Genética poblacional y filogenia de la hormiga esclavista *Rossomyrmex minuchae*

TESIS DOCTORAL

OLIVIA Mª SANLLORENTE BOLINCHES

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MEMORIA PRESENTADA POR LA LICENCIADA
OLIVIA Mª SANLLORENTE BOLINCHES
PARA OPTAR AL GRADO DE DOCTOR EN BIOLOGÍA

Fdo. Olivia Mª Sanllorente Bolinches

Directores de la tesis

Fdo. Dra. Francisca Ruano Díaz **Fdo. Dr. Alberto Tinaut Ranera**

Ad astra per aspera

A mi familia:
Mis padres, que desde que nací me han apoyado en todo
Juan Diego, que es mi vida
Mi hermano Tomás, lejos pero siempre ahí
Blas, Manoli y Bea, que me acogieron con cariño

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1. LAS ESPECIES RARAS

Definición

Según Rosenzweig y Lomolino (1997), se hablaría de rareza cuando existe un bajo número total de individuos de una especie, por tanto cuando hay una baja abundancia. Ampliando esta definición, la rareza estaría determinada por una baja densidad poblacional y una distribución geográfica restringida. Anteriormente, Gaston (1994) propuso ya una definición parecida basada en el uso del menor cuartil poblacional, aquel que contiene el 25% de las especies con las abundancias más bajas o los rangos de distribución poblacional menores. Otra definición quizá menos práctica, es aquella dada por Kunin y Gaston (1997) que consideran la rareza como el estado actual de un organismo, el cual por una combinación de factores biológicos y/o físicos está restringido en número o en área a un nivel inferior al de la mayoría de organismos de entidades taxonómicas comparables. Por tanto, se podría afirmar que no existe una definición universalmente aceptada de qué se considera una especie rara, aunque todas apuntan a un bajo número de individuos y una distribución limitada.

Por otra parte, se deben de separar las consideraciones de amenaza de las definiciones de rareza para una especie. Como se ha dicho anteriormente, según los parámetros biológicos, se favorece una definición de rareza basada en la abundancia y/o rango de distribución. Hay especies que por su historia evolutiva son raras pero en principio no tienen amenazada su supervivencia; sin embargo, las especies amenazadas implicarían casi siempre rareza porque han visto mermadas sus poblaciones. También se suele confundir rareza con endemicidad, siendo este último un término biogeográfico que no tiene porqué implicar baja densidad o una pequeña distribución geográfica (simplemente se es endémico de un lugar cuando sólo se está en dicho lugar). Por último, hay que tener en cuenta que muchas especies que son raras en ciertos lugares, son comunes en otros, mientras que otras especies son raras en cualquier lugar. Consecuentemente, se deben estudiar en profundidad la distribución de una especie y los factores que condicionan dicha distribución antes de considerarla rara.

Características

En general, las especies raras se diferencian de las especies abundantes porque suelen presentar algunas de las siguientes características (Kunin y Gaston 1997):

- Tendencia a practicar la autogamia y cruzamiento restringido
- Baja inversión en reproducción
- Menor capacidad de dispersión
- Niveles bajos de polimorfismo genético
- Poca capacidad competitiva
- Ocupación de un nicho ecológico pequeño y utilización de recursos ya de por sí poco abundantes
- Situadas en niveles tróficos altos, lo que redunda en una menor abundancia
- Presentan un tamaño corporal mayor y por tanto menor abundancia

Las especies raras van a presentar por tanto, unas dinámicas poblacionales particulares diferentes de las especies abundantes o comunes.

2. GENÉTICA POBLACIONAL DE LAS ESPECIES RARAS

La importancia del tamaño poblacional

Se considera que una población ideal es aquella que cumple fundamentalmente los siguientes requisitos (modificado de Hartl y Clark 1997; Frankham *et al* 2002):

- Los organismos son diploides
- Mismo número de machos que de hembras
- Todos los individuos con la misma probabilidad de contribuir en la descendencia de la siguiente generación (apareamiento aleatorio)
- Tamaño poblacional grande y constante (sin migración)
- Ausencia de mutación
- Generaciones no solapadas (discretas)

Sin embargo, en la naturaleza no existen poblaciones que se comporten como ideales, sino que lo normal es encontrar frecuencias alélicas muy diferentes de las estimadas dado un tamaño poblacional. Estas diferencias se deben fundamentalmente a que la deriva génica afecta como si hubiera un número inferior de individuos censados (Hartl y Clark 1997). Se ha constatado que los modelos se ajustan más a la realidad cuando se considera el tamaño efectivo de la población. Este concepto surge de la idea de que no todos los individuos tienen la misma probabilidad de contribuir con sus genes a la siguiente generación (Groom *et al* 2006). El tamaño efectivo poblacional (Ne) se define como el tamaño de la población ideal (N) que resultará en la misma cantidad de deriva génica, endogamia o pérdida de hererocigosis que la población actualmente considerada (Hartl y Clark 1997). Sin embargo, el tamaño efectivo poblacional no se considera un buen indicador de la pérdida de diversidad alélica dentro de las poblaciones, ya que por ejemplo, dos poblaciones con el mismo tamaño poblacional pueden sufrir un cuello de botella y perder diferentes cantidades de diversidad genética, o bien una haber sufrido un cuello de botella y la otra no y tener el mismo tamaño poblacional pero no la misma diversidad genética.

De acuerdo con Frankham (2005), el tamaño poblacional es el factor más influyente en las poblaciones pequeñas y el azar tiene un papel predominante, siendo los efectos de la selección mínimos e incluso nulos. Por el contrario, en poblaciones grandes la mutación, la selección y la migración son factores determinísticos mientras que los efectos del azar son mínimos. Las poblaciones pequeñas pierden diversidad genética en cada generación dado que la endogamia se vuelve inevitable, por lo que la respuesta de la selección frente a cambios en su ambiente, debería reducirse respecto a las grandes poblaciones. Esto se ha comprobado en numerosos trabajos sobre diversos grupos de seres vivos como las moscas de la fruta, ratones, gallinas o el maíz (Frankham *et al* 2002).

Según estos autores, las poblaciones pequeñas, las distribuidas en islas y las especies amenazadas suelen mostrar niveles muy bajos de diversidad genética. El nivel de diversidad va a variar según el proceso predominante (selección, mutación, migración…). La diversidad genética puede reducirse como consecuencia de un fuerte cuello de botella (drástica reducción del tamaño poblacional normalmente produciendo sus efectos en una sola generación) pero también debido al mantenimiento de un tamaño poblacional pequeño a lo largo del tiempo (efectos a lo largo de varias generaciones). Los cuellos de botella drásticos son bastante

infrecuentes mientras que una moderada restricción del tamaño poblacional es algo bastante común. La rapidez o lentitud en que ocurre esta pérdida de variabilidad también va a depender del tiempo de generación (siendo la pérdida más rápida cuanto más corto es el tiempo de generación). Además, un tamaño poblacional pequeño no sólo es perjudicial por las consecuencias genéticas adversas y el aumento del riesgo de extinción, sino también porque el proceso evolutivo en estas pequeñas poblaciones es muy diferente del de las grandes poblaciones, por lo que se debe de tener especial cuidado con estos problemas asociados al tamaño poblacional (Frankham *et al* 2002).

Finalmente, cuando el tamaño poblacional es reducido, el impacto de la varianza demográfica en la tasa de crecimiento poblacional aumenta y puede desembocar en un estocástico efecto Allee (Engen *et al* 2001). Allee (1949) observó que muchas especies sufrían un descenso en su tasa de crecimiento conforme sus poblaciones alcanzaban tamaños pequeños o bajas densidades. Los factores que determinan el efecto Allee son la endogamia (como pérdida de heterocigosidad), la demografía estocástica y la reducción en las interacciones cooperativas (Courchamp *et al* 1999).

Flujo génico y fragmentación

La capacidad de los individuos para dispersarse es un rasgo fundamental de la historia vital que modela la distribución de la variabilidad genética dentro y entre poblaciones, siendo crucial para asegurar la supervivencia de las mismas (Clémencet *et al* 2005). El flujo génico se define como el movimiento de genes de una población a otra (Groom *et al* 2006). El intercambio de genes entre poblaciones homogeniza las frecuencias alélicas entre dichas poblaciones y determina los efectos relativos de la selección y la deriva génica. Un elevado flujo génico excluye la adaptación local (fijación de alelos) y por tanto impedirá el proceso de especiación (Slatkin 1987; Hartl y Clark 1997; Groom *et al* 2006). Por otra parte, el flujo génico genera nuevo polimorfismo en las poblaciones e incrementa el tamaño poblacional efectivo local (es decir, la capacidad de resistir cambios aleatorios en las frecuencias alélicas), por lo que se opone a la deriva génica estocástica. Sin embargo, las poblaciones pequeñas y aisladas suelen presentar un escaso flujo génico, estando sujetas consecuentemente a la deriva génica y por tanto a la fijación de mutaciones deletéreas (Balloux y Lugon-Moulin 2002).

El flujo génico está relacionado con el tamaño poblacional. Todos los efectos causados por reducción del hábitat se vuelven más severos en el caso de hábitats fragmentados que en aquellos no fragmentados (Frankham *et al* 2002). La fragmentación del hábitat implica dos procesos: (1) una reducción en el área total del hábitat y (2) la creación de parches separados y aislados a partir de una distribución continua y mayor. Por tanto, la fragmentación del hábitat conduce a reducciones totales en los tamaños poblacionales de la mayoría de las especies y a una reducción en la migración (flujo génico) entre parches. El impacto de la fragmentación depende del nivel de flujo génico entre los fragmentos, lo cual a su vez depende de varios factores:

- Número de fragmentos poblacionales
- Distribución de los tamaños poblacionales en los fragmentos
- Patrón de distribución geográfica o espacial de las poblaciones
- Distancia entre fragmentos
- Capacidad de dispersión de los individuos
- Tasas de migración entre fragmentos
- Ambiente de la matriz entre los fragmentos y su impacto en la dispersión
- Tiempo desde la fragmentación

En un hábitat fragmentado, la migración restringida entre poblaciones, así como el tamaño poblacional potencialmente pequeño van a conducir a una elevada diferenciación genética entre dichas poblaciones (Clémencet *et al* 2005). Las diferencias en las frecuencias alélicas entre poblaciones fragmentadas son mayores en fragmentos pequeños que en grandes y ocurre más rápidamente. Los fragmentos poblacionales completamente aislados, por carecer de flujo génico, son las formas más severas de fragmentación y las más fáciles de estudiar. Esta forma de fragmentación guarda muchos paralelismos con poblaciones de islas oceánicas. Las poblaciones insulares difieren en sus alelos más o menos de una forma aleatoria. Esto se debe a que cuando una población se subdivide, los alelos individuales y los genotipos se distribuyen entre los fragmentos al azar; por tanto, dichos fragmentos serán genéticamente diferentes del que existía al inicio (Frankham *et al* 2002). Esta misma situación se da en las poblaciones de la alta montaña, que pueden ser consideradas como islas por estar generalmente igual de aisladas entre sí que las poblaciones insulares (Lomolino *et al* 2005).

En resumen, se considera que la fragmentación influye en la genética poblacional en dos pasos:

1. Fragmentación que resulta en una subdivisión genética inicial de una población.

2. Diversificación acumulativa por deriva génica y endogamia a lo largo del tiempo en cada uno de los fragmentos poblacionales.

Endogamia

La endogamia ocurre cuando individuos emparentados se aparean y se produce frecuentemente como consecuencia de un pequeño tamaño poblacional. Así pues, un individuo se considera endógamo si sus padres estaban más emparentados entre sí que dos individuos escogidos al azar. Una población en la que ocurre la endogamia presenta una frecuencia mayor de individuos homocigotos que una población con apareamiento aleatorio (Halliburton 2004). La endogamia tiene consecuencias deletéreas en todos los aspectos de la reproducción y la supervivencia, incluyendo la producción espermática, la capacidad de apareamiento, fecundidad de las hembras, supervivencia de los juveniles, cuidados maternales, edad de madurez sexual y supervivencia de adultos. A este fenómeno se le denomina depresión endogámica y tiene un impacto inmediato mientras que la pérdida de diversidad genética debida a otros factores, impacta a largo plazo, asociada a cambios ambientales (Frankham 2005).

Por otro lado, las poblaciones con apareamiento aleatorio a menudo presentan una enorme carga genética en forma de genes letales o deletéreos que se mantienen en los individuos heterocigotos (Kunin y Gaston 1997). Sin embargo, cuando las poblaciones han estado históricamente cruzadas al azar dentro de una situación de endogamia, estos alelos deletéreos han estado expuestos a la selección en los individuos homocigotos. Con el tiempo, la endogamia y la selección deberían purgar a la población de gran parte de su carga genética, especialmente de alelos letales, produciendo entonces menores niveles de depresión endogámica (Lacy 1992; Keller y Waller 2002; Halliburton 2004). En base a esto, hay que tener en cuenta que la depresión endogámica afectará más a poblaciones históricamente grandes que se han vuelto pequeñas recientemente (Groom *et al* 2006).

Existen una serie de parámetros estadísticos que ayudan a estimar el nivel de endogamia de una población. En primer lugar, el coeficiente de endogamia (f) mide la probabilidad de que dos alelos en un locus de un individuo sean idénticos por ascendencia (iguales porque descienden de un ancestro común en alguna generación anterior). A su vez, existen una serie de coeficientes de endogamia derivados (Wright 1951) para medir la distribución de la variación genética dentro de una especie como la desviación del equilibrio Hardy-Weinberg: F_{IS} como desviación del equilibrio debido al apareamiento no aleatorio dentro de cada población, F_{ST} para medir la diferencias alélicas entre poblaciones dentro de especie (diferencias debidas a una subdivisión) y F_{IT} como desviación del equilibrio considerando las poblaciones en su conjunto.

Cuellos de botella

Los cuellos de botella son procesos que implican reducciones drásticas en el tamaño poblacional y que resultan en pérdidas de heterocigosis, diversidad alélica y polimorfismo. Sin embargo, la conclusión de que una población tiene baja diversidad porque ha experimentado uno o más cuellos de botella es inferencial (Cornuet y Luikart 1996; Dinerstein y McCracken 1990). Los análisis genéticos se realizan después de que ocurra la reducción poblacional, por tanto causa y efecto entre baja diversidad y tamaño poblacional pequeño no se ha demostrado. Una baja diversidad en el presente no indica cuanta diversidad tenía la población en el pasado. Actualmente hay controversia en si se ha sobrevalorado el efecto de los cuellos de botella en la literatura. Los cuellos de botella deben ser muy pequeños y repetidos en el tiempo o bien mantenerse durante varias generaciones para que se produzca una erosión importante en la heterocigosis.

Para estar seguros de que ha ocurrido un evento de cuello de botella, deben conocerse los tamaños poblacionales históricos (lo cual no suele ser posible) por lo que los métodos de estima en ausencia de estos datos serían muy útiles (Cornuet y Luikart 1996; Luikart *et al* 1998). Normalmente, se recurre a las frecuencias alélicas, ya que métodos como los de ADN microsatélite permiten trabajar con niveles de polimorfismo fácilmente detectables. Los alelos raros (los que aparecen en baja frecuencia) se pierden rápidamente en los cuellos de botella y especialmente en poblaciones pequeñas, lo que podría explicar niveles de diversidad alélica

bajos (Cornuet y Luikart 1996; Luikart *et al* 1998). Estos alelos raros contribuyen poco a la variación genética total, sin embargo, podrían ser importantes en determinadas situaciones de cambio en las condiciones naturales de las poblaciones. Por tanto, cuando ocurre un cuello de botella en una población y el tamaño poblacional está reducido significativamente, la diversidad de alelos (especialmente los de baja frecuencia) se reduce más rápidamente que la heterocigosis. Recuperar la variabilidad genética y heterocigosis de una población que ha sufrido un cuello de botella es un proceso muy largo y probablemente imposible de conseguir (Halliburton 2004).

Deriva génica

La deriva génica es el cambio aleatorio en las frecuencias alélicas entre generaciones debidas al error de muestreo. Es decir, el número finito de genes transmitidos a la descendencia será una muestra imperfecta de las frecuencias alélicas presentes en los progenitores. Como resultado, se produce una pérdida de variación genética (Hartl y Clark 1997). La deriva génica es un ejemplo de un proceso estocástico en el que no se puede predecir el resultado porque está afectado por elementos aleatorios. Incluso dentro de una población muy grande, el destino de un alelo raro estará determinado por la deriva génica. Aún así, cuanto más pequeña es una población, mayores son los cambios en las frecuencias alélicas debido a la deriva génica, produciendo una mayor pérdida en la variación genética (aumento de homocigosis), reduciendo su eficacia biológica y su potencial evolutivo (Halliburton 2004; Frankham *et al* 2002).

La heterocigosis en un locus con dos alelos es máxima cuando los dos alelos tienen la misma frecuencia (0.5). La frecuencia de un alelo particular tiene la misma probabilidad de aumentar que de disminuir por deriva; por tanto, la heterocigosis aumentará si la frecuencia alélica tiende a 0.5 y descenderá si tiende a 0 o a 1. Se espera que la deriva génica produzca una pérdida de variación genética de generación en generación y en última instancia provocará que se pierdan todos los alelos salvo uno, que permanecerá fijado (Frankham *et al* 2002).

3. LA CONSERVACIÓN DE LAS ESPECIES RARAS

Las especies raras han atraído la atención de los conservacionistas en los últimos tiempos. A menudo se sabe muy poco de las especies raras y se toman medidas de manejo sin la información adecuada. Es una práctica habitual el utilizar datos de otras especies bien conocidas emparentadas con las especies raras para el manejo de estas últimas. Esta práctica introduce una fuente potencial de errores ya que no hay dos especies idénticas. Sin embargo, hay veces que estos datos son los únicos disponibles y por tanto son indispensables. Es esencial conocer los patrones de diferencias entre las especies raras y las abundantes para poder corregir este sesgo a la hora de aplicar un plan de manejo a una especie rara (Kunin y Gaston 1997).

De acuerdo con los anteriores autores, ciertas especies son raras debido a alguna de estas circunstancias:

- Son intolerantes a ciertos factores ambientales
- Ocupan un nicho ecológico extremo
- Dependen de un hábitat raro a causa de cambios climáticos
- Pertenecen a un taxón con alta selectividad de hábitat
- Son producto de especiación centrífuga
- Están influenciadas por dinámicas caóticas
- Son vulnerables a nuevas interacciones competitivas o de explotación

Hay especies que siempre han sido raras. Es posible que una especie con una larga historia evolutiva de rareza se haya hecho menos vulnerable a la extinción. Sin embargo, otras especies son raras desde hace poco tiempo y estarían en mayor riesgo que las que históricamente han sido raras. Si se confirman estas diferencias, los esfuerzos de conservación deberían concentrarse en las especies raras recientes, sobre todo aquellas que lo son por causas antropogénicas (Kunin y Gaston 1997).

En relación a esto, la UICN (Unión Internacional para la Conservación de la Naturaleza) ha definido unos criterios para clasificar las especies según el grado de amenaza para su supervivencia. Las cuatro categorías existentes son: especies críticamente amenazadas,

amenazadas, vulnerables y en bajo riesgo. Estas categorías están basadas en los principios de la biología de poblaciones ampliamente desarrolladas por Mace y Lande (1991), los cuales definieron que una especie amenazada es aquella con un elevado riesgo de extinción dentro de un intervalo de tiempo pequeño. Por ejemplo, una especie críticamente amenazada tiene un riesgo de extinción del 50% en 10 años o en tres generaciones, según sea más largo (Frankham *et al* 2002).

Según este sistema revisado, es poco probable que una especie rara se considere como amenazada a menos que haya observaciones de un declive continuado en sus poblaciones o razones de peso que evidencien un futuro declive. Sólo las especies con tamaños poblacionales totales muy pequeños (menos de 1000 individuos adultos y sexualmente maduros) pueden ser listados como amenazados si no hay una probabilidad de declive. Las especies de las que únicamente se conozca que presentan una distribución geográfica muy restringida solo pueden ser catalogadas como las de menor rango de amenaza (vulnerable). Esto se debe a que no hay una evidencia clara de descenso en la abundancia o rango de distribución. Una vez se compruebe este nivel de amenaza, se podrá aumentar la categoría de amenaza (Kunin y Gaston 1997).

Un factor que puede confundir en este punto es cuando las poblaciones han sido siempre raras y por tanto estarían en menor riesgo de extinción que aquellas que han sido reducidas recientemente. Por tanto, la rareza por sí sola no es suficiente predictor de la probabilidad de extinción de una especie (Gaston 1994). Además, las poblaciones pueden tener baja abundancia o pequeño rango de distribución por causas de muestreo: por estar censadas en un momento y lugar donde están en recesión de una anterior situación de normalidad o bien por estar al borde de su rango geográfico y por tanto ocupando hábitats subóptimos. (Kunin y Gaston 1997).

Mantener el potencial evolutivo de las especies amenazadas es un objetivo fundamental en la biología conservacionista. Los niveles de heterocigosis están relacionados directamente con la reducción en la fitness poblacional vía depresión endogámica. De media, las especies amenazadas tienen solo un 60% de la heterocigosis alélica que presentan sus parientes cercanos con poblaciones grandes y alrededor de la mitad del número de alelos (Frankham *et al* 2002).

Los biólogos conservacionistas coinciden en que los cuellos de botella poblacionales deben ser evitados en especies amenazadas ya que aumentan las tasas de endogamia, pérdida de variación genética y fijación de alelos ligeramente deletéreos (Cornuet y Luikart 1996). La teoría de la genética de poblaciones predice que la subdivisión de una población históricamente continua resultará en la divergencia a través de la pérdida de variabilidad genética debido a la deriva génica. A medida que el tamaño poblacional decrece, otros factores genéticos y/o ambientales podrían eventualmente conducir a las poblaciones individuales a la extinción (Frankham *et al* 2002). Por ello, es importante comprobar la variación genética en las poblaciones actuales para poder estudiar la viabilidad de cada una de ellas y para estimar la cantidad de variación genética presente en el conjunto de la especie (MacAvoy *et al* 2007).

4. IMPLICACIONES EN LOS INSECTOS SOCIALES

La vida en sociedad puede afectar al nivel de amenaza de las especies de insectos sociales por su biología y genética poblacionales particulares (Chapman y Bourke 2001). En una colonia, la reproducción la llevan a cabo una o unas pocas hembras emparentadas entre sí y fecundadas por uno o pocos machos, siendo la mayoría de los individuos de la colonia estériles. Esto implica que el tamaño efectivo poblacional podría ser menor que el número total de individuos de dicha colonia por varios órdenes de magnitud.

Por otro lado, los insectos sociales pertenecientes al orden Himenópteros presentan haplodiploidía, siendo los machos haploides (producidos por huevos sin fertilizar) y las hembras diploides (procedentes de huevos fertilizados). La determinación del sexo parece debida a un solo locus multialélico; los individuos heterocigotos para este locus resultan en hembras, mientras que los homocigotos y hemicigotos se desarrollan en machos. Los machos homocigotos para el gen determinante del sexo suponen una carga genética al producir esperma diploide y descendencia triploide (Crozier y Pamilo 1996). Por tanto, una consecuencia genética de un tamaño efectivo poblacional pequeño es la producción de machos diploides (homocigotos para el locus determinante del sexo). Esto ocurre cuando una hembra copula con un macho que comparte con ella algún alelo del gen para la determinación sexual. Dado que los machos diploides son estériles y no realizan ningún trabajo beneficioso

para la colonia, provocan un impacto negativo en la supervivencia y crecimiento de la misma (Pamilo y Crozier 1997; Cook y Crozier 1995).

El tamaño efectivo poblacional en especies haplodiploides es equivalente a las tres cuartas partes de especies diploides para el mismo tamaño total, con lo que presentan menor diversidad genética que los organismos diploides (Hedrick y Parker 1997). Se ha constatado la existencia de una relación inversa entre la pérdida de diversidad genética y la ploidía, siendo la probabilidad de pérdida de diversidad en especies haplodiploides proporcionalmente mayor que en diploides (Frankham *et al* 2002). Estas diferencias se deben a que la tasa de pérdida de diversidad genética va a depender del número de copias de cada gen por individuo (Bever y Felber 1994).Además, las frecuencias de mutaciones deletéreas van a ser menores bajo selección natural y por tanto, presentarán menor depresión endogámica que las especies diploides. Todo ello determina que el manejo de especies haplodiploides requiere mayores tamaños poblacionales para mantener una proporción equivalente de diversidad genética que en diploides aunque haya menor preocupación por la depresión endogámica (Hedrick y Parker 1997).

A este respecto, en las especies monogínicas (las que presentan una reina por hormiguero) se puede considerar que el tamaño efectivo poblacional es igual al número de hormigueros, mientras que en las poligínicas (varias hembras por hormiguero), sería mayor, así como la heterocigosis. Por otro lado, la poliginia va a afectar a la tasa de dispersión, siendo menor que en monoginia. Esto es así puesto que en las especies poligínicas la dispersión está a menudo restringida al quedarse las reinas jóvenes en el hormiguero materno o formando nuevos por gemación en los alrededores, mientras que en las especies monogínicas, las hembras se suelen dispersar volando y fundan colonias independientes (Mäki-Petäys *et al* 2005).

5. EL CASO DE LA HORMIGA *ROSSOMYRMEX MINUCHAE*, PARÁSITA ESCLAVISTA

Rossomyrmex minuchae Tinaut, 1981 (Hymenoptera: Formicidae) es una hormiga esclavista endémica de las altas cumbres del Sureste de la Península Ibérica. Se localiza entre los 1900 y

los 2100 m de altitud en Sierra Nevada, Sierra de Gádor y Sierra de los Filabres. Su única especie hospedadora es *Proformica longiseta* Collingwood, 1978 (Hymenoptera: Formicidae), también endémica del sureste español, si bien sus poblaciones son más abundantes y su distribución altitudinal mayor (se encuentra entre los 1800 y los 2700m). Sin embargo, *R. minuchae* sólo aparece en las partes más inferiores de esta distribución, lo cual es más notable en Sierra Nevada, donde el rango de distribución de la hospedadora es mayor por la mayor altitud de dicha sierra pero sin embargo, la distribución en altitud de la parásita no aumenta. Este hecho parece señalar que la especie parásita tiene una cota altitudinal que coincide con las máximas altitudes de las sierras de Gádor y Filabres, pero no en el caso de Sierra Nevada. La densidad de sus hormigueros es muy baja, con una media de 0.002 nidos por m^2 (Ruano 2000; Zamora-Muñoz et al 2003). La especie se encuentra catalogada como vulnerable por la UICN y también aparece en el Libro Rojo de los Invertebrados Españoles (Galante y Verdú 2006).

Los hormigueros de *R. minuchae* se fundan de forma independiente, es decir, una hembra fecundada sale del hormiguero materno y se dirige sola a la búsqueda de un hormiguero hospedador ya existente, en donde se instalará e iniciará un nuevo hormiguero (Ruano 2000; Ruano *et al* 2005). Este proceso de toma de control de un hormiguero hospedador se denomina usurpación, ya que la hembra parásita acaba asumiendo el rol de la hembra o hembras hospedadoras. La usurpación en esta especie se consigue gracias a que las hembras poseen en su glándula de Dufour una sustancia altamente repelente para la especie hospedadora, lo que le facilita poder infiltrarse en el hormiguero sin ser atacada (Ruano *et al* 2005). Una vez dentro, la hembra parásita acabará con la vida de la/s hembra/s de *P. longiseta* (puesto que se trata de una especie poligínica) quedando ella sola como reina del hormiguero y única en producir descendencia. Las obreras de *P. longiseta* seguirán trabajando para ella como si nada hubiera cambiado: recolectando comida, aseándola, cuidando de su prole, etc.

Las obreras de *P. longiseta* tienen un periodo de actividad amplio, comienza en mayo y termina a finales de septiembre (Fernández-Escudero *et al* 1997). Sin embargo, las obreras de *R. minuchae*, no se dedican a las tareas propias de las obreras sino que su única función consiste en buscar otros hormigueros hospedadores para asaltarlos y así ir reponiendo mano de obra. De esta forma, las obreras parásitas tienen un periodo de actividad menor, apareciendo de junio hasta agosto (Ruano y Tinaut 1999). El asalto en esta especie es diferente al de la mayoría de hormigas esclavistas, en las que gracias al empleo de feromonas,

se forman columnas de obreras para atacar los nidos hospedadores y donde se originan violentos combates entre las especies implicadas (Talbot 1967; Topoff y Zimmerli 1991). Cuando una obrera de *Rossomyrmex* localiza un potencial nido hospedador, vuelve a su hormiguero y recluta a otra obrera a la que transporta hacia el nido a asaltar. Una vez enseñado el hormiguero objetivo, ambas vuelven al suyo y realizan el mismo proceso de transporte de otra obrera hacia el objetivo. El reclutamiento es un proceso exponencial que dura de media 80 minutos y acaba de forma brusca cuando se alcanza un número crítico de obreras parásitas (entre 60 y 90). En un momento dado y posiblemente ante una señal, todas las obreras empiezan a excavar alrededor del hormiguero y rápidamente empieza el asalto (Ruano y Tinaut 2004). Las obreras de *P. longiseta* del nido asaltado, se muestran muy nerviosas y tratan de huir del escenario, no habiéndose observado combates generalizados entre ellas en el exterior del nido asaltado (Ruano y Tinaut 1999). Alrededor de 24 horas después de que empezara el reclutamiento, empiezan a salir las *Rossomyrmex* del hormiguero asaltado con su botín, el cual consiste en pupas, larvas, huevos, pequeñas obreras hospedadoras e incluso gastros de aquellas huéspedes más grandes. El proceso de transporte del botín al nido materno lleva gran parte del día y hasta parte de los dos siguientes (Ruano y Tinaut 1999). Como consecuencia del asalto, el nido queda básicamente sin nueva generación de obreras al expoliarse de huevos y pupas, pero por otro lado, las bajas de obreras o incluso de algunas reinas parece que no impide que aproximadamente el 45% de los hormigueros asaltados puedan reemprender su actividad al año siguiente (Zamora-Muñoz et al 2003).

La baja agresividad de *P. longiseta* hacia su parásita se ha constatado que sólo se da en las poblaciones simpátridas, mientras que en aquellas zonas donde no se ha observado la presencia de *R. minuchae*, la reacción de las primeras es fuertemente agresiva hacia las últimas y finalmente siempre ganan las esclavistas (Zamora-Muñoz et al 2003). Los últimos estudios señalan que paralelamente a este diferente comportamiento entre *P. longiseta* simpátridas y alopátridas, existe una diferenciación a nivel de hidrocarburos cuticulares. Así, existen mayores diferencias entre las *Proformica* de las dos zonas que entre las simpátridas y las *Rossomyrmex.* Parece ser que esta similitud en los perfiles entre parásitas y potenciales hospedadoras sería beneficioso para estas últimas, ya que al reducirse la agresividad en los encuentros se favorece la supervivencia de los nidos asaltados (Errard *et al* 2006).

Finalmente, la biología reproductiva de *R. minuchae* (Ruano y Tinaut 2005) no es menos singular que su modo de vida, ya que presentan un comportamiento reproductor muy estereotipado. Los machos salen del hormiguero en primer lugar por la mañana (entre las 10 y las 12 horas) y vuelan. Posteriormente salen las hembras (de 12 a 15 horas), que se quedan cerca del nido materno esperando a que acudan machos. Este comportamiento de las hembras recuerda al típico síndrome de llamada de las hembras descrito por Hölldobler y Bartz (1985) en el cual, las hembras emiten una feromona sexual atrayente para los machos. En el caso de *Rossomyrmex*, no se ha podido comprobar dicha emisión de feromona, sino que se ha observado que las hembras permanecen a la espera hasta que llega un macho a copular o bien se alcanza la hora límite de espera, regresando al nido hasta el día siguiente en que volverán a salir. Si acuden machos, rápidamente localizan a las hembras y copulan con ellas, pudiendo copular un mismo macho con más de 10 hembras hermanas en la misma mañana. No parece existir ningún comportamiento de elección por parte de la hembra. Nada más copular, las hembras vuelven inmediatamente al hormiguero mientras que los machos se quedan en el exterior en espera de más hembras para copular. Pasado el horario de cópulas, las hembras que han copulado empiezan a salir para fundar su propio hormiguero (a partir de las 17 horas). Si hay machos cerca del nido, permanecen inmóviles ante la presencia de estas hembras y seguirán así hasta la hora de las cópulas del día siguiente.

Por tanto, esta forma de vida tan particular junto al hecho de que las dos poblaciones conocidas hasta 2004 de *Rossomyrmex minuchae* constaban de pocos hormigueros detectados (35) y que la producción de sexuados es ciertamente irregular (no todos los hormigueros producen sexuados cada año y si lo hacen es en bajo número; Ruano 2000; Ruano y Tinaut 2005), planteó dudas sobre la supervivencia de la especie. Concretamente, cabía la posibilidad de que estuviera atravesando un cuello de botella con la consiguiente pérdida de variabilidad genética y aumento de endogamia así como estar sufriendo un posible lastre genético en forma de machos diploides como consecuencia de esta situación. Así pues, se hizo urgente un análisis genético en profundidad para intentar explicar la actual situación de las poblaciones de esta hormiga tan escasa e intentar favorecer las condiciones óptimas para su conservación, que es lo que ha conducido a la realización de esta tesis.

6. GENERALIDADES DEL PARASITISMO SOCIAL

Antes de continuar, cabe señalar qué se entiende por parásito social, puesto que la especie objeto de estudio de esta tesis, *Rossomyrmex minuchae*, se ha dicho que es esclavista, una modalidad del parasitismo social. De acuerdo con Tinaut y Ruano (1999), se considera que existe parasitismo social cuando una sociedad de insectos vive a expensas de otra sociedad también de insectos pero de diferente especie. Por tanto, que la especie parásita así como la hospedadora sean sociales es requisito indispensable.

Dentro de esta definición, se pueden encontrar diversas modalidades, ampliamente descritas por Wilson (1971); aquí consideraremos dos grandes grupos:

1. Parasitismo temporal: ocurre cuando la especie parásita necesita de la hospedadora únicamente en el momento de fundar nuevas colonias. La reina parásita ocupa el lugar de la hospedadora (bien matándola o expulsándola) y su descendencia es cuidada por las obreras hospedadoras hasta que estas van desapareciendo de forma natural, quedando finalmente solo la reina y obreras parásitas.

2. Parasitismo permanente: a diferencia del anterior, en este caso la especie parásita depende de la hospedadora toda su vida. Existen dos tipos de parasitismo permanente: inquilinismo (la especie parásita suele carecer de casta obrera y la reina normalmente cohabita con la reina hospedadora) y esclavismo (como el parasitismo temporal pero las obreras hospedadoras o "esclavas" son repuestas mediante asaltos a otros hormigueros de la especie huésped llevados a cabo por las obreras parásitas o "esclavistas").

La vida social aparece en termitas, avispas, abejas y hormigas, sin embargo, el parasitismo social aquí definido solo se encuentra en algunas familias de los tres últimos grupos, todos ellos himenópteros. Dentro de esto, el esclavismo es exclusivo de las hormigas (Tinaut y Ruano 1999).

El parasitismo social aparece en varios momentos independientes de la evolución, lo cual nos indica que este modo de vida ha surgido como estrategia común bajo determinadas

circunstancias y no que a partir de un primer proceso se derivaron el resto de especies parásitas (Buschinger 1986). Una de las circunstancias que más pueden haber influido en ello es que la fundación de un nuevo hormiguero por parte de una solitaria reina es un proceso peligroso. Esto puede evitarse si la reina entra en una colonia ya formada de su misma especie y es capaz de dejar allí su descendencia; este comportamiento sería el origen de la poliginia (cuando varias hembras participan en la descendencia de una misma colonia). No en vano, se considera que la poliginia sería en realidad un autoparasitismo (Bolton 1986; Rüppel y Heinze 1999). A partir de este comportamiento, no es difícil que se dé el salto de que una hembra mate al resto y así ser la única en producir descendencia, conduciendo hacia la aparición de una especie parásita. Este argumento está fuertemente apoyado por el hecho de que muchas especies parásitas tienen hospedadoras poligínicas (Buschinger 1986) y que las relaciones filogenéticas entre especies parásitas y sus hospedadoras son a menudo muy cercanas, lo que se conoce como la regla de Emery (Emery 1909). La hormiga objeto de estudio de esta tesis, *R. minuchae*, se sabe que está próxima filogenéticamente a su hospedadora, *P. longiseta* (Hasegawa *et al* 2002), si bien su relación precisa respecto a *Cataglyphis* y la del resto del género *Proformica* permanece incierta. Por esta razón, en esta tesis se aborda también el estudio filogenético de numerosas especies de estos tres géneros: *Rossomyrmex, Cataglyphis y Proformica* para comprobar sus relaciones filogenéticas y con ello el origen de la aparición del parasitismo en este grupo.

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OBJETIVOS GENERALES

Puesto que la hormiga esclavista *Rossomyrmex minuchae* cumple varias premisas que ponen su supervivencia en entredicho, en esta tesis nos interesamos por los siguientes aspectos poblacionales, evolutivos y conservacionistas:

1. Estudio de diversos parámetros poblacionales en *R. minuchae* (diversidad genética, consanguinidad, heterocigosis,…) mediante el análisis de ADN microsatélite y mitocondrial.

2. Comparación de la genética poblacional de *R. minuchae* con la de *R. anatolicus* y *R. quandratinodum*, especies de amplia distribución en las llanuras de Turquía y Kazajstán, respectivamente.

3. Para cada especie de *Rossomyrmex,* estudiar paralelamente la estructura genética de las respectivas especies esclavizadas del género *Proformica*, así como otros parámetros (como los hidrocarburos cuticulares) de forma que podamos entender los mecanismos de coevolución que han tenido lugar entre ellas.

4. Establecer la filogenia del género *Rossomyrmex*, así como las relaciones filogenéticas entre las especies hospedadoras.

5. De acuerdo con los resultados obtenidos en los objetivos anteriores, determinar el grado de amenaza de *R. minuchae* y las posibles medidas de protección a adoptar.

CAPÍTULO 1

Insect Science en prensa

Nest composition and worker relatedness in three slave-making ants of the genus *Rossomyrmex* Arnoldi and their *Proformica* Ruzsky hosts (Hymenoptera, Formicidae)

En este artículo analizamos y comparamos la composición y arquitectura de los hormigueros así como el parentesco entre las obreras de tres especies de hormigas esclavistas: *Rossomyrmex anatolicus*, *R. minuchae* y *R. quandratinodum*. La estructura de las colonias es un carácter importante en las hormigas, especialmente en las sociedades mixtas en las que parásito y hospedador cohabitan un mismo hormiguero. También se aportan datos de sus respectivas hospedadoras de vida libre, *Proformica korbi, P. longiseta* y *P. sp*. Para nuestro estudio integramos un procedimiento de excavación meticuloso con uno genético. Concluimos que tanto el número medio de parásitas como de esclavas es particular a cada especie esclavista, mientras que la profundidad depende de la arquitectura del hormiguero hospedador. El género *Rossomyrmex* parece ser monogínico y monándrico mientras que *Proformica* muestra diferencias en el número de reinas y frecuencia de cópulas. *R. quandratinodum* presenta rasgos distintivos en la composición de los hormigueros (proporción hospedador/parásito: P/R) y arquitectura. Estas diferencias podrían deberse a diferentes niveles de parasitismo en el proceso de asalto o al intento de las hospedadoras de evitar ser parasitadas.

Nest composition and worker relatedness in three slave making ants of the genus *Rossomyrmex* Arnoldi and their *Proformica* Ruzsky hosts (Hymenoptera, Formicidae)

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ABSTRACT

In this paper, we analyse and compare nest composition and architecture as well as worker relatedness in three related species of slave-making ants: *Rossomyrmex anatolicus*, *R. minuchae*, and *R. quandratinodum*. Colony structure within nests is an important trait in ants, especially in the case of mixed societies: when host and parasite coexist in the same nest. Data for their respective free-living hosts: *Proformica korbi, P. longiseta and P. sp,* are also provided. For our study we integrated a meticulous excavation procedure with a genetic method. We conclude that the average number of parasites as well as of slaves is species-specific whereas nest depth depends more on nest architecture of the host. The genus *Rossomyrmex* seems to be monogynous and monandrous whereas *Proformica* shows differences in the number of queens and mating frequency. *R. quandratinodum* shows different traits in nest composition (host/parasite ratio: P/R) and architecture that probably point out to some differences in parasitism: raid process or avoidance of parasitism.

KEY WORDS: Nest composition, nest architecture, *Proformica*, relatedness, *Rossomyrmex*, slave-making ants.

INTRODUCTION

A basic aspect in the study of social insects is to know their colony structure and especially the number of queens, as this trait can be "like some anatomical features ....., shaped by multiple influences during evolution…" (Wilson 1993). Many ant species are monogynous (single queen colonies) while others are polygynous (several queens). In this sense, polygyny is considered to arise from monogyny through adoption of new queens in different ecological and physiological conditions (Goropashnaya *et al* 2001) such as nest site limitation or high risks associated with independent nest founding. Polygyny is also associated with weaker nestmate recognition than under monogyny (Bourke & Franks 1995). Thus, estimating worker-worker relatedness may approximate the average effective number of reproducing queens in colonies as well as mating frequency (Savolainen & Sëppa 1996). This method is helpful given that queens are often missing during excavation of nests and can mislead data. Moreover, other traits are fundamental for the knowledge of social structuring, such as the number of workers and the physical architecture of the nest, being both also modulated by multiple influences during evolution. The number of workers may be a direct effect of the number of queens (Elmes & Keller 1993) while the physical architecture of the nest is determined by environmental variables, but also both traits involve a major phylogenetic component common to a genus or related

genera, as these usually share similar nest architectures (Ruano & Tinaut 1993; Mikheyev & Tschinkel 2004) and composition (Baroni-Urbani *et al*. 1978; Baroni-Urbani & Pisarski 1978).

In the case of parasite-host societies in which two species, generally from different genera, live together in the same nest, the interest of knowing these traits has even more implications. Cohabitation would engender conflicts of interest given that in most cases obligated parasite workers do not perform colony maintenance tasks (Hölldobler & Wilson 1990). Also, they may present different preferences for a specific nest architecture because of a different phylogenetic constraint. Therefore, parasitized and free living nests could differ in composition and architecture.

The ants of the genus *Rossomyrmex* Arnoldi are slave-makers that invade nests of the genus *Proformica* Ruzsky to steal brood that then grow as slaves lacking their own queen (Arnoldi 1932; Ruano & Tinaut 1999). There are four species known in the genus *Rossomyrmex*: *R. proformicarum* Arnoldi from the Caspian steppes (Russia), *R. minuchae* Tinaut in Sierra Nevada (Spain), *R. quandratinodum* Xia and Zheng in the region of Urumchi (China) and *R. anatolicus* Tinaut from the Anatolian steppes (Turkey). The parasitic life, the short activity period, and the low number of raids per nest are assumed to occur in the whole genus (Marikosvky 1974, Ruano & Tinaut 1999, 2004). These traits together with the isolated and distant distribution areas of the genus make them very difficult to find and work with. Probably for this difficulty their biology and distribution are known only fragmentarily (Arnoldi 1932, Marikovsky 1974). In this later paper Marikovsky referred to *R. proformicarum* but we consider that these data must be applied to *R. quandratinodum* (Tinaut *et al.* 2008). More recently a considerable number of articles have been focused on different aspects of the biology and behaviour of *R. minuchae* (see Ruano & Tinaut references), being at present the best-known species of the genus. For *R. anatolicus*, only the aspects related to its taxonomy are known to date (Tinaut 2007).

In the present paper, we provide new data and analyse differences in nest composition (number of slaves and slave-makers), worker relatedness and inner nest architecture in *R. anatolicus*, *R. quandratinodum* and *R. minuchae* and also in the non parasitized nests of their respective *Proformica* host species. With relatedness estimates we would like to obtain information on colony structure that could have gone unnoticed by randomly excavation of nests and also to check the reliability of our excavating method for finding queens.

MATERIAL AND METHODS

Field methods

Five nests of *Rossomyrmex anatolicus* were excavated in May 2008 from two different populations in Anatolia (Turkey). The first corresponds to the type location: Belembaçi Beli (37º53'N, 32º22'E), northwest of Konya, where we collected three complete nests. The second comes from Ziyaret Tepesi (38º49'N, 36º54'E), in the province of Sivas where Schulz and Sanetra (2002) had mentioned the presence of *R. minuchae* but where we only found *R. anatolicus* after excavating two complete nests and another one partially (not included in this paper). Moreover, two free living nests of *Proformica korbi* (Emery), host of *R. anatolicus*, were excavated in Belembaçi Beli. For *R. quandratinodum* three nests were excavated in June 2007 located in the steppes between the Ily and the Charyn rivers (43º22'N, 49º01'E), about 200 Km Northeast from Almaty (Kazakhstan), near the Charyn canyon. Also, data of Marikovsky on four nests were included in this analysis (nests 1-4 in Table 1). In this region, another two free-living nests of the host species called *Proformica sp* were excavated. Seven nests of *R. minuchae* and another seven nests of free-living *P. longiseta* Collingwood studied here were located in Sierra Nevada (Granada, Spain; 37º07'N, 32º54'W) and excavated between 1993 and 1997.

The excavation procedure of the nest, similar to that described in Plaza and Tinaut (1989), consisted in making a deep rectangular trench of about 20 cm far from the entrance of the nest. From this position, we moved forward through the nest with uniform vertical cuts, in layers, so that we can easily detect over its length the main gallery and the chambers. This method allowed us to follow the main gallery to the end and therefore its chambers, giving us an expected high certainty that the nests have been excavated completely including the queen(s). The whole population, including the brood if existed, was collected with an aspirator. The entire procedure under standard conditions required about 1.5-2 hours. Collected ants were counted and installed in the laboratory or stored in ethanol at 99%.

Table 1. Colony structure, nest depth and worker relatedness of mixed colonies. Data of nests 1-4 of *R. quandratinodum* come from Marikovsky 1974, the rest are our own. – indicates the absence of queen. Relatedness for *R. minuchae* was calculated from 26 nests not included in the table.

	Locality	Queens	*Rossomyrmex* workers	*Proformica* workers	P/R ratio	Nest depth (cm)	*Rossomyrmex* Relatedness
R. minuchae	Spain						
Nest 1		--	116	537	4.63	--	--
Nest 2		1	142	414	2.92	40	--
Nest 3		1	64	387	6.05	--	--
Nest 4		1	122	803	6.58	42	--
Nest 5		1	235	2252	9.58	36	--
Nest 6		1	81	414	5.11	32	--
Nest 7		1	128	710	5.55	33	--
Mean ± SE		1	126.86 ± 20.77	788.14 ± 251.34	5.77 ± 0.77	36.60 ± 1.64	0.72 ± 0.10
R. quandratinodum	Kazakhstan						
Nest 1 (Marikovsky)		--	200	650	3.25	132	--
Nest 2 (Marikovsky)		--	23	65	2.83	120	--
Nest 3 (Marikovsky)		1	76	594	7.82	88	--
Nest 4 (Marikovsky)		1	53	84	1.58	48	--
K1N1		1	23	43	1.87	85	0.83
K1N2		--	15	21	1.4	50	0.40
K1N3		1	21	30	1.43	38	0.36
Mean ± SE		1	58.71 ± 24.97	212.43 ± 106.23	2.88 ± 0.87	80.14 ± 13.87	0.53 ± 0.13
R. anatolicus	Turkey						
T1N2		1	95	389	4.09	30	0.78
T1N3		1	133	893	6.71	43	0.75
T1N4		1	66	621	9.41	22	0.73
T2N1		1	50	362	7.24	29	0.84
T2N2		1	49	177	3.61	30	0.48
Mean ± SE		1	78.60 ± 15.94	488.40 ± 123.31	6.21 ± 1.07	30.80 ± 3.40	0.72 ± 0.03

Genetic analyses

Five to eight workers per colony were genetically analysed. Total DNA was extracted from workers using the Puregene DNA Isolation Kit (Gentra Systems). *Rossomyrmex* workers were genotyped at eleven microsatellite loci: Ccur11, Ccur46, Ccur63b, Ccur76, Ccur89, Ccur99, FE11, FE19, FE21, FE37 and FE51 (Pearcy *et al*. 2004; Gyllenstrand *et al.*. 2002). Proformica workers were genotyped at seven loci: Ccur11, Ccur26, Ccur63, Ccur76, FE19, FE37 and FL12 (Chapuisat 1996). Microsatellites were amplified by PCR in a 10µL reaction containing 2.5 ng of DNA, 0.2 nM of each primer, 0.25 nM of each dNTP, 1 x MBL buffer and 2.5mM of MgCl2 (overall), 0.5 u/µL of MBL Taq DNA Polimerase and 7 µL of distilled water. PCR conditions were 94ºC for 3 min to denature the samples, followed by 11 touch down temperature cycles of denaturing at 94ºC for 15 s, annealing at 55-45ºC for 30 s and extension at 72ºC for 30s, followed by 30 cycles at 94ºC for 15s, 50ºC for 15s and 72ºC for 30s. PCR products were genotyped at Unidad de genómica. Scai (University of Córdoba) using an ABI310 sequencer. We scored genotypes using GENESCAN software v 3.7. Genetic data of *R.*

minuchae and *P. longiseta* do not correspond to excavated nests but to more recent samplings in the same population (summers 2004-2007). Relatedness for *R. minuchae* was calculated over 26 nests and 10 nests were used for *P. longiseta*.

Statistical analyses

Most of the variables studied followed a normal distribution, nevertheless, because of the relative low number of samples (19 *Rossomyrmex* nests and 12 of *Proformica*) (Tables 1 and 2), we applied the Kruskal-Wallis test to detect differences in means, lately testing significance of differences between groups by mean of U-Mann-Whitney test. Spearman rank correlation was applied to test correlation among variables (STATVIEW 5.0). All measures are expressed in the text as mean ± SE. Worker nestmates relatedness was calculated weighting nests equally using the software RELATEDNESS 5.0 (Queller & Goodnight 1989). Standard errors were obtained by jackknifing over loci. Expected and observed values were compared with *t*-tests.

Table 2. Colony strucrure, nest depth and worker relatedness in free-living nests of *Proformica*. – indicates the absence of queen. Relatedness for *P. longiseta* was calculated from 10 nests not included in the table.

	Locality	Queens	Workers	Nest depth (cm)	Relatedness
Proformica longiseta	Spain				
Nest 1		1	478	30	--
Nest 2		3	565	16	--
Nest 3		4	316	45	--
Nest 4		1	470	42	--
Nest 5		4	453	48	--
Nest 6		1	622	55	--
Nest 7		--	69	55	--
Mean ± SE		2.33 ± 0.57	424.71 ± 69.46	41.57 ± 5.35	0.27 ± 0.02
Proformica sp	Kazakhstan				
PK1N4		1	359	130	0.66
PK1N2		--	218	110	0.69
Mean ± SE		1	288.5 ± 70.50	120 ± 10	0.67 ± 0.02
Proformica korbi	Turkey				
PT1N1		1	178	12	0.20
PT1N2		1	88	15	0.09
Mean ± SE		1	133 ± 45.00	13.50 ± 1.50	0.14 ± 0.05

RESULTS

Nest architecture

Nests of *Rossomyrmex* consisted in one entrance with several shallow galleries (1-5 cm deep), leading to a single vertical gallery with small lateral chambers. This vertical gallery ended in a final chamber, slightly larger than the others, in which the queen usually appeared. In relation to nest depth, Marikovsky provided no specific value for *R. quandratinodum (= R. proformicarum sensu* Marikovsky*)*, but his data on chamber distribution implied a depth of around 70-100 cm, what is consistent with the nests that we excavated in the same study area (Table 1). There were significant differences for nest depth among the *Rossomyrmex* species considered (Kruskal-Wallis Test P = 0.005), with *R. quandratinodum* being the species with significantly the deepest nests (U Mann-Whitney test vs *R. minuchae* P = 0.012; vs *R. anatolicus* P < 0.01), whereas no significant differences for this variable were found between *R. minuchae* and *R. anatolicus* (Fig. 1).

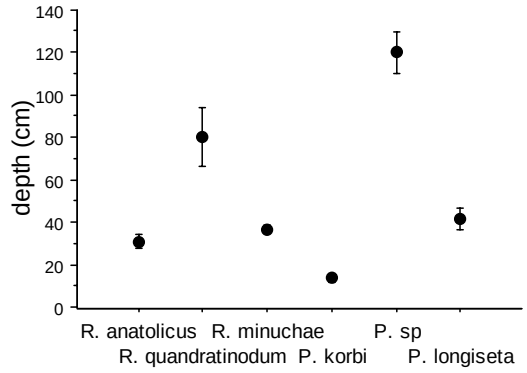

Figure 1. Depth of mixed and free-living nests (bars mean SE).

Nest architecture of free-living *Proformica* was similar to those nests parasitized by their corresponding *Rossomyrmex*. Overall, we found no significant differences in depth between parasitized and non parasitized nests (Kruskal-Wallis test; P > 0.5). We found significant differences in nest depth among *Proformica* species (Kruskal-Wallis test; P = 0.02) being *P. sp* the species with the deepest nests (120 ± 10 cm) and *P. korbi* (host of *R. anatolicus*) that with the shallowest (13.5 ± 1.5 cm). *P. longiseta,* host of *R. minuchae*, had intermediate nest depths (41.6 ± 5.3 cm) (U Mann-Whitney test *P. longiseta* vs *P. korbi and P. sp*, P = 0.04) (Table 2, Fig. 1).

Nest composition

In 15 *Rossomyrmex* nests one queen was found whereas in the others (4) there was no one (Table 1). The mean overall number of workers found in parasitized nests was 89.30 for *Rossomyrmex* and

497.15 for *Proformica*. Nests of *R. minuchae* were the most populated for parasites and slaves, followed by *R. anatolicus*. In *R. quandratinodum*, excavated nests generally consisted of a very low number of workers of both species, being similar only to the lowest data from Marikovsky (1974) (Table 1). We found significant differences in the number of workers in the nests of each *Rossomyrmex* species (Kruskal-Wallis test; P = 0.04). Significance is due principally to differences between *R. minuchae* and *R. quandratinodum* (U Mann-Whitney test P = 0.02), being *R. anatolicus* intermediate (Fig. 2). Nevertheless, the relationship between the number of *Proformica* slave and *Rossomyrmex* slave-maker workers (P/R ratio) significantly varied among species (Kruskal-Wallis test; P = 0.03). Again, means were similar between *R. anatolicus* and *R. minuchae* and both were significantly much higher than in *R. quandratinodum* (Fig. 3) (U Mann-Whitney test P = 0.03).

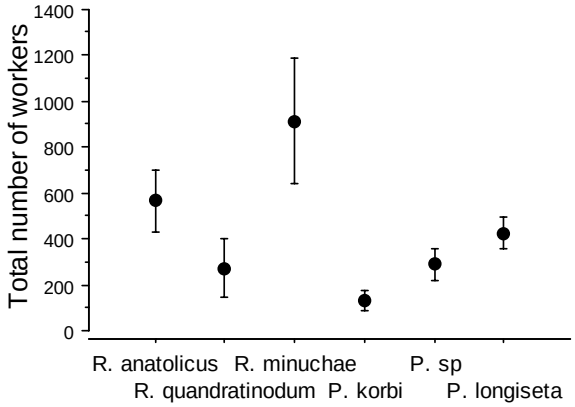

Figure 2. Number of workers in mixed and free-living nest (bars mean SE)

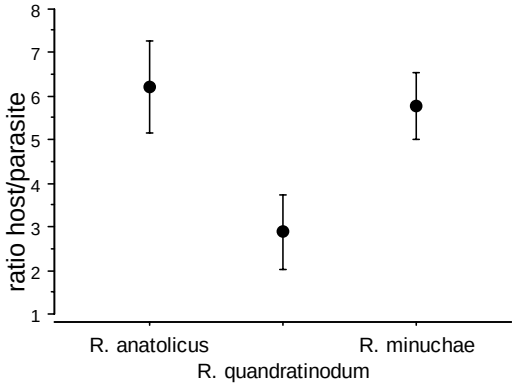

Figure 3. Mean ratio of *Proformica* and *Rossomyrmex* workers per nest (bars mean SE).

From the host species, and as a result of the excavation procedure, all but *P. longiseta* seemed to be monogynous (Table 2). *Proformica korbi* had the lowest number of workers and the highest corresponded to *P. longiseta* (Fig. 2). However, these differences were not significant (Kruskal-Wallis test P = 0.13).

Overall there was no correlation between the number of workers in a nest and its depth (Spearman rank correlation Rho = - 0.120, P = 0.53).

Worker relatedness

Intracolony worker relatedness was quite low in *R. quandratinodum* (Table 1) although did not differ significantly from the other *Rossomyrmex* values (Kruskal Wallis test, P > 0.05), meaning they were close to that expected under single mating by queens (0.75). Conversely, we found significant differences among the three host species (Kruskal Wallis test; P = 0.03). Obtained values of relatedness for *P. longiseta* (Table 2) are in accordance with polygyny (0.27 ± 0.02) as well as the observed number of queens in the nests (2.33 ± 0.56). *P. korbi* and *P. sp* nests had only one queen, what was reflected in a high value of relatedness not different from 0.75 just for *P. sp* (0.67 ± 0.02; t-test P = 0.19). In the case of *P. korbi* the low values (0.14 ± 0.05) were similar to *P. longiseta* (Mann-Whitney U test; P = 0.08) meaning that in fact nests must be polyandrous and even polygynous. *P. longiseta* had significantly lower levels of worker nestmate relatedness than monogynous and monoandrous *P. sp* (Mann-Whitney U test P = 0.03) although we found no significant differences between *P. korbi* and *P. sp* (Mann-Whitney U test P = 0.12).

DISCUSSION

Nest architecture

By comparing the general nest architecture and depth of mixed societies of *Rossomyrmex* and *Proformica* with monospecific nests of *Proformica*, we found that they do not differ significantly in each pair host-parasite, indicating that after the usurpation *Rossomyrmex* does not produce any change in nest architecture. The little effect of the parasite on the original nest architecture of the host differs from other slave makers like *Polyergus samurai* Yano, which presents larger nests than its unparasitized host (Tsuneoka 2008). Nest architecture in all cases fits to the little already known for *Rossomyrmex, Proformica* (Marikovsky 1974; Tinaut & Fernández Escudero 1993) and even for *Cataglyphis* (Plaza & Tinaut 1989; Ruano & Tinaut 1993) maybe as a consequence of their phylogenetic relationship (Hasegawa *et al*. 2002). In relation to nest depth, *R. anatolicus-P. korbi* have the shallowest nests whereas the deepest are clearly that of *R. quandratinodum-P. sp.* When this data is compared with the number of workers living in the nests of each species, it appears that nest depth is not directly related to colony size (apart from the minimum space for the whole colony to live in). So other factors apart from the number of workers must influence nest depth in these species (climate, soil structure, etc).

Nest composition

In a mixed society formed by a slave-maker species and its host, the number of parasite workers usually grows gradually, depending on the age of the nest, to reach a maximum determined by environmental factors but also by a phylogenetic component (see data on Baroni-Urbani *et al*. 1978; Baroni-Urbani & Pisarski 1978). However, in the case of enslaved workers that lack their own queen, their number will depend on intrinsic traits of the parasite species such as the number of raids per year and their magnitude. The number of slave workers could depend also on stochastic factors like the number of workers in the raided nests, their accessibility, and/or aggressiveness (Zamora *et al*. 2003). In addition, the number of ants in a sampled nest can vary depending on the moment of the excavation; by chance this could take place before or after an assault, so that worker numbers would vary markedly from one sampling date to another. In the case of the genus *Rossomyrmex*, although the activity period is more or less known (Arnoldi 1932, Marikovsky 1973; Ruano & Tinaut 1999), it is not for the number of raids per nest or the exact moment in the activity period (Ruano & Tinaut 1999). According to our results, the number of host and parasite workers is variable within a species. However, we found significant differences at the species level for the number of parasite workers in each nest and the proportion of slave and parasite workers. The host-parasite ratio (P/R) is not a fixed parameter, but quite variable instead (Table 1) and independent of total numbers. In the three species studied, the ratio is similar for *R. minuchae* and *R. anatolicus* (Fig. 3) but significantly lower in *R. quandratinodum*. However, there are no differences in the number of workers in free living nests for the three host species. Obtained values for the number of workers are similar to other slavemakers and free living hosts, such as *Polyergus lucidus* and its *Formica* hosts (King & Trager 2007) but much lower than for others like *Polyergus samurai* and its host *Formica japonica* (Tsuneoka 2008). The proportion of *Rossomyrmex* workers for the three species is similar to the slavemakers mentioned above and is related to their inability to perform worker tasks.

Our observations on *R. minuchae* indicate that raids are long (2-3 days) and what it is stolen depends basically on the population of the assaulted nest so that generally the whole brood is harvested. Thus, our results point that on average *R. quandratinodum* should show lower levels of raid efficiency given that its nests present significantly less enslaved workers than the other parasites but there are no differences when comparing free living nests for the number of workers. This can be due to two reasons: *R. quandratinodum* performs fewer raids per season otherwise *P. sp* can avoid raids more efficiently than the other *Proformica* species (escaping before the assault starts, pulling down the galleries, repelling the parasites, etc). Unfortunately, at present the exact circumstances of raiding behaviour remain unknown and further studies would be needed to shed light to it. Therefore our results would show different outcomes to the arms race as a result of the selective pressure of the slave-making ants on different host species.

Worker relatedness

According to relatedness estimates *R. minuchae* and *R. anatolicus* were as expected monogynous and generally monoandrous (Ruano & Tinaut 2005). The relatively low values of nestmate relatedness among workers in *R. quandratinodum* may be due to the occurrence of intraspecific raids (as we observed in the field while excavating one of the nests) or multiple mating by the queen because only one or no queen was found in all its nests (polygyny should therefore be excluded). The cases in which the queen was not found (4 nests) the reason was presumed to be because of accidental death or age of the nest. Intraspecific raids were also observed in *R. minuchae*, so we can assume this is a common trait to the genus and to other ants like *Pogonomyrmex* (Gadau *et al* 2003) or *Myrmecocystus* (Kronauer *et al* 2002).

Proformica longiseta was consistently polygynous and according to Fernandez-Escudero *et al* (2002), polyandrous. On the contrary, *P. sp* seems to be monogynous and apparently monoandrous. Data on *P. korbi* are misleading with one queen per nest but similar values of worker nestmates relatedness to *P. longiseta* but not significantly lower than *P. sp*; nevertheless polygyny and polyandry are presumed to occur in this species. Our results on *P. sp* are not surprising given that other species of *Proformica* are in fact monogynous, such as *P. ferreri* (unpublished data) or *P. nasuta* (Stumper 1957).

In general, our sampling method has proven to be quite reliable for the number of workers as well as for the queens at the moment of the excavation. In this sense, worker nestmate relatedness estimated from genetic data confirmed our sampling results in monogynous species.

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CAPÍTULO 2

Enviado a *Conservation Genetics*

Extreme population differentiation in a vulnerable slave-making ant with fragmented distribution

En biología de la conservación comprender los niveles de diferenciación poblacional y endogamia son importantes cuestiones, especialmente para los himenópteros sociales con tamaños poblacionales pequeños y fragmentados. Las poblaciones aisladas son más vulnerables a la pérdida genética y la extinción que aquellas que presentan distribuciones extensas y continuas. Sin embargo, las poblaciones pequeñas no siempre proceden de una dramática reducción de su hábitat. Por tanto, determinar el origen del aislamiento poblacional y el nivel actual de diversidad genética de una especie es crucial para su conservación. *Rossomyrmex minuchae* es una hormiga esclavista de distribución parcheada en el sureste español clasificada como vulnerable por la UICN. Por el contrario, las otras tres especies del género presumiblemente presentan distribuciones más uniformes. En este trabajo comparamos la diversidad genética y la estructura poblacional de *R. minuchae* con la de otras dos especies del género: *R. anatolicus* and *R. quandratinodum* y concluimos que aunque la diversidad genética de *R. minuchae* es baja, no hay evidencias de un reciente declive o cuello de botella, sugiriendo un proceso de fragmentación gradual y natural. También encontramos una diferenciación poblacional extrema para marcadores nucleares y mitocondriales, y aislamiento por distancia a escala local. A pesar de ciertas evidencias de endogamia y baja variación genética dentro de las poblaciones, no encontramos apenas machos diploides, el patrón esperado bajo condiciones de endogamia en himenópteros con determinación complementaria del sexo por un locus. Esto podría significar que el sexo está determinado por otro mecanismo y por tanto, los machos diploides no reflejarían el nivel de endogamia en esta especie. Basándonos en los datos genéticos disponibles, sostenemos que de continuar con un tamaño poblacional pequeño, los efectos negativos de la endogamia y la baja variabilidad genética son probables en el futuro. Sugerimos que se considere como opción de manejo una política de flujo génico artificial enfocada al incremento de la variación intrapoblacional.

EXTREME POPULATION DIFFERENTIATION IN A VULNERABLE SLAVEMAKING ANT WITH FRAGMENTED DISTRIBUTION

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ABSTRACT

Understanding levels of population differentiation and inbreeding are important issues in conservation biology, especially for social Hymenoptera with fragmented and small population sizes. Isolated populations are more vulnerable to genetic loss and extinction that those with extended continuous distributions. However, small populations do not always come from a dramatic reduction of their habitat. Thus, determining the origin of population isolation and current genetic variation of a species is crucial for its conservation. *Rossomyrmex minuchae* is a slave-making ant with patchy distribution in South Eastern Spain and is classified as vulnerable by the IUCN. In contrast, the other three known species of the genus are presumed to show more uniform distributions. In this work we compare the genetic diversity and population structure of *R. minuchae* with two other species of the genus: *R. anatolicus* and *R. quandratinodum* and conclude that although genetic diversity of *R. minuchae* is low, there is no evidence of a recent bottleneck, suggesting a gradual and natural fragmentation process. We also show extreme population differentiation at nuclear and mitochondrial markers, and isolation by distance at a local scale. Despite some evidence for inbreeding and low genetic variation within populations, we found almost no diploid males, the expected pattern under conditions of inbreeding in Hymenoptera with single locus complementary sex determination. This could mean that the sex is determined by another mechanism and thus diploid male frequency would not reflect the level of inbreeding in this species. On the basis of the genetic data available, we argue that continued low population size means that the detrimental effects of inbreeding and low genetic variation are likely in

the future. We suggest that a policy of artificial gene flow aimed at increasing within population variation is considered as a management option.

Key words: *Rossomyrmex minuchae*, slave-making ant, vulnerable species, population differentiation, fragmented versus continuous distribution.

INTRODUCTION

Terrestrial invertebrates are important components of ecosystems and their effective conservation raises issues distinct from those raised by vertebrates (Samways 1994, 2005; Thomas 1994; New 1995). Because invertebrates are not one homogeneous group, but a highly diverse group with enormous variation in life history and biology, their effective conservation requires a tailored approach. For example, social insects are arguably one of the most important invertebrate groups ecologically, and their ecological success is largely because they are social (Wilson 1971), but sociality can also be a problem when population size declines (Chapman and Bourke 2001). First, the limitation of reproduction to a few individuals per nest means there is a large disparity between census and effective population size. Thus, even though densities of individuals appear high the effective population size can be low. This can lead to an underestimation of demographic threats. Second, if sex is determined by a single locus complementary system (sl-CSD) (Cook and Crozier 1995; van Wilgenburg et al. 2006) a decrease in genetic variation will increase the likelihood of individuals being homozygous at the sex-determining locus with the effect that many diploid males are produced. As diploid males do not work and are generally sterile (Cook and Crozier 1995; Pamilo and Crozier 1997), their production damages colony productivity and increases extinction risk by an order of magnitude above that in inbred diploids (Zayed and Packer 2005). However, species with regular inbreeding may have alternative mechanisms of sex determination which lowers diploid male load (Crozier 1971; Beukeboom 1995).

Social parasites such as slave-makers rely on host species and are almost always rare relative to their host (but see Fischer-Blass et al. 2005). Because of this low density they are particularly susceptible to demographic and genetic problems associated with sociality. They are therefore important species to assess the impact of genetic threats to social insects as a whole. Furthermore, social insects at risk of extinction are often social parasites or slave-makers (http://www.iucnredlist.org) and so there are direct reasons for understanding more about their biology and ecology. Although slave-makers are not

'keystone' species in ecosystems, their highly specialized behaviour makes them worthy of conservation in their own right.

Rossomyrmex minuchae is a scarce slave-making ant species that invades nests of *Proformica longiseta* to steal brood that develop as slaves (Ruano and Tinaut 1999). Its conservation importance is for two reasons. First, it uses a unique method of slave-raiding, where slave-maker workers carry fellow workers to the target host nest rather than relying on chemical trail following (Ruano and Tinaut 1999). Second, it is endemic to south-western Spain and currently known from only three populations, each about 40-100 km apart (Sierra Nevada, Sierra de Gador and Sierra de Filabres; Ruano et al. 2007). The species is listed as vulnerable in the IUCN Red List of Threatened Species and as endangered in the Red Book of Spanish Invertebrates (Martínez-Ibáñez et al. 2006). In addition to this scarcity, there are other aspects of its biology that are likely to impact on genetic variation. Excavations suggest that *R. minuchae* nests are monogynous (Ruano and Tinaut 2005; Tinaut et al. in press) so that if queens mate singly each colony would contain only three haploid genomes. Furthermore, Ruano and Tinaut (2005) found that males are attracted by female calling but, uncommonly for ants, individual males were observed mating with multiple sister females. The result of this mating behaviour is that new nests would be more related than if each sister female mated with a different male. Finally, in a given year only about 35% of the nests produce sexual offspring (Ruano and Tinaut 2005), further lowering the effective population size and making it difficult for females to attract males (Ruano and Tinaut 2005). Together these suggest that *Rossomyrmex minuchae* may be particularly vulnerable to genetic threats and so serve as an important case study on the genetic effects of small population size in ants.

Our aim is to identify likely survival risks for *R. minuchae* populations. Specifically, we assess the degree of genetic differentiation between populations, investigate inbreeding and whether diploid males occur, estimate the effective mating frequency of queens, and examine evidence for recent bottlenecks. Based on these analyses we make conservation recommendations. To do so we collected genetic samples from all known populations. Our sample sizes are necessarily small but they are also a remarkably complete sampling of a rare ant, with all known nests of the species sampled. We also compared *R. minuchae* genetic diversity and population structure to two species of the same genus (*R. anatolicus* and *R. quandratinodum*). These species are found in the semi arid steppes of Central Asia (Anatolian desert in Turkey and Charyn plains in the east of Kazakhstan) and likely have more continuous distributions than *R. minuchae*. As species with isolated populations are more prone to genetic drift, inbreeding and extinction than those with extended continuous distributions (Frankham et al. 2002; Keller and Waller 2002), the Asian species should exhibit higher levels of genetic variation but lower population structuring than *R. minuchae*. All three *Rossomyrmex* species are monogynous and have similar mating and raiding behaviour (Marikovsky 1974; Ruano and Tinaut

1999; Tinaut et al. in press), so differences in population genetics cannot be simply due to variation in these life-history traits.

MATERIAL AND METHODS

Study areas and field work

A total of 64 nests (Table 1) were sampled during the summers of 2004, 2006 and 2007 from the three known populations of *Rossomyrmex minuchae*: Sierra de Gador (SG*: n*=30 nests), Sierra de los Filabres (SF: *n*=8 nests) and Sierra Nevada (SN: *n*=26 nests, Fig. 1). These populations are located in high mountains at 1900-2100 m a.s.l. and separated from each other by about 50Km. For all nests we recorded UTM coordinates using a hand-held GPS (Garmin) and calculated straight line distances between nests by trigonometry. Workers (mean/range=5.7/4-8 per nest; Table 1) were collected at nest entrances and stored in ethanol at 99%. Due to the short period of emergence of sexuals, during the course of our sampling only four nests in SN (the best monitored population) were detected producing sexual offspring; we sampled *n*=31 males (mean/range=7.8/7-8 per nest) from these nests that were stored frozen at -80ºC until later dissection and genotyping. We dissected all males to assess seminal vesicle status. Males were considered reproductive when no morphological abnormality was observed and if seminal vesicles occupied the length of the last abdominal segment.

Table1. Number of sampled nests (N), number of workers analysed, allelic richness for an equalized sample of 3 nests (A), gene diversity (H_S) and inbreeding coefficient (F_{IS}) of the three *Rossomyrmex* species studied. SE for F_{IS} was not reliable to calculate (see text).

Species	**Population**	**N**	**Workers**	**A ± SE**	**H_S ± SE**	**F_{IS}**
R. minuchae	SG	30	157	2.004 ± 0.337	0.366 ± 0.102	0.188
	SF	8	41	2.129 ± 0.235	0.446 ± 0.076	0.176
	SN	26	170	1.669 ± 0.210	0.254 ± 0.079	0.093
R. anatolicus	BB	4	32	2.458 ± 0.351	0.499 ± 0.303	0.069
	ZT	3	24	3.212 ± 0.335	0.684 ± 0.276	0.089
R. quandratinodum	CC	3	24	2.286 ± 0.227	0.486 ± 0.069	0.211

Two populations of *R. anatolicus* (Fig.1) separated by 425 Km were sampled in Turkey: Belembaçi beli, Konya province (BB; n=4) and Ziyaret tepesi, Sivas province (ZT; n=3). One nest was sampled in 2006 and the other six in 2008. For *R. quandratinodum* only three nests could be sampled in June 2007 in the South eastern plains of Kazakhstan (near the Charyn canyon, named CC) (Fig.1). For both species 8 workers per nest were analysed.

Fig. 1. Study areas: Spain (with three *Rossomyrmex minuchae* populations), Turkey (with two *R. anatolicus* populations) and Kazakhstan (one *R. quandratinodum* population).

Microsatellite genotyping and mitochondrial sequencing

Total DNA was extracted from workers using the Puregene DNA Isolation Kit (Gentra Systems). *Rossomyrmex* workers were genotyped at twelve microsatellite loci using primers from *Cataglyphis cursor*: Ccur11, Ccur26, Ccur46, Ccur63b, Ccur76, Ccur89 and Ccur99 (Pearcy et al. 2004), *Formica exsecta*: FE11, FE19, FE37 and FE51 (Gyllenstrand et al. 2002) and *Formica lugubris*: FL12 (Chapuisat 1996). Males were genotyped at a subset of five loci (Ccur11, Ccur46, Ccur63b, FE19 and FE51). All loci were amplified by PCR in a 10μL reaction containing about 2.5 ng of DNA, 0.2 nM of each primer, 0.25 nM of each dNTP, 1 x Qiagen buffer and 2.5mM of MgCl2 and 0.5 u of *Taq* (Qiagen). PCR conditions were an initial denaturing step of 94ºC, followed by 11 'touch-down' cycles of: 94ºC for 15s, 55-45ºC for 30s (-1 ºC per cycle) and 72ºC for 30s, followed by 30 cycles of: 94ºC for 15s, 50ºC for 15s and 72ºC for 30s. PCR products were sized using an ABI3100 sequencer and Genemapper software v 3.5.

We amplified about 791bp of mitochondrial cytochrome oxidase I (COI) from one randomly selected worker per nest of *R. minuchae* (*n*=64) using primers developed for *F. exsecta*: COI exsecta 2F: GGATCNCCAGANATNGCTTANCCTCG and COI exsecta 2R: TAATNGCAAAAACNGCTCCTA designed by B. Holzer (University of Lausanne) and the same reaction conditions and temperature profile used for microsatellites. PCR products were cleaned with QIAquick PCR purification kit (spin column format) before sequencing either by a commercial company (Microsynth, Switzerland or Macrogen, Korea) or by Servicio de Genómica (University of Córdoba, Spain). We aligned resulting sequences using ClustalW and investigated sequence differences with MEGA version 3.1 (Kumar et al. 2004).

Genetic diversity and population structure

Because workers within nests are related, and so not genetically independent, we constructed 1000 resampled datasets by randomly selecting 1 worker per nest using the program RESAMPIND (pers. comm. Jérôme Goudet). Each resampled dataset was tested for departure from Hardy-Weinberg equilibrium, genotypic disequilibrium (the independence of loci), gene diversity (H_S, the expected heterozigosity), allelic richness and allele frequencies with the program FSTAT 2.9.3.2 (Goudet 2002). All reported values of statistics, their standard errors and p values are averages over all resampled data sets. Allelic richness (with a standardised sample size of 3 nests per population) and H_S were compared among populations and species using STATISTICA 7. We tested for genetic differentiation among populations using *F*-statistics (Weir and Cockerham 1984) also with FSTAT. F_{IS} and F_{ST} were calculated over all populations in each species except F_{ST} for ***R. quandratinodum***, which was excluded from calculations because we could only sample one population. Pairwise F_{ST} was also estimated for all population pairs of ***R. minuchae***. Means and standard errors were jackknifed over loci and significant levels were determined by randomization (1000 times) of alleles for F_{IS} and genotypes for F_{ST}. In the case of pairwise F_{ST} 600 permutations were performed applying Bonferroni corrections. Standard errors for F_{IS} and pairwise F_{ST} are not provided by the program and an estimate of the 1000 resampled datasets would be meaningless (the greater the datasets, the smaller the error), so we do not give a value for this. As the range of possible F_{ST} values depends on the level of within population genetic variation, we calculated a standardized F'_{ST} (Hedrick 2005) with the program RecodeData v.0.1 (Meirmans 2006).

To investigate the genetic structure of ***R. minuchae*** at a local scale, we tested for isolation by distance among colonies within populations by regression of a matrix of genetic distances (F_{ST} /1- F_{ST}) against a matrix of geographical distances (Rousset 1997) with the program GENEPOP (Raymond and Rousset 1995) in its web implementation (version 3.3). Significance levels were analysed using Mantel tests (Mantel 1967) for each correlation using 10000 permutations.

Population bottlenecks

Also, in order to know whether the low density of nests within the isolated populations of ***R. minuchae*** was due to recent bottlenecks, we performed the qualitative graphical method (Luikart et al 1998), which requires no information on historical population size or historical levels of genetic variation.

Mating system

We explored the mating system of *Rossomyrmex minuchae* by inferring queen and mate genotypes from the worker offspring data and workers were assigned to patrilines using the software MATESOFT 1.0 (Moilanen et al. 2004). Given that we never found more than a single queen per nest during

excavations of *R. minuchae* (Ruano and Tinaut 1999; Tinaut et al. in press), whenever worker offspring did not fit to monogyny, colonies were excluded from further analyses because the presence of strange genotypes probably reflects intraspecific raids, a behaviour observed in this species (pers. obs.). We tested whether social structure was consistent with monandry (Ruano and Tinaut 2005; Tinaut et al. in press). If several queen genotypes were possible, we used a conservative approach and selected the queen genotype with the lowest number of patrilines that could explain workers genotypes. There were no colonies where worker offspring could not be assigned unambiguously to a patriline. We calculated the effective mating frequency according to Pamilo (1993) and the non-detection error, due to males sharing the same genotype according to Boomsma and Ratnieks (1996). Relatedness between queens and their mates was calculated with the programme RELATEDNESS 5.0 (Queller and Goodnight 1989) and, for cases where queens were not singly-mated, between males mated to the same queen. In the calculations we estimated allele frequencies separately for each population, weighted nests equally and estimated standard errors by jackknifing over loci.

Sex-biased dispersal

We tested for sex-biased dispersal in SN and SG, but not in SF because too few nests were sampled. Similar to our isolation by distance analysis, we tested for a correlation between pairwise kinship coefficients and geographic distance matrices using Mantel tests. Kinship coefficients (F_{ij}) were estimated for both queens and their mates using the program SPAGeDi (Hardy and Vekemans 2002) and analysed in separate Mantel tests. Where a queen was multiply mated we included all male genotypes and set their distance as zero. We chose F_{ij} as it is insensitive to ploidy or inbreeding and so suitable for comparing the spatial genetic structure of males and queens (Hardy et al. 2008). We conducted Mantel tests with 10000 permutations for significance levels in FSTAT.

Male ploidy and parentage

Males that were heterozygous at one locus or more were considered diploid. To estimate the probability of non detection of diploid males (i.e. diploid males homozygous at all loci) we determined the proportion of females having a homozygous genotype at all the loci genotyped in males.

We estimated the proportion of worker-produced males using genotype data with males classified as worker-produced if they carried at least one allele not present in their mother's genotype. The probability of detecting a worker produced male (N_a) was calculated as in Foster et al. (2001) and the percentage of total males produced by workers (WPM) as in Brunner et al. (2005).

All results are expressed as mean ± SE.

RESULTS

Genetic diversity

We found no significant deviation from Hardy-Weinberg expectations in any of the populations/species or evidence for associations between alleles at different loci. As shown in Tables 2 and 3, locus Ccur26 did not amplify in SG and SF populations of *R. minuchae* nor Ccur76 and FE11 in *R. anatolicus*. There was considerable differentiation between populations in *R. minuchae* with six loci being monomorphic in SN, three in SG and two in SF. SN was the population with the lowest percentage of private alleles (29.6%; Table 2). The percentage of private allele was intermediate in SF (37.5%) and the highest (56.4%) in SG. In *R. anatolicus* and *R. quandratinodum* only locus (FL12) was monomorphic and it was fixed for a different allele than in *R. minuchae*. Over all loci, *R. minuchae* and *R. anatolicus* shared 12 alleles, *R. minuchae* and *R. quandratinodum* 11 alleles and *R. anatolicus* and *R. quandratinodum* 10 alleles (Table 3).

Table2. Allele distribution among *R. minuchae* populations (number of sampled nests). Private alleles marked in bold. NA corresponds to not amplified locus.

Locus	**SG (30)**	**SN (26)**	**SF (8)**
Ccur11	**263, 265**	283, 285, 287, 289	**235, 253, 257**, 283, 285, 287, 289, **291, 293**
Ccur26	NA	139	NA
Ccur46	171, 173, **175, 179, 185, 187, 189**	**161**, 171, 173	**157**, 171
Ccur63	173**, 179, 181, 193,** 195, 197, 199	199, 201**, 203, 205**	173, **175, 191**, 195, 197,199, 201
Ccur76	164, 166	164	**154, 158**, 164, 166
Ccur89	**137**	**125**	**141**
Ccur99	114, 120	120	114, **118**, 120
FE11	285	289	285, 289
FE19	**213, 215,** 217, 219, **225, 227, 229**	219, 221, **223**	**211**, 217, 219, 221
FE37	**115**, 121, 123, **125**	119, 121	119, 121, 123
FE51	110, **112, 114, 116, 118**	**100, 102,** 104**, 106**, 110	**96**, 104, **108**, 110
FL12	99	99	99

Table3. Allele distribution among the three *Rossomyrmex* species (number of sampled nests). Private alleles marked in bold. NA corresponds to not amplified locus.

Locus	***R. minuchae* (64)**	***R. anatolicus* (7)**	***R. quandratinodum* (3)**
Ccur11	235, 253, 257, **263, 265, 283, 285, 287, 289, 291, 293**	**239, 243, 245, 247, 249, 269**	235, 253, 257
Ccur26	**139**	101, **107, 109, 111, 113, 115**	**97, 99**, 101
Ccur46	157, **161, 171, 173, 175, 179, 185, 187, 189**	151, **153, 155**, 157	**147, 149**, 151
Ccur63	173, 175, 179, 181, **191, 193, 195, 197, 199, 201, 205**	173, 175, **177,** 179, 181,**183**	**171**, 173
Ccur76	**154, 158**, 164, **166**	NA	164, **174, 184**
Ccur89	125, **137, 141**	117, 119, **121**, 125	117, 119, **123**
Ccur99	114, 118, 120	**96, 104**, 114, **116**, 118, 120, **122, 124**	**92, 98, 100,102**, 118
FE11	285, 289	NA	285, 289
FE19	211, 213, **215**, 217, 219, 221, **223, 225, 227, 229**	**187, 197, 203, 205, 207**, 213, 219, 221	211, 217, 219
FE37	**115, 119, 121, 123, 125**	**103, 105, 107**, 109, 111	109, 111
FE51	96, **100, 102, 104, 106, 108, 110, 112, 114, 116, 118**	88, **90, 92, 94**, 96	**86**, 88
FL12	**99**	89	89

The overall allelic richness (A) was 1.93 (± 0.23 SE) for *R. minuchae*, 2.84 (± 0.32 SE) for *R. anatolicus* and 2.29 (± 0.23 SE) for *R. quandratinodum* (Table 1). The mean allelic richness was similar in the three species (Kruskal-Wallis test, H = 4.89; P = 0.09) while *R. anatolicus* had a significant higher value than *R. minuchae* (LSD test, t = -2.33; P = 0.032). No significant differences in allelic richness were found among *R. minuchae* populations (ANOVA test, F = 0.80; P = 0.46) or between both *R. anatolicus* populations (t = -1.55; P = 0.14).

The mean gene diversity (H_S) in *R. minuchae* was 0.35 (± 0.08 SE), 0.657 (± 0.07 SE) in *R. anatolicus* and 0.49 (± 0.07 SE) in *R. quandratinodum* (Table 1). As for allelic richness, H_S in *R. anatolicus* was significantly higher than in *R. minuchae* and also than in *R. quandratinodum* (Kruskal-Wallis test, H = 8.30; P = 0.016). No significant differences in H_S were detected among the three *R. minuchae* populations (ANOVA test, F = 1.19; P = 0.32) and both *R. anatolicus* populations (Wilcoxon test, W = 61; P = 0.077).

Population structure

The high level of differentiation found for the distribution of alleles in *R. minuchae* was also reflected in other measures. The overall F_{ST} value was 0.605 (± 0.096 SE; P = 0.001) and pairwise estimates ranged from a maximum of 0.650 between SG and SN to 0.508 between SN and SG (Table 4). These values are very high given that the maximum value of F'_{ST}, which is equal to the mean within population level of homozygosity (Hedrick 2005), was 0.680.

Table4. Pairwise F_{ST} in *R. minuchae* (above diagonal). Significance from zero (below diagonal) is Bonferroni corrected * P < 0.05.

	SG	SF	SN
SG		0.5079547	0.6499504
SF	*		0.5243215
SN	*	*	

There was also some significant genetic differentiation at a local scale. In SG the genetic distance between colonies increased significantly with increasing geographic distance, providing clear evidence of isolation by distance (Mantel test, P = 0.002), whereas in SN the differentiation was not significant (Mantel test, P = 0.064). SF had insufficient sampled nests to permit a robust test.

In *R. anatolicus*, there was a moderate and not significant genetic differentiation between the two populations sampled (F_{ST} = 0.233 ± 0.090 SE; P = 0.068). None of the populations in any species showed significantly positive values of inbreeding (F_{IS} all tests P > 0.05) (Table 1). However, the

power of our analysis was relatively low because of limited sample sizes (in particular for *R. anatolicus* and *R. quandratinodum*).

Population bottlenecks

The analysis of allele frequency classes showed that for all three *R. minuchae* populations rare alleles (those with frequencies lower than 0.1) were the most common. This is contrary to the expected pattern for bottlenecked populations where alleles of intermediate frequency classes should be overrepresented as rare alleles are more likely to be lost during the bottleneck. There is therefore no evidence for a distortion in allele frequencies stemming from recent bottleneck in any of the studied populations (Figure 2).

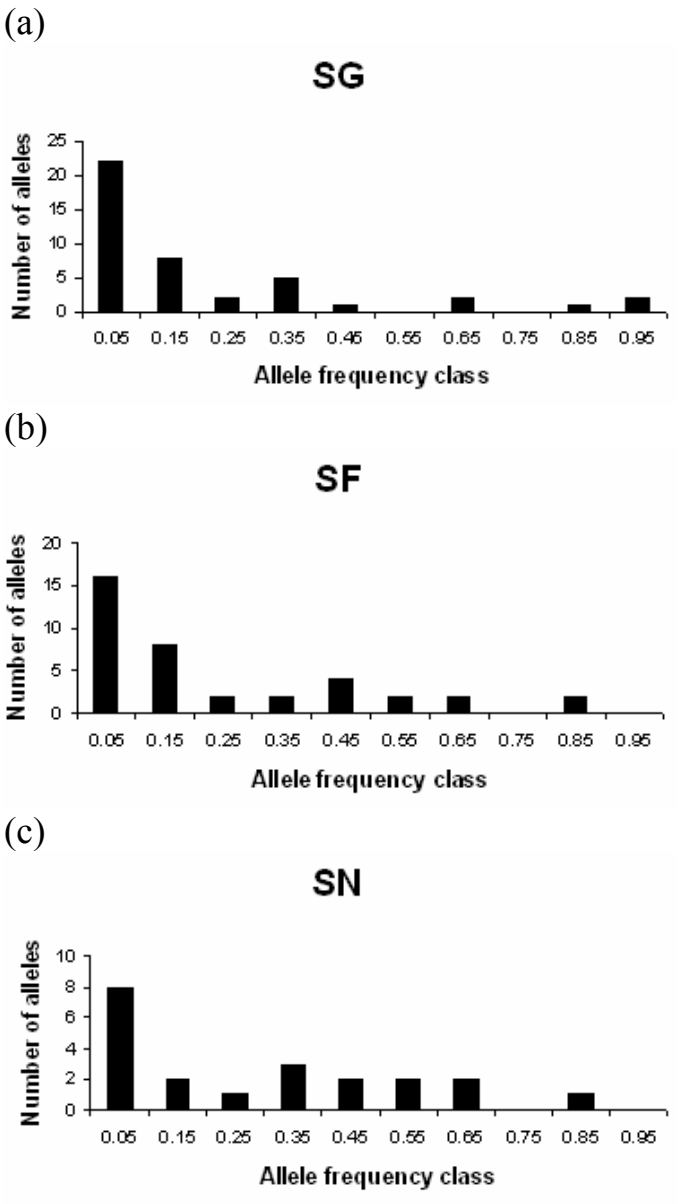

Fig. 2. Allele frequency distribution from the three known populations of *R. minuchae*: SG (a), SF (b) and SN (c).

Mitochondrial sequences

Alignment of 791 bp of COI from 64 *R. minuchae* workers revealed seven haplotypes, three in SG (GenBank accession numbers GU147935, GU147936 and GU181201), two in SN (accession nos. GU147937 and GU181204) and two in SF (accession nos. GU181202 and GU181203). Within populations, haplotypes differed only by one or two (in SG) base pairs but when comparing among populations, haplotypes were differentiated: 3.4% between SN and SG, 2.6% between SF and SG and 1.1% between SF and SN.

Mating system of R. minuchae

There was only one nest (in SF) in which worker genotypes violated the assumption of monogyny, suggesting that intraspecific raids are not common (1 out of 64 nests). In 51% of the nests queens were singly mated and in 38% they were doubly mated (Figure 3). Higher mating frequencies were found in only seven nests (3 in six nests and 5 in one nest). The effective mating frequency was similar in the three populations (mean/range=1.33/1.28-1.38; the likelihood of two patrilines having identical multilocus haplotypes was higher in SF (0.195), than in SN or SG (0.074 and 0.033 respectively). The relatedness between queens and their mates related was not significantly different from zero: r = 0.153 ± 0.247 in SN (t = 0.506, df = 41, P = 0.615) in SG, r = 0.134 ± 0.323 (t = 0.322, df = 38, P = 0.750) and r = -0.057 ± 0.267 in SF (t = -0.208, df = 9, P = 0.840) i. In addition, males mated to the same queen were on average not related to each other as relatedness between them was not significantly greater than zero (r = 0.343 ± 0.171; t = 1.467, df = 24, P = 0.155 for SG, r = 0.262 ± 0.317; t = 0.709, df = 12, P = 0.492 for SN and r = 0.315 ± 0.229; t = 0.1.048, df = 5, P = 0.342 for SF).

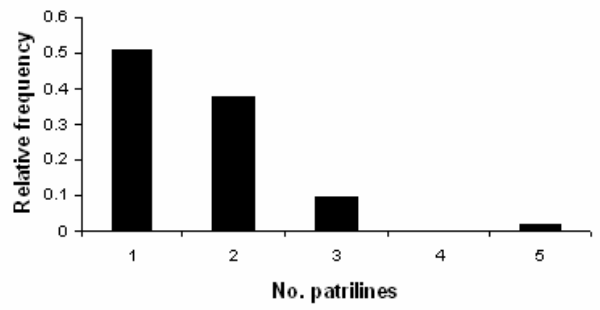

Fig. 3. Number of patrilines observed in *R. minuchae* nests.

Analysis of isolation by distance for queens and their mates separately showed population differences, with significant isolation by distance for males and queens in SG (n_{males}=52; r^2 = 0.084; P < 0.01;

n_{queens}=30; r^2 = 0.047; $P < 0.01$), however, no trend was found in SN (n_{males}=39; r^2 = 0.002; P = 0.20; n_{queens}=26; r^2 = 0.009; P = 0.09).

Male ploidy and parentage in R. minuchae

Only one of the 31 males (3.2%) was found to be diploid and only at one locus (Ccur63b) (Table 4). As 85% of the genotyped workers were heterozygous for at least for one locus, the probability of detecting diploid males was high. Dissections showed normal seminal vesicles development in all males, including the diploid male.

Males produced by workers were found in three of the four colonies that reared males. The overall proportion of worker-produced males was 40%, with values ranging from 0 to 72% per colony (Table 5).

Table 5. Males produced by *R. minuchae* workers. Nd is the number of males carrying an informative allele, Nm is the number of haploid genotyped males, Na is the number of assignable males and WPM is the percentage of males produced by workers

Nest	Nd	Nm	Na	WPM
A	2	6	3.14	64
B	3	8	4.18	72
C	0	8	4.02	0
D	1	8	4.06	25

DISCUSSION

Our analysis reveals that the three populations of *Rossomyrmex minuchae* are genetically very distinct from each other at both microsatellite loci and mtDNA sequences. Even on a local scale differentiation was evident with significant isolation by distance in one of the populations and marginally in another, suggesting dispersal is restricted to some extent. The level of differentiation found in *R. minuchae* is higher than reported for other scarce and isolated ant species (Goropashnaya et al. 2001; Mäki-Petäys et al. 2005; Mäki-Petäys and Breen 2007), a finding that points to long-term isolation of its populations, rather than isolation because of recent habitat fragmentation (Sundström et al. 2005). This latter point is supported by our bottleneck analysis which provided no evidence of recent population reductions. The finding of extreme differentiation in *R. minuchae* can probably be accounted by its enslaved 'host' *Proformica longiseta* being restricted to high altitude habitats and that the three populations have been isolated since the intervening habitat became inhospitable because of warming during some of the Pleistocene interglacials.

Genetic differentiation on such a local scale is surprising given that it generally only occurs in polygynous species (Ross and Keller 1995) where mated queens disperse on foot from their natal nest. In monogynous species there are only very few reports of high structuring, a notable exception being *Cataglyphis cursor* where colonies reproduce by fission (Clémencet et al. 2005). However, we found a significant isolation by distance for males and females in SG (but no significant relationship in SN), suggesting that dispersal is likely limited in both male and female *R. minuchae*. Interestingly in most ants dispersal is typically male-biased (Sundström et al. 2003; Hardy et al. 2008), although extremely limited male dispersal has been reported in *Cardiocondyla* ants because some males are wingless (ergatoid) and mate in their own nest (Schrempf et al. 2005). But winged dispersing males (Heinze et al. 1998) are also produced so it is unknown to what extent males contribute to gene flow in *Cardiocondyla*. The finding of limited male dispersal is in line with the low proportion of *R. minuchae* colonies observed producing sexuals each year (Ruano and Tinaut 2005), which means that few nests would have available queens to mate with and so, males should tend to congregate in the same nests regardless they can fly long distances. Therefore, related males may well congregate outside the same nests waiting for unmated females to emerge. As well as low male dispersal, single males mating repeatedly with different queens from the same nest (Ruano and Tinaut 2005) would likely add to population viscosity. Overall there is little doubt that dispersal of both sexes is restricted and populations are highly structured. In contrast, gene flow in two slave making ants (*Harpagoxenus sublaevis* and *Protomognathus americanus*, Brandt et al. 2007) showed male-biased gene flow and female phylopatry (wingless queens). Mating and dispersal behaviour therefore seem to vary among parasitic ants.

Given that the populations are effectively isolated, that within population gene flow of both sexes is restricted and that numbers of nests within populations are small, one would expect evidence of inbreeding. Consistent with this, F_{IS} was positive (although not significantly so). Interestingly, despite the evidence of some inbreeding diploid males were rare with only one out of 31 morphological males diploid. This low level of diploid male production may have a number of explanations. First, the frequency of diploid males may be underestimated as only adult males were genotyped. Since in some species diploid males are selectively removed before adulthood (Woyke 1963) they may have remained undetected. Second, *R. minuchae* may not have a simple slCSD mechanism of sex determination. Although the data of most social Hymenoptera studied seem compatible with slCSD (van Wilgenburg et al. 2006) some species regularly undergoing inbreeding probably have other mechanisms of sex determination (Buschinger 1989). Indeed, *Cardiocondyla obscuriour*, an ant with intranidal brother-sister mating, successfully produces haploid male eggs and adults, so slCSD can be reasonably excluded (Schrempf et al. 2006). Another possible strategy to reduce inbreeding would be queens mating more than once and as estimated here, doubly mated queens are not uncommon.

However, further studies will be required to determine the mechanisms of sex determination in *R. minuchae*.

The levels of genetic variation in *R. minuchae* were low in comparison to *R. anatolicus*, which had higher levels of allelic richness and gene diversity. In addition, the levels of genetic differentiation between populations of *R. anatolicus* were smaller than among *R. minuchae* populations despite an almost ten-fold greater distance (425 km vs ~50km). This likely reflects a history of long-term fragmentation for *R. minuchae*, compared to a more continuous distribution for *R. anatolicus*. Similar patterns have been reported between small and isolated populations of *Formica lugubris* from Ireland and continuous populations from Finland (Mäki-Petäys and Breen 2007). What is clear is that levels of genetic variation within populations of *R. minuchae* are low compared to its closely related Turkish species. Given that we could only collect three nests of *R. quandratinodum*, we could not test population structure and so, further studies are needed.

How do our findings influence the possibility of managing of *R. minuchae* populations to ensure their survival? The genetic differentiation between the three known populations is sufficient for them to be considered separate sub-species, perhaps even species, as they show considerable differentiation at mtDNA and at multiple nuclear loci, including many fixed differences. As such, conventional wisdom would have them managed as separate units (Moritz 1994) so as to conserve the genetic diversity among populations (e.g. Loftis et al. 2009; Mockford et al. 2007; Pabijan et al. 2005). In contrast, detrimental effects of inbreeding in small populations are widely known (Frankham et al. 2002; Keller and Waller 2002), so one must balance the idea of preserving genetic diversity – and the *status quo* - with the possibility of alleviating detrimental effects by introducing new alleles from another population. Importantly, this issue is relevant to any declining and fragmenting social species with genetically differentiated populations, such as *Plagiolepis xene* (Trontti et al. 2006) or *Bombus muscorum* (Darvill et al. 2006). The degree of nuclear differentiation between known populations in *R. minuchae* suggests that valuable new genetic variation could be introduced into each population by translocating nests or sexual offspring. However, the risks of potentially negative effects such as out-breeding depression or disease transmission would need to be carefully considered before such management was undertaken. Although we did not find direct evidence for inbreeding negative effects, the small sample size and the apparently low dispersal of sexuals suggest that the future for these ants is bleak. Deciding between preserving conventional management units or being more adventurous and interventionist depends on what is deemed more important: conserving genetically distinct units based on genetic markers or conserving ants with a highly specific slave raiding behaviour?

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CAPÍTULO 3

Enviado a *Journal of Evolutionary Biology*

A geographical mosaic of coevolution in a slave-making host-parasite system

La teoría de la coevolución en mosaico geográfico predice diferencias locales en las comunidades dependiendo del contexto de la interacción. Esto implica: (1) puntos calientes coevolutivos (zonas de simpatría) y puntos fríos (zonas de alopatría); (2) selección en mosaico dado que la variación espacial en la eficacia biológica sirve para describir la selección recíproca; (3) mezcla de caracteres debidas al flujo génico, por lo que algunos encuentran adaptaciones locales y otros maladaptación. Nosotros probamos esta teoría en las poblaciones aisladas de la hormiga esclavista *Rossomyrmex minuchae* y en su hospedadora *Proformica longiseta* estudiando los hidrocarburos cuticulares (CHCs, relacionados con el reconocimiento entre compañeras de nido y la agresividad) y microsatélites. Encontramos perfiles de CHC propios de cada población parásita pero diferencias en la similitud de las hospedadoras, confirmando la existencia de un mosaico geográfico coevolutivo. Además, *P. longiseta* muestra un mayor flujo génico que *R. minuchae*, lo que junto con la fuerte presión selectiva de reducir las agresiones de la parásita, sugieren que la migración es beneficiosa para *P. longiseta*, ya que está localmente adaptada a su parásita.

A GEOGRAPHICAL MOSAIC OF COEVOLUTION IN A SLAVE-MAKING HOST–PARASITE SYSTEM

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Running title: Host–parasite mosaic of coevolution

ABSTRACT

The geographical mosaic theory of coevolution predicts differences in local communities depending on the context of the interaction. It involves: (1) Coevolutionary hot spots (zones of sympatry) and cold spots (zones of allopatry); (2) Selection mosaic as spatial variation in fitness functions describing reciprocal selection; and (3) Trait remixing conducted by gene flow, for which some find local adaptations, whereas others point to maladaption. We tested this theory in isolated populations of the slave-making ant, *Rossomyrmex minuchae,* and its host, *Proformica longiseta,* by studying cuticular hydrocarbons (CHCs, related to nestmate recognition and aggressiveness) and microsatellites. We found population-specific parasite CHCs profiles but differences in host similarity, confirming the existence of a geographical mosaic of coevolution. Furthermore, *P. longiseta* showed a higher gene flow than *R. minuchae*, which, together with the strong selection pressure of reducing parasite aggressions, suggest that migration is beneficial for *P. longiseta* as it is locally adapted to its parasite.

KEY WORDS: Cuticular hydrocarbons, gene flow, geographical mosaic, coevolution, *Rossomyrmex minuchae, Proformica longiseta,* host–parasite system.

INTRODUCTION

Over the 20 last years, a number of convincing examples of coevolution between parasites and their hosts have demonstrated the importance of coevolution as an evolutionary process, shaping the organization of communities and influencing the diversification of life (Thompson, 1999a, 2005). According to the geographical theory of coevolution, the outcome of interactions and the degree of specialization vary among physical and biotic environments, and depend on the community context in which the interaction takes place (Thompson, 1994, 1999b).

The geographical mosaic theory of coevolution (GMTC) predicts differences in the advance or trajectory of the coevolutionary process among local communities due to their composition and the strength of ecological selection pressures through competition and resource availability. Gomulkiewicz *et al.* (2007) proposed some ideas to test the GMTC to detect:

1. Coevolutionary hot spots (zones of sympatry) and cold spots (zones of allopatry). The GMTC assumes that fitness interactions among species vary geographically in intensity, with regions in which reciprocal selection occurs (hot spots) and other regions in which a species is completely unaffected by the other (cold spots).

2. Selection mosaic refers to spatial variation in the interspecific frequency-dependent fitness functions describing reciprocal selection among interacting species. In this case, we tried to investigate whether a selection mosaic exists in nestmate recognition in a host–parasite system. Nestmate recognition is a key element in the organization of social insects. It is the ability to distinguish among individuals, which is essential to avoid competition, predation, and parasitism (Wilson, 1971). The maintenance of societies is achieved via a recognition system based on chemical odors on the cuticle. Hydrocarbons are thought to play a crucial role in nestmate recognition (Lenoir *et al*., 1999; D'Ettorre *et al.,* 2002; Howard & Blomquist, 2005; Ozaki *et al.,* 2005; Hefetz, 2007) and constitute a species-specific chemical signal for some social insects (Copren *et al.,* 2005; Martin *et al.,* 2008). Among the strategies employed by social parasites to integrate into their host colony are the existence and/or acquisition of similar chemical profiles between the social parasites and their hosts (see Lenoir *et al.,* 2001; Akino, 2008). Cuticular distances mediating recognition between host and parasites are positively correlated with geographical distance and aggression (Zamora-Muñoz *et al.,* 2003; Errard *et al.,* 2006; Nash *et al.,* 2008; Ugelvig *et al.,* 2008).

3. Trait remixing (Thompson, 2005; Gomulkiewicz *et al.,* 2007): The pattern of this mosaic changes locally with time when animal populations change, especially when migration occurs. Some previous studies have pointed that gene flow is very important for local coevolution as a main source of genetic

variation. However, researchers do not agree on the effect of migration on coevolution: some find that gene flow enables beneficial adaptations at a particular time and space (Forde *et al.,* 2004; Morgan *et al.,* 2005). In patches where the parasite can migrate at a higher rate than the host, the parasite will be locally adapted to its host and vice versa, whereas if both species have similar migration rates, no local adaptation is expected (Gandon *et al.,* 1996). Other studies point that absent or restricted gene flow is essential for local coevolution, given that migration homogenizes populations. Thus, selection may be weak in patches where host and parasite coincide and the widespread species is maladapted to the other (Nuismer *et al.,* 2003; Nash *et al.,* 2008).

In this work, we tested the GMTC for the current three known isolated *Rossomyrmex minuchae* populations (Tinaut *et al.,* 2007) and its host *Proformica longiseta*, using both chemical (cuticular hydrocarbons CHCs) and molecular traits (microsatellites). We predicted that the CHC profiles (cuticular distances) of both host and parasite are population-specific, showing a selection mosaic for this trait, with different degrees of mimicry as an evidence for local mosaic of coevolution taking place in three different scenarios independently. The outcomes of these interspecific interactions could differ among communities because of different selection pressures (Thompson, 2005), expected in the separated populations. To determine whether trait remixing for CHCs has taken place, the gene flow (inferred from microsatellites) among populations for both species was compared. An asymmetry in the gene flow between species can elucidate the current situation of the geographic mosaic of coevolution.

MATERIALS AND METHODS

Study area and field work

Rossomyrmex minuchae is an endemic ant that can be found in the mountains of South-Eastern Spain (Tinaut *et al.,* 2007). It has always been found in arid steppes partially covered by snow in winter, with summer being very hot, sunny, and dry.

Live ants were collected with an aspirator during summer 2006. Only three populations of this species are known to date: the first one in Sierra Nevada (Sierra Nevada 1 and 2 (SN1, SN2) and Casillas Rojas (CR) locations), the second one in Sierra de Gador (Fuente Alta (FA) and Pinos (P) locations), and the last one in Sierra de los Filabres (F) (Fig. 1). For the *R. minuchae* nests, we recorded UTM coordinates using a GPS (Garmin) and calculated the straight-line distances between nests by trigonometry. For *P. Longiseta,* a single UTM coordinate per subpopulation was recorded given that the nest distances were shorter than the GPS sensibility. The distance between subpopulations varied

from 190 m to 61 km (Table 1). In all these localities, for chemical analyses, we sampled *R. minuchae* nests with their corresponding *P. longiseta* slaves and free-living *P. longiseta* nests. A total of 26 *R. minuchae* mixed nests and 31 free-living *P. longiseta* were sampled (Table 2). For genetic analyses, up to five workers per nest were used (23 nests for the parasite and 31 for the host).

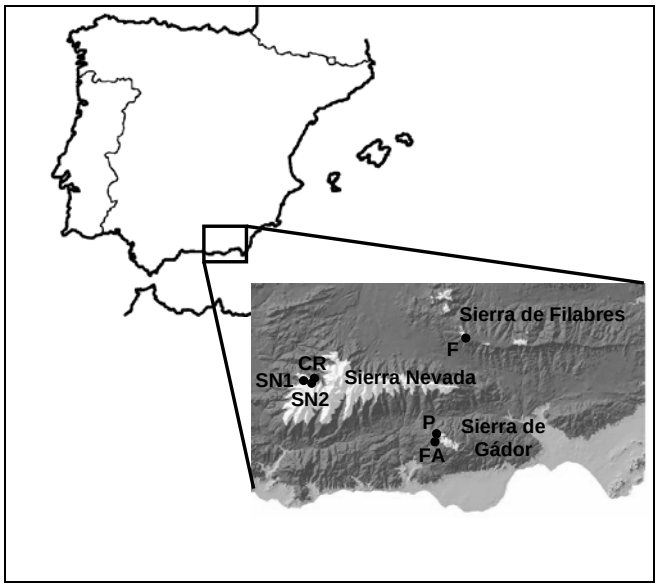

Figure 1. Location of the three studied sympatric populations and subpopulations.

Table 1. Geographic distance in meters among populations and subpopulations considered: Sierra Nevada (SN1, SN2, and CR), Sierra de Gador (FA and P), and Sierra de Filabres (F).

	FA	P	F	SN1	SN2	CR
FA	0					
P	415	0				
F	39559	39146	0			
SN1	61351	61177	60797	0		
SN2	60734	60557	60315	620	0	
CR	60815	60636	60271	564	190	0

Table 2. Nests and samples (in brackets) used for chemical analyses.

Population	Locality	*Rossomyrmex* nests (R)	Enslaved *Proformica* nests (E)	Free-living *Proformica* nests (FL)
Sierra Nevada	SN1	14-17-30 (11)	14-17-30 (15)	11-12-13 (15)
Sierra Nevada	SN2	56-64-65-68 (20)	56-64-65-68 (20)	11-12-13-14-15 (25)
Sierra Nevada	CR	1-2-3 (15)	1-2-3 (15)	1-2-3 (15)
Filabres	F	2-3-4-5-6 (25)	2-3-4-5-6 (25)	11-12-13-14-15-16-17-18-19-20 (50)
Gador	FA	14-15-17-22-23-24 (30)	14-15-17-22-23 (22)	11-13-14-15-16 (21)
Gador	P	13-21-24-27-28 (25)	13-21-24-27-28 (21)	11-13-14-15-16 (25)

Chemical and genetic analyses

Ants were frozen at −18ºC for 1 h and then immersed for 10 min in 1 ml of pentane. The extracts were stored at −18ºC until analyses were carried out. Cuticular content was identified using pools of at least 20 workers, by combined gas chromatography/mass spectrography (Turbomass system, Perkin-Elmer, Norwalk CT, USA, operating at 70 eV) using a nonpolar DB-5 fused silica capillary column. Samples were run using a temperature program from 150ºC (2 min of initial hold) to 300ºC at 5ºC min^{-1}, with 10 min of final hold. Quantification was achieved by gas chromatography using the same FID-GC and temperature program. When possible, five individual extracts were used for each colony. When the individual extracts were not sufficiently concentrated, they were pooled by two or three. As the database was very large, we used the mean for each colony. Identification of substances was made according to previous published data for *R. minuchae* and its host in Sierra Nevada (Errard *et al*., 2006), and completed with a more precise analysis of small peaks.

The total DNA was extracted from the workers using Puregene DNA Isolation Kit (Gentra Systems). *Rossomyrmex* workers were genotyped at 11 microsatellite loci: Ccur11, Ccur46, Ccur63b, Ccur76, Ccur89, Ccur99, FE11, FE19, FE21, FE37, and FE51 (Pearcy et al. 2004; Gyllenstrand et al. 2002). *Proformica* workers were genotyped at seven loci: Ccur11, Ccur26, Ccur63, Ccur76, FE19, FE37, and FL12 (Chapuisat, 1996). Microsatellites were amplified by PCR in a 10-µL reaction containing 2.5 ng of DNA, 0.2 nM of each primer, 0.25 nM of each dNTP, 1× MBL buffer, 2.5mM of $MgCl_2$ (overall), 0.5 µ/µL of MBL Taq DNA polymerase, and 7 µL of distilled water. PCR conditions were 94ºC for 3 min to denature the samples, followed by 11 touch-down temperature cycles of denaturing at 94ºC for 15 s, annealing at 55–45ºC for 30 s, and extension at 72ºC for 30s, followed by 30 cycles at 94ºC for 15 s, 50ºC for 15 s, and 72ºC for 30 s. PCR products were genotyped at Unidad de genómica. Scai (University of Córdoba) using an ABI310 sequencer. We scored genotypes using GENESCAN software v 3.7.

Statistics

As there were great qualitative and quantitative differences in the chemical profiles (too many zeros or very low values), a discriminant analysis was not applicable, and therefore, we performed cluster analysis with Euclidean distances and Ward method on the percentages of all the peaks (see Elmes *et al.*, 2002). As it was not possible to normalize the data, differences in hydrocarbon composition for free-living hosts and parasites were tested using Kruskal–Wallis analysis. The analyses were carried out with STATISTICA 7 for Windows.

Pairwise genetic distances (F_{ST}) among *R. minuchae* nests were calculated with FSTAT 2.9.3.2. as well as Mantel tests (10000 permutations) to correlate geographical, genetic ($F_{ST}/1–F_{ST}$), and chemical distances.

Differences in hydrocarbon composition among colonies and populations were quantified by modifying the standard genetic distance of Nei (1987), as shown in Dronnet *et al.* (2006). Differences in the Nei Index among the parasite and free-living nests were considered as a measure of adjusted coevolution in the proposed arms race.

Gene flow among the three populations was estimated according to Barton and Slatkin (1986), based on the distribution of rare alleles, using GENEPOP software (Raymond & Rousset, 1995) in its web implementation. We considered the best approximation to be the corrected estimate from the closest regression line provided by the program.

RESULTS

1. Hot and cold spots

The current distribution of both species was confirmed by exhaustive sampling around the known populations and in other potential biotopes across the Iberian Peninsula. The host was found to be widely distributed between 1650 and 2900 m a.s.l., but the parasite was located at 1900–2200 m a.s.l (hot spots). Therefore, many areas may be empty of parasites where the potential host occurs (cold spots). Furthermore, the host was not found at altitude lower than 1650 m a.s.l. Thus, the distribution of *P. longiseta* presented hot spots when sympatric with *R. minuchae,* and cold spots when allopatric.

2. Selection mosaic

A total of 46 peaks were identified including a series of n-alkanes (C23–C34), namely, mono, dimethyl, and trimethyl alkanes (Table 3). These data confirm the previous findings (Errard *et al*., 2006). However, only two alkenes were found (C24:1 and C26:1) in small quantities. Furthermore, we did not observe any aldehydes or esters. The major peaks (>5%) were completely different between Sierra Nevada + Gador and Filabres. In Sierra Nevada and Gador, all major peaks had more than 28 carbons, whereas in Filabres, the major peaks were also found in the C24/C27 region (Fig. 2).

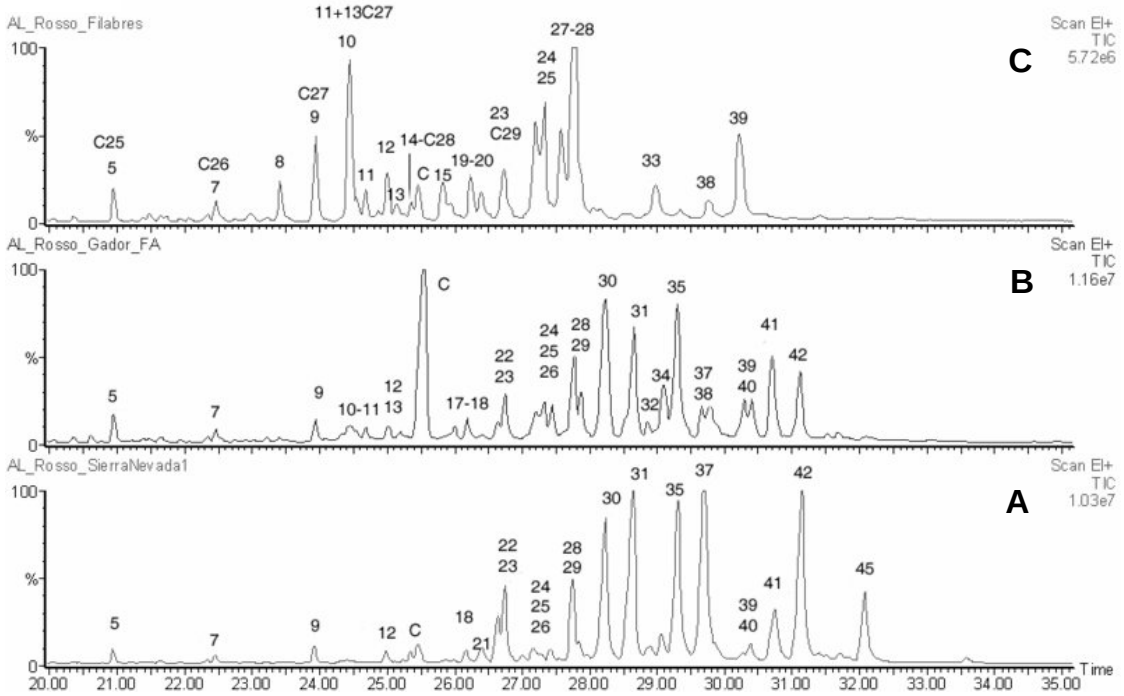

Figure 2. Gas chromatograms (GS-MS) of total body washes of 20 pooled workers of *R. minuchae* and free-living *P. longiseta* from Sierra Nevada (A), Gador (B), and Filabres (C). C is squalene contaminant

We found a strong positive correlation between chemical and geographic distances among the populations (Mantel test, $r = 0.56$; $P = 0.01$). However, there was no correlation within the populations (Mantel tests, all $P > 0.05$), indicating that if the distance between the populations was more, then their hydrocarbons diverged more. On the contrary, inside the populations, this pattern was not evident.

Cluster analysis indicated that the *R. minuchae* populations appeared clearly separated with three chemotypes – the Filabres chemotype being separated from Gador and Sierra Nevada (Fig. 3). Gador and Sierra Nevada were also well differentiated, but inside the populations, the subpopulations were not segregated.

Exactly the same groups were observed when slaves were added. Nevertheless, in the same colony, the slave-maker and the slaves were rarely together (it appeared only in one case, SN1-30) (Fig 4).

The CHCs of the potential hosts (free-living nests surrounding the parasitized nests) clustered in four groups: Filabres were again completely separated, and Gador, Sierra Nevada, and an intermediate heterogeneous group were formed by some colonies of Gador or Sierra Nevada (Fig. 5).

CHCs differences between parasite and host, an indicator of the ability to decrease aggression towards the host, were lower in Sierra Nevada population, being higher at the same level in Gador and Filabres (Kruskal–Wallis test, $P = 0.007$) (Fig. 6).

From all these results, we can conclude that a selection mosaic occurs in the three different populations, in which *P. longiseta* and *R. minuchae* are sympatric (hot spots).

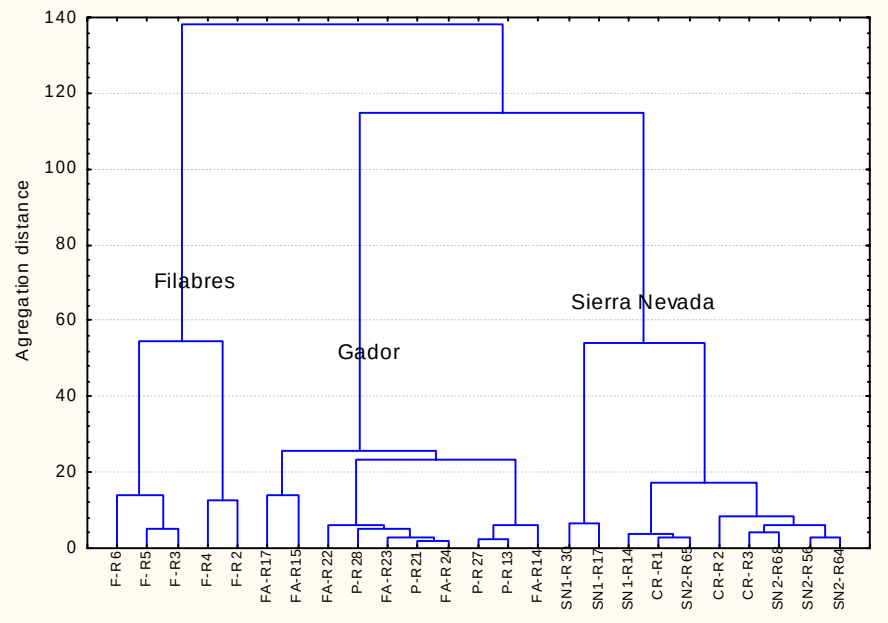

Figure 3. Cluster analysis of *R. minuchae* in the three isolated populations (Euclidian distances and Ward method).

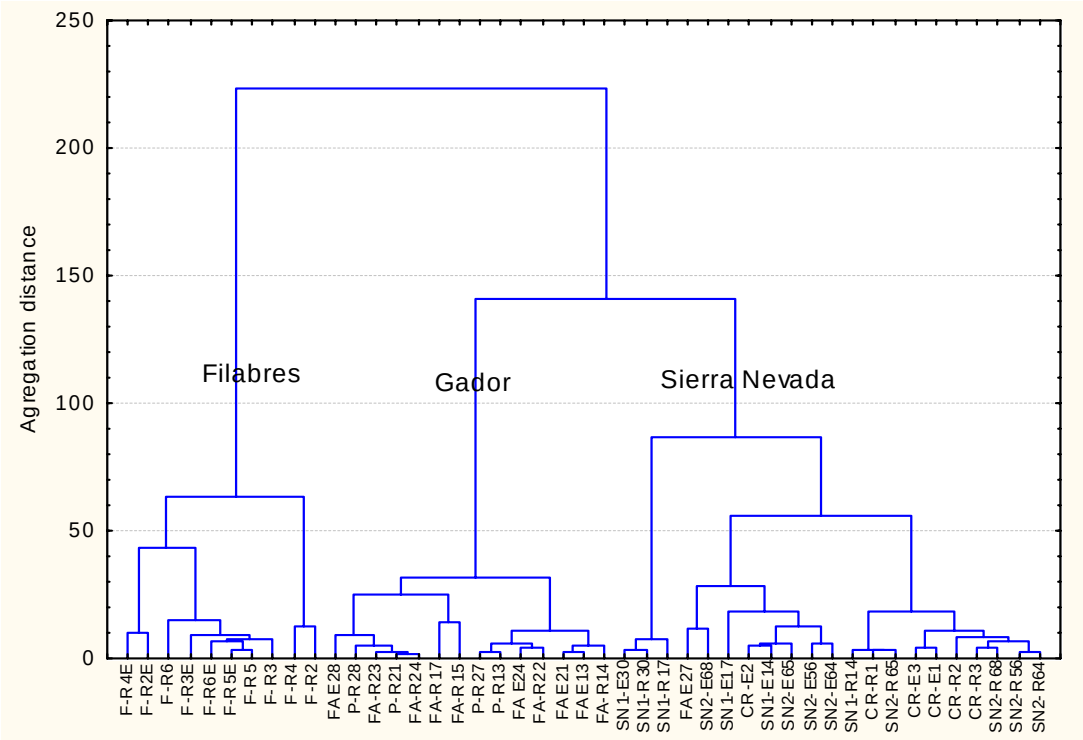

Figure 4. Cluster analysis of *R. minuchae* and the slaves in the three isolated populations (Euclidian distances and Ward method).

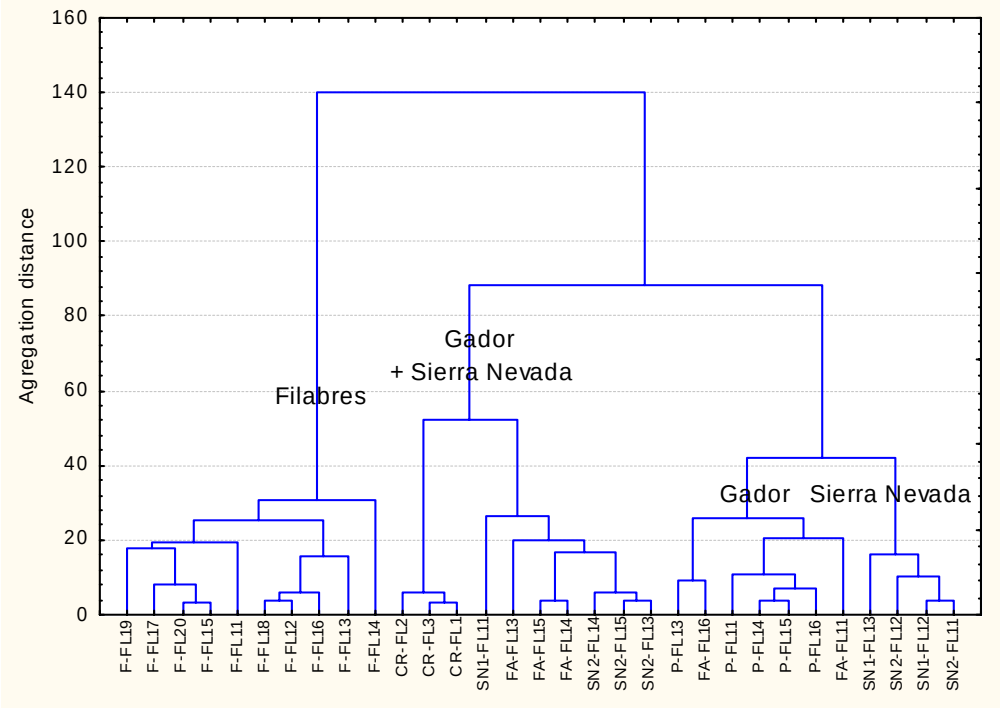

Figure 5. Cluster analysis of free-living *P. longiseta* (Euclidian distances and Ward method).

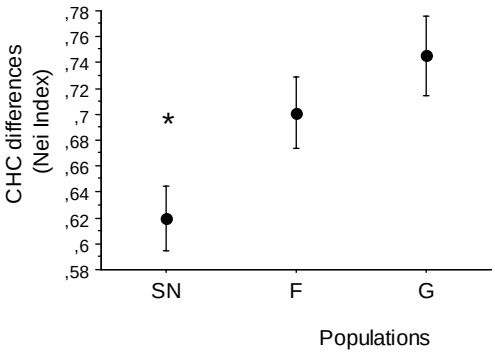

Figure 6. Mean CHC differences (Nei Index) between free-living host and parasite nests, compared among populations. The asterisk indicates a significant difference between SN and F + G (Kruskal–Wallis test).

3. Trait remixing

The estimated number of *R. minuchae* migrants after correction for size was 0.076, whereas this value for *P. longiseta* was 1.538. Although Whitlock and McCauley (1999) warned about the inaccuracy of gene-flow estimates based on indirect measures such as microsatellite data, they also considered that these estimates are likely to be accurate within certain limits. Considering that the differences in the gene flow between *R. minuchae* and *P. longiseta* are very high, there is a good probability that they are indicators of differences in the migration rates between the species.

Among populations, there were strong positive correlations between CHC profiles and genetic differences (Mantel test, $r = 0.52$; $P = 0.01$), as well as between genetic and geographic distances (Mantel test, $r = 0.88$; $P = 0.01$). However, we found no correlation when comparing these variables within populations (Mantel tests, all $P > 0.05$), at least for our sample sizes. Thus, *R. minuchae* populations were chemically and genetically differentiated, with population-specific CHC profiles. In contrast, the nests within each sierra were found to be very similar for both traits.

DISCUSSION

1. Hot and cold spots

The three populations (Sierra Nevada, Sierra de Gador, and Sierra de Filabres) have been isolated since the intervening habitat became inhospitable because of a warming period during one of the Pleistocene interglacial periods. According to the current distribution of the genus (Tinaut *et al.,* 2008), *Rossomyrmex* and its *Proformica* host would have arrived at the Iberian Peninsula during the Pliocene, coinciding with any of the reported invasions of vertebrates from Central Asia (Arribas *et al.,* 2001). It is possible that during the climatic fluctuations of the Pleistocene, their populations went up and down the mountains, with respect to the minimum and maximum glacial periods, getting together at low altitude at some of the glacial maximums. The degree of differentiation suggests that the three populations have been isolated much longer than the time since the last glacial maxima (younger Dryas), which was reported to be warmer than previous ones (Lomolino *et al.,* 2005), and probably might not have been cold enough to reunite the *Rossomyrmex* populations. Population differentiation in the Iberian Peninsula during the Pleistocene has also been reported in some species of birds, such as the azure-winged magpie (Fok *et al.,* 2002) and stonechats (Illera *et al*., 2008) as well as among insects (Ribera & Vogler, 2004; Cánovas *et al*., 2008).

2. Selection mosaic

The only way for hosts to evolve resistance is to avoid being parasitized in the first place (Brandt *et al.,* 2005). This can be achieved by reliable enemy recognition and reducing the overall success of raids. The similarity in the CHC profiles that we found between the parasite and its sympatric host population appears to be related to the behavior of *R. minuchae* that are less aggressive towards *P. longiseta* than with the allopatric counterparts (Zamora-Muñoz *et al*., 2003). This behavior towards the parasite must be advantageous for *P. longiseta,* because a closer odor to the parasite would reduce intracolony aggressions and a similar pattern is expected for nonparasitized workers. Zamora-Muñoz *et al.* (2003) found that *P. longiseta* workers always died when fighting *R. minuchae,* and therefore,

this aggressive strategy would be too costly when the nest is under attack. However, if *P. longiseta* workers did not fight the parasite, *R. minuchae* became less aggressive during raids, allowing the survival of a fragment of the colony.

The three *R. minuchae* populations appeared well differentiated in the chemical cluster, and even the CHCs distances between the slave-maker and potential slaves were different in one population (Sierra Nevada) with respect to the other two (Gador and Filabres). This may indicate a different ability of the host to resist slave-maker invasions, leading to a different level of aggression against the parasite, as it has been observed in other cases or species (Zamora *et al*., 2003; Nash *et al*., 2008; Ugelvig *et al*., 2008). Different host–parasite aggression patterns have been proved between different host species and among populations of the same host species in other host–parasite systems (Bauer *et al*., 2009). Therefore, we predict different host–parasite aggression patterns in different populations of the *P. longiseta–R. minuchae* system, which must be investigated further.

Slave-maker and slaves appeared clustered together, but in the same colony, both were rarely together. This result confirms our previous data showing CHCs similarity between host and parasite, but not an exact congruence (Errard *et al.*, 2006) as each species has its own profile.

Potential hosts appeared clustered together for Sierra Nevada and Gador with an intermediate CHCs position. This CHCs mixed populations could be the result of less selective pressure for a determined cuticular odor in the absence of the parasite. Allopatric populations appeared to be more similar, despite geographical distances.

3. Trait remixing

Gene flow appeared to be clearly higher in the host species (*P. longiseta*) than in the parasite (*R. minuchae*). The obtained number of migrants of *P. longiseta* may be slightly overestimated, because the females are wingless and hence, dispersal is carried out mainly by haploid males (Berg *et al.,* 1998). However, gene flow should not be much lower, because this finding is supported by previous studies showing weak genetic differentiation among its populations and the absence of isolation by distance within populations (Fernandez-Escudero *et al.,* 2001). In the case of the parasite, males and females are winged and dispersal is reported for both sexes (Ruano & Tinaut., 2005). Thus, estimates of gene flow may be more accurate than those for the host. Nevertheless, the great differences in estimates obtained for both species are very likely to indicate a strong migration restriction for *R. minuchae,* when compared with *P. longiseta*.

To determine whether *R. minuchae* or *P. longiseta* have their CHCs adapted to one another, the gene flow and the previous results comparing profiles of allopatric and sympatric host populations are crucial. In this case, allopatric *P. longiseta* showed higher differences in the CHC profiles than sympatric *P. longiseta* and its parasite (Errard *et al.,* 2006), and allopatric potential hosts showed a higher level of aggression than in sympatric populations (Zamora *et al.,* 2003). A selection pressure is presumed to cause these differences between the sympatric and allopatric host populations, and also among sympatric populations as demonstrated in this study, evolving in different geographical mosaics. We consider that the strongest selective pressure for *P. longiseta* must be the presence of the parasite, as environmental conditions are basically the same in all the populations. An interesting point in this interaction is the isolation of *R. minuchae* populations when compared with *P. longiseta*. The main source of variation for the parasite should be genetic drift and/or mutation, whereas the gene flow seems to be almost inexistent. On the other hand, the host species is expected to be more influenced by migration due to its large distribution range. Thus, our results are in agreement with Gandon *et al.* (1996), because the species with the highest migration rate (*P. longiseta*) appears to be locally adapted to the other and no evidence for maladaptation has been found. This conclusion is also in accordance with the genetic diversity found in both species (Fernández-Escudero *et al*., 2001; O. Sanllorente, R.L. Hammond, F. Ruano, L. Keller & A. Tinaut, unpublished data), which is higher in the host, consequently leading to better adaptation ability.

Thus, by confirming the existence of hot and cold spots, the selection mosaic in CHC among populations and a higher gene flow in *P. longiseta* than in *R. minuchae*, reflecting a better ability to the host than the parasite, we assessed the existence of a geographical mosaic of coevolution in this host–parasite system.

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Table 3. Mean of cuticular hydrocarbons in each population of *R. minuchae* (R), *P. longiseta* slaves (S), and free-living (FL). SE values given in brackets.

nº	Name	SN-R	SN-S	SN-FL	F-R	F-S	F-FL	G-R	G-S	G-FL
1	**C23**	1.65 (0.81)	3.05 (1.44)	4.35 (2.54)	3.54 (1.03)	4.11 (0.81)	4.3 (2.61)	2.75 (1.44)	3.1 (1.15)	2.97 (1.61)
2	**C24:1**	1.83 (1.1)	4.89 (2.3)	6.93 (2.45)	3.09 (1.46)	6.59 (1.24)	5.82 (2.3)	3.11 (1.81)	4.58 (2.53)	7.18 (4.6)
3	**C24**	1.32 (0.78)	3.87 (3.63)	4.83 (2.74)	1.58 (0.82)	3.16 (0.59)	2.98 (1.4)	1.6 (0.88)	2.56 (1.41)	3.61 (2.26)
4	**$_{6}$C24**	0.45 (0.41)	1.79 (1.74)	1.59 (1.05)	0.51 (0.53)	0.75 (0.57)	0.63 (0.42)	0.63 (0.53)	1.1 (0.85)	1.24 (0.92)
5	**C25**	1.96 (0.85)	4.53 (1.62)	6.74 (3.41)	4.55 (1.27)	6.26 (1.41)	5.35 (1.73)	2.97 (1.58)	4.03 (1.99)	6.07 (3.07)
6	**C26:1**	1.1 (0.55)	2.91 (0.94)	4.12 (1.4)	1.61 (0.96)	3.71 (0.76)	2.98 (1.09)	1.81 (1.09)	2.62 (1.43)	4.2 (2.68)
7	**C26**	1.74 (0.81)	4.67 (1.79)	5.93 (2.17)	3.34 (0.92)	5.88 (1.32)	5.77 (1.63)	2.47 (1.34)	3.59 (1.85)	5.89 (3.05)
8	**$_{2}$C26**	0.42 (0.26)	1.09 (0.48)	1.68 (0.43)	2.08 (1.6)	2.8 (1.67)	1.6 (1.02)	0.7 (0.39)	0.95 (0.49)	1.57 (1.12)
9	**C27**	2.24 (0.79)	4.65 (1)	5.91 (2.07)	7.96 (2.21)	9.59 (4.07)	11.62 (2.29)	2.67 (1.17)	3.62 (1.72)	5.46 (2.51)
10	**$_{11+13}$C27**	0.25 (0.21)	0.49 (0.61)	0.2 (0.29)	8.78 (2.85)	5.36 (1.8)	6.28 (2.38)	1.32 (0.58)	1.15 (0.72)	0.42 (0.37)
11	**$_{5}$C27**	0.14 (0.1)	0.21 (0.22)	0.52 (0.27)	1.81 (0.53)	1.23 (0.27)	1.22 (0.51)	1.14 (0.39)	0.97 (0.53)	0.62 (0.66)
12	**$_{3}$C27**	0.77 (0.27)	0.57 (0.2)	0.31 (0.27)	3.29 (0.63)	1.37 (0.77)	1.98 (0.87)	1.27 (0.56)	0.9 (0.64)	0.56 (1.51)
13	**$_{5,15+5,17}$C27**	0.7 (0.45)	1.75 (0.93)	1.95 (0.9)	1.5 (0.91)	1.25 (0.69)	0.53 (0.52)	1.43 (0.57)	1.31 (0.72)	1.13 (1.16)
14	**C28**	1.42 (0.58)	2.93 (0.88)	4.34 (1.48)	3.1 (1.1)	2.78 (1.26)	4.69 (1.01)	1.37 (0.54)	1.93 (0.6)	4.53 (2.4)
15	**$_{8,12+8,14+10,12}$ C28**	0.35 (0.14)	0.15 (0.16)	0.1 (0.17)	2.49 (0.71)	1.76 (0.18)	1.71 (0.57)	0.97 (0.68)	0.71 (0.69)	0.14 (0.23)
16	**$_{7}$C28**	0 (0)	0.01 (0,03)	0 (0)	0.74 (0.16)	0.46 (0.29)	0.5 (0.26)	0 (0)	0 (0)	0 (0)
17	**$_{6}$C28**	0.19 (0.13)	0.09 (0.11)	0.18 (0.34)	0 (0)	0 (0)	0 (0)	0.86 (0.23)	0.74 (0.26)	0.26 (0.27)
18	**$_{4}$C28**	0.62 (0.45)	0.75 (0.61)	0.63 (0.43)	0.4 (0.9)	0.29 (0.65)	0.18 (0.57)	1.27 (0.4)	1.21 (0.24)	1 (0.34)
19	**$_{10,12+10,14}$ C28**	0.25 (0.4)	0.35 (0.65)	0.29 (0.52)	1.53 (1.05)	1.04 (0.89)	1.75 (1.03)	0 (0)	0 (0)	0 (0)
20	**$_{7,11,19}$C27**	0 (0)	0.02 (0.07)	0 (0)	1.33 (0.63)	1.24 (0.41)	0.88 (0.54)	0 (0)	0 (0)	0 (0)
21	**$_{6,10}$C28**	0.66 (0.18)	0.5 (0.27)	0.36 (0.34)	0 (0)	0 (0)	0 (0)	0.47 (0.19)	0.71 (0.69)	0.24 (0.37)
22	**$_{4,8+4,10+4,12}$ C28**	2.02 (1)	1.7 (0.85)	1.92 (0.92)	1.12 (0.93)	1.87 (0.12)	1.2 (0.51)	1.48 (0.57)	1.18 (0.51)	0.91 (0.52)
23	**C29**	5.19 (3.37)	4.62 (2.27)	5.17 (2.17)	2.41 (2.3)	1.8 (0.99)	2.99 (1.3)	2.69 (1.71)	3.52 (1.7)	6.13 (4.29)
24	**$_{11+13+15}$C29**	1.16 (0.27)	0.74 (0.31)	0.72 (0.44)	6.55 (2.12)	4.94 (0.88)	5.05 (1.74)	1.9 (0.72)	1.64 (0.71)	1.09 (0.72)
25	**$_{7}$C29**	0.16 (0.08)	0.04 (0.07)	0.06 (0.14)	4.76 (1.31)	3.98 (1.28)	5.57 (4.39)	1.93 (0.59)	1.76 (0.45)	1.21 (0.79)
26	**$_{5}$C29**	0.72 (0.37)	0.39 (0.26)	0.39 (0.26)	7.94 (11.11)	0 (0)	0 (0)	1.31 (0.53)	1.4 (0.29)	1.1 (0.56)
27	**$_{11,15}$C29**	0 (0)	0.02 (0.07)	0 (0)	3.09 (2.94)	7.96 (6.03)	4.51 (4.8)	0 (0)	0 (0)	0 (0)
28	**$_{3}$C29**	5.05 (0.8)	3.74 (1.78)	4.53 (2.25)	0 (0)	0 (0)	0 (0)	5.02 (1.65)	5.66 (1.8)	4.57 (2.92)
29	**$_{5,9+7,17}$C29**	0 (0)	0.67 (2.11)	0 (0)	9.59 (9.35)	9.6 (6.73)	9.51 (5.76)	0 (0)	0 (0)	0 (0)
30	**$_{8+10}$C30**	6.86 (3.66)	5.17 (2.62)	3.29 (3.25)	0.82 (0.91)	1.38 (1.06)	1.62 (0.85)	14.54 (3.01)	12.01 (3.13)	10.73 (6)
31	**$_{8,12+8,14}$C30**	11.71 (2.3)	8.19 (3.51)	6.7 (2.82)	0.16 (0.22)	0.2 (0.26)	0.2 (0.17)	6.22 (2.88)	5.95 (2.5)	3.41 (3.22)
32	**$_{4}$C30**	0.66 (0.29)	0.47 (0.35)	1.91 (2.38)	0 (0)	0 (0)	0 (0)	1.03 (0.27)	1 (0.34)	0.8 (0.39)

33 **9,11,16C29**	0 (0)	0.03 (0.1)	0 (0)	1.5 (1.51)	1.75 (1.72)	1.27 (0.8)	0 (0)	0 (0)	0 (0)
34 **6,14C30**	1.46 (0.39)	1.06 (0.52)	1.62 (3.47)	0 (0)	0.13 (0.18)	0.06 (0.1)	3 (0.98)	2.73 (0.93)	1.39 (1.23)
35 **4,8+4,10C30**	8.83 (2.58)	6.55 (2.61)	3.09 (2.71)	0 (0)	0 (0)	0 (0)	8.81 (2.44)	7.07 (2.01)	5.59 (3.44)
36 **C31**	0 (0)	0 (0)	0 (0)	0.46 (0.39)	0.57 (0.55)	0.9 (0.53)	0 (0)	0 (0)	0 (0)
37 **9,17C31**	13.19 (4.29)	9.1 (4.12)	5.42 (2.85)	1.39 (0.85)	0.96 (0.91)	1.58 (1)	1.55 (0.4)	1.6 (0.45)	1.35 (1.18)
38 **9+11+13+15 C31**	0.74 (0.72)	0.31 (0.28)	2.11 (2.63)	0.5 (0.75)	0.28 (0.48)	0.13 (0.29)	1.85 (0.67)	1.67 (0.73)	1.7 (0.93)
39 **9,19C31**	0.35 (0.15)	0.32 (0.24)	0.35 (0.29)	4.52 (2.71)	4.05 (1.9)	4.1 (1.79)	2.56 (1.32)	2.41 (1.2)	1.52 (1.54)
40 **5,17C31**	1.2 (0.33)	0.87 (0.46)	0.61 (0.53)	1.97 (2.48)	0.58 (1.23)	0.47 (1.45)	2.97 (0.8)	2.65 (0.74)	2.04 (1.26)
41 **8+10C32**	3.32 (0.75)	2.63 (1.53)	1.24 (1.31)	0 (0)	0.24 (0.26)	0.04 (0.12)	7.13 (1.35)	6.1 (1.69)	6.04 (3.11)
42 **8,14+8,16C32**	14.25 (5.32)	10.46 (4.64)	4.82 (3.97)	0 (0)	0 (0)	0.01 (0.03)	6.02 (1.49)	4.84 (1.71)	3.14 (2.83)
43 **6,14C32**	0.23 (0.17)	0.07 (0.06)	2.36 (3.84)	0 (0)	0.07 (0.16)	0.03 (0.07)	0.52 (0.29)	0.41 (0.31)	0.1 (0.15)
44 **15+17C33**	0.27 (0.21)	0.07 (0.09)	0.07 (0.11)	0 (0)	0.01 (0.03)	0 (0)	0.51 (0.28)	0.51 (0.55)	0.06 (0.12)
45 **9,17C33**	3.78 (2.17)	3.2 (1.7)	1.6 (1.65)	0 (0)	0 (0)	0.01 (0.03)	0.12 (0.07)	0.12 (0.09)	0.01 (0.03)
46 **10,14+10,16 C34**	0.8 (0.63)	0.3 (0.28)	1.1 (1.39)	0 (0)	0.01 (0.02)	0 (0)	0.02 (0.03)	0.01 (0.02)	0 (0)
	100	100	100	100	100	100	100	100	100
Total Alkanes	15.51 (7.99)	28.33 (12.62)	37.26 (12.58)	26.93 (10.03)	34.14 (11.01)	38.59 (12.5)	16.53 (8.66)	22.33 (10.42)	34.66 (19.19)
Methyl alkanes	21.78 (8.99)	18.57 (11.3)	19.43 (15.85)	38.18 (23.41)	23.09 (10)	24.81 (13.42)	42.41 (12.16)	38.77 (13.41)	32.98 (20.76)
Dimethyl alkanes	59.77 (20.49)	45.28 (23.27)	32.27 (26.37)	28.85 (23.66)	31.23 (21.03)	26.92 (18.63)	36.14 (12.7)	31.69 (12.55)	20.98 (17.15)
T**rimethyl alkanes**	0 (0)	0.02 (0.07)	0 (0)	1.33 (0.63)	1.24 (0.41)	0.88 (0.54)	0 (0)	0 (0)	0 (0)
Alkenes	2.94 (1.66)	7.79 (3.24)	11.05 (3.85)	4.71 (2.42)	10.29 (2)	8.8 (3.39)	4.92 (2.9)	7.2 (3.96)	11.38 (7.28)

CAPÍTULO 4

En preparación

Mitochondrial COI reveals population isolation in a vulnerable slave-making ant

En este trabajo tratamos de evaluar la dispersión de las hembras y el tiempo en que se produjo el aislamiento poblacional de *Rossomyrmex minuchae*, una especie esclavista vulnerable con poblaciones pequeñas y aisladas, endémica del sureste español. Para ello analizamos un fragmento del gen mitocondrial COI y encontramos que las poblaciones estaban muy diferenciadas entre sí y no compartían ningún haplotipo. El número de haplotipos dentro de cada población fue bajo, lo que pudo influir en la no detección de aislamiento por distancia, el escenario más plausible dado el reducido tamaño poblacional y la posible influencia de factores abióticos en la usurpación de hormigueros. Nuestros resultados sugieren que el aislamiento poblacional parece haber ocurrido en los dos periodos interglaciales anteriores. Su conservación se vuelve todavía más difícil ya que cada población podría ser considerada como una unidad evolutiva independiente.

Mitochondrial COI reveals population isolation in a vulnerable slave-making ant

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ABSTRACT

In this study we try to assess female dispersal and time since population isolation in *Rossomyrmex minuchae*, a vulnerable slave-making ant with small isolated populations endemic to south eastern Spain. To do so we analysed a fragment of the mitochondrial gene COI and found that populations were highly differentiated from each other and shared no haplotypes. Within populations the number of haplotypes was low, what could influence in failing to detect isolation by distance, the most plausible scenario given the reduced population sizes and the possible influence of abiotic conditions on colony usurpation. Our results suggest that population isolation seems to have occurred in the two previous interglacial periods. Conservation becomes more difficult as each population may be considered as an evolutionary independent unit.

KEY WORDS: *Rossomyrmex minuchae*, COI, female dispersal, population isolation.

INTRODUCTION

Social insects, as ants, are more prone to population declines and extinction than non socials due to two main factors (Pamilo and Crozier 1997; Champan and Bourke 2001): (1) their effective population sizes are not proportional to their biomass because only few individuals will reproduce (in monogynous species the effective population size is equal to the number of nests since there is in each nest a unique fertile queen); (2) ants are haplodiploid, this condition imposes that males are haploid, thus reducing genetic variation and favouring the effects of genetic drift (Hedrick and Parker 1997; Frankham et al. 2002). One way to avoid this situation is to disperse from the natal nest in order to enhance the opportunities to mate with a non relative. That is the typical rule for ants, showing winged males and females that usually perform nuptial flights (Bourke and Franks 1995). However, notable

exceptions can be found in many polygynous species in which females are wingless and present a strong phylopatry (daughter queens remain in their natal nests or found new colonies by budding: nearby and accompanied by some workers), leaving the task of dispersal to males (e.g. Chapuisat et al. 1997; Gyllenstrand and Seppä 2003; Seppä et al. 2006). This behaviour can easily lead to local genetic differentiation or viscosity (e.g. Seppä and Pamilo 1995; Chapuisat et al. 1997). In other ant groups males are the wingless sex and show intranidal mating, being in some cases the females the dispersers (e.g. Heinze et al. 2007).

Rossomyrmex minuchae is an endemic slave-making ant with small and isolated populations in three high mountains (above 2000 m.a.s.l.) of south eastern Spain. The species is considered as vulnerable by the IUCN Red List of Threatened Species and as endangered in the Red Book of Spanish Invertebrates (Martínez-Ibáñez et al. 2006). Then, it is necessary an exhaustive study of its population genetics to determine the most adequate conservation policies. *R. minuchae* parasitizes nests of *Proformica longiseta*, another endemic ant to the high mountains of the South East of Spain but with higher densities than the parasite (with an average of 0.06 ± 0.26 SD nest/m^2 for the host against 0.002 ± 0.002 SD nest/m^2 for the parasite; per. obs.). In *R. minuchae* both sexes are winged but males show an unusual behaviour: they fly very short distances and when detect a nest producing females, stay there until they die; in contrast, females fly after mating and when lose their wings, they continue on foot until they find a host nest to invade (Ruano and Tinaut 2005), being unknown the distance females can travel from their natal nest to the target one.

In this work we studied the genetic structure at the mitochondrial COI gene of the three known populations of *R. minuchae*. As mitochondrial DNA is maternally inherited, it can be useful to study female dispersal. Our aim was to conduct an in-depth study of mitochondrial variation using as high sample sizes as possible and including ants from all populations in order to know: (1) the population genetic variation; (2) whether populations are viscous at mitochondrial DNA; (3) whether any of the populations show shared haplotypes otherwise populations should be clearly differentiated; (4) the time of the most recent common ancestor of each population and thus how long have the populations been separated.

MATERIAL AND METHODS

Sampling and molecular analyses

Ants were collected from 2004 to 2007 and preserved in absolute ethanol. We analysed one worker per nest, having a total of 62 sampled nests (Table 1) from three populations in three mountains in south eastern Spain separated by about 60 Km: Sierra Nevada, Gador and Filabres. DNA was

extracted following the indications of the Puregene DNA Isolation Kit (Gentra Systems). For the analysis of mitochondrial DNA, we amplified a fragment of 791 bp from the mitochondrial cytochrome c oxidase subunit 1 gene using the primers COI exsecta 2F: GGATCNCCAGANATNGCTTANCCTCG and COI exsecta 2R: TAATNGCAAAAACNGCTCCTA designed by B. Holzer (University of Lausanne). PCR amplifications were carried out in a 10μL reaction containing 2.5 ng of DNA, 0.2 nM of each primer, 0.25 nM of each dNTP, 2.5mM of MgCl2 (overall) and 0.5 u of Taq DNA Polimerase. PCR products were purified with the AccuPrep PCR Purification Kit (Bioneer) and sequenced in an ABI Prism 310.

Table 1. Number of sampled nests (N), number of haplotypes (nh), haplotype diversity (hd), nucleotide diversity (π) of COI in each population and GenBank accession numbers for the mitochondrial sequences.

Population	N	nh	hd	π (%)	GenBank accession nos.
Gador	28	3	0.315	0.048	GU147935, GU147936, GU181201
Filabres	8	2	0.25	0.032	GU181202, GU181203
Sierra Nevada	26	2	0.077	0.01	GU147937, GU181204

Statistical procedures

Chromatograms were first checked by eye for base call accuracy using the program Chromas Lite 2.01 (Technelysium Pty Ltd). Alignment of the sequences was conducted with ClustalW using BioEdit version 5.0.6 (Hall 1999). Haplotypes were identified with the aid of the software DNASP v. 5 (Librado & Rozas 2009). The resulting mitochondrial DNA haplotypes were analysed for sequence variation using the software ARLEQUIN 2.000 (Schenider et al. 2000). A minimum spanning tree for haplotypes was constructed using pairwise differences. Genetic structuring was assessed by conducting an analysis of molecular variance (AMOVA, Excoffier et al. 1992) and significance was obtained by performing 1000 permutations of data. Overall as well as pairwise differentiation between populations (Φ_{ST}) were estimated by permuting haplotypes between populations. Also, Tajima's test of selective neutrality was calculated under the infinite sites model. Mantel tests with 10000 permutations were performed for each population to investigate for genetic viscosity (a matrix of geographical distances against a matrix of pairwise genetic distances) with the software FSTAT 2.9.3.2 (Goudet 2002). The time of the most recent common ancestor (tmrcm) was estimated using the program BEAST v.1.4.8 (Drummond and Rambaut 2007). Divergence time was set to 1.5% (as calculated by Quek et al. 2004) and the most appropriate substitution model inferred from MODELTEST 3.7 (Posada and Crandall 1998). Two independent 10,000,000 steps MCMC analyses were performed and convergent chains were combined and analysed with TRACER v. 1.4.1 (Rambaut and Drummond 2007).

RESULTS

Molecular variation

All sequences were translated into amino acids according to the invertebrate mitochondrial genetic code and no stop codons were detected. We found a total of 7 mitochondrial COI haplotypes ranging 2-3 in each population (Table 1), usually with a most frequent one and another extremely rare (Fig. 1). Not a single haplotype was found in more than one population. The number of polymorphic sites was 28 whereas the number of substitutions was 29. No indels were found. Base composition was A-T biased (67.6%) with the highest proportion at the second codon position (81.2%). Tajima's test did not detect any deviation in the distribution of variation from expected under a model of neutral infinite alleles.

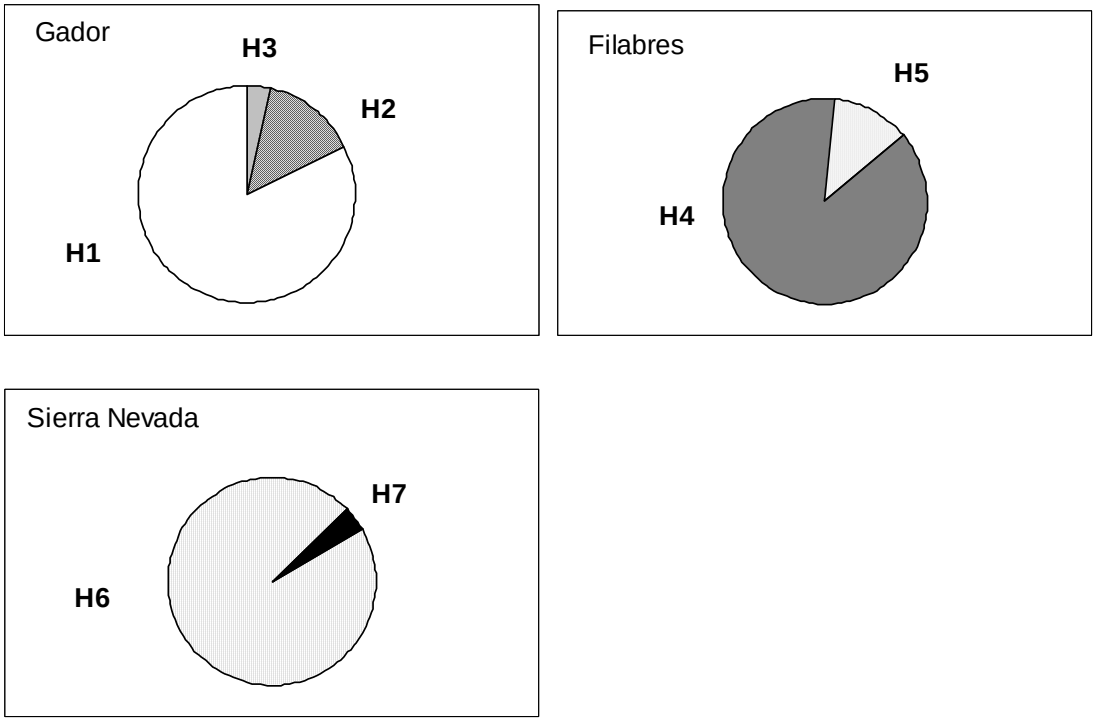

Fig. 1. Haplotype frequencies in each population.

Population structure

Haplotype differences between populations were highly significant (Table 2). The AMOVA analysis confirmed that 98.86 % of these differences occurred among populations ($P < 0.01$) while only a 1.14% was due to intrapopulation variation. The minimum spanning tree also grouped haplotypes in the three populations considered (Fig. 2). Mantel tests did not show evidences for genetic viscosity in

any of the populations (all $P > 0.05$), so that geographical distance was not proportional to genetic differences. The age estimate for the most recent common ancestor (tmrca) suggests that divergence occurred first in Gador approximately 226,000 years ago, during the interglacial Mindel-Riss. Sierra Nevada and Filabres seem to have diverged more recently, possibly in the interglacial Riss-Würm, with tmrca for Filabres of 152,000 years and 133,000 for Sierra Nevada.

Table 2. Pairwise Φ_{ST} (below diagonal) and their significance (above diagonal; * means $P < 0.05$)

	Gador	Filabres	Sierra Nevada
Gador		*	*
Filabres	0.984		*
Sierra Nevada	0.990	0.986	

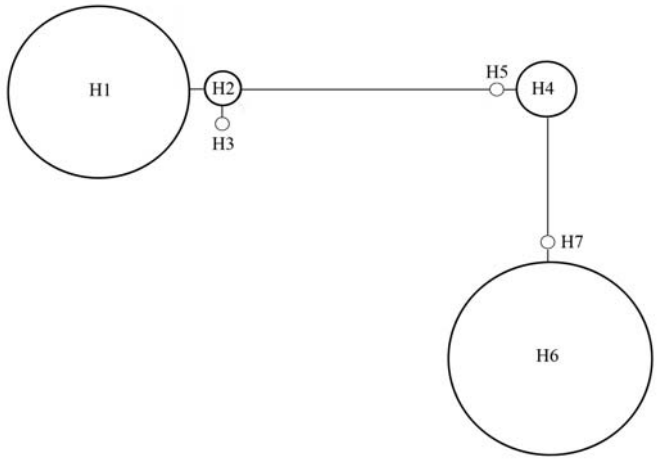

Fig. 2. Minimum spanning network of haplotypes. Length is proportional to the number of substitutions and areas of circles reflect the relative frequency of each haplotype.

DISCUSSION

In this work we analysed 62 independent nests of *Rossomyrmex minuchae* and only 7 haplotypes were found. Each population has a few private haplotypes (two or three) but within them, there is one very frequent haplotype and the rest are very rare. This suggests that populations have gone through a continued scenario of small population size and genetic drift that led to the loss of less frequent haplotypes.

Analyses of isolation by distance failed to find genetic viscosity within populations, what could be interpreted as not restricted female dispersal within populations, especially in Gador and Filabres with higher values of haplotype and nucleotide diversities (Table 1); however, this result could also be explained by a lack of power of the method given the low number of haplotypes found per population. Restricted female dispersal has been documented from other monogynous ants (and even in slave

makers) but usually in these cases genetic differentiation at short distances was evident (Foitzik and Herbers 2001; Clémencet et al. 2005). A possible explanation for a short range dispersal could be a restriction imposed by the slave making condition of *R. minuchae*: females should find a mature *Proformica* nest to initiate their own and flying too far would be disadvantageous if females land on areas where there are no host nests or their density is low, thus the possibilities of successfully invading a host nest would not be high. However, host nest density seems quite uniform in areas between 1800-2800 m of altitude (Tinaut and Fernández-Escudero 1993), so it is likely that other abiotic factors can influence the survival of *R. minuchae*. By dispersing short distances, females could increase their chances to find a host nest to invade with the adequate abiotic conditions. This comes from the fact that their maternal nest has survived in an area surrounded by other host nests from which slaves have been periodically provided; therefore, it should be more probable to find suitable host nests near the maternal nest than in far distances. On the other hand, migration between populations by females seems rather improbable due to the large geographic distances (around 60 Km) and the fact that populations do not share any haplotype.

Analyses of genetic differences reflected high differences among populations but they were very low at the intrapopulation level. Analyses of divergence time among populations supported these results, suggesting that the isolation process started before the Riss glaciation separating the Gador population and continued during the interglacial Riss-Würm leading to the separation of Filabres and Sierra Nevada populations. Note that interglacials act as a separating force in this species, living in the high mountains, like at present, whereas cold periods may lead to temporary re-union of populations on the side of the mountains, making trait mixing possible. However, Riss and Würm glaciations did not seem to have lead to the join of the three populations.

The existence of isolated and clearly differentiated populations makes the conservation of *R. minuchae* even more difficult: not only population size is a problem to cope with, but also the preservation of the singularity of each of its populations, as they possibly constitute evolutionary significant units (Moritz 1994). Crossing of individuals policies would enhance variability but would impoverish genetic differences and particularities that characterise each population.

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CAPÍTULO 5

En preparación

Population genetic structure in three host species of the ants *Proformica*

Los hormigueros del género *Proformica* son generalmente poligínicos mientras que las reinas son ápteras y crean nuevos nidos por gemación; por tanto, probablemente muestran una dispersión restringida y una fuerte estructuración poblacional. Por el contrario, el género parásito *Rossomyrmex* es monogínico, ambos sexos son alados y presentan fundación independiente de colonias. Las hormigas esclavistas dependen de sus especies hospedadoras para sobrevivir y por tanto su impacto deberá estar constreñido a la densidad de nidos hospedadores. En este trabajo investigamos la estructura poblacional en tres especies hospedadoras de *Rossomyrmex* (*P. longiseta* en el sureste de España, *P. korbi* en Turquía central y *P. sp* en el sureste de Kazajstán) con distintos patrones de distribución y número de reinas para ver si distintos perfiles de distribución (parcheado y continuo) reflejarían diferencias en la genética poblacional y las implicaciones de la estructura poblacional de las hospedadoras sobre sus esclavistas. Encontramos que la distribución geográfica influye enormemente en la estructura poblacional de *P. longiseta* aunque la diversidad genética aparentemente no se ve afectada. Esto implica que el parásito solo podría verse afectado si estas diferencias genéticas entre poblaciones están relacionadas con algún cambio en la susceptibilidad a ser esclavizadas.

Population genetic structure in three host species of the ants *Proformica*

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ABSTRACT

Nests of the genus *Proformica* are generally polygynous whereas queens are wingless and create new nests by budding; therefore they are likely to show restricted dispersal and strong population structure. In contrast, the parasite genus *Rossomyrmex* is monogynous, both sexes are winged and show independent colony founding. Slave-making ants depend on their host species to survive and thus their impact should be constrained by host natural history and genetical structure. We investigated the population genetic structure in three host species of *Rossomyrmex* (*P. longiseta* in Southeastern Spain, *P. korbi* in Central Turkey and *P. sp* in Southeastern Kazakhstan), with different distribution patterns and number of queens, to see whether different distribution profiles (patchy and continuous) would reflect differences at population genetics and the implications of the host population structure on their slavemakers. We found that the geographic distribution strongly influences the population structure of *P. longiseta* although genetic diversity is apparently not affected. This implies that the parasite might only be affected if these genetic differences among populations are related to any change in their susceptibility to be enslaved.

KEY WORDS: *Proformica*, genetic structure, host, slave-making ant, polygyny, *Rossomyrmex*

INTRODUCTION

In social insects, especially ants, dispersal strategy is usually closely associated with social organization. In polygynous species (multiple queen colonies), queens commonly mate near their maternal nests and found new colonies by budding (Keller 1991; Ross and Carpenter 1991; Pamilo et al. 1997), a process whereby young mated queens leave their natal nest by feet and start a new colony nearby with the help of a worker force. This results in a low dispersal rate and a strong structuring of ant populations (Ross and Keller 1995; Seppä and Pamilo 1995; Ross 2001). This strategy presumably evolved in habitats which pose high costs on queens that attempt to found a colony independently, like in cold climates or under nest site limitation (Buschinger 1986; Bourke & Franks 1995; Brandt et al. 2005). In contrast, in monogynous species, nuptial flight and independent colony founding are the normal traits, leading to no population structuring.

Proformica is an ant genus of around 26 species typical from arid habitats in Central Asia and the Mediterranean area (Dubovikoff 2005; Bolton et al. 2006). Within these areas this genus usually appears in locations characterized by a strong seasonality (hot and dry summers in contrast with long and cold winters) that imposes a considerable hibernation period (Marikovsky 1974; Fernández-Escudero et al. 1998). The genus is usually polygynous, although some species are mostly monogynous (Tinaut et al. in press). Consistent with frequent polygyny, the genus *Proformica* presents wingless queens and founds new colonies by budding (Fernández-Escudero and Tinaut 1999; Fernández-Escudero et al. 2001), and female dispersal is likely restricted. Dispersal restriction may lead to genetic differentiation among geographical locations. This could shape a marked population structure. By contrast, males present a normal winged development and thus most gene flow would depend on them. An interesting feature of the genus *Proformica* is that at least four species are hosts of the slave-making ants *Rossomyrmex*. Social parasites are eusocial species that exploit the social system of another such a species (Buschinger 1986; Hölldobler and Wilson 1990). In social parasitism, host and parasite usually have more similar population parameters than in other types of parasitism, as in the case of bacterial microparasites (Brandt et al. 2005). A social parasite is considered slavemaker when parasite workers perform raids to replenish the labor force (Buschinger 1986; Blatrix and Herbers 2003). In slavemaking ants, frequent slave raids exert strong selection pressures on their hosts (Fischer-Blass et al. 2006), but on the other hand parasites are dependant on host density as its colonies constitute future supplies. The

ants *Rossomyrmex* seem to be not very aggressive against host adults: do not kill host workers unless hosts fight them, what would permit nests to survive after raids (Marikovsky 1974; Zamora-Muñoz et al. 2003).

Proformica longiseta is an endemic polygynous ant from the South East of Spain parasitized by *Rossomyrmex minuchae*. Its distribution is patchy, restricted to the mountains higher than 1800m (Fernández-Escudero and Tinaut 1999). *R. minuchae* is strictly monogynous with independent colony founding and winged males and females (Ruano and Tinaut 2005). Sanllorente et al. (submitted) found strong genetic differentiation among three populations of *R. minuchae* in three separated mountains; however, for *P. longiseta* previous studies suggested some genetic structuring but without isolation by distance in one locality within Sierra Nevada (Seppä et al. 2006), nevertheless no genetic study has focused in all its distribution area. The study of the genetic structure of host and parasite is relevant as it allows comparison of population traits between two genera with similar recent evolutive histories but with different reproductive strategies that interact. For instance, a marked genetic structure in the host could be a response of local adaptation to the parasite, what at the same time, could hinder the parasite to adapt fast enough to the host, as they are engaged in a coevolutionary arms race (Zamora-Muñoz et al. 2003). In addition, the other *Proformica* host species are basically unknown; they inhabit open steppes in Central Asia and are presumed to have quite big and continuous distributions (Marikovsky 1974), but no study on their genetic structure has been carried out yet.

The goals of this paper are three-fold: (1) to study the genetic variability and population differentiation through the whole distribution range of *P. longiseta*; (2) to make a first attempt to assess the population genetics of *P. korbi* (from Turkey) and *P. sp* (from Kazakhstan), with continuous distributions, and (3) to compare the Spanish and the Asian host species in order to determine whether different distribution profiles (patchy and continuous) imply differences at population. Our results will be discussed in the light of what is known for the parasite and their possible consequences in conservation (low genetic diversity in hosts could imply a lower evolutionary potential whereas strong population differences could create behavioural and/or physiological barriers among populations, thus limiting parasite expansion).

MATERIAL AND METHODS

Field work

A total of 106 nests in eleven populations of *P. longiseta* were collected in summer 2006 from four different mountains in Southeastern Spain: Sierra Nevada, Sierra de Gador, Sierra de los Filabres and Sierra de Cazorla (Fig. 1; Table 1). Mean number of nests sampled in each of these populations was 9.64 (range 7-10). The populations studied are separated from 15 to 100 Km and were named as P followed by a number. In the case of *P. korbi*, 8 nests were sampled from two different Turkish localities (BB and ZT) distant by about 500 Km in May 2006 and 2008 (Fig 2; Table 1). Finally, 18 nests of *P. sp* were sampled in two sites of Kazakhstan (Fig.2; Table 1) separated by about 100 Km (BK and CC) in June 2007. Five to eight ant workers were collected from each nest with an aspirator and stored in absolute ethanol.

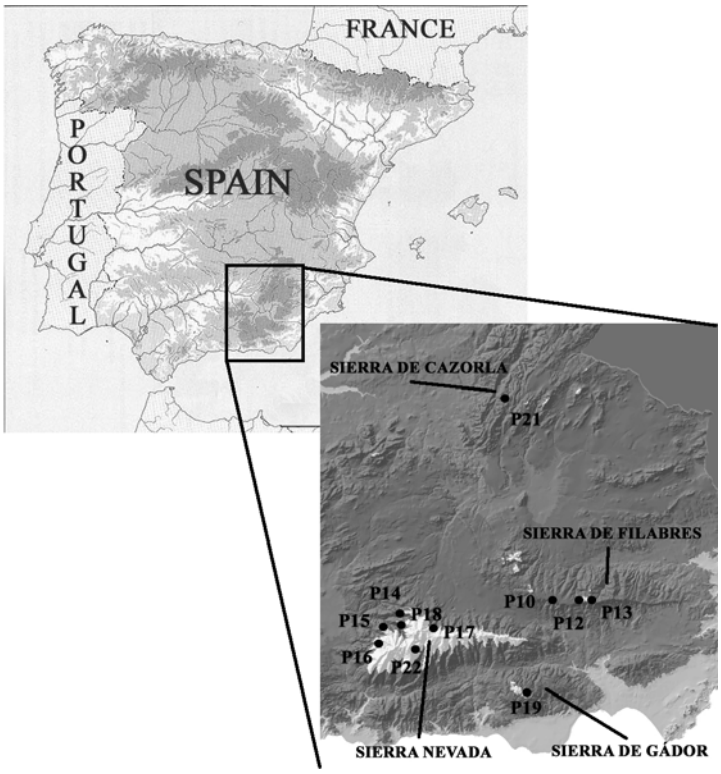

Fig. 1. Sampling localities for *Proformica longiseta*.

Table 1. Sampling sites, number of nests (N), number of workers, allelic richness (A), gene diversity (H_S) and inbreeding coefficient (F_{IS}).

Species	Location	Populations	N	Workers	A ± SE	Hs ± SE	Fis
P. longiseta	Sierra Nevada	P14,P15,P16,P17,P18,P22	60	300	2.725 ± 0.283	0.609 ± 0.085	0.047
	Sierra de Gádor	P19	7	35	2.772 ± 0.263	0.722 ± 0.074	0.070
	S. Filabres.	P10,P12,P13	29	145	2.316 ± 0.291	0.526 ± 0.102	0.037
	S. Cazorla	P21	10	50	2.248 ± 0.313	0.526 ± 0.127	0.047
P. korbi	Belembaşi beli	BB	3	15	2.417 ± 0.235	0.657 ± 0.084	0.127
	Ziyaret teppesi	ZT	5	25	2.957 ± 0.180	0.790 ± 0.050	0.059
P. sp	Big Kalkan	BK	12	96	2.706 ± 0.258	0.698 ± 0.083	0.123
	Charyn canyon	CC	6	48	2.454 ± 0.335	0.603 ± 0.113	0.205

Fig. 2. Study sites of *P. korbi* (Turkey) and *P. sp* (Kazakhstan).

Molecular analysis

Total DNA was extracted from workers using the Puregene DNA Isolation Kit (Gentra Systems). *Proformica* workers were genotyped at seven microsatellite loci using primers from *Cataglyphis cursor*: Ccur11, Ccur26, Ccur63b and Ccur76 (Pearcy et al. 2004), *Formica exsecta*: FE19 and FE37 (Gyllenstrand et al. 2002) and *Formica lugubris*: FL12 (Chapuisat 1996). Microsatellites were amplified by PCR as in Tinaut et al. 2009 (in press). PCR products were genotyped at Unidad de genómica Scai (University of Córdoba) using an ABI310 sequencer. We scored genotypes using GENESCAN software v 3.7.

Mitochondrial DNA variation was studied in the cytochrome oxidase subunit 1 region (COI). We used primers developed for *Formica exsecta* designed by B. Holzer (University of Lausanne): COI exsecta 2F: GGATCNCCAGANATNGCTTANCCTCG and COI esxecta 2R: TAATNGCAAAAACNGCTCCTA following the same PCR protocol as for

microsatellites. Sequences were obtained at Servicio de secuenciación of the department of Genetics (University of Granada) and aligned with CHROMAS LITE 2.01 (Technelysium Pty Ltd).

Genetic diversity

Given that worker nestmates are related, we constructed 1000 resampled datasets by randomly selecting one worker per nest using the program RESAMPIND (pers. comm. Jérôme Goudet) and for each dataset we tested for departure from Hardy-Weinberg equilibrium and genotypic disequilibrium. Also, gene diversity (H_S, the expected heterozigosity according to Hardy-Weinberg expectations), allelic richness and allele frequencies were calculated with FSTAT 2.9.3.2 (Goudet 2002). Allelic richness (with a standardised sample size of 2 diploid individuals) and H_S were compared among the three species with Kruskal-Wallis or ANOVA tests (depending on normality of data) and among populations within species with ANOVA or t-test using STATGRAPHICS plus 4.1.

Population structure

To investigate the genetic structure, we calculated *F*-statistics (Weir and Cockerham 1984) using FSTAT. F_{IS} and F_{ST} were estimated over all populations as well as pairwise F_{ST} for all population pairs. Means and standard errors were jackknifed over loci and significant levels were determined by randomization (1000 times) of alleles for F_{IS} and genotypes for F_{ST}. For pairwise F_{ST} 900 permutations applying Bonferroni corrections were performed. Local genetic isolation by distance was tested in *P. longiseta* by regression of a matrix of genetic distances (θ /1- θ) against a matrix of unlogged geographical distances (Rousset 1997) also with FSTAT. Significance levels were analysed using Mantel tests (Mantel 1967) for each correlation using 10000 permutations.

For *P. longiseta* mitochondrial genetic structuring was assessed by performing an analysis of molecular variance (AMOVA, Excoffier et al. 1992) grouping the samples geographically (Sierra Nevada, Sierra de Gador, Sierra de los Filabres and Sierra de Cazorla) with the program ARLEQUIN 2.0 (Schenider et al. 2000). Significance was obtained by performing 1000 permutations of data. Additionally, a minimum spanning network was constructed for all *Proformica* haplotypes

RESULTS

Genetic diversity

We found no significant deviation from Hardy-Weinberg expectations in any of the populations or evidences for linkage disequilibrium. Overall allelic richness (A) was 4.366 (± 0.830 SE) for *P. longiseta*, 3.145 (± 0.304 SE) for *P. korbi* and 4.598 (± 0.812 SE) for *P. sp* (Table 1). Mean allelic richness did not differ significantly among the three species (Kruskal-Wallis test, H = 0.208; *P* = 0.901) or among their populations (for *P. longiseta* ANOVA test, F = 0.87; P = 0.469; for *P. korbi t*-test, t = -1.823; *P* = 0.093; for *P. sp* t-test, t = -0.595; P = 0.563),). In addition, the three species showed similar levels of genetic diversity (Table 1; ANOVA test, F = 0.78; P = 0.475) and we found no population differences either (for *P. longiseta* ANOVA test, F = 1.79; P = 0.177; for *P. korbi* and *P. sp* t-tests, t = -1.005; P = 0.334; and t = 0.68; P = 0.509 respectively).

Population structure

The inbreeding coefficient (F_{IS}) was not significantly different from zero in any of the species or populations studied (all $P > 0.05$; Table 1), although *P. sp* presented tendency to heterozigous excess ($P = 0.057$ in BK and $P = 0.069$ in CC).

Estimated overall F_{ST} in *P. longiseta* was moderate and significant ($\theta = 0.185 \pm 0.036$ SE; $P = 0.002$). Some of the pairwise differences among the eleven populations of *P. longiseta* were not significant (Table 2). However, we found no association between genetic differentiation and geographic distance among the populations considered (Mantel test, $P = 0.420$). Similar values of genetic structuring, but not significantly, were detected in the populations of *P. korbi* ($F_{ST} = 0.178 \pm 0.062$ SE; $P = 0.110$) and *P. sp* ($F_{ST} = 0.128 \pm 0.05$ SE; $P = 0.075$).

Table 2. Pairwise F_{ST} in the eleven populations of *P. longiseta* studied (above diagonal) and their significances (below diagonal). NS means not significant differences while * means $P < 0.05$.

	P10	P12	P13	P14	P15	P16	P17	P18	P19	P21	P22
P10	0	0.0996909	0.1495476	0.3140734	0.2397168	0.2295908	0.3083737	0.2042091	0.2719894	0.2573525	0.3453744
P12	*	0	0.0200449	0.2524371	0.1829202	0.1854586	0.243925	0.1493655	0.2181697	0.2290235	0.2763598
P13	*	*	0	0.2629388	0.2028515	0.1959013	0.2688509	0.1663812	0.2181026	0.2489942	0.2871496
P14	*	*	*	0	0.150201	0.1417137	0.0707947	0.0613981	0.0830398	0.2065283	0.2417697
P15	*	*	*	*	0	0.1064998	0.112104	0.0376351	0.1548468	0.1379182	0.2576929
P16	*	*	*	*	*	0	0.1146212	0.1013352	0.0991886	0.135876	0.1752708
P17	*	*	*	*	*	*	0	0.0616465	0.083157	0.1644225	0.1798687
P18	*	*	*	NS	NS	*	*	0	0.0815905	0.1532368	0.2008985
P19	NS	*	NS	NS	NS	NS	*	NS	0	0.168417	0.1970197
P21	*	*	*	*	*	*	*	*	NS	0	0.2697089
P22	*	*	*	*	*	*	*	*	NS	*	0

A single mitochondrial haplotype was found in each population of *P. longiseta* studied and therefore the analysis was performed at two levels (among populations and among geographical groups). The hierarchical AMOVA analysis revealed that most of the mitochondrial variation was due to differences within the geographical groups considered (98%) and not among them (Table 3). In agreement with this, the haplotype network (Fig. 3) showed no relationship between haplotypes and concrete mountainous locations. Therefore, mitochondrial analyses are in accordance with microsatellite results, confirming population structuring but not because of a strong geographical effect. For *P. sp* three haplotypes were found in BK and two in CC while *P. korbi* showed population-specific haplotypes (Fig. 3).

Table 3. Two-level hierarchical analyses of mitochondrial molecular variance (AMOVA) in *P. longiseta*. No haplotype variation was found within populations.

	d.f.	Percentage variation	*P*
Among geographical groups	3	1.95	0.391
Among populations within groups	8	98.05	<0.001

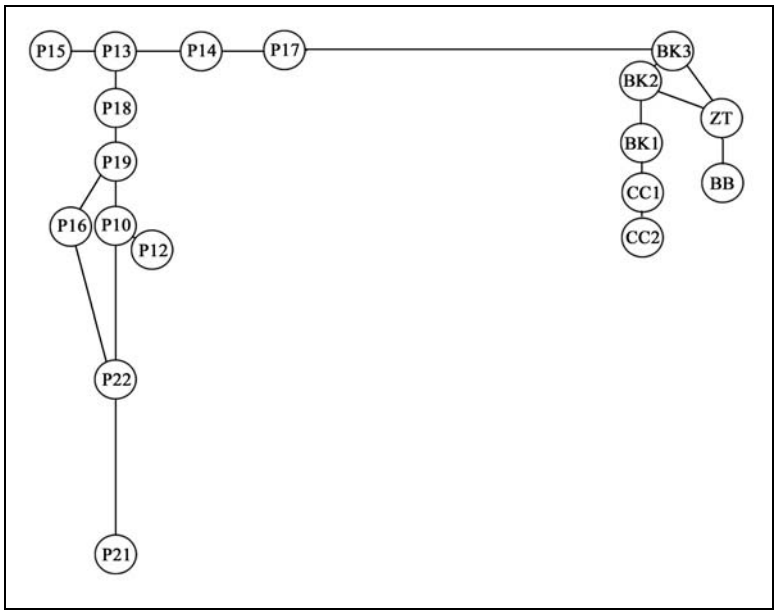

Fig. 3. Minimum spanning network of *Proformica* haplotypes. Length of the branches is proportional to genetic distance.

DISCUSSION

Population structure of *Proformica longiseta*

Nuclear and mitochondrial genetic variation in *P. longiseta* was studied across all of its distribution range. Our results revealed moderate and significant overall genetic structuring (F_{ST} = 0.185) among the eleven populations, however a geographic distribution of these differences was not at all evident. This lack of geographical pattern of genetic differentiation is also supported by the absence of isolation by distance among the populations studied (P = 0.42) and by the AMOVA analysis, pointing that 98% of the mitochondrial genetic structuring is due to differences within geographical groups (Table 3). Local differences are probably a consequence of the colony founding behaviour of the species, given that budding promotes restricted queen dispersal (Bourke & Franks 1995). As males are winged, they are supposed to disperse farther than females, however, it would not be sufficient to genetically homogenize populations separated at least 15 Km (our minimum sampling range). The observed pattern of genetic structuring in *P. longiseta* widely differs from that found for its parasite, *R. minuchae* which was highly influenced by geographic isolation (Sanllorente *et al* submitted). Population structure in *P. longiseta* could be explained by a higher genetic diversity in an ancient continuous population than for the parasite; afterwards when this ancient population was fragmented in the present four isolated sierras, genetic variability was better maintained in the host species due to the higher population size. The lack of significant differentiation among sierras may reflect that genetic drift has not been powerful enough to select for private alleles (in the case of nuclear DNA) or to establish strong differences among sierras (in mitDNA). Therefore, it is not surprising that some populations at separated sierras share more alleles by chance than others closer geographically; a similar situation was found in island populations of *Formica exsecta* (Sundström et al. 2003), where there was isolation by distance within islands but not among them. Despite the patchy distribution, *P. longiseta* does not show evidences for any important reduction in genetic diversity as reflected in allelic richness, H_S and the coefficient of inbreeding.

Population structure of Asian *Proformica* species

Proformica korbi presents similar levels of gene diversity and allelic richness to *P. longiseta*. In contrast, we found no significant genetic differences between both populations (F_{ST} = 0.178; P = 0.110). These results would agree with a more continuous distribution than in *P. longiseta* despite dependent colony founding. The haplotype network (Fig. 3) shows that *P. korbi* and *P. sp* are closer whereas *P. longiseta* is quite distant, which would reflect a more recent differentiation between both Asian species than between these and the Spanish.

In *Proformica sp* nuclear genetic results are similar to those in *P. korbi* and *P. longiseta*, which is interesting given that the Kazak species is apparently monogynous (Tinaut et al. in press) and thus is expected to have a different genetic structure from its polygynous counterparts. More concretely, single queen colonies are predicted to form panmictic populations with no inbreeding or genetic structure (Bourke and Franks 1995; Pamilo et al. 1997). However, this species would not behave as a typical monogynous ant given that its queens are presumably wingless and therefore dispersal would be as restricted as in the polygynous species of *Proformica*. A similar pattern of restricted female dispersal has also been found in *Cataglyphis cursor* (Clémencet et al. 2005; Pearcy and Aron 2006), a monogynous ant species related to the genus *Proformica* (Hasegawa et al. 2002). Moreover, *P. sp* presents slight differences in the inbreeding coefficients, which although not significantly higher than zero (F_{IS} = 0.123 in BK and 0.205 in CC), were close to deviate in both populations. This suggests that some queens may mate with related males, which could be due to restricted male dispersal. Anyway, further ecological studies are needed to asses mating behaviour in *P. sp* and thus explain this apparent tendency to exceed the proportion of heterozygous individuals. Finally, it is remarkable that this species is monogynous despite living in an arid and extreme habitat, where polygyny is assumed to be more adaptive (Buschinger 1986; Bourke & Franks 1995; Brandt et al. 2005).

Comparison of Asian and the Spanish *Proformica*

To the light of our results, we found evidences supporting that the geographic pattern strongly influences the population structure of the genus *Proformica*, as reflected in the lack of population differentiation at microsatellites in both Asian species with continuous

distributions. In contrast, *P. longiseta* is spread in mountains with subpopulations separated by microgeographical barriers (valleys and rivers), thus making dispersal more difficult, even for winged males. However, population differentiation has not leaded (by now) to a decrease in genetic variation or evidences of inbreeding.

Implications for the slave makers

In general, the observed levels of genetic variation in the three species of *Proformica* are lower than those found in other host species as *Leptothorax* (Blatrix and Herbers 2003; Fischer-Blass et al. 2006), a trait that could be linked to the different population size and nest density of both genera.

Rossomyrmex minuchae, the species parasitizing *P. longiseta* shows levels of genetic variation typical for a social parasite (Sanllorente et al submitted) and this situation is not likely to decline because of the genetic diversity of its host. Our results evidence that *P. longiseta* populations show normal levels of genetic variation (with no evidences for deviations from expected under random mating) also implying that its distribution should be quite continuous. Also, as the habitat where it lives has almost no economical interest and is usually within protected areas, the probability of degradation is low and thus the maintenance of the distribution range would be easily fulfilled. However, it remains unclear whether the differences among populations detected in *P. longiseta* would affect their susceptibility to be enslaved, in fact only some small populations of P. longiseta are parasitized.

The Turkish slave maker (*R. anatolicus*) is likely to be spread (Sanllorente *et al* submitted) and as evidenced in this study, should have no problems to find host nests. *P. korbi* inhabits steppes in Central Turkey of little human interest, so its populations are not at a high fragmentation or degradation risk. *P. sp* appears to present large populations, some of them in protected areas (BK) or at natural interest sites (CC) leading to some extent future maintenance. Consequently, the parasite *R. quandratinodum* may not be affected by the genetic structure of its host. However, a more precise study on mating behaviour is needed to exclude a possible inbreeding depression in *P. sp*.

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CAPÍTULO 6

En preparación

Phylogeography of the slave-making ants *Rossomyrmex* and their *Proformica* hosts

El género de hormigas *Proformica* es muy común en los semi-desiertos Euroasiáticos y unas pocas especies son parasitadas por las hormigas esclavistas *Rossomyrmex*. Sin embargo, la relación filogenética entre parásita y hospedadora aún no está aclarada y la filogenia de cada grupo tampoco es conocida. El propósito de este trabajo consiste en dos objetivos principales: (1) determinar si el género parásito deriva del hospedador de las emparentadas hormigas desérticas *Cataglyphis*; (2) determinar el centro de origen de *Proformica* y *Rossomyrmex*, que predecimos estaría en Asia central dado el elevado número de especies en esta región. Nuestros resultados nuestra predicción para el área ancestral pero fallan al discernir una relación clara entre los tres géneros emparentados; aún así, hospedadoras y parásitas son géneros monofiléticos mientras que algunas especies ibéricas de *Proformica* no se diferencian bien con COI.

Phylogeography of the slave-making ants *Rossomyrmex* and their *Proformica* hosts

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ABSTRACT

The ant genus *Proformica* is very common to Eurasian semi-deserts and a few species are parasitized by the slave making ants *Rossomyrmex*. The phylogenetic relationship between host and parasite remains unclear and the phylogeny within each genus is currently unknown. The aim of this work was two-fold: (1) to determine whether the parasite genus descends from the host or from the related desert ants *Cataglyphis* and (2) to determine the center of origin of both *Proformica* and *Rossomyrmex*, which we predict to be Central Asia given the higher number of species in this area. Our results confirmed our prediction for the ancestral area but failed to discern a clear relationship among the three related genera; nevertheless hosts and parasites are monophyletic genera whereas some Iberian *Proformica* species are not well differentiated for COI.

KEY WORDS: Phylogeography, *Rossomyrmex*, *Proformica*, *Cataglyphis*, COI.

INTRODUCTION

In the Miocene (- 23.8 to –5.3 Mya) global biological change consisted in the expansion of open vegetation systems mainly represented by grasslands (Jacobs et al. 1999; Retallack 2001). This was due to a general drying process of the climate that meant an increase in aridity especially in Eurasia, helped by the uplift of the Himalayas and the closure of Tethys (Crowley and North 1991; Culver and Rawson 2000). Species associated to arid habitats were then favoured and likely to spread throughout the affected territory, in this case from Eastern Eurasia to Western Mediterranean (Strömberg et al. 2007); these migration flows were maintained in several episodes until the transition Pliocene-Pleistocene (-1.8 Mya) (Arribas et al. 2001). The invasion of central asian elements to western Europe

before the Pleistocene has been also demonstrated in plants (Koch et al. 2006). In these migration flows, not only big vertebrates or plants migrated, but also undoubtedly many insects. One of these could be the ant genus *Proformica*, a forager very common and highly adapted to the Eurasian semi-deserts. Frequently used criteria for determining the center of origin of a taxon are the greatest variety of forms (species) and density of distribution (Crisci et al. 2003). According to this, Central Asia would be the center of origin of *Proformica* given the current high levels of species richness and abundance in this area (Collingwood & Heatwole 2000; Pfeiffer et al. 2003) and from here it would colonise west, where occurs at a lower diversity and frequency. Similar patterns of decreasing number of species distributed from Central Asia to Europe are also found in other insects (Ribera and Blasco-Zumeta 1998; Omoto et al. 2003) as well as vertebrates (Illera et al. 2008).

To our knowledge there are four species of *Proformica* parasitized by ants of the genus *Rossomyrmex*, all obligate slave makers: *P. epinotalis* parasitized by *R. proformicarum* (Arnoldi 1928) in the North shore of the Caspian Sea (Russia), *P. sp* and *R. quandratinodum* (Xia and Zheng 1995) are found between China and Kazakhstan, *P. korbi* and *R. anatolicus* (Tinaut 2007) in the plains of Anatolia (Turkey) and *P. longiseta*, with a patchy distribution in high mountains of Southeastern Spain parasitized in three of them by *R. minuchae* (Tinaut 1981). The biology and population genetics of host and parasite have been specially studied in Spain (Ruano and Tinaut 1999; Zamora-Muñoz et al. 2002; Ruano et al. 2005; Ruano and Tinaut 2005; Sanllorente et al submitted), although preliminary studies have also been carried out in Kazakhstan and Turkey (Marikovsky 1974; Tinaut et al. *in press*; Sanllorente et al. *in prep*). Slave making ants are interesting species to study as they are supposed to arise from intraspecific parasitism and therefore would be generally closely related to their host species (Buschinger 1986). This phenomenon is called Emery's rule. Hasegawa *et al* (2002) provided the first genetic evidence for the phylogenetic position of *Rossomyrmex*, clearly separated from *Polyergus*, another slave maker previously considered as a sister genus (Buschinger 1990; Hölldobler and Wilson 1990; Agosti 1994). The same authors found that the genus *Proformica* was phylogenetically close to *Rossomyrmex* and to the desert ants *Cataglyphis* although their relationship within the clade remains unclear.

In the present paper we want to study two main goals: (1) the phylogenetic relationship among *Cataglyphis, Proformica* and *Rossomyrmex* to determine the ancestral genus and the origin of the parasite; (2) to infer the possible ancestral area and dispersal procedure of parasite and host.

MATERIAL AND METHODS

Sampling and laboratory procedures

Three species of *Rossomyrmex* were collected from 74 nests (Table 1); also 76 nests of *Proformica* were sampled, including hosts as well as non parasitized species. We included in the analysis sequences of four species of *Cataglyphis* (from GenBank) that contained the same region of COI considered in this study. As close genera according to Moreau et al. (2006) we considered *Formica exsecta* and *Oecophilla smaragdina*, whereas *Myrmica rubra* was used as outgroup (Moreau et al. 2006; Brady et al. 2006). Also, a *P. ferreri* GenBank sequence was added to the analysis given that we only collected from two different localities (Table 1) and a *P. nasuta* from GenBank was included too. Sampling was carried out during activity periods in summers 2006-2008 and ants were preserved in 99% alcohol. For genetic procedures only one individual per nest was analysed.

Table 1. Species, number of nests sampled and locality (or Genebank access number) used for analyses.

Genus	species	No. nests	Locality/Genebank accession number
Rossomyrmex	*minuchae*	26	Sierra Nevada (Spain)
	minuchae	30	Sierra de Gador (Spain)
	minuchae	8	Sierra de Filabres (Spain)
	anatolicus	4	Belembasi beli (Turkey)
	anatolicus	3	Ziyaret tepesi (Turkey)
	quandratinodum	3	Charyn canyon (Kazakhstan)
Proformica	*longiseta*	26	Sierra Nevada (Spain)
	longiseta	9	Sierra de Gador (Spain)
	longiseta	6	Sierra de Filabres (Spain)
	longiseta	2	Sierra de Cazorla (Spain)
	korbi	3	Belembaşi beli (Turkey)
	korbi	5	Ziyaret tepesi (Turkey)
	kazakhstan	12	Big Kalkan (Kazakhstan)
	kazakhstan	6	Charyn canyon (Kazakhstan)
	kazakhstan	1	Charyn river (Kazakhstan)
	ferreri		AB010935.1
	ferreri	1	Sierra Tejeda (Spain)
	ferreri	4	Guadalajara (Spain)
	nasuta		DQ353311.1
	portugal	4	Serra da Estrela (Portugal)
Cataglyphis	*ibericus*		AB010939.1
	floricola		AB010936.1
	rosenhaueri		AB010933.1
	velox		AB010932.1
Formica	*exsecta*	1	Buriatia (Russia)
Oecophilla	*smaragdina*		AB503208.1
Myrmica	*rubra*		DQ074387.1

Total DNA was extracted following the indications of the Puregene DNA Isolation Kit (Gentra Systems). For the analysis of mitochondrial DNA, we amplified an internal region of 576 pb of the COI gene using the primers COI exsecta 2F: GGATCNCCAGANATNGCTTANCCTCG and COI esxecta 2R: TAATNGCAAAAACNGCTCCTA designed by B. Holzer (University of Lausanne). PCR amplifications were carried out in a 10μL reaction containing 2.5 ng of DNA, 0.2 nM of each primer,

0.25 nM of each dNTP, 2.5mM of MgCl2 and 0.5 u of Taq DNA Polimerase. PCR conditions were 94ºC for 3 min to denature the samples, followed by 11 touch down temperature cycles of denaturing at 94ºC for 15 s, annealing at 55-45ºC for 30 s and extension at 72ºC for 30s, followed by 30 cycles at 94ºC for 15s, 50ºC for 15s and 72ºC for 30s.PCR products were purified with the AccuPrep PCR Purification Kit (Bioneer) and sequenced in an ABI Prism 310.

Data analysis

Chromatograms were first checked by eye for base call accuracy using the program Chromas Lite 2.01. Alignment of the sequences was conducted with ClustalW using BioEdit version 5.0.6 (Hall 1999). The most suitable substitution model was determined using MODELTEST 3.7 (Posada and Crandall 1998) that compares 56 models of DNA substitution. Sequence variation and substitution patterns were analysed with the program MEGA version 4 (Tamura et al. 2007) as well as the Neighbour-Joining tree. Maximum likelihood (ML) trees were performed with PAUP* version 4.0 b10 (Swofford 2003) and node support was assessed with 100 bootstrap replicates. Bayesian analyses were carried out using MrBayes version 3.1(Ronquist and Huelsenbeck 2003) and ran for 500,000 generations with trees sampled each 250 generations.

The most probable ancestral area and sequence of dispersal was estimated with the program DIVA v. 1.1 (Ronquist 1996), which considers the possibility of vicariance, dispersal and extinction events. Bayesian topology was used to estimate the ancestral geographic distribution based on current distributions. We defined five geographical distributions and each sample was assigned to one of them: (H) Holartic, (A) Central Asia, (T) Turkey, (I) Iberian Peninsula and (B) Betic sierras. In this analysis we excluded the genera *Myrmica*, *Oecophylla* and *Cataglyphis* (given that for phylogeny we could only consider Iberian species as were the only ones with the same COI region studied in this work, and thus were not representative of the whole genus).

RESULTS

Sequence variation

All sequences were translated to amino acids according to the invertebrate mitochondrial genetic code and no stop codons were detected. Base composition was A-T biased (68%), being the third codon position the highest (88%) and the first position the lowest (56%). Over the 576 nucleotide positions, 238 were variable with 193 parsimony informative polymorphic sites. Transitions (Ti) were proportional to transversions (Tv) with an average ratio Ti/Tv of ca. 1.5 (Fig. 1). Substitutions were

most abundant at the third codon positions (77.6%) and least abundant at the second positions (3%). Excluding sites with gaps and missing, seven different haplotypes were found for *Rossomyrmex minuchae*, two for *R. quandratinodum* and a single one for *R. anatolicus*. For *Proformica longiseta* we found 13 haplotypes whereas for *P. korbi*, *P. sp* and *P. ferreri* two haplotypes were found for each species. The mean distance within the genus *Rossomyrmex* haplotypes was 0.056 (± 0.007 SE, Kimura-2 parameter) and within *Proformica* haplotypes 0.075 (± 0.007 SE, Kimura-2 parameter). Mean distances among species groups are shown in Table 2.

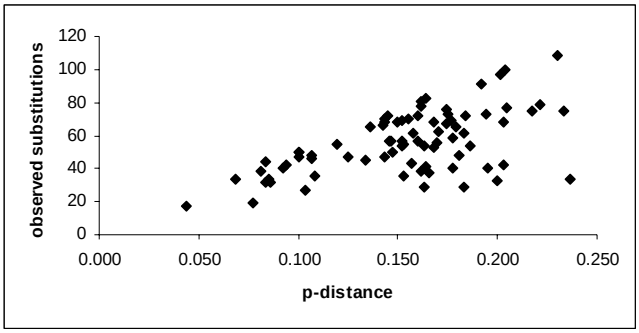

Fig. 1. Transitions plotted against transversions for all pairwise sequences and codon positions.

Phylogenetic analyses

According to MODELTEST the best model that explained our data under the Akaike information criterion (AIC) was the general time-reversible model with invariable sites and a rate of variation among sites (GTR+I+G). The NJ tree (Fig. 2) supported the monophyly of genera *Rossomyrmex* and *Proformica*, whereas polyphyly of *Cataglyphis* was suggested. *Myrmica, Formica* and *Oecophilla* were supported as outgroups. In all cases it seemed that *Cataphyphis* would be the ancestral group whereas *Proformica* and *Rossomyrmex* would be derived. The genus *Rossomyrmex* is well differentiated into the morphologically-described species, with the Kazak and Turkish species more related than the Spanish. *R. minuchae* appears clearly separated in correspondence with its isolated geographical distribution. In contrast, the genus *Proformica* is separated in five clear groups: *P.korbi, P. kazakhstan*, a group formed by two presumed *P. longiseta* haplotypes and another from Portugal, *P. nasuta* and finally a mixed clade containing *P. longiseta* and *P. ferreri* haplotypes. The bootstrap values within this mixed clade were high. A very similar result was obtained when performing trees with Bayesian inference (Fig. 3) or ML (Fig. 4), only differing in the *Proformica* mixed clade. Finally, optimal reconstruction of unconstrained analysis in DIVA inferred 9 dispersal events and the root node (genus *Formica*) included all areas. The estimated ancestral area of both genera *Proformica* and *Rossomyrmex* resulted in either Central Asia or Turkey (Fig. 5).

Table 2. Mean distances (Kimura-2) and standard errors (in parenthesis) among species groups for COI. Below diagonal using all positions and above diagonal only first and second codon positions.

	Myrmica	*P. longiseta*	*P. portugal*	*P. ferreri*	*P. sp*	*P. korbi*	*Cataglyphis*	*R. minuchae*	*R. quandratinodum*	*R. anatolicus*	*Formica*	*P. nasuta*	*Oecophylla*
Myrmica		0.058	0.060	0.067	0.057	0.066	0.069	0.084	0.079	0.079	0.066	0.057	0.069
		(0.012)	(0.013)	(0.013)	(0.013)	(0.013)	(0.013)	(0.015)	(0.015)	(0.015)	(0.013)	(0.013)	(0.013)
P. longiseta	0.195		0.013	0.023	0.024	0.017	0.032	0.050	0.051	0.051	0.039	0.021	0.063
	(0.021)		(0.004)	(0.005)	(0.007)	(0.005)	(0.006)	(0.011)	(0.011)	(0.011)	(0.001)	(0.006)	(0.013)
P. portugal	0.202	0.086		0.026	0.019	0.016	0.033	0.047	0.045	0.045	0.039	0.014	0.064
	(0.023)	(0.012)		(0.006)	(0.006)	(0.006)	(0.008)	(0.011)	(0.011)	(0.011)	(0.010)	(0.007)	(0.014)
P. ferreri	0.217	0.068	0.100		0.035	0.031	0.044	0.061	0.062	0.062	0.053	0.033	0.073
	(0.024)	(0.007)	(0.013)		(0.008)	(0.007)	(0.007)	(0.011)	(0.011)	(0.011)	(0.011)	(0.007)	(0.013)
P. sp	0.204	0.109	0.106	0.120		0.019	0.034	0.043	0.043	0.042	0.043	0.022	0.058
	(0.023)	(0.014)	(0.016)	(0.015)		(0.006)	(0.007)	(0.010)	(0.010)	(0.010)	(0.010)	(0.007)	(0.012)
P. korbi	0.195	0.085	0.081	0.100	0.094		0.031	0.041	0.042	0.042	0.036	0.025	0.058
	(0.022)	(0.011)	(0.012)	(0.012)	(0.013)		(0.007)	(0.010)	(0.011)	(0.011)	(0.010)	(0.008)	(0.012)
Cataglyphis	0.221	0.134	0.143	0.142	0.150	0.136		0.051	0.044	0.044	0.046	0.038	0.061
	(0.023)	(0.012)	(0.015)	(0.013)	(0.016)	(0.014)		(0.010)	(0.010)	(0.010)	(0.010)	(0.008)	(0.011)
R. minuchae	0.236	0.168	0.163	0.181	0.162	0.178	0.187		0.021	0.020	0.062	0.048	0.055
	(0.026)	(0.019)	(0.020)	(0.020)	(0.019)	(0.021)	(0.019)		(0.007)	(0.007)	(0.013)	(0.011)	(0.012)
R. quandratinodum	0.233	0.164	0.164	0.180	0.153	0.160	0.175	0.104		0.005	0.058	0.046	0.052
	(0.027)	(0.019)	(0.021)	(0.021)	(0.020)	(0.020)	(0.019)	(0.015)		(0.004)	(0.013)	(0.011)	(0.012)
R. anatolicus	0.230	0.153	0.145	0.158	0.146	0.147	0.160	0.077	0.044		0.052	0.045	0.051
	(0.027)	(0.018)	(0.019)	(0.019)	(0.019)	(0.019)	(0.018)	(0.012)	(0.010)		(0.012)	(0.011)	(0.012)
Formica	0.203	0.157	0.152	0.168	0.171	0.152	0.155	0.203	0.183	0.170		0.043	0.039
	(0.024)	(0.018)	(0.019)	(0.019)	(0.021)	(0.018)	(0.017)	(0.023)	(0.023)	(0.021)		(0.011)	(0.010)
P. nasuta	0.192	0.084	0.083	0.100	0.106	0.092	0.152	0.183	0.144	0.143	0.147		0.064
	(0.023)	(0.011)	(0.014)	(0.013)	(0.015)	(0.013)	(0.016)	(0.022)	(0.019)	(0.019)	(0.020)		(0.014)
Oecophylla	0.204	0.166	0.164	0.174	0.184	0.177	0.175	0.200	0.178	0.161	0.125	0.162	
	(0.025)	(0.019)	(0.020)	(0.020)	(0.022)	(0.021)	(0.018)	(0.024)	(0.022)	(0.020)	(0.016)	(0.019)	

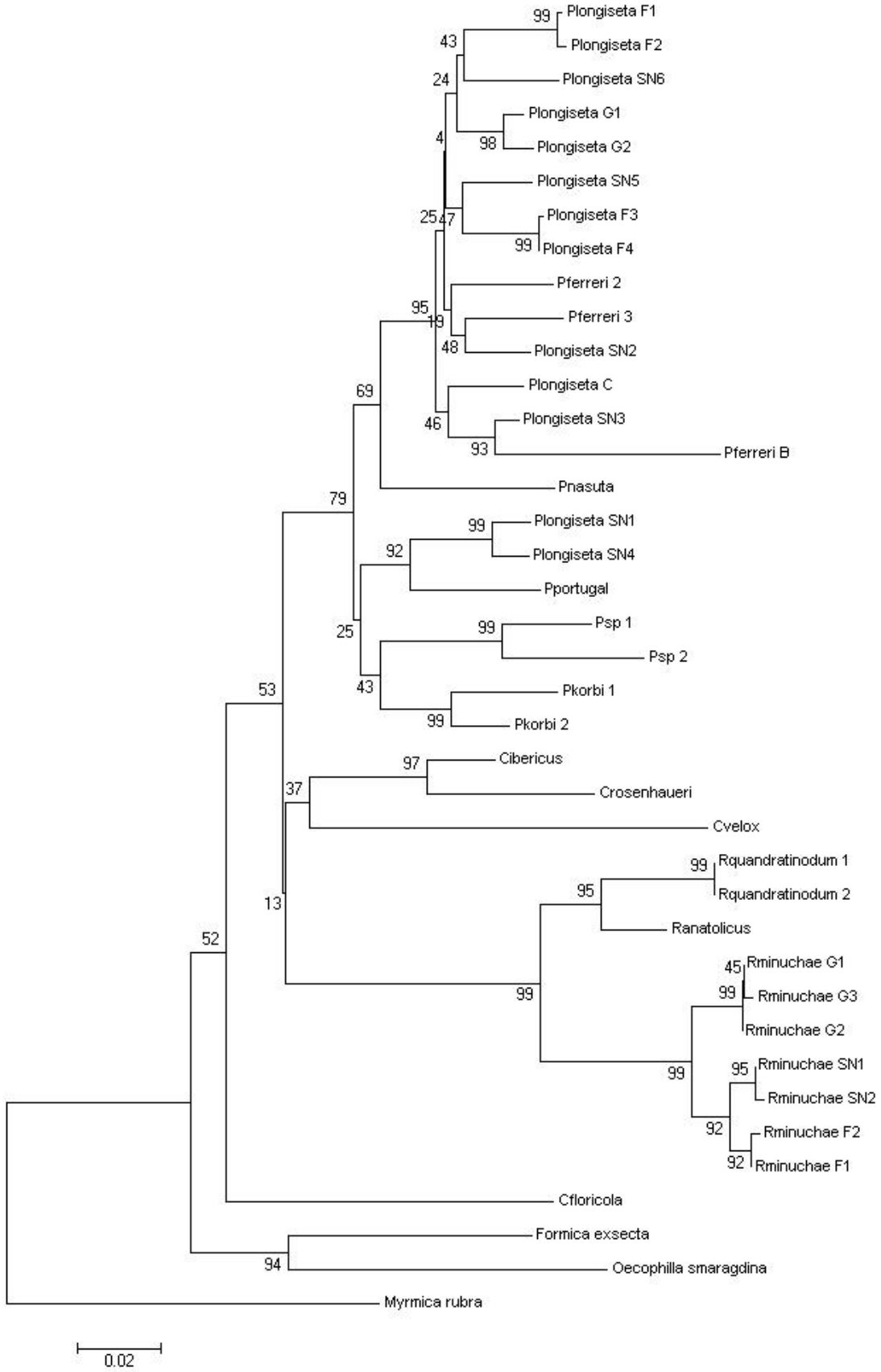

Fig. 2. Neighbour-Joining tree with bootstrap values estimated from 5000 replicates. *P. longiseta* and *R. minuchae* localities are named as SN (meaning Sierra Nevada), G (Sierra de Gador), F (Sierra de Filabres) and C (Sierra de Cazorla). Genebank sequences are marked with a final B in the haplotype name (*P. ferreri* and *F. exsecta*) or a final H (*R. minuchae*).

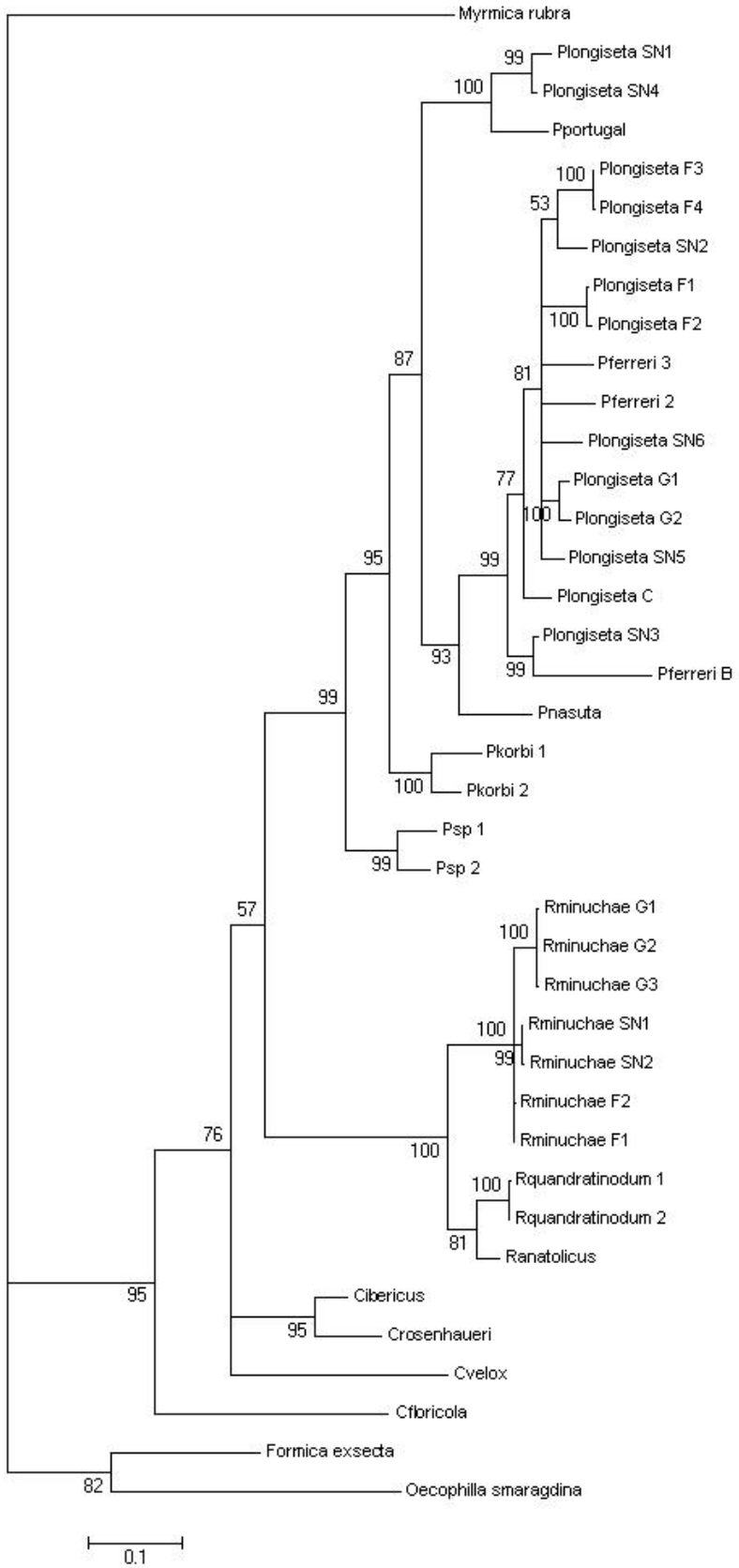

Fig. 3. Bayesian inference tree. For *P. longiseta* and *R. minuchae* localities are noted as SN (meaning Sierra Nevada), G (Sierra de Gador), F (Sierra de Filabres) and C (Sierra de Cazorla). Genebank sequences are marked with a final B in the haplotype name (*P. ferreri* and *F. exsecta*) or a final H (*R. minuchae*).

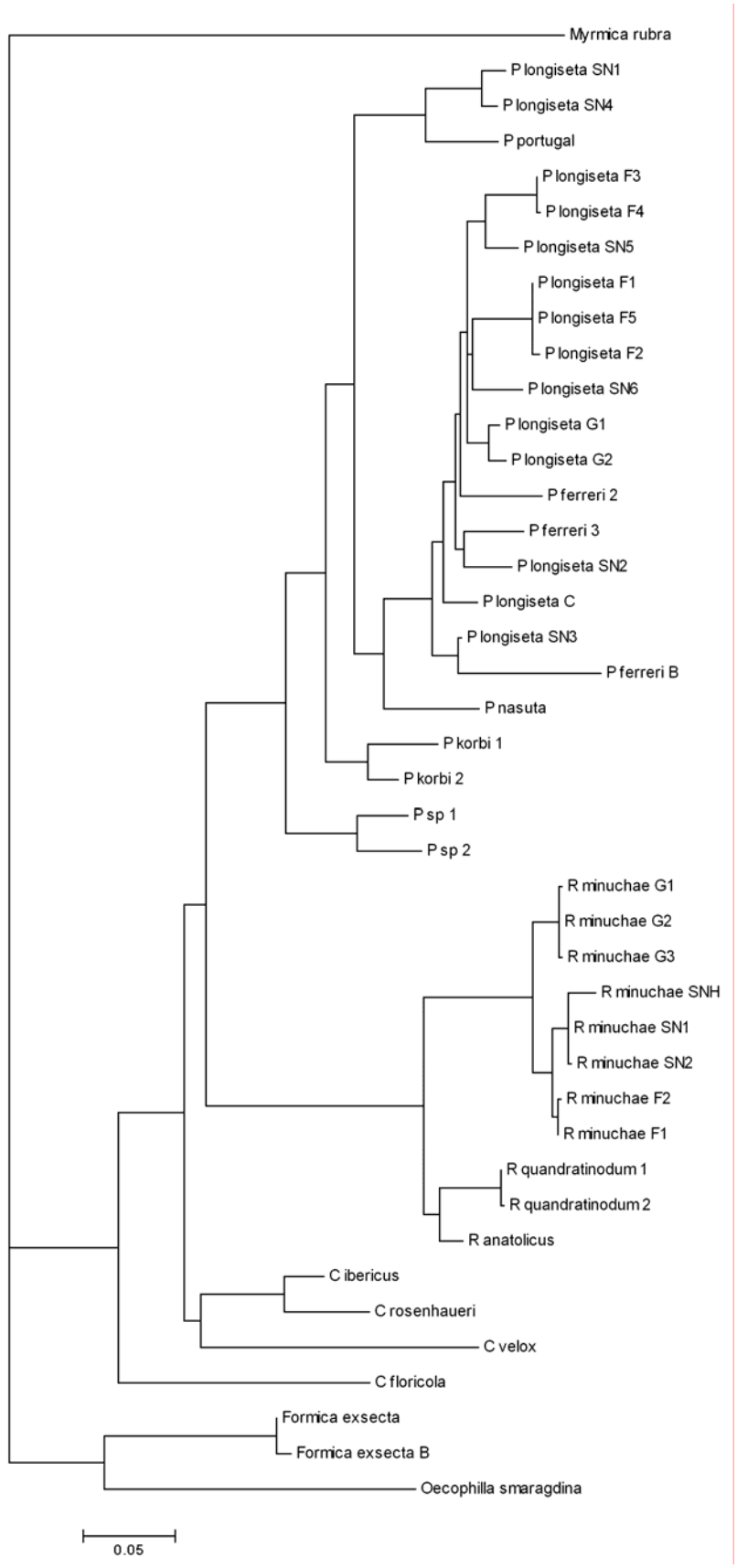

Fig. 4. Maximum likelihood (ML) tree. For *P. longiseta* and *R. minuchae* localities are noted as SN (meaning Sierra Nevada), G (Sierra de Gador), F (Sierra de Filabres) and C (Sierra de Cazorla). Genebank sequences are marked with a final B in the haplotype name (*P. ferreri* and *F. exsecta*) or a final H (*R. minuchae*).

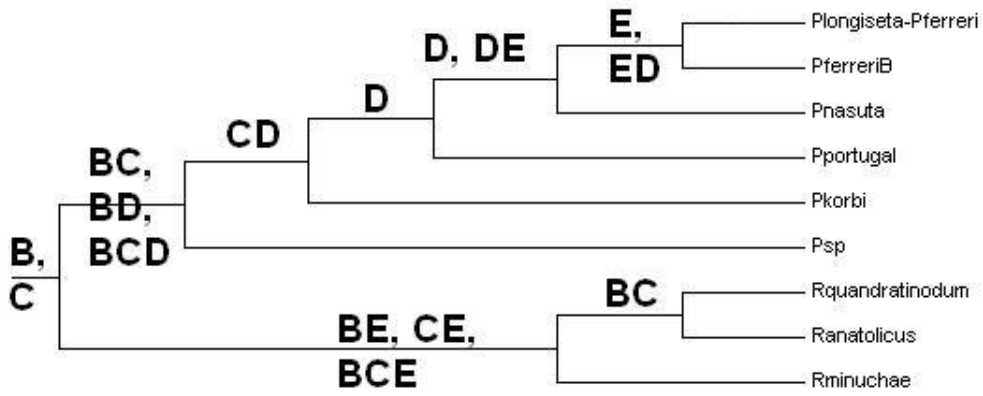

Fig. 5. Simplified tree showing ancestral distributions of the main nodes according to Diva for *Proformica* and *Rossomyrmex*. Distributions are named as follows: (B) Central Asia; (C) Turkey; (D) Iberian Peninsula; (E) Betic sierras (South Eastern Spain).

DISCUSSION

Systematic overview

On the whole, all three tree methods yielded similar results with minor deviations. *Cataglyphis* appears to be the ancestral group with *Proformica* and *Rossomyrmex* derived. The origin of the parasite genus *Rossomyrmex*.seems to be independent of the host genus *Proformica* and thus, not following Emery's rule, a similar result to Hasegawa et al. (2002). The phylogeny of *Cataglyphis* appears to be quite complex and we could have a biased result because of the low number of species considered in our analyses and the great variability in male genitalia. Therefore, we would need more species to discern whether *Rossomyrmex* is originated within *Cataglyphis*. Nevertheless, *Rossomyrmex* is undoubtedly out of *Proformica*'s clade, which constitutes one of the more evident cases of exceptions to the Emery's rule at least in its strict sense (Poulin 1998).

In all cases the genus *Rossomyrmex* forms a monophyletic group with 100% bootstrap support. Also, the analyses confirmed the morphology-based classification of the three species considered here, being genetically well differentiated from each other. *R. quandratinodum* and *R. anatolicus* (both Asian species) show smaller distances between them than with *R. minuchae* (Spanish species), which is probably due to the geographical distance among the species distribution, much larger for *R. minuchae*. The fragmented distribution in three sierras of *R. minuchae* is well reflected in phylogeny too, with three monophyletic branches (in NJ and ML analyses) or two monophyletic branches and two independent samples of Sierra de Filabres (Bayesian tree). These results point to a highly restricted migration among populations and a possible strong genetic drift, results previously found for microsatellites (Sanllorente et al submitted). This situation implies that the species would beneficiate from important conservation policies.

The genus *Proformica* is also monophyletic according to the three methods performed. In all cases the Asian host species (*P. korbi* and *P. kazakhstan*) are clearly independent with a strong bootstrap support as well as the Mediterranean species *P. nasuta*. But surprisingly, a group formed by two populations of *P. longiseta* from Sierra Nevada and a population of *Proformica* from Serra da Estrela (Portugal) appeared clustered together in whatever analysis we performed and with a very high bootstrap. This result probably suggests a cryptic speciation process that took place without an apparent morphological change, although distribution of this cryptic species must be studied carefully. The rest of *Proformica longiseta* and *P. ferreri* samples appear together in a mixed clade with different branching and bootstrapping depending on the method. Grouping of the samples does not show a geographic pattern for any of the samples. Our results could be interpreted as a recent speciation process so that COI alone is not a good genetic marker to differentiate between these species or among populations. Otherwise the conventional morphologically-based criteria for differentiating both species would be wrong and both would belong to the same species (synonymy) but probably with differences at the subspecies level. In fact the systematic of the genus *Proformica* is still very weak and a more in depth morphological analysis is needed.

Cataglyphis floricola is separated from the other three *Cataglyphis* species in all analyses, what could reflect that this genus is in fact polyphyletic. However, there is controversy in the classification of this genus and many methods have been proposed to differentiate among the many extant species (Agosti 1990; Knaden et al. 2005; Dahbi et al. 2008). The aim of this work was not a reclassification of this genus so we will not discuss our results further, however we consider that a wide-range phylogeny including distant species is needed to assess the monophyly of *Cataglyphis* given that many studies assume this situation and only focus on a few related species when analyse data.

Phylogeography

The genera *Rossomyrmex* and *Proformica* seem to share an Asian origin according to DIVA analyis, which is supported by the basal position of the Eastern species in both cases. This confirms our suspect that a first dispersal event is likely to have colonised Anatolia from Central Asia whereas a second dispersion should have occurred heading West through the Mediterranean region. Afterwards, speciation processes must have happened in refugia, especially in the South East of the Iberian Peninsula in the Quaternary while glaciations could have driven central European populations to extinction (Hewitt 1996; 1999). During warm periods some of the surviving populations of *Proformica* may have migrated in altitude following stepparian flora and cold winter conditions not found below whereas others adapted to a warmer climate at lower altitudes. The first group would speciate to *P. longiseta*, well adapted to high mountains and the second would become *P. ferreri*,

spreading along the Iberian Peninsula at medium altitudes. For some reason, the parasite *R. minuchae* followed the first group of hosts and was constrained to mountains higher than 2000 metres. Due to its small population sizes and scarce dispersal ability, its populations remained isolated, favouring COI divergence and differentiation.

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DISCUSIÓN GENERAL

Los trabajos realizados en esta tesis nos han permitido conocer a fondo el estado genético de las poblaciones de *Rossomyrmex minuchae* así como de su hospedadora *Proformica longiseta* en todo su rango conocido de distribución con vistas a la conservación de *R. minuchae* como elemento endémico y singular de las Béticas. Así mismo, hemos podido saber más de las especies de ambos géneros, apenas estudiadas de Asia Menor y Central, para así tratar de entender el origen, el proceso de especiación y dinámicas poblacionales en función de la orografía (montañas o estepas) y el rango de distribución de sus poblaciones (fragmentadas o continuas).

1. Estructura poblacional de *R. minuchae*

Con los datos genéticos de parentesco entre obreras hemos descubierto que un 49% de las hembras de *R. minuchae* sulen copular varias veces y por tanto no son monándricas estrictas como se pensaba a partir de observaciones en el campo (Ruano y Tinaut 2005). Dichas observaciones previas sobre el sistema de apareamiento apuntaban a un elevado riesgo de endogamia, pero con los datos obtenidos no podemos asegurar que las poblaciones presenten depresión endogámica. Sin embargo, hemos confirmado las sospechas de que los machos efectivamente se desplazan distancias mucho más cortas de las esperadas en la generalidad de hormigas pese a ser alados. Esta circunstancia favorece que machos procedentes del mismo hormiguero copulen con hembras hermanas e incluso con una misma hembra, con lo que las posibilidades de endogamia aumentan. Un indicativo normalmente aceptado del nivel de endogamia en insectos sociales es la aparición de machos diploides (puesto que la determinación genética del sexo es habitualmente debida a un solo locus) pero sorprendentemente apenas encontramos indicios de la presencia de machos diploides (3.2%) a pesar de que se favorecía dicha circunstancia. Por tanto, debe existir algún mecanismo que evite la aparición de machos adultos diploides en *R. minuchae*, bien por eliminación de las larvas o pupas o bien porque en esta especie el sexo no lo determine un solo locus, como se ha demostrado en otras hormigas con elevados índices de endogamia (Schrempf et al. 2006).

Las tres poblaciones conocidas (Sierra Nevada, Gádor y Filabres) han resultado ser genéticamente muy diferentes, tanto a nivel de ADN nuclear como mitocondrial. Sin duda, se trata de una consecuencia del reducido tamaño poblacional y del aislamiento geográfico, ambos favoreciendo la acción de la deriva génica y divergencia entre poblaciones. De esta

forma, se han ido fijando alelos propios de cada población y perdiéndose otros, dando lugar además, a una variabilidad genética en general baja para hormigas, si bien no hay muchas especies documentadas con poblaciones tan pequeñas (Trontti et al. 2006). Además, podemos concluir que la situación actual no ha sido causada por cuellos de botella en ninguna de sus poblaciones, sino a una progresiva reducción del tamaño poblacional por procesos naturales.

2. Estructura poblacional de *P. longiseta*

La especie hospedadora cuenta con poblaciones de mayor tamaño y además con un rango de distribución mucho más amplio que la parásita. Los resultados sobre variabilidad genética confirman que se trata de una especie que no se encuentra amenazada. Sin embargo, tiene la peculiaridad de no presentar diferenciación genética según la distancia geográfica, de tal forma que poblaciones muy alejadas pueden resultar más similares genéticamente entre sí que poblaciones cercanas (lo contrario de lo que ocurre en *R. minuchae*). Esto podría ser debido a que las poblaciones actuales procederían de una gran población ancestral continua y que la deriva génica no ha sido lo suficientemente potente como para fijar diferencias geográficas, sino que en cada sistema montañoso o incluso en diferentes valles dentro de los sistemas montañosos, se han ido dando procesos locales de fijación de alelos. Dicha diferenciación local ha sido favorecida por el proceso de formación de nuevas colonias que se da en la generalidad del género *Proformica* (la gemación: las hembras se dispersan andando acompañadas por una cohorte de obreras), imponiendo una restricción a la capacidad dispersora de las hembras. Los machos por su parte son alados, recayendo en principio sobre ellos el papel de la dispersión; sin embargo, la orografía de las montañas no favorece dicha tarea y por tanto, la distancia que puedan recorrer parece también reducida a la vista de la alta diferenciación genética existente entre algunas poblaciones separadas tan solo 15 Km entre sí.

3. Coevolución en mosaico geográfico

R. minuchae solo habita en puntos muy localizados del rango de distribución de *P. longiseta* y además sus poblaciones están muy alejadas geográficamente, por tanto, el flujo génico (posibilidad de intercambiar genes de una población a otra) sería a priori mucho menor que en la hospedadora. En nuestro estudio encontramos que esta diferencia se eleva a dos órdenes de magnitud y que las diferencias genéticas debidas a la distancia geográfica están correlacionadas con las diferencias químicas de sus hidrocarburos cuticulares. Dicho de otro

modo, los perfiles de hidrocarburos cuticulares son diferentes en cada población de *Rossomyrmex* como ocurre a nivel genético. Al analizar los mismos compuestos en *P. longiseta*, encontramos que se daba una situación análoga a su parásita: cada población presentaba unos perfiles diferenciados y además, más similares a su correspondiente parásita que a las otras poblaciones de su misma especie. Por tanto, al encontrar mayores similitudes interespecíficas (entre parásito y hospedador) a nivel local que intraespecíficas (entre poblaciones de la misma especie), nos hace pensar que en cada localidad se ha producido un proceso de coevolución independiente.

Los hidrocarburos cuticulares son muy importantes para la comunicación y reconocimiento específico en las hormigas (Lenoir et al. 2001), circunstancia especialmente relevante en el caso del parasitismo. Por trabajos anteriores ya sabíamos que en la relación *R. minuchae-P. longiseta* los hidrocarburos juegan un papel fundamental en la agresividad y la posibilidad de supervivencia de las colonias asaltadas, siendo ventajoso para las hospedadoras no enfrentarse a las esclavistas y por tanto, presentar perfiles químicos más parecidos a éstas que a otras poblaciones de su misma especie no parasitadas (Zamora-Muñoz et al. 2003; Errard et al. 2006). A la luz de los resultados obtenidos, podemos pensar que los hidrocarburos de *P. longiseta* se han adaptado a los perfiles locales de sus parásitas (puesto que la presión selectiva es mayor en la hospedadora que en *R. minuchae*, estando su supervivencia en juego). Por tanto, en este sistema parásito-hospedador, un mayor flujo génico ha aumentado las posibilidades de adaptación de la especie hospedadora. Este resultado es interesante ya que actualmente existe un debate sobre si el flujo génico favorecería o impediría el proceso adaptativo (Morgan et al. 2005; Nash et al. 2008).

4. Comparación con las especies asiáticas

La comparación entre especies cercanas con distinto patrón de distribución es una herramienta especialmente útil en conservación, puesto que se pueden comprobar los niveles de variabilidad genética y estructura poblacional de una especie de la que se sospeche que esté amenazada. En nuestro caso comparamos la situación de *R. minuchae*, de distribución parcheada en tres montañas aisladas, con la de *R. quandratinodum* (no *R. proformicarum* como creía Marikovsky) y *R. anatolicus*, de distribución presumiblemente continua en las estepas de Kazajstán y Turquía respectivamente. En primer lugar, observamos que pese a un tamaño de muestra menor que en la especie ibérica, las poblaciones estudiadas de *R.*

anatolicus mostraban mayor variabilidad genética que *R. minuchae* y además que poblaciones muy alejadas geográficamente (500 Km) eran muy parecidas entre sí, apoyando la hipótesis de que sus poblaciones estarían distribuidas de forma más o menos continua. En el caso de *R. quandratinodum*, las diferencias no resultaron significativas seguramente debido al bajo número de nidos estudiados (3), ya que sus niveles eran similares a los más altos hallados en *R. minuchae* con mayor tamaño muestral.

Por otra parte, las tres especies parecen tener un comportamiento reproductor similar, con nidos monogínicos y hembras que copularían en general una o dos veces. Además, parece ser también característico que en principio parasiten a una sola especie de *Proformica* y no modifiquen el tamaño ni el diseño de los hormigueros a los que parasitan. Sin embargo, sí parece haber diferencias en el número y la proporción de obreras hospedadoras y parásitas, siendo llamativamente bajos en *R. quandratinodum* que por el contrario presenta los nidos más profundos. Por tanto, la profundidad del nido (y presumiblemente el tamaño) no sería directamente proporcional al número de hormigas y además existirían diferencias específicas en la eficacia de los asaltos, bien porque las hospedadoras estén por delante en la carrera de armamentos o bien por otros factores.

En cuanto a las *Proformica* hospedadoras, como ya se ha comentado difieren en la profundidad de sus nidos y el número de obreras, estando ambas variables no correlacionadas. Por otra parte, *P. sp* (oriunda de Kazajstán) parece ser monogínica a diferencia de las otras dos especies aunque la fundación de nuevas colonias bien podría ser dependiente, pues nos parece que se correspondería con el carácter ancestral del género. Finalmente, a pesar de la diferente distribución geográfica de las especies asiáticas y la ibérica, no encontramos evidencias de menor variabilidad ni endogamia; si bien las diferencias genéticas locales detectadas en *P. longiseta* no las hemos hallado en las otras dos especies seguramente por la compleja orografía de las béticas, en comparación con las inmensas llanuras en donde viven las especies asiáticas, aunque un estudio más detallado sería necesario para confirmar esta afirmación.

5. Filogenia de *Rossomyrmex* y *Proformica*

Establecer la filogenia de los géneros *Rossomyrmex* y *Proformica* tiene diversos puntos de interés: ver la validez de las diferentes especies y/o poblaciones de *Rossomrymex* y de

Proformica; dilucidar el posible origen biogeográfico del género *Rossomyrmex* y por tanto del parasitismo, así como tratar de aclarar si en este género se cumple la regla de Emery por la que el parásito derivaría del hospedador. Nuestros resultados basados en el análisis de un fragmento de la citocromo oxidasa 1 (COI) muestran que ambos géneros son monofiléticos y muy posiblemente compartirían un ancestro común, con lo que seguirían la regla pero de forma laxa (derivaría el género en conjunto pero no las especies de forma independiente). Por desgracia, con las muestras analizadas no hemos podido aclarar la relación de estos dos grupos con el género *Cataglyphis*, próximo filogenéticamente (Hasegawa et al. 2002; Moreau et al. 2006). Por tanto, más análisis serán necesarios para aclarar la posición relativa de estos tres clados y si *Rossomyrmex* deriva efectivamente de *Proformica* o bien de *Cataglyphis*.

A nivel de género, las tres especies esclavistas aparecen bien delimitadas mientras que las hospedadoras muestran problemas a la hora de separar *P. longiseta* y *P. ferreri*, lo que puede ser debido a un proceso reciente de especiación que no ha dado tiempo a fijar diferencias para este marcador. Además, encontramos un posible caso de especiación críptica al aparecer agrupadas dos poblaciones de P. longiseta con P. Portugal.

Por último, el origen geográfico de ambos géneros parece situarse en Asia central o bien en Anatolia.

6. Estado de conservación

Con todos los resultados anteriormente expuestos, podemos elucidar el estado conservación de *R. minuchae*. En principio la especie no se enfrenta a un peligro inmediato que amenace sus poblaciones: la especie hospedadora es abundante y con elevados niveles de variabilidad con lo que no sería un factor limitante; no hemos detectado cuellos de botella recientes que hayan provocado una reducción drástica de diversidad alélica; el hábitat que ocupa no presenta interés económico ni está en lugares de paso de infraestructuras antrópicas; tampoco hemos evidenciado síntomas directos de depresión endogámica. Sin embargo, no podemos descartar que la especie sea vulnerable a la extinción dado que: el sistema de apareamiento favorece la endogamia (algunas hembras copulan con machos emparentados con ellas y algunas hembras copulan con machos hermanos); las poblaciones son muy pequeñas por lo que fácilmente podrían desaparecer si se dan ciertas circunstancias adversas (calentamiento global); todos los nidos no producen sexuados cada año y si lo hacen no siempre de ambos

sexos, pudiendo por estocástica haber años con reducidas posibilidades de cópulas y por tanto, de formación de nuevos hormigueros (un proceso ya de por sí peligroso para las hembras y con reducido éxito según Ruano 2000). Además, la gran diferenciación genética entre poblaciones hace que el intercambio de individuos entre éstas (como medida de manejo y gestión de especies) pueda comprometer las particularidades poblacionales, no siendo una medida de conservación aceptable dado el riesgo actual.

Por tanto, con los datos de los que disponemos actualmente, proponemos mantener el estatus de especie vulnerable y que se inicien políticas de protección en las áreas donde se ha detectado la presencia de *R. minuchae*. Dicha medida no sería difícil de llevar a cabo dado que las poblaciones son reducidas y ya se encuentran en zonas de algún modo protegidas: Parque Nacional (en Sierra Nevada), Parque Natural (en Filabres) y Lugar de Interés Comunitario (en Gádor). Así, se trataría en algunos casos de aumentar el nivel de protección y en general de evitar la alteración del hábitat: repoblaciones, paso de carreteras, apertura o ampliación de carriles, tránsito de motocross y quads, etc.

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CONCLUSIONES

Con respecto a la estructura genética poblacional:

1. Las poblaciones de *Rossomyrmex minuchae* (Sierra Nevada, Sierra de Gádor y la recientemente encontrada en la Sierra de los Filabres) presentan entre sí diferencias genéticas significativas, sin duda debidas a su aislamiento geográfico, por lo que para su conservación habría que considerarlas como unidades independientes.

2. El parentesco encontrado entre algunos machos de *R. minuchae* que copularon con una misma hembra así como el encontrado entre algunas hembras y sus parejas, parecen apuntar a la existencia de depresión endogámica. Sin embargo, el impacto de machos diploides parece escaso. El sexo en esta especie podría no estar determinado por un solo locus.

3. No hemos encontrado evidencias de que un evento reciente de cuello de botella haya producido la actual situación de baja diversidad genética en *R. minuchae*, puesto que un considerable número de alelos raros se han mantenido en cada población. El aislamiento no fue simultáneo sino que debió ocurrir de forma gradual durante dos interglaciares del Pleistoceno.

4. *R. minuchae* presenta un flujo génico mucho más restringido que su hopedadora *P. longiseta*, con mayores tamaños poblacionales. Analizando los hidrocarburos uniculares de ambas especies obtuvimos grandes similitudes parásito-hospedador para cada población pero diferencias entre poblaciones, lo que sugiere que la especie con mayor migración (*P. longiseta*) se ha adaptado a la otra (dado que las alopátridas son más diferentes que las simpátridas y sus parásitas).

5. La especie presente en las llanuras de Kazajstán, *R. quandratinodum* y la descubierta en 2007 en Turquía, *R. anatolicus*, mostraron similitudes con *R. minuchae* en la no modificación de los nidos parasitados y en ser monogínicas. Una particularidad de la especie kazaja es que presenta una menor proporción de esclavas.

6. Los resultados genéticos obtenidos evidencian que *Rossomyrmex anatolicus* presenta una distribución amplia y ausencia de diferenciación entre poblaciones, así como una mayor diversidad génica que *R. minuchae*, de distribución fragmentada en montañas. Los datos de *R. quandratinodum* apuntan al mismo resultado que para *R. anatolicus*. Por tanto, el tipo de distribución: fragmentada o continua, afecta a la estructura genética de *Rossomyrmex*.

7. No existe un comportamiento reproductor único en las especies parasitadas por *Rossomyrmex*, ya que *Proformica sp* (hospedadora de *R. quandratinodum*) presentaba nidos mucho más profundos que las otras especies hospedadoras; además resultó ser monogínica y monándrica, a diferencia de *P. longiseta* (poligínica y poliándrica) y *P. korbi* (hospedadora de *R. anatolicus*, monogínica y poliándrica)

8. La genética poblacional de *P. longiseta* difiere de la de su parásita por la marcada ausencia de estructuración geográfica pero fuerte estructuración local. Este resultado podría deberse al comportamiento filopátrico de las hembras al fundar nuevos nidos, favoreciendo la diferenciación genética local por deriva.

9. Por el contrario, *P. korbi* y *P. sp* muestran evidencias de que su distribución es amplia, con ausencia de diferenciación genética entre poblaciones, lo cual concuerda con los resultados obtenidos para sus respectivas parásitas.

Con respecto a la filogenia:

10. El género *Rossomyrmex* parece haber evolucionado de forma temprana desde el origen de *Proformica* o bien haberse originado a partir de *Cataglyphis* de forma independiente de *Proformica*. *Rossomyrmex* y *Proformica* son géneros claramente monofiléticos.

11. Las especies *P. longiseta* y *P. ferreri* no pueden separarse de forma clara basándose en el gen COI exclusivamente, pudiendo deberse a una especiación aún en proceso. También encontramos evidencias de una especie críptica de *Proformica* presente en Sierra Nevada (España) y una nueva especie en la Serra da Estrela (Portugal).

Con respecto al estado de conservación de *R. minuchae*:

12. La especie es efectivamente vulnerable a la extinción y por tanto proponemos que se realicen políticas de protección de su hábitat que impidan que sus poblaciones se reduzcan por causa antrópica.

CONCLUSIONS

With respect to population genetic structuring:

1. The populations of *Rossomyrmex minuchae* (Sierra Nevada, Sierra de Gádor and the recently discovered in Sierra de Filabres) show significant genetic differences among them, surely because of their geographic isolation and so they should be considered as independent units for conservation.

2. The relatedness found among some R. minuchae males mated to the same queen as well as that found among some queens and their mates suggest some inbreeding depression. However, the impact of diploid males seems scarce. Sex in this species could not be by a single-locus determination.

3. We detected no evidences for a recent event of bottleneck as the cause for the current situation of low population density in *R. minuchae* given that a considerable number of rare alleles are maintained in each population. Isolation should have occurred in a gradual way during two Pleistocene interglatials.

4. *R. minuchae* presents a much more reduced gene flow than its host P. longiseta, with higher population sizes. The analyses of cuticular hydrocarbons of both species showed great parasite-host similitudes in each population but differences among populations, what suggests that the species with the highest migration (P. longiseta) has adapted to the other (as the alopatric populations are more different than the sympatric and their parasites).

5. The species inhabiting the plains of Kazakhstan, *R. quandratinodum* and the discovered in Turkey in 2007, *R. anatolicus*, were similar to *R. minuchae* in that they do not modify parasitized nests and are monogynous. A particularity of the Kazak species is that is has a lower proportion of slaves.

6. Our genetic results evidence that *Rossomyrmex anatolicus* shows a wide distribution and a lack of population differentiation, along with a higher genetic diversity than *R. minuchae*, with a fragmented distribution in mountains. Data on *R. quandratinodum* point to the same results to those of *R. anatolicus*. Therefore, the type of distribution: fragmented or continuous affects the genetic structure of *Rossomyrmex*.

7. There is not a unique mating system in the hosts of *Rossomyrmex* given that *Proformica sp* (host of *R. quandratinodum*) presented nests much deeper than the other host species; in addition, it was monogynous and monoandrous, contrary to *P. longiseta* (polygynous and polyandrous) and *P. korbi* (host of *R. anatolicus*, monogynous and polyandrous).

8. Population genetics of P. longiseta differs from its parasite's due to the lack of geographic structuring but strong local structure. This result could be a consequence of the phylopatric behaviour of females in colony founding, favouring local genetic differentiation by genetic drift.

9. On the contrary, P. korbi and P. sp show evidences for a spread distribution with absence of genetic differentiation among populations, what is in accordance with the results obtained for ther corresponding parasites.

With respect to the phylogeny:

10. The genus Rossomyrmex seems to have evolved early since the origin of Proformica otherwise it evolved from *Cataglyphis* independently of *Proformica*. *Rossomyrmex* and *Proformica* are undoubtedly monophyletic genera.

11. *P. longiseta* and *P. ferreri* species can not be clearly separated if we consider the COI gene exclusively as there can be a process of speciation. We also found evidences for a criptic Proformica species in Sierra Nevada (Spain) and a new species in Serra da Estrela (Portugal).

With respect to the conservation status of *R. minuchae*:

12. The species is indeed vulnerable to extiction and therefore we encourage protection policies to be made in its habitat that prevent from population reductions due to human activities.