80 km). This study investigates a possible correlation between increased prevalence of *A. apis* in honey bee colonies and the practice of small-scale migratory beekeeping.

2. Materials and methods

2.1. Sampling

Samples (20 to 25 honey bee workers) were taken from the inner frames of 46 beehives of *A. m. iberiensis* during spring (2015), when the incidence of *A. apis* is higher (Flores et al., 1996; Borum and Ulgen, 2008). From these 46 beehives, 21 were stationary (8 belonging to an apiary at the locality of Miravetes and 13 from our research apiary at the, University of Murcia, Espinardo Campus); the remaining 25 beehives were moved yearly around 60 km from their original locations (10 from Loma Ancha, 6 from EL Llano and 9 from Pantano Puentes migratory apiaries) (Fig. 1).

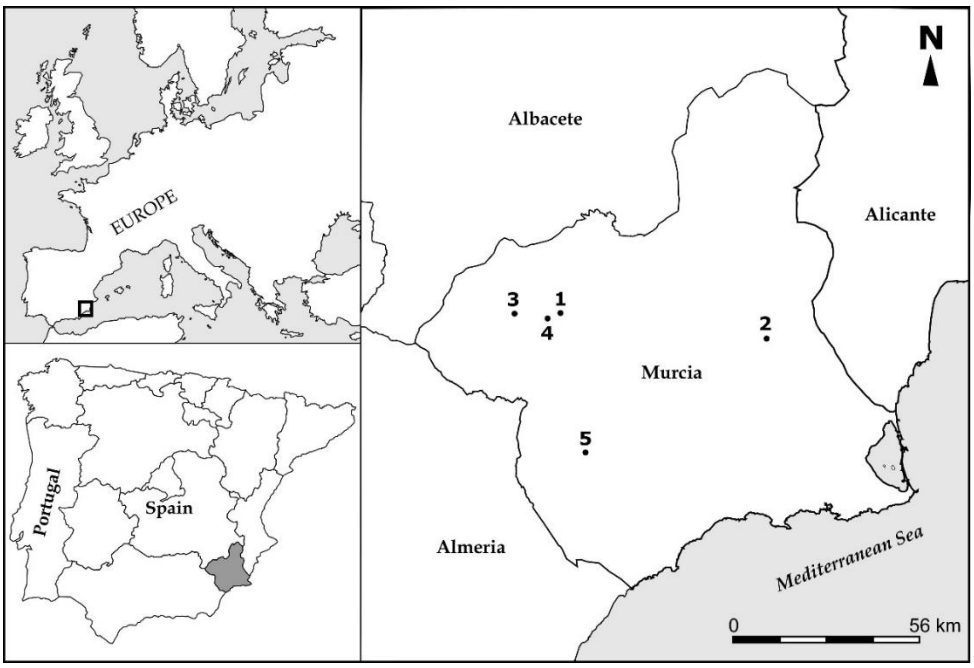

Fig. 1. Location of the apiaries sampled in Murcia (SE Spain). Stationary hives were located in 1 (Miravetes) and 2 (Espinardo Campus), while migratory hives were located in 3 (Loma Ancha), 4 (El Llano) and 5 (Pantano Puentes).

2.2. DNA extraction and PCR amplification

Different protocols were tested to optimise DNA extraction and PCR amplification of the fungus *A. apis*. DNA was first extracted at the individual level using two legs of each worker honey bee following either the Ivanova et al. (2006) or Chelex® methods (Evans et al., 2013). At the colony level, two tests were performed: one consisted on a macerate of five workers per colony, from which DNA was extracted by following a modified protocol from Martín-Hernández et al. (2007), and the second consisted of a pool of ten legs (two from each of the five workers), from which DNA was extracted with a modified Chelex® method by adding 200 µl of 5% Chelex® and 10 µl of proteinase K (10 mg/ml). DNA extraction yields obtained from each protocol were assessed by using NanoDrop 1000 Spectrophotometer (NanoDrop Technologies).

The rDNA region of *A. apis* was amplified using the primers 3F1F: 5'-TGT CTG TGC GGC TAG GTG-3' y AscoAII2R: 5'-GAW CAC GAC GCC GTC ACT-3' (James and Skinner,

2005) in a thermocycler PTC 100 (MJ Research), by adding 2 µl of DNA of each sample to a final reaction volume of 15 µl. The programme cycle used was as follows: denaturation for 3 min at 94 ºC; 36 cycles of 30 s at 96 ºC, 30 s annealing at 55 ºC and extension for 30 s at 68 ºC; then a final elongation step of 20 min at 65 ºC (modified from Yoshiyama and Kimura, 2011). Positive (DNA extracted directly from the fungus) and negative controls were included in each reaction. Amplicon size was confirmed after electrophoretic separation on 1.5% agarose gels.

2.3. Statistical analysis

Correlation between the variables "stationary" and "migratory" and “presence of *A. apis*" was analysed using the Pearson chi square test and the Fisher exact statistic for contingency tables (SPSS 15.0 programme).

3. Results

DNA concentration obtained from the different tested protocols was appropriate for PCR amplification in all cases, yielding from 9 to 115 ng/µl. The highest extraction yields were obtained with the Chelex® protocol at both individual and colony levels. All subsequent colony samples were processed by following this protocol adapted to pools of legs of honey bee workers.

Samples selected from a colony with chalkbrood symptoms (Fig. 2) to set up the amplification reaction of the ITS region of *A. apis*, yielded amplicons of the expected size (around 500 base pairs).

Fig. 2. Chalkbrood at the entrance of the beehive, indicating the development of the disease.

The next step was to detect *A. apis* in the migratory and stationary apiaries at the colony level. The fungus *A. apis* was detected in all 25 colonies belonging to the three studied migratory apiaries. In the case of stationary apiaries, 50% of the colonies from Miravetes and 77% of the colonies from Espinardo Campus were positive for the presence of *A. apis* (Fig. 3). In total, 100% of migratory colonies and 62% of stationary colonies were positive for the fungus. The chi-square test showed a significant relationship (χ^2 = 11.53, gl = 1 and *p*= 0.001) between the presence of *A. apis* and the beekeeping management of the colonies: a higher prevalence of *A. apis* was detected in migratory apiaries when compared to stationary ones.

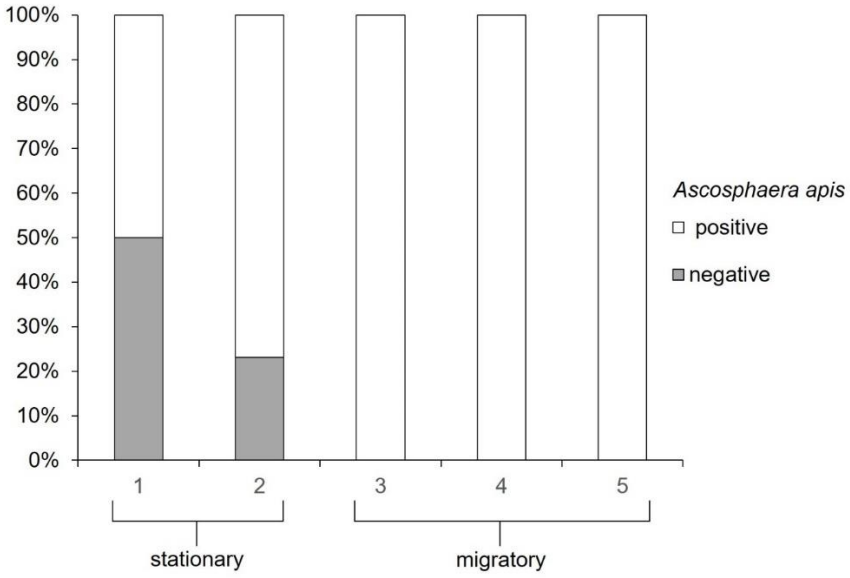

***Fig. 3. Percentage of hives in which* Ascosphaera apis *was detected. Numbers refer to the locations from** Fig. 1.*

4. Discussion

One of the drivers of pollinator decline at the global level is the dispersion of pathogens and parasites; therefore, the study of the prevalence of these organisms is of increasing interest. In this study, we detected the fungus *A. apis* by extracting DNA with the Chelex method directly from adult worker honey bees sampled from brood combs. This method is widely used in population genetic studies of the honey bee (Evans et al., 2013), and it has also proven to be useful for detecting the fungus both at the individual and colony levels. This is particularly relevant in the case of social insects, as population studies include large number of samples, hence the need for cheap, fast, and effective molecular tools to monitor individual or colony health and exposures.

Notably, the percentage of colonies positive for *A. apis* in the stationary apiary at the Espinardo Campus was higher (77%) than that found in the apiary of a private beekeeper

(50%). Colonies at the University are subjected to continuous management and experimentation procedures. It is possible that the higher proportion of infected colonies is the result of fungus transmission through the use of contaminated beekeeper material, because spores can accumulate in all parts of the beehives and in all beehive products (e.g. foundation wax, stored pollen and honey), and remain viable for at least 15 years (Gilliam, 1986). It is therefore recommended to carry out management procedures with clean instruments, to minimise the dispersion of the fungus spores and the possible development of the disease. Likewise, combs should be replaced yearly to avoid transfer of combs between potentially infected beehives (Flores et al., 2005a,b).

The results of the statistical analysis confirm the initial hypothesis about the effects of migratory beekeeping on the dispersion of the fungus *A. apis.* The percentage of detection of the pathogen was significantly lower in stationary than in migratory colonies (50-77% stationary, 100% migratory). The intensive management of migratory colonies makes them prone not only to a higher prevalence of the fungus, but also rapid dispersal of spores across distant areas; both transport stress and spore contamination may explain the high incidence of chalkbrood disease in moved colonies mentioned by Aronstein and Murray (2010). During migratory events, apiaries dedicated to economic exploitation need constant supervision and management, including handling with the same equipment, which increases the possibility of contagion from one colony to another. In addition, because of continuous management to produce more honey, colonies may suffer a nutritional deficiency that increases the likelihood of disease development, since shortage of essential amino acids will impair the correct functioning of the immune system (Di Pasquale et al., 2013). Other undesirable factors that affect migratory colonies in relation to a higher incidence of *A. apis* fungus are stress upon honey bees resulting from truck noise and vibrations, changing temperatures among the visited sites (Simone-Finstrom et al., 2016) and a higher drift of workers during migratory operations (Fries and Camazine, 2001).

Although adult honey bees are not susceptible to chalkbrood disease, they can transmit the pathogen within and between beehives, because fungal spores carried by foraging honey bees are passed to nursing honey bees that then feed larvae with contaminated food (Aronstein and Murray, 2010). This possibility should be taken in consideration by beekeepers, as one of the drivers of pollinator decline at the global level is the dispersion of pathogens and parasites.

4. Conclusion

Beekeepers should find a balance between economic benefits of migrating colonies (even at a small-scale) for a continuous honey harvesting and the impact of spreading pathogens and stressing the colonies that increases the incidence of diseases.

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Chapter IV

(Capítulo IV)

The effect of migratory beekeeping on the prevalence of pathogens in honey bee colonies and on their genetic composition

The effect of migratory beekeeping on the prevalence of pathogens in honey bee colonies and on their genetic composition

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Abstract

Migratory management is a widely-extended practice aimed to increase the yield of beekeeping products and pollination services. However, it represents a stress factor for the colonies, affecting their fitness and enhancing the spread and proliferation of diseases, and in Spain also a factor of genetic homogenization for Iberian honeybee populations. To analyze the extent of these effects, we tracked the health condition and the genetic composition in a field experiment comparing stationary and migratory colonies belonging to hobbyist and professional beekeepers, sharing the same environmental conditions but differing in management (stationary *vs*. migratory) and with a different genetic background. We studied the prevalence of important pathogens (*Varroa destructor*, *Nosema* spp. and DWV) in four different times: before migratory beekeeping operation, two weeks after transport ot the hives, at the end of the migratory period and two weeks after the return of migratory hives. It was found an increasing incidence of *Varroa destructor* and *Nosema ceranae* in migratory colonies but not in DWV load. Temporal changes in genetic diversity were also detected, although irrespective of the group of hives (migratory *vs.* stationary), suggesting that stressors other than management individually affect the genetic diversity of the colonies.

Keywords: Migratory beekeeping, managed colonies, *Apis mellifera*, Parasitic prevalence, Stressors, Genetic diversity, *Varroa destructor*, *Nosema* spp., Deformed Wing Virus (DWV).

1. Introduction

The importance of the honey bee (*Apis mellifera* L.) is related to its crucial role in the pollination of wild plant species and in agroecosystems (Klein et al. 2007; Aizen et al. 2009; Rader et al. 2009). The economic benefits of beekeeping are derived both from the products generated, including honey, wax, pollen and royal jelly, and the pollination service that honey bees provide to many crops (Gallai et al. 2009). This pollination service is even more important in the case of migratory beekeeping, as crops and wild plants from different regions and different flowering seasons benefit from honey bee foraging behavior (FAO 2009; Lautenbach et al. 2012).

There has been a decline in honey bee populations in North America and Europe over the last three decades, with beekeepers reporting unexpected high winter losses of about 30% among managed bees in the last decade (vanEngelsdorp et al. 2007, 2008; Lee at al. 2015; COLOSS 2009). To date, large-scale surveys of managed honey bee populations in the US and Europe have been unable to identify a single factor responsible for this colony loss, leading researchers to hypothesize that a combination of factors are acting in synergy to compromise bee survival (Oldroyd 2007; Cox-Foster

and vanEngelsdorp 2009; Higes et al. 2010; vanEngelsdorp and Meixner 2010; Daniat et al. 2012; Potts et al. 2016). Among the most important factors that have a negative impact on the longevity of honey bee colonies are parasites: primarily the mite *Varroa destructor* Anderson and Trueman 2000 (Sammarato et al. 2000; Rosenkranz et al. 2010) and its associated viruses (Tentcheva et al. 2004; Chen and Siede 2007; Mondet et al. 2014) as the Deformed Wing Virus (DWV: de Miranda and Genersch 2010), and pathogens as the microsporidia from the genus *Nosema* (Klee et al. 2007; Paxton et al. 2007; Higes et al. 2008; Chen and Huang 2010), and several other bacterial and fungal brood pathogens (Cox-Foster et al. 2007; Aronstein and Murray 2010; Genersch 2008). Other factors believed to enhance the detrimental effects of these parasites and pathogens are pesticide exposure (Desneux et al. 2007), poor nutrition (Brodschneider and Crailsheim 2010), reduced genetic diversity (Mattila and Seeley 2007) and queen failure (vanEngelsdorp et al. 2013), as well as beekeeper expertise (Jacques et al. 2017) and current bee management practices like migratory beekeeping (VanEngelsdorp et al. 2012).

In Spain, migratory beekeeping is a common practice due to the marked seasonality of the Mediterranean climate. About 80% of the 2.5 million of Spanish colonies are moved in an annual cycle (data from 2015; REGA 2016) that involves transportation over distances of at least 400 km in summer, from the southern and warmer regions of Spain to the northern regions with a milder climate and later flowering season. To date, the potentially harmful effects of migratory beekeeping have seldom been investigated (Traynor et al. 2016; Simone-Finstrom et al. 2016). Honey bee confinement, vibration and noise, and marked changes in colony temperature are among the negative influences on colonies associated with such practices. In fact, long distance transportation has been shown to affect honey bee physiology (Ahn et al. 2012). Moreover, the installation of colonies in new pollination locations might increase their exposure to pesticides (vanEngelsdorp et al. 2012; Mullin et al. 2010; Traynor et al. 2016) and pathogens (Zhu et al. 2014; Jara et al. submitted). These colonies must also adapt to new environmental conditions and potential stressors, including orientation cues, daily oscillations in temperature, humidity and wind regimes. Indeed, a study of the impact of migratory management on bee health and their stress response showed a significant decrease in lifespan and an increase in oxidative stress in migratory as opposed to stationary worker honey bees in the USA (Simone-Finstrom et al. 2016). Migratory practices also lead to higher drifting rates (Nelson and Jay 1989), which increases the horizontal transmission of pathogens and parasites (Fries and Camazine 2001). In fact, transportation and pollination services were recently proposed to increase the prevalence and abundance of *Nosema ceranae* (Zhu et al. 2014) and some viruses (Welch et al. 2009) in *A. mellifera* worker bees.

The extended practice of migratory beekeeping in Spain represents an evident risk for disease spread and a higher prevalence of pathogens. This practice also favors gene flow

between migratory and stationary colonies over a large geographic scale in the Iberian Peninsula (Cánovas et al. 2011; Jara et al. 2015). At the intra-colony level, the environmental conditions of the different settlements of apiaries and the proliferation of pathogens may also affect the genetic diversity in colonies, as both are important selective factors (vanEngelsdorp and Meixner 2010; Spivack and Reuter 2001; Chávez-Galarza et al. 2013). Furthermore, there is accumulating evidence that genetic variation can influence host susceptibility to pathogens (Reed and Frankham 2003; Spielman et al. 2004; Whitehorn et al. 2011).

As a result, we set out to determine the effects of migratory beekeeping on the prevalence of important pathogens (*Varroa destructor*, *Nosema* spp. and DWV) in honey bee colonies in Spain. The hypothesis tested was that stress factors associated to migratory management affect colony health and disease transmission, resulting in a higher prevalence of pathogens and viruses in the migratory colonies. We also aimed to determine whether migratory beekeeping has an effect on the genetic diversity in the individual colonies, comparing the changes in genetic diversity between migratory and stationary colonies from the same apiary. It is hypothesized that factors associated to migratory beekeeping favor particular patrilines and alleles within the colonies, depending on their selective value against external stressors prevailing in the migratory areas. Additionally, changes in the genetic composition of colonies due to queen replacement and its subsequent mating in the region of migration are also expected; a practice that represents a significant mechanism of genetic flow and homogenization of honey bee colonies over a large geographic distance in Spain.

2. Material and Methods

2.1. Determination of parasites and pathogens

Honey bee colonies

Ten hives from the apiary located at the Campus of the University of Murcia (in the Southeast of Spain) were divided into two groups of five hives. While one group remained stationary (UM-S) during the experiment, the second was moved (UM-M) to the region of Soria (Northern-Central Spain) during the summer (June - October 2015: Fig. 1). This latter group was moved together with another five hives belonging to a professional beekeeper from Murcia (PB-M) who usually carries out migratory beekeeping. The transported colonies were settled in Soria not far from a stationary apiary of a hobbyist beekeeper (SO-S), thereby sharing the same environmental conditions. At the beginning of October, the UM-M and PB-M colonies returned to Murcia, transported together from Murcia to Soria and back in the same truck.

This experimental design was established to assess the effects of migratory beekeeping by comparing groups of migratory (UM-M and PB-M) and stationary (UM-S and SO-S) hives, when: (i) sharing the same genetic background but different management strategies (UM-M *vs.* UM-S); (ii) with different genetic backgrounds but with the same management (UM-M *vs.* PB-M); and (iii) settled in the same location (in Soria) sharing the same environmental conditions, but with different genetic backgrounds (UM-M *vs* PB-M) and different management strategies (SO-S).

Sampling dates

The 20 colonies included in the experiment were sampled on four different occasions in 2015: in May before moving the hives from Murcia to Soria (T_0); in June, two weeks after transportation and settling the migratory hives in Soria (T_1); in October, at the end of the migratory season (T_2); in November, two weeks after transportation of hives back to Murcia (T_3). The stationary colonies from Soria (SO-S) were only sampled during the migratory season (T_1 and T_2), when the UM-M and PB-M hives were in their proximity (Fig. 1).

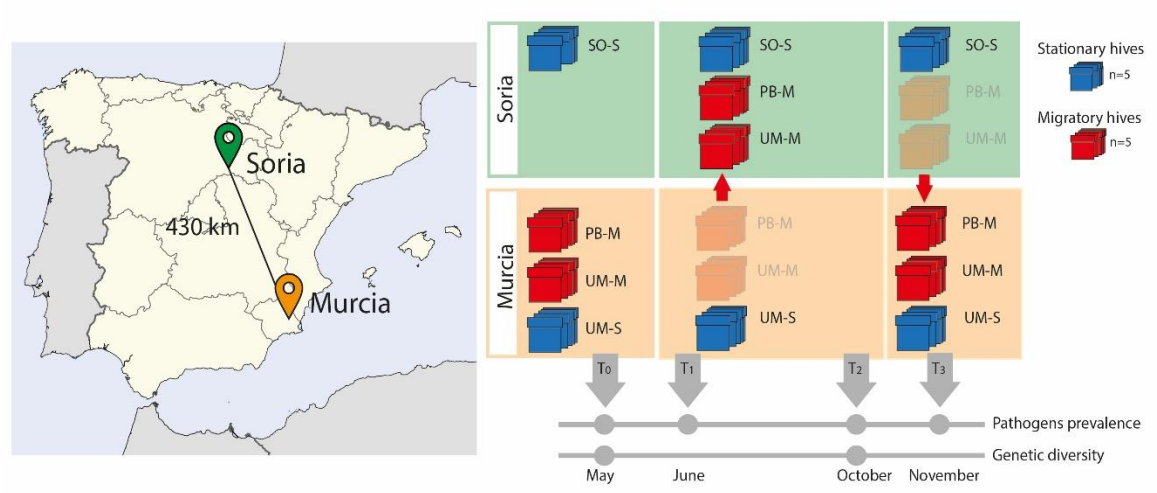

Fig. 1: Graphical representation of the sampling design

Sample collection

Adult honey bee workers were taken from combs located between those with sealed brood and those with fresh honey in order to sample both, nursing and foraging workers (Jack et al. 2016). Samples were kept either in absolute ethanol at -20 °C or frozen directly at -80 °C.

Varroa destructor *detection*

The infestation rate of *V. destructor* was estimated from about 200 worker honey bees per colony at each sampling time. A total of 70 samples were evaluated: 15 colonies (5 UM-M, 5 PB-M and 5 UM-S) and 4 sampling times, plus 5 colonies of the SO-S apiary sampled at T_1 and T_2. *Varroa* mites were dislodged from honey bee workers preserved in ethanol and the proportion of infested individuals per colony was calculated by dividing the number of mites counted by the number of honey bees in each sample (Dietemann et al. 2013).

Extraction and purification of DNA and RNA

Simultaneous RNA and DNA extraction was carried out to detect *Nosema* spp. and to quantify DWV. As the gut is a major site of accumulation for viruses and other pathogens (de Miranda et al. 2013), honey bee abdomens were macerated in AL buffer (Jara et al. 2016). From each colony and sampling time, 22 honey bee workers were used to determine the presence of pathogens. The abdomen of each honey bee was removed and placed in one well of a 96-well plate containing glass beads (2 mm; Sigma®), and they were then macerated individually in 180 µl of AL buffer (Qiagen® 19075) and shaken in a Tissuelyser (Qiagen®) for 6 min at 30Hz. Afterwards, the homogenate of each abdomen was transferred to another plate (Deepwell, Eppendorf®) and incubated for 10 min with 20 µl of proteinase K (10 mg/ml) and 200 µl of the AL buffer at 70 °C, shaking at 300 rpm.

DNA and RNA purification was performed simultaneously in a Biosprint 96 workstation (Quiagen®) following the BS96 DNA Tissue extraction protocol. Total nucleic acids (RNA and DNA) were eluted in 100 µL of elution buffer and aliquots of 75 µL of this eluted product were frozen until further processing to detect *Nosema* spp. The remaining 25 µL obtained from each honey bee from the same colony and sampling time were pooled (Highfield et al. 2009), and any genomic DNA was completely removed by digestion with DNase I (Quiagen® kit 79254). This DNA-free pooled RNA (10 µl) was used to generate cDNA (hereafter referred to as pooled cDNA) using the iScriptTM cDNA Synthesis Kit (Biorad®). Negative and positive controls were run in parallel for each step (maceration, nucleic acid extraction and reverse transcription).

Nosema *spp. detection*

Aliquots of 2.5 µl of the total nucleic acids eluted from the abdomen of each individual honey bee were used to detect *N. apis* and *N. ceranae*. PCR reactions were performed as described previously (Martín-Hernández et al. 2012), using an internal PCR control to determine the reliability of the analysis. The prevalence per colony was estimated from the number of *Nosema* positive honey bee samples divided by the total number (22) of honey bees analyzed (Fries et al. 2013).

Quantification of DWV

Quantification of the viral load of each colony and time was performed by qPCR in a 384-well plate using the LightCycler® 480 Real Time PCR System (Roche®). The qPCR was carried out in a total volume of 10 µl containing 2x LightCycler® 480 Probe Master, 0.3 µM of the primers DWV958F: CCTGGACAAGGTCTCGGTAGAA and DWV9711R: ATTCAGGACCCCACCCAAAT, 0.1 µM of the FAM marked probe DWV9627T: CATGCTCGAGGATTGGGTCGTCGT (Chantawannakul et al. 2006) and 2.5 µl of the pooled cDNA. The qPCR conditions were 95 °C for 10 min, and 45 cycles of 95 °C 10 s and 60 °C 40 s (annealing temperature). Each sample was run in duplicate and the average *Cq* values were calculated. Positive samples were only considered when the average *Cq* value was < 40 (Budge et al. 2010; 2015). A known amount of DWV PCR product was run in parallel to obtain a standard curve that was used to convert the *Cq* data to the initial concentration of DWV RNA in the reaction tube. Positive and negative PCR controls were also included. The online tool (http://cels.uri.edu/gsc/cndna.html) was utilized to calculate the equivalence between amount (ng) of DWV RNA and DWV copy number in each qPCR reaction. The viral loads per honey bee were calculated for each colony and sampling time by averaging the amount of RNA extracted from 80.5 mg of honey bee abdomens. We determined the average amount of RNA in a single honey bee and thus, the viral load, by taking into account the elution volume and the average weight of a honey bee (Highfield et al. 2009). Prior to data analysis, virus copy number was log10 transformed to account for the exponential distribution of the data (de Miranda et al. 2013). Because zero values cannot be log transformed, negative samples were assigned a hypothetical *Cq* value of 40 and later converted to the theoretical virus copy number as described above.

Number of combs

The number of combs with capped brood per colony was annotated for every colony at each of the four sampling times, as a qualitative measure of their strength and general health status (Budge et al. 2015). This also served to assess the extent to which this characteristic, often evaluated by beekeepers when checking health and vitality status of the colonies, is related to pathogen prevalence.

Statistical analysis

The calculation and graphical representation of the different descriptive measures was implemented with the R v. 3.2.2 software (R Core Team, 2015). The normal distribution of the data obtained of each group of colonies and the equality of variance between the groups of data were tested with the Kolmogorov–Smirnov normality test and the F-test of equality of variances respectively. Thereafter, T-tests, or Mann–Whitney U test if required, were used to compare means between groups. All these analyses were carried out in PAST v.3.14 (Hammer et al. 2001). In collapsing colonies,

the number of brood combs was considered as zero, and the pathogen prevalence and viral load were considered as not available (NA) at the sampling times subsequent to colony collapse. Possible correlations between the different variables were assessed with the Pearson's correlation test sing PAST v.3.14.

A Principal Component Analysis (PCA) was also performed with the PAST v.3.14 software to compare the relative weight of the different variables (brood combs, pathogens prevalence and virus load), as well as to assess the explanatory power of these variables on the dispersion of the colonies in function of the type of beekeeping management.

2.2. Genetic diversity and patrilineal composition of the colonies

Sampling

The effect of migratory beekeeping on the genetic diversity of the colonies was analyzed in the ten colonies from the research apiary at the University of Murcia (UM-S and UM-M groups) which have been placed in the same site for more than ten years. Honey bee worker were sampled at T_0 (May) prior to the movement of the five migratory UM-M hives to Soria, and at T_2 (October) after the migratory season (Fig. 1). Samples of 45 worker honey bees per colony and sampling time were preserved in absolute ethanol at -20 °C until processed.

Microsatellite amplification and detection

Two analyses were performed to compare the accuracy in the detection of patrilines within one colony focusing on a total of 12 loci: a first one involved data from a multiplex of five loci A113, A7, Ap43, Ap55 and B124 (Evans et al. 2013); and a second analysis including seven more microsatellite loci A79, A8, A88, Ac11, Ap224, Ap249 and Ap274 (Chahbar et al. 2013). The results of these two analyses yielded the same number of patrilines and thus, the determination of the number of patrilines and genetic diversity were taken from the analysis of the five microsatellite loci alone as in Hernández-García et al. (2009).

Total DNA from each worker honey bee was extracted using the Chelex® method (Evans et al. 2013) and 2 µl of this DNA was used for multiplex microsatellite amplification in a total volume of 10 µl, containing: 1X reaction buffer, 1.2 mM $MgCl_2$, 0.3 mM of each dNTP, 0.4 µM of each primer (one of each pair fluorescence labelled), and 1.5 units of Taq polymerase (Biotools®). The annealing temperature was set at 54 °C and the PCR products were visualized by capillary electrophoresis (ABI-3730, Applied Biosystems®), scoring individual alleles using GeneMapper v3.7 software (Applied Biosystems®).

Microsatellite data analysis

The following population genetic parameters were calculated for each group of colonies (migratory and stationary) and at each sampling time (T_0 and T_2) based on allele frequency and using the GenAlex v. 6.41 software (Peakall and Smouse 2006, 2012): expected heterozygosity (*He*), number of different alleles (Na), number of effective alleles (Ne), and number of private alleles (Pa).

Fisher's exact and Pearson's X^2 tests were applied to detect changes in allele frequencies between T_0 and T_2, per each locus and colony group (stationary and migratory). The tests were carried out in R v. 3.2.2 and “fisher.test” and “chisq.test” functions were utilized, respectively. Fisher's tests included all the alleles at each locus, while to meet Cochran’s conditions, the Pearson's X^2 test only included those alleles with ten or more observations (considering both sampling times). To test the significance of the variation in the genetic composition of the two groups of colonies between T_0 and T_2, population pairwise F_{ST}, estimated through a "weighted" analysis of variance (Cockerham 1973; Weir and Cockerham 1984) was calculated in Arlequin v. 3.5 (Excoffier et al. 2005). Also, a G-test or maximum likelihood statistical significance test for population differentiation (Raymond and Rousset 1995; Rousset 2008) was carried out in Genepop On the Web (http://genepop.curtin.edu.au/genepop_op3.html), and Lositan (Antao et al. 2008) and Bayescan (Foll and Gaggiotti 2008) programs were run to investigate whether selection affected any of the loci analyzed.

Honey bee worker genotypes were analyzed with the Colony 2.0.5 software (Wang 2004; Wang and Santure 2009; Jones and Wang 2010) to unravel the patrilineal composition of each colony at each sampling time. Events of queen replacement and bee drifting were also extrapolated with the software.

Furthermore, discriminant analysis of principal components (DAPC: Jombart et al. 2010) based on the worker genotypes, was carried out using the “Adegenet R” package (Jombart, 2008) in R v. 3.2.2., in order to assess and graphically represent the changes in the clustering of the worker honey bees sampled from each colony before (T_0) and after (T_2) the migration period.

3. Results

3.1. Parasite and pathogen assays

Varroa destructor

A low prevalence of *V. destructor* was detected in all the colonies at the beginning of the study (T_0 mean values 4.5 ± 6.2% in the migratory colonies and 4.0 ± 2.3% in the stationary colonies). No significant changes ($p > 0.05$) were observed after transportation to Soria (T_1 5.7 ± 11.1% and 3.0 ± 4.0% respectively), although a significant increase ($z = -2.1650$ $p = 0.0304$) in the prevalence of *Varroa* was detected in migratory colonies after the summer period (T_2 26.1 ± 29.2%), while the prevalence in stationary colonies remained low (T_2 5.2 ± 6.8%). Indeed, the former displayed a significantly higher mite prevalence than the stationary colonies at T_2 ($t = 2.2049$ $p = 0.0415$: Fig. 2a), with colonies 1M (UM-M), 7M (PB-M) and 10M (PB-M) registering the highest infestation rate at T_2 (42%, 83% and 61%, respectively: Suppl. material Table 1).

When comparing migratory and stationary colonies from the University of Murcia (same genetic background), a trend towards a higher prevalence of *Varroa* mites ($t= 1.5030$ $p= 0.0775$) was observed in the migratory colonies at T_2 (UM-M 18.0 ± 14.2% *vs.* UM-S 8.0 ± 9.1%). This difference was less apparent after the colonies returned to Murcia at T_3 ($p > 0.05$), due to a decrease in *V. destructor* prevalence in these migratory group of colonies (UM-M 8.56 ± 11.04%), while prevalence remained stable in the stationary colonies (UM-S 5.8% ± 3.9). Migratory colonies from Murcia with different genetic backgrounds, UM-M and PB-M, displayed a distinct response to *V. destructor*, whereby the PB-M colonies suffered a stronger increase in *Varroa* prevalence (from T_0 5.12 ± 8.57% to T_2 36.28 ± 41.98%) than the UM-M colonies (from T_0 3.78 ± 2.28 to T_2 17.96 ± 9.06%) in the migratory period. The standard deviation was also higher in the PB-M group, evidence of the large differences in *Varroa* prevalence among individual colonies within the group (Suppl. material Table 1). Stationary colonies from Soria (SO-S) that shared their location and environmental conditions with UM-M and PB-M colonies from June to October (T_1 and T_2), showed the lowest prevalence of *V. destructor* in June (T_1 0.28 ± 0.63%), which increased mildly in October (T_2 4.58 ± 4.59%); a prevalence similar to that registered in the UM-S group at this time (T_2 5.76 ± 9.06%).

Nosema *spp.*

Of a total of 1413 worker honey bees analyzed by PCR, *Nosema spp.* were detected in 10.9%. *N. ceranae* was the species detected in 84% of the infected workers, *N. apis* in 8% and co-infection with both species was detected in the remaining 8%. Co-infection increased from T_0 to T_1 (Suppl. material Table 1), yet since the *N. apis* infestation and co-infection rates were low and unsuitable for proper statistical analysis, the following data mainly refer to *N. ceranae* infestation (Fig. 2b).

The prevalence of the microsporidium was distinct among the colonies at the beginning of the experiment due to the random selection of the hives analyzed. The prevalence of *Nosema* in migratory colonies (9.9 ± 10.03%) was higher than in stationary colonies (1.7 ± 1.7%) at T_0 (Fig. 2b). Moreover, at T_1 the prevalence of *N. ceranae* increased significantly (z= -2.3866 *p*= 0.017) in migratory colonies (T_1 26.8 ± 15.74%) but not in the stationary hives (*p* > 0.05), as UM-S colonies maintained a lower prevalence (T_1 0.9 ± 2.0%). Therefore, statistically higher *Nosema* parasitism was detected in migratory as opposed to stationary colonies at T_1 (z= -3.1841 *p*= 0.0015). After the summer period (T_2), the prevalence of this microsporidium tended to decrease (*p* > 0.05) in the two groups of colonies, yet the migratory colonies (T_2 18.1 ± 15.54%) still maintained a higher prevalence of *N. ceranae* (z= -2.8917 *p*= 0.0038) than the stationary colonies (T_2 2.1 ± 3.15%). Two weeks after their transportation back to Murcia (T_3), the prevalence of *N. ceranae* continues its decreasing tendency in the migratory colonies (*p* > 0.05: Fig. 2b).

When comparing UM-M and UM-S (the same genetic background), the prevalence of *Nosema* was significantly higher in the migratory colonies (UM-M) at all the sampling times, but particularly at T_1 (z= -2.4866 *p*= 0.0129). Infestation rates of 16.8 ± 9.9% and 0.9 ± 2.03% were registered in UMU-M and UM-S respectively, in T_1. Between the two groups of migratory colonies (of different background), the PB-M colonies had a greater prevalence of *N. ceranae* (T_1 36.7 ± 14.57%) than the UM-M colonies after they were transported to Soria (T_1 16.8 ± 9.9%, z = -2.0889 *p* = 0.0367).

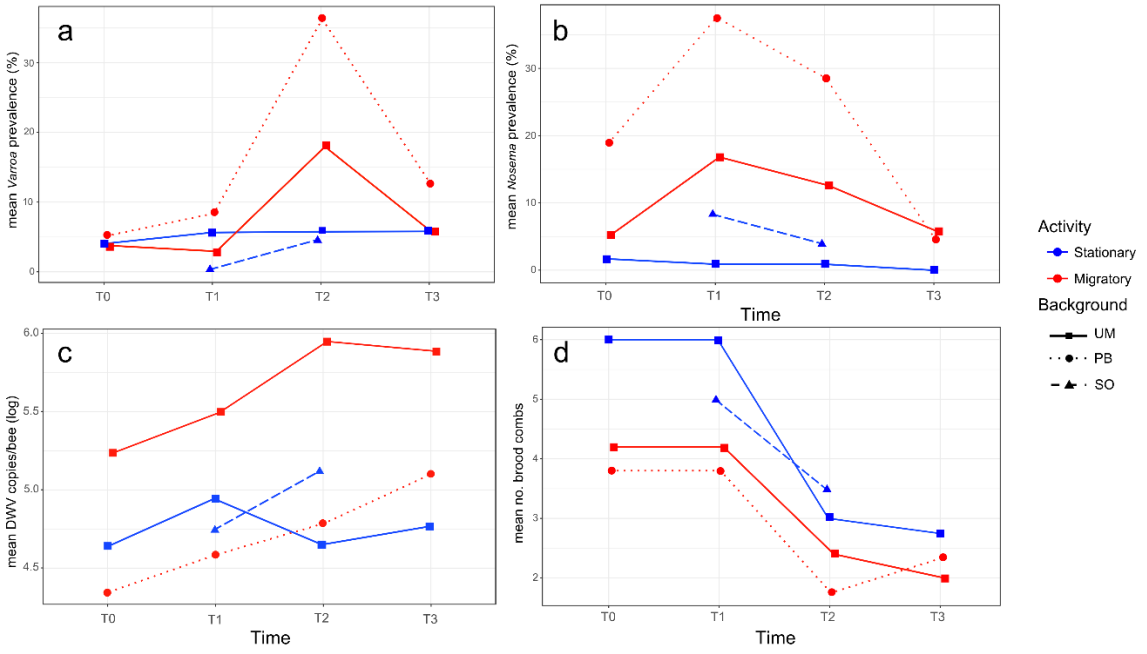

Fig. 2: The average data per colony group and sampling time of the variables: a) prevalence of* V. destructor*; b) prevalence of* N. ceranae*; c) log of the DWV load (DWV copies/bee); d) number of brood combs.

DWV loads

From the 65 pools of RNA extracted from 22 worker honey bees per colony, and at the distinct sampling times (5 NA due to colony collapse), DWV was detected in 35 (53.9%), with mean viral loads ranging from $2.22x10^4$ to $6.89x10^6$ DWV copies/bee (Suppl. material Table 1). Due to the random selection of the hives, the initial viral load at T_0 differed widely among the colonies irrespective of their management. However, at T_1 the average DWV load tended to increase, albeit not significantly ($p > 0.05$), for the migratory and stationary colonies. After the migratory period, at T_2 there were no clear trends in DWV load between the stationary and migratory colonies (Fig. 2c). In general, the largest differences were detected among the individual colonies over the entire study period (T_0 to T_3), although a temporal fluctuation at the colony level was also observed irrespective of the management group.

The UM-M and UM-S colonies registered a distinct initial situation, with a tendency (although not significant, $p > 0.05$) towards higher DWV loads in UM-M (T_0 $8.13x10^5$ ± $1.38x10^6$ DWV copies/bee) than in UM-S (T_0 $6.58x10^4$ ± $7.14x10^4$ DWV copies/bee). In June (T_1) both groups of colonies showed a slight increase in DWV copies/bee ($p > 0.05$), while at T_2 (at the end of the migration period) the DWV load had increased in UM-M (T_2: $1.4x10^6$ ± $1.5x10^6$ copies/bee) whereas it had decreased in UM-S (T_2 $4.4x10^4$ ± $6.0x10^6$ copies/bee). Nevertheless, no significant differences ($p > 0.05$) were found in the DWV load between the two groups at T_2 due to the high standard deviation registered. Likewise, similar viral loads were found in the two groups of colonies at T_3 (Fig. 2c).

For migratory colonies that differed in their genetic background (UM-M and PB-M), the initial situation was also distinct. The DWV viral load was below the detection threshold in all the PB-M colonies at T_0, while the UM-M colonies registered an average viral load of $4.45x10^4$ ± $2.47x10^4$ DWV copies/bee. However, despite the initial differences, both groups showed a tendency towards a higher DWV load at T_1 (UM-M $6.7x10^5$ ± $9.6x10^5$ and PB-M $2.4x10^4$ ± $1.5x10^4$ copies/bee) and T_2 (UM-M $1.4x10^6$ ± $1.5x10^6$ and PB-M $1.8x10^5$ ± $3.4x10^5$ copies/bee), while at T_3 the DWV load tended to decrease slightly in UM-M ($8.1x10^5$ ± $6.3x10^5$ copies/bee) and it tended to increase in PB-M ($7.6x10^5$ ± $1.3x10^6$ copies/bee: $p > 0.05$ in both cases).

In the case of the SO-S colonies established close to the migratory area, a tendency towards an increased DWV load was observed from T_1 ($8.2x10^4$ ± $1.4x10^5$ copies/bee) to T_2 ($7.9x10^5$ ± $1.7x10^6$ copies/bee: $p > 0.05$), while in the colonies settled in Murcia (UM-S) a tendency towards a decrease in DWV load was registered in the same period (T_1 $1.1x10^5$ ± $1.36x10^5$ to T_2 $4.4x10^4$ ± $6.0x10^6$ copies/bee :$p > 0.05$).

Brood combs

There was a tendency towards a slightly higher mean number of brood combs in stationary as opposed to migratory colonies at the beginning of the experiment (T_0 5.5 ±

2.1 and 4.0 ± 3.0, respectively: $p > 0.05$). The same number of brood combs was also found at T_1, two weeks after the transportation of the migratory colonies to Soria. At T_2, the 6M colony (from the PB-M group) collapsed and fewer brood combs were detected after the summer period in both groups of colonies (T_2 3.2 ± 1.6 and 2.1 ± 1.0, respectively: Fig. 2d). This decrease was significant in the stationary group (t = 2.6800 p = 0.0157), although at T_3, after transporting the migratory hives back to Murcia, a similar low mean number of brood combs was observed in the migratory and stationary colonies (2.14 ± 0.7 and 2.8 ± 1.9, respectively). Colonies 1M (UM-M), 4S (UM-S), 6 M and 7M (PB-M) had collapsed by that time (T_3).

Correlation analysis

As expected, a Pearson's correlation test (Fig. 3) between the variables analyzed and the sampling times highlighted a positive correlation in the number of brood combs between T_0 and T_1 (same number of brood combs, r = 1 p = 0.0000), and also between T_2 and T_3 (r = 0.6615 p = 0.0010). However, no correlation was found between the number of brood combs recorded in T_1 and that observed in T_2 ($p > 0.05$).

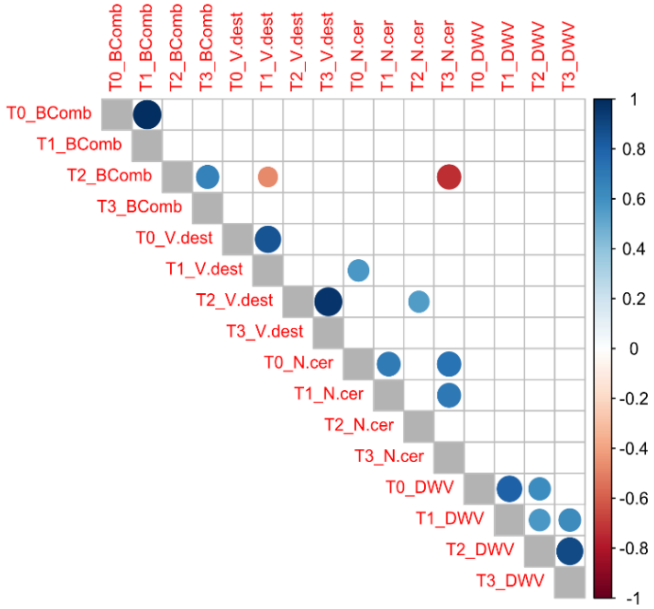

Fig. 3: Pearson's correlation tests between the variables analyzed at each sampling time: number of brood combs,* V. destructor *and* N. ceranae *prevalence, and DWV load.

An increased prevalence of *V. destructor* at T_1 was negatively correlated (r = -0.4715 p = 0.0416) with the number of brood combs at the next sampling time (T_2). This negative correlation was stronger in migratory (r = -0.67238 p = 0.033165) than in stationary colonies (r = -0.47149 p = 0.04156). Similarly, this negative correlation was also found in migratory colonies (r = -0.69053 p = 0.039471), whereby the prevalence of *V. destructor* at T_2 was negatively related to the mean number of brood combs at T_3. Likewise, the

lower number of brood combs at T_2 appeared to be strongly correlated to the higher prevalence of *N. ceranae* a few weeks later (T_3 r = -0.73142 *p* = 0.0105), although there was no correlation between the number of brood combs and the DWV loads (*p* > 0.05). The prevalence of *N. ceranae* at T_0 appeared to be positively correlated to the *V. destructor* prevalence at T_1 (r= 0.57064 *p*= 0.0263) and T_2 (r= 0.55137 *p*= 0.0144), yet there was no correlation between *Varroa* or *Nosema* prevalence and DWV load.

PCA analysis

A first PCA analysis, including all the colonies and taking as variables the number of brood combs, *Varroa* and *Nosema* prevalence, and DWV viral load at the four sampling times (Suppl. material Table 1), allowed us to assess the relative weight of each variable in the clustering of the colonies throughout the study (T_0-T_3), without making any prior assumption regarding beekeeping management. Eigenvalues of the two principal components explained 75.42% of the overall variability. The prevalence of *V. destructor* and *N. ceranae* at T_2 (PC 1) and the prevalence of *N. ceranae* at T_1 (PC 2) were the variables with the strongest influence on the dispersion of the colonies in the PCA (Fig. 4). The dispersion of the migratory colonies was based on the most prevalent pathogens (3M, 8M, 6M and 9M along the T1_*N. ceranae* vector, 1M along T2_*N. ceranae*, and 5M, 7M and 10M along T2_*V. destructor* vector), while the dispersion of the stationary colonies was not affected by these vectors. A second PCA with only the data from UM-M and UM-S colonies (the same genetic background) showed a similar result with same variables influencing the dispersion, i.e.: *V. destructor* and *N. ceranae* at T_2, and the prevalence of *N. ceranae* at T_1 (data not shown).

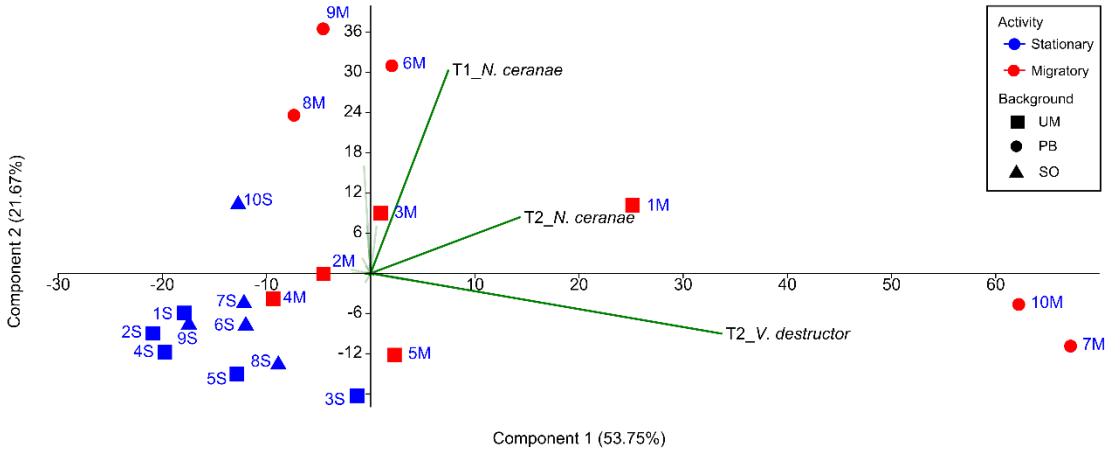

Fig. 4: The Scatterplot of the colonies shows dissimilar dispersion of stationary and migratory colonies according to the rates of parasitism

3.2. Genetic diversity and patrilineal composition of the colonies

Genetic diversity

In order to assess the impact of beekeeping management on the genetic variation of the colonies, population genetic parameters were inferred from the genotypes of 45 worker honey bees per colony (UM-M and UM-S, five colonies each), before (T_0) and after migration (T_2). Temporal variation of these parameters was not statistically significant in the migratory or stationary colonies ($p > 0.05$), although differences were observed in the mean number of alleles, in the number of private alleles and in the genetic diversity in some individual colonies (e.g., 1M, 3M, 4M and 4S: Table 1). In general, the greatest differences in genetic diversity were found among individual colonies (e.g., 4M *vs.* 5M or 2S *vs.* 3S), irrespective of their management (migratory and stationary) or sampling time (T_0 and T_2).

To further analyze the temporal differences between T_0 and T_2, Fisher's and X^2 Pearson´s tests were performed for each locus (Table 2). Significant differences were detected in the frequency of the alleles at the five loci analyzed within the group of migratory colonies, whereas in the stationary colonies significant differences in the allelic frequency were only evident at the Ap43 and A7 loci. Moreover, the population differentiation analyzed with a pairwise F_{ST} and Exact G tests showed significant differences between T_0 and T_2 only in the migratory colonies (Table 2). Nevertheless, the Lositan and Bayescan analyses did not show any outlying locus susceptible to evolutionary selection for any of the two groups of colonies.

	He-T0	He-T2	Na-T0	Na-T2	Ne-T0	Ne-T2	Pa-T0	Pa-T2
Migratory mean	**0.49**	**0.47**	**4.6**	**3.8**	**2.40**	**2.21**	**0.04**	**0.12**
1M	0.61	0.56	4.8	4.6	2.60	2.50	0.2	0
2M	0.61	0.60	4.4	4.0	2.64	2.51	0	0
3M	0.48	0.48	5.4	3.8	2.37	2.26	0	0.2
4M	0.60	0.53	6.2	4.2	3.03	2.38	0	0.4
5M	0.15	0.18	2.2	2.6	1.33	1.40	0	0
Stationary mean	**0.54**	**0.55**	**5.0**	**4.6**	**2.53**	**2.48**	**0.04**	**0.04**
1S	0.52	0.56	4.8	5.0	2.31	2.46	0	0.2
2S	0.38	0.36	4.4	3.4	2.04	2.01	0	0
3S	0.64	0.64	5.2	5.0	2.95	2.67	0	0
4S	0.58	0.57	5.8	4.2	2.71	2.56	0.2	0
5S	0.57	0.60	4.8	5.6	2.62	2.70	0	0
Total mean	**0.52**	**0.51**	**4.8**	**4.2**	**2.46**	**2.35**	**0.04**	**0.08**

Table 1: Population genetic parameters of migratory and stationary colonies at the four sampling times.

		Fisher's exact test	Pearson's X^2 test	Exact G Test	Population pairwise FSTs
Migratory	A113	0.00253*	*P*= 0.06071 (X^2=10.57; df=5)		
	A7	0.006968*	*P*= 0.4594 (X^2=1.56; df=2)		
	Ap43	0.00031**	*P*= 0.004462* (X^2=20.57; df=7)		
	Ap55	0.000005***	*P*= 0.0000009*** (X^2=36.08; df=5)		
	B124	0.00068**	*P*= 0.00213* (X^2=22.44; df=7)		
	Total			*P*=0.0000000*** (X^2=∞; df=10)	*P* =0.00901* (F_{ST}=0.00367)
Stationary	A113	0.8859	*P*= 0.7251 (X^2=2.84; df=5)		
	A7	0.03473*	*P*= 0.01441* (X^2=12.43; df=4)		
	Ap43	0.00099**	*P*= 0.00107* (X^2=27.7; df=9)		
	Ap55	0.4102	*P*= 0.2229 (X^2=4.38; df=3)		
	B124	0.07931	*P*= 0.07686 (X^2=12.81; df=7)		
	Total			*p*= 0.00342374* (X^2=26.3; df=10)	*P* =0.24324 (F_{ST}=0.00080)

Table 2: Results of the Fisher's and X^2 Pearson's tests per locus, and the Exact G test for population differentiation and the population pairwise F_{ST} by time (T_0 vs. T_2) in function of beekeeping management (migratory/stationary).

Patriline analysis

Paternal lines within the colonies and the relationships among the worker offspring were studied at T_0 and T_2. The number of patrilines differed strongly among the individual colonies as opposed to between the colony groups (migratory *vs.* stationary), ranging from 7 to 16 in the migratory, and from 9 to 19 in the stationary colonies. The temporal variation in the number and frequency of patrilines was observed in individual colonies from both groups.

The number of patrilines changed in most of the colonies from T_0 to T_2 (Table 3). Despite the equivalent number of patrilines in colonies 5M, 2S and 4S, a replacement of some patrilines was detected between T_0 and T_2, with some low frequency patrilines no longer detected and new patrilines recorded. At the colony level, some patrilines were more frequently found at one sampling time than at another. Despite the trends

observed in favor of some patrilines rather than others in both individual migratory and stationary colonies, no selective patterns could be detected related to the different management groups.

Queen replacement and drifting

Colony software detected honey bee queen replacement in the migrating colonies M1, M3 and M4, and this replacement was seemingly related to the genetic differences observed between T_0 and T_2. In colony M1, worker offspring from the new queen was only detected at T_2, suggesting that the replacement of the queen occurred in Soria, during the migratory period. For colonies M3 and M4, worker offspring from the new queen was already present at T_0, which indicates that the replacement and mating of the new queen took place in Murcia prior to transportation of the colonies to Soria. Drifting workers (Table 3) were detected in five colonies at T_0 (three migratory and two stationary) and in two colonies (both migratory) at T_2. The sample size was too small to accurately assess the effect of the sampling time or the colony management on the number of drifters or the queen replacement events detected in the colonies.

	Number Patrilines		**Queen events**		**Drifting workers**	
Colony	T0	T2	T0	T2	T0	T2
1M	11	10	1			
2M	10	11			1	
3M	24	11	1			
4M	15	9		1	4	2
5M	4	4			1	1
1S	13	12				
2S	8	8			3	
3S	13	15				
4S	9	9			5	
5S	17	18				

Table 3. Number of patrilines, queen replacement events and drifting workers per colony and time (T_0 and T_2).

DAPC analysis

A discriminant analysis of principal components based on the worker genotypes showed a distinct clustering of the samples between T_0 and T_2 in some colonies (Fig. 4). In general, differences in the dispersion of the clustering data between these two times were more pronounced in migratory colonies due to queen replacement, mainly in the case of the colonies M1, M3 and M4 (Fig. 5).

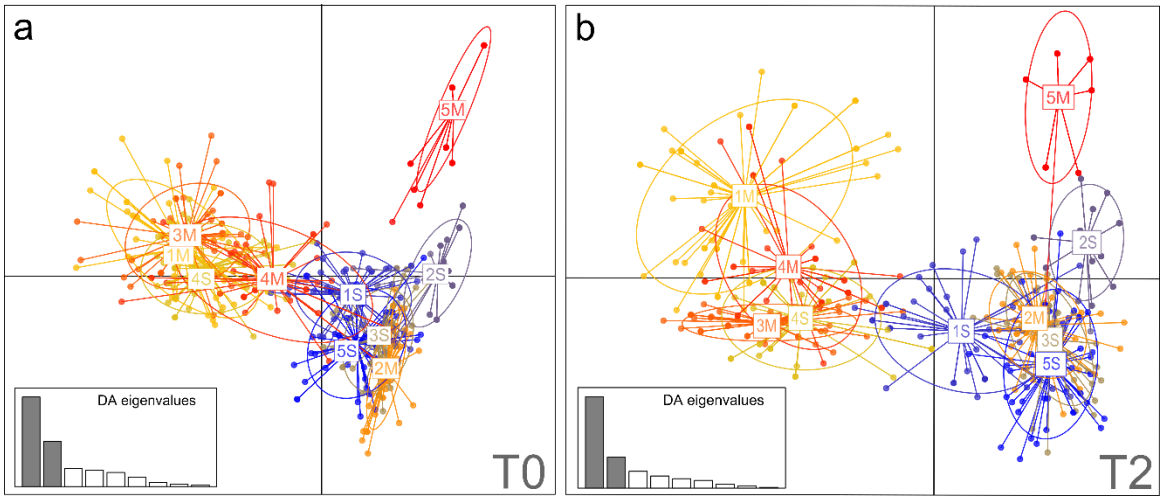

Fig.5: Discriminant analysis of principal components based on worker genotypes

4. Discussion

4.1. Effects of migratory beekeeping on the colony health and disease transmission

This study provides further insights into the impact of migratory beekeeping practices on honey bee health (Ahn et al. 2012; Simone-Finstrom et al. 2016), and on the prevalence of pathogens and diseases in the colonies (Zhu et al. 2014, Jara et al. submitted; Cavigli et al. 2016). Our major finding is that migratory beekeeping and its associated stress leads to higher *V. destructor* and *N. ceranae* prevalence in the colonies, a result that partially confirms our initial hypothesis.

Changes in Varroa destructor *prevalence*

In the case of *V. destructor*, the type of colony management (migratory *vs.* stationary) seems to influence the proliferation of the mite during the summer months. Although the two groups of colonies display similar low *Varroa* prevalence in May (T_0) and June (T_1), a significantly higher prevalence of *V. destructor* was recorded in migratory colonies compared to the stationary colonies after the summer period (T_2). *V. destructor* population dynamics are known to be highly influenced by the host population dynamics (Rosenkranz et al. 2010). Indeed, the increase of mites in migratory colonies may be partially explained by the existence of sufficient brood combs (Calis et al. 1999; Martin 2001), which is thanks to the mild weather conditions in Soria and the availability of ample resources. However, colonies that are permanently located in Soria did not show such a large increase in *Varroa* mite, despite sharing the same environmental conditions. Thus, we believe that stresses associated to migratory management also contributed to a high prevalence the pathogen. For example, phoretic infestation by mites from highly

infected colonies to healthy ones during migratory operations have been noted (Nelson and Jay 1989), a clear case of horizontal pathogen transmission (Fries and Camazine, 2001). Genetic background is another factor to be considered in terms of the susceptibility of colonies to pathogens (Behrens et al. 2011; Jara et al. 2012, 2015; Huang et al. 2013; Francis et al. 2014). This could explain the stronger increase in *V. destructor* prevalence after summer (T_2) in the colonies of the professional beekeeper from Murcia than in our migratory colonies, even though they were transported together, located in the same migratory area in Soria and showed similar *Varroa* infestation rates at the beginning of the experiment.

Changes in Nosema *spp. prevalence*

Nosema ceranae was more prevalent than *N. apis*, in consonance with previous reports in Spain (Martín-Hernández et al. 2012; Jara et al. 2012, 2015) and elsewhere (Klee et al. 2007; Paxton et al. 2007; Fries 2010). Despite the low rates of *N. apis* infestation, the prevalence of combined infections of the two *Nosema* species increased more in the migratory than in the stationary colonies (Annex X). This increase is worth noting as it has been demonstrated that combined *Nosema* infection significantly decreases honey bee survival when compared to single species infection (Milbrath et al. 2015). *N. ceranae* was significantly more prevalent in migratory than in stationary colonies in June (T_1), just two weeks after the hives were transported to Soria. Its prevalence was also high in October (T_2), although the mean infestation rates were lower. Hence, transportation and pollination services are apparently related to the prevalence and abundance of *N. ceranae* in *A. mellifera* workers, as reported previously (Zhu et al. 2014). It was hypothesized that transportation and the ensuing pollination weakened the immune system of bees, rendering them more susceptible to infection. Stressors that affect the honey bee immune system and that thereby favor pathogen proliferation include truck vibration, noises, marked changes of temperature during hive transport and the release of *Nosema* spores from infected bees that die during transportation (Webster 1993; Malone et al. 2001; Klee et al. 2007; Giersch et al. 2009). At the physiological level, migratory management has a negative impact on honey bee lifespan and oxidative stress (Simone-Finstrom et al. 2016), as well as on individual bee development and physiology (Ahn et al. 2012), favoring the spread of pathogens.

The significant increase in *Nosema* spp. from T_0 to T_2 was not found at T_3, at the beginning of November after the migratory colonies had returned to Murcia. A factor that may have counterbalanced the negative effects of transportation is the better weather encountered by the bees on their return to their original location. During their stay in Soria, the temperatures were often close to 10 °C and rainfall was frequent (e.g., in mid-October, in the two weeks before their return, 40% of rainy days and a mean temperature of 11.2 °C). Cold temperatures and bee confinement is thought to enhance the prevalence and density of *Nosema* spp. inside colonies (Bailey 1955; OIE 2008;

Martín-Hernández et al. 2009; Jara et al. 2012; Fries 2010; Traver et al. 2012; Retschnig et al. 2017). The reduced possibility of bees flying out and defecating due to bad weather favors the spread of *Nosema* within the colony, as horizontal transmission occurs predominantly through trophallaxis and oral-fecal routes (Higes et al. 2010; Fries 1993; 2010). When the bees returned to Murcia they encountered only 10% of rainy days and a mean temperature of 18.5 °C, weather conditions that favor a lower prevalence of *Nosema*.

Moreover, a higher prevalence of *Nosema* spp. was found in PB-M compared to MU-M at T_1 and T_2. However, since a higher prevalence of *Nosema* in the PB-M bees was detected at the beginning of the study, it is not clear whether genetic differences between the two groups of colonies might underlie this difference in *Nosema* prevalence.

Changes in DWV load

In terms of DWV load, the virus was detected in 53% of the samples analyzed, as reported in France for adult *A. m. mellifera* bees from March to November (Tentcheva et al. 2004). No clear trends were found between stationary and migratory colonies during our survey and the largest differences were found among individual colonies rather than between colony groups. The viral loads detected ranged from, $2.22x10^4$ to $6.89x10^6$ DWV copies/bee, comparable to those reported in asymptomatic colonies in other studies employing a similar methodology (Highfield et al. 2009; Fievet et al. 2006). Despite the evident differences in DWV load among colonies in May (T_0), a tendency towards a greater viral load (although not significant) was detected in the two groups of colonies (migratory and stationary) in June (T_1). Seasonal load variation of DWV is known to closely follow that of the *Varroa* mite, due to the role of this mite as a vector of DWV (de Miranda and Genersch 2010; Runckel et al. 2011). Thus, DWV load usually increases as the bee season progresses (Sumpter and Martin 2004; Tentcheva et al. 2004; Gauthier et al. 2007) and *V. destructor* parasitism augments, mainly in spring and early summer (Rosenkranz et al. 2010).

Changes in number of brood combs

We found a significant reduction in the number of brood combs present in the colonies in October (T_2), probably reflecting the increase of *Varroa* infestation. Indeed, higher infestation rates of *V. destructor* at T_1 were negatively correlated with the strength of the colony at T_2 (inferred from the number of brood combs), both in migratory and stationary colonies. This result agrees with a number of studies where *V. destructor* is identified as the main factor affecting colony strength (Budge et al. 2015). Indeed, migratory colonies that collapsed before the end of the study had a high prevalence of *Varroa* mites. In addition, colonies with fewer brood combs at T_2 also had higher levels of *Nosema* a few weeks later (T_3), after their return to Murcia.

Correlation among variables

A positive correlation was detected between *V. destructor* and *N. ceranae* infestation rates, consistent with previous studies showing that week colonies (less than five brood combs) are more frequently associated with higher numbers of pathogens in adult bees (Budge et al. 2015; vanEngelsdorp et al. 2009; Cornman et al. 2012; Ravoet et al. 2013). Unexpectedly, no correlation was found between *V. destructor* prevalence and DWV load, in contrast to many studies that point to *Varroa* as the most important vector for DWV, positively affecting the DWV copy number in bees (Bowen-Walker et al. 1999; Shen et al. 2005; Hedtke et al. 2011; Prisco et al. 2011; Francis et al. 2013). However, some studies have shown a lag in the correlation (Mondet et al. 2014) between the mite and the virus (Meixner et al. 2014), suggesting that the epidemic's dynamics are unique to individual colonies, or even to different times during the season (Prisco et al. 2011).

PCA analysis

As discussed here, a number of stressors associated with migratory management negatively affect the colony's heath, leading to increased pathogen spread. As summarized by the PCA analyses, the prevalence of *N. ceranae* in June (T_1), and the prevalence of *V. destructor* and *N. ceranae* in October (T_2), appeared to be the variables that most strongly influence the dispersion of migratory and stationary colonies.

4.2. Effects of migratory beekeeping on the colony health and disease transmission

Changes in the genetic diversity

Migratory beekeeping had only a weak impact on the genetic diversity of the colonies. Short migrations may be insufficient to produce changes on the overall genetic diversity (as depicted by the lack of outlier loci), although trends were evident within the colonies in relation to allele frequency. This is exemplified by the fact that the strongest differences in genetic parameters were found between individual colonies irrespective of the management group or the sampling time T_0–T_2.

Changes in patriline composition

Temporal changes in allele frequencies and in the patriline composition of the colonies were also higher within each management group than between groups, suggesting a strong influence of stressors other than management that affect the colonies individually. Stressor like pathogen prevalence and climatic condition are important selective forces that shape the genetics of colonies (Spivack and Reuter 2001; vanEngelsdorp and Meixner 2010; Chávez-Galarza et al. 2013). Furthermore, there is accumulating evidence that genetic variation can influence host susceptibility to

pathogens (Reed and Frankham 2003; Spielman et al. 2004; Whitehorn et al. 2011). Previous studies found different incidence of *N. ceranae* (Burgeois et al. 2012) and bacterial pathogens (Palmer and Oldroyd 2003) among patrilines inside the colonies; as well as changes in allele frequencies and signal of selection determined by *Varroa* and *Nosema* parasitism (Oxley et al. 2010; Behrens et al 2011; Huang et al. 2013; Jara et al. 2015; Chávez-Galarza et al. 2013). As climatic conditions were shared by all migratory colonies in this study, temporal genetic variation may rather be influenced by the prevalence and the synergic effects of the particular combination of pathogens affecting each colony (Yang and Cox-Foster 2007; Antúnez et al. 2009; Costa et al. 2011; Runckel et al. 2011; Nazzi et al. 2012; Martin et al. 2013; Aufauvre et al. 2014; Milbrath et al. 2015). These stressors, along with queen replacement events detected in some migratory colonies, may be the causes of the significant genetic differences detected by the Fisher's and Pearson's tests in migratory colonies between T_0 and T_2.

Queen replacement and drifting

Queen replacement, as well as brood diseases, high *Varroa* prevalence and DWV load have been proposed as the main factors leading to colony collapse in migratory beekeeping operations (vanEngelsdorp et al. 2013). Moreover, queen replacement during migration and the subsequent mating of the queen in migratory areas, as occurred here with the 1M colony, have been also highlighted as an important factor for genetic homogenization and the loss of local adaptation in the Iberian honey bee populations (Cánovas et al. 2011; Jara et al. 2015). However, the true impact of each individual stressor could not be weighed here due to the difficulty in controlling all the variables affecting the colonies in field conditions.

This study is a first attempt to identify the effects of migratory operations on bee colonies under field conditions, where the combined interactions of stress factors may have a significant effect on the colonies. Together, these findings indicate that genetic diversity is not notably affected by the type of beekeeping management. However, further studies will be necessary to fully untangle these complex interactions between management, genetic background, environment and other factors related to the status of the colony itself, weighing the effect of each factor on the honey bee colonies.

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Supplementary Material

Table 1. Data obtained for each colony in the four sampling times (T_0-T_3)

		Brood combs (No.)				*V.destructor* (%)				*N. ceranae* (%)				*N. apis* (%)				*Co-infections by* both *Nosema spp*				DWV copies/bee			
Code	**Group**	**T0**	**T1**	**T2**	**T3**	**T0**	**T1**	**T2**	**T3**	**T0**	**T1**	**T2**	**T3**	**T0**	**T1**	**T2**	**T3**	**T0**	**T1**	**T2**	**T3**	**T0**	**T1**	**T2**	**T3**
1UM	**M**	0	0	3	0	2.6	1.4	42	NA	16.7	32	4	NA	0	0	0	NA	0	0	0	NA	2.22E+04	2.22E+04	2.15E+06	NA
2UM	**M**	8	8	2	2	5.7	4.6	10.8	5.8	0	15.8	8.7	4.5	0	0	0	0	0	0	0	0	1.11E+05	1.80E+05	3.28E+06	2.26E+06
3UM	**M**	6	6	2	2	1.2	5.6	7.8	3.4	9.1	13.6	32	4.5	0	0	0	9.1	0	4.5	0	0	3.22E+05	4.10E+06	5.13E+05	2.55E+06
4UM	**M**	3	3	3	3	8.7	2	9.4	4.4	0	13.6	0	0	0	0	9.1	0	0	0	0	0	3.56E+06	1.70E+06	6.89E+06	1.07E+06
5UM	**M**	4	4	2	1	0.7	1.1	19.8	8.5	0	4.5	9.1	4.8	0	0	0	0	0	0	0	0	5.14E+04	1.06E+05	2.22E+04	5.75E+04
6PB	**M**	6	6	0	NA	20.3	36.7	NA	NA	28.6	31.8	NA	NA	0	0	NA	NA	0	4.5	NA	NA	2.22E+04	2.22E+04	NA	NA
7PB	**M**	4	4	0	0	2.2	4.8	82.7	NA	0.0	18.2	27.3	NA	0	0	0	NA	4.5	4.5	0	NA	2.22E+04	4.47E+04	2.22E+04	NA
8PB	**M**	1	1	2	3	0.3	0.4	0.9	4.9	5.6	36.8	11.5	4.8	0	0	0	0	0	15.8	0	0	2.22E+04	2.22E+04	2.22E+04	2.22E+04
9PB	**M**	8	8	2	2	0	0	0.6	0	22.7	40.9	22.7	9.1	0	4.5	4.5	0	0	9.1	0	0	2.22E+04	4.32E+04	2.22E+04	2.22E+04
10PB	**M**	0	0	3	2	2.8	0	60.9	32.9	0.0	21.7	44	0	20	0	0	0	12	0	4	0	2.22E+04	9.00E+04	1.27E+06	4.14E+06
1UM	**S**	8	8	3	0	3.9	4.5	0	5.1	4.5	4.5	4.5	0	0	0	0	0	0	0	0	0	3.33E+04	5.53E+05	5.91E+04	2.22E+04
2UM	**S**	4	4	3	3	3	12.4	0	0.8	4	0	0	0	0	0	0	0	0	0	0	0	4.32E+04	2.22E+04	2.22E+04	2.22E+04
3UM	**S**	6	6	5	4	4.6	2	20.7	10.1	0	0	0	0	0	0	0	0	0	0	0	0	2.22E+04	2.22E+04	2.22E+04	2.22E+04
4UM	**S**	5	5	0	0	7.4	6.3	0	NA	0	0	0	NA	0	0	0	NA	0	0	0	NA	2.22E+04	4.84E+04	2.22E+04	NA
5UM	**S**	7	7	4	4	1.2	3	8.1	7.2	0	0	0	0	0	0	0	0	0	0	0	0	2.08E+05	3.94E+05	2.74E+05	1.06E+06
6SO	**S**		2	4			0	1.6			0	16			0	4			0	0			2.22E+04	2.22E+04	
7SO	**S**		5	3			0	6.1			10	0			0	0			0	0			2.22E+04	2.46E+05	
8SO	**S**		3	5			1.4	12			0	0			0	0			0	0			6.13E+05	6.89E+06	
9SO	**S**		7	2			0	1.4			4.5	0			0	0			0	0			7.16E+04	4.66E+04	
10SO	**S**		8	NA			0	1.8			27.3	0			0	0			0	0			2.22E+04	2.22E+04	

General conclusions

(Conclusiones generales)

General conclusions

Chapter I: Linking evolutionary lineage with parasite and pathogen prevalence in the Iberian honey bee

1. No significant relationship was observed between the parasitism by *Varroa destructor* and the colony evolutionary lineages in both sampling years, 2006 and 2010, what suggests an independent distribution pattern of the mite of that of the evolutionary lineages (A or M) of *A. m. iberiensis* in Spain. This finding is consistent with results previously reported for the original host *A. cerana*.

2. No relationship was detected between *Nosema ceranae* infection and the evolutionary lineage of the infected colonies in both sampling years, 2006 and 2010. However, *N. apis* infected M lineage colonies with higer prevalence than those of the A lineage in 2006. It is no clear whether this preference of *N. apis* for bees of the M lineage is the result of the preference of the parasite for the Atlantic climatic conditions that prevail in the northern third of the Iberian Peninsula, the area where the M lineage naturally occurs. Alternatively, it might be that co-evolution between *N. apis* and bee populations of the M lineage is more advanced than that with bee populations of the A lineage.

3. The infestation pattern of *Nosema* spp. microsporidia described for 2006 significantly shifted in 2010, as the number of infested colonies by *N. ceranae* (alone or mixed with *N. apis*) markedly increased, while those exclusively infected by *N. apis* decreased. Further studies are needed to investigate the interaction between both species of *Nosema*, as co-infections may be only temporary situations that will evolve to the exclusion of *N. apis* under particular circunstances.

Chapter II: Stable genetic diversity despite parasite and pathogen spread in honey bee colonies

1. The results from this study indicate that the genetic diversity among honey bee colonies in Spain was stable over the two years surveyed, 2006 and 2010. This possibly is due to the multidrone mating system of the queens, and the high gene flow between colonies across most regions of Spain, derived from an extensive migratory beekeeping practice.

2. The initial hypothesis that the increased incidence of parasites (*V. destructor*) and pathogens (*N. apis and N. ceranae*) in recent years was associated with a decrease in genetic diversity levels (*He*) of the Iberian honey bee population was not corroborated.

3. Significant differences in allele frequencies (G test) were detected between colonies sampled in 2006 and 2010, as well as between colonies parasitized by *Varroa* and/or *Nosema* and non-parasitized ones. Furthermore, we identified two outlier loci, Ap249 and B124, related to genes that respond to stress and that could potentially reflect selective processes.

Chapter III: Effect of small-scale migratory movements on the dispersion of *Ascosphaera apis* in *Apis mellifera iberiensis* colonies

1. DNA extraction and PCR amplification of the fungus *Ascosphaera apis* in worker adult bees have been optimised. From the tested protocols, better DNA extraction and PCR amplification yields were obtained with the Chelex® extraction method followed by a modified PCR protocol from Yoshiyama and Kimura (2011).
2. We found a positive correlation between migratory beekeeping (subjected to small-scale regional level movements) and a higher prevalence of the fungus.
3. This result is of concern for beekeepers, and highlight the need of finding a balance between the economic benefits of migrating colonies (even at a small-scale) for a continuous honey harvesting, and the impact of spreading pathogens and stressing the colonies, as both factors increase the incidence of diseases.

Chapter IV: The effect of migratory beekeeping on the prevalence of pathogens in honey bee colonies and on their genetic composition

1. The study provides further understanding into the impact of migratory beekeeping practices on honey bee health and pathogens spread. Our major finding is that migratory beekeeping and its associated stress lead to higher *V. destructor* and *N. ceranae* prevalence in the colonies, a result that partially confirms our initial hypothesis.
2. Conversely, any clear trend was found in the DWV load comparison between stationary and migratory colonies during our survey, as the largest differences were found among individual colonies rather than between colony groups. Furthermore, no correlation was detected between DWV and *V. destructor*, in agreement with previous studies that show a lag in the correlation between the mite and the virus, suggesting that the dynamic of the disease is unique to individual colonies.
3. Migratory beekeeping had only a weak impact on the genetic diversity of the colonies. Short migrations may be insufficient to produce changes on the overall

genetic diversity. This is exemplified by the fact that the strongest differences in genetic parameters were found between individual colonies, irrespective of the management group or the sampling time. It seems that stressors other than management have been involved in these results, what should be further investigated.

Annexes

(Anexos)

Other publications of the author (SCI)

PLOS ONE

RESEARCH ARTICLE

Population Genetics of *Nosema apis* and *Nosema ceranae*: One Host (*Apis mellifera*) and Two Different Histories

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Abstract

Two microsporidians are known to infect honey bees: *Nosema apis* and *Nosema ceranae*. Whereas population genetics data for the latter have been released in the last few years, such information is still missing for *N*. *apis*. Here we analyze the patterns of nucleotide polymorphism at three single-copy loci (*PTP2*, *PTP3* and *RPB1*) in a collection of *Apis mellifera* isolates from all over the world, naturally infected either with *N*. *apis* (*N* = 22) or *N*. *ceranae* (*N* = 23), to provide new insights into the genetic diversity, demography and evolution of *N*. *apis*, as well as to compare them with evidence from *N*. *ceranae*. Neutral variation in *N*. *apis* and *N*. *ceranae* is of the order of 1%. This amount of diversity suggests that there is no substantial differentiation between the genetic content of the two nuclei present in these parasites, and evidence for genetic recombination provides a putative mechanism for the flow of genetic information between chromosomes. The analysis of the frequency spectrum of neutral variants reveals a significant surplus of low frequency variants, particularly in *N*. *ceranae*, and suggests that the populations of the two pathogens are not in mutation-drift equilibrium and that they have experienced a population expansion. Most of the variation in both species occurs within honey bee colonies (between 62%-90% of the total genetic variance), although in *N*. *apis* there is evidence for differentiation between parasites isolated from distinct *A*. *mellifera* lineages (20%-34% of the total variance), specifically between those collected from lineages A and C (or M). This scenario is consistent with a long-term host-parasite relationship and contrasts with the lack of differentiation observed among host-lineages in *N*. *ceranae* (< 4% of the variance), which suggests that the spread of this emergent pathogen throughout the *A*. *mellifera* worldwide population is a recent event.

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Introduction

The genus *Nosema* (Fungi, Microsporidia, Dihaplophasea, Dissociodihaplophasida Nosematidae; Nägeli, 1857) contains over eighty species [1,2] typically found in arthropods. Two species, *N. apis* and *N. ceranae*, parasitize the Western honey bee, *Apis mellifera*. *N. apis* Zander, 1909 is a globally distributed pathogen that was identified in this host more than a hundred years ago [3], whereas *N. ceranae* was described at the end of the twentieth century [4]. This latter species, although initially discovered in the Asian honey bee *Apis cerana* [4], was recently proved to infect *A. mellifera* [5,6], and since then it has been found worldwide in this new host [7,8,9,10,11], as well as in several other *Apis* [12,13] and *Bombus* species [14,15]. Both pathogens are transmitted through the ingestion of spores during feeding, grooming and trophallaxis [16,17]. Once in the gut, they invade the ventricular cells causing disease, but the clinical and epidemiological characteristics of the parasitization by either species are different; the infection by *N. apis* (type A nosemosis) does not usually cause the death of the colonies and is characterized by dysentery, general weakening of the adults, locomotion impairment and crawling [18]. These symptoms are not present in *N. ceranae* infections (type C nosemosis) [19], which produce alterations in the temporal polyethism, foraging activity and life span of infected bees [20,21,22]. Although the same could also be true for *N. apis* [23], the higher prevalence of *N. ceranae* throughout the year in temperate climates [24,25]–in contrast with that of *N. apis*, which usually displays seasonal peaks [26]–, induces a chronic stress on the colony that may eventually lead to its collapse [20,27,28]. This effect, whose potential relationship with the large scale depopulation phenomenon is still matter of debate ([24,28], or [29,30] for a different point of view), is much more dramatic in Mediterranean countries, especially in Spain, where climatic conditions and/or beekeeping practices seem to increase the impact of *N. ceranae* on honey bee colonies reviewed in [31].

Genetic data revealed that *N. apis* and *N. ceranae* are highly divergent at the nucleotide level (average nonsynonymous divergence of 10%; [32]) and that there has been considerable gene shuffling since the split from their common ancestor [33], evidencing that they are not very close relatives within the genus *Nosema* [34,35].

The genetic characterization of *N. ceranae* populations in *A. mellifera* has been achieved in the last few years by analyzing different components of the ribosomal DNA (rDNA) [36,37], single-copy genes [32,38,39,40,41] and whole genomes [42]. The most relevant conclusions of these studies are that i) *N. ceranae* isolates obtained from individual honey bees exhibit multiple alleles at single copy loci [38,39], ii) most of the variation resides within honey bee colonies [39,42], iii) there is no differentiation among geographically distant isolates [36,38,39,42], iv) this pathogen has experienced a recent demographic expansion in *A. mellifera* [38,39,42], and v) there is evidence for low, but significant, levels of recombination [36,38,40,41,42].

In contrast, there is little information about the population genetics of *N. apis*. Although a few sequence data have been released in public databases, most of them remain unpublished (e.g. PopSets 723438493, 698364701, 225055863 from GenBank) and/or involve the analysis of rDNA [43,44,45] that harbors multiple copies in the *N. apis* genome [33]. These are organized as tandemly repeated units, each of them consisting of a small (SSU) and large (LSU) subunits separated by an internal transcribed spacer (ITS), and an intergenic spacer (IGS) [45,46] (see [33] for a slightly different organization). The redundancy of these arrays usually promotes the conservation of rDNA sequences through different mechanisms (concerted evolution [47], and/or birth-and-death processes [44]) that preserve their important role in mRNA translation. However, in the case of *N. apis* and *N. ceranae*, rDNA copies are highly diverse [33,36,48] and, although the reasons for the existence of differently expressed rRNA copies are still to be determined [37], the presence of such heterogeneous units complicates the assessment of the

levels of polymorphism [44,49], as within-genome heterogeneity is hard to disentangle from between-individuals diversity. This limits the use of ribosomal loci to estimate the genome-wide patterns of variability.

Here we report a population genetic analysis conducted to address questions that are central to our understanding of the recent evolutionary history of *N. apis*, such as: what are the levels of genetic variation of this parasite? Is there any genetic evidence for a long-term association between *N. apis* and *A. mellifera*? Is the *N. apis* population panmictic or is there any sign of geographical structure? With this aim, the sequences of three single copy genes were studied in a collection of *N. apis* and *N. ceranae* isolates obtained from *A. mellifera* colonies from all over the world. These loci had been previously studied in *N. ceranae* [32,39,40] and their patterns of polymorphism used to yield new insights into this parasite's populations. Our results, along with these previous data, provide the first comparative analysis of the patterns of genetic variation of both pathogens in the same host species.

Material and Methods

Samples

N. apis was isolated from twenty two naturally infected *A. mellifera* colonies from eleven countries worldwide: Algeria, Argentina, Canada, Chile, Germany, Hungary, the Netherlands, Poland, Slovenia, Spain, and Turkey (S1 Table). A similar number of *N. ceranae* isolates (N = 23) were collected from *A. mellifera* colonies from 17 countries: Algeria, Argentina, Australia, Brazil, Canada, Chile, Croatia, Greece, Hawaii (USA), Hungary, Japan, the Netherlands, Slovenia, Solomon Islands, Spain, Taiwan, and continental United States of America (S2 Table).

Ethics statement

No specific permits were required for the described studies, which did not involve endangered or protected species.

DNA extraction, PCR amplification, cloning and sequencing

DNA was extracted from homogenized pools of 15–20 honey bees from each colony. This was carried out as in [50], using the BioSprintTM 96 DNA Blood Kit (QIAgen, Izasa, Barcelona, Spain). The reagents used in this process were tested by PCR to check for the presence of potential contamination with *N. apis*, *N. ceranae* or honey bee DNA in each round of extractions. The identity of *Nosema* species was determined by specific PCR amplification of the *16S rDNA*, as in [51]. No co-infections were detected in these samples.

Specific primers were designed with Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) using sequences of each species as query (KE647054.1: 13328–14116—locus tag NAPIS_ORF00435—for *PTP2*, KE647278.1: 2294–4285—locus tag NAPIS_ORF01922—for *PTP3* and DQ996230.1 for *RPB1* in *N. apis*, and XM_002995356.1 for *RPB1* in *N. ceranae*, respectively). Primer pairs for amplification of *PTP2*, *PTP3* and *RPB1* in *N. apis* were: `PTP2 Na-F (CTGCTACAGCACCGCCATTA) and PTP2 Na-R (TGGGGTTTAATCTTGCTTTTTCCA)`, `PTP3 Na-F (AGACAAGGTGTTTCTC CAAAAGA) and PTP3 Na-R (GCAAGGTTTTCTTCTGTTGCC) and RPB1 Na-F (GTTAA GAGCAGAAGATGATCTAAC) and RPB1 Na-R (CTGATAATTTGTTTTCCTGTCCAATA)`, respectively. Primer pairs to amplify *RPB1* in *N. ceranae* were those published in [32].

PCR amplification, cloning and sequencing procedures were performed as in [39]. PCR annealing temperatures were adjusted for each of the primer pairs. These were 59.0°C, 58.0°C

and 56.5°C for *PTP2*, *PTP3* and *RPB1* in *N. apis* and 55°C for *RPB1* in *N. ceranae*, respectively. Each round of PCR amplification included negative and positive controls (PCR components with no template DNA, and PCR components + DNA extracted from *N. ceranae*–or *N. apis*–positive isolates, respectively).

Sequences were checked for accurate base calling using CodonCode Aligner (CodonCode Corporation, Dedham, MA, USA) and aligned with MUSCLE [52] with their reference sequences to determine the nucleotide positions at each locus. The alignments were manually corrected with BioEdit [53] and the sequences submitted to GenBank (S1 and S2 Tables).

Apis mellifera: lineage assignment

Determination of the *A. mellifera* evolutionary lineage was performed by sequence analysis of the intergenic region between the $tRNA^{leu}$ and the cytochrome oxidase II (cox2) gene as described previously [54]. DNA was extracted from a pair of legs using the Chelex® method [55]. The intergenic $tRNA^{leu}$-cox2 region was PCR-amplified in a thermocycler PTC 100 (MJ Research) in a total volume of 12.5 μL with KapaTaq DNA Polymerase (KAPA BIOSYSTEMS), containing 2 μL of DNA template, 200 μM total dNTP, 1 X Reaction Buffer, 0.5 U/rxn KapaTaq DNA Polymerase, 1.5 mM $MgCl_2$, 0.4 μM of each primer (E2 and H2, [56]). The thermocycler program used was: 94°C (5 min); 35 cycles of a 45 s denaturation at 94°C, a 45 s elongation at 48°C, a 60 s extension at 62°C; and a final extension step at 65°C for 20 min. Amplicons were sequenced with the primer E2 (Secugen S.L., Madrid, Spain). Each sequence was manually checked for base calling and a multiple sequence alignment was performed with the MEGA program, version 6 [57]. Evolutionary lineages were determined by comparison with sequences deposited in GenBank (lineage C: JQ977699, JF723946; lineage M: HQ337441, KF274627; lineage A: EF033650, JQ746693).

Sequence analyses

Nucleotide diversity in *N. apis* and *N. ceranae* was estimated at synonymous and non-synonymous sites with DnaSP v5.10.02 [58]. Sites with alignment gaps were excluded from the analyses. π [59] and θ_W [60] were calculated applying the Jukes-Cantor correction [61]. π, the average number of pairwise differences between sequences, is sensitive to the frequency of polymorphisms and complementary to the estimate of θ_W, which measures the levels of variability by counting the number of segregating sites, independently of their frequency, and thus giving more weight to rare variants. The Tajima´s *D* test [62] compares the two statistics. If the population is in mutation-drift equilibrium, π and θ_W are expected to have same value, and *D* should be equal to zero. Negative *D* values reflect an excess of low frequency variants (greater θ_W), which under neutrality can be interpreted as evidence for a recent population expansion. According to Tajima's considerations on the different distributions followed by *D* at individual or pooled loci [62], the statistical significance of the deviation from neutral expectations for individual genes was determined using DnaSP (which assumes a beta distribution). When several unlinked regions of DNA were combined to describe the patterns of polymorphism of a species (pooled loci) this significance was calculated by applying Tajima´s formulae and assuming a normal distribution [62]. The possibility of a population expansion was further investigated by applying the Fu's F_S [63], as implemented in DnaSP v5.10.02. Its significance was assessed by comparing the observed values with a null distribution generated by 1,000 coalescent simulations. The number of net nucleotide substitutions per site between populations, *D*a [59], was also estimated with DnaSP.

The program MLHKA [64] allows to test for selection at individual loci in a multilocus framework by comparing the relative amounts of within and between-species synonymous

variation across unlinked loci. [65]. The patterns of diversity at the genes used in this study were compared with those observed at other loci with similar data available (*actin*, *Hsp70*, *HSWP4*, and *SWP30*) [32,38,39]. Genes that did not exhibit enough sequence identity between *N. apis* and *N. ceranae* to be confidently assigned as orthologs were discarded from the analysis (*NCER_100064*, *NCER_100070*, *NCER_100533*, *NCER_100768*, *NCER_101165* and *NCER_101600*; [38]), as was *PTP1* [39], which is tightly linked to *PTP2* [48,66]. Rates of synonymous and nonsynonymous divergence between *N. apis* and *N. ceranae* sequences for these loci were estimated using the Yang and Nielsen method [67], as implemented in the software PAML v 4.8a [68].

Lower bounds of the recombination rate were estimated using two different statistics under the assumption of the infinite sites model (i.e. each segregating site has mutated only once): *Rm*, the minimum number of recombination events, is based on the four-gamete test [69], which infers a recombination event if all four possible two-locus haplotypes occur in the sample, and *Rh* [70], which bounds the number of recombination events by calculating the difference between the number of haplotypes in the sample and the number of segregating sites. Both statistics were calculated with RecMin [70] (http://www.stats.ox.ac.uk/~myers/RecMin.html). The population scaled recombination rate (ρ) at the three loci was estimated applying the composite-likelihood method of Hudson [71], adapted to finite-sites model (to account for sites that might have experienced more than one mutation), as implemented in LDhat v2.0 [72]. Since the likelihood of observing recombination is dependent on the order of sites, the statistical significance of a non-zero rate of recombination was evaluated with a permutation test, in which the maximum composite likelihood was calculated under random permutation of the physical position of the variants (1000 permutations) [72]. *Nosema* parasites are commonly found as single-cell diplokaryons, so that they harbor a minimum of two haploid genomes. Thus, the number of haploid genomes is assumed to be 2 x $2N_e$, and $\rho = 4N_e r$.

Haplotype diversity was estimated with DnaSP v5.10.02, the statistics K_{ST}^* [73] and S_{nn} [74], were used to investigate the population structure. Their significance was assessed using permutation tests (1000 replicates).

An analysis of molecular variance (AMOVA) was performed with Arlequin 3.5 [75] and the significance of covariance components was checked by applying non-parametric permutation procedures (3000 permutations).

Haplotype networks were generated with Network 4.6.1.0 (Fluxus Technology, http://www.fluxus-engineering.com/sharenet.htm) using the Median-Joining algorithm, which allows for more than one different nucleotide per site. The epsilon parameter was set to 0, 10 and 20 in successive runs in order for the resulting network to include all possible shortest trees in the resulting network. Since no significant differences were observed, only those networks generated with epsilon = 0 are presented. The Connection Host criterion was used as distance calculation method. The Reduced Median algorithm (with $r = 2$) was applied to obtain a simplified network containing all shortest trees. All networks were redrawn manually.

Identification of mating and meiotic genes

The identification in *N. apis* and *N. ceranae* of the components of a sex-related locus [76] and an inventory of genes involved in meiosis [77] was performed by means of Blastp and SQR Sequence Search (https://www.ncbi.nlm.nih.gov/Structure/seqr) using as queries protein sequences from other microsporidia such as *Encephalitozoon cuniculi*, *Enterocytozoon bieneusi*, *Antonospora locustae* and *Nosema bombycis*.

Results

Genetic diversity

The genetic variability of *N. apis* samples was initially assessed at *PTP2*, *PTP3* and *RPB1* in seven naturally infected *A. mellifera* colonies from Algeria, Argentina, Canada, Slovenia, Spain ($N = 2$) and Turkey (dataset^{A} in Table 1 and S1 Table). To increase the resolution of the analysis, and given that the levels of diversity were similar for the three genes (see below), the *RPB1* locus was randomly selected to enlarge the former dataset with sequences of 15 additional samples from Canada ($N = 2$), Chile ($N = 2$), Germany ($N = 3$), Hungary, Netherlands ($N = 2$), Poland ($N = 3$), Slovenia ($N = 2$), (dataset^{A+B} in Table 1 and S1 Table).

The three genes displayed similar levels of synonymous variation in *N. apis* (pooled π_S values of 1.17%, 1.27% and 1.50%, for *PTP2*, *PTP3* and *RPB1*^{A}, respectively; these values were estimated by pooling all the sequences of each locus). The enlarged *RPB1* dataset (*RPB1*$^{A+B}$) produced comparable results ($\pi_S = 1.68\%$) and was used hereafter. It is interesting to note that these pooled values are twice the observed average diversity across the seven samples (0.80%, 0.85% and 0.82% for *PTP2*, *PTP3* and *RPB1*^{A}, respectively). This discrepancy could reflect some level of differentiation among isolates and was further investigated in the "Population structure" section below.

The patterns of variation at these loci were also studied in *N. ceranae* (Table 2 and S2 Table). The pooled π_S values for *PTP2*, *PTP3* and *RPB1* were 1.00%, 0.85% and 1.58%, respectively and, in contrast to what was observed in *N. apis*, these estimates were very similar to the average diversities across samples (0.95%, 0.82% and 1.42%, for the same loci, respectively).

In both species the levels of polymorphism at nonsynonymous positions were much lower than those observed at synonymous sites (Tables 1 and 2) and θ_W estimates were usually higher than those of π (resulting in pooled negative Tajima's *D* values, especially in *N. ceranae*).

To verify if the observed patterns diversity and divergence at these three genes departed significantly from those of other genomic loci with available population data (*actin*, *Hsp70*, *HSWP4*, and *SWP30*) we performed a maximum likelihood HKA test [64]. *Actin* and *Hsp70* displayed lower diversity relative to their divergence levels (likelihood ratio test, $LRT = 18.5$, $P < 0.001$). No significant deviations were detected at any the other five loci. In addition, the ratio of nonsynonymous to synonymous divergence (d_N/d_S) was below unity for the seven loci, ranging from 0.02–0.04 for *actin*, *Hsp70* and *RPB1* to 0.21 for *SPW30* and *HSPW4* (S3 Table).

Considering the evidence for a recent population expansion in *N. ceranae* [32,38,39,42], we examined this possibility in *N. apis* by applying two alternative tests: Tajima's *D* [62] and Fu's F_S [63]. The former test can be used to compare the frequency spectrum of variants with neutral expectations, and revealed an excess of low frequency synonymous variants in *N. apis*. Although this deviation was not significant at individual loci (Table 1), it was significant when data from *PTP2*, *PTP3* and *RPB1* were combined ($D_S = -1.82$, $P = 0.034$). Similar results were obtained in *N. ceranae*, where the combined data revealed a significant excess of low frequency synonymous variants ($D_S = -2.93$, $P < 0.002$). The F_S test [63] provided additional evidence for a significant excess of haplotypes in *N. apis PTP2* and *RPB1* genes, as compared with neutral expectations (Table 3). *N. ceranae* sequences obtained from *A. mellifera* displayed a similar pattern (Table 3).

Given the possibility that the *N. apis* population was subdivided in two demes (see the "Population structure" section below), one in lineage A honey bees (from Africa and the Iberian Peninsula) and another one in the European honey bee lineages C and M, the *D* and F_S statistics were estimated separately in both groups. Tajima's *D* for lineage C isolates was significant both at synonymous and nonsynonymous sites (-2.18, $P < 0.015$ and -3.30, $P < 0.0001$, respectively), but no significant skew was observed at synonymous sites among isolates collected

Table 1. Nucleotide diversity at three loci from *N. apis*: *PTP2*, *PTP3* and *RPB1*.

			PTP2							*PTP3*							*RPB1*						
	Isolate	Origin	*N*	π_S	θ_{WS}	D_S	π_A	θ_{WA}	D_A	*N*	π_S	θ_{WS}	D_S	π_A	θ_{WA}	D_A	*N*	π_S	θ_{WS}	D_S	π_A	θ_{WA}	D_A
[A]	52	SLO	9	0.31	0.51	-1.36	0.00	0.00	NA	5	0.00	0.00	NA	0.00	0.00	NA	9	1.78	1.42	1.03	0.15	0.25	-1.68
	174	TUR	10	0.28	0.49	-1.40	0.15	0.26	-1.67	5	1.05	1.25	-1.05	0.34	0.41	-1.09	10	0.71	1.02	-1.28	0.11	0.19	-1.67
	204	ARG	8	0.00	0.00	NA	0.09	0.14	-1.31	5	1.42	1.68	-1.09	0.13	0.10	-1.22	11	0.72	0.65	0.34	0.20	0.23	-0.49
	381	CAN	9	0.31	0.51	-1.36	0.12	0.20	-1.51	10	0.53	0.93	-1.56	0.24	0.30	-0.76	11	1.22	2.13	-1.90*	0.32	0.51	-1.59
	399	SPA	7	1.60	1.69	-0.34	0.16	0.23	-1.36	7	1.02	1.42	-1.43	0.12	0.09	1.34	7	0.14	0.20	-1.01	0.12	0.17	-1.36
	410	SPA	10	1.79	1.46	0.85	0.15	0.26	-1.67	13	1.22	0.84	1.37	0.13	0.20	-1.23	12	1.06	0.63	2.28*	0.07	0.14	-1.63
	854	ALG	10	1.30	0.98	1.23	0.04	0.07	-1.11	5	0.70	0.83	-0.97	0.17	0.20	-0.97	8	0.12	0.18	-1.05	0.03	0.05	-1.05
	Pooled [A]		63	1.17	1.91	-1.53	0.10	0.58	-2.51***	50	1.27	2.14	-1.20	0.20	0.57	-1.91*	68	1.50	1.81	-0.55	0.25	0.74	-2.08*
[B]	59	SLO															8	1.97	1.30	2.29*	0.29	0.32	-0.34
	264	POL															10	1.94	1.36	1.78	0.18	0.14	0.85
	266	POL															10	1.96	1.70	0.58	0.21	0.29	-1.19
	268	POL															10	1.93	1.89	1.03	0.17	0.14	0.70
	363	CHI															8	2.09	1.66	1.10	0.18	0.16	0.58
	380	CAN															8	1.80	1.48	0.93	0.19	0.21	-0.52
	382	CAN															10	1.83	1.19	-2.16*	0.23	0.24	-0.23
	411	CHI															10	0.86	1.36	-1.64	0.16	0.29	-1.80*
	529	SLO															9	1.66	1.77	-0.37	0.09	0.15	-1.51
	569	HUN															10	1.93	1.36	1.69	0.20	0.19	0.14
	1074	GER															8	2.07	1.48	1.77	0.17	0.26	-1.60
	1098	GER															10	1.32	1.36	-0.21	0.18	0.24	-1.04
	1099	GER															10	0.79	0.85	-0.33	0.15	0.19	-0.94
	1511	NED															10	2.03	1.53	1.31	0.10	0.10	0.02
	1735	NED															9	0.11	0.18	-1.09	0.06	0.10	-1.36
	Pooled [A+B]																208	1.68	2.78	-1.16	0.22	1.37	-2.53***

[A], Seven isolates used to estimate *N. apis* diversity;

[B], 15 additional isolates used for further analysis of *RPB1*;

ALG: Algeria, ARG: Argentina, CAN: Canada, CHI: Chile, GER: Germany, HUN: Hungary, NED: Netherlands, POL: Poland, SLO: Slovenia, SPA: Spain, TUR: Turkey; *N*: number of cloned sequences; π_S and π_A, pairwise nucleotide diversity at synonymous and nonsynonymous sites expressed as percentage, respectively [59]; θ_{WS} and θ_{WA}, nucleotide site variability based on the number of synonymous and nonsynonymous segregating sites expressed as percentage, respectively [60]; the average number of synonymous and nonsynonymous sites analyzed across loci were 155.6 and 582.4, respectively; D_S and D_A, Tajima´s *D* [62] at synonymous and nonsynonymous sites, respectively; NA: not available; statistical significance of Tajima´s *D*:

*, $P < 0.05$;

***, $P < 0.001$.

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Table 2. Nucleotide diversity at three loci from *N. ceranae*: *PTP2*, *PTP3* and *RPB1*.

		PTP2 [a]							*PTP3* [a]							*RPB1* [b]						
Isolate	**Origin**	***N***	π_S	θ_{WS}	D_S	π_A	θ_{WA}	D_A	***N***	π_S	θ_{WS}	D_S	π_A	θ_{WA}	D_A	***N***	π_S	θ_{WS}	D_S	π_A	θ_{WA}	D_A
3	AUS															8	1.68	1.66	0.24	0.00	0.00	NA
4	AUS															8	0.81	1.23	-1.67*	0.13	0.19	-1.53
57	SPA	9	1.16	1.02	0.54	0.12	0.14	-0.58	8	1.10	1.08	-0.92	0.07	0.06	0.33	10	1.08	1.13	-0.24	0.05	0.09	-1.40
169	BRA	7	0.66	0.56	0.69	0.15	0.16	-0.27	9	1.14	1.07	0.24	0.22	0.28	-0.91	9	1.46	1.00	1.90	0.00	0.00	NA
253	SPA	10	1.53	1.47	0.11	0.12	0.21	-1.56	12	0.76	0.77	-0.06	0.29	0.46	-1.52	10	1.90	1.61	0.69	0.05	0.09	-1.40
376	CAN	8	0.60	0.53	0.41	0.08	0.07	0.33	6	0.70	0.76	-0.45	0.08	0.07	0.85	7	1.59	1.48	0.26	0.00	0.00	NA
377	CAN	6	1.11	1.21	-0.47	0.13	0.17	-1.13	8	0.88	1.34	-1.64	0.16	0.18	-0.30	10	1.87	1.61	0.41	0.05	0.09	-1.40
440	HUN	8	0.70	0.53	1.10	0.08	0.07	0.33	9	0.39	0.64	-1.51	0.13	0.17	-0.94	9	1.26	1.17	0.25	0.00	0.00	NA
526	NED	8	0.97	0.80	0.84	0.16	0.15	0.24	10	0.99	0.82	0.75	0.24	0.27	-0.38	7	1.85	1.48	1.18	0.07	0.10	-1.24
531	SLO	13	0.93	0.67	1.20	0.13	0.12	0.10	5	0.94	1.11	-1.09	0.21	0.22	-0.17	7	1.72	1.30	1.55	0.04	0.05	-1.01
839	ALG	7	0.40	0.56	-1.24	0.11	0.16	-1.24	10	0.35	0.62	-1.56	0.09	0.16	-1.56	7	0.00	0.00	NA	0.04	0.05	-1.01
911	TWN	6	0.74	0.60	1.03	0.30	0.34	-0.68	10	0.53	0.62	-0.51	0.14	0.16	-0.76	10	1.23	1.13	0.33	0.00	0.00	NA
912	SPA	8	0.87	1.33	-1.60	0.14	0.22	-1.45	12	0.70	0.96	-1.02	0.21	0.30	-1.17	6	0.61	0.80	-1.30	0.13	0.17	-1.23
1175	CRO	9	0.66	0.51	0.98	0.12	0.14	-0.58	11	0.57	0.79	-1.03	0.13	0.16	-0.51	7	1.99	1.86	0.27	0.04	0.05	-1.01
1244	ARG	7	1.06	0.85	1.11	0.09	0.08	0.56	11	0.40	0.59	-1.11	0.12	0.16	-0.75	10	1.59	1.45	0.36	0.05	0.09	-1.40
1251	HI	10	0.76	0.49	1.74	0.15	0.14	0.22	10	1.08	1.07	-0.54	0.30	0.43	-1.36	8	1.50	1.05	1.89	0.10	0.15	-1.45
1299	GRE	10	1.18	0.98	0.75	0.33	0.41	-0.84	12	1.06	0.96	0.33	0.11	0.10	0.22	8	0.97	1.23	-1.04	0.10	0.15	-1.45
1319	HI	7	1.06	0.85	1.11	0.09	0.08	0.56	5	1.17	1.39	-1.12	0.18	0.22	-1.05	11	1.55	1.09	1.62	0.05	0.09	-1.43
1324	HI	8	1.45	1.07	1.50	0.10	0.07	1.17	12	0.91	0.77	0.59	0.13	0.15	-0.38	6	1.51	1.20	1.39	0.13	0.17	-1.23
1610	USA	5	0.84	0.66	1.46	0.12	0.09	1.22	11	0.81	0.79	0.04	0.11	0.10	0.20	10	1.76	1.77	-0.10	0.05	0.09	-1.40
1994	CHI	11	1.09	1.41	-0.94	0.25	0.46	-1.90*	12	0.86	0.77	0.39	0.10	0.15	-1.38	10	1.69	1.29	1.28	0.05	0.09	-1.40
2032	SOL	12	1.20	0.92	1.03	0.14	0.13	0.15	11	1.11	1.78	-1.62	0.22	0.31	-1.22	10	1.39	1.12	0.90	0.10	0.18	-1.67
KI	JAP															8	1.60	1.23	1.35	0.00	0.00	NA
Pooled		169	1.00	2.19	-1.46	0.15	0.88	-2.34**	194	0.82	3.50	-2.21**	0.17	1.35	-2.49***	196	1.58	3.19	-1.54	0.05	0.78	-2.71***

[a], Sequence data from [39];

[b], sequence data for isolates 440, 1251 and 1324 from [32];

ALG: Algeria, ARG: Argentina, AUS: Australia, BRA: Brazil, CAN: Canada, CHI: Chile, CRO: Croatia, GRE: Greece, HI: Hawaii (USA), HUN: Hungary, JAP: Japan, NED: Netherlands, SLO: Slovenia, SOL: Solomon Islands, SPA: Spain, TWN: Taiwan, USA: United States of America; *N*: number of cloned sequences; π_S and π_A, pairwise nucleotide diversity at synonymous and nonsynonymous sites expressed as percentage, respectively [59]; θ_{WS} and θ_{WA}, nucleotide site variability based on the number of synonymous and nonsynonymous segregating sites expressed as percentage, respectively [60]; the average number of synonymous and nonsynonymous sites analyzed across loci were 178.3 and 650.7, respectively; D_S and D_A, Tajima´s *D* [62] at synonymous and nonsynonymous sites, respectively; NA: not available; statistical significance of Tajima´s *D*:

*, $P < 0.05$;

**, $P < 0.01$;

***, $P < 0.001$.

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Table 3. F_S test for detecting population expansion.

	Fs					
Dataset	***PTP2***		***PTP3***		***RPB1***	
N. apis	-18.66	***	-8.17	ns	-87.22	***
Lineage A	-1.52	ns	0.46	ns	-3.54	*
Lineage C	-13.24	***	-5.70	**	-34.15	***
Lineage M					-0.77	ns
N. ceranae [a, b]	-34.35	***	-120.89	***	-77.55	***

Fs: Fu's *Fs* [63]; The significance was evaluated by comparing the values of the statistic, and the observed levels of recombination per gene, with a null distribution generated by 1,000 coalescent simulations (ns, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$);

[a], sequence data for *PTP2* and *PTP3* from [39];

[b], sequence data for isolates 440, 1251 and 1324 (*RPB1*) from [32];

the remaining ones are from this work. Lineages A, C and M indicate the evolutionary lineage of the honey bee colonies where the isolates come from (isolate 410 was excluded from this analysis).

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from honey bees of lineages A or M (D_S = -0.19 and 1.83, ns; respectively.). The F_S test produced a similar scenario (Table 3).

Recombination

The estimate of the population scaled recombination rate (ρ) was significantly greater that zero at *PTP2* and *RPB1* in *N. apis* (Table 4) and several recombination events were detected at these two loci (*Rm* = 2 and 6, *Rh* = 2 and 14, respectively).

The outcome of these tests did not change after removing singleton mutations, which usually contain little information and could cause interferences in the assessment of recombination. In addition, the four-allele combinations expected after a recombination event were found both in individual samples (e.g. AT, TT, AC and TC at positions 276 and 453 of *PTP2* in

Table 4. Statistics used to detect recombination in *N. apis*.

Dataset	Locus	*N*	*Rm*	*Rh*	*ρ*	*P*
N. apis	*PTP2*	63	2	2	36	*
	PTP3	50	0	0	2	ns
	RPB1	208	6	14	11	***
N. ceranae	*PTP2* [a]	169	5	10	68	ns
	PTP3 [a]	194	3	6	61	*
	RPB1 [b]	196	6	12	14	*

N, number of cloned sequences; *Rm*, minimum number of recombination events [69]; *Rh*: lower bound on the number of recombination events [70] (http://www.stats.ox.ac.uk/~myers/RecMin.html); *ρ*, estimate of the population scaled recombination rate; *P*, probability of Lkmax $\leq$ estimated in a likelihood permutation-based test as implemented in LDhat [72]; ns, non-significant,

*, $P < 0.05$ and ***, $P < 0.001$;

[a], sequence data from [39];

[b], sequence data for isolates 440, 1251 and 1324 from [32];

the remaining ones are from this work.

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sample 854 –haplotypes 1, 15, 23 and 24, respectively–; S4 Table) and in samples from different populations (e.g. CC, CT, TC and TT at positions 771 and 963 of $RPB1^{A+B}$; S7 Table), further supporting the existence of low, although statistically significant, levels of recombination in *N. apis*. Likewise, evidence for recombination was found in *N. ceranae*, although in this case it only reached statistical significance for *PTP3* and *RPB1* (Table 4).

Sex and meiotic loci

A Blastp analysis of the genomes of *N. apis* and *N. ceranae* revealed the presence of several components of a sex-related locus [78] that encode a *triose phosphate transporter* (*TPT*), a *high-mobility group* (*HMG*), and an *RNA helicase* (with accession numbers EQB61312, EQB61310, EQB60627 and KKO76186, KKO76188, KKO75161, respectively). In both species *TPT* and *HMG* were syntenic and harbored an additional predicted ORF between them, whereas the *RNA helicase* was unlinked to the former.

These genomes also contained meiotic genes, although not all of them presented orthologs in both species (S8 Table). However, it must be noted that these results should be taken with caution due to the difficulty of distinguishing orthologs encoding different members of gene families (i.e. *Smc1* and *Smc4* in *N. ceranae*; S8 Table).

Population structure

N. apis exhibited high levels of haplotype diversity at the three loci under study (*Hd* = 0.79–0.91). To explore the distribution of the haplotypes among samples these were plotted into networks (Fig 1), revealing the following patterns: (i) all loci presented a reduced number of common haplotypes (e.g. h1 in *PTP2* and in *PTP3*, and h2 and h5 in *RPB1*); (ii) most other haplotypes differed from these by a reduced number of substitutions (usually one or two); and (iii), there was a hinted association among haplotypes obtained from Spanish and Algerian samples. For example, *PTP2* haplotypes h15, h16, h17 were shared by the three samples from these two countries (Fig 1A), and most other closely linked haplotypes (e.g. h14, h18, h19, h21, h22 and h24) were found in either of them. A similar effect was observed for *PTP3* haplotypes h3 and h13 (Fig 1B), and h2 of *RPB1* (Fig 1C). This raised the possibility of a structuring of the parasite and host populations, which was not apparent in *N. ceranae* networks (S1 Fig), and it was further explored by determining the evolutionary lineage of all sampled honey bee colonies and by quantifying the relative contribution of three covariance components to the observed parasite haplotype diversity: (i) covariance within isolates (i.e., within honey bee colonies), (ii) among isolates obtained from honey bees of the same evolutionary lineage, and (iii) among host evolutionary lineages. Most *N. apis* isolates were collected from *A. mellifera* colonies of lineage C, except isolates 399 and 854 (from Spain and Algeria) which were of lineage A, and isolates 382 (Canada) and 411 (Chile) which belonged to lineage M (S1 Table). A third isolate from Spain, 410, displayed mixed results, with evidence for the presence of honey bees of both lineages A and M, probably due to drifting workers and the existence of colonies of both evolutionary lineages in the same apiary (S1 Table).

The analysis of molecular variance revealed a structured *N. apis* population, where between 20 and 34% of the total variance at the three loci corresponded to differences among host-lineages ($P < 0.05$, in permutation tests; Table 5). These results held irrespective of whether sample 410 was considered as belonging to lineage A or M. Differences among haplotypes of the same isolate accounted for the best part of the variance (between 62 and 70%), and differences among isolates of the same lineage represented $\leq$11% of the variance. In *N. ceranae* the differentiation of haplotypes within isolates was even greater (between 91 and

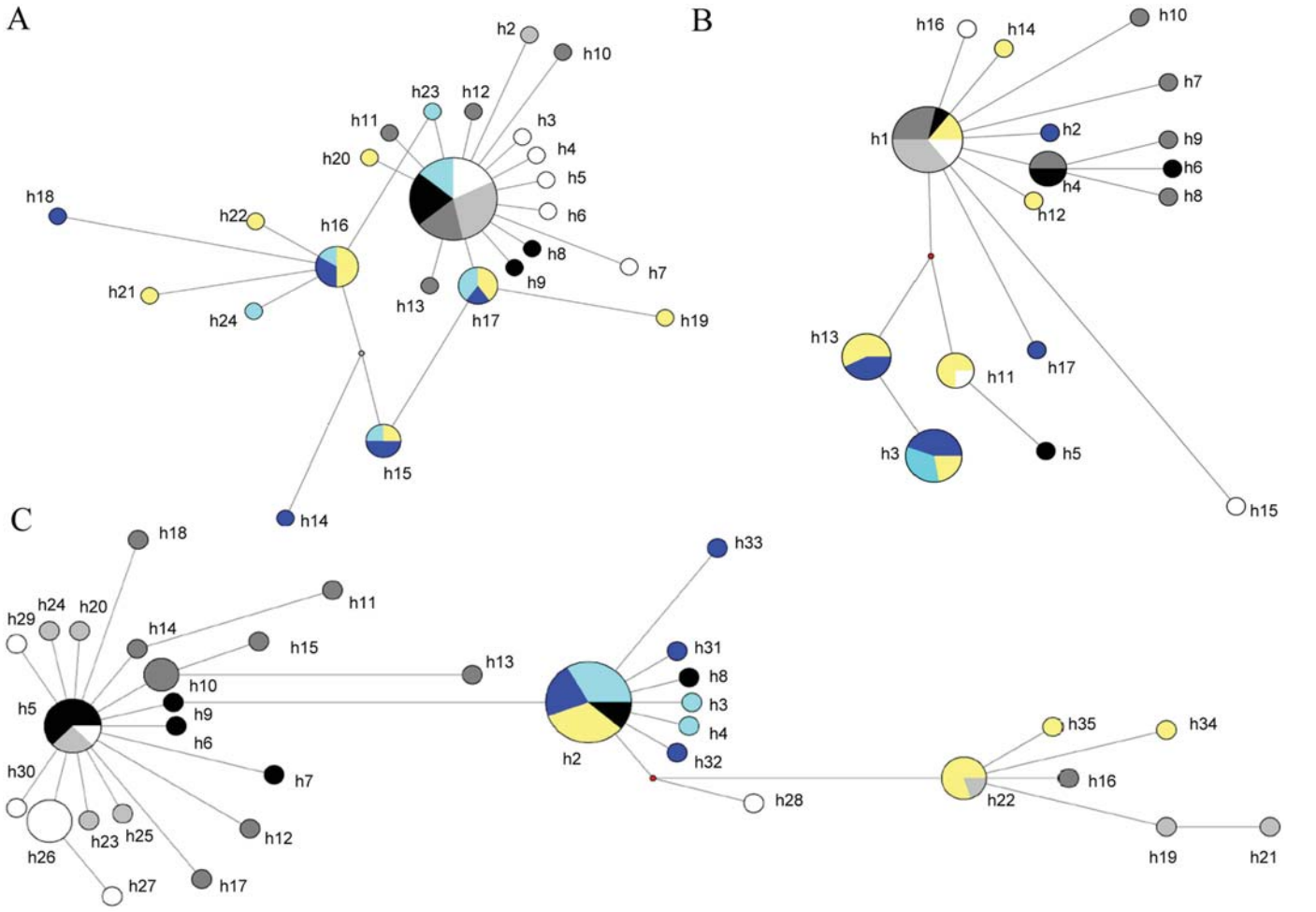

Fig 1. Median-joining haplotype network for three *N. apis* loci according to their geographical origin and *A. mellifera* lineage: *PTP2* (A), *PTP3* (B) and *RPB1* (C). Haplotypes are depicted by circles, the width being proportional to their frequencies. Color codes are as follows; blue: lineage A (light blue: isolate 854 (Algeria); dark blue: isolate 399 (Spain)); yellow: lineages A/M (isolate 410 (Spain)); greyscale: lineage C (black: isolate 204 (Argentina); dark grey: isolate 381 (Canada); light grey: isolate 52 (Slovenia); white: isolate 174 (Turkey)); red dots represent median vectors (hypothesized haplotypes required to connect existing sequences within the network with maximum parsimony).

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99%) but, contrastingly, the differences among host-lineages were not significant as they represented just a tiny fraction of the observed variance (< 4%, Table 5).

A pairwise analysis of *N. apis* differentiation between host lineages uncovered that most variation occurred between lineage A and the other two lineages, which were otherwise indistinguishable (Table 6). K_{ST}^*, which measures the average pairwise differences within populations with respect to the total, revealed significant differentiation between *N. apis* sequences obtained from *A. mellifera* colonies of lineages A and C (K_{ST}^* between 0.09 and 0.25, $P < 0.001$, Table 6) or between A and M ($K_{ST}^* = 0.30$, $P < 0.001$). Similarly, S_{nn}, which estimates how often related sequences are found in the same population, reached significant values between group A and C ($S_{nn} = 0.82–0.93$, $P < 0.001$) and between A and M ($S_{nn} = 1.00$, $P < 0.001$). Both tests failed to detect significant differences between sequences obtained from lineages C and M.

The divergence between two populations is a direct function of the mutation rate times twice the number of generations since their split. Considering that: (i) the net divergence (*D*a)

Table 5. Analysis of molecular variance (AMOVA) in *N. apis* haplotypes according to *A. mellifera* lineages.

Dataset	Locus	Source of variation	d.f.	*SS*	*VC*	*% var*	*P*
N. apis	*PTP2*	Among lineages	1	17.2	0.51	32.6	*
		Among isolates within lineages	5	6.1	0.02	1.3	ns
		Within isolates	56	58.7	1.05	66.1	***
	PTP3	Among lineages	1	13.9	0.50	33.8	*
		Among isolates within lineages	5	6.8	0.07	4.6	*
		Within isolates	43	38.9	0.90	61.6	***
	RPB1	Among lineages	2	54.3	0.56	19.6	**
		Among isolates within lineages	19	94.1	0.32	10.8	***
		Within isolates	186	374.3	2.01	69.6	***
N. ceranae	*PTP2* [a]	Among lineages	2	6.0	0.04	3.5	*
		Among isolates within lineages	17	20.5	0.01	0.9	ns
		Within isolates	149	166.3	1.12	95.6	ns
	PTP3 [a]	Among lineages	2	242.1	-13.96	-2.2	ns
		Among isolates within lineages	17	14014.6	20.29	3.2	ns
		Within isolates	174	109558.1	629.64	99.0	ns
	RPB1 [b]	Among lineages	2	10.0	0.04	1.9	ns
		Among isolates within lineages	20	63.9	0.14	6.7	*
		Within isolates	173	339.7	1.96	91.4	**

[a], Sequence data from [39];

[b], sequence data for isolates 440, 1251 and 1324 from [32];

the remaining ones are from this work; isolate 410 was considered as if sampled from a lineage A honey bee colony (see text); d.f., degrees of freedom; *SS*, sum of squares; *VC*, variance components; % var, percentage of variation; *p*, probability of a random variance component value $\leq$ observed value, in 3024 permutations;

*, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$; ns, non-significant.

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between *N. apis* from lineage A and C/M colonies is 0.002. (ii) The number of spores can double as fast as every 24 hours (e.g. [79,80]). (iii) The substitution rate in these pathogens is about two times faster than that observed in other fungi (as estimated for *Encephalitozoon cuniculi* [81]), and that (iv) the per site mutation rates in fungi is of the order of 7.2×10^{-11} for *Neurospora crassa* or 2.2×10^{-10} for *Saccharomyces cerevisiae* [82], the split between the two parasite populations can be dated between 6,200 and 19,000 years ago.

Discussion

Here we report a population genetic analysis of *N. apis* based on the study of the patterns of diversity of three unlinked single copy genes: *PTP2* and *PTP3*, which encode polar tube proteins (reviewed in [83]), and the largest subunit of the RNA polymerase II (*RPB1*), a housekeeping gene that has been frequently used as phylogenetic marker in microsporidian species [84,85]. The levels of synonymous variation at these unlinked coding genes should be a good proxy for the extant neutral variation of the species [86]. The fact that they are single copy markers makes them a preferred choice than the commonly used ribosomal loci, as substantial levels of genetic variation and recombination between paralogous rDNA copies have been previously reported in microsporidia [36]. So far only Ironside [44] has published diversity data for a single copy locus (*RPB1*) in *N. apis*.

Table 6. Analysis of population differentiation in *N. apis* according to *A. mellifera* lineages.

Dataset	Locus	Host Lineages	K_{ST}*	*P*	S_{nn}	*P*
N. apis	*PTP2*	A & C	0.16	***	0.82	***
	PTP3	A & C	0.25	***	0.95	***
	RPB1	A & C	0.09	***	0.93	***
		A & M	0.30	***	1.00	***
		C & M	0.00	ns	0.81	ns
N. ceranae	*PTP2* [a]	A & C	0,02	**	0,69	*
		A & M	0,02	*	0,55	ns
		C & M	0,00	ns	0,69	ns
	PTP3 [a]	A & C	0,00	ns	0,67	*
		A & M	0,01	*	0,61	**
		C & M	0,00	ns	0,73	**
	RPB1 [b]	A & C	0.02	*	0.68	ns
		A & M	0.00	ns	0.54	ns
		C & M	0.00	ns	0.76	ns

[a], sequence data from [39];

[b], sequence data for isolates 440, 1251 and 1324 from [32];

the remaining ones are from this work; isolate 410 was excluded; K_{ST}* [73], estimates the amount of within-deme nucleotide diversity relative to the overall diversity; S_{nn} [74], measures how often related sequences are found in the same deme; *P*, significance in permutation tests: ns, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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The three loci analyzed displayed similar average levels of synonymous diversity (π_S), about 1% (Table 1), analogous to what was found for these and other loci in *N. ceranae* [32,38,39,41], and somewhat higher than those estimated for *RPB1* (0.41%) in cloned sequences from a single isolate of *N. apis* [44]. In terms of diversity at all sites *N. ceranae* and *N. apis* displayed values of the order of 0.40%, which are lower than those described in *N. bombycis* (1.83%, [44]), but higher than those of other microsporidia of the genus *Hamiltosporidium* (between 0.06% and 0.28%, [87]).

Although it has been postulated that the polar tube proteins could be factors of virulence and thus subject to adaptive selection [88], the MLHKA test revealed that the relative levels of diversity and divergence of the *PTP* loci do not differ from those observed at three other unlinked loci (including a housekeeping gene, *RPB1*), which suggests that they evolve under the effect of similar evolutionary forces. Consistently low d_N/d_S values can be reconciled with a predominant effect of purifying selection over the seven loci. Although the large synonymous divergence between these species means that these results should be taken with caution, the fact that it applies to all loci supports that the genes used in the current study are a good proxy of the patterns of variation across the parasites' genome.

The detection of substantially lower variation (π_A) coupled with significantly negative Tajima's D_A values at nonsynonymous sites at the three loci in both species indicate that amino acid replacement variants are readily removed from the populations, which reflects that these loci are likely to be functional and subject to purifying selection, as previously suggested in *N. apis* [44] and *N. ceranae* [32,36,38,39,44] for these and other genes. This fits well with the finding of just 29 putative pseudogenes in the genome of *N. ceranae* [42], which indicates that the majority of coding sequences retained in these reduced genomes [33,48] are essential for the survival of these parasites. The relatively lower variability at a*ctin* and *Hsp70* can probably be

attributed to the effect of negative selection at linked deleterious sites (background selection) at these loci [89], which is consistent with the strong purifying selection—low d_N/d_S values—observed in these highly conserved genes [90,91] and the low recombination rates reported for these parasites [40] (see below).

The results of the Tajima´s *D* test at silent sites revealed an overall excess of low frequency variants in the two parasite species (Tables 1 and 2). Although some of these could correspond to nucleotide misincorporations introduced during the PCR process (despite the use of a high-fidelity enzyme blend), previous assays using either invariant DNA templates [38,39] or single DNA molecules [40], confirmed that the vast majority of the mutations detected in *N. ceranae* were actually present in the sample mixture, and that no error-prone bias was brought throughout the procedure. The same pattern was also observed at the genomic level [42], so there are no reasons to think that this would be different in *N. apis*.

All isolates presented substantial levels of nucleotide diversity (Tables 1 and 2). In fact, many of them harbored various distinct haplotypes, sometimes as many as nine (e.g. S7 Table). Given that the three genes are present as a single copy in the genome of both parasites, there are two possible and non-mutually exclusive explanations for the observed within-isolate variation: one is to assume that the two nuclei present in each cell are diploid, as it has been recently proposed for *N. ceranae* [42], so that they can harbor up to four different haplotypes for each loci, and the other is the existence of genetic heterogeneity among parasites in each host colony (mixed infections) [32,39,41].

At any rate, the accumulation of alleles at low frequencies observed in these two species is compatible with a demographic growth, in which most mutations present in the expanding populations have a recent origin and, therefore, are rare [92]. The greater D_S value obtained for *N. ceranae* in the combined sample (D_S = -2.93, $P < 0.002$), suggests that the expansion of this population might have taken place more recently or it has been more accentuated than that experienced by *N. apis* (D_S = -1.82, $P < 0.034$). This would agree with its recent jump to *A. mellifera* and spread throughout the worldwide distribution range of its new host [32,38,39,41,42].

A. mellifera originated between 6 and 8 million years ago somewhere in Asia, where all other species of the genus are confined, and from where it expanded to its historical geographic distribution range across sub-Saharan Africa, Europe and Western Asia [93,94]. The species now comprises several locally adapted and anatomically distinct subspecies, which split between 0.3 and one million years ago and can be clustered into four major groups: lineage A, includes subspecies that can be found in Africa and the Iberian Peninsula; lineage M is distributed along Western and Northern Europe; lineage C, in South Eastern Europe, and lineage O, in the Middle East and Western Asia [93,94]. Our results suggest that between 20% and 34% of the genetic variance of the *N. apis* population corresponds to differences between samples collected from honey bee colonies of different lineages (Table 5). It should be noted that the sampling scheme might influence the observed frequency spectra of variants, as the retrieval of alleles from distant locations of a structured population is likely to cause departures from neutral expectations assuming panmixia. The reduced between-sample variation in *N. ceranae* means that this effect is unlikely in this species, but the evidence for genetic differentiation between *N. apis* collected from different host lineages (lineage A *vs*. C or M) suggests that the assumed panmixia might not be met. The separate analysis of the parasite subpopulations revealed that only those isolated from European honey bees of lineage C depart from mutation-drift equilibrium.

Remarkably, the observed structure of the parasite population does not match that of the host: lineages C and M display the highest differentiation amongst the four *A. mellifera* lineages [93,94]. In contrast, *N. apis* isolates from these lineages are genetically indistinguishable. This lack of a full correspondence between the structures of parasite and host populations suggests

that they only share a fraction of their demographic history. Indeed, the split of the *N. apis* populations retrieved from honey bee lineages A and C (or M) was dated between 6,200 and 19,000 years ago, that is just after the Last Glacial Maximum, about 20,000 years ago [93]. Thus, a reduction of the parasite's geographic distribution range during the last glacial period might have prompted the isolation of the two populations. Contrastingly, the absence of genetic differentiation between the parasites from lineages C and M suggests that this population has spread across Europe recently (which is also consistent with the results of the Tajima´s *D* and *Fs* tests), and much later than the honey bees did. This expansion might have been associated with the practice of beekeeping by humans, whose origin has been traced to the Middle East and Egypt about five thousand years ago [95], and also with the human-driven colonization by *A. mellifera* of the New World, East Asia and Oceania in the last few centuries [94,96].

Evidence for low levels of recombination from nucleotide variation data had been previously reported [32,36,38,39,41,42], and further supported by Single Genome Amplification (SGA) analysis, in *N. ceranae* [40]. This new evidence for a second *Nosema* species, suggests that recombination might be a common feature of the genus. Genetic exchange between chromosomes is crucial in the evolution of organisms, and its detection has important consequences since it can generate new genetic combinations that result in individuals better adapted to confront novel environments or hosts.

If exclusively clonal reproduction is assumed, high levels of genetic differentiation would be expected between homologous sequences in the two nuclei of each individual (an adaptation of the Meselson effect for a diplokaryon), as observed in the asexual microsporidian *Hamiltosporidium tvarminnensis* [87]. But the absence of genetic structuration of the haplotypes retrieved in each colony, along with the observed neutral diversity within samples and the evidence for genetic recombination, suggest that there might exist mechanisms for occasional genetic flow between the nuclei. Whether this exchange takes place between nuclei of the same cell or between those of different cells during the multinucleated stages of the merogonia [97] remains to be determined.

Genetic exchange can occur during meiosis in sexual stages of the life cycle but also during parasexual processes such as mitotic crossover, non-homologous recombination and gene conversion. Although both mechanisms have been proposed in microsporidians (e.g.*Encephalitozoon cuniculi* [98], *Hamiltosporidium magnivora* [87], *Nematocida* [99] or *Nosema* spp. [36,44,100]), their occurrence and frequency are still a matter of debate as, in absence of cytological observation, the outcomes of these processes are difficult to distinguish [101,102]. The finding of a putative sex-related locus in *N. ceranae* and in *N. apis* goes in line with similar evidence in other microsporidians [76,87,99,103], but it should not be taken as a definite proof of sexual reproduction, which would require the presence of idiomorphs of this locus in different isolates and their expression during the mating phase [76]. The same could be said regarding the existence of core meiotic genes in these genomes [76,99,103,104] whose detection, although suggestive that meiosis may occur, does not ensure their functionality during this process [103].

The genetic diversity patterns at the three loci analyzed in this study suggest that *N. apis* and *N. ceranae* have experienced different recent evolutionary histories and provide new data on the relationship between these parasites and the honey bees that host them. In addition, they extend the evidence for genetic recombination to a second species of the genus, further supporting the idea that mechanisms of genetic exchange between chromosomes play an important role in modeling the genetic configuration of these organisms. However, further studies are needed in order to determine the extent to which the observed patterns extend to other parts of these species' genomes, to elucidate the molecular mechanisms responsible for the observed recombination and whether or not it implies sexual reproduction.

Supporting Information

S1 Fig. Median-joining haplotype network for three *N. ceranae* loci according to their *A. mellifera* lineage: *PTP2* (A), *PTP3* (B) and *RPB1* (C). Haplotypes are depicted by circles, the width being proportional to their frequencies (only shared haplotypes are named). Color codes are as follows; blue: lineage A (isolates 839 (Algeria), 57 and 253 (Spain), 169 (Brazil)); yellow: lineage M (isolates 912 (Spain), 526 (Netherlands), 1251 (Hawaii)); grey: lineage C (isolates 1244 (Argentina), 3 and 4 (Australia), 376 and 377 (Canada), 440 (Hungary), 531 (Slovenia), 911 (Taiwan), 1175 (Croatia), 1299 (Greece), 1319 and 1324 (Hawaii), 1610 (USA), 2032 (Solomon Islands), 1994 (Chile), KI (Japan)); red dots represent median vectors (hypothesized haplotypes required to connect existing sequences within the network with maximum parsimony).
(TIF)

S1 Table. Origin and accession numbers of *N. apis* sequences obtained from *A. mellifera* honey bees.
(XLS)

S2 Table. Origin and accession numbers of *N. ceranae* sequences obtained from *A. mellifera* honey bees.
(XLS)

S3 Table. Ratio of nonsynonymous to synonymous divergence (d_N/d_S) between *N. apis* and *N. ceranae* (Yang and Nielsen method).
(XLS)

S4 Table. Number of occurrences and nucleotide variants of $PTP2^A$ haplotypes from *N. apis*.
(XLS)

S5 Table. Number of occurrences and nucleotide variants of $PTP3^A$ haplotypes from *N. apis*.
(XLS)

S6 Table. Number of occurrences and nucleotide variants of $RPB1^A$ haplotypes from *N. apis*.
(XLS)

S7 Table. Number of occurrences and nucleotide variants of $RPB1^{A+B}$ haplotypes from *N. apis*.
(XLS)

S8 Table. Meiotic genes in different microsporidian species.
(XLS)

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Author Contributions

Conceived and designed the experiments: CB XM MH RM-H. Performed the experiments: TG-M LJ. Analyzed the data: CB XM TG-M LJ PDR. Contributed reagents/materials/analysis tools: MH RM-H CB XM PDR. Wrote the paper: CB XM MH RM-H PDR.

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ORIGINAL PAPER

A geometric morphometric and microsatellite analyses of *Scaptotrigona mexicana* and *S. pectoralis* (Apidae: Meliponini) sheds light on the biodiversity of Mesoamerican stingless bees

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Abstract Geometric morphometrics and molecular methods are effective tools to study the variability of stingless bee populations and species that merit protection given their worldwide decline. Based on previous evidence of cryptic lineages within the *Scaptotrigona* genus, we tested the existence of multiple evolutionary lineages within the species *S. mexicana* and we investigated the status of *S. pectoralis*. By analyzing their population structure, we found differences between the Pacific and Atlantic populations of each of these species, although geometric morphometrics of the wing only confirmed these results in *S. mexicana*. There was a tendency towards enhanced genetic differentiation over larger distances in the Atlantic populations of both species but not in the Pacific populations. These results revealed a pattern of differentiation among evolutionary units and a specific distribution of genetic diversity within these *Scaptotrigona* species in Mesoamerica, suggesting the need for future taxonomic revisions, as well as activities aimed at management and conservation.

Keywords *Scaptotrigona* · Stingless bees · Geometric morphometrics · Microsatellites · Evolutionary units

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Introduction

Stingless bees (Hymenoptera: Apidae: Meliponini) are eusocial, haplodiploid insects that carry out ecologically and economically important pollinating activity, especially in Neotropical regions (Slaa et al. 2006). Most of the diversity of the tribe Meliponini is concentrated in this region, with approximately 42 endemic genera (Camargo and Pedro 2013) that include around 400 species (Michener 2007). One of these genera is *Scaptotrigona* Moure, 1942, which consists of 24 species extending from Mexico to Argentina (Michener 2007). Three *Scaptotrigona* species are found in Mexico: *S. mexicana* Guérin, 1845 the distribution of which extends to Costa Rica; *S. pectoralis* Dalla Torre, 1896 the distribution of which reaches Panama; and the endemic *S. hellwegeri* Friese, 1900 (Ayala 1999; Camargo and Pedro 2013). These three species are managed for honey production (Manzo 2009; González-Acereto 2012; Ayala et al. 2013) but at present, only *S. mexicana* and *S. pectoralis* contribute to the pollination of crops like avocado (Ish-Am et al. 1999).

Given their important contribution to agriculture, the commercial use of these species is augmenting (Albores-González et al. 2011), potentially influencing the gene flow among populations due to hive translocation (Jaffé et al. 2016; Santiago et al. 2016). Like many other insects, these species are also being affected by extensive deforestation and the extension of monocrops in these areas (Freitas et al. 2009; Stout and Morales 2009). These phenomena may potentially drive a decline in stingless bee populations and they may provoke endogamous depression, an increase in homozygosity and the ensuing loss of genetic diversity (Zayed 2009). Hence, the lack of information about their richness, diversity, genetic status and distribution is an important obstacle to ensure their protection and to

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establish appropriate conservation programs (Brown and Paxton 2009; Freitas et al. 2009). These programs should include measures avoiding the translocation of hives and to define which populations should remain isolated to preserve genetic integrity.

The existence of cryptic species of stingless bees has been proposed (Tavares et al. 2007; Francisco et al. 2008; May-Itzá et al. 2012) and thus, the total number of species could be higher than anticipated (Michener 2007). The number of cryptic species will probably increase as more molecular methods are made available and more powerful morphological analyses are employed. In the last decade, geometric morphometric analysis of wings has proven to be a good tool to clarify discrepancies within species and to identify bees (for example honeybees: Bouga et al. 2011; da Silva et al. 2015; or bumblebees: Barkan and Aytekin 2013). In stingless bees, this approach has proved to be very sensitive to characterize species and even to differentiate populations (Ferreira et al. 2011; Francoy et al. 2011; Lima Junior et al. 2012; Nunes et al. 2013; Bonatti et al. 2014), although genetic information should be incorporated to more rigorously define species and to resolve taxonomic problems (Schlick-Steiner et al. 2010). Species and population identification using genetic markers has advanced significantly, and population parameters important for the conservation of endangered species can now be derived from the use of molecular markers (Frankham et al. 2002; Hedrick 2005). Among these, microsatellite loci have good statistical resolution to characterize demographic events within populations (Luikart and England 1999), and they have been successfully used within the Meliponini to characterize genetic variability (Arias et al. 2006; Francisco et al. 2006; Fernandes et al. 2012) and issues like the genetic characteristics of drone congregation areas (Kraus et al. 2008; Mueller et al. 2012).

Studies of morphological and/or molecular markers in the genus *Scaptotrigona* have suggested the existence of genetically distinct evolutionary lineages (or cryptic species). A study on *S. hellwegeri* found signs of ongoing speciation, indicating that two populations distributed at different altitudes along the Trans-Mexican Volcanic Belt and the Pacific coast should be treated as separate units (Quezada-Euán et al. 2012). The existence of cryptic species was also proposed within *S. mexicana* colonies in two regions located at opposite extremes of the species distribution range in Mexico (Veracruz and Chiapas: Hurtado-Burillo et al. 2013). Recently, up to five distinct clusters (considered as units of management and conservation) with high rates of genetic diversity were described in another *Scaptotrigona* species (*S. xanthotricha*, which is widely distributed in the Brazilian Atlantic rainforest: Duarte et al. 2014).

As such, here we aimed to resolve taxonomic issues in *S. mexicana* and *S. pectoralis*, two species that share an equivalent distribution, as well as similar biological and ecological aspects, but that are managed distinctly. Specifically, we used geometric morphometry and microsatellite markers to assess the population structure and the molecular diversity of *S. mexicana* and *S. pectoralis*, and to test the existence of various evolutionary units within *S. mexicana*. In addition, these tools were used to investigate the species status of *S. pectoralis* in the light of the diversity observed in this genus.

Materials and methods

Sampling

This study was carried out in Mexico and northern Guatemala, within the distribution area of *S. mexicana* and *S. pectoralis*. Colonies were sampled at 15 sites between 2008 and 2011 (Table 1; Fig. 1), and most *S. mexicana* samples (70 colonies in total) were provided by stingless beekeepers that maintain colonies in clay pots or in their original trunks, either on their own property or in the vicinity (meliponaries). *S. pectoralis* samples (33 colonies in total) were obtained from non-managed wild colonies located in native forests. Adult worker bees were collected at the entrance of each colony and preserved in absolute ethanol at −20 °C. Voucher specimens were deposited in the insect collection of the Animal Biology Laboratory at the Veterinary Faculty of the University of Murcia (Spain).

Geometric morphometric analysis

One to six specimens from 70 *S. mexicana* and 32 *S. pectoralis* colonies were analysed (Table 1). Only the right wing of each specimen was used and they were photographed with a Spot Insight Firewire digital camera (Sterling Heights, USA) adapted to a Zeiss Stemi 2000C Trinocular Zoom Stereomicroscope (Thornwood, USA). We manually plotted twelve homologous landmarks of the wing vein intersections (Fig. 2) using the tpsDig software, (version 2.17: Rohlf 2013). MorphoJ software (version 1.06c: Klingenberg 2011) was used to analyze the images, which were first Procrustes aligned (Bookstein 1991) in order to identify the points of shape variation. Mean values of the specimens from each colony were used to perform the analyses at the colony level. Principal component (PCA) and canonical variate (CVA) analyses were performed with these data.

Mahalanobis distances between groups of colonies were defined by the PCA analysis and were obtained with the

Table 1 Details of *S. mexicana* and *S. pectoralis* sampling in Mexico and Guatemala

Species	Locality, state, country	Coordinates		Map code	N1	N2	Cluster
		Latitude	Longitude				
S. mexicana	Cacahoatan, Chiapas, Mexico	14.996	−92.167	10	8	8	*Sm1*
S. mexicana	Tapachula, Chiapas, Mexico	14.979	−92.266	12	16	8	*Sm1*
S. mexicana	Tuxtla Chico, Chiapas, Mexico	14.906	−92.261	9	21	22	*Sm1*
S. mexicana	Chilcuahuta, Hidalgo, Mexico	20.331	−99.232	1	–	1	*Sm2*
S. mexicana	Melchor de Mencos, Peten, Guatemala	17.066	−89.150	15	11	11	*Sm2*
S. mexicana	Cuetzalan del Progreso, Puebla, Mexico	20.017	−97.522	4	–	1	*Sm2*
S. mexicana	Tuzamapan de Galeana, Puebla, Mexico	20.065	−97.576	2	–	1	*Sm2*
S. mexicana	Coatepec, Veracruz, Mexico	19.451	−96.959	5	6	6	*Sm2*
S. mexicana	Coyulta, Veracruz, Mexico	20.248	−97.658	3	8	9	*Sm2*
Total *S. mexicana*					70	67	
S. pectoralis	Merida, Yucatan, Mexico	20.861	−89.624	14	8	8	*Sp2*
S. pectoralis	Tlaltetela, Veracruz, Mexico	19.314	−96.901	6	4	5	*Sp2*
S. pectoralis	Palenque, Chiapas, Mexico	17.511	−91.993	13	3	3	*Sp2*
S. pectoralis	Tapachula, Chiapas, Mexico	14.966	−92.261	8	8	8	*Sp1*
S. pectoralis	Tuxtla Chico, Chiapas, Mexico	14.937	−92.167	7	9	9	*Sp1*
Total *S. pectoralis*					32	33	

Map code refers to the number labelling each locality in Fig. 1. N1 = number of colonies used for geometric morphometrics analysis; N2 = number of colonies used for microsatellite analysis. The cluster assigned to each locality is also indicated

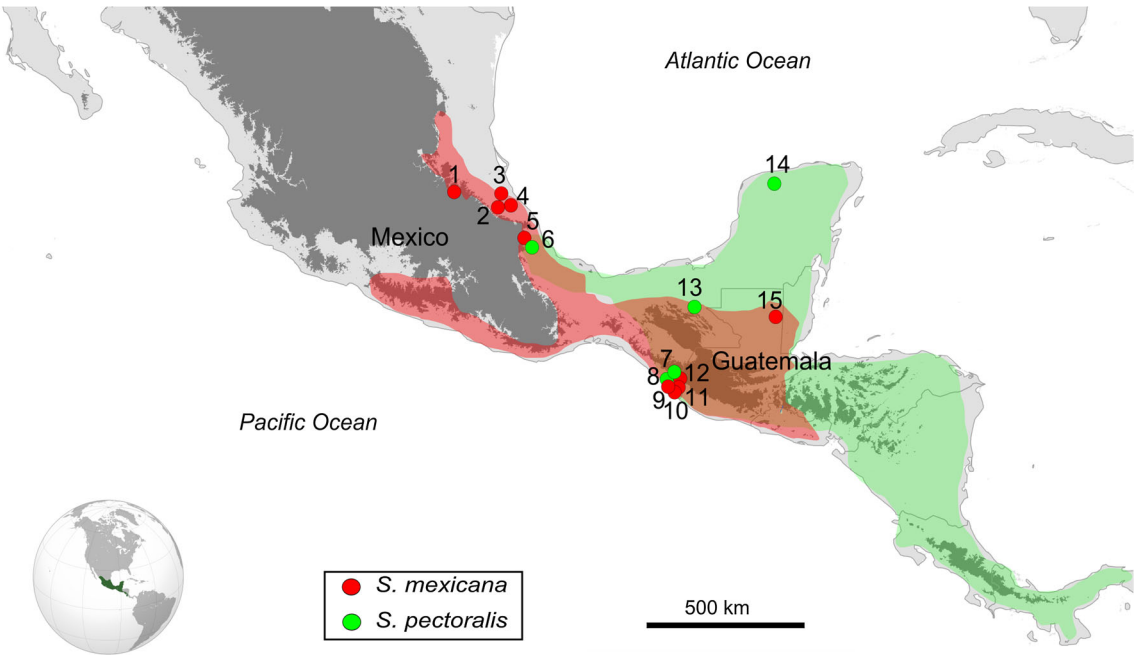

Fig. 1 Localities in Mexico and Guatemala where specimens were collected: *red dots* represent *S. mexicana* and *green dots*—*S. pectoralis* colonies. *Numbers* correspond to the sampling code used in Table 1. Distribution ranges have been shadowed: in *red*—*S. mexicana*, in *green*—*S. pectoralis* and in *light brown* the area where the two species co-exist. (Color figure online)

MorphoJ software. We also performed a Mantel test to investigate whether the Procrustes-fitted landmark co-ordinates of the wings varied with the geographic distance in each group using Past 3.08 software (Hammer et al. 2001).

DNA extraction and microsatellite amplification

DNA was extracted from the leg of one worker bee per colony (*S. mexicana*, N = 67; *S. pectoralis* N = 33:

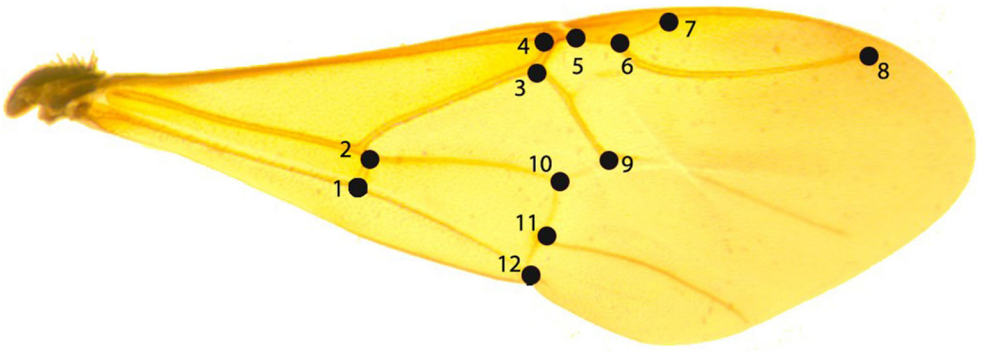

Fig. 2 Worker wing with 12 landmarks in the vein junctions

Table 1) using the DNeasy tissue kit (QIAGEN). Seven microsatellite loci were amplified in three reactions (two of them multiple), two of which were microsatellites originally described in *S. postica* (T4–171 and T7–5; Paxton et al. 1999), four in *Melipona bicolor* (Mbi278, Mbi259, Mbi254 and Mbi201; Peters et al. 1998) and one in the bumblebee *Bombus terrestris* (B124; Estoup et al. 1995). The T4–171, T7–5 and B124 loci have already been used successfully in *S. mexicana* (Kraus et al. 2008; Mueller et al. 2012) and *S. hellwegeri* (Quezada-Euán et al. 2012).

PCR reactions were carried out in 12.5 µl with PureTaqTM Ready-To-Go TM PCR beads (GE Healthcare) in a PTC-200 Thermal Cycler (Biorad). The amplified fragments (microsatellite alleles) were detected with an ABI prism 3100 sequencer (Applied Biosystems) and scored with Genemapper software (version 3.7: Applied Biosystems).

Molecular data analyses

The number of clusters (K) was estimated with the STRUCTURE software (version 2.2: Pritchard et al. 2000) using a Bayesian model-based method, basing the results on simulations of 80,000 burn-in steps and 1,000,000 MCMC (Markov Chain Monte Carlo) algorithm iterations. Five runs for each K-value ($K = 1 - 8$) were used to estimate the most likely value of K, and the runs were performed without any prior information about the origin of samples using the "Admixture Model" and considering allele frequencies correlated among populations. The number of clusters defined by the ΔK value described previously (see Evanno et al. 2005) was inferred by STRUCTURE HARVESTER (Earl and vonHoldt 2012). CLUMPP (v1.1.2: Jakobsson and Rosenberg 2007) was used to obtain the average permutated individual and population Q-matrices of the best K value. The *distruct* software (v1.1: Rosenberg 2004) was used to graphically display the CLUMPP results.

The dataset describing the genotypes included in each cluster was analyzed with MICRO-CHECKER (Van Oosterhout et al. 2004) to detect scoring incongruities and possible null alleles. GENALEX (Peakall and Smouse 2006) was used to calculate population genetics parameters per locus and cluster in each species (the number of detected and private alleles, and the expected heterozygosity). Given the different number of individuals sampled for each cluster, a rarefaction analysis gave an objective estimate of allelic richness (i.e. the number of alleles: Leberg 2002) and it was carried out with the HP-RARE 1.0 program (Kalinowski 2005). Differences between clusters were estimated using the two-tailed Mann–Whitney U Test (Mann and Whitney 1947).

Hardy–Weinberg and genotypic linkage equilibria, and the population differentiation using the Fisher's exact probability test were calculated in GENEPOP (Raymond and Rousset 1995a, b). All probability values were adjusted for multiple comparison tests using sequential Bonferroni adjustments (Rice 1989).

Pairwise R_{ST} values were calculated as a measure of genetic distance in ARLEQUIN 3.5 (Excoffier and Lischer 2010), calculating R_{ST} pairwise values to estimate the connectivity and patterns of gene flow between clusters given that F_{ST} values might underestimate the differentiation in highly structured populations (Balloux and Lugon-Moulin 2002) like those of *Scaptotrigona* species (Quezada-Euán et al. 2012; Hurtado-Burillo et al. 2013). The correlation between genetic and geographic distances within each cluster was analyzed with a Mantel test, as implemented in GENALEX, to test whether allelic frequencies varied in function of a pattern of isolation by distance (IBD).

Results

Geometric morphometrics

PCA analysis of the 12 wing landmarks generated 20 relative warp measurements. The first 11 factors of these measurements had eigenvalues greater than one and explained 95.46 % of the total variability in the data. The first two PC values explained 64.52 % of the total variability (PC1

Table 2 Microsatellite variation in *S. mexicana* (*Sm1* and *Sm2*) and *S. pectoralis* (*Sp1* and *Sp2*). Sample size (N), number of detected alleles (Na) and number of private alleles (Npa) (within brackets after rarefaction) and expected (He) heterozygosity per locus. Mean values ± SE are shown for each cluster

Cluster	Locus	N	Na	Npa	He
Sm1	Mbi254AAG	32	2 (1.71)	0 (0.01)	0.26
	Mbi259AAG	37	4 (2.06)	0 (0.36)	0.41
	Mbi278AAG	37	1 (1)	0 (0)	0
	Mbi201AAG	37	2 (1.46)	1 (0.46)	0.15
	T4-171	35	9 (4.24)	4 (3)	0.80
	T7-5	35	9 (3.77)	6 (2.23)	0.73
	B124	32	10 (4.3)	6 (4.02)	0.78
	Mean ± SE	35 ± 0.9	5.3 ± 1.5 (2.65 ± 0.4)	2.43 ± 1.1 (1.44 ± 0.5)	0.45 ± 0.1
Sm2	Mbi254AAG	24	2 (1.99)	0 (0.28)	0.50
	Mbi259AAG	28	4 (2.39)	0 (0.69)	0.43
	Mbi278AAG	28	2 (1.88)	1 (0.88)	0.38
	Mbi201AAG	28	1 (1)	0 (0)	0
	T4-171	23	6 (3.3)	1 (2.06)	0.64
	T7-5	25	7 (3.84)	4 (2.30)	0.76
	B124	27	8 (3.08)	4 (2.80)	0.57
	Mean ± SE	26.1 ± 0.8	4.3 ± 1.0 (2.50 ± 0.3)	1.43 ± 0.7 (1.29 ± 0.3)	0.47 ± 0.1
Sp1	Mbi254AAG	15	4 (2.41)	2 (0.57)	0.51
	Mbi259AAG	17	2 (1.83)	1 (1.29)	0.33
	Mbi278AAG	17	2 (1.71)	0 (0.05)	0.25
	Mbi201AAG	17	1 (1)	0 (0)	0
	T4-171	13	4 (3.38)	0 (1.01)	0.70
	T7-5	13	1 (1)	0 (0)	0
	B124	17	1 (1)	0 (0)	0
	Mean ± SE	15.6 ± 0.7	2.1 ± 0.5 (1.76 ± 0.3)	0.43 ± 0.3 (0.4 ± 0.2)	0.3 ± 0.1
Sp2	Mbi254AAG	9	2 (1.89)	0 (0.06)	0.35
	Mbi259AAG	16	3 (2.63)	2 (2.09)	0.60
	Mbi278AAG	16	2 (1.93)	0 (0.27)	0.40
	Mbi201AAG	16	1 (1)	0 (0)	0.00
	T4-171	15	10 (5.28)	6 (2.91)	0.88
	T7-5	16	2 (1.95)	1 (0.95)	0.43
	B124	16	3 (2.20)	2 (1.20)	0.52
	Mean ± SE	14.9 ± 1.0	3.3 ± 1.1 (2.41 ± 0.4)	1.58 ± 0.8 (1.1 ± 0.3)	0.5 ± 0.1

explained 49.31 % and PC2 15.21 %) and a PCA identified three groups, two within *S. mexicana* (herein named *Sm1* and *Sm2*) and one with the *S. pectoralis* colonies. The graphic representation showed that the colonies from the Pacific coast (Chiapas, *Sm1*) were placed in quadrant 4 while the rest of the colonies from the Atlantic coast (Veracruz in Mexico and Peten in Guatemala, *Sm2*) were mainly placed in quadrant 3. Conversely, the samples of *S. pectoralis* were dispersed in quadrants 1–2 (Fig. 3).

The Mahalanobis distances between the groups confirmed the differentiation between *Sm1* and *Sm2* (4.847), although this value was around half of that observed between *S. pectoralis* and *Sm1* (8.477) or *Sm2* (8.171). In addition, the Mantel test showed no significant correlation between morphological and geographical distances in each putative species (*Sm1*: $R = -0.13$, $p = 0.99$; *Sm2*: $R = 0.012$, $p = 0.3$ and *S. pectoralis*: $R = 0.010$, $p = 0.39$).

Microsatellites

The average genotyping error rate for the seven microsatellite loci assessed in this study was <5 %. Less than four loci were amplified from two of the 67 *S.*

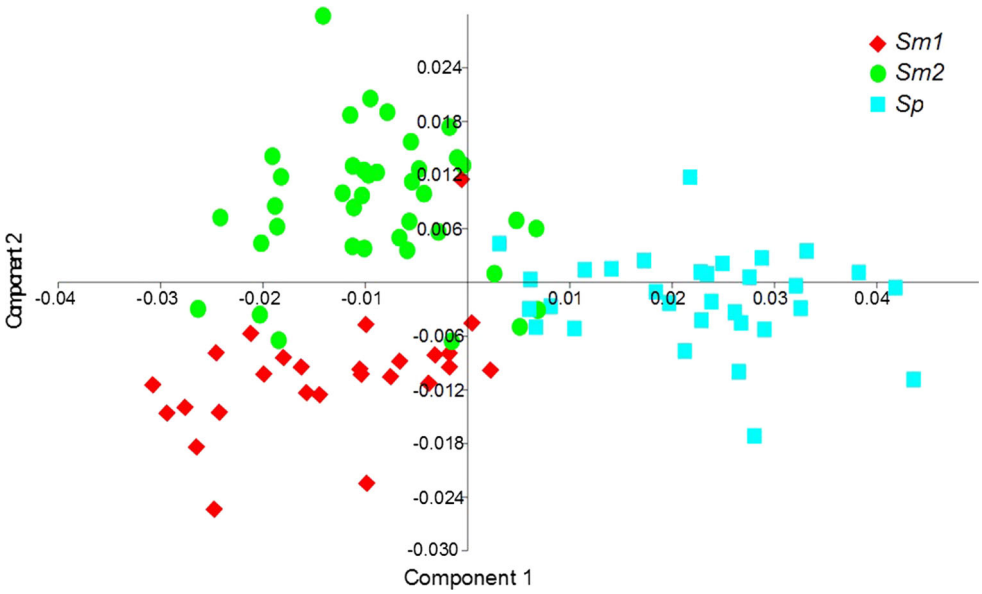

Fig. 3 Distribution of the average scores of *Scaptotrigona* colonies against principal components 1 and 2 of the principal component analysis (PCA) based on geometric morphometry data. *Sm* named the two clusters detected within *S. mexicana* and *Sp* referred to *S. pectoralis* colonies

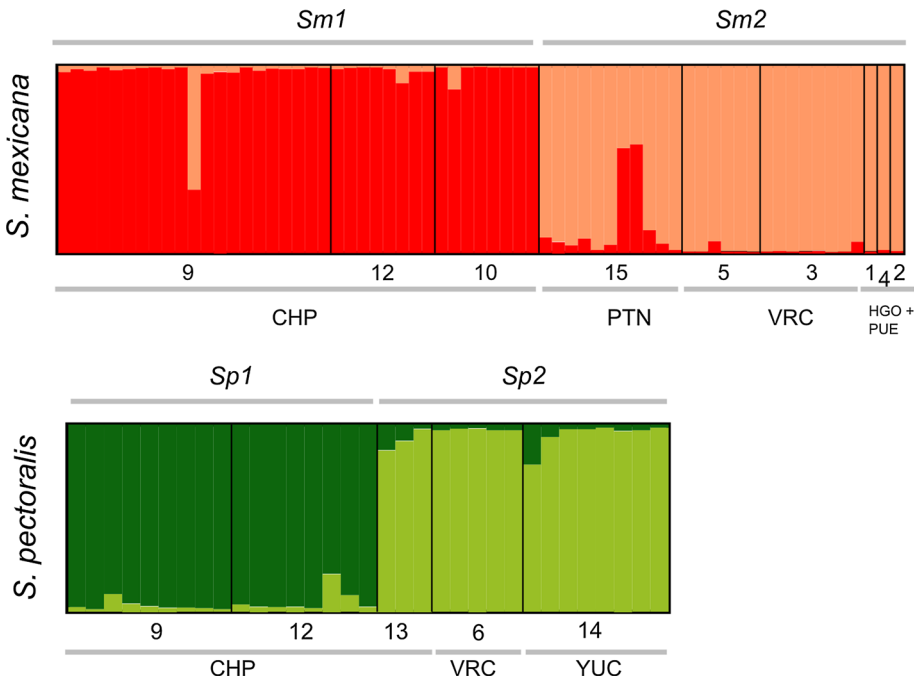

Fig. 4 Results of the Bayesian clustering showing the most probable number of clusters (K = 2) for the two species. Division of specimens into *colored segments* represents the assignment probability of that specimen to each of the K clusters (*red—Sm1*; *light red—Sm2*; *dark green—Sp1*; *light green—Sp2*). *Numbers* correspond to the sampling code used in Table 1 and acronyms to the name of the state (*CHP* Chiapas, *PTN* Petén, *VRC* Veracruz, *HGO* Hidalgo, *PUE* Puebla, *YUC* Yucatán). (Color figure online)

mexicana specimens and thus, they were excluded from the analysis. In a first step, we looked for signs of genetic structure in each species. The highest posterior probability of the data set was detected when assuming two clusters (K = 2) in both species: *S. mexicana* (*Sm1* and *Sm2*) and *S. pectoralis* (*Sp1* and *Sp2*) (Fig. 4). *S. mexicana* clusters were congruent with the geometric morphometry data: *Sm1* (colonies from Chiapas) and *Sm2* (colonies from Veracruz and Petén, including in this case three more colonies, two from Puebla and one from Hidalgo). In contrast to the morphological results, *S. pectoralis* was divided into two clusters: *Sp1* (colonies from southern Chiapas: Tapachula and Tuxtla Chico) and *Sp2* (colonies from Veracruz, Yucatan and northern Chiapas: Palenque). These clusters showed a geographic pattern for both species, splitting Pacific (*Sm1* and *Sp1*) and Atlantic populations (*Sm2* and *Sp2*), and they were used to estimate the population genetic parameters.

There was no evidence of scoring error due to stuttering or allele dropout, yet there appeared to be null alleles at

locus T4-171 in *Sm2*, and at loci Mbi254-AAG and B124 in *Sp2*. These loci had a higher percentage of unsuccessfully genotyped individuals (3.5, 4.5 and 1.5 %, respectively), a fact that was taken into account to explain several population parameters.

The number of alleles of the seven microsatellite loci ranged from one to ten across all the samples (Table 2). The average allele number after rarefaction was higher in the *Sm1* (2.65 ± 0.4) than in *Sm2* (2.50 ± 0.3) populations, and in the *Sp2* (2.41 ± 0.4) than in *Sp1* (1.76 ± 0.3) populations, and fewer private alleles (i.e. alleles unique to each cluster, Npa) were evident after rarefaction. The highest Npa was detected in *Sm1* within *S. mexicana*, (1.44 ± 0.5), whereas for *S. pectoralis* the highest Npa was detected in *Sp2* (1.1 ± 0.3). All comparisons were not statistically significant ($p > 0.05$). Gene diversity was measured as the expected heterozygosity (He) and it was higher in the *Sm2* than in the *Sm1* population, and in the *Sp2* than in the *Sp1* population, although these difference were not significant.

The *Sm1* cluster was in Hardy–Weinberg equilibrium before Bonferroni correction ($p > 0.05$ for each locus), whereas in the *Sm2* population one locus (T4-171) did not show Hardy–Weinberg equilibrium even after Bonferroni correction, highlighting a marked excess of homozygotes that was probably due to the presence of null alleles. A similar result was obtained in the *S. pectoralis* populations as all loci were in Hardy–Weinberg equilibrium in the *Sp1* population whereas *Sp2* did not show such equilibrium at those loci with null alleles (Mbi254AAG and B124). The loci were generally out of linkage disequilibrium after Bonferroni correction for each cluster, with the exceptions of 4 out of the 15 pairwise comparisons in *Sm2*.

A Fisher's exact test of population differentiation showed highly significant genetic differences between the clusters of each species. The pairwise R_{ST} was higher between *Sm1* and *Sm2* ($R_{ST} = 0.266$, $p < 0.0001$) than between *Sp1* and *Sp2* ($R_{ST} = 0.198$, $p < 0.0001$). The IBD was evaluated with the Mantel test for each cluster and there was a significant correlation between genetic and geographic distance in the Atlantic populations of both species (*Sm2* $r = 0.383$, $p = 0.001$; and *Sp2* $r = 0.477$, $p = 0.001$), but no correlation in the Pacific populations (*Sm1* $r = -0.034$, $p = 0.177$; and *Sp1* $r = 0.158$, $p = 0.079$).

Discussion

Our results demonstrate that diversity in the *Scaptotrigona* genus is higher than expected in Mesoamerica. The genetic and geometric morphometric analyses fully support the existence of two evolutionary significant units (ESUs) within *S. mexicana* (*Sm1* and *Sm2*), with a Pacific and Atlantic distribution, respectively. By contrast, *S. pectoralis* population differentiation is evident through genetic analysis (*Sp1* and *Sp2*) but not so clearly by geometric morphometry, suggesting that *S. pectoralis* is in an incipient stage of population differentiation.

The clusters found within *S. mexicana* resembled two ESUs with different distributions, one dispersed along the Pacific coast of Mexico (*Sm1*) and the other along the Atlantic Mexican coast up to the North of Guatemala (*Sm2*). These data support previous results suggesting the existence of a cryptic species within *S. mexicana* based on high divergence between the populations identified by means of the barcoding method (Hurtado-Burillo et al. 2013). The results obtained here support this hypothesis, since both the molecular and morphological approaches discriminate the two ESUs. A multidisciplinary approach such as that performed here also proved to be useful in other population studies on stingless bees species (Mendes et al. 2007; Gonçalves 2010; Francoy et al. 2011; May-Itzá et al. 2012; Bonatti et al. 2014) and on honey bees, albeit at the sub-species level in the latter (Oleksa and Tofilski 2015).

The differentiation between *Sm1* and *Sm2* is evident through several population parameters. First, geometric mophometry of the wings indicates a significant phenotypic differentiation of two separate groups. Second, while both *S. mexicana* clusters share alleles, the presence of private alleles in both the Pacific *Sm1* and Atlantic *Sm2* populations, together with different allelic frequencies at some loci, indicate genetic differences between these two evolutionary units. Although microsatellite markers usually show less allelic diversity when used in species other than those they have been designed for (Borges et al. 2010), they yielded appropriate information in analyses of other *S. mexicana* populations (Kraus et al. 2008; Mueller et al. 2012) and *S. hellwegeri* (Quezada-Euán et al. 2012). Finally, the R_{ST} value (0.266) points to significant divergence within *S. mexicana* units. While this R_{ST} value could reflect an IBD effect that restricts gene flow among colonies, it is not conclusive of complete separation into two species. Introgression events between ESUs of *S. mexicana* (*Sm1* and *Sm2*: Fig. 4) have been observed in both Atlantic and Pacific populations, possibly the result of natural gene flow. However, human influence should not be ignored as *S. mexicana* is one of the species traditionally managed in Mesoamerican cultures and our samples were obtained from stingless beekeepers. Although movements of hives over large distances are uncommon in Mesoamerica (May-Itzá et al. 2012; González-Acereto pers. comm.), genetic traces of human-mediated transportation have been observed elsewhere for other stingless bees (Byatt et al. 2016).

Genetic differentiation was also observed for *S. pectoralis*, with microsatellite analysis defining two clusters (*Sp1* and *Sp2*) and confirming the data obtained with mitochondrial markers (Hurtado-Burillo et al. 2013). The clustering obtained previously through the barcoding approach also suggested some degree of differentiation within *S. pectoralis*, although the intraspecific divergence did not fully support the existence of distinct genetic lineages. Since extant geometric morphometric data does not corroborate this differentiation, the two clusters in *S. pectoralis* may not be ESUs but rather, units of management and conservation, as in the case of Brazilian populations of *Scaptotrigona xanthotricha* (Duarte et al. 2014). In *S. pectoralis*, the failure to detect introgression suggests there has been no recent gene flow between the two clusters. This could be due to the limited sampling (n = 33) and or the fact that the colonies sampled were wild, unmanaged colonies located in native forests.

The homogeneous genetic structure detected in distant populations of Atlantic clusters, despite the presence of three introgressed *S. mexicana* specimens (one from Chiapas in *Sm1* and two from Petén in *Sm2*), and especially in the Pacific clusters of both species, may be related to the reproductive behavior of this group of bees. This behavior favors gene flow within nearby populations due to the migration of males from several colonies to form male congregation areas. This reproductive strategy is used by *S. mexicana* (Kraus et al. 2008) and it is an effective mechanism to avoid inbreeding (Mueller et al. 2012). Such homogeneity contrasts with the population structure observed in the Pacific and Atlantic clusters of each species, which may be explained by the influence of a geographical barrier to their distribution, the Sierra Madre, as seen in other Meliponini (May-Itzá et al. 2010, 2012), Hemiptera (Dorn et al. 2009), Coleoptera (Anducho-Reyes et al. 2008) and even in terrestrial birds within the same area (Álvarez and Morrone 2004; Yáñez-Ordóñez et al. 2008). Furthermore, molecular data indicate that recent evolutionary processes like IBD also affect the genetic diversity observed within the *Sm2* and *Sp2* colonies sampled at two extremes of the Atlantic axis. By contrast, the lack of IBD in Pacific *Sm1* and *Sp1* is probably due to the close proximity of the colonies. In addition, the lack of a significant relationship between geographic and morphometric distances suggests that wing shape may not be a neutral marker and that it is affected by selective pressures (Reed and Frankham 2001). In these stingless bees, not only the presence of geographic barriers but also the limited dispersion of the colonies influences the differentiation between populations.

Stingless bees are important elements for the preservation of ecosystems as they are the most frequent visitors of many native plants, including economically important local crops in Mesoamerica (Russell et al. 2005; Morandin and Winston 2006; Ayala et al. 2013). A major threat to stingless bees is the loss of tropical forests, which implies a reduction of potential nesting sites and foraging areas (Foley et al. 2005; Venturieri 2009; May-Itzá et al. 2010). Deforestation may drastically affect the effective size of bee populations as they remain isolated in fragments (Brown and Albrecht 2001). The effect of landscape fragmentation may be more drastic on stingless bees as these insects have some of the lowest dispersal rates, reflecting the mother–daughter colony attachment that persists in the process of swarming (Engels and Imperatriz-Fonseca 1990). This feature of stingless bee reproduction could become maladaptive when habitats are destroyed, as colonies and individuals may be unable to bridge the deteriorated landscapes (Zayed 2009). Indiscriminate use of pesticides is another factor that may affect stingless bee populations, especially those that frequently visit crops (Valdovinos-Núñez et al. 2009). The effect of human-induced translocation of colonies is also particularly relevant to species exploited for honey production (Jaffé et al. 2016), as is the case of *Scaptotrigona*. Avoiding the movement of colonies is crucial to preserve the genetic integrity of ecotypes adapted to particular geographic areas. Thus, it is also important to educate farmers and other individuals through conservation programs that promote the preservation of natural habitats for the reproduction of colonies.

In conclusion, the combined use of genetic and morphological techniques indicates that only the *S. mexicana* colonies from both coasts are distinct evolutionary significant units. A requisite to establish valid species affirmation is congruence among several independent lines of evidence (Schlick-Steiner et al. 2010). Accordingly, further studies that include data from more samples that more extensively cover the distribution area of these two ESUs and that address other aspects of their biology will be necessary to confirm their taxonomic status. In any case, the results presented here favor the promotion of conservation measures not only for the *Sm1* and *Sm2* populations but also, for the *Sp1* and *Sp2* populations, which should be treated as separate units in order to avoid inbreeding and a loss of diversity (González-Acereto et al. 2006). Programs and strategies focused on maintaining the diversity of these bees should be set-up in order to preserve their genetic diversity.

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SNPs selected by information content outperform randomly selected microsatellite loci for delineating genetic identification and introgression in the endangered dark European honeybee (*Apis mellifera mellifera*)

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Abstract

The honeybee (*Apis mellifera*) has been threatened by multiple factors including pests and pathogens, pesticides and loss of locally adapted gene complexes due to replacement and introgression. In western Europe, the genetic integrity of the native *A. m. mellifera* (M-lineage) is endangered due to trading and intensive queen breeding with commercial subspecies of eastern European ancestry (C-lineage). Effective conservation actions require reliable molecular tools to identify pure-bred *A. m. mellifera* colonies. Microsatellites have been preferred for identification of *A. m. mellifera* stocks across conservation centres. However, owing to high throughput, easy transferability between laboratories and low genotyping error, SNPs promise to become popular. Here, we compared the resolving power of a widely utilized microsatellite set to detect structure and introgression with that of different sets that combine a variable number of SNPs selected for their information content and genomic proximity to the microsatellite loci. Contrary to every SNP data set, microsatellites did not discriminate between the two lineages in the PCA space. Mean introgression proportions were identical across the two marker types, although at the individual level, microsatellites' performance was relatively poor at the upper range of *Q*-values, a result reflected by their lower precision. Our results suggest that SNPs are more accurate and powerful than microsatellites for identification of *A. m. mellifera* colonies, especially when they are selected by information content.

Keywords: *Apis mellifera mellifera*, dark European honeybee, honeybee conservation, introgression, microsatellites, SNPs

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Introduction

The western honeybee, *Apis mellifera* L., is currently distributed worldwide. However, prior to the human-assisted global expansion, this species was confined to western Asia, Middle East, Africa and Europe (Ruttner 1988; Chen *et al.* 2016). In such an environmentally heterogeneous range, the honeybee has differentiated into 31 currently recognized subspecies (Ruttner 1988; Sheppard & Meixner 2003; Meixner *et al.* 2011; Chen *et al.* 2016), which have been grouped into four main evolutionary lineages: M (western European), C (eastern European), O (Middle Eastern) and A (African) (Ruttner 1988). This vast diversity has been increasingly threatened by major factors including habitat loss and fragmentation, pesticides and spread of pests and pathogens (Potts *et al.* 2010; Van Engelsdorp & Meixner 2010). An additional, but less publicized, threat comes from honeybee queen (legal or illegal) trade and intensive queen breeding. Large-scale movements of commercial queen strains, usually of the beekeepers-favoured C-lineage ancestry, represent a risk for local populations, not only because they may bring new parasites and pathogens or more virulent strains of established parasites and pathogens (Mutinelli 2011; Muñoz *et al.* 2014a; McMahon *et al.* 2016), but also because of introgressive hybridization (Jensen *et al.* 2005; Soland-Reckeweg *et al.* 2009; Muñoz *et al.* 2014b; Pinto *et al.* 2014).

There are growing concerns that intensified queen breeding and trade may promote gene flow between

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native and commercial strains leading to an irremediable loss of diversity adapted to local conditions (De la Rúa *et al.* 2009; 2013; Büchler *et al.* 2014). This is the case for the M-lineage *A. m. mellifera* (the dark European honeybee), which in a substantial portion of its native range in western Europe is gravely threatened by C-derived (mainly *A. m. ligustica* and *A. m. carnica*) introgression (Jensen *et al.* 2005; Soland-Reckeweg *et al.* 2009; De la Rúa *et al.* 2009; Nedić *et al.* 2014; Pinto *et al.* 2014). In an attempt to reverse this threat, several conservation programmes have been implemented across Europe (De la Rúa *et al.* 2009; Muñoz *et al.* 2015). An efficient management of these programmes requires, however, molecular tools capable of reliably identifying pure-bred *A. m. mellifera* colonies in a cost- and time-effective manner.

While a variety of molecular markers, including RFLPs (Hall 1990), AFLPs (Suazo & Hall 1999), RAPDs (Hunt & Page 1995) and STSs (Arechavaleta-Velasco *et al.* 2003), have been employed in honeybee research, microsatellites (aka short tandem repeats, STRs) have indisputably been the marker of choice over the last 15–20 years (reviewed by Meixner *et al.* 2013). Numerous studies have demonstrated their usefulness in unravelling the signatures of historical and contemporary human-driven events in the native (Franck *et al.* 1998, 2001; Garnery *et al.* 1998; De la Rúa *et al.* 2001, 2003, 2006; Sušnik *et al.* 2004; Bodur & Kence 2007; Dall'Olio *et al.* 2007; Miguel *et al.* 2007; Muñoz *et al.* 2009; Cánovas *et al.* 2011; Coroian *et al.* 2014; Francis *et al.* 2014; Nedić *et al.* 2014; Uzunov *et al.* 2014; Péntek-Zakar *et al.* 2015) and introduced (Clarke *et al.* 2002; Pinto *et al.* 2005; Galindo-Cardona *et al.* 2013; Rangel *et al.* 2016) distributional range of the honeybee. Microsatellites have been particularly useful for identifying introgression of C-derived genes into gene pools of native honeybees and monitoring conservation programmes of *A. m. mellifera* in the Danish island of Læsø (Jensen *et al.* 2005), in the French region of Landes (Strange *et al.* 2008), in the eastern part of Switzerland (Soland-Reckeweg *et al.* 2009) and in the north-eastern part of Poland (Oleksa *et al.* 2011), of *A. m. carnica* in Slovenia (Sušnik *et al.* 2004), of *A. m. iberiensis* in the Canary islands (Muñoz *et al.* 2012) and of *A. m. siciliana* in the Filicudi and Vulcano islands (Muñoz *et al.* 2014c).

Single-nucleotide polymorphisms (SNPs) represent the most recent addition to the molecular toolkit for honeybee genetic analysis and are rapidly becoming popular among honeybee scientists. SNPs have been used to scrutinize the evolutionary history of the honeybee (Whitfield *et al.* 2006; Wallberg *et al.* 2014; Chen *et al.* 2016), to search for footprints of selection (Zayed & Whitfield 2008; Spötter *et al.* 2012; Chávez-Galarza *et al.* 2013; Harpur *et al.* 2014; Wallberg *et al.* 2014; Fuller *et al.* 2015; Chen *et al.* 2016; Wragg *et al.* 2016), to dissect the evolutionary complexities of the Iberian honeybee hybrid zone (Chávez-Galarza *et al.* 2015) and to examine genomewide recombination patterns (Wallberg *et al.* 2015). At a more practical level, the potential of SNPs for identifying African-derived genes in the European stock of North America (Chapman *et al.* 2015) and C-derived genes in *A. m. mellifera* in western Europe for commercial and conservation purposes has also been investigated (Pinto *et al.* 2014; Muñoz *et al.* 2015). However, whether SNPs can replace microsatellites for identifying genetic stocks needs to be addressed. While as a usually biallelic marker, the per locus information content of a SNP is lower than that of a multiallelic microsatellite, this drawback can be offset by employing large numbers of SNPs, whose identification is greatly facilitated in the genomics era. The average number of random SNPs required to equal the information content of random microsatellites has been estimated to be two to 10 times, depending largely on the question under scrutiny (Kalinowski 2002; Thalamuthu *et al.* 2004; Herráez *et al.* 2005; Liu *et al.* 2005; Schopen *et al.* 2008; Gärke *et al.* 2012). The ratio can, however, be lowered if informative SNPs selected from a larger panel are employed instead of randomly selected SNPs (Glover *et al.* 2010; Gärke *et al.* 2012; Ozerov *et al.* 2013).

Given the high number of SNPs available for the honeybee (Whitfield *et al.* 2006; Spötter *et al.* 2012; Pinto *et al.* 2014; Chapman *et al.* 2015), the challenge is to identify the most informative. Several approaches can be implemented to measure the contribution of single SNPs, which can then be ranked by information content (Muñoz *et al.* 2015). In this context, a subset of SNPs, the so-called ancestry informative markers (AIMs), displays large allele frequency differences between populations. Ranking the most informative SNPs allows one to design reduced SNP panels that correctly assign individuals to ancestry origin. Reduced SNP panels have been used by others to delineate the genetic structure of honeybee populations in Canada (Harpur *et al.* 2015) and identify Africanized honeybees in North America (Chapman *et al.* 2015), and by us to estimate introgression in *A. m. mellifera* populations in Europe (Muñoz *et al.* 2015). Building from this previous study, here we compared the resolving power of a widely utilized 11-microsatellite set to detect structure and introgression with that of reduced SNP sets that were selected from a genomewide data set using two criteria: (i) the ranking in terms of information content and (ii) genomic proximity to the 11 microsatellites. Using these criteria, five reduced SNP sets (48, 96 and 144 top-ranked AIMs previously identified by Muñoz *et al.* 2015; and the closest five and 10 SNPs to each of the 11 microsatellites) were built and compared with

the 11-microsatellite set using principal component analysis (PCA) and a model-based clustering algorithm implemented in STRUCTURE (Pritchard *et al.* 2000). The ultimate goal of this study was to appraise the feasibility of a SNP-based alternative to microsatellites that can be used for identifying pure-bred *A. m. mellifera* genetic stock for breeding and for assisting management of conservation centres across Europe.

Methods

Samples

A total of 113 haploid males representing single colonies of three subspecies (*A. m. mellifera*, *A. m. ligustica* and *A. m. carnica*) was collected across Europe in 2010 and 2011. Seventy-seven were collected from the native range of the M-lineage subspecies *A. m. mellifera* in England, France, Belgium, Denmark, the Netherlands, Switzerland, Scotland and Norway, of which 64 originated from protected conservation areas and 13 from unprotected areas. The remainder made up a reference collection of 36 haploid males representing C-lineage diversity sampled in the native range of *A. m. ligustica* ($N = 17$) in Italy and *A. m. carnica* ($N = 19$) in Serbia and Croatia. Further details of sampling and DNA extraction procedures are provided in the study by Pinto *et al.* (2014) and Muñoz *et al.* (2015).

Microsatellite genotyping

Eleven widely utilized microsatellites were amplified in two multiplex PCR reactions. Plex-1 consisted of the five loci recommended in the Coloss Beebook (Dietemann *et al.* 2013), which is becoming the standard manual for honeybee research, namely A7, A113, Ap43, Ap55 and B124 (Evans *et al.* 2013). Plex-2 combined A8, A88, Ac11, Ap224, Ap249 and Ap274, which have been used to detect structure and introgression in different honeybee subspecies and populations (Chahbar *et al.* 2012; Coroian *et al.* 2014; Muñoz *et al.* 2014b; Nedić *et al.* 2014; Uzunov *et al.* 2014). Each reaction (10 μL) contained 1.2 mM $MgCl_2$, 0.3 mM of each dNTP, 0.4 μM of each forward and reverse primer, 1.5 U of *Taq* DNA polymerase (Biotools B&M Labs, Madrid, Spain) and 2 μL of extracted DNA. PCR amplification was performed at 95 °C for one 5-min cycle followed by 30 cycles at 95 °C for 30 s, either 54 °C (plex-1) or 50 °C (plex-2) for 30 s, 72 °C for 30 s and a final step at 72 °C for 30 min. PCR products were analysed on an ABI® 3730 automated DNA sequencer (Applied Biosystems, Waltham, USA) and sized with an internal standard. Alleles were subsequently scored using GENEMAPPER® v3.7 software (Applied Biosystems, Foster City, USA).

SNP genotyping

A total of 1536 SNP loci evenly spaced across the 16 honeybee linkage groups were genotyped using Illumina's BeadArray Technology and the Illumina GoldenGate® Assay with a custom Oligo Pool Assay (Illumina, San Diego, USA) following manufacturer's protocols. Upon the filtering process, 353 SNPs were removed because they did not meet the quality criteria for analysis (see Chávez-Galarza *et al.* 2013 for details). Allele frequencies were calculated for the remaining 1183 biallelic SNPs in each population using the program PLINK (Purcell *et al.* 2007).

Comparison analysis for the detection of population structure and introgression

The two types of molecular markers were examined by comparing the 11 microsatellites with the genomewide 1183 SNPs (hereafter named reference SNP set) and with five SNP sets. These included three reduced SNP sets formed by the top-ranked 48, 96 and 144 informative SNPs (hereafter named 48, 96 and 144 AIMs) identified by Muñoz *et al.* (2015) and two reduced SNP sets formed by the 55 and 110 SNPs closest to the 11 microsatellites (hereafter named 55 and 110 closest SNPs). The physical map of these loci shows that the 144 AIMs are distributed across the 16 honeybee linkage groups providing good genome coverage, in stark contrast to the 11 microsatellite loci and corresponding 110 closest SNPs, which only mark seven linkage groups (Fig. 1).

Genetic diversity was assessed for each subspecies from the microsatellite and the six SNP data sets using unbiased estimates of gene diversity (Nei 1987) and allelic richness (Petit *et al.* 1998). The mean number of alleles (Na), number of effective alleles (Ne) and unbiased diversity (uh) were computed using GENALEX 6.501 (Peakall & Smouse 2006, 2012), whereas allelic richness (Rs) was computed after rarefaction using HP-RARE 1.1 (Kalinowski 2005). Differentiation was estimated using the standardized G'_{ST} measure proposed by Hedrick (2005), which allows comparison between markers with different levels of genetic variation. Global and pairwise G'_{ST} values were estimated across the three subspecies and seven data sets with GENALEX 6.501.

The resolving power of the microsatellite set and the five reduced SNP sets to detect population structure and introgression was compared with the reference SNP set using both PCA and a model-based clustering approach. PCA was performed on a normalized matrix of individuals' genotypes to generate two-dimensional PCA and to visualize the stability of population assignment produced by all sets. PCA was implemented in the PAST

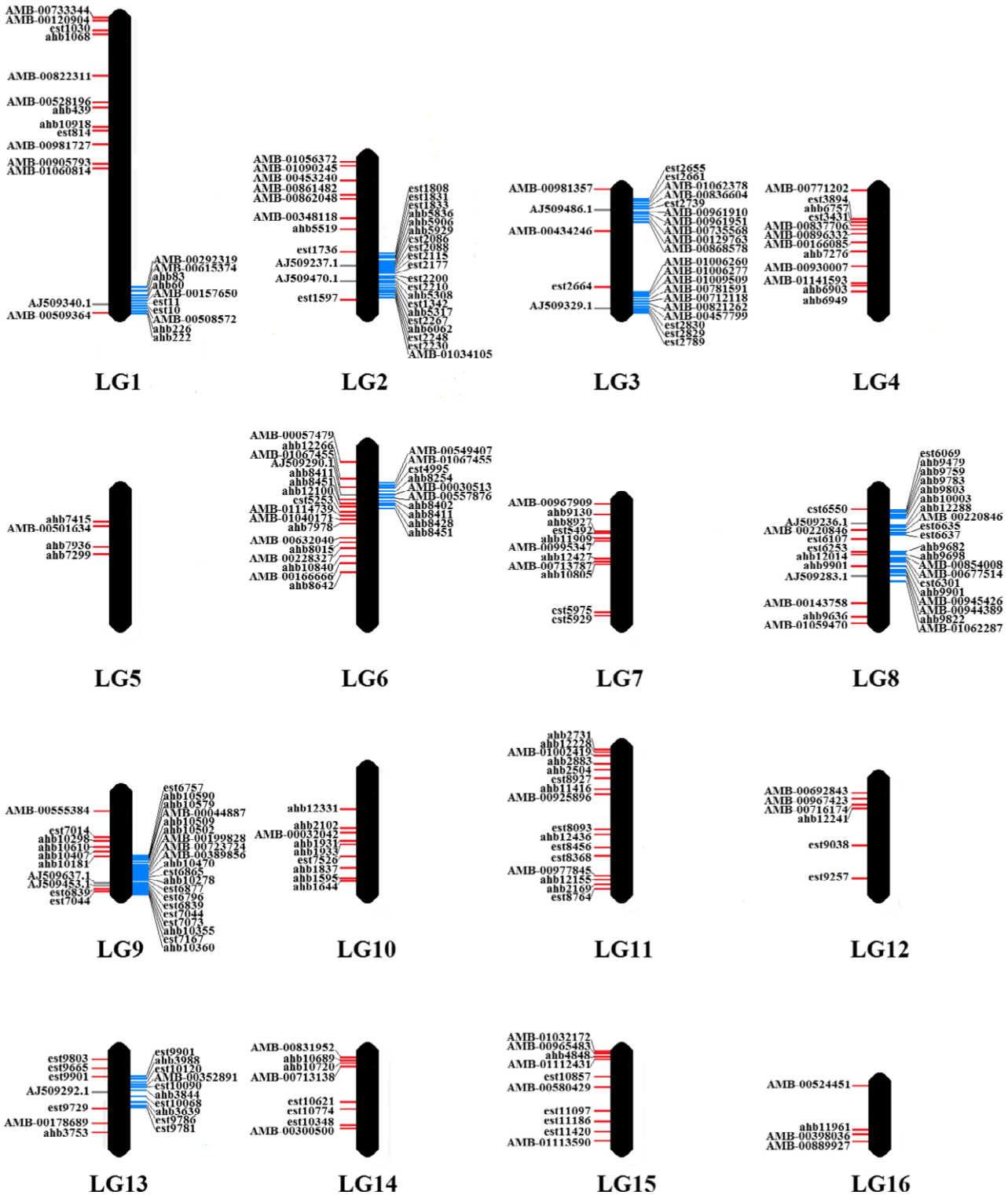

Fig. 1 Physical map of the 16 honeybee linkage groups showing the genomic positions of the 11 microsatellites (coded as AJ509XXX.1) marked in black, the 55 and 110 closest SNPs (five and 10 flanking each of the 11 microsatellite loci) marked in blue and the top-ranked 144 AIMs (includes the 48 and 96 AIMs) marked in red. The map was depicted from the honeybee genome sequence available at http://www.ncbi.nlm.nih.gov/projects/mapview using the Map Viewer tool. [Colour figure can be viewed at wileyonlinelibrary.com]

software (Hammer *et al.* 2001). The model-based Bayesian clustering algorithm implemented in STRUCTURE 2.3.3 (Pritchard *et al.* 2000) was employed to infer membership or introgression proportions (*Q*-value). The number of ancestral clusters (*K*) was estimated using the admixture ancestry and correlated allele frequency models with the unsupervised option. The program was set up for 750 000 Markov chain Monte Carlo iterations after an initial burn-in of 250 000. Over 20 independent runs for each *K* (from 1 to 5) were performed to confirm consistency across runs. The output was exported into STRUCTURE HARVESTER v0.6.93 (Earl & Von Holdt 2012), and the estimation of the most probable *K* was calculated as described by Evanno *et al.* (2005). The Greedy algorithm, implemented in the software CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007), was used to compute the pairwise 'symmetric similarity coefficient' between pairs of runs and to align the 20 runs for each *K*. Differences in diversity, $F_{ST,}$ and *Q*-values between data sets and subspecies were assessed for statistical significance using the Tukey test implemented in PAST.

The precision of each marker type and set was tested against the reference SNP set by calculating linear regression coefficients (r^2) and the standard deviations of the differences between introgression proportions. Finally, the accuracy of the different sets was estimated via percentage of absolute error of introgression estimates in relation to the reference SNP set.

Results

In this study, the resolving power of microsatellites and SNPs to detect population structure and introgression was compared on 113 honeybee individuals representing three honeybee subspecies (*A. m. mellifera*, *A. m. ligustica* and *A. m. carnica*) of the two evolutionary lineages (M and C) native to Europe.

Genetic diversity and differentiation

As expected, the SNP loci were biallelic, whereas the 11 microsatellite loci were multiallelic with the number of microsatellite alleles per locus ranging from two (Ap274) to 21 (A7). The mean number of microsatellite alleles per locus varied with subspecies, being 8.7, 3.6 and 4.7 for *A. m. mellifera*, *A. m. carnica* and *A. m. ligustica*, respectively. This wide variation across subspecies may be in part explained by the variable sample size and geographical distribution of samples. A summary of diversity measures (Na, Ne, uh and Rs) inferred from the microsatellite and the six SNP data sets for the three subspecies are shown in Table S1 (Supporting information). Diversity varied across subspecies with the highest values obtained for *A. m. mellifera*. Diversity measures obtained with the 11 microsatellites were significantly higher than those estimated by SNPs ($0.0002 \leq P$-value ≤ 0.0066, Tukey test; Table S2, Supporting information).

Global and pairwise G'_{ST} values shown in Table 1 revealed variable levels of differentiation across markers and subspecies. Global G'_{ST} was lower for microsatellites (0.6371), and corresponding 55 (0.6274) and 110 (0.6172) flanking SNPs, than for any reduced SNP panel (0.8889, 0.8966 and 0.9044 for 144, 96 and 48 AIMs, respectively). As expected, pairwise G'_{ST} values showed the lowest differentiation for the two C-lineage subspecies *A. m. ligustica* and *A. m. carnica* (0.1336 for microsatellites and 0.1008 for the reference SNP data set) and the largest between the M-lineage *A. m. mellifera* and the two C-lineage subspecies (0.8098, 0.8082 for microsatellites and 0.7523, 0.7630 for the reference SNPs).

Table 1 Global and pairwise G'_{ST} values estimated by microsatellites and SNPs for the European honeybee subspecies *A. m. mellifera*, *A. m. ligustica* and *A. m. carnica*

		Pairwise G'_{ST}		
Set	Global G'_{ST}	*A. m. mellifera* vs. *A. m. ligustica*	*A. m. mellifera* vs. *A. m. carnica*	*A. m. ligustica* vs. *A. m. carnica*
11 STRs*	0.6371	0.8098	0.8082	0.1336
55 SNPs†	0.6274	0.6874	0.7162	0.1408
110 SNPs‡	0.6172	0.7008	0.6901	0.1270
48 AIMs§	0.9044	0.9651	0.9658	0.0242
96 AIMs§	0.8966	0.9623	0.9641	0.0168
144 AIMs§	0.8889	0.9581	0.9616	0.0245
1183 SNPs¶	0.6682	0.7523	0.7630	0.1008

*11 Microsatellite loci.

†55 Closest SNPs to the 11 microsatellites.

‡110 Closest SNPs to the 11 microsatellites.

§Reduced panels of top-ranked 48, 96 and 144 informative SNPs (AIMs—ancestry informative markers) identified by Muñoz et al. (2015).

¶Reference SNP data set.

Principal component analysis (PCA)

The results of PCA partitioning by the two markers and the reduced SNP sets are shown in Fig. 2. PCA grouping obtained with the microsatellites differed considerably from each of those obtained with the SNPs. The two main PCA components generated from the 11 microsatellites (Fig. 2a) showed a pronounced overlap of the 113 individuals representing the three *A. mellifera* subspecies and did not distinguish the M and C divergent lineages.

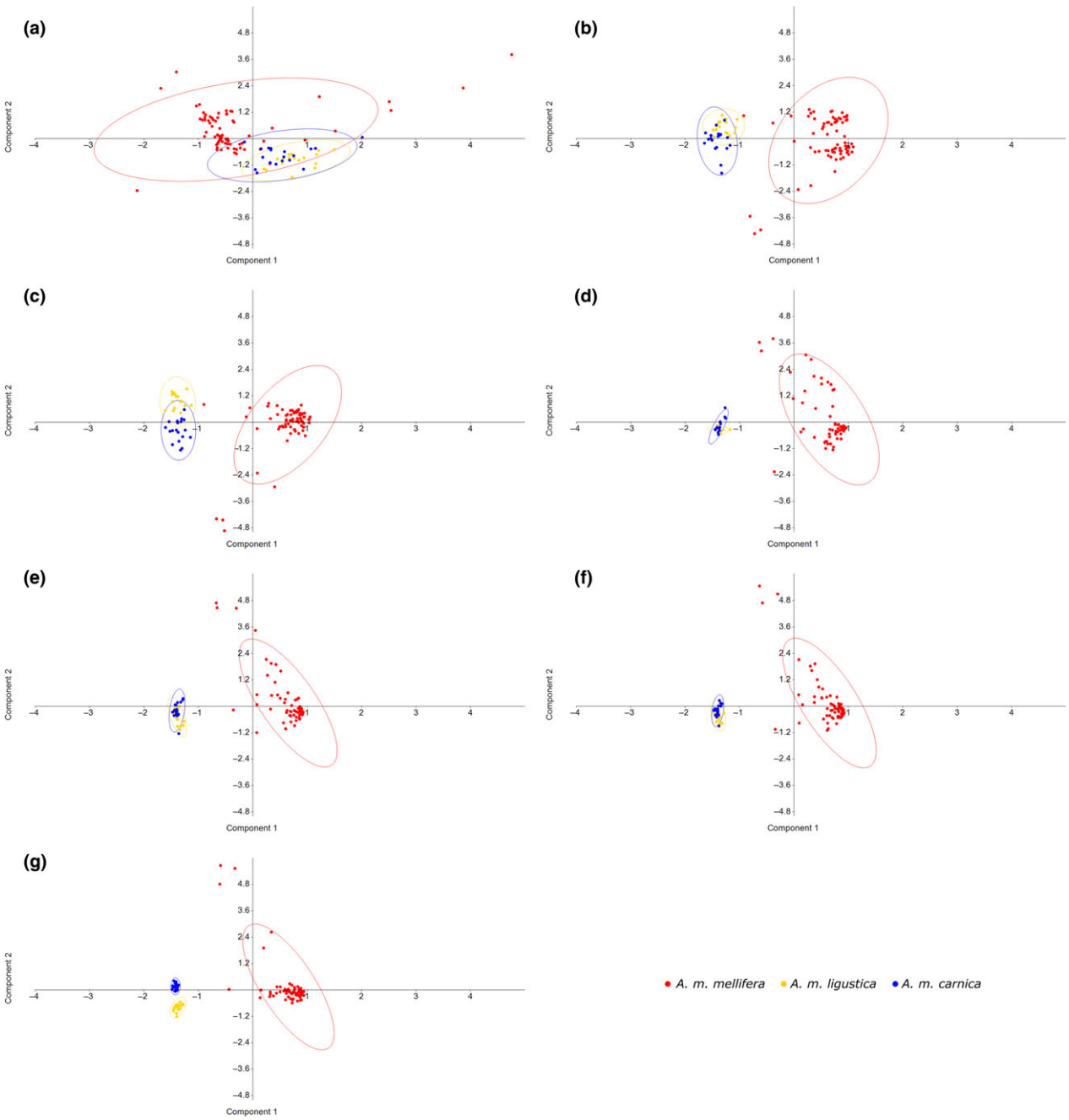

Fig. 2 Principal component analysis (PCA) of (a) 11 microsatellites, (b) 55 closest SNPs, (c) 110 closest SNPs, (d) 48 AIMs, (e) 96 AIMs, (f) 144 AIMs and (g) reference SNP set (d-g taken from Muñoz *et al.* 2015 for comparison purposes). PCAs show the 77 individuals sampled in the native range of the M-lineage honeybee subspecies *A. m. mellifera* in western Europe (marked in red), the 36 individuals sampled in the native range of the C-lineage subspecies *A. m. ligustica* (marked in yellow) and *A. m. carnica* (marked in blue) in eastern Europe. [Colour figure can be viewed at wileyonlinelibrary.com]

Contrasting with microsatellites, all SNP sets were able to distinguish individuals of M-lineage ancestry and C-lineage ancestry, although the degree of overlap varied. A greater overlap was observed for the 55 and 110 closest SNP data sets (Fig. 2b,c) than for the three AIM sets (Fig. 2d–f), which showed a separation pattern more similar to that exhibited by the reference SNP data set (Fig. 2g). The first two PCA components estimated using the 11 microsatellite, the 55 and the 110 closest SNP data sets, and the reference SNP data set explained 53.15, 51.70, 49.95 and 49.80% of the total variances, respectively. Higher values were obtained for the 48, 96 and 144 AIM data sets with 77.50, 76.29 and 75.35% of the total variance explained, respectively.

Clustering analysis with STRUCTURE

Membership proportions (Q) were inferred from microsatellites and SNPs for the 113 honeybee individuals using STRUCTURE (Fig. S1, Supporting information). The ΔK method (Evanno *et al.* 2005) indicated that $K = 2$ was the most likely number of genetic clusters, for both markers and for all data sets tested (Fig. S2, Supporting information). The 36 individuals originating from the C-lineage distributional range in eastern Europe formed one tight cluster with Q-values estimated by the reference SNP and the 11 microsatellite sets all at or above 0.9490 and 0.9217, respectively (Table 2, Table S3, Supporting information). The other cluster contained the 77 individuals sampled from protected and unprotected apiaries across the M-lineage *A. m. mellifera* native range in western Europe. Consistent with a previous report (Pinto *et al.* 2014), these individuals exhibited a wide array of Q-values denoting variable levels of C-lineage introgression (Fig. S1, Table S3, Supporting information).

While estimates of introgression proportions (inferred from Q-values) for the 77 individuals varied across markers and data sets, differences among them were more pronounced for the upper than for the lower range of Q-values (Table S3, Supporting information). For example, the five uppermost Q-values estimated by the reference SNP data set (or the AIMs) ranged from 0.3400 to 0.6902, whereas those estimated by microsatellites ranged from 0.7543 to 0.9602. Q-value differences $>|0.10|$ between these data sets were exhibited by 20 individuals (9 positive and 11 negative values), of which 14 were among the 20 most introgressed (Fig. S3, Supporting information). Nonetheless, when mean introgression proportions (Table 2) were compared across marker types and data sets for the 77 individuals, none of the pairwise comparisons revealed to be significant (P-value ≥ 0.9972, Tukey test; Table S4, Supporting information), despite the higher dispersion of data points observed for microsatellites (SD = 0.2222, Table 2).

Precision and accuracy

Membership proportions in the *A. m. mellifera* cluster estimated by the different data sets were further examined using linear regression (Table 3, Fig. S4, Supporting information). When microsatellites were regressed against the SNP data sets, the highest regression coefficient was obtained for the 55 closest SNPs ($r^2 = 0.6986$, Table 3), which suggests that even though the five flanking SNPs are on average 330425.5 ± 263719.9 bp away from each of the 11 microsatellite loci, and considering the extremely high recombination rate in honeybees (Wallberg *et al.* 2015), they are capturing the same information as microsatellites. On the other hand, when microsatellites and the five reduced SNP data sets were regressed against the reference SNP data set, which with its 1183 loci represent the best genome coverage, the lowest regression coefficient was produced by the microsatellite data set ($r^2 = 0.6202$, Table 3).

Precision and accuracy in estimating C-derived introgression into *A. m. mellifera* varied between marker types and data sets (Fig. 3). The standard deviations of

Table 2 Statistics of Q-values inferred from STRUCTURE for the individuals sampled in the M-lineage *A. m. mellifera* native range in western Europe ($N = 77$) and in the C-lineage *A. m. ligustica* and *A. m. carnica* native range in eastern Europe ($N = 36$)

	M-lineage group			C-lineage group		
Set	Max.	Min.	Mean ± SD	Max.	Min.	Mean ± SD
11 STRs	0.9602	0.0090	0.1177 ± 0.2222	0.9910	0.9217	0.9762 ± 0.0205
55 SNPs	0.8185	0.0050	0.1239 ± 0.1921	0.9940	0.8885	0.9727 ± 0.0272
110 SNPs	0.7790	0.0030	0.1230 ± 0.1825	0.9950	0.8842	0.9768 ± 0.0291
48 AIMs	0.6765	0.0040	0.1129 ± 0.1573	0.9960	0.9315	0.9822 ± 0.0170
96 AIMs	0.6706	0.0020	0.1075 ± 0.1531	0.9980	0.9487	0.9763 ± 0.0161
144 AIMs	0.6592	0.0020	0.1077 ± 0.1462	0.9980	0.9452	0.9746 ± 0.0142
1183 SNPs	0.6902	0.0010	0.1131 ± 0.1496	0.9990	0.9490	0.9829 ± 0.0123

Set	Parameter	11 STRs	55 SNPs	110 SNPs	48 AIMs	96 AIMs	144 AIMs
11 STRs	Slope a	–	0.7225	0.6703	0.5355	0.5190	0.4961
	Intercept b	–	0.0388	0.0441	0.0498	0.0464	0.0492
	r^2	–	0.6986	0.6661	0.5723	0.5678	0.5684
1183 SNPs	Slope a	0.5301	0.7392	0.7910	0.9259	0.9623	1.0103
	Intercept b	0.0507	0.0215	0.0158	0.0086	0.0097	0.0043
	r^2	0.6202	0.9014	0.9317	0.9484	0.9695	0.9758

Table 3 Parameters and coefficients obtained by the linear regression analysis of C-lineage introgression proportions in *A. m. mellifera*

Q-value differences were higher for microsatellites than for the five reduced SNP panels (Fig. 3a), which indicates that precision of the microsatellites was the lowest. When comparing among SNP data sets, the 55 and 110 closest SNPs provided less precise estimates than the AIMs, despite the identical number of loci included in the two groups of the reduced panels. Accuracy was high for the six sets, but the mean accuracy of microsatellites (91.84%) was lower than that provided by SNPs, which ranged from 95.17%, for the 55 closest SNPs, to 98.23%, for the 144 AIMs. It is noteworthy that at the individual level, microsatellite accuracy for the upper range of *Q*-values was highly variable and below 80% for 11 individuals, suggesting that mean values should be interpreted with caution (Fig. 3b). In summary, the SNP sets provided more accurate introgression estimates than the microsatellite set, especially when they were selected by their information content (Muñoz *et al.* 2015).

Discussion

Reliable molecular tools for detecting C-lineage introgression and genetic identification of pure-bred *A. m. mellifera* colonies are crucial to effectively manage conservation centres not only for restoring and preserving genetic identity and diversity but also for increasing adaptively important traits of this endangered honeybee subspecies. While the PCR-RFLP of the intergenic $tRNA^{leu}$-cox2 mitochondrial DNA region has proved to be a powerful and cost-effective tool for monitoring *A. m. mellifera* conservation centres in France (Bertrand *et al.* 2015), its maternal inheritance precludes identification of

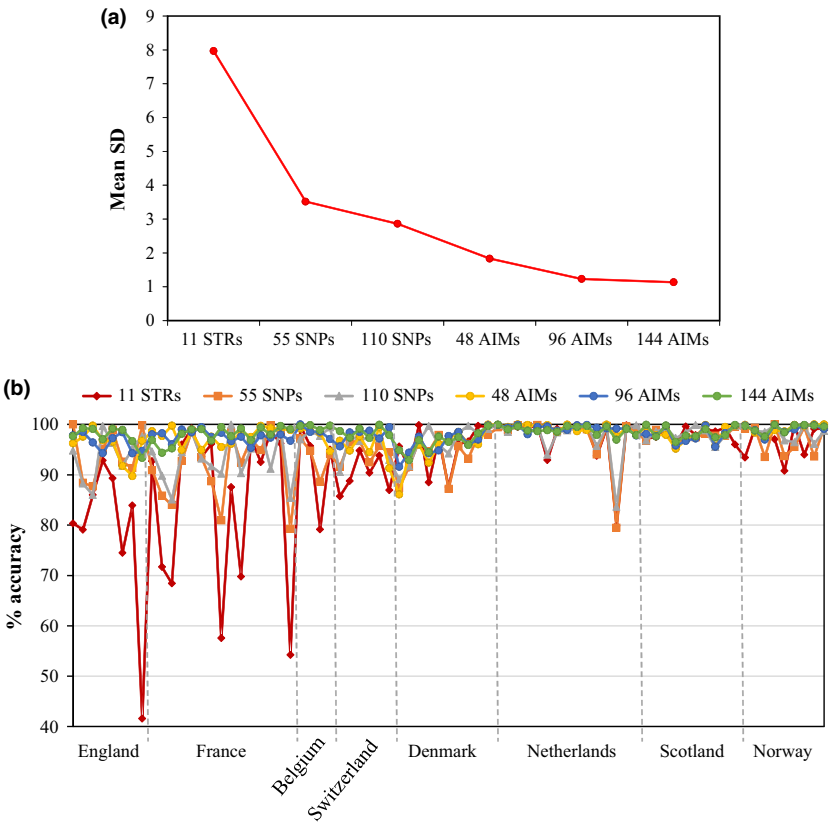

Fig. 3 (a) Precision estimates obtained from standard deviations (SD) of the differences between introgression inferred from the six sets in relation to the reference SNP set. The data set included the microsatellites (11 STRs) and the reduced SNP panels containing the 5 and 10 flanking SNPs of each microsatellite locus (55 and 110 closest SNPs) and the top-ranked AIMs (48, 96 and 144 AIMs). (b) Accuracy of the six data sets for each of the 77 *A. m. mellifera* individuals. Individuals are ordered as in Fig. S1 (Supporting information), which shows their introgression proportions. [Colour figure can be viewed at wileyonlinelibrary.com]

male-directed C-lineage introgression. Of the 77 *A. m. mellifera* colonies examined in this study, 76 carried haplotypes of M-lineage ancestry and one was a maternal descendant of a C-lineage colony (Pinto *et al.* 2014). However, these colonies exhibited variable levels of nuclear C-lineage introgression suggesting that a full identification of pure-bred *A. m. mellifera* requires biparentally inherited markers.

Microsatellites are still the most popular molecular tool for monitoring nuclear C-lineage introgression across *A. m. mellifera* conservation centres in Europe (L. Garnery, P. Kryger and G. Soland, pers. comm.). However, the benefits of using SNPs over microsatellites have been increasingly reported for many organisms (Väli *et al.* 2008, 2010; Glover *et al.* 2010; Hauser *et al.* 2011; Rašić *et al.* 2014), and honeybees are no exception. In this study, comparisons between different marker types and data sets showed that reduced sets of top-ranked informative SNPs (Muñoz *et al.* 2015) provide higher power in resolving the two highly divergent western (M) and eastern (C) European lineages, and are more accurate at estimating introgression proportions than microsatellites or their flanking SNPs (55 and 110 closest SNP sets). Our findings add to an increasing list of studies showing that SNPs outperform microsatellites in a variety of applications (Karlsson *et al.* 2007; Hauser *et al.* 2011; Gärke *et al.* 2012; Moore *et al.* 2014; Rašić *et al.* 2014; Puckett & Eggert 2016). This suggests that although biallelic SNPs provide lower information per locus than multiallelic microsatellites, the drawback can be offset by using a disproportionally larger number of randomly selected SNPs than microsatellites (Herráez *et al.* 2005; Liu *et al.* 2005; Narum *et al.* 2008; Hauser *et al.* 2011; Ciani *et al.* 2013; Rašić *et al.* 2014) or, instead, by using a reduced number of SNPs selected by information content (Rosenberg *et al.* 2003; Liu *et al.* 2005; Tokarska *et al.* 2009; Gärke *et al.* 2012; Hess *et al.* 2011; Oserov *et al.* 2013; this study).

Diversity estimates were lower for SNPs than for microsatellites (Table S1, Supporting information), which is expected given the disparity in the number of alleles per locus between the two markers (biallelic *versus* multiallelic). Global differentiation values obtained with the standardized G'_{ST} were identical across the two marker types (Table 1), although they were considerably higher for the reduced SNP data sets. This is an expected result given that the SNP loci of the reduced panels were selected by their highest resolution power for discriminating subspecies of the divergent M and C evolutionary lineages (Muñoz *et al.* 2015). The degree of differentiation between M and C lineages was high for both marker types and in the range reported by other honeybee studies (Whitfield *et al.* 2006; Harpur *et al.* 2012).

The PCA showed that subspecies partitioning differs substantially between the two markers (Fig. 2). Specifically, microsatellites exhibited lower clustering in the PCA space than SNPs, an unexpected result given their widely claimed higher power in detecting population clustering (Herráez *et al.* 2005; Liu *et al.* 2005; Livingstone *et al.* 2011; Ciani *et al.* 2013; DeFaveri *et al.* 2013; Ross *et al.* 2014). While the number of loci used here is in the range of other studies (Narum *et al.* 2008; Schopen *et al.* 2008; Glover *et al.*2010; Hauser *et al.* 2011; Hess *et al.* 2011; Livingstone *et al.* 2011), it is possible that the power of microsatellites was limited by the low genome coverage (only seven of the 16 honeybee chromosomes). However, the 55 and 110 closest SNP sets, which sample the same chromosomes, provided a lineage separation in the PCA space identical to that of the 144 AIMs, which are spread across the 16 chromosomes (Fig. 1). An alternative explanation for the poor lineage separation provided by microsatellites is homoplasy. Due to allele size constraints and high mutation rates, homoplasy is expected to occur relatively often in microsatellites, a problem that is aggravated with increasing divergence times (Kimura & Crow 1964; Estoup *et al.* 2002). Although geographically close, the two European lineages (M and C) are the most divergent among the four honeybee lineages (Garnery *et al.* 1992; Wallberg *et al.* 2014). Accordingly, it is possible that convergence of allele size has obscured lineage differentiation. This hypothesis is supported by a simulation study showing that for moderate to high levels of divergence, SNPs have generally greater power than microsatellites in detecting structure (Haasl & Payseur 2011).

While all reduced SNP data sets were able to separate the two lineages, *A. m. carnica* and *A. m. ligustica* could only be unambiguously distinguished by the 1183 SNP data set (Fig. 2g). This is not a surprising result given that the AIM panels were selected by their high discriminatory power in separating variation between and not within lineages. Furthermore, in accordance with the simulations of Haasl & Payseur (2011), as divergence time decreases, an exponential increase in the number of SNP loci is required for population separation. On the other hand, these authors found that in the presence of low levels of divergence, microsatellites may outperform SNPs, which was not the case here. It is possible that the 11 microsatellite loci were not sufficient to distinguish *A. m. carnica* from *A. m. ligustica*, as suggested by recent surveys using 25 microsatellite loci that resolved a number of C-lineage subspecies (Francis *et al.* 2014; Uzunov *et al.* 2014).

The results of STRUCTURE provided further insights into the relative performance of the two marker types. Although mean introgression proportions (inferred from *Q*-values) into *A. m. mellifera* estimated by microsatellites and SNPs were identical, at the individual level there were some major discrepancies, mostly in the upper

range of *Q*-values. The honeybee individuals 8 and 18 are two examples of highly skewed positive and negative microsatellite introgression estimates, respectively (Fig. S3, Supporting information). This finding has important implications for conservation programmes because decision-making works at the individual colony level; the decision of whether these two colonies would be maintained or removed from the protected population would be determined by the marker used for colony identification.

Precision and accuracy were lowest for microsatellites and highest for the SNP panel containing the largest number of top-ranked AIM loci. When comparing among SNP sets, both precision and accuracy were lowest for the two SNP sets flanking microsatellites, which indicate that SNPs carefully selected by their discriminatory power perform better than equivalent numbers of unselected SNPs. While mean accuracy was high across markers and data sets, at the individual level there was a trend showing lower accuracy at the upper range of *Q*-values, especially for microsatellites, suggesting that mean values can be misleading and are of little help for monitoring conservation programmes.

In summary, our results showed the superiority of SNPs in distinguishing the two European evolutionary lineages and estimating C-lineage introgression, especially when they are selected by their information content. These findings, together with high throughput, ease of analysis, transferability between laboratories, low genotyping error and low per locus genotyping cost (Vignal *et al.* 2002; Brumfield *et al.* 2003; Morin *et al.* 2004), make SNP markers more compliant to the test of tracking introgression, promising to supersede microsatellites in *A. m. mellifera* conservation programmes across Europe.

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M.A.P., D.H., I.M., P.D.L.R. and J.S.J. designed the project; L.J., M.A.P. and J.S.J. performed research and laboratory work; I.M., D.H. and J.C.-G. analysed the data; M.A.P. and I.M. prepared the manuscript with input from J.S.J and P.D.L.R.

Data accessibility

Microsatellite and SNP genotypes are deposited in Dryad: http://dx.doi.org/10.5061/dryad.5vp20.

Supporting Information

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

Table S1 Diversity measures estimated by the 11 microsatellites and the six different SNP data sets for each of three subspecies *A. m. mellifera*, *A. m. ligustica* and *A. m. carnica*. Na - mean number of alleles, Ne - effective number of alleles, uh - unbiased diversity, and Rs - allelic richness

Table S2 *P*-values obtained with the Tukey test for the comparisons of the diversity measures between marker types and data sets of the three subspecies *A. m. mellifera*, *A. m. ligustica* and *A. m. carnica*

Table S3 Membership proportions (*Q*-values) in the C-lineage cluster, inferred from STRUCTURE, for the 77 *A. m. mellifera* individuals sampled in western Europe (M-lineage) and for the 17 *A. m. ligustica* and 19 *A. m. carnica* individuals sampled in eastern Europe (C-lineage). Sampling locations of *A. m. mellifera* marked in bold represent unprotected apiaries; the remaining sampling locations represent apiaries under conservation management. The 113 individuals are ordered as in Fig. S1 (Supporting information)

Table S4 *P*-values obtained with the Tukey test for pairwise comparisons of C-lineage introgression proportions into *A. m. mellifera*

Fig. S1 Clusters identified by the Bayesian-based software STRUCTURE (Pritchard *et al.* 2000) for 77 individuals sampled in the native range of the M-lineage honeybee subspecies *A. m. mellifera* in western Europe and the 36 individuals sampled in the native range of the C-lineage subspecies *A. m. ligustica* (N = 17) and *A. m. carnica* (N = 19) in eastern Europe. Individual membership proportions (*Y*-axis) were inferred from **(a)** 11 microsatellites, **(b)** 55 closest SNPs, **(c)** 110 closest SNPs, **(d)** 48 AIMs, **(e)** 96 AIMs, **(f)** 144 AIMs, and **(g)** reference SNP data set. Each individual is represented by a bar, which is partitioned into two coloured segments that represent the individual's estimated membership proportions in K = 2 optimal number of clusters, as determined by the ΔK method (Evanno *et al.* 2005)

Fig. S2 Determination of the optimal number of genetic clusters (K) using the ΔK method (Evanno *et al.* 2005) for **(a)** 11 microsatellites, **(b)** 55 closest SNPs, **(c)** 110 closest SNPs, **(d)** 48 AIMs, **(e)** 96 AIMs, **(f)** 144 AIMs, and **(g)** reference SNP sets

Fig. S3 Differences of *Q*-values inferred from the six data sets in relation to those of the reference SNP set for each of the 77 individuals sampled in the native range of the M-lineage honeybee subspecies *A. m. mellifera* in western Europe (see *Q*-values in Table S3, Supporting information). The six data sets included the 11 microsatellites (11 STRs) and the reduced SNP panels containing the 5 and 10 flanking SNPs of each microsatellite locus (55 and 110 closest SNPs) and the top-ranked informative SNPs (48, 96, and 144 AIMs). Individuals (ID 1 to 77) are ordered as in Fig. S1 (Supporting information), which shows introgression proportions and geographical origin of the 77 individuals

Fig. S4 C-lineage introgression into *A. m. mellifera* inferred with STRUCTURE from the 11 microsatellites plotted as linear regressions against the **(a)** 55 closest SNPs, **(b)** 110 closest SNPs, **(c)** 48 AIMs, **(d)** 96 AIMs, **(e)** 144 AIMs, and **(f)** reference SNP sets; and from the reference SNPs against the **(g)** 55 closest SNPs, **(h)** 110 closest SNPs, **(i)** 48 AIMs, **(j)** 96 AIMs, **(k)** and 144 AIMs