Universitat de les Illes Balears

Estudio filogeográfico de especies vegetales del Mediterráneo Occidental

- Tesis Doctoral -

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CERTIFICAN:

Que Dña. **Arántzazu Molins Piqueres** ha realizado bajo su dirección en el Laboratorio de Ecología Molecular del CREAF y en el Laboratorio de Biología Molecular del Jardí Botànic de la Universitat de València el trabajo que, para optar al grado de Doctor en Biología, presenta el título:

ESTUDIO FILOGEOGRÁFICO DE ESPECIES VEGETALES DEL MEDITERRÁNEO OCCIDENTAL

Considerando concluida la presente memoria, autorizamos su presentación a fin de que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste, firmamos el presente certificado en Palma de Mallorca, a 30 de noviembre de 2009.

Dra. Maria Mayol   Dr. Josep A. Rosselló

Quiero agradecer a mis directores Maria y Pep el haberme permitido entrar en el maravilloso mundo de la Filogeografía y el tiempo invertido en mi. Por sus consejos que no siempre he sabido valorar y por los momentos vividos.

Agradecer a mi familia el apoyo, el cariño y el esfuerzo realizado durante tantos años y que me ha permitido llegar donde estoy.

De igual manera quisiera agradecer al Jardí Botànic de la Universitat de València y al CREAF de la Universitat Autònoma de Barcelona, el haberme permitido usar sus instalaciones para poder realizar esta tesis.

A mis compañeros del Jardín, los que son y los que han sido, por todo el tiempo compartido y por todo el tiempo vivido, por la ayuda brindada y por las cervezas!

A todo el personal del Jardí, el haberme echado una mano cuando lo he necesitado. Gracias Jaime y Pepe por vuestras sonrisas y ánimos.

Quisiera también agradecer a todos aquellos que desinteresadamente me han prestado su ayuda, en el campo, muestreando por mi, indicándome donde localizar poblaciones, regalándome fotos, haciendo recuentos, secuenciando, aportando ideas… Miquel Àngel Conesa, Maurici Mus, Gianluigi Bacchetta, Gonzalo Nieto, Guillem Alomar, Josep Lluís Gradaille, Magdalena Vicens, Pere Fraga, Néstor Torres, Toni Muñoz, Cati Artigues, Marcela Rosato, Eugenio y Ana, Amparo Lázaro, Jorge Sánchez Balibrea (ANSE) …

Y a mis amigos, que siempre he tenido a mi lado queriéndome y apoyándome.

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INTRODUCCIÓN GENERAL

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La cuenca Mediterránea: un "punto caliente" de biodiversidad vegetal

La cuenca Mediterránea es uno de los 25 enclaves de biodiversidad mundial más notable (Médail & Quézel, 1997; Myers *et al.*, 2000), que incluye aproximadamente unas 25.000 especies de plantas vasculares nativas. El origen y evolución de la diversidad vegetal en esta región se atribuye a una serie de factores, como son una larga y compleja historia paleogeológica y paleoclimática, una gran heterogeneidad ecogeográfica, y el impacto más reciente de la actividad humana (Blondel & Aronson, 1999; Thompson, 2005).

La configuración actual de los territorios que forman parte de la cuenca Mediterránea es el resultado de la superposición de múltiples procesos geológicos. En la región oriental, las cuencas del mar Jónico y Levantino corresponden a los restos de los inicios del mar Neotethys Mesozoico (Garfunkel, 2004). Por su parte, la región occidental (que incluye las cuencas del mar Tirrénico, Ligúrico, Provenzal, Argelino y de Alborán) comenzó a formarse durante el Oligoceno, hace unos 30 millones de años, durante la convergencia de las placas Africana y Euroasiática (Dewey *et al.*, 1973; Dercourt *et al.*, 1986; Krijgsman, 2002). Otros eventos geológicos importantes que han tenido lugar en esta región han sido la orogenia Alpina, la dispersión de diversas microplacas tectónicas durante el Terciario, y el cierre de los corredores marinos entre el Atlántico y el Mediterráneo que dio lugar a la crisis del Messiniense (Thompson, 2005). Por ejemplo, en el Mediterráneo Occidental, la historia de la microplaca Cirno-Sarda resulta crítica para una correcta interpretación del elemento endémico presente en esta región. En el Oligoceno Tardío (30-35 Ma), las montañas del Pirineo oriental, los macizos de Maures-Esterel, el extremo occidental de los Alpes, juntamente con los territorios que hoy conforman las Islas Baleares, Córcega, Cerdeña, Sicilia, Calabria, la cordillera Bética, y las Kabilias en el norte de África, estaban agrupados formando lo que se conoce como el macizo Hercínico (Alvarez, 1972; Rosenbaum *et al.*, 2002; Speranza *et al.*, 2002). A partir del Oligoceno y durante todo el Mioceno, las diferentes microplacas empezaron a desgajarse del margen continental, rotando en dirección sureste en el sentido contrario a las agujas del reloj, hasta que alcanzaron sus posiciones actuales (Rosenbaum *et al.*, 2002; Speranza *et al.*, 2002). Por otra parte, durante el Mioceno Tardío (8-6 Ma), diversos procesos de

subducción condujeron al cierre de los corredores Bético y Rifeño que conectaban el océano Atlántico con el mar Mediterráneo, lo que desencadenó la desecación de este último durante lo que se conoce como la crisis salina del Messiniense (ca. 5.96-5.33 Ma; Duggen *et al.*, 2003). Todos estos cambios geológicos han provocado la sucesiva fragmentación-unión de los territorios del Mediterráneo Occidental, a medida que se creaban o desaparecían las barreras para la dispersión, lo que ha favorecido el aislamiento entre poblaciones y la creación de nuevas especies.

Además de los notables cambios geológicos que han afectado a la región Mediterránea, la evolución de la biodiversidad en este área ha estado marcada por una serie de complejos eventos paleoclimáticos, entre los que cabe destacar la instauración definitiva del clima mediterráneo durante el Plioceno (ca. 3.2-2.8 Ma) y la alternancia de períodos fríos y templados durante las glaciaciones del Pleistoceno (1.8-0.01 Ma; Suc, 1984). Durante los períodos fríos del Cuaternario, los diferentes territorios de la cuenca Mediterránea ofrecieron refugio a multitud de especies animales y vegetales (Hewitt, 1999, 2000, 2004), permitiendo la persistencia a largo plazo de sus poblaciones. Los repetidos episodios de contracción-expansión de las áreas de distribución de muchas especies durante este período han tenido un gran impacto sobre la estructura espacial actual de las poblaciones, tanto europeas como mediterráneas. A diferencia de las migraciones a gran escala que se produjeron durante la recolonización de los territorios del norte en los períodos interglaciares, los desplazamientos en las regiones del sur fueron mucho menores, implicando básicamente cambios en la distribución altitudinal de las especies (Hewitt, 2001) y, muy probablemente, el aislamiento prolongado de sus poblaciones (Hampe & Petit, 2005). Por tanto, la estabilidad y aislamiento de las poblaciones mediterráneas durante los ciclos glaciares han favorecido la aparición de nuevas especies en este área a través de procesos de deriva y de adaptación local (Hampe & Petit, 2005).

Por otro lado, la especie humana ha estado presente en el Mediterráneo durante los últimos milenios, alterando profundamente la estructura de las comunidades y hábitats naturales. El mosaico de hábitats resultante de la actividad antrópica también ha favorecido el aislamiento genético y la variación en las presiones de selección locales, lo que en algunos casos puede haber promovido la diferenciación poblacional y, en última instancia, la especiación (Thompson, 2005).

Marcadores moleculares: su aplicación al conocimiento de la evolución vegetal en el Mediterráneo

En las últimas décadas, el desarrollo de diversos marcadores moleculares ha marcado un hito en los estudios evolutivos, ya que ha permitido el análisis de la variabilidad genética y su estructuración a diferentes niveles de divergencia biológica (Avise, 1994). Todo ello ha supuesto un notable avance en la comprensión de los mecanismos evolutivos, así como su interacción con los diferentes procesos históricos y ecológicos. Por otra parte, la aplicación de la teoría de la coalescencia al estudio de los procesos microevolutivos en las últimas décadas ha supuesto un enorme avance conceptual, ya que ha permitido inferir los patrones genealógicos de relación entre los diferentes alelos de una población. Esto ha posibilitado la evaluación del cambio genético a través del tiempo, permitiendo el análisis de la microevolución como un proceso histórico dinámico, cambiando a través del tiempo en el seno de una especie. Una de las aplicaciones más exitosas de los métodos genealógicos en poblaciones naturales es el campo de la Filogeografía (Avise *et al.*, 1987; Avise, 2000). Basada en la asociación entre la distribución geográfica de las variantes alélicas y sus relaciones genealógicas, esta disciplina permite inferir la historia evolutiva de las poblaciones, subespecies y especies. Las bajas tasas de mutación del ADN cloroplástico limitaron, inicialmente, el número de trabajos filogeográficos en plantas (Schaal *et al.*, 1998). No obstante, en los últimos años el número de regiones cloroplásticas potenciales para estudios filogenéticos o filogeográficos se ha incrementado considerablemente (véanse Shaw *et al.*, 2005; Shaw *et al.*, 2007). Ello ha permitido un progresivo aumento en el número de publicaciones sobre esta temática en las últimas décadas.

Así, el uso de marcadores moleculares neutros ha permitido constatar la importancia de la cuenca Mediterránea como reservorio de diversidad genética (Petit *et al.*, 2003), y ha puesto de manifiesto la existencia de diversos refugios glaciares en este área (penínsulas Ibérica, Balcánica e Itálica), además de demostrar la importancia de estos refugios para la posterior recolonización del continente europeo después del último máximo glacial (Comes & Kadereit, 1998; Taberlet *et al.*,1998; Hewitt, 1996, 1999, 2000).

No obstante, y a pesar de la importancia de la flora mediterránea para la biodiversidad global, existe un número sorprendentemente escaso de estudios sobre el origen de la notable riqueza específica en esta región (Comes & Abbott, 1999;

Thompson, 1999; Vargas *et al.*, 1999; Gielly *et al.*, 2001), los cuales siempre han sido planteados sobre una perspectiva temporal contemporánea o reciente, es decir desde el Pleistoceno. Por otra parte, la aplicación de las técnicas moleculares al estudio de la evolución de los organismos insulares en esta región es relativamente escasa, a pesar de la importancia que presentan estos ecosistemas en la diversidad general de la región. Las numerosas islas presentes en el Mediterráneo constituyen uno de los centros más importantes de diversidad vegetal en este área, con una elevada proporción de plantas endémicas y de poblaciones aisladas desde el punto de vista genético (Médail & Quézel, 1997; Médail & Diadema, 2009). La proporción de endemismos en las grandes islas del Mediterráneo (Baleares, Cerdeña, Chipre, Córcega, Creta, Sicilia) oscila entre el 10-12% (Médail, 2008) y, por consiguiente, estos ecosistemas tienen un papel central en las investigaciones sobre las dinámicas temporales y espaciales de la diversidad en esta región. Sin embargo, los estudios moleculares centrados en especies vegetales de islas continentales del Mediterráneo son relativamente escasos, aunque han ido aumentando en los últimos años (Hurtrez-Boussès, 1996; Affre & Thompson, 1997; Affre *et al.*, 1997; Sales *et al.*, 2001; Widén *et al.*, 2002; Bittkau & Comes, 2005; López de Heredia *et al.*, 2005; Edh *et al.*, 2007; Mansion *et al.*, 2008; Salvo *et al.*, 2008; Falchi *et al.*, 2009; Rosselló *et al.*, 2009).

Los pocos estudios existentes en la actualidad indican que las especies vegetales presentes en las islas del Mediterráneo (i) presentan niveles de diversidad genética elevados, y que ésta se encuentra altamente estructurada (Affre & Thompson, 1997; Bittkau & Comes, 2005; López de Heredia *et al.*, 2005; Edh *et al.*, 2007; Falchi *et al.*, 2009), (ii) ello sugiere que el flujo génico entre poblaciones es escaso, incluso en especies con elevada capacidad de dispersión, por lo que los procesos de deriva parecen determinantes en los patrones de diversidad observados (Widén *et al.*, 2002, Bittkau & Comes, 2005; Edh *et al.*, 2007).

Sin embargo, aspectos biogeográficos cruciales como cual es el origen de las especies endémicas compartidas entre diferentes archipiélagos del Mediterráneo han sido objeto de escasos estudios empíricos. Por ejemplo, la distribución actual de los endemismos compartidos entre Baleares, Córcega y Cerdeña, tradicionalmente considerados como paleoendemismos (p.ej. *Arum pictum*, *Helicodiceros muscivorus*, Araceae; *Cymbalaria aequitriloba*, Scrophulariaceae; *Naufraga balearica*, Apiaceae; *Soleirolia soleirolii*, Urticaceae; *Thymus herba-barona*, Lamiaceae), ha sido

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interpretada por los estudios biogeográficos clásicos como el resultado de las antiguas conexiones de estos territorios durante el Oligoceno, hace unos 30 millones de años (Greuter, 1995; Quézel, 1995; Thompson, 2005). Sin embargo, hasta el momento sólo unos pocos estudios han proporcionado evidencias empíricas que apoyen el origen Terciario de algunos endemismos tirrénicos (p.ej. *Helicodiceros muscivorus* y *Arum pictum*; Mansion *et al.*, 2008).

Otro aspecto que ha sido escasamente abordado por los estudios existentes en la actualidad es el efecto de las regresiones y transgresiones marinas ocurridas durante los ciclos glaciales de Cuaternario (Fairbridge, 1961, 1962), y que afectaron significativamente a las líneas costeras del Mediterráneo durante este período. Dependiendo de los territorios, estas oscilaciones del nivel del mar pueden haber alcanzado entre +100 m/-150 m sobre el nivel actual (Cuerda, 1975; Van Andel & Shackleton, 1982; Gràcia *et al.*, 2001), lo que habría causado drásticos reajustes de los territorios insulares. Desafortunadamente, hasta el momento son pocos los estudios que evaluen los efectos de estos cambios eustáticos del nivel del mar sobre la distribución de la diversidad genética y, por tanto, sobre la evolución de las biota insulares del Mediterráneo. Los pocos ejemplos incluyen la lagartija balear *Podarcis lilfordi* (Terrasa *et al.*, 2009) o el complejo *Nigella arvensis* en las islas del mar Egeo (Bittkau & Comes, 2005).

Por último, muchas de las especies vegetales presentes en el Mediterráneo presentan distribuciones disyuntas este-oeste (Davis & Hedge, 1971). Sin embargo, a pesar de las nuevas herramientas moleculares y de análisis existentes en la actualidad, el estudio de los factores históricos causantes de estos patrones disyuntos es relativamente escaso, particularmente a nivel infraespecífico. La utilización de marcadores moleculares neutros ha puesto de manifiesto la existencia de marcados patrones filogeográficos este-oeste en el caso de *Microcnemum coralloides*, una especie presente en la Península Ibérica, Turquía, Siria, Armenia e Irán (Kadereit & Yaprak, 2008), y en el caso de *Buxus balearica*, cuya distribución actual se restringe a la Península Ibérica, Baleares, Cerdeña, Norte de África y Anatolia (Rosselló *et al.*, 2007). Sin embargo, los resultados obtenidos por estos autores necesitan ser validados con un mayor número de estudios centrados en estas cuestiones.

OBJETIVOS Y ESTRUCTURA DE LA TESIS

Así, a pesar del floreciente aumento en los estudios destinados a dilucidar el origen de la diversidad vegetal en la cuenca Mediterránea, es todavía evidente la escasez de trabajos orientados hacia las cuestiones antes mencionadas. Todo ello nos ha llevado a plantear el presente trabajo de investigación con el objetivo general de profundizar en el conocimiento general de los procesos evolutivos causantes de la diversificación vegetal del Mediterráneo, y su relación con los factores históricos y antropogénicos que han afectado a esta región. Para ello se ha analizado la estructuración de la diversidad genética en tres especies vegetales utilizando marcadores moleculares del ADN cloroplástico. Debido a su estructura simple y no recombinante, su tipo de herencia generalmente uniparental, y la existencia de intrones y espaciadores intergénicos no sometidos a constricciones evolutivas significativas, estos marcadores han sido los más usados en estudios filogeográficos en plantas (Schaal *et al.*, 1998). Frente al ADN nuclear, el cloroplástico presenta tiempos de coalescencia más cortos, y son buenos indicadores de cuello de botella y de efecto fundador (McCauley, 1995) ya que son más sensibles que los nucleares a los procesos de deriva genética.

La elección de los sujetos de estudio se ha centrado en aquellas especies que pueden proporcionar datos relevantes sobre determinados aspectos evolutivos de la flora mediterránea escasamente explorados en la actualidad. Así, la presente tesis se organiza en tres bloques diferentes, dependiendo de la especie considerada:

- En el primer bloque, que corresponde al Capítulo 1, se analizan los patrones de estructuración genética en *Senecio rodriguezii* (Asteraceae), una especie endémica de las Islas Baleares (Mallorca, Menorca) que presenta una distribución restringida a la costa de estas dos islas. El análisis de 28 poblaciones de esta especie a lo largo de su rango de distribución nos ha permitido investigar el papel de la historia paleogeográfica del archipiélago en la estructuración genética que presenta actualmente la especie, con especial atención al papel de las transgresiones y regresiones marinas del Pleistoceno.

- Un segundo bloque, formado por los Capítulos 2 y 3, está centrado en el análisis de los niveles de variación presente en el ADN cloroplástico de *Buxus balearica*

(Buxaceae), una especie con una distribución altamente fragmentada y restringida a diversas poblaciones del oeste (Península Ibérica, Baleares, Cerdeña, Norte de África) y del este (Anatolia) de la cuenca Mediterránea. En el Capítulo 2 se han analizado 18 poblaciones para investigar si su distribución actual es debida a la fragmentación de una distribución anterior más amplia de la especie o si, por el contrario, el patrón disyunto observado es fruto de procesos de dispersión a larga distancia o de introducciones deliberadas. Los resultados obtenidos se han interpretado en relación a la historia paleogeológica y paleoclimática de la región. Por otra parte, se ha verificado la validez de la cita que documentaba esta especie en las montañas costeras de Cartagena, una localidad alejada más de 200 km de la población más cercana (en Andalucía), y que se basaba en un único pliego de herbario depositado en el Real Jardín Botánico (Madrid). El análisis del ADN cloroplástico presente en la muestra de herbario, juntamente con el resto de poblaciones naturales de la especie, nos ha permitido inferir las causas más probables de la presencia de *B. balearica* en esta localidad (Capítulo 3).

- Un tercer bloque lo constituye el Capítulo 4, centrado en la estructuración de la variabilidad genética presente en *Thymus herba-barona* (Lamiaceae), una especie endémica de Baleares (Mallorca), Córcega y Cerdeña. Los resultados obtenidos del análisis de 15 poblaciones de la especie nos ha permitido evaluar el papel de los diferentes procesos evolutivos (migración, selección, deriva) en los patrones de diversidad genética observados. Del mismo modo, se ha examinado el papel de las conexiones Terciarias de estos territorios en el origen de la distribución actual de la especie.

Estos cuatro capítulos han sido redactados en inglés y se presentan con la estructura de artículos científicos, de manera que pueden ser consultados de manera independiente del resto de capítulos. Por último, el hecho de que estas tres especies presenten áreas de distribución contrastadas, que abarcan todo el Mediterráneo (*Buxus balearica*), las islas Tirrénicas (*Thymus herba-barona*), o las islas Gimnesias (*Senecio rodriguezii*), nos ha permitido realizar un análisis comparativo del grado de estructuración genético a diferentes escalas geográficas, e inferir el tipo de procesos más relevantes en función de la escala considerada. Esta comparación se ha

realizado en el capítulo dedicado a la Discusión general de los resultados obtenidos. El último apartado lo constituye el capítulo dedicado a las Conclusiones, donde se exponen las conclusiones generales derivadas de esta Tesis Doctoral.

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CAPÍTULO 1

Phylogeographic structure in the coastal species *Senecio rodriguezii* (Asteraceae), a narrowly distributed endemic Mediterranean plant

This chapter has been published in *Journal of Biogeography* 36: 1372–1383 (2009).

Abstract

Our goals were (1) to assess the levels of chloroplast DNA variation in a narrowly distributed plant restricted to continental islands, (2) to ascertain whether a phylogeographic structure is present in plants restricted to coastal linear systems, and (3) to interpret the results in the light of the known palaeogeography of the Eastern Balearic Islands (Majorca and Minorca) in the Western Mediterranean basin. Our Sampling included 134 individuals from 28 populations of *Senecio rodriguezii* covering the full species range. Sequences of the chloroplast genome (*trn*T-*trn*L spacer) were obtained and parameters of population genetic diversity and substructure were determined (h_s h_t, G_{st}). The geographical structure of genetic variation was assessed by an analysis of molecular variance (AMOVA). Additionally, a spatial AMOVA (SAMOVA) was used to identify groups of populations that were geographically homogeneous and maximally differentiated from each other. Finally, a pattern of isolation by distance was assessed by testing the correlation between the matrix of pairwise Φ_{ST} values and the matrix of geographical distances between pairs of populations using a Mantel test. Seven haplotypes were detected in *S. rodriguezii*. Only two of them were shared between islands, all of the others were restricted to Majorca (two) or Minorca (three). Overall, we found high levels of genetic diversity and significant geographical structuring of cpDNA markers. Most of the variation detected can be attributed to differences among populations (84.6%), but there was also a significant differentiation between the islands. Our results support the view that the Balearic Islands constitute a reservoir of genetic diversity, not only for widespread Mediterranean taxa, but also for endemic ones. The intraspecific genetic structure found in *Senecio rodriguezii* suggests that its population history was dominated by both expansion and contraction events. This has resulted in a species that is highly structured genetically, showing very few shared haplotypes between islands and a high number of haplotypes restricted to small geographical areas within the islands. Changes in habitat availability and dynamic processes of population fragmentation and connectivity due to repeated cycles of sea-level changes during the Quaternary are the possible underlying factors that have shaped the cpDNA pool of this endemic species on a regional scale.

INTRODUCTION

Cyclical changes in the Earth's circumnavigation of the sun have resulted in episodes of global cooling, or Ice Ages, since the Precambrian era (Hays *et al.*, 1976). In all likelihood, these glacial and interglacial periods have shaped the distribution of the

Earth's biota through geological time (Hewitt, 2004). However, the profound impact of these climatic changes can only be deduced with certainty from the biological footprints recovered from the most recent episodes of global cooling during the Pleistocene, either from the fossil record (Willis & Niklas, 2004) or from the scrutiny of the phylogeographic history of selected species (Kadereit *et al.*, 2004).

The availability of suitable case studies has allowed for the assessment of the biological impact of the Pleistocene climatic changes on the distribution of plant taxa and on their genetic structure (Lascoux *et al.*, 2004), including (1) the identification of glacial refuges and colonization history after the Last Glacial Maximum of the Würm glaciation (Taberlet *et al.*, 1998), (2) the role of hybridization and the establishment of hybrid zones in shaping the current distribution of organellar markers (McKinnon *et al.*, 2004), and (3) the impoverishment of the gene pool of several species due to repeated cycles of area fragmentation and population bottlenecks (Nasri *et al.*, 2007).

One of the effects of the Pleistocene Ice Ages was the worldwide eustatic change in the sea level driven primarily by patterns of glaciation and deglaciation (Fairbridge, 1961, 1962). Studies are consistent in describing the overall picture, but not in the details concerning the low standing of the sea level. Levels between 85 and 130 met lower than present day have been suggested, with some regional variation ascribable in part to local tectonic and hydrographic effects (Dillon & Odale, 1968; Moerner, 1971).

Quaternary sea level changes along the coastal margins could have had a strong influence in shaping the population distribution and genetic structure of many insular plants through sea-level oscillations due to eustatic changes that were linked to glacial/interglacial cycles. These effects would have been even more acute in islands located near continental areas, many of which were joined at low sealevel stands (during stadials) by land bridges with the mainland.

Also, distant archipelagos could be affected, as recurrent changes in coastlines could have promoted the lumping and splitting of islands. Accordingly, the Pleistocene Ice Ages have probably influenced the genetic structure of insular plants through historical changes that have promoted or heavily restricted their intraspecific gene flow, even though these plants are not located in alpine or montane environments. These

effects have been demonstrated in plants living in simple (one island) insular systems that have been connected to the mainland (e.g., Taiwan; Chiang & Schaal, 2006). However, case studies from more complex palaeogeographic scenarios (archipelagos that have undergone re-delimitations of island boundaries) are rarely available (e.g., Bittkau & Comes, 2005).

The Balearic Islands are continental islands, meaning that they were connected to the continent before their genesis and did not originate through volcanic activity, but rather by active and complex tectonic events. Both Balearic sub-archipelagos (Eastern: Majorca, Minorca and Cabrera; Western: Ibiza and Formentera) have remained isolated since the opening of the Gibraltar Strait (about 5.3 Ma; Gautier *et al.*, 1994). Thus, during all of the Quaternary period the Balearic Islands have been true islands with no land connections to other Mediterranean islands (such as the Corsica-Sardinia archipelago) or bordering territories (i.e. the Iberian Peninsula, North Africa, and South France). Climatic changes during the Quaternary period have led to sea level changes, mediated by glacio-eustatic oscillations that have modified the shape and size of the emerged Balearic lands (e.g., Hillaire-Marcel *et al.*, 1996; Vesica *et al.*, 2000). Absolute dating of endokarstic features (phreatic speleothems) from the coastal caves of Majorca by means of physico-chemical methods (Th/U) accurately reflects a complex succession of shifts in the sea level during the Middle and Upper Pleistocene and Holocene (from 3,900 yr BP to more than 350,000 yr BP). In fact, the reconstruction of a eustatic curve for the last 300,000 yr BP reveals the existence of sea level fluctuations greater than 18 m in amplitude within time spans lower than 10,000 years (Ginés *et al.*, 2001).

A maximum transgression during the Lower Pleistocene was a rise in the sea level up to +90 m with respect to the current coast line (Figure 1), causing (1) the splitting of the present-day island of Majorca into two major islands, and (2) the fragmentation of the island of Minorca into many smaller islets of disparate shapes and sizes (Cuerda, 1975; Gràcia *et al.*, 2001). By contrast, a maximum regression during the Upper Pleistocene lowered the sea level about -130 m below current level (Cuerda, 1975; Gràcia *et al.*, 2001), giving rise to the formation of a single land mass including Majorca, Minorca and Cabrera islands (Figure 1). Emerged lands of this single insular

territory were estimated to be nearly 9.600 km^2, about twice the size of the current landmass of the Eastern archipelago. The formation of the present-day insular configuration of the Eastern Balearic archipelago took place recently in evolutionary times. It has been hypothesized that Majorca and Minorca have become completely isolated from each other only after the LGM (ca. 18,000 Ka) (Cuerda, 1975).
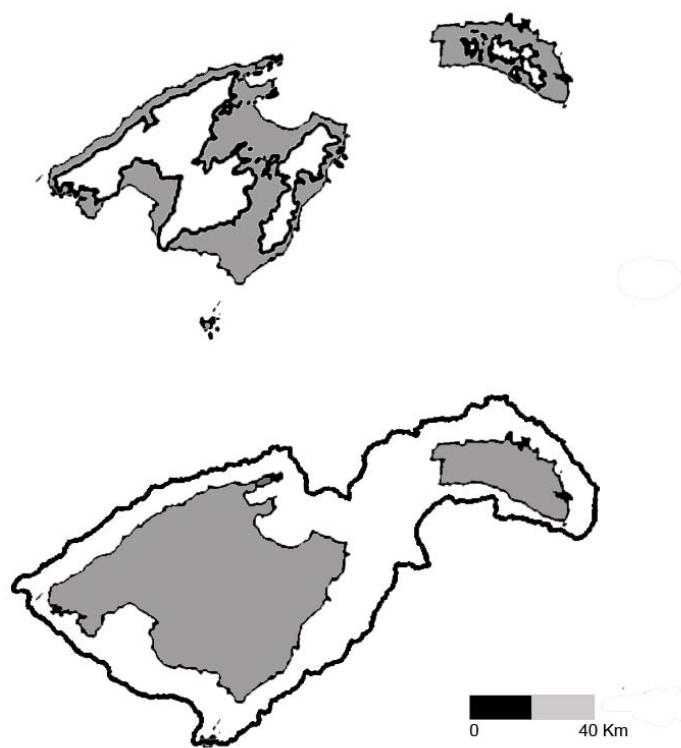
Figure 1 Approximate geographic configurations of Majorca, Minorca and Cabrera islands during the maximum transgression (above) and maximum regression (below) during the Pleistocene. Modified from Gràcia *et al.* (2001). The current distribution of emerged lands is shown (in grey).

This complex palaeogeographic scenario, with recurrent reconfigurations of the insular landscapes and environments, could have left detectable genetic footprints in the coastal plant endemic assemblages, as a consequence of dynamic processes, such as the expansion of populations into newly formed coastland areas and the extinction or reduction in size of some populations due to the flooding of the habitat.

Thus, we were interested to assess whether a substantial phylogeographic structure could be retrieved from local species with a restricted coastal distribution showing fragmented populations. Coastal plants have been shown to be appropriate for addressing patterns of spatial genetic structure on relatively large scales (Clausing *et al.*, 2000; Franks *et al.*, 2004; Ortiz *et al.*, 2007).

We selected a Balearic endemic, *Senecio rodriguezii* Willk. ex J.J. Rodr. (Asteraceae), present on two Eastern islands (Majorca and Minorca) as a model system to assess the phylogeographic structure in narrowly distributed plants from the Balearic Islands. A priori, a strong pattern of population differentiation and structure concerning molecular markers was expected, mirroring the dynamic Quaternary palaeogeographic history of the Balearic Islands. On the other hand, *S. rodriguezii* seeds bear a pappus that facilitates wind dispersal, potentially over long distances, and this might have caused a lack of a phylogeographic signal in this species. Hence, it is interesting to investigate whether or not seed dispersal has blurred the patterns of population differentiation due to the recurrence of Quaternary vicariance. Our specific goals were (1) to assess the levels of cpDNA variation in a narrowly distributed plant restricted to continental islands, (2) to investigate the presence of phylogeographic structure in plants restricted to linear systems in islands, and (3) to interpret the results in light of the Quaternary palaeography of the Balearic archipelago.

MATERIALS AND METHODS

The plant species

Senecio rodriguezii is an annual or short-lived perennial plant with succulent leaves and stems that exists in discontinuous, fragmented populations close to sea level in Majorca and Minorca. To a lesser extent, it can be found up to 150 m a.s.l. in coastal slopes with strong maritime influence. This species mostly lives in open rocky places although rarely it will colonize compact sandy soils. Bagging experiments conducted on cultivated plants showed that *S. rodriguezii* is self-compatible. It is a diploid species (2n=20) belonging to a Mediterranean species complex closely related to *S. vulgaris* L. (*Senecio* sect. *Senecio*; Alexander, 1979). The species can be easily differentiated

from the remaining Mediterranean *Senecio* taxa by the conspicuous colours of the ray (white instead of yellow) and tube flowers (pink instead of yellow) (Figure 2).
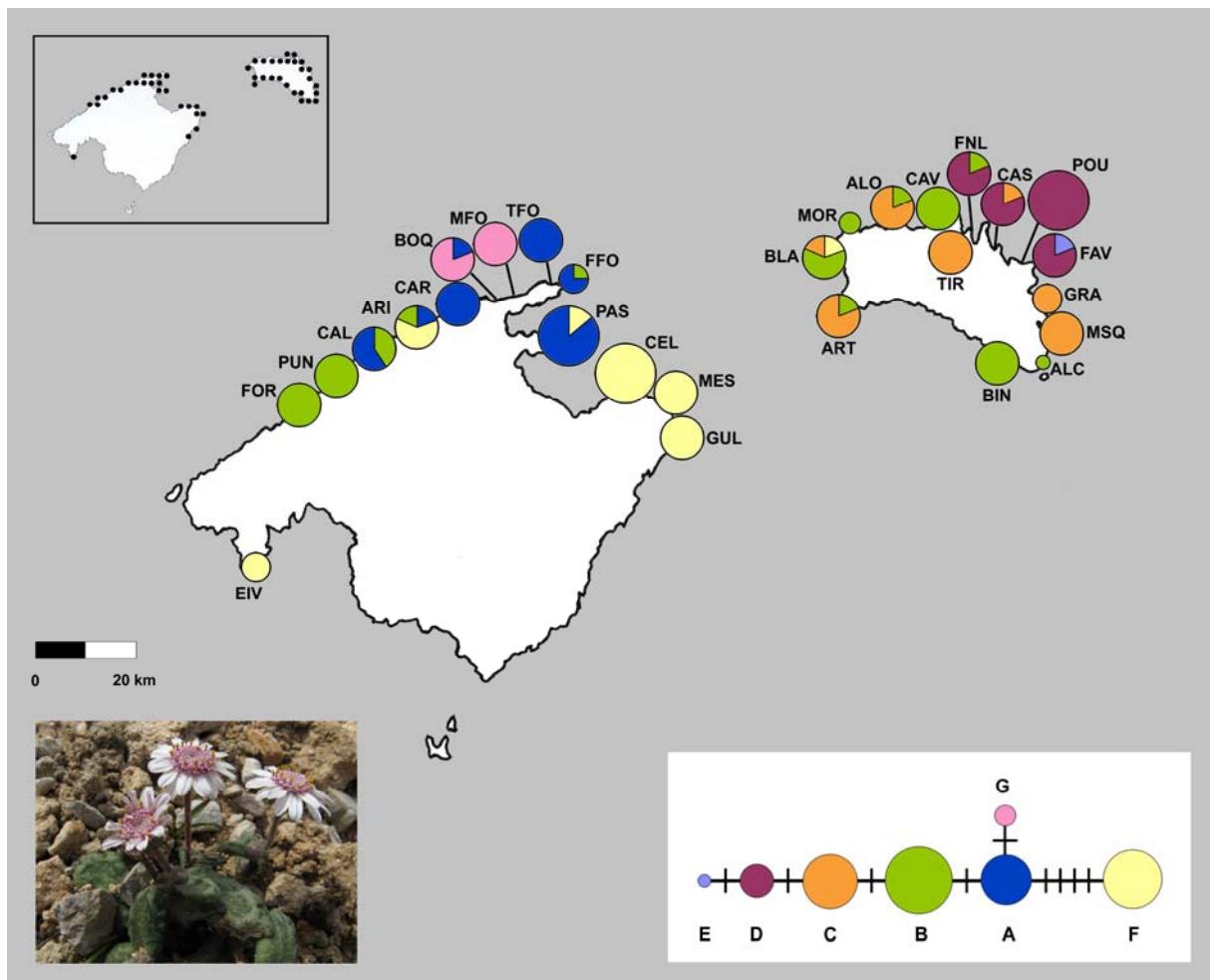
Figure 2 Geographical distribution and parsimony network relationships of seven chloroplast haplotypes found in *Senecio rodriguezii*. For population codes, see Table 1. The size of circles is proportional to the number of individuals carrying a given haplotype. The detailed distribution of the species is shown in the inset.

However, phylogenetic relationships within Mediterranean *Senecio* sect. *Senecio* using nuclear ribosomal ITS sequences were inconclusive (Comes & Abbott, 2001). *Senecio rodriguezii* was included within a well-supported polytomy together with *S. leucanthemifolius* Poir., *S petraeus* Boiss. & Reuter and *S. squalidus* L., and all showed very similar ribotypes (Comes & Abbott, 2001). The chloroplast DNA data

(although based on a limited sample size) did not differentiate *S. rodriguezii* from the widespread diploids such as *S. glaucus* L., *S. leucanthemifolius*, *S. rupestris* Waldst. & Kit. and *S. vernalis* Waldst. & Kit. (Comes & Abbott, 2001). One of these species, *S. leucanthemifolius*, was recently located as a single population on the Eastern coast of Majorca (Gil *et al.*, 1996).

Plant sampling and DNA extraction

Twenty-eight representative populations of *S. rodriguezii,* covering the entire range of the species, were sampled in the field (Table 1, Figure 2). As a rule, between three to six individuals were collected per population (average sample size per population was 4.78). Three populations of *S. leucanthemifolius* (one from Sardinia, one from Majorca, and one from Ibiza; n=5 in each population) were also sampled for comparative purposes. Fresh and healthy leaves from a total of 134 individuals were dried in silica gel and stored at room temperature until DNA extraction. Total genomic DNA was isolated and purified using the DNeasy™ Plant Minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

DNA amplification and sequencing

The *trn*T-*trn*L intergenic spacer was amplified using the universal primers *A* and *B* described in Taberlet *et al.* (1991). PCR reactions were performed in 50 µl, containing 1X reaction buffer, 0.001% BSA, 2 mM $MgCl_2$, 0.5 mM of each dNTP, 0.6 µM of each primer, approximately 50-100 ng of genomic DNA and 3 units of DNA polymerase (NETZYME™ DNA Polymerase, NEED S.L., Spain). Thermal cycling started with a denaturation step at 94ºC lasting 2 min, followed by 30 cycles each comprising 50 s denaturation at 94ºC, 50 s annealing at 55ºC, and 1.5 min elongation at 72ºC, and ended with a final elongation cycle of 3 min at 72ºC. Amplifications were carried out on an ABI GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, California). PCR products were visualized on 2% agarose gels, purified using the High Pure PCR Product Purification Kit (Roche, Germany) and sequenced with an ABI 3100 Genetic Analyzer using the ABI BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California). Samples were sequenced in

both the forward and reverse directions. The sequences obtained were compared to GenBank DNA sequence databases using BLAST (Altschul *et al.*, 1997) and aligned with CLUSTAL X vs. 1.83 (Thompson *et al.*, 1997).

Table 1 Site locality names, site codes, samples sizes (N), and haplotypes recorded in each site. The geographical location of each population is represented in Figure 1. * Sample not included in the calculation of population genetic diversity parameters.

Origin	Locality	Code	Altitude (m)	N	Haplotypes
Majorca	Banc d´Eivissa	EIV	1	4	F
	Sa Foradada	FOR	20	5	B
	Ses Tres Puntes	PUN	1	5	B
	Sa Calobra	CAL	15	5	A, B
	Cala Ariant	ARI	30	5	A, B, F
	Cala Carbó	CAR	10	5	A
	Cala Boquer	BOQ	50	5	A, G
	Mirador de Formentor	MFO	60	5	G
	Tunel de Formentor	TFO	160	5	A
	Faro de Formentor	FFO	170	4	A, B
	Es Mal Pas	PAS	2	6	A, F
	Sa Font Celada	CEL	2	6	F
	Cala Mesquida	MES	5	5	F
	Es Gulló	GUL	5	5	F
Minorca	Cap d´Artrutx	ART	15	5	B, C
	Cala d'en Blanes	BLA	5	5	B, C, F
	Cala Morell	MOR	70	3	B
	Els Alocs	ALO	5	5	B, C
	Arenal de Cavalleria	CAV	10	5	B
	Tirant	TIR	5	5	C
	Cala Fornells	FNL	10	5	B, D
	Arenal d´en Castell	CAS	10	5	C, D
	Pou d´en Caldes	POU	25	6	D
	Cap de Favaritx	FAV	20	5	D, E
	Cala Mesquida	MSQ	5	5	C
	Es Grau	GRA	15	4	C
	Torre d'Alcalfar*	ALC	20	1	B
	Binidalí	BIN	25	5	B

Data analysis

Measures of genetic diversity and population differentiation were calculated with two different analyses: one taking into account the frequencies of haplotypes (HAPLODIV software; Pons & Petit, 1995), and one incorporating both the haplotype frequencies and the genetic distances between haplotypes (HAPLONST software; Pons & Petit, 1996).

The geographical structure of genetic variation was assessed by analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) using ARLEQUIN vs. 2.000 (Schneider *et al.*, 2000). The total genetic variance was partitioned into covariance components at different hierarchical levels under two hypotheses: (1) without regional grouping, therefore just between and within populations; (2) with regional grouping, between islands, populations within each island, and individuals within populations. The significance levels of the variance components were obtained by non-parametric permutation using 10,000 replicates. In addition, SAMOVA (spatial analysis of molecular variance) was used to identify groups of populations that were geographically homogeneous and maximally differentiated from each other using the software SAMOVA vs. 1.0 (Dupanloup *et al.*, 2002). This method is based on a simulated annealing procedure that maximises the proportion of total genetic variance due to differences between groups of populations, and also leads to the identification of genetic barriers between these groups. The most likely number of groups (K) was identified by repeatedly running the program for 10,000 iterations for $K \in \{2, ..., 5\}$ using 500 random initial conditions, and retaining the largest Φ_{CT} values (i.e., the largest proportion of total genetic variance due to differences between groups) as predictors of the best grouping of populations (Dupanloup *et al.*, 2002). Finally, pairwise Φ_{ST} values were computed using ARLEQUIN vs. 2.000 (Schneider *et al.*, 2000), and significance levels of the estimated values were obtained by permutation using 10,000 replicates. A pattern of isolation by distance was assessed by testing the correlation between the matrix of pairwise Φ_{ST} values and the matrix of geographical distances between pairs of populations using a Mantel test (10,000 permutations) implemented in the MANTEL vs. 2.0 package (Liedloff, 1999). However, as *S. rodriguezii* is a coastal species it shows a linear distribution range, and thus migration

might occur mainly along the coast. For this reason the distances between populations were also determined following the coastal line, and the isolation by distance was tested again as described above.

Unrooted statistical parsimony networks were constructed using TCS vs. 1.21 software (Clement *et al.*, 2000). Root probabilities were also calculated using the TCS program following the method of Castelloe & Templeton (1994).

RESULTS

Sequence and haplotype variation

The length of the *trn*T-*trn*L spacer ranged between 559 bp and 565 bp, and resulted in an aligned matrix of 570 bp. No intragenomic polymorphisms (heteroplasmy), previously found in the related *S. vulgaris* (Frey *et al.*, 2005) were detected in this study. Site mutations were detected at alignment positions 124, 201 and 349. Insertion deletion polymorphisms included a 7-bp mutation and a mononucleotide poly-A stretch composed of between 13-18 nucleotides. These mutations defined seven haplotypes (A to G) that are depicted in Table 2. Two haplotypes (F, G) were characterised by site and indel mutations. By contrast, haplotypes A-E differed only in the mononucleotide length. Only two haplotypes were shared between the two islands: haplotype B (the most frequent and widespread haplotype, 25.37%) and haplotype F, although the latter was very rare in Minorca (a single individual). All of the other haplotypes were either restricted to Majorca (A and G) or Minorca islands (C, D, and E). Sampled populations harboured between one to three haplotypes (Table 1, Figure 2). With a single exception (haplotype E), all haplotypes were shared by at least two populations. More than half of the sampled populations (17) were composed of only a single haplotype; nine populations had two haplotypes, and only two contained three haplotypes. The most distant populations sharing the same haplotype (F) were EIV and BLA, which were separated by a linear distance of ca. 250 km (Table 2, Figure 2). The individuals of *S. leucanthemifolius* from Majorca and Sardinia were haplotype F, whereas haplotype A was present in the population from Ibiza. Parameters of genetic diversity and structure for *S. rodriguezii* are given in Table 3.

Table 2 Characterization of *Senecio rodriguezii* haplotypes, based on the polymorphic sites of the *trn*T -*trn*L intergenic cpDNA spacer, found in this study. The frequencies of the cpDNA haplotypes and their geographical origin are indicated. D_{MIN} and D_{MAX} refer to the minimum and maximum lineal distances (km), respectively, separating any two populations sharing the same haplotype.

Haplotype	Polymorphic sites					Frequency			D_{MIN}	D_{MAX}
	124	201	254-260	261-279	349	MA	ME	Total		
A	T	C	-	A_{14}	T	0.1716	-	0.1716	3.7	48.1
B	T	C	-	A_{15}	T	0.1045	0.1492	0.2537	18	164.1
C	T	C	-	A_{16}	T	-	0.1791	0.1791	5	89
D	T	C	-	A_{17}	T	-	0.1343	0.1343	4	37
E	T	C	-	A_{18}	T	-	0.0074	0.0074	-	-
F	T	G	TTTTTAT	A_{13}	C	0.1791	0.0074	0.1865	5.8	243.5
G	C	C	-	A_{14}	T	0.0671	-	0.0671	1.6	1.6

Table 3 Parameters of population genetic diversity and substructure in *Senecio rodriguezii*. Estimates of genetic diversity and differentiation measures for ordered (v_s, v_t, N_{st}) and unordered (h_s, h_t, G_{st}) haplotypes are provided, together with their standard deviations. h_s and v_s, intrapopulation diversity, h_t and v_t, total diversity, G_{st} and N_{st}, degree of differentiation among populations.

	Majorca	Minorca	Total value
No. of populations	14	14	28
No. of individuals	70	64	134
No. of haplotypes	4	5	7
No. of private haplotypes	2	3	-
Average no. of haplotypes	5.07	4.85	4.96
No. of polymorphic sites	5	4	5
h_s	0.181 ± 0.0708	0.208 ± 0.0684	0.194 ± 0.0484
h_t	0.763 ± 0.0392	0.722 ± 0.0314	0.840 ± 0.0200
G_{st}	0.763 ± 0.0963	0.712 ± 0.0935	0.769 ± 0.0580
v_s	0.124 ± 0.0628	0.265 ± 0.1271	0.153 ± 0.0536
v_t	0.767 ± 0.0762	0.718 ± 0.0899	0.841 ± 0.1219
N_{st}	0.838 ± 0.0871	0.631 ± 0.1380	0.818 ± 0.0686
N_{st}-G_{st}	0.075	-0.081	0.049

Overall, high levels of cpDNA genetic diversity were detected across all *Senecio* populations, with h_s = 0.194 ± 0.048 and h_t = 0.840 ± 0.02. Significant geographical structuring of cpDNA markers, based both on unordered and ordered alleles, was found in Majorca (G_{st} = 0.763 ± 0.096, N_{st} = 0.838 ± 0.087), Minorca (G_{st} = 0.712 ± 0.093, N_{st} = 0.631 ± 0.138) and across all samples (G_{st} = 0.769 ± 0.058, N_{st} = 0.818 ± 0.068). Significant phylogeographic structure was deduced from the cpDNA data from Majorca (N_{st}>G_{st}; *U*= 3.37, *P*<0.05), but not for Minorca (N_{st}=G_{st}; *U*= -0.72, *P*>0.05), and the whole data set ($N_{st=}G_{st}$; *U*= 0.54, *P*>0.05).

Hierarchical partitioning of variation within and between populations

Genetic variation in *S. rodriguezii* was highly structured geographically. The results obtained from AMOVA showed that most of the cpDNA variation found in this species can be attributed to differences between populations (84.6%, Table 4).

When islands were analysed separately, Majorca showed a stronger population differentiation than Minorca (83.3% vs. 65.21%, respectively). However, the distances between populations where significantly higher in Majorca than in Minorca (mean distance between Majorca populations: 76.2 and 36.7 km, for geographical and linear distances, respectively; among Minorca populations: 48.4 and 21.31 km; ANOVA: $P <$ 0.001 for both geographic and linear distances). These greater distances could lead to a reduced gene flow and consequently increased differentiation between Majorcan populations. On the other hand, when variation between regions (islands) was taken into account, AMOVA found that 44.75% of the cpDNA variation was distributed between the islands and 43.17% between populations within the islands (Table 4). Thus, approximately half of the variation found in *S. rodriguezii* was due to differences between the islands.

Table 4 Analysis of molecular variance (AMOVA) based on *trn*T-*trn*L sequence data for *Senecio rodriguezii*. (a) Assuming no regional differentiation. (b) Two islands (Majorca, Minorca). * $P <$ 0.001 (significant after 10,000 permutations).

	Source of variation	df	Sum of squares	Variance components	Percentage of variation
(a)	Among populations	27	147.71	1.103	84.60*
	Within populations	106	21.28	0.200	15.40
(b)	Among islands	1	53.49	0.743	44.75*
	Among populations within islands	26	94.22	0.717	43.17*
	Within populations	106	21.28	0.200	12.08*

Spatial AMOVA (SAMOVA) did not allow us to clearly identify one single group of maximally differentiated populations, as F_{CT} values increased progressively with increasing values of K (F_{CT} values ranging from 0.729 to 0.825; Figure 3). The first level of divergence (K=2) revealed a well-defined group including those populations from Majorca with a high frequency of the divergent F haplotype (CEL, EIV, GUL, MES), which were differentiated from the remaining populations (72.9%; Figure 3). The composition of the groups detected by SAMOVA with increasingly larger values of K mainly corresponded to the geographical distribution of haplotypes (Figure 3).
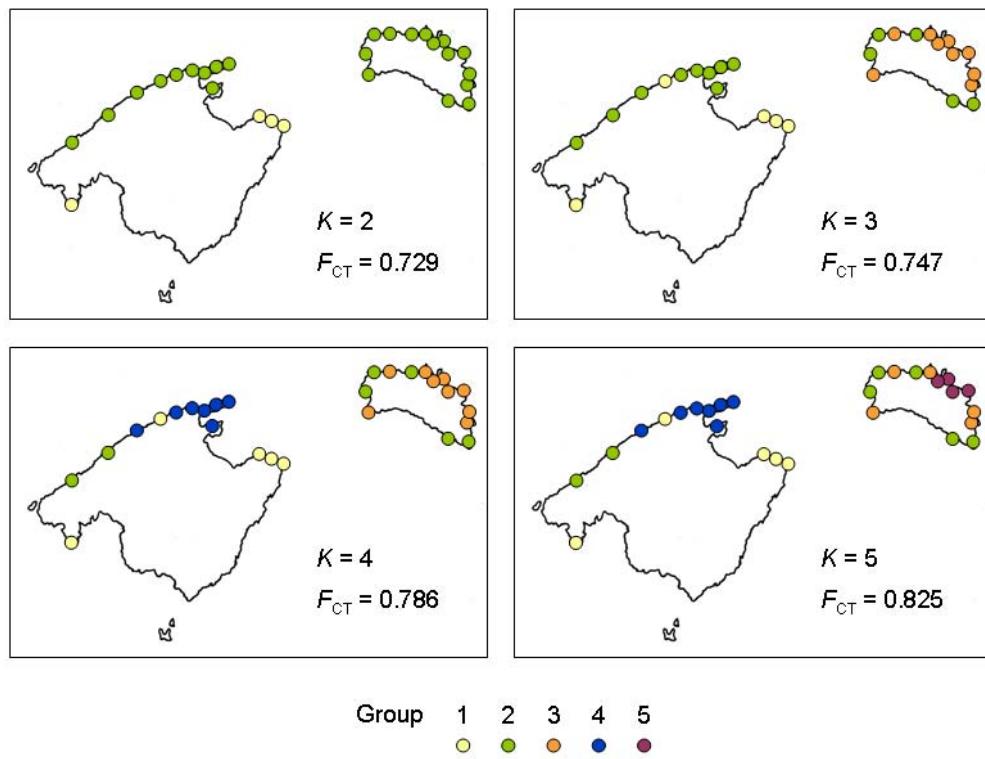
Figure 3 Groups of *Senecio rodriguezii* defined by the spatial analysis of molecular variance (SAMOVA). The maximum value for F_{CT} was obtained for $K = 5$. The composition of groups detected was highly coincident with the geographical distribution of haplotypes.

No significant correlations between genetic (Φ_{ST}) and either geographic or coastal distances were found within the islands (Mantel tests for Majorca: r = 0.1921, *P* = 0.067; r = 0.1949, *P* = 0.070; Mantel tests for Minorca: r = -0.1235, *P* = 0.166; r = -0.0322, *P* = 0.430). However, there were weak but significant positive relationships between the genetic and geographic distances (r = 0.2998, *P* < 0.001; r = 0.3073, *P* < 0.001) when the whole data set was analysed.

Phylogenetic relationships among haplotypes

Statistical parsimony analysis at the 95% confidence limit initially resolved two separate networks (one including haplotype F and the other with the remaining haplotypes) that could only be joined with a non-parsimonious connection of four steps (Figure 2). Four haplotypes (A, B, C, D) were nested in the network as interior nodes whereas three (E, F, G) occupied tip clades and were connected to the network by one (E, G) and four (F) mutational steps. Missing intermediate haplotypes in the network were only identified between A and F haplotypes. The highest root probability was assigned by TCS to haplotype B (P = 0.286), which is central within the network, showed the highest frequency (0.253) and was widespread on both islands.

DISCUSSION

Genetic variation in island plants: a caution

There have been very few studies on levels of intraspecific cpDNA differentiation in narrowly distributed plants from the Mediterranean basin. The scarcity of these records are due to the persisting perceptions that (1) rare species show on average low levels of overall genetic variation (Stebbins, 1942; Hamrick & Godt, 1989), (2) insular species tend to show low values of genetic diversity as compared with close continental relatives (Frankham, 1997), and (3) the cpDNA genome is an unsuitable target for assessing genetic markers for intraspecific analysis due to its relative slower mutation rate compared to nuclear DNA (Wolfe *et al.*, 1987). However, these general expectations seem to be lineage-specific (Banks & Birky, 1985; Soltis *et al.*, 1992; McCauley, 1995; Gitzendanner & Soltis, 2000), and the detected levels of cpDNA variation depend to a large extent on the recent evolutionary history of the species (e.g., Vendramin *et al.*, 1999, 2008; Jaramillo-Correa *et al.*, 2006). Furthermore, the use of more informative genotyping techniques (such as DNA sequencing), rather than indirect genotyping by RFLP approaches, has increased the number of species showing extensive intraspecific DNA variation (e.g., Widmer & Baltisberger, 1999; Dobeš *et al.*, 2004).

Our survey on the *trn*T-*trn*L spacer of the narrow endemic *S. rodriguezii* detected seven haplotypes, which is similar to the number of haplotypes found in other congeneric species, such as *S. halleri* (seven; Bettin *et al.*, 2007) and *S. leucanthemifolius* var. *casablancae* (five; Coleman & Abbott, 2003), or the widespread Mediterranean *S. gallicus*, *S. glaucus*, and *S. leucanthemifolius* (five; Comes & Abbott, 1999). It could be argued that the relatively high allelic richness found in *S. rodriguezii* is due to the use of different experimental approaches (sequencing in this study versus RFLPs using cloned or PCR-amplified fragments). This could be a reasonable explanation since the five haplotypes found in *S. rodriguezii* only differ in the microsatellite region. However, it should be pointed out that identical levels of cpDNA variation have been reported in some species when RFLPs and microsatellite markers have been compared (Waters & Schaal, 1991; Provan *et al.*, 1999). Other parameters of genetic diversity estimated for *S. rodriguezii* also indicate that this species is not genetically depauperate (h_s = 0.194, h_t = 0.840). Similar results of cpDNA genetic diversity have been obtained in other narrowly distributed, endemic species from the Western Balearic Islands (e.g. *Crepis triasii*, *Naufraga balearica*, *Hippocrepis balearica*; A. Molins, M. Mayol & J.A. Rosselló, unpublished data), and other continental islands (Chiang & Schaal, 2006, and references therein). Contrary to our expectations, the emerging picture is that the Balearic Islands are a reservoir of genetic diversity, both nuclear and organellar, not only for widespread Mediterranean taxa (López de Heredia *et al.*, 2005), but also for narrowly distributed endemics (Sales *et al.*, 2001). Thus, more complex evolutionary histories, rather than a simple hypothesis based on genetic bottlenecks due to founder effects, should be invoked to explain the origin and evolution of plant species from continental islands.

Genetic differentiation in a wind-dispersed plant

Similar to the wind-dispersed diaspores found in many Asteraceae, the cypselas of *S. rodriguezii* show a distinct plumose pappus that acts as a drag-enhancing parachute suitable for effective dispersal. Predictions based on a wide range of supporting data suggest that wind-dispersed taxa should exhibit low values of population differentiation, at least for nuclear markers (Nybom & Bartish, 2000). However, cpDNA

data suggest apparent reduced dispersability in this species since most of the genetic variance found in *S. rodriguezii* can be attributed to variation between populations (AMOVA = 84.6%; G_{st} = 0.77) and about half of this variation is due to differences between populations within islands. These parameters of genetic (cpDNA) differentiation between populations are significantly higher when compared to those reported in the two congeneric species (1) *S. halleri* (G_{st} = 0.39), an alpine species with fragmented populations that survived glaciations in the nunatak mountains (Bettin *et al.*, 2007), and (2) the Moroccan coastal endemic *S. leucanthemifolius* var. *casablancae* (Φ_{ST} = 0.30; Coleman & Abbott, 2003). *Senecio rodriguezii* also shows the highest values by far of population differentiation when compared to other coastal species with a linear, fragmented distribution (Franks *et al.*, 2004; Fievet *et al.*, 2007; Ortiz *et al.*, 2007; Piñeiro *et al.*, 2007).

The restricted distribution of haplotypes D, G and E located in populations separated by short coastal distances (less than 40 km; Table 2, Figure 2) and the presence of island-specific haplotypes (Majorca and Minorca are separated by a minimum distance of 36 km) strongly suggest that the diaspores of S*. rodriguezii* have a very ineffective dispersal ability, despite showing a well-developed and functional pappus. Based on phylogeographic data the possession of a pappus has not been considered as an effective means of long-distance dispersal in *S. halleri* (Bettin *et al.*, 2007) and the closely related *S. leucanthemifolius* (Coleman & Abbott, 2003). Significant reduction of dispersal ability may develop relatively quickly in small, isolated natural populations (Cody & Overton, 1996) and has been documented for species living on both oceanic (Carlquist, 1974) and continental islands (Cody & Overton, 1996; Fresnillo & Ehlers, 2007). Further, selection against dispersal in insular species might be enhanced in strictly coastal species since diaspores with high dispersal ability may be blown off the islands or dispersed to unsuitable habitats surrounding the existing populations (Olivieri *et al.*, 1995; Cody & Overton, 1996). The fact that Majorca had stronger population differentiation and geographical structuring of cpDNA markers than Minorca could be explained by differences in morphological traits (length and shape of cypsela and/or pappus) linked to dispersal. Unfortunately, we lack data on morphological variation of propagules in *S. rodriguezii* to check this hypothesis. On the

other hand, distances between populations were significantly higher in Majorca than in Minorca, which could have led to a reduced gene flow and, consequently, increased differentiation between Majorcan populations. Different topographical and/or ecological features along the coasts of Majorca and Minorca (e.g. the presence of elevated calcareous platforms and hills in the north of Majorca are unsuitable for *S. rodriguezii*) may have historically constituted natural barriers to gene flow that have favoured population differentiation of cpDNA markers.

Phylogeographic structure in a palaeodynamic insular system

The pattern of population differentiation in *S. rodriguezii* is not random but suggests an association between the genealogy and the geographical distribution of cpDNA haplotypes (Figure 2). This population structuring has been modulated by a balance between historical processes linked to the dynamic Quaternary palaeogeography of the Eastern Balearic Islands (fragmentation) and to past and contemporary recurrent gene flow involving long-distance dispersal (range expansion, colonisation). Because (1) with few exceptions, maximum distances separating any two populations sharing the same haplotype are low (Table 2), (2) only two haplotypes (B, F) are shared by Majorca and Minorca, and (3) parameters of population genetic structure (e.g., G_{st}, N_{st}) are high, seed dispersal in *S. rodriguezii* has not counteracted the patterns of population differentiation drawn by the recurrent Quaternary vicariance.

However, disentangling the contribution of each factor influencing the geographic distribution of haplotypes in *S. rodriguezii* is not an easy task. First, there is no prior knowledge of the species history based on the fossil record. Second, the use of statistical analyses in phylogeographic studies to distinguish processes and events shaping spatial genetic structuring throughout the complex evolutionary histories of natural populations is sometimes very complicated. For example, one of the most popular methods for reconstructing the demographic history of spatially distributed populations (nested clade phylogeographic analysis (NCPA); Templeton, 1998) has been criticised by several authors. Independent analyses have shown that clade statistics implemented by the NCPA method can be significantly biased, yielding more

false positives (type I error) than expected under a number of scenarios (Knowles & Maddison, 2002; Petit & Grivet, 2002; Panchal & Beaumont, 2007).

One of the significant findings of this study is the lack of a phylogeographic break between populations from Majorca and Minorca, despite the fact that five out of seven haplotypes are restricted to single islands. In fact, SAMOVA analysis did not identify two well-defined groups corresponding to Majorca and Minorca populations at any level of divergence (from K=2 to K=5). In addition, the assessment of areas where cpDNA haplotype frequency changes and genetic barriers are more robust, using Monmonier's (1973) maximum difference algorithm, failed to detect a significant barrier between Majorca and Minorca islands (data not shown). On the other hand, when genetic variance was partitioned into covariance components at different hierarchical levels under the hypothesis of regional grouping (between islands, between populations within each island, and between individuals within populations), AMOVA analysis showed that about the same percentage of the variation found in *S. rodriguezii* was due to differences between islands (44.75%) and between populations within islands (43.17%). These results could be explained by the fact that complete isolation of Majorca and Minorca from the precursor island (Figure 1) was a relatively recent event in evolutionary times (c. 13,000 yr BP; Cuerda, 1975) and might not have left a significant phylogeographic footprint in *S. rodriguezii*. The presence of too few clades in the recovered parsimony haplotype network and the high similarity shown by most of the haplotypes (differing by very few mutations except for the divergent haplotype F), might explain the lack of significant phylogeographic structure in those parameters (e.g. N_{st}) that are based on the distance between haplotypes (Pons & Petit, 1996).

A rate of 1.24×10^{-9} synonymous substitutions per site and year has been reported for different chloroplast loci (Gaut, 1998; Xiang *et al.*, 2000). Assuming a similar substitution rate at noncoding cpDNA regions, it is likely that haplotypes A, G and F (differing by substitutions and indels) were present in the precursor island (i.e. the united lands of Majorca, Minorca and Cabrera) and have not evolved *in situ* within the islands. Moreover, estimated mutation rates at cpSSR loci (between 3.2×10^{-5} and 7.9×10^{-5} s/s/y; Provan *et al.*, 1999) implies a recent origin of haplotypes differing in

the number of poly-A stretches (B-E) following the separation of Majorca and Minorca islands. Assessing the ancestry of the cpDNA haplotypes present in *S. rodriguezii* is not free of uncertainties. On the one hand, the coalescent theory predicts that interior nodes of a gene network represent ancestral haplotypes from which derived genotypes, located at tip nodes, evolve (Crandall & Templeton, 1993). Statistical parsimony identifies haplotype B as the ancestral genotype in *S. rodriguezii*, albeit with a moderate probability. However, outgroup comparison did not support this answer, because accessions from the closely related *S. leucanthemifolius* from Majorca, Ibiza, and Sardinia had haplotypes A and F, suggesting their ancestrality, despite the fact that the latter is located on the tips of the network. Sharing cpDNA haplotypes among members of Mediterranean *Senecio* sect. *Senecio* is the rule rather than the exception, and reticulation and lineage sorting have been invoked to explain it (Comes & Abbott, 1999, 2001). Interestingly, populations of *S. rodriguezii* from eastern Majorca (CEL, MES, GUL), where the single known population of *S. leucanthemifolius* is sympatrically located, are fixed for haplotype F. Past cytoplasmic capture of cpDNA from *S. leucanthemifolius* cannot be ruled out. However, apart from current sympatry, there is no evidence from floral and foliar morphology or nuclear markers (ribosomal ITS sequences, J.A. Rosselló & A. Molins unpublished data) that introgression has occurred in *S. rodriguezii.* Thus, incomplete lineage sorting is left as the most viable hypothesis for the presence of haplotype F in this species.

Irrespective of what method is used to root the network, there is full agreement that the closely related haplotypes C, D, E and G are derived. Interestingly, putative ancestral haplotypes (A, B, F) show a wider distribution than derived ones and are mainly located on abrupt territories with elevated calcareous hills or platforms (N and NE Majorca, W and E Minorca), suggesting that these areas could have served as refuges throughout the transgressive sea-level cycles. Furthermore, the northern and central coasts of Minorca, to which the derived haplotypes D and E are restricted, were probably colonised from nearby propagule sources after the separation of Majorca and Minorca. Finally, the fixed presence of haplotype F in the isolated EIV population from Majorca suggests a link with the distant eastern populations (CEL, MES, GUL). This implies the extinction of many intermediate populations from the eastern and southern

coasts of Majorca, although the possibility of a rare event of long-range dispersal cannot be discarded.

CONCLUSIONS

Senecio rodriguezii is a genetically highly structured species showing very few shared haplotypes between islands and a high number of haplotypes restricted to small geographical areas within the islands. This scenario appears to be shaped by historical processes and restricted gene flow that have left detectable genetic footprints in this coastal species. Changes in habitat availability and dynamic processes of population fragmentation and connectivity drawn by repeated cycles of conspicuous sea level changes during the Quaternary are potential underlying reasons locally shaping the cpDNA pool of this endemic species. It would be interesting to analyse the cpDNA variation and structure in other Eastern Balearic, strictly coastal endemic species showing similar distribution patterns (such as *Launaea cervicornis* and *Polycarpon colomense*) to see whether they share congruent phylogeographic patterns.

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CAPÍTULO 2

Lack of a west-east divide in the disjunct Mediterranean shrub *Buxus balearica* suggests ancient hybridization with its sister species *B. sempervirens*

Capítulo 2: Lack of a west east divide in the disjunct Mediterranean shrub *Buxus balearica* suggests ancient
hybridization with its sister species *B. sempervirens*

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Abstract

The extreme disjunct distribution of taxa between the west and east Mediterranean basin has been reported for hundreds of taxonomic and phylogenetically unrelated organisms. However, very few studies have assessed the phylogeographical pattern of Mediterranean plants showing intraspecific disjunctions and inferred the historical causes explaining them. We have assessed the levels of chloroplast DNA variation in the shrub *Buxus balearica*, showing an east-west Mediterranean disjunction, (i) to assess the levels of cpDNA variation in a narrowly distributed shrub showing no obvious adaptations to long-range dispersal, (ii) to investigate the presence of phylogeographic structure in Mediterranean disjunct plants restricted to glacial refugia, and (iii) to interpret the results in light of the palaeography of the Mediterranean basin, and to shed light on the origin of the disjunct distribution of the species. Eighteen representative populations of *B. balearica* covering the entire range of the species were sampled and sequences of the chloroplast *trn*T-*trn*L spacer were obtained for 108 individuals. Nine haplotypes were detected in *B. balearica*. About half of the detected haplotypes were present in Eastern Mediterranean samples (A-D) and five were detected in Western Mediterranean populations (E-I). Low levels of intrapopulation diversity were detected in *B. balearica* and, except two populations from Anatolia, all sampled populations showed a single haplotype. When variation between regions was taken into account, AMOVA found that only 10.10% of the cpDNA variation was distributed between the Eastern and Western Mediterranean. The haplotype network obtained did not support a phylogeographic break between Western and Eastern Mediterranean; instead, a well-defined group including those populations from the Balearic and Sardinia islands was differentiated from the remaining populations. Regional sharing of chloroplast haplotypes between *B. balearica* and the sister species *B. sempervirens* at Anatolia and at the Iberian Peninsula and North Africa suggest that ancient and independent chloroplast capture of *B. sempervirens* haplotypes by *B. balearica* in continental areas could be invoked as a likely explanation to explain the lack of a west-east divide in the disjunct Mediterranean shrub.

INTRODUCTION

Disjunctions in the ranges of species have fascinated biologists since they were first detected and their interpretation has long been regarded as one of the central and heated problems of plant biogeography (Raven, 1972; Thorne, 1972). Dispersal across pre-existing barriers and vicariance through fragmentation and isolating events have

been often contrasted as competing processes primarily responsible for these biological disjunctions (e.g. Stace, 1982). However, recent methods of biogeographical reconstruction using DNA markers within a phylogenetic and phylogeographic context recognize the potential of both processes to explain singular patterns of biotic diversity (Riddle *et al.*, 2008), and the emerging question is about discovering their relative frequencies (Zink *et al.*, 2000; Yoder & Nowak, 2006).

The extreme disjunct distribution of taxa between the west and east Mediterranean basin has been reported for hundreds of taxonomic and phylogenetically unrelated organisms, including several animal phylla (Ribera & Blasco-Zumeta, 1998; Sanmartín 2003, and references therein), lichenized fungi (Egea & Alonso, 1996), bryophytes (Casas *et al.*, 1981), and vascular plants (Davis & Hedge, 1971; Kadereit & Yaprak, 2008, and references therein).

The current distribution and population structure of Mediterranean plant species has been shaped by a combination of complex geological and climatic changes presumably dating back to the Miocene, when a negative hydric balance caused the desiccation of the Mediterranean sea during the Messinian salinity crisis (about 5.8-5.3 My BP; Gautier *et al.*, 1994). However, molecular signatures from modern Mediterranean populations of some taxa suggest that older palaeogeographic events from the Oligocene (about 30-25 million years BP) may be invoked to trace back their current genetic structure (Magri *et al.*, 2007). Moreover, the contrasting climate changes occurred during the Quaternary, modulated by the glacial and interglacial periods, is believed to have played a substantial role in shaping the distribution of the earth biota (Hewitt, 2004). These events, together with anthropogenic processes linked to the arrival, evolution and dispersal of our species and its ancestors in Europe (Blondel & Aronson, 1999) could have played more recent essential factors contributing to the genetic diversity, population structure, and range shift for most Mediterranean species.

In plants, the scrutiny of the phylogeographic history of selected species has allowed the understanding of the biological impact of the Pleistocene climatic changes, shaping the distribution of glacial refuges, the corridors of plant migrations, the patterns of population differentiation and, ultimately, the temporal frames of speciation. Intense range dynamics associated to several cycles of area expansion and

fragmentation has been postulated to have occurred on plants with distinctly different distribution patterns, ecological demands and life stories. Changes in habitat availability, population fragmentation and connectivity, and interspecific gene flow due to environmental changes during the Quaternary are the possible underlying factors that have shaped the genetic pool and the phylogeographical structure of most species on a regional scale.

In the Mediterranean basin, molecular footprints of ancient range shifts and population bottlenecks, and inferences about glacial refuges contributing to the colonization history of widespread European taxa after the Last Glacial Maximum of the Würm glaciation have been revealed by the phylogeographic analysis of nuclear and cytoplasmic markers, mainly in tree and long-lived shrub species (Taberlet *et al.*, 1998). However, it is surprising that very few work has been devoted to assess the phylogeographical pattern of Mediterranean plants showing intraspecific disjunctions and to infer the historical causes explaining them. Clear East-West phylogeographic breaks were detected in *Microcnemum coralloides* (Chenopodiaceae) and *Buxus balearica* (Buxaceae) using molecular markers (Rosselló *et al.*, 2007; Kadereit & Yaprak, 2008). Unfortunately, these results were substantiated on a very limited data set with no intrapopulation sampling (Kadereit & Yaprak, 2008) or inferred from nuclear multigene families (ITS) showing rampant intragenomic paralogy (Rosselló *et al.*, 2007).

The genus *Buxus* is widely represented on most continents but only two extant sister species (*B. balearica* Lam. and *B. sempervirens* L.; von Baltazhar *et al.*, 2000) showing a mostly non-overlapping range are present in Europe and North Africa (Figure 1)*.* The common box (*B. sempervirens*) is widely present throughout Southern and Western-central Europe, North Africa and Western Asia, on dry and base rich (chalk and limestone) soils, usually in mesophyllous forests, mixed with deciduous species or forming pure populations. By contrast, the Balearic box (*B. balearica*) shows an east-west Mediterranean disjunction, occurring in few and fragmented populations (Figure 1), including southeastern Iberian Peninsula, the Balearic Islands, Sardinia, North Africa (Riff, Middle Atlas, High Atlas and Saharian Atlas), and southern Anatolia.

We selected *B. balearica* as a model system to assess the phylogeographic structure in Mediterranean plants showing east-west disjunctions. We used chloroplast

DNA markers in order to study the underlying causes explaining these contrasting distributions. A priori, a strong pattern of population differentiation and structure concerning molecular markers is expected if the extant range is relict of a wider distribution area that was fragmented due to climatic turnover, stochastic catastrophic events, and anthropogenic activities through the extinction of many populations linking the now disjunct Western and Eastern Mediterranean sites. On the other hand, dispersal over long distances or anthropogenic introductions might have caused a lack of a phylogeographic signal in this species. Hence, it is interesting to investigate whether or not historical factors have shaped the patterns of population differentiation in a species whose distribution mirror the Mediterranean glacial refugia (Médail & Diadema, 2009) and that has not contributed to the current northern European biota after the Last Glacial Maximum of the Würm glaciation.

Our specific goals were (1) to assess the levels of cpDNA variation in a narrowly distributed shrub showing no obvious adaptations to long-range dispersal, (2) to investigate the presence of phylogeographic structure in Mediterranean disjunct plants restricted to glacial refugia, (3) to interpret the results in light of the palaeography of the Mediterranean basin, and to shed light on the origin of the disjunct distribution of the species.

MATERIALS AND METHODS

Sampling collection and DNA extraction

Eighteen representative populations of *B. balearica* covering the entire range of the species were sampled in the field (Table 1, Figure 1). Between five to seven individuals for each population were analyzed. Two populations of the sister species *B. sempervirens* were also sampled (one from the Iberian Peninsula and one from Anatolia, n=5 in each population) for comparative purposes. Total genomic DNA was isolated and purified from silica gel-dried specimens using the DNeasy[TM] Plant Minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Amplification of cpDNA, sequencing and alignment

The *trn*T-*trn*L intergenic spacer was amplified using the universal primers *A* and *B* described in Taberlet *et al.* (1991). PCR reactions were performed in 50 µl, containing 1X reaction buffer, 0.001% BSA, 2 mM MgCl$_2$, 0.5 mM of each dNTP, 0.6 µM of each primer, approximately 50-100 ng of genomic DNA and 3 units of DNA polymerase (NETZYME™ DNA Polymerase, NEED S.L., Spain). Thermal cycling started with a denaturation step at 94ºC lasting 2 min, followed by 30 cycles each comprising 50 s denaturation at 94ºC, 50 s annealing at 53ºC, and 1.5 min elongation at 72ºC. Amplification ended with a final elongation cycle of 3 min at 72ºC. Amplifications were carried out on an ABI GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, California). PCR products were checked for concentration on 2% agarose gels, purified using the High Pure PCR Product Purification Kit (Roche, Germany) and sequenced with an ABI 3100 Genetic Analyzer using the ABI BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California). Samples were sequenced in both forward and reverse directions. Available *trn*T-*trn*L accessions from *Buxus* were retrieved from GenBank and used for comparative purposes. Sequences were aligned with CLUSTAL X vs. 1.83 (Thompson *et al.*, 1997) and manually adjusted.

Completeness of sampling

The completeness of haplotype sampling was determined by means of Stirling probability distribution (Dixon, 2006) using an online interface for calculating posterior probabilities of haplotype sampling available at the website http://www.botanik.univie.ac.at/plantchorology/haplo.htm.

Genetic diversity and population differentiation

Measures of genetic diversity and population differentiation were calculated with two different analyses: one taking into account the frequencies of haplotypes (HAPLODIV software; Pons & Petit, 1995), and one incorporating both the haplotype frequencies and the genetic distances between haplotypes (HAPLONST software; Pons & Petit,1996).

Occurrence of a significant phylogeographic structure was inferred by testing if G_{st} (coefficient of genetic variation over all populations) and N_{st} (equivalent coefficient taking into account the similarities between haplotypes) were significantly different by use of 1,000 permutations using PERMUT (Pons & Petit, 1996).

The geographical structure of genetic variation was assessed by analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) using ARLEQUIN vs. 2.000 (Schneider *et al.*, 2000). The total genetic variance was partitioned into covariance components at different hierarchical levels under two hypotheses: (1) without regional grouping, therefore just between and within populations; (2) with regional grouping, that is, between major biogeographic territories (Iberian Peninsula-North Africa, Western Mediterranean islands, Anatolia), between populations within each region, and between individuals within populations. The significance levels of the variance components were obtained by non-parametric permutation using 10,000 replicates. In addition, SAMOVA (spatial analysis of molecular variance) was used to identify groups of populations that were geographically homogeneous and maximally differentiated from each other using the software SAMOVA vs. 1.0 (Dupanloup *et al.*, 2002). This method is based on a simulated annealing procedure that maximizes the proportion of total genetic variance due to differences between groups of populations, and also leads to the identification of genetic barriers between these groups. The most likely number of groups (K) was identified by repeatedly running the program for 10,000 iterations for $K \in \{2, ..., 7\}$ using 500 random initial conditions, and retaining the largest Φ_{CT} values (i.e., the largest proportion of total genetic variance due to differences between groups) as predictors of the best grouping of populations (Dupanloup *et al.*, 2002). In addition, the assessment of areas where cpDNA haplotype frequency changes and genetic barriers are more robust were assessed using Monmonier's (1973) maximum difference algorithm as implemented in the BARRIER vs. 2.2 software (Manni *et al.* 2004).

Table 1 Site locality names, site codes, samples sizes (*N*), and haplotypes (*h*) recorded in each site. The geographical location of each population is represented in Figure 1.
* Sample not included in the calculation of population genetic diversity parameters.

| Locality	Code	Coordinates		Altitude	*N*	*h*
B. balearica						
North Africa						
Talembote (Morocco)	TA	5º16'26'' W	35º16'06'' N	160	7	H
Jebha (Morocco)	J	4º45'27'' W	35º02'55'' N	150	6	H
Boulemane (Morocco)	B	4º37'14'' W	33º25'43'' N	900	6	H
Cirque de Jaffar (Morocco)	CJ	4º54'50'' W	32º34'17'' N	1,100	7	H
Gorges du Todra (Morocco)	GT	5º35'09'' W	31º35'51'' N	1,000	7	H
Iberian Peninsula						
Frigiliana (Spain)	M	3º52'39'' W	36º46'33'' N	350	5	H
Cuevas de Nerja (Spain)	MM	3º50'45'' W	36º45'46'' N	150	7	H
Velez de Benaulla (Spain)	GR	3º22'47'' W	36º51'08'' N	1,200	7	H
Rágol (Spain)	AL	2º41'30'' W	36º59'03'' N	440	7	I
Balearic Islands						
Ternelles (Majorca)	TE	2º57'54'' E	39º53'46'' N	650	6	G
Andratx (Majorca)	AN	2º23'42'' E	39º31'31'' N	75	7	G
Artá (Majorca)	AR	3º21'01'' E	39º46'43'' N	340	6	F
Cap Ventós (Cabrera)	CV	2º57'54'' E	39º09'43'' N	50	7	F
Sardinia						
Carbonia	CE	8º42'00'' E	39º20'24'' N	150	7	E
Monte Tassua	MT	8º42'01'' E	39º20'25'' N	150	6	E
Anatolia						
Adrasan, Antalya (Turkey)	AD	30º30'55'' E	36º19'45'' N	300	6	A (4), B (2)
Süphandere, Feke (Turkey)	FK	35º50'46'' E	37º59'17'' N	750	7	C
Antakya, Hatay (Turkey)	AK	36º10'35'' E	36º12'26'' N	150	7	C (6), D (1)
B. sempervirens						
Ribes del Freser (Spain)	PI	2º06'16'' W	42º18'17'' N	1,000	5	H
Tevekkeli, Kahraman Maras (Turkey)	MR	36º58'60''E	37º28'60''N	550	5	C

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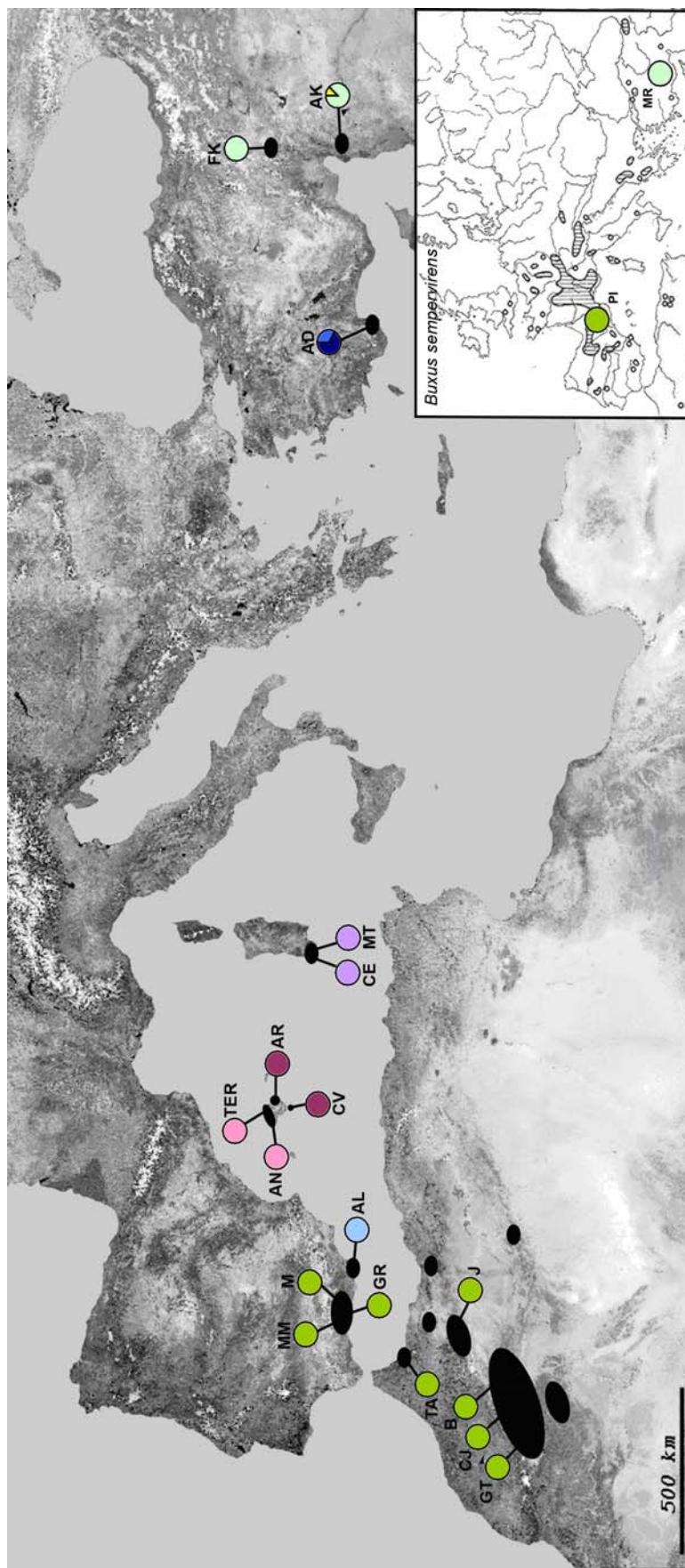
Figure 1 Geographical distribution of nine chloroplast haplotypes found in *Buxus balearica*. For population codes, see Table 1. The detailed distribution of the *Buxus sempervirens* is shown in the inset.

Finally, pairwise Φ_{ST} values were computed using ARLEQUIN vs. 2.000 (Schneider *et al.*, 2000), and significance levels of the estimated values were obtained by permutation using 10,000 replicates. A pattern of isolation by distance was assessed by testing the correlation between the matrix of pairwise Φ_{ST} values and the matrix of geographical distances between pairs of populations by means of a single Mantel test implemented in GENETIX vs. 4.04 software (Belkhir *et al.*, 2001) using 1,000 random permutations to test for statistic significance. The geographical distances were computed between all sampling locations using the geographical coordinates (see http://jan.ucc.nau.edu/~cvm/latlongdist.html).

Phylogeographic estimation

Unrooted statistical parsimony networks were constructed using TCS vs. 1.21 software (Clement *et al.*, 2000). Insertions and deletions were treated as a fifth state, regardless of their length. Root probabilities were calculated using the TCS program following the method of Castelloe & Templeton (1994), and also using *B. microphylla* as an outgroup. Ambiguities in the cladogram were present in the form of a loop. To solve it, predictions from coalescent theory (frequency, topological, and geographical criterions; Crandall & Templeton, 1993) were used to decide among alternative solutions.

We used Nested Clade Phylogeographic Analysis (NCPA) to test for significant association of haplotypes with geographical location, and to disentangle the main historical and contemporary processes that determine a species evolution and geographical distribution (Templeton *et al.*, 1995; Templeton, 1998). The use of NCPA has been criticized by some authors (Knowles & Maddison, 2002), but it remains a powerful method of reconstructing phylogeographic histories in species where prior information does not exist (Templeton, 2004). The NCPA was performed using ANECA (Panchal, 2007; available at http://www.rubic.rdg.ac.uk/~mahesh/software.html) a fully automated implementation of TCS vs. 1.18 (Clement *et al.*, 2000) and GeoDis vs. 2.2 (Posada *et al.*, 2000) software. ANECA automate the nesting algorithm and the published inference key (available at http://darwin.uvigo.es/software/geodis.html), and link together the various components of the NCPA analysis.

RESULTS

Sequence and haplotype variation

The length of the *trn*T-*trn*L spacer ranged between 660 bp and 685 bp, and resulted in an aligned matrix of 706 bp. Polymorphic sites included three point mutations, five indels (ranging from 4 to 25-bp long) and variations at two mononucleotide poly-A and poly-T stretches composed of between 9-11 and 9-10 nucleotides, respectively. These mutations defined nine haplotypes (A to I) that are depicted in Table 2. Applying the Stirling probability distribution to the data (9 haplotypes, 108 plants), which calculates a posterior probability distribution of the total number of haplotypes including those which may not have been sampled, it was estimated that all haplotypes were probably found with a probability of 95%.

Excepting two populations from Anatolia showing two haplotypes, all sampled populations harboured only a single haplotype (Table 1, Figure 1). About half of the detected haplotypes were present in Eastern Mediterranean samples (A-D) and five were detected in Western Mediterranean populations (E-I). Haplotypes H, the most frequent (46.34%) and widespread, and I were present in the Iberian accessions; the former was the single haplotype detected in North African populations. Two haplotypes, G and F, were found in samples from the Balearic Islands and haplotype E characterized the Sardinian populations. Haplotype C was the most frequent (10.57%) chlorotype in the Eastern Mediterranean and was present in two (FK, AK) out of the three sampled populations. Interestingly, samples of *B. sempervirens* from the Iberian Peninsula and Anatolia shared the most frequent haplotypes (H and C, respectively) found in *B. balearica* in these territories. Haplotype H was also present in a *B. sempervirens* sample coming from the Bonn botanical garden AY145357.

Table 2 Characterization of the *Buxus balearica* haplotypes based on the polymorphic sites of the *trn* T-*trn* L intergenic cpDNA spacer. A dash (-) indicates insertion-deletion events. The frequencies of the cpDNA haplotypes and their geographical origin are indicated. WM= Iberian Peninsula and North Africa; MI= Mediterranean islands; AN= Anatolia.

Haplotype	Polymorphic sites										Frecuency			
	72-78	178	187-211	212-216	225	228-237	253-259	390-411	429	541-544	WM	MI	AN	Total
A	TTTAAT	T	-	TAAAA	T	$[T]_{10}$	-	$[A]_{10}$	G	-	-	-	0.0325	0.0325
B	-	T	-	TAAAA	T	$[T]_{10}$	-	$[A]_{10}$	G	-	-	-	0.0163	0.0163
C	-	T	-	TAAAA	T	$[T]_{9}$	-	$[A]_{9}$	A	-	-	-	0.1057	0.1057
D	-	T	AATTTCTATAAAAATATAATTTCTA	TAAAA	T	$[T]_{9}$	-	$[A]_{9}$	A	-	-	-	0.0081	0.0081
E	-	G	-	-	G	$[T]_{10}$	TATTTAA	$[A]_{11}$	A	-	-	0.0894	-	0.0894
F	-	T	-	-	G	$[T]_{9}$	TATTTAA	$[A]_{11}$	A	-	-	0.1057	-	0.1057
G	-	G	-	-	G	$[T]_{9}$	TATTTAA	$[A]_{11}$	A	-	-	0.1057	-	0.1057
H	-	T	-	TAAAA	T	$[T]_{9}$	-	$[A]_{9}$	A	GGTC	0.4634	-	-	0.4634
I	-	T	-	TAAAA	T	$[T]_{10}$	-	$[A]_{10}$	A	-	0.0732	-	-	0.0732

Hierarchical partitioning of variation within and between populations

Parameters of genetic diversity and structure for *B. balearica* are given in Table 3. Overall, low levels of intrapopulation diversity were detected in *B. balearica* across all populations, with h_s = 0.046 ± 0.032 and v_s = 0.010 ± 0.007. Genetic variation in Balearic box was highly structured geographically, based both on unordered and ordered alleles (G_{st} = 0.943 ± 0.039, N_{st} = 0.987 ± 0.009). However, a significant phylogeographic structure was not deduced from the whole data set (N_{st} = G_{st}; U= 1.41, *P*>0.05).

The results obtained from AMOVA showed that, when no regional grouping was considered, most of the cpDNA variation found in this species can be attributed to differences between populations (98.86%, Table 4). When variation between regions was taken into account, AMOVA found that only 10.10% of the cpDNA variation was distributed between the Eastern and Western Mediterranean. Instead, most of the variation among groups was revealed (80.06%) when the hypothesis involving three regional units (Iberian Peninsula and North Africa, Western Mediterranean islands, Anatolia) was considered.

Spatial AMOVA (SAMOVA) did not allow us to clearly identify one single group of maximally differentiated populations, as F_{CT} values increased progressively with increasing values of *K* (F_{CT} values ranging from 0.810 to 0.991). The first level of divergence (*K*=2) revealed a well-defined group including those populations from the Balearic and Sardinia islands showing the divergent E, F and G haplotypes, which were differentiated from the remaining populations (81.03%). When three groups were considered (*K*=3) the populations of AL (Iberian Peninsula) and AD (Anatolia) were split from the Iberian-North African-Anatolian group.

Table 3 Parameters of population genetic diversity and substructure in *Buxus balearica*. Estimates of genetic diversity and differentiation measures for ordered (v_s, v_t, N_{st}) and unordered (h_s, h_t, G_{st}) haplotypes are provided, together with their standard deviations. h_s and v_s, intrapopulation diversity, h_t and v_t, total diversity, G_{st} and N_{st}, degree of differentiation among populations. WM= Iberian Peninsula and North Africa; MI= Mediterranean islands; AN= Anatolia.

	WM	MI	AN	Total value
No. of populations	9	6	3	18
No. of individuals	61	39	20	120
No. of haplotypes	2	3	4	9
No. of private haplotypes	2	3	4	9
Average no. haplotypes	6.56	6.50	6.67	6.56
No. of polymorphic sites	3	2	5	10
h_s	-	-	-	0.046± 0.0328
h_t	-	-	-	0.792± 0.0849
G_{st}	-	-	-	0.943± 0.0394
v_s	-	-	-	0.010± 0.0075
v_t	-	-	-	0.794± 0.1005
N_{st}	-	-	-	0.987± 0.0090
N_{st}-G_{st}	-	-	-	0.044

The composition of the groups detected by SAMOVA with increasingly larger values of *K* mainly corresponded to the geographical distribution of haplotypes. In no case SAMOVA identified a phylogeographic break between Western and Eastern Mediterranean populations. A significant correlations between genetic (Φ_{ST}) and geographic distances were found as evidenced by the Mantel test (r = 0.426, *P* < 0.01).

Table 4. Analysis of molecular variance (AMOVA) based on *trn*T-*trn*L sequence data for *Buxus balearica*. (a) Assuming no regional differentiation; (b) Eastern Mediterranean versus Western Mediterranean; (c) three regional units (Iberian Peninsula and North Africa, Western Mediterranean islands, Anatolia). * *P* < 0.001 (significant after 10,000 permutations), ns = not significant.

	Source of variation	df	Sum of squares	Variance components	Percentage of variation
(a)	Among populations	17	211.51	1.895	98.86*
	Within populations	100	2.19	0.022	1.14
(b)	Among regions	1	19.19	0.208	10.10 ns
	Among populations within regions	16	192.31	1.833	88.84*
	Within populations	100	2.19	0.022	1.06*
(c)	Among regions	2	161.11	2.136	80.06*
	Among populations within islands	15	50.40	0.510	19.12*
	Within populations	100	2.19	0.022	0.82*

Phylogenetic relationships among haplotypes

TCS calculated a 95% parsimony connection limit of 13 steps for the nine haplotypes resulting in a network with a single loop (Figure 2), which was solved according to predictions from coalescent theory (Crandall & Templeton, 1993). Five haplotypes (B, C, E, G, I) were nested in the network as interior nodes whereas four (A, D, F, H) occupied tip clades. Missing intermediate haplotypes in the network (five) were identified between E-I and C-I haplotypes, all other haplotypes were one mutational step apart from each other. The highest root probability was assigned by TCS to the Eastern Mediterranean haplotype C (*P* = 0.286). The nested design for the NCPA is shown in Figure 2 and the inferred historic processes are given in Table 5. Both, at the one-step-level and the second clade level, NCPA revealed allopatric fragmentation for the haplotypes present in the Western Mediterranean islands. Conversely, long-distance colonisation or past fragmentation followed by range expansion was inferred for the continental haplotypes. On the level of the entire parsimony network NCPA provided conclusive evidence that the main historic process explaining the cpDNA lineages for *B. balearica* is allopatric fragmentation.

Table 5 Inferred historical processes affecting genetic structure of *Buxus balearica* populations based on nested clade analysis.

| Clade	χ^2-statistic	Probability	Inference chain	Inferred pattern
1-1	71,08	<0.001	1-2-11-12-13 YES	Long-distance colonisation possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion.
1-3	26,00	<0.001	1-19 NO	Allopatric fragmentation.
2-1	158,00	<0.001	1-2-11-12-13-14 NO	Long-distance colonisation and/or past fragmentation (not necessarily mutually exclusive).
2-2	38,00	<0.001	1-19 NO	Allopatric fragmentation.
Total	117,00	<0.001	1-19 NO	Allopatric fragmentation.
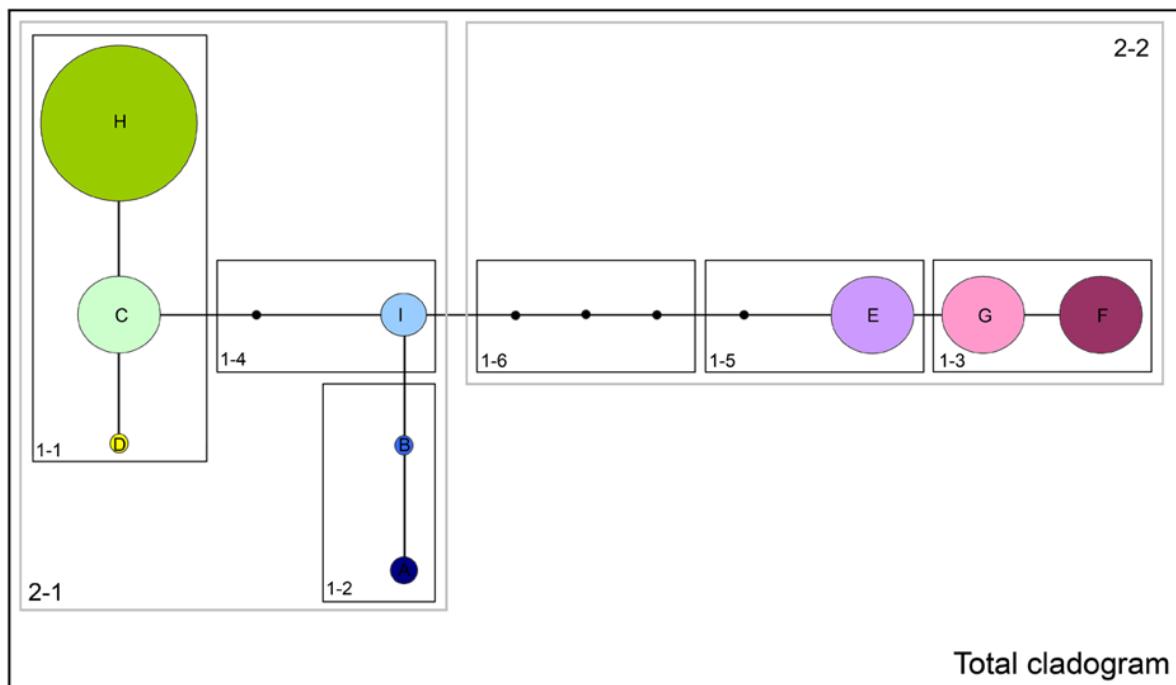
Figure 2 The nested design for the NCPA of nine chloroplast haplotypes found in *Buxus balearica.*

DISCUSSION

The distribution of vascular plant species and landscape vegetation thorough the Mediterranean basin show a profound biogeographic differentiation that is more significantly related to a longitudinal east-west divide rather than a latitudinal split into northern-southern shores (Thompson, 2005, and references therein). This has been substantiated either by narrative and intuitive views or by numerical phytogeographical approaches comparing the floristic affinities of selected territories (Junikka *et al.*, 2006). Moreover, morphological east-west differentiation and vicariance has been suggested for some putative, closely related sister taxa showing disjunct distributions (e.g. *Pinus halepensis-P. brutia*, *Quercus coccifera-Q. calliprinos*, *Cyclamen repandum* subspecies; Thompson, 2005, and references therein).

Significantly, this organismal and ecological biogeographic pattern is also mirrored by intraspecific molecular genealogies documented from a number of strict Mediterranean plant species showing continuous distributions. Thus, contrasting molecular differentiation between western and eastern populations has been reported not only for terrestrial species, including *Ficus carica* (Khadari *et al.*, 2005), *Olea europaea* subsp. *europaea* (Besnard & Bervillé, 2000; Breton *et al.*, 2006) and *Laurus nobilis* (Rodríguez-Sánchez *et al.*, 2009), but also for marine seagrasses like *Posidonia oceanica* (Micheli *et al.*, 2005). Furthermore, major genetic subdivisions reflecting a west-east phylogeographic break throughout the Mediterranean have even been reported for submediterranean (e.g., *Anthyllis montana*, Kropf *et al.*, 2002) and widespread Euro-Asiatic inland (e.g., *Frangula alnus,* Hampe *et al.*, 2003; *Hedera helix*, Valcárcel *et al.*, 2003) or coastal taxa (e.g., *Eryngium maritimum*, *Halimione portulacoides*; Kadereit *et al.*, 2005).

This biogeographic break might be intimately linked to the extremely complex geological history, palaeogeography and paleoclimatology of the Mediterranean basin that fragmented and merged biotas as dispersal barriers appeared and disappeared through time. Two distinct large domains are recognized: the western part (including the Tyrrhenian, Ligurian, Provencal, Algerian, and Alboran basins) that started to form during the Oligocene (ca. 34-30 Ma BP) in an overall convergence setting between the African and Eurasian plates (Dewey *et al.*, 1973; Dercourt *et al.*, 1986; Krijgsman,

2002), and the eastern part (including the Ionian, Herodotus, and Levant basins) that represent remnants of the Early Mesozoic Neotethys Ocean (Garfunkel, 2004).

Furthermore, the glacial episodes of the Quaternary (starting 2.6 Ma; Webb & Bartlein, 1992; Lambeck *et al.*, 2002), and most recently the last glacial maximum (around 23.000-18.000 yr BP), has had major impacts on the present-day distribution of European species and their lineages (Hewitt, 2004). The long-term isolation of populations within geographically separate major refugia at the Mediterranean (Iberian, Italian, and Balkan Peninsulas; Taberlet *et al.*, 1998) might shaped differentially the spatial structuring and genetic differentiation of intraspecific lineages in Mediterranean plants, further enhancing the distinctiveness of biogeographic breaks in this area.

Thus, empirical data drawn from many sources would predict the existence of a cpDNA split encompassing the western and eastern populations of *B. balearica*. Contrary to expectations and to the results obtained using nuclear ribosomal nuclear markers (Rosselló *et al.*, 2007), this west-east divide is not supported by our cpDNA data set and, interestingly, a new unrecognized phylogeographic pattern emerged (Western islands vs. continental Mediterranean areas).

Conflicting genealogical signals retrieved by nuclear and cpDNA markers could be caused by several processes. These could be in principle explained by the inherent contrasting properties of the nuclear and cytoplasmic genomes analyzed (e.g., different patterns of evolution, heritage, and recombination), stochastic factors (incomplete lineage sorting), as well as the questionable utility of the ribosomal ITS region as a suitable marker due to the presence of multiple copies and loci within the nuclear genome (Álvarez & Wendel, 2003; Nieto Feliner & Rosselló, 2007). However, such conflict is generally explained by interspecific gene flow resulting in more or less complex scenarios of hybridization and introgression. Regional sharing of chloroplast haplotypes across morphospecies is a consequence of hybridization and could be expected if directional introgression has occurred via asymmetric flow of pollen, (e.g. Rieseberg & Soltis, 1991; McKinnon *et al.*, 2001; Petit *et al.*, 2004; Albaladejo *et al.*, 2005). Interestingly, *B. sempervirens* and *B. balearica* shares some haplotypes at western (haplotype H) and eastern (haplotype C) populations, where both species grow at close proximity. Moreover, the presence of wild populations of *B. sempervirens*

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has not been ever recorded at the Balearic Islands and Sardinia, the regions were species-specific haplotypes of *B. balearica* occurs.

Thus, ancient and independent chloroplast capture (i.e., introgression followed by relatively rapid fixation of an introgressed haplotype) of *B. sempervirens* haplotypes by *B. balearica* in both Western and Eastern Mediterranean basin could be invoked as a likely explanation to reconcile the facts that the nuclear multigene ITS family show species-specific markers, no signs of hybridization throughout the *B. balearica* range, and a west-east phylogeographic break (Rosselló *et al.*, 2007). Similar processes involving extensive gene flow has been invoked to blur the phylogeographic, but not the phylogenetic signal, in other Mediterranean woody plants like *Olea europaea* L. (Rubio de Casas *et al.*, 2006). Additional support for the hybridization scenario here proposed for *B. balearica* comes from morphology. Benedí (1997) reported that populations of this species from the Iberian Peninsula and North Africa differ from those present in the Balearic Islands by the presence of hairy shoots and a lower style/capsule length ratio, two of the discriminating features used to identify *B. sempervirens* from *B. balearica*.

Both, nuclear and chloroplast markers strongly reject the hypothesis that, given the highly appreciated use of the Balearic box wood by humans for several purposes in prehistoric times (e.g., charcoal, manufacture, ritual; Piqué, 1999a, 1999b; Piqué & Noguera, 2002), anthropogenic introductions in historical times be responsible of the disjunct pattern seen in *B. balearica*. Furthermore, the fixed presence of a private cpDNA haplotype in Sardinia strongly suggests its wild origin in the island, in contrast with earlier views supporting an alien status by anthropogenic introductions (Gennari, 1864). This contrast with problems posed by the delimitation of the natural area of the related *B. sempervirens* for which doubt has often been thrown on its indigenity in countries of temperate Europe like England, France or Belgium and introductions from medieval times have been postulated for most of the current stations located in northern France (Decocq *et al.*, 2004).

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CAPÍTULO 3

Ancient DNA suggest unexpected relationships from an extinct population of *Buxus balearica* from SE Spain

Abstract

A single herbarium specimen collected in 1943 is the only biological voucher sustaining the presence of the shrub *Buxus balearica* in Cartagena (SE Spain), where it is though to be extinct and protected by regional laws. Recovery plans devised for this species included the reintroduction of ex situ stocks in several points of Cartagena Mountains to establish self-sustaining populations. DNA analysis (*trn*T-*trn*L region of the chloroplast genome) from the ancient herbarium specimen revealed a haplotype restricted to the wild populations of the Balearic Islands. These unexpected results disagree with the geographic structure of the cpDNA and phylogeography of *B. balearica.* Overall evidence from the palaeogeography of the Western Mediterranean and from the seed dispersal syndrome shown by this species does not support a relict status for the Cartagena specimen. Rather, it points to a likely anthropogenic introduction or to the falsification of the herbarium specimen from material originating from Mallorca.

INTRODUCTION

The reintroduction of propagules to reinforce the existing populations or to restore those already extirpated is one of the key conservation strategies of ex situ management aimed to ensure recovery of wild populations (Wieren, 2006). The establishment of self-sustaining populations that retain the genetic diversity necessary to undergo adaptative evolutionary changes can counteract dramatic declines in distribution and abundance originated by biological causes and anthropic activities.

However, conservation strategies involving translocation of populations and the reinforcement of declining populations is a complex issue that is not free of criticisms (Green, 1981; Dodd & Seigel, 1991; Hodder & Bullock, 1997; Storfer, 1999). The problem is even more acute when the restoration involves wild extinct taxa or locally extirpated populations since several case-studies have shown that the choice of ex situ stocks is vital to ensure reintroduction success (Templeton, 1986; Rhymer & Simberloff, 1996; Hufford & Mazer, 2003; Honjo *et al.*, 2008). Molecular markers could be of great help to improve conservation programs involving reintroductions by assessing the genetic diversity of the ex situ collections and their suitability to avoid mixing of genetically distinct populations (Gray, 2002; Sinclair *et al.*, 2005). The later

might disturb the evolutionary history of the endangered taxa by a loss of local adaptation or the breakup of coadapted gene complexes (Hufford & Mazer, 2003).

Buxus balearica Lam., the Balearic box, is a circum-Mediterranean shrub showing a disjunct area and fragmented populations in southern Anatolian Peninsula, North Africa, southern Iberian Peninsula, the Balearic Islands, and Sardinia. The reasons underlying this distribution are not fully understood, but climatic turnover, stochastic catastrophic events (e.g. fires), and anthropogenic activities could have shaped the distribution of *B. balearica* since Holocene times through the extinction of many populations linking the now disjunct Western and Eastern Mediterranean ranges.

Some authors have documented a significant reduction in the box pollen fossil record in the Balearic Islands during the Holocene. They suggested a climatic change to explain its synchronous and conspicuous rarefaction from the woody landscape (Yll *et al.*, 1997, 1999; Pérez-Obiol *et al.*, 2002). Even, pollen and wood remains documented the presence of *B. balearica* in Minorca until 2,000 years ago, where it is now extinct (Burjachs *et al.*, 1994; Piqué 1999a, 1999b). There is also evidence of the rarefaction of *B. balearica* populations in the Western Mediterranean in recent times. Its distributional range has drastically decreased in historical times (19th century) in Majorca as its extremely hard, dense wood was highly appreciated for carving, turnery, engraving blocks and inlay work, and because of its use as a means of obtaining charcoal (Marès & Vigineix, 1880). Lastly, the species is in risk of extinction in Sardinia where only a few individuals are known from only two close populations (Biondi *et al.*, 1996).

In the Iberian Peninsula the Balearic box is restricted to few populations in the south, where it is endangered and protected under regional laws (Anonymous, 1994). A single herbarium specimen preserved in the Botanical Garden of Madrid (Spain) documented the presence of *B. balearica* in coastal mountains near Cartagena (Murcia region), about 200 km away from the closest known populations from Andalusia. The herbarium sheet was collected in 1943 and it is the only reference documenting the presence of *B. balearica* in Cartagena, where it has not been found again. For conservation purposes, *B. balearica* was catalogued as extinct in the wild for the Murcia region (Anonymous, 2003). This legal figure implied the redaction of a reintroduction plan aimed to evaluate its viability and to made eventual actions for its

recovery. In recent years, conservation programs aimed to restore the apparently extinct population of Cartagena has been devised. These include the introduction of individuals in several points of Cartagena Mountains to establish self-sustaining populations. The choice of ex situ stocks of *B. balearica* to create the new populations is a challenge.

Recent phylogeographic studies using chloroplast DNA (cpDNA) markers have shown the presence of two allopatric haplotypes in Southern Spain (Molins *et al.*, unpublished data), the closest continental territory from Cartagena where extant *B. balearica* populations are located. In plant species, the geographical distribution of cpDNA haplotypes is often restricted (reviewed by Soltis *et al.*, 1997; Newton *et al.*, 1999). The incorporation of ancient DNA into conservation genetics holds a lot of potential and DNA from museum specimens has been sown to provide reliable, alternative data source in conservation studies (Leonard, 2008, and references therein).

The goal of this study is to use historical DNA from a herbarium collection to assess the cpDNA genotype of the extinct population of *B. balearica* in Cartagena. This knowledge, put in a phylogeographic context, could be relevant to guide management actions concerning the reintroduction of this species at this locality.

MATERIALS AND METHODS

Plant accessions

Leaf tissue from the single herbarium specimen collected in an unspecified site near Cartagena was analyzed (MA 405069). Data previously obtained from 18 populations of *B. balearica* covering the entire range of the species and two populations from the related *B. sempervirens* were used for comparative purposes (see Chapter 2). Additional herbarium specimens and three field samples originating from the Iberian Peninsula and North Africa, increasing the sampling coverage in these territories, were also analyzed (Table 1).

DNA extraction from herbarium specimens

Fresh and silica-gel dried samples from *B. balearica* and *B. sempervirens* were previously analyzed in our lab (Rosselló *et al.*, 2007, Molins *et al.*, unpublished data). Accordingly, in order to avoid lab and PCR contaminations, DNA extraction (and later molecular work) of the Murcia sample was performed in a molecular biology lab located in other institution (Institute of Biomedicine, Valencia) and where no work with any plant DNA was previously made. Total genomic DNA was isolated and purified using the DNeasy^{TM} Plant Minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Duplicate extractions of the Murcia sample were made and a DNA extraction control involving all reagents but no DNA sample was included to check for contamination.

Table 1 Site locality names, codes, samples sizes (*N*), and haplotypes (*h*) recorded in samples of *B. balearica*. The geographical location of each population is represented in Figure 1.

Accessions	Code	Voucher	*N*	*h*
Iberian Peninsula				
Cartagena	MU	MA 405069	1	G
Cortijo de los Hornajos	HO	HVAL 11411	1	I
Barranco de las Losas	LO	HVAL 1264	1	I
Cuevas de Nerja	MM	VAL	7	H
Barranco Calailla	NE	VAL	1	H
Velez de Benaulla	GR	VAL	7	H
Barranco del río Verde	VE	MGC 56719	1	H
Los Guajanes	GU	MGC 64508	1	H
Almuñecar	ÑE	MGC 34406	1	H
Rio de la Miel	MI	MGC 65377	1	H
North Africa				
Oued Laou (Morocco)	OU	RNG 21847	1	H
El Ksiba to tinghir (Morocco)	KS	RNG	1	H
Beni Snassen (Morocco)	BE	RNG	1	I

DNA amplification and sequencing

A 800 bp fragment of the *trn*T-*trn*L intergenic spacer was amplified using the universal primers *A* and *B* described in Taberlet *et al*. (1991) according to the PCR conditions described in Chapter 2. Initial amplifications using an Eppendorf Mastercycler personal thermal cycler resulted in very low PCR yields or no visible product at all, but these were not discarded. In order to increase the efficiency of the PCR we designed a set of internal primers, amplifying overlapping fragments of lower size (Tables 2 and 3), aimed to be conducted by nested PCR on the initial amplifications using the *A* and *B* primers.

Table 2 Sequences of PCR primers used in this study.

Primer name	Sequence (5' to 3')	Reference
A	CATTACAAATGCGATGCTCT	Taberlet *et al*. (1991)
B	TCTACCGATTTCGCCATATC	Taberlet *et al*. (1991)
BOJR1	CTTATCCTTTCCTTAGGTTAGG	This work
BOJF2	TAGTTATAGCGGATCGGC	This work
BOJR3	CTTGCCTATCTTCTTTCATC	This work

Table 3 Primer combinations used and size of the fragments amplified in the herbarium sample from Cartagena.

Fragment	Size	Primer combination
1	800	*A* / *B*
2	350	*A* / BOJR1
3	350	BOJF2 / BOJR3

Three µl of the first PCR rounds were used in 50 µl PCR reactions containing 1X reaction buffer, 0.001% BSA, 2 mM $MgCl_2$, 0.5 mM of each dNTP, 0.6 µM of each primer, approximately 50-100 ng of genomic DNA and 3 units of DNA polymerase (Biotools DNA Polymerase, Spain). Thermal cycling started with a denaturation step at 94ºC lasting 2 min, followed by 30 cycles each comprising 50 s denaturation at 94ºC,

50 s annealing at 53ºC, and 1.5 min elongation at 72ºC. Amplification ended with a final elongation cycle of 3 min at 72ºC. To check for PCR reproducibility and to further exclude spurious amplifications originating from exogenous DNA, amplifications were made in duplicate in different labs by independent workers, using separate PCR reagents stocks (excepting the internal primers) and thermal cyclers (ABI GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, California). All PCR reactions were repeated twice to verify the reliability of the results obtained. PCR products were checked for concentration on 2% agarose gels, purified using the High Pure PCR Product Purification Kit (Roche, Germany) and sequenced with an ABI 3100 genetic Analyzer using the ABI BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California). The sequences obtained were compared to GenBank DNA sequence databases using BLAST (Altschul *et al.*, 1997) and aligned with CLUSTAL X vs. 1.83 (Thompson *et al.*, 1997).

RESULTS

DNA from the Cartagena sample was highly degraded showing no signals of intact DNA (>20kb). The observed smear include fragments from 200 to 900 bp. PCR amplifications of the whole trn*T*-trn*L* resulted in very faint bands. Attempts to clone these amplicons failed and the low yield of the products prevented their direct sequencing. However, nested PCR from these amplifications using internal primers were successful and could be reproduced in two labs by independent personal and PCR conditions.

Sequencing of products from independent PCR always yielded the same results. The Cartagena sample showed no diagnostic mutation when compared to the haplotypes recovered from other *B. balearica* samples. It belonged to haplotype G, restricted to the box populations from northern Mallorca (Table 4, Figure 1). The additional herbarium samples analyzed conformed well to the geographic distribution of the known haplotypes. Thus, samples from North Africa showed haplotype H, the only genotype known in this area to date. Samples from south Spain included haplotypes H and I, agreeing with the phylogeographic split detected between Western and Eastern Iberian populations (see Chapter 2).

Table 4 Characterization of the *Buxus balearica* haplotypes based on the polymorphic sites of the *trn*T-*trn*L intergenic cpDNA spacer. A dash (-) indicates insertion-deletion events.

Haplotype	Polymorphic sites									
	66-72	178	187-211	212-216	225	228-238	259-265	397-408	430	547-550
A	1^a	T	-	1^c	T	$[T]_{10}$	-	$[A]_{10}$	G	-
B	-	T	-	1^c	T	$[T]_{10}$	-	$[A]_{10}$	G	-
C	-	T	-	1^c	T	$[T]_9$	-	$[A]_9$	A	-
D	-	T	1^b	1^c	T	$[T]_9$	-	$[A]_9$	A	-
E	-	G	-	-	G	$[T]_{10}$	1^d	$[A]_{11}$	A	-
F	-	T	-	-	G	$[T]_9$	1^d	$[A]_{11}$	A	-
G	-	G	-	-	G	$[T]_9$	1^d	$[A]_{11}$	A	-
H	-	T	-	1^c	T	$[T]_9$	-	$[A]_9$	A	1^e
I	-	T	-	1^c	T	$[T]_{10}$	-	$[A]_{10}$	A	-

Notes: 1^a =TTTTAAT, 1^b =AATTTCTATAAAAATATAATTTCTAT, 1^c = TAAAA, 1^d = TATTTAA, 1^e =GGTC
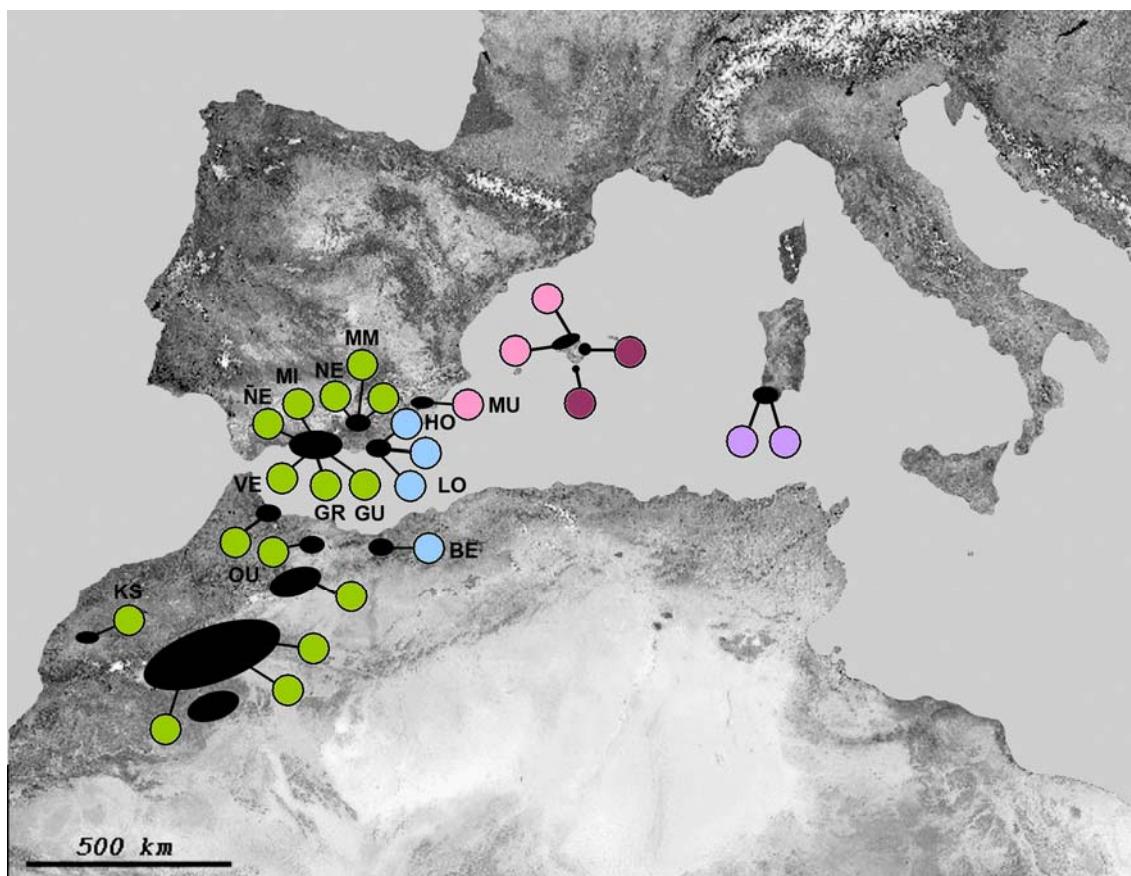
Figure 1 Distribution of cp DNA haplotypes found in *B. balearica* samples from the Western Mediterranean basin. Data samples not analysed in this study are taken from Molins *et al*., unpublished data (see Chapter 2). Haplotype characterization is given in Table 4.

DISCUSSION

Reliability of the recovered ancient DNA sequences

Success in recovering DNA sequences from the 65 years old specimen of *B. balearica* agree with other studies reporting PCR amplifications of cpDNA fragments from plant herbarium specimens up to about 200 years old (Savolainen, 1995, up to 109 years old; Drábková, 2002, up to 74 years old; De Castro & Menale, 2004, up to 193 years old; Jankowiak *et al.*, 2005, up to 100 years old). Contamination of ancient DNA with exogenous DNA and molecular damage given rise to erroneous sequences are two of the most serious potential problems associated to the ancient DNA research (Pääbo *et al.*, 2004).

Several precautions and criteria used in this work, agreeing with standard criteria (Pääbo *et al.*, 2004), suggest that the *trn*T-*trn*L sequences derived from authentic ancient DNA obtained from the herbarium specimen: DNA extractions and PCR manipulations were performed in two independent labs where no previous plant DNA extractions or PCR amplifications of plant DNA extracts were made; the herbarium specimen contained enough endogenous DNA detectable with routinely molecular techniques; blank extraction controls and negative PCR controls indicating lack of exogenous DNA were included; repeated amplifications from several extracts yielded consistent and reproducible results; the length of the PCR products is within the range of the DNA length extracted from the herbarium sample; the results were reproduced in a second laboratory by independent workers and identical sequences were obtained. Furthermore, all cpDNA sequences were identical to a previous haplotype previously identified in *B. balearica* (Molins *et al.*, unpublished data, see Chapter 2). This strength the reliability of the cpDNA retrieved and suggests that it is not an artifact due to nucleotide misincorporations in the PCR amplification process induced by damage in the ancient DNA template.

The origin of the Balearic box specimen from Cartagena

Chloroplast DNA variation in *B. balearica* is highly structured and parameters of population differentiation revealed a low level of genetic divergence between populations located within each of the three major regional units where the species is

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located, i.e. North Africa and Iberian Peninsula, Balearic Islands and Sardinia, Anatolia Peninsula (Molins *et al.*, unpublished data, Chapter 2). In fact, AMOVA analysis showed that the greatest genetic variation was attributable to regional differences, and most of the variation was found among insular and continental territories, whereas the remaining diversity was distributed among populations and among individuals within populations. Thus, the finding that the herbarium sample from continental Spain shares the same cpDNA haplotype with Balearic samples is intriguing and contrary to expectations obtained from the phylogeography. When a rare plant species is found in fragmented populations this may correspond either to natural relicts' outliers or artificial introductions planted by man; however it is usually difficult to find objective criteria that undoubtedly solve this question (Decocq *et al.*, 2004).

The disjunct area shown by haplotype G might be explained either as a consequence of a vicarious split or originated by dispersal events, but other explanations involving anthropogenic introductions or labelling errors of the herbarium specimen are also likely. The vicarious hypothesis implies that the Cartagena sample is a remnant of a past distribution that was present before the fragmentation of a Palaeozoic mountain chain (the Hercynian belt) that was situated in Iberia and southern Europe during the Early Oligocene (about 30-35 My BP). Corsica, Sardinia, the Balearic Islands, the Betic Mountains of Spain and the Riff Mountains of Morocco, the Kabylies (in the Atlas Mountains of Algeria), and Calabria (in the southern tip of the Italian peninsula) are all remnants of this Palaeozoic massif. According to tectonic reconstructions, the Balearic-Kabylies microplate and the Corsica-Sardinia-Calabria microplate rifted off from Iberia and southern Europe at ca. 30-28 Ma (Alvarez *et al.*, 1974; Rosenbaum *et al.*, 2002). From around 25 Ma, the Balearic-Kabylies microplate rotated clockwise until the Balearic archipelago reached its current position (ca. 21 Ma) and separated from the Kabylies terrane. The persistence of the same lineage in the continent and in the Balearic Islands for over 30 my implies an evolutionary stasis that is difficult to substantiate taking into account that (1) haplotype E, and not haplotype G, appears to be the most ancestral one in the insular clade (see Figure 2, Chapter 2), and (2) several mutational hotspots, including a microsatellite region, prone to duplication and deletion events appears to be present in the *trn*T-*trn*L spacer (see Table 4).

The dispersal hypothesis would imply a long range dispersal of seeds from (presumably) the Balearic Islands to the continent. Documented examples of back-colonization of the continent from islands are known, but scanty, from the Macaronesian islands (Carine *et al.*, 2004). However, *B. balearica* has a poor seed dispersal capacity since fruits are dry and dehiscent, and seed cases open suddenly ejecting small black seeds up to a few meters away (Rosselló *et al.*, 2007). In fact, seeds rain followed a typical leptokurtic distribution and the number of seeds dispersed decreased with distance from the parent plant (Lázaro *et al.*, 2006). More interesting, seeds bear an elaisome suggesting ant dispersal; however, the seeds do not attain long distances from the source plant and myrmecory must be minimal compared to seed predation (Lázaro *et al.*, 2006). Furthermore, most populations of *B. balearica* showed little or negligible recruitment and possess only a low number of juvenile individuals, implying that great losses during the dispersal and post-dispersal phases may be seriously limiting its population regeneration (Lázaro *et al.*, 2006). Thus, available biological data do not support, but can not reject, the hypothesis that the extinct Cartagena population of Balearic box is the result of a recent (Pleistocene) long range dispersal of propagules from the Balearic Islands.

Alternative hypotheses explaining the presence of the insular cpDNA genotype at Cartagena is based on the assumption that wild Balearic box was never present at this area. The natural area of many species having economical interest has experienced an artificial expansion by man, by way of plantation or cultivation. Given the highly appreciated use of the Balearic box wood by humans for several purposes, an anthropogenic introduction of the Cartagena specimen in historical times from the Balearic Islands should not be discarded. In fact similar problems concerning the natural area delimitation of the related *B. sempervirens* have been reported. Doubt has often been thrown on its indigenity in countries of temperate Europe like England, France or Belgium and introductions from medieval times have been postulated for most of the current stations in northern France (Decocq *et al.*, 2004).

Lastly, some authors have cast doubts about the authenticity of the herbarium specimen from Cartagena on the basis that it shows a rather vague geographical indication of the plant collection and does not include collector name (Benedí & Vicens, 1996; Benedí, 1997). Reports on falsifications of the herbarium labels indicating untrue

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localities to lead competitors astray, to gain undue merit, or to increase the economic value of the specimens being sold to scientific institutions, have been indicated worldwide (e.g. Standley, 1927; Bradlaugh-Bonner, 1972), including Spanish herbaria (Benedí, 1987; Benedí & Sáez, 1996; Montserrat, 2000). This could also apply to the specimen from Cartagena, since the cpDNA haplotype found in this sample is widespread and it is present in the most accessible populations from the Balearic Islands; the eastern population from Artà was not discovered until 1997 (Fraga *et al.*, 1997) and Cabrera Island was under the control of the Spanish army from 1916 to 1991 and had severe restrictions for accessing.

CONCLUSIONS

On the basis of environmental similarities Sánchez-Gómez *et al.* (2002) suggested that the target populations of *B. balearica* to be selected for eventual reintroductions to Cartagena should originate from Sierra de Gádor (Southern Spain). CpDNA analysis from the single biological remain sustaining the presence of this species in Cartagena indicated that, on the contrary, its closest relatives are from the Balearic Islands (Western Mallorca). This is an unexpected result based on the known geographic structure of the cpDNA variation and phylogeography of the Balearic box. Overall evidence from the palaeogeography of the Western Mediterranean and from the seed dispersal syndrome shown by this species does not support a relict status for the Cartagena specimen. Rather, it points to a likely anthropogenic introduction or to the falsification of the herbarium specimen from material originating from Mallorca.

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CAPÍTULO 4

Chloroplast DNA variation in *Thymus herba-barona* (Lamiaceae): insights into common palaeogeographical history of the Balearic Islands, Corsica and Sardinia

Abstract

The aim of this study was to examine the phylogeographical patterns in the Tyrrhenian endemic *Thymus herba-barona* in order to gain more insights into its evolutionary history. Our specific goals were: (1) to evaluate the relative importance of gene flow, selection and drift in structuring the patterns of genetic variation observed; and (2) to test whether the current distribution of the species is compatible with the ancient connections of the Tyrrhenian islands (Balearic Islands, Corsica and Sardinia), in the Western Mediterranean basin. Our sampling included 106 individuals from 15 populations covering the full species range. Sequences of the chloroplast genome (*trn*T-*trn*L spacer) were obtained and population genetic parameters were determined. Partition of haplotype variation was studied using AMOVA, and a SAMOVA was used to identify major groups within the species. Relationships among haplotypes were determined using parsimony networks, and a Nested Clade Phylogeographic Analysis (NCPA) was used to infer the evolutionary history of the species. Divergence times between major clades were estimated using a coalescent-based method (MIDV). Seventeen haplotypes were detected in *Th. herba-barona*. Only two of them were shared between Corsica and Sardinia, and the others were restricted to Majorca (one), Corsica (four) or Sardinia (ten). Most of the genetic variation detected was due to differences among populations (67%), supporting that gene flow among populations is very restricted. Haplotype network supported the occurrence of three main clades, the ancestral one being geographically restricted to the Gennargentu massif, in Sardinia, while the two derived ones were relatively widespread. A total of eighteen missing haplotypes were inferred, and the coalescent-based analyses indicated large divergence times and very limited ongoing gene flow between major clades. Inferred evolutionary history of the species was characterized by an early range expansion event, followed by successive fragmentation episodes. The observed level of cpDNA differentiation in *Th. herba-barona* supports an ancient origin for the species, and suggests that long-distance dispersal events have played a minor role in the species' evolutionary history. Several episodes of fragmentation seem to be determinant in the phylogeographical pattern observed, and suggest that genetic drift has acted historically as a major evolutionary force driving plant evolution in the Western Mediterranean basin.

INTRODUCTION

The Mediterranean basin is characterized by a high plant diversity and endemism (Greuter, 1991), being recognized as a global biodiversity hotspot (Myers *et al.*, 2000; Thompson, 2005). This high biodiversity is due, in part, to the fact that Mediterranean

areas served as refugia for many taxa during the Pleistocene glaciations (Médail & Diadema, 2009), allowing the long-term persistence of populations, which have in turn favoured the formation of new species. The numerous islands present in the Mediterranean constituted major refugial areas, and are nowadays a significant component of its plant diversity, with a large number of narrowly endemic taxa (Médail & Quezel, 1997, 1999). In addition, the highly fragmented insular landscapes have promoted geographical and genetic isolation among plant populations, favouring allopatric speciation via selection and/or genetic drift (Thompson, 2005). In spite of the central role that islands play to understand the evolution of plant biodiversity in the Mediterranean, and despite a long tradition of studies based on the endemic species, it is only recently that the Mediterranean islands have regained interest from evolutionists, as shown by several studies on the evolution of the Aegean (Affre & Thompson, 1997; Widén *et al.*, 2002; Bittkau & Comes, 2005; Edh *et al.*, 2007), the Balearic (Sales *et al.*, 2001; López de Heredia *et al.*, 2005; Molins *et al.*, 2009; Rosselló *et al.*, 2009) and the Corso-Sardinian floras (Mansion *et al.*, 2008; Salvo *et al.*, 2008; Falchi *et al.*, 2009).

The Balearic Islands, Corsica and Sardinia are, together with Sicily, the larger islands of the Western Mediterranean basin (the Tyrrhenian islands). They have been identified as one of the ten hotspots of plant diversity in the Mediterranean (Médail & Myers, 2004), with about 5,000 vascular plants, of which 360 are endemics (Médail & Quezel, 1997; Jeanmonod & Gamisans, 2007; Médail & Diadema, 2009). These islands have a complex paleogeographic origin, linked to important tectonic movements. According to geodynamic reconstructions of the Western Mediterranean since the last 30 million years, the territories currently found in southern France, the Balearic Islands, Corsica, Sardinia, Kabylies (Algeria), Calabria, Sicily, northeastern Spain and Rif/Betic Range were all connected forming an Oligocenic land known as the Hercynian massif (Alvarez *et al.*, 1974; Westphal *et al.*, 1976; Rosenbaum *et al.*, 2002; Speranza *et al.*, 2002). Successive splitting of this massif in the Late Oligocene and the south-eastward rotation of land masses during the Miocene led to the current position of the different microplates. The distribution of several endemic plants shared between the Balearic Islands, Corsica and Sardinia, termed paleoendemisms (e.g. *Arum pictum* L. f., *Arenaria balearica* L., *Cymbalaria aequitriloba* (Viv.) A. Cheval.,

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Delphinium pictum Willd., *Helicodiceros muscivorus* Engl., *Naufraga balearica* Constance & Cannon, *Soleirolia soleirolii* (Req.) Dandy, *Teucrium marum* L., *Thymus herba-barona* Loisel.), has been attributed to the ancient connections among these territories (Contrandriopoulos, 1981; Greuter 1995; Quézel, 1995; Thompson, 2005). To date, however, only very few empirical studies provide explicit support to the ancient origin of some Hercynian endemic plants, such as *Helicodiceros muscivorus* and *Arum pictum* (Mansion *et al.*, 2008) and, in numerous instances, this has fuelled speculations about their status as paleoendemics of likely Tertiary origin (Comes, 2004).

Thymus herba-barona is a perennial plant endemic from Majorca, Corsica and Sardinia. Together with *Thymus nitens* Lamotte, restricted to the South of France, constitutes the subsection *Pseudopiperellae* (sect. *Serpyllum*), a small assemblage of thyme species endemic to the Western Mediterranean basin (Jalas, 1971). In Corsica and Sardinia it is a common plant growing in mountainous and sub-mountainous zones at an altitude of 800–2,000 m (Camarda, 1978; Camarda & Valsecchi, 1990). In Sardinia it is less widespread than in Corsica, and only occurs in an almost continuous fashion in the Gennargentu massif and Marghine-Goceano region (Camarda & Valsecchi, 1990; Figure 1). In the Balearic Islands, however, the only known population of Majorca (Serra d'Alfàbia, ca. 900 m) has approximately 150 individuals and its area of occupancy is only about 100 m^2 (Mayol *et al.*, 1990; 1998). Until the discovery of the Balearic population, it has been postulated that *Th. herba-barona* was a paleoendemic plant originated by hybridization between unknown ancestral species (Contandriopoulos, 1962). The fact that the species was restricted to only one small population in Majorca could be interpreted as a rare long-distance seed dispersal event from Corsica or Sardinia, or a human-mediated introduction of the plant in the island. Nevertheless, in contrast to previous chromosome counts reported from Corsica (2n = 56, tetraploid cytotype; Contandriopoulos, 1962) or Sardinia (2n = 84, hexaploid cytotype; Diana-Corrias, 1980), individuals from Majorca were found to be diploid (2n = 28; Mayol *et al.*, 1990), which suggests an ancient origin for the Balearic population. Other evidence supporting the relict nature of this population are the morphological differences of the Balearic plants when compared to those inhabiting Corsica and Sardinia, which has led to the recognition of different entities within the

species, either at the subspecific and the specific level. Thus, Mayol *et al.* (1990) propose the recognition of the Balearic plants at the infraspecific level (*Th. herba-barona* subsp. *bivalens* Mayol, L. Sáez & Rosselló), whereas Camarda (2003) considers that morphological discontinuities between the plants inhabiting the different islands warranted the recognition of three separated entities at the species level: *Th. herba-barona* from Corsica, *Th. catharinae* Camarda from Sardinia, and *Th. bivalens* (Mayol, L. Sáez & Rosselló) Camarda, from Majorca.
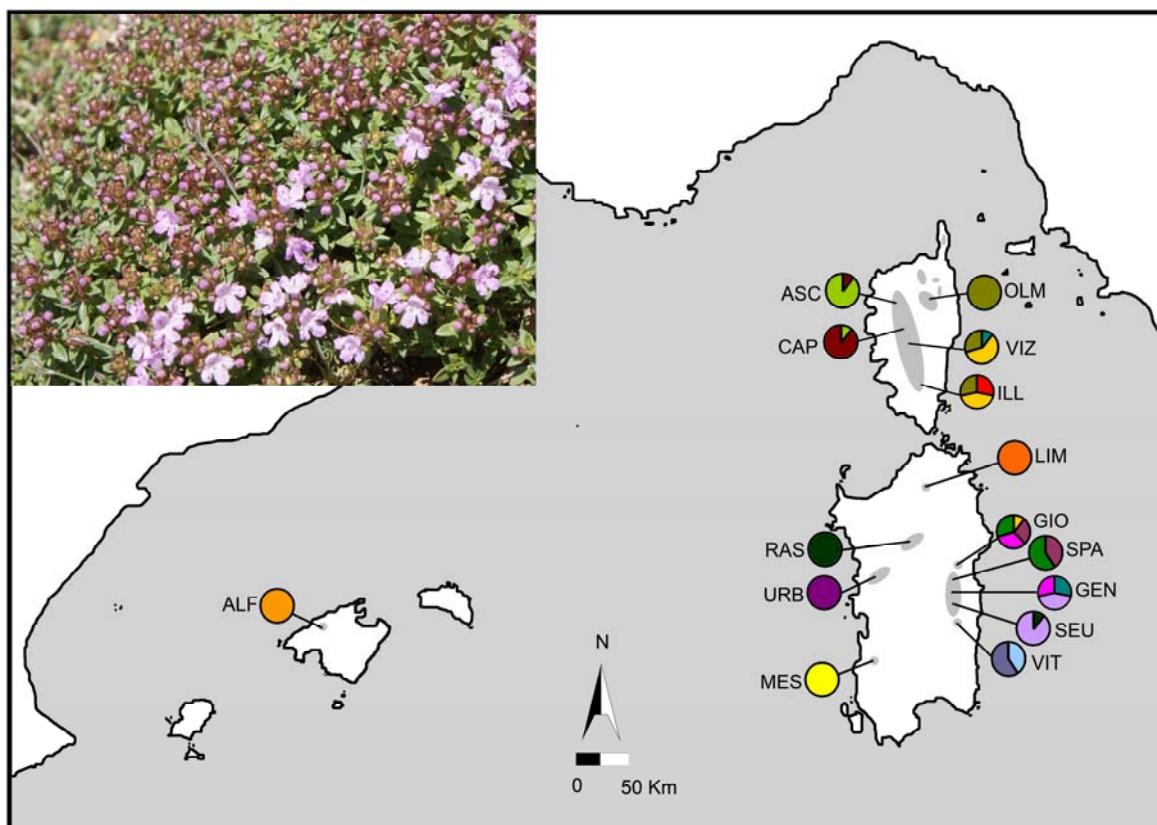
Figure 1 Geographical distribution of the seventeen chloroplast haplotypes found in this study for *Thymus herba-barona*. For population codes, see Table 1. The grey area indicates the approximate distribution range of the species.

In this study we used cpDNA sequences to examine the phylogeographical pattern of *Th. herba-barona* throughout their whole distribution range, in order to gain more insights into its evolutionary history. The main objectives of this work were: (1) to determine the relative role of the different evolutionary processes in shaping genetic

variation; (2) to test whether the disjunct distribution of this Tyrrhenic endemism is due to a vicariant origin congruent with the Oligocene geological splitting of the ancient Hercynian massif, or other biological and/or anthropogenic processes must be invoked to explain its current distribution.

MATERIALS AND METHODS

Plant sampling and DNA extraction

A total of 106 samples of *Th. herba-barona* were collected from 15 populations covering the entire species range (Figure 1, Table 1). Seven to eight individuals were analysed for each locality. Leaves were dried in silica gel and stored at room temperature until being processed. Total genomic DNA was isolated and purified from 20 mg of dried leaf tissue using the DNeasy™ Plant Minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quantity and quality of DNA was checked by running 5 μL of each sample in 1% agarose gels.

DNA amplification, sequencing and alignment

Amplification and sequencing of the *trn*T-*trn*L intergenic spacer was conducted using the universal primers *A* and *B* described in Taberlet *et al.* (1991). PCR reactions were performed in 50 µL, containing 1 X PCR buffer, 0.0001% BSA, 2 mM MgCl$_2$, 0.2 mM of each dNTP, 0.6 µM of each primer, approximately 50-100 ng of genomic DNA and 3 units of DNA polymerase (NETZYME™ DNA Polymerase, NEED S.L., Spain). Thermal cycling started with a denaturation step at 94ºC lasting 2 min, followed by 30 cycles each comprising 50 s denaturation at 94ºC, 50 s annealing at 53ºC, and 1.5 m elongation at 72ºC. Amplification ended with a final elongation cycle of 3 m at 72ºC. Amplifications were carried out on an ABI GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, California). PCR products were checked for concentration on 2% agarose gels, purified using the High Pure PCR Product Purification Kit (Roche, Germany) and sequenced with an ABI 3100 Genetic Analyser using the ABI BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California). Samples were sequenced in both forward and reverse directions. The sequences obtained were compared to GenBank sequence

Table 1 Location, description and codes of the fifteen *Thymus herba-barona* populations included in this study. The geographical location of each population is represented in Figure 1. *N*, sample size. The number of individuals carrying a given haplotype are shown between brackets.

Origin	Locality	Code	Altitude (m)	*N*	Haplotypes
Majorca	Alfàbia	ALF	990	7	O (7)
Corsica	Olmi	OLM	775	7	H (7)
	Haut Ascò	ASC	1,825	8	K (7), N (1)
	Lago di Capitello	CAP	1,850	7	K (1), N (6)
	Bocca d´Illarata	ILL	905	7	H (2), L (3), M (2)
	Colle di Vizzavona	VIZ	1,150	7	H (2), I (1), L (4)
Sardinia	Monte Rasu	RAS	1,000	7	J (7)
	Badde Urbara	URB	930	7	C (7)
	Montarbu di Seui	SEU	1,305	7	D (6), J (1)
	Monte Santa Vittoria	VIT	1,120	7	E (4), F (3)
	Monte Spada	SPA	1,300	7	B (3), G (4)
	Monte Novo S. Giovanni	GIO	1,210	7	A (2), B (2), G (2), L (1)
	Bruncu Spina	GEN	1,800	7	A (2), D (3), I (2)
	Monte Linas	MES	975	7	P (7)
	Monte Limbara	LIM	1,200	7	Q (7)

databases using BLAST (Altschul *et al.*, 1997) and aligned with CLUSTAL X vs. 1.83 (Thompson *et al.*, 1997). To confirm that *trn*T-*trn*L is evolving in a neutral manner, sequence variation was tested for significant deviation from neutrality using Tajima's *D* (Tajima, 1989) and Fu's F_S (Fu, 1997) tests, considering all individuals analysed as a single population. Both statistics were calculated using ARLEQUIN vs. 2.000 software (Schneider *et al.*, 2000).

Genetic diversity and structure analysis

The completeness of haplotype sampling based on the number of individuals analysed was estimated using the Stirling probability distribution method proposed by Dixon (2006). Parameters of genetic diversity and differentiation were estimated following the methods described by Pons & Petit (1995, 1996), using the programs HAPLODIV and HAPLONST (availables at http://www.pierroton.inra.fr/genetics/labo/Software/), both at the species and the island level (Corsica and Sardinia). Within-population diversity, total diversity and level of population differentiation, as well as their standard errors, were computed both by taking the distance between haplotypes into account (v_s, v_t, N_{st}) and by ignoring genetic distance between them (h_s, h_t, G_{st}). Values of N_{st} and G_{st} were compared using the *U*-statistics, to test for the existence of a phylogeographical structure (N_{st} significantly higher than G_{st}, Pons & Petit, 1996).

The geographical structure of genetic variation was assessed by the analysis of molecular variance (AMOVA, Excoffier *et al*., 1992) using ARLEQUIN vs. 2.000 (Schneider *et al*., 2000). The total genetic variance was partitioned into covariance components at different hierarchical levels under two hypotheses: (1) all locations were treated as a single group to determine the amount of variation partitioned among and within populations; and (2) locations were grouped according to their origin to determine the amount of variation attributable to differences between islands, populations within each island, and individuals within populations. The significance levels of the variance components were obtained by non-parametric permutation using 10,000 replicates.

In addition, the approach proposed by Dupanloup *et al*. (2002) was used to define groups of populations that were geographically homogeneous and genetically differentiated from each other (spatial analysis of molecular variance; SAMOVA). The most likely number of groups (*K*) was identified by repeatedly running the program SAMOVA vs. 1.0 (Dupanloup *et al*., 2002) from *K* = 2 to *K* = 10, using 500 random initial conditions and performing 10,000 iterations. The largest F_{CT} values (i.e. the largest proportion of total genetic variance due to differences between groups) were choosen as predictors of the best grouping of populations (Dupanloup *et al*., 2002).

Haplotype network and nested clade analysis

A haplotype network was constructed using statistical parsimony (Templeton *et al.*, 1992) with the program TCS vs. 1.21 (Clement *et al.*, 2000). Insertions and deletions were treated as a fifth character state, and coded as a single mutational event (Simmons *et al.*, 2001). Since one of the polymorphisms detected consisted of a poly-A repeat, being more prone to homoplasy (Ingvarsson *et al.*, 2003), an alternative haplotype network was also constructed excluding this site from the analysis. Two network ambiguities (loops) were resolved using predictions based on coalescent theory according to the rules outlined in Crandall & Templeton (1993) and Pfenninger & Posada (2002). The resulting haplotype network was nested into hierarchical clades using the automated implementation of Nested Clade Phylogeographic Analysis (NCPA, Templeton *et al.*, 1995) provided by the program ANECA vs. 1.1 (Panchal, 2007). ANECA software implements both TCS vs. 1.18 and GEODIS vs. 2.2 (Posada *et al.*, 2000), the inference key dated 11th November 2005, and automates the inference process, providing a framework for replicating analyses in an objective way.

Divergence time estimate

After major clades of the network were identified, we estimated divergence times between the clades using the program MDIV (Nielsen & Wakeley 2001; available at http://people.binf.ku.dk/rasmus/webpage/mdiv.html). MDIV uses a Markov Chain Monte Carlo (MCMC) approach that allows the joint estimation of the posterior distribution of a variety of parameters: the divergence time since two populations diverged from a common ancestral population ($T = t_{div1}/ N_e$), the time to most recent common ancestor (TMRCA $= t_{div2}/ N_e$), the migration rate between populations since divergence ($M = N_e m$), and the relative size of each of the two current populations ($\theta = 2N_e\mu$), where N_e is the female-effective population size, m is the migration rate, and μ is the per locus mutation rate. Initial runs were tested under a finite sites (HKY) model of evolution and default priors M = 10, T = 20 and θ = 10, to explore the posterior distribution of scaled migration rate (M) and divergence time (T). Initial analysis indicated that migration among major clades was nearly zero, so we reanalysed the data with the migration prior set to M = 0 and the max T = 20. MDIV analyses were run for 2×10^6 generations following a burn-in period of 500,000 generations, and the

analysis was repeated three times to ensure convergence upon the same posterior distributions for each of the parameter estimates. The values of θ, scaled migration rate, and scaled time of divergence with the highest posterior probability were considered as the best estimates. These values were converted into years before present as $t_{div} = (T\theta)/2\mu$, and assuming a mutation rate of 1.1–2.9 x 10^{-9} nucleotide substitutions per site per year (Wolfe *et al.* 1987; Clegg *et al.* 1994).

RESULTS

Patterns of genetic diversity and structure

Sequences ranged from 673 to 690 bp, with a total aligned length of 700 bp. A neutral pattern of variation was observed when we applied both Tajima's (D = 1.781, P > 0.5) and Fu's tests (F_S = 2.313, P > 0.5). Thirteen polymorphic sites were detected, identifying a total of 17 haplotypes (Table 2). This was the most likely number of haplotypes estimated using the Stirling probability, suggesting that we have sampled all existing haplotypes with a 95% of certainty. Only two haplotypes were shared between islands, haplotype I and haplotype L, which were present in both Corsica and Sardinia (Figure 1, Table 1). All the analysed individuals from Majorca shared the same haplotype O. In Sardinia, all populations located in the Gennargentu massif were polymorphic, harbouring between two to four haplotypes, while the remaining Sardinian populations were all fixed for a single haplotype (Figure 1). Five out of the twelve haplotypes present in Sardinia were shared among two different populations (haplotypes A, B, D, G and J, Figure 1). All populations located in Corsica were polymorphic with a single exception (OLM), and four of the six haplotypes found in this island were shared by at least two populations (haplotypes H, K, L and N, Figure 1).

Parameters of genetic diversity and structure are given in Table 3. At the species level, total genetic diversity detected in *Th. herba-barona* was high, with very similar values for ordered and unordered haplotypes (v_t = 0.977 ± 0.0575 and h_t = 0.979 ± 0.0082, respectively). However, we found quite lower values of within-population variation, with an average haplotypic diversity values of h_s = 0.334 ± 0.0858 and v_s = 0.376 ± 0.1058, based on unordered or ordered alleles, respectively (Table

Table 2 Characterization of *Thymus herba-barona* haplotypes, based on the polymorphic sites of the *trn*T-*trn*L intergenic cpDNA spacer, found in this study. The frequencies of the cpDNA haplotypes and their geographical origin are indicated. Numbers 0 and 1 in the sequences indicate indels; 0 represent absence and 1 presence of sequences cited by uppercase letters.

Haplotype	Polymorphic sites													Frequency			
	46	112-116	125-135	246	273	386	399-418	419	422	463	554	595	663-669	Majorca	Corsica	Sardinia	Total
A	T	1[a]	1[b]	A	0	0	A_{16}	0	G	C	T	G	0	-	-	0.038	0.038
B	T	0	1[b]	T	0	0	A_{14}	0	G	C	T	G	0	-	-	0.048	0.048
C	T	1[a]	1[b]	T	0	0	A_{15}	0	G	C	T	G	0	-	-	0.067	0.067
D	T	1[a]	0	T	0	0	A_{14}	0	G	C	T	A	0	-	-	0.086	0.086
E	T	1[a]	1[b]	T	0	0	A_{21}	0	G	T	T	G	0	-	-	0.038	0.038
F	T	1[a]	1[b]	T	0	0	A_{20}	0	G	T	T	G	0	-	-	0.028	0.028
G	T	0	1[b]	T	0	0	A_{12}	1[e]	T	C	A	G	0	-	-	0.057	0.057
H	T	0	1[b]	T	0	0	A_{10}	0	T	C	A	G	0	-	0.105	-	0.105
I	T	0	1[b]	T	0	0	A_{16}	0	T	C	A	G	0	-	0.009	0.019	0.028
J	T	0	1[b]	T	0	0	A_{11}	0	T	C	A	G	0	-	-	0.076	0.076
K	T	0	1[b]	T	0	0	A_{15}	0	T	C	A	G	0	-	0.068	-	0.068
L	T	1[a]	1[b]	A	1[c]	1[d]	A_{11}	0	G	C	T	G	1[f]	-	0.066	0.009	0.075
M	T	0	1[b]	A	1[c]	0	A_{18}	0	G	C	T	G	0	-	0.019	-	0.019
N	A	1[a]	1[b]	A	0	0	A_{13}	0	G	C	T	G	0	-	0.065	-	0.065
O	A	1[a]	1[b]	A	0	0	A_{11}	0	G	C	T	G	0	0.067	-	-	0.067
P	A	1[a]	1[b]	A	0	0	A_{15}	0	G	C	T	G	0	-	-	0.067	0.067
Q	A	1[a]	1[b]	A	0	0	A_{16}	0	G	C	T	G	0	-	-	0.067	0.067

Notes: a = CAGAA; b = TACCAATAAAT; c = T; d = T; e = TAA; f = AAATCTA

3). Genetic differentiation among all fifteen populations was high, and was independent of the genetic distance between haplotypes (G_{st} = 0.659 ± 0.0899, N_{st} = 0.615 ± 0.0962; N_{st} = G_{st}, U = -0.81, P > 0.05). This was in accordance with the AMOVA results, revealing that about 67% of the chloroplast variation in *Th. herba-barona* was explained by differences among locations (Table 4). Genetic differentiation among islands was low (7.71%) and non-significant (Table 4).

Table 3 Estimates of genetic diversity and differentiation measures for ordered (v_s, v_t, Nst) and unordered (h_s, h_t, Gst) haplotypes in *Thymus herba-barona*, and their standard deviations. h_s and v_s, intrapopulation diversity, h_t and v_t, total diversity, G_{st} and N_{st}, pairwise differentiation between populations.

	Majorca	Corsica	Sardinia	Total value
No. of populations	1	5	9	15
No. of individuals	7	36	63	106
No. of haplotypes	1	6	12	17
No. of private haplotypes	1	4	10	-
Average no. of haplotypes	1	7.20	7.00	7.07
No. of polymorphic sites	-	9	13	13
h_s	-	0.393 ± 0.1410	0.339 ± 0.1189	0.334 ± 0.0858
h_t	-	0.887 ± 0.0460	0.976 ± 0.0179	0.979 ± 0.0082
G_{st}	-	0.557 ± 0.1731	0.653 ± 0.1264	0.659 ± 0.0899
v_s	-	0.467 ± 0.1708	0.333 ± 0.1347	0.376 ± 0.1058
v_t	-	0.871 ± 0.1053	0.976 ± 0.0721	0.977 ± 0.0575
N_{st}	-	0.464 ± 0.1685	0.659 ± 0.1371	0.615 ± 0.0962
N_{st}-G_{st}	-	-0.093	0.006	-0.044

When islands were analysed separately, Sardinia revealed a stronger population differentiation than Corsica, as shown by the AMOVA (72.12 vs. 49.53%, Table 4) and HAPLONST analyses (G_{st} = 0.653 vs. 0.557, N_{st} = 0.659 vs. 0.464, Table 3). Neither Corsica (N_{st} = G_{st}, U = -0.39, P > 0.05) nor Sardinia (N_{st} = G_{st}, U = 0.14, P > 0.05) revealed significant patterns of phylogeographic structure, indicating that groups of related haplotypes were not restricted to particular geographical regions. These

results were in agreement with those obtained from the SAMOVA approach, since F_{CT} values were similar for all *K* analysed (range from 0.439 to 0.560), suggesting that there is not a single group of geographically homogeneous and maximally differentiated populations (data not shown).

Table 4 Analysis of molecular variance (AMOVA) based on *trn*T-*trn*L sequence data for *Thymus herba-barona*. (a) Assuming no regional differentiation, (b) Three islands (Majorca, Corsica, Sardinia), (c) Corsica, (d) Sardinia. * *P* < 0.001 (significant after 10,000 permutations), ns = not significant.

Source of variation		df	Sum of squares	Variance components	Percentage of variation
(a)	Among populations	14	233.64	220.740	66.93*
	Within populations	91	99.27	109.086	33.07
(b)	Among islands	2	46.00	0.26299	7.71 ns
	Among populations within islands	12	187.64	205.887	60.33*
	Within populations	91	99.27	109.086	31.96*
(c)	Among populations	4	54.21	164.997	49.53*
	Within populations	31	52.12	168.145	50.47
(d)	Among populations	8	133.43	225.794	72.12*
	Within populations	54	47.14	0.87302	27.88

Haplotype network and nested clade analysis

TCS estimated a 95% connection limit of two mutational steps, leaving seven haplotypes unincorporated. By manually increasing the connection limit it was possible to connect all of them with a non-parsimonious connection of six steps (Figure 2). Multiple missing haplotypes (18) were inferred in the network. Two closed loops were detected, one linking haplotypes B, C, and two non-detected haplotypes, and the other linking two missing haplotypes. These network ambiguities could be resolved using the frequency and geographical criteria, and ultimately did not affected nesting design or conclusions inferred from the analysis. The hierarchical nested design identified 18 one-step, eight two-step, and three three-step clades (Figure 2). The interior clade 3-1
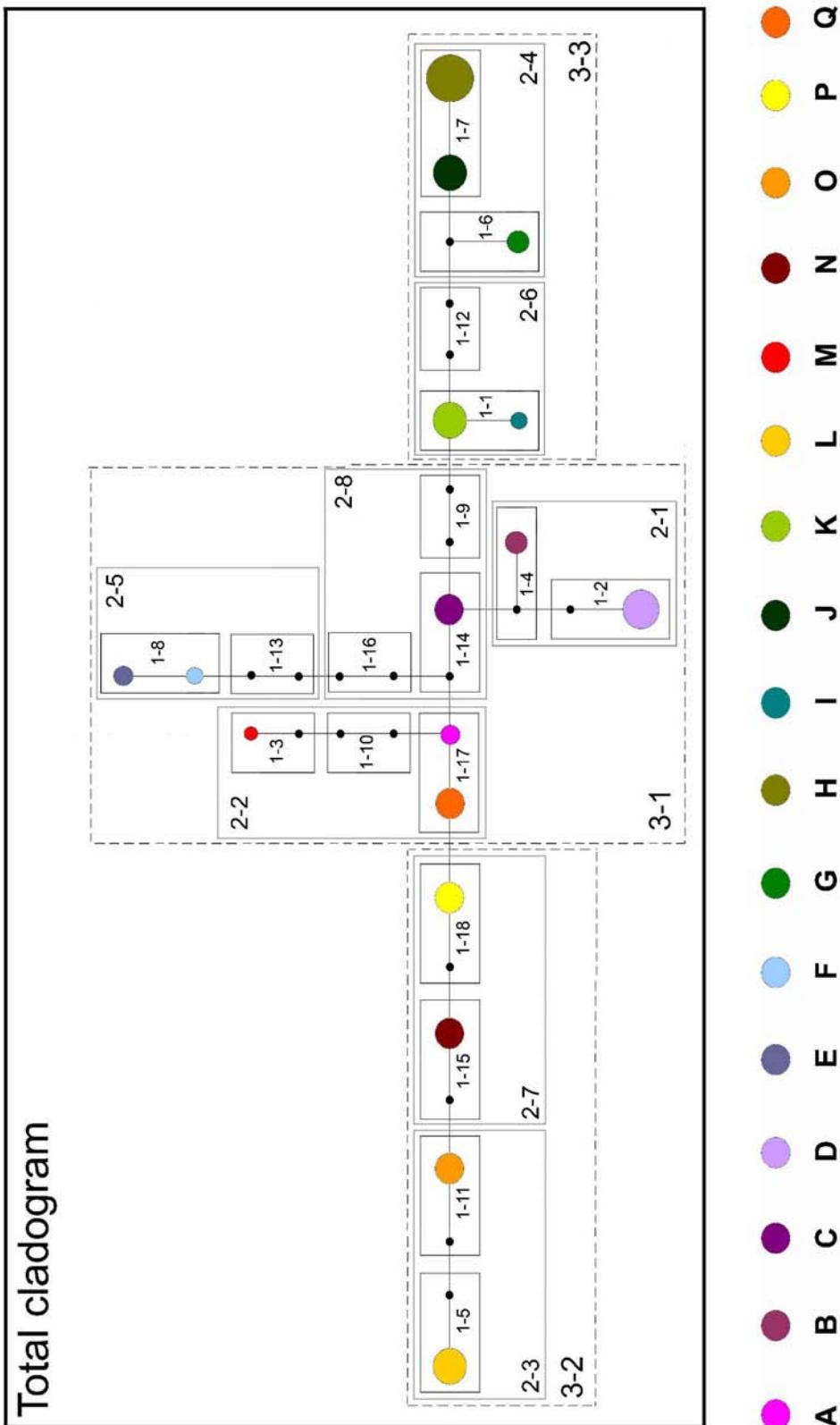
Figure 2 Parsimony network showing the relationships among cpDNA haplotypes detected in *Thymus herba-barona*. The size of the circle is proportional to the frequency of each haplotype in the total sample. Colors are the same as in Figure 1. Each line in the network represents one mutational step. Small black circles represent missing haplotypes that were not observed in the data. Hierarchically nested clades are indicated by boxes and numbered clade designations

comprised eight haplotypes (A, B, C, D, E, F, M, Q) that were distributed along the Gennargentu massif, northern Sardinia and southern Corsica (Figures 1 and 2). Clades 3-2 and 3-3 showed an external position in the network, and comprised four (L, N, O, P) and five (G, H, I, J, K) haplotypes, respectively (Figure 2).

The former haplotype group was present in Majorca (O), Corsica (L, N) and Sardinia (L, P), while the latter was widely distributed in Corsica (H, I, K) and Sardinia (G, I, J). NCPA identified twelve clades for which the null hypothesis of no geographic structuring of haplotypes could be rejected (Table 5). Higher-level (older) clades indicated that the evolutionary history of *Th. herba-barona* was characterized by an early range expansion event, followed by more recent fragmentation episodes, the main process detected for younger clades (Table 5). The alternative network was obtained when the polymorphic poly-A repeat was not taken into account was highly congruent with these results (Figure 3).
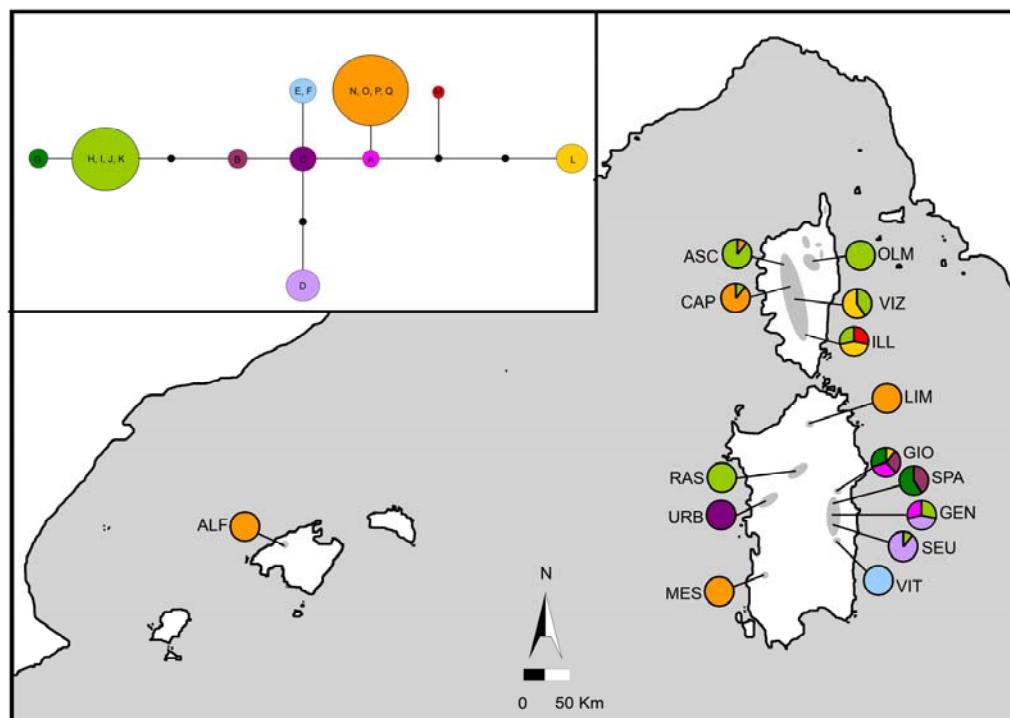

Figure 3 Alternative parsimony network showing the relationships among cpDNA haplotypes when the poly-A repeat was not take into account. The size of the circle is proportional to the frequency of each haplotype in the total sample. Colors are the same as in Figure 1. Each line in the network represents one mutational step. Small black circles represent missing haplotypes that were not observed in the data.

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Table 5 Inferred historical processes affecting genetic structure of *Thymus herba-barona* populations based on nested clade analysis.

Clade	χ^2-statistic	Probability	Inference chain	Inferred event
1-1	11.00	<0.05	1-19-20-2-11-12 No	Contiguous range expansion
1-7	19.00	<0.001	1-19-20-2-11-12-13-14 No	Long distance colonisation and/or past fragmentation
1-17	11.00	<0.01	1-19 No	Allopatric fragmentation
2-1	13.00	<0.01	1-19 No	Allopatric fragmentation
2-2	13.00	<0.05	1-19 No	Allopatric fragmentation
2-3	15.00	<0.001	1-19-20-2-11-12-13-14 No	Long distance colonisation and/or past fragmentation
2-4	24.00	<0.001	1-2-11-12 No	Contiguous range expansion
2-7	14.00	<0.001	1-19 No	Allopatric fragmentation
3-1	109.62	<0.001	1-19-20-2-3-5-15 No	Long distance colonisation and/or past fragmentation
3-2	29.00	<0.001	1-2-11-12 No	Contiguous range expansion
3-3	32.86	<0.001	1-2-3-4 No	Restricted gene flow with isolation by distance
Total cladogram	141.20	<0.001	1-2-11-12 No	Contiguous range expansion

Divergence time between the high-level clades of Thymus herba-barona

The coalescent-based analyses indicated that our data were consistent with a model of large divergence times with very limited gene flow between major clades, rather than a model of recurrent gene flow. Estimates of T for all the pairwise comparisons were similar (clades 3-1/3-2 = 2.00; clades 3-1/3-3 = 2.40; clades 3-2/3-3 = 4.00), suggesting that all the events occurred approximately at the same time. Assuming mutation rates of 1.1–2.9 x 10^{-9} s/s/y, an estimate of divergence time between clades 3-1/3-2, clade 3-1/3-3 and clades 3-2/3-3 was placed during the Middle Pliocene-Lower Pleistocene, at 3.26-1.23, 3.66-1.39 and 3.90-1.48 million years ago, respectively. For TMRCA, estimates were slightly greater, with values ranging from 4.96-1.88, 5.52-2.09 and 5.99-2.27 million years among clade pairs 3-1/3-2, 3-1/3-3 and 3-2/3-3, respectively.

DISCUSSION

The distribution of genetic variation: the relative role of gene flow, selection and drift

The results obtained in this study for *Thymus herba-barona* indicate high levels of genetic diversity, both at the species and the within-population level (h_t = 0.979; h_s = 0.334, respectively). We detected a total of 17 cpDNA haplotypes, which is similar to the number of haplotypes found in the widespread Mediterranean *Cistus creticus* L. on Corsica and Sardinia (16 haplotypes; Falchi *et al.*, 2009). There is a general perception that endemic island plant species often have reduced genetic diversity compared to more common and widespread species (Frankham, 1997), but this trend is not consistent, and the scarcity of examples from the Western Mediterranean basin prevent further corroboration of this prediction. For example, very high genetic variation has been detected in some narrowly distributed, endemic species from the Balearic Islands, such as *Crepis triasii* or *Hippocrepis balearica* Jacq. (21 and 18 haplotypes, respectively; our unpublished data), or in *Cyclamen creticum*, endemic to the island of Crete (Affre & Thompson, 1997). Thus, there is a clear need of studies seeking to investigate genetic diversity patterns present in endemic plants from this region.

In addition to the high within-population diversity, we found that approximately 67% of the chloroplast variation in *Th. herba-barona* was found between populations, suggesting a limited extent of gene flow among the studied populations. Substantial levels of genetic structure have been reported for other Mediterranean island endemics, such as *Brassica insularis* (G_{st} = 0.107; allozyme markers; Hurtrez-Boussès, 1996), *Cyclamen creticum* (G_{st} = 0.170; allozyme markers; Affre & Thompson, 1997), *Nigella arvensis* alliance (F_{st} = 0.814; cpDNA; Bittkau & Comes, 2005), *Brassica cretica* (F_{st} = 0.628 and 1.000; nuclear and chloroplast microsatellites, respectively; Edh *et al.*, 2007), *Centaurea horrida* (R_{st} = 0.158; nuclear microsatellites; Mameli *et al.*, 2008) or *Senecio rodriguezii* (G_{st} = 0.769; cpDNA; Molins *et al.*, 2009). Significant population divergence is expected to develop in species occurring in spatially isolated populations and with restricted dispersal capability, circumstances that are likely to reduce gene flow and enhance random genetic drift. Recently, drift has been invoked as a major evolutionary force driving plant diversification in the Aegean region, being much more influential on population structure than cytoplasmic gene flow (Widén *et al.*, 2002, Bittkau & Comes, 2005; Edh *et al.*, 2007). In the case of *Th. herba-barona*, our results are consistent with this hypothesis, since most of the haplotypes found were exclusive to only one population (C, E, F, M, O, P, Q) or were restricted to a few geographically close localities (A, B, D, G, K, N). Moreover, smaller and more isolated populations (ALF, MES, URB, RAS, LIM, OLM; Figure 1) were all randomly fixed for a single haplotype, a result that fit theoretical expectations suggesting that small populations are more prone to genetic drift as a consequence of random sampling effects. In contrast, most of the Corsican populations and those from the Gennargentu massif (Sardinia), where the species is more widely distributed, harboured at least two different haplotypes (Figure 1), and genetic divergence among Corsican populations was much lower than those from Sardinia (49.53 vs. 72.12%, Table 4). All this evidence suggest that genetic drift might acted as a major evolutionary force determining the patterns of genetic variability and structure observed, but that higher gene flow has contributed to lower its effects in those areas where the species occurs in large and less isolated populations.

The dynamics of cytoplasmic genomes (cpDNA and mtDNA) could also be influenced by natural selection acting elsewhere in the molecules, since all portions

(neutral or adaptive) of the genome are inherited as single units. In gynodioecious species, where the impairment of the pollen formation in functional females results from interactions between the nuclear and the mitochondrial genomes (CMS: cytoplasmic male sterility; reviewed in Ivanov & Dymshits, 2007), selection targeting mitochondrial genes associated with CMS could influence cpDNA evolution as well, owing to gametic disequilibrium between cpDNA and mtDNA (Olson & McCauley, 2000; McCauley & Olson, 2003). Because in many gynodioecious species, such as *Thymus vulgaris* L., female monoecious plants produce more viable seeds than hermaphrodites (Thompson & Tarayre, 2000), this could result in an increased frequency of the mtDNA carrying a given CMS factor, but also in the frequency of the cpDNA haplotypes present in the same individuals. This kind of jointly evolution of cpDNA and mtDNA lineages might further complicate the understanding of forces that influence cpDNA evolutionary dynamics. To our knowledge, there is no report on the reproductive system of *Th. herba-barona*, but preliminary studies involving the single Balearic population (ALF) have detected coexisting hermaphrodite and female individuals, suggesting that it is also a gynodioecious species (J.M. Iriondo, Universidad Rey Juan Carlos, Spain, pers. comm.). Hence, we cannot rule out the possibility that such kind of hitchhiking-like effect could indeed contributed to the structuring of genetic variation in some populations. The fact that all small and highly isolated populations are fixed for a single haplotype, however, suggest an important effect of genetic drift relative to other evolutionary processes, such as gene flow or selection. In any case, a detailed understanding of the role of cytoplasmic selection in this species requires specific studies dealing with this question.

Evolutionary history: vicariant origin or long-dispersal processes?

A striking result of this study is the lack of a phylogeographical break between populations from the different islands, despite the fact that fifteen out of seventeen haplotypes were restricted to one of them (one to Majorca, four to Corsica and ten to Sardinia, Table 3). The lack of phylogeographic structure was also evident within major islands (Corsica and Sardinia), meaning that similar haplotypes were not geographically close to each other. This could be explained by the fact that haplotypes belonging to clades 3-2 and 3-3 (differing from each other by at least six mutations)

were widely distributed, and present at the same time in several populations (Figures 1 and 2).

In contrast, haplotypes from clade 3-1 were mainly restricted to the Gennargentu massif in Sardinia. This fact, together with the interior position of the clade 3-1 in the network, is consistent with a model of range expansion, where the older (interior) haplotypes are confined to the ancestral preexpansion area, and the younger (derived) haplotypes are geographically widespread (Templeton *et al.*, 1995). Thus, the oldest event inferred from NCPA in the population history of *Th. herba-barona* was a range expansion from the Gennargentu massif (Sardinia) to the rest of territories. After this range expansion, inferred population history from lower-level (younger) clades supports the occurrence of several fragmentation episodes (Table 5).

To set an approximate time frame for the above-mentioned processes can be complicated in the absence of any fossil record. In these cases, coalescent-based methods has proved to be a valuable tool for estimating demographic parameters, such as effective population size, migration rates and divergence times (Knowles & Maddison, 2002; Nielsen & Beaumont, 2009). Under the slower mutation rates considered (1.1×10^{-9} s/s/y), coalescent estimates of the time to common ancestry (TMRCA) among three-step clades ranged from 5.99 to 4.96 million years ago. Reconciliation between the inferred ages and the paleogeological history of the Mediterranean basin suggest that an ancestor of *Th. herba-barona* dispersed from Sardinia to Corsica and Majorca islands during the desiccation of the Mediterranean Sea between 5.96 and 5.33 million years ago (the Messinian salinity crisis; Krijgsman *et al.*, 1999). After this date, the estimated time divergence between major clades (3.90-3.26 Myrs BP) suggests that several fragmentation episodes took place within all islands, probably related to the onset of the Mediterranean climatic regime during the Pliocene (ca. 3.2 Myrs BP; Suc, 1984) and the alternation of warm and cold periods during the Pleistocene glaciations (1,8 Myrs-15,000 yrs BP). The progressive warming and drying of the climate has caused high levels of extinction in the pre-existing flora, as well as an important shift in the distribution of many species (Thompson, 2005). Mesophilous plants were replaced by thermophylous taxa or displaced to higher altitudes, leading to the fragmentation and isolation of formerly well connected areas. Populations of *Th. herba-barona* are found today at high altitude (above 800 m),

suggesting that the recent climate has prevented these populations to occur at lower altitudes, and therefore has strongly limited gene flow among populations. This is congruent with the lower among-population differentiation found in Corsica, where mountain areas occurring above the 1,000 m are much more frequent than in Majorca or Sardinia.

The Messinian salinity crisis has been advocated as one of the main events in shaping biogeographical patterns for both animal and plant species in the Western Mediterranean (e.g., Bocquet *et al.*, 1978; Kiefer & Bocquet, 1979; Stöck *et al.*, 2008). During this time, it has been postulated that land bridges between different islands and the continent could have acted as corridors allowing the exchange of taxa. However, land connections among the Balearic Islands and the Corso-Sardinian archipelago have not been documented during the Messinian salinity crisis (Alvarez *et al.*, 1974; Rosenbaum *et al.*, 2002). Hence, a long-distance dispersal event must be invoked to explain the occurrence of *Th. herba-barona* in the Balearic Islands if we the accept that the range expansion of the species has taken place during this period. Nevertheless, a rare long-distance dispersal event do not agree with the levels of ploidy reported for the species, since individuals from Majorca are diploids (2n = 28; Mayol *et al.*, 1990), and populations from Corsica and Sardinia are tetraploids (2n = 56; Monte Reinoso, Vizzavona, Monte Foscu; Contandriopoulos, 1962) and hexaploids (2n = 84; Gennargentu; Diana-Corrias, 1980), respectively. New chromosome counts made on material from other Corsican and Sardinian populations (our unpublished data) confirmed the presence of the tetraploid cytotype in Corsica (Lago di Capitello), and revealed the presence of the diploid cytotype in Corsica (Haut Ascò), and the tetraploid cytotype in Sardinia (Bruncu Spina, Gennargentu). Based on this new evidence, a rare long-distance colonization from diploid populations in Corsica to the Balearic Islands cannot be discarded, although it seems very unlikely given the strong genetic divergence and reduced gene flow detected among populations.

Indeed, the accuracy of the divergence time estimates depends on the reliability of the substitution rate assumed. The mutation rates used to convert scaled time of divergence into years before present has been inferred from cultivated plants (e.g. tobacco, wheat, rice; Wolfe *et al.*, 1987; Clegg *et al.*, 1994), whose sequence divergence per million years could be faster than those of wild Mediterranean plants.

Since the Mediterranean region has provided stable climatic conditions for species persistence over long timescales (Hampe & Petit, 2005), and the tempo of evolutionary rates may be largely determined by the rate of environmental change in the context in which a species lives, this may have prevented the accumulation of morphological and molecular variation in some lineages. Some recent studies provide good examples of such kind of molecular stasis. In the case of *Quercus suber* L., Magri *et al.* (2007) report just five distinct haplotypes for more than 100 populations covering the whole distribution range of the species, using an extensive set of 14 chloroplast microsatellite markers. Moreover, Magri and colleagues suggest that the extremely clear-cut geographical distribution of haplotypes is consistent with the break-up and separation of the Hercynian massif during the Oligocene and Miocene, between 25 and 15 million years ago, and since then they have persisted in a number of separate microplates without detectable cpDNA variation. The highly structured pattern of the five distinct haplotypes found in *Cephalaria squamiflora* (Sieber) Greuter, an endemic plant restricted to few islands from the Western (Balearic Islands, Sardinia) and Eastern Mediterranean (Crete and some Aegean islands), is also attributed by Rosselló *et al.* (2009) to the Oligocenic land connections between these territories, despite no more than four mutational steps were detected among the most distant haplotypes. Finally, Dubreuil *et al.* (2008) report a total lack of variation using several chloroplast markers for *Ramonda myconi* (L.) Rchb., a Tertiary relict plant species restricted to the NE Iberian Peninsula. The authors suggest that the low mutation rates associated with the chloroplast genome, together with the extremely slow population dynamics documented for the species, might have prevented the accumulation of mutations in this Tertiary relict.

Hence, it is probable that long periods of stasis favouring the preservation of ancestral molecular variants in different lineages has been a much more common situation than previously thought in the Mediterranean region. In the case of *Th. herba-barona*, there are several reasons suggesting an ancient origin for the phylogeographic pattern observed. First, the high cpDNA diversity found is likely explained by the ancient presence of the species, which would have allowed the accumulation of a high number of mutations. Second, the inference of multiple (18) missing intermediate haplotypes in the network (Figure 2) support a long evolutionary separation of

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populations leading to the extinction of several ancestral haplotypes. The pattern of molecular variation detected could also be explained by past geological connections of the Hercynian Islands (Balearic Islands, Corsica and Sardinia) during the mid-Tertiary period (Oligocene-Miocene). The current distribution of the species, restricted to a high mountain habitats, probably represent relict populations from once more widespread mesophytic ancestors during more favourable climatic periods. Tectonic events and progressive climate warming has likely resulted in the fragmentation and reduction of the species' range, leading to the extinction of local populations and many ancestral haplotypes, and favouring the random fixation of haplotypes in those small or geographically isolated extant populations due to increased genetic drift.

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DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

Existen pocos estudios sobre los niveles de diferenciación genética citoplásmica (ADN cloroplástico) presentes en plantas con distribución restringida y propias de la cuenca Mediterránea. Hay diversas razones que podrían explicar la escasez de dichos estudios. En primer lugar podría deberse a que las especies raras muestran en general unos bajos niveles de variación genética (Stebbins, 1942; Hamrick & Godt, 1989), si bien éstos han sido detectados mediante la aplicación de marcadores nucleares. Además, e incidiendo en este punto, ha sido repetidamente postulado que las especies insulares tienden a mostrar bajos niveles de diversidad genética si se comparan con sus ancestros continentales, o bien con poblaciones conespecíficas del continente (Frankham, 1997). Finalmente, se ha sugerido que las tasas de variación del genoma cloroplástico, significativamente más bajas que las halladas en el ADN nuclear (Wolfe *et al.*, 1987), no son las más adecuadas para estudios microevolutivos a niveles intraespecíficos en especies estenócoras.

Sin embargo, estas expectativas no parecen de aplicación general y se ha sugerido que más bien son específicas de linajes (Banks & Birky, 1985; Soltis *et al.*, 1992; McCauley, 1995; Gitzendanner & Soltis, 2000) y que la detección de los niveles de variabilidad del ADN cloroplástico dependen en buena medida de la evolución histórica reciente de la especie así como de los procesos estocásticos asociados (p.ej., Vendramin *et al.*, 1999, 2008; Jaramillo-Correa *et al.*, 2006). Por otra parte, el uso de técnicas de genotipado directo, mucho más informativas (como la secuenciación de DNA), en lugar del genotipado indirecto (como los RFLPs) ha aumentado el número de especies en los que se ha detectado unos niveles relativamente altos de variación intraespecífica (p.ej., Widmer & Baltisberger, 1999; Dobeš *et al.*, 2004).

En los trabajos que han constituido nuestro proyecto doctoral hemos comprobado la validez de tales aseveraciones. Así, en nuestro estudio del endemismo de distribución reducida *Senecio rodriguezii* hemos detectado siete haplotipos, un número que es similar al número de haplotipos encontrado en otras especies congenéricas como *S. halleri* (siete; Bettin *et al.*, 2007) y *S. leucanthemifolius* var. *casablancae* (cinco; Coleman & Abbott, 2003), o de las especies afines distribuidas por toda la cuenca del Mediterráneo como *S. gallicus*, *S. glaucus*, y *S.*

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leucanthemifolius (cinco; Comes & Abbott, 1999). Se podría argumentar que la alta riqueza encontrada en *Senecio rodriguezii* podría deberse al uso de diferentes aproximaciones experimentales (en nuestro estudio, secuenciando la región del espaciador *trn*T-*trn*L en lugar de utilizar RFLPs en fragmentos heterólogos clonados o bien amplificados por PCR usando cebadores universales). Esto podría ser una explicación razonable a los cinco haplotipos encontrados en *Senecio rodriguezii* que difieren sólo en la longitud de la región microsatélite hallada en dicho espaciador. No obstante, se han detectado niveles muy semejantes de variación en algunas especies cuando se han comparado las técnicas de RFLPs y los marcadores microsatélites (Waters & Schaal, 1991; Provan *et al.*, 1999).

Los resultados obtenidos en este estudio de *Thymus herba-barona* también indican unos niveles de diversidad genética significativamente altos, ya sea a nivel de especie como a nivel poblacional (h_t = 0.979; h_s = 0.334, respectivamente). El número de haplotipos cloroplásticos detectados (17) es similar al encontrado en especies de amplia distribución Mediterránea como es el caso de *Cistus creticus* L. en Córcega y Cerdeña (16 haplotipos; Falchi *et al.*, 2009). Por lo tanto, la predicción general de que las plantas endémicas de islas a menudo tienen una diversidad genética reducida comparándolas con especies comunes y ampliamente distribuidas (Frankham, 1997) no parece consistente a tenor de los resultados obtenidos en esta tesis. Es más, resultados similares de diversidad genética cloroplástica se han obtenido en otras especies de distribución restringida en las Islas Baleares occidentales (Molins, Mayol & Rosselló, datos no publicados; Tabla 1), así como en otras especies de islas continentales (Taiwan; Chiang & Schaal, 2006, y referencias allí incluidas).

En contra de lo esperado, las Islas Baleares parecen constituir un reservorio de diversidad genética, tanto nuclear como organular, no sólo para especies de amplia distribución en el Mediterráneo (*Quercus* sp. pl.; López de Heredia *et al.*, 2005), sino también para los endemismos de distribución restringida (nuestros resultados, así como Sales *et al.*, 2001). Todo ello podría ser el resultado de unas historias evolutivas complejas, en contraste con las hipótesis simples de colonización insular que asumen cuellos de botella demográficos y genéticos debido al efecto fundador, y que han sido sugeridas para explicar el origen y evolución de plantas de islas oceánicas.

Tabla 1 Parámetros de diversidad genética obtenidos del estudio de diferentes endemismos baleáricos (Molins *et al.*, datos no publicados).

	No. de poblaciones (individuos)	No. de haplotipos	h_s	h_t	G_{st}
Crepis triasii	25 (164)	21	0.274	0.845	0.676
Hippocrepis balearica	28 (146)	18	0.249	0.904	0.725
Polycarpon colomense	25 (109)	3	0.030	0.563	0.946
Naufraga balearica	5 (139)	3	0.091	0.767	0.882
Thymelaea velutina	11 (55)	7	0.127	0.527	0.759
Erodium reichardii	18 (86)	6	0.075	0.832	0.910

De todos modos, hay que destacar que estos niveles de diversidad genética elevados lo son básicamente a nivel regional, cuando se tienen en cuenta todas las poblaciones analizadas, ya que los niveles de diversidad genética intrapoblacional tienden a ser mucho más bajos (Tabla 1). Estos resultados concuerdan con lo que se espera para las poblaciones de especies situadas en los refugios glaciales meridionales, donde el aislamiento prolongado en un escenario de pequeños enclaves adecuados para su supervivencia habría comportado un flujo génico escaso entre poblaciones (Hewitt, 2001; Petit *et al.*, 2003; Hampe & Petit, 2005; Thompson, 2005), y ello habría dado lugar a unos niveles excepcionalmente altos de diversidad genética a nivel regional.

Los resultados obtenidos en esta memoria concuerdan con estas predicciones: las tres especies analizadas presentaron valores de diferenciación interpoblacional elevados (*S. rodriguezii*, G_{st} = 0.77; *B. balearica*, G_{st} = 0.94; *Th. herba-barona*, G_{st} = 0.66). Niveles significativos de estructuración genética han sido aportados en otros endemismos de las islas mediterráneas como *Brassica cretica* (F_{st} = 0.628 y 1.000; microsatélites nucleares y cloroplásticos, respectivamente; Edh *et al.*, 2007), *Brassica insularis* (G_{st} = 0.107; aloenzimas; Hurtrez-Boussès, 1996), *Cyclamen creticum* (G_{st} = 0.170; aloenzimas; Affre & Thompson, 1997), el complejo de *Nigella arvensis* (F_{st} = 0.814; DNA cloroplástico; Bittkau & Comes, 2005), o *Centaurea horrida* (R_{st} = 0.158; microsatélites nucleares; Mameli *et al.*, 2008).

Las predicciones teóricas indican que se espera que se desarrolle una divergencia poblacional significativa en aquellas especies con poblaciones aisladas y con una capacidad de dispersión restringida, ya que estas circunstancias conducen a un flujo génico interpoblacional reducido, aumentando así la probabilidad de incrementar los efectos de la deriva. Diversos estudios empíricos recientes llevados a cabo en especies insulares del Egeo sugieren que la deriva génica sería una de los principales procesos evolutivos que han dado lugar a la elevada diversificación vegetal presente en este área (Widén *et al.*, 2002; Bittkau & Comes, 2005; Edh *et al.*, 2007).

En el caso de las tres especies analizadas en nuestro estudio, los resultados son consistentes con esta hipótesis, ya que los valores de diferenciación encontrados son significativamente elevados. Además, en el caso de *S. rodriguezii* y de *Th. herba-barona*, muchos de los haplotipos encontrados fueron exclusivos de una población o estuvieron restringidos a territorios geográficos, lo que indicaría un flujo génico escaso incluso a escala muy local. Todas estas evidencias sugieren que la deriva genética puede haber actuado como una de las principales fuerzas evolutivas que determinan los patrones de variabilidad genética y su estructuración en las especies analizadas.

Por último, los resultados obtenidos en esta Tesis Doctoral indican que no existen patrones generales de diversidad genética y estructuración en función de la escala considerada. Por ejemplo, *S. rodriguezii* presentó unos niveles de diferenciación similares que los de *Th. berba-barona* (G_{st} = 0.77 y G_{st} = 0.66, respectivamente), aún cuando esta última especie presenta una distribución geográfica más amplia. De igual modo, el mayor grado de diferenciación encontrado para *B. balearica* (G_{st} = 0.94) sólo es válido cuando se incluyen todas las poblaciones muestreadas, pero se reduce en gran medida (~20%) cuando se calcula la divergencia interpoblacional dentro de las tres regiones detectadas (Península Ibérica-Norte de África, Islas del Mediterráneo Occidental, Anatolia). Estos resultados sugieren que los niveles de variabilidad en el ADN cloroplástico dependen en buena medida de la historia evolutiva de cada especie analizada. Así, la elevada estructuración hallada en el caso de *S. rodriguezii* parece altamente influenciada por los cambios históricos en cuanto a disponibilidad de hábitat como consecuencia de las oscilaciones en el nivel del mar durante las glaciaciones Cuaternarias. En el caso de *Th. herba-barona*, los niveles de diferenciación y variabilidad detectados sugieren la superposición de diversos acontecimientos históricos antiguos y recientes, que habrían conducido a una

progresiva fragmentación del área de distribución de la especie desde el Oligoceno (hace 25-30 Ma). En el caso de *B. balearica*, la incongruencia entre los patrones detectados con marcadores nucleares y cloroplásticos sugiere que los procesos de hibridación interespecífica pueden haber sido determinantes en los patrones de diversidad cloroplástica detectados.

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CONCLUSIONES

CONCLUSIONES

Del estudio de la variabilidad molecular de tres especies de distribución Mediterránea (*Senecio rodriguezii*, *Thymus herba-barona* y *Buxus balearica*) utilizando marcadores del ADN cloroplástico se han podido establecer las siguientes conclusiones:

1) En general las poblaciones de endemismos insulares de distribución restringida no presentan evidencias de depauperación genética y presentan niveles de variabilidad molecular como mínimo comparables a congéneres de distribución continental.

2) Los datos empíricos disponibles cuestionan los paradigmas actuales de que los organismos vegetales de las islas oceánicas y continentales presentan similares niveles bajos de diversidad genética.

3) La estructuración de la diversidad genética se manifiesta a escalas geográficas muy locales. Esto implica que, para los marcadores citoplasmáticos, los procesos históricos de fragmentación poblacional y de deriva genética condicionan más su estructuración que el flujo genético contemporáneo.

4) Probablemente, las expansiones y contracciones poblacionales de endemismos restringidos y que presentan una distribución litoral como consecuencia del cambio de los niveles eustáticos en el Cuaternario han modulado su actual estructuración filogeográfica.

5) No se ha detectado una significativa correspondencia entre los actuales límites geográficos insulares y la distribución de los haplotipos cloroplásticos. Ello es probablemente consecuencia de los cambios paleogeográficos acaecidos en el Mediterráneo Occidental como consecuencia de la dinámica de los ciclos glaciales e interglaciares del Cuaternario.

6) De igual modo no se ha constatado que las especies que presentan vectores de dispersión diferentes presenten historias filogeográficas discordantes y sugieren que éstos no son, en principio, los máximos condicionantes de dicha estructuración.

7) Los espaciadores intergénicos del ADN cloroplástico presentan niveles suficientes de variación para ser utilizados en estudios filogeográficos a escalas regionales en el Mediterráneo. No se ha constatado que, a estos niveles, las mutaciones estructurales y las regiones de microsatélites presenten homoplasias que interfieran en la reconstrucción filogenética, por lo que se aconseja su inclusión en tales estudios.

8) La hibridación constituye uno de los potenciales efectos distorsionadores de la filogeografía de especies Mediterráneas. La señal filogeográfica detectada en *Buxus balearica* sugiere que las islas continentales del Mediterráneo no sólo constituyen focos activos de microespeciación, sino que además son zonas de refugio para linajes que han sido extirpados en diversas zonas debido a una captura cloroplástica.

9) Las islas continentales del Mediterráneo constituyen excelentes laboratorios donde detectar aspectos microevolutivos a escalas locales que contrastan significativamente con los que han sido postulados en islas oceánicas.