FACULTAD DE BIOLOGÍA

DEPARTAMENTO DE BOTÁNICA Y FISIOLOGÍA VEGETAL

Filogeografía y conservación de especies endémicas mediterráneo-occidentales con distribución disyunta

TESIS DOCTORAL

Javier Bobo Pinilla

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Filogeografía y conservación de especies endémicas mediterráneo-occidentales con distribución disyunta

Memoria presentada por

Javier Bobo Pinilla

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Prof. Dra. Mª Montserrat Martínez Ortega

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Prof. Dr. Julio Peñas de Giles

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La Doctora **Mª. Montserrat Martínez Ortega**, Catedrática de Botánica de la Universidad de Salamanca y el Doctor **Julio Peñas de Giles**, Profesor Titular de Botánica de la Universidad de Granada,

AUTORIZAN, la presentación, para su lectura, de la Tesis Doctoral titulada "***Filogeografía y conservación de especies endémicas mediterráneo-occidentales con distribución disyunta***", realizada por D. **Javier Bobo Pinilla**, bajo su dirección, en la Universidad de Salamanca.

Y para que así conste a los efectos legales, expiden y firman el presente certificado en Salamanca, a 10 de junio de 2019.

Fdo. M.ª Montserrat Martínez Ortega

Fdo. Julio Peñas de Giles

JUSTIFICACIÓN:

Esta tesis se enmarca en los proyectos de investigación del Ministerio de Ciencia e Innovación **CGL2010-16357 (Filogeografía y conservación de flora endémica de hábitats isla: las especies íberonorteafricanas de *Moehringia* sect. *Pseudomoehringia*)** y **REN2003-09427 (Desarrollo de herramientas aplicables a la definición de criterios y diseño de estrategias de conservación de endemoflora del SE árido Ibérico)**, y en el proyecto de investigación de la Consejería de Innovación, Ciencia y Tecnología de la Junta de Andalucía **RNM1067 (Conservación de flora endémica y amenazada de habitats frágiles: las zonas áridas y altas montañas de Andalucía)**, dirigidos por los directores de esta tesis y representando una colaboración investigadora entre las universidades de Salamanca y Granada; en este marco y tras los trabajos de Sara Barrios de León (Tesis Doctoral sobre conservación en "hábitats isla"), Jaume Seguí Colomar (Trabajo de Fin de Master), Ana Robles González (Trabajo de Fin de Master), Nelida Padilla García (Trabajo de Fin de Master) y el propio trabajo de Fin de Master del autor de esta tesis, se pretenden resolver las cuestiones aún no resueltas acerca de las especies en las que el Grupo de Investigación Reconocido BIOCONS (Grupo de Investigación en Biodiversidad, Sistemática y Conservación de Plantas Vasculares y Hongos) ha focalizado los esfuerzos, *Arenaria balearica, Astragalus edulis* y el complejo *Arenaria* sect. *Pseudomoehringia*; todas ellas endemismos mediterráneos con una distribución disyunta y, en mayor o menor medida, con alguna preocupación acerca de su estado de conservación.

La presente Tesis Doctoral está elaborada en el formato de compendio de artículos/publicaciones según la normativa aprobada por la Comisión de Doctorado y Posgrado de la Universidad de Salamanca el 15 de febrero de 2013 y consta de las siguientes publicaciones:

ARTÍCULO 1: **PHYLOGEOGRAPHY OF AN ENDANGERED DISJUNCT HERB: LONG-DISTANCE DISPERSAL, REFUGIA AND COLONIZATION ROUTES**

Javier Bobo-Pinilla[1,2], Julio Peñas de Giles[3], Noemí López-González[1,2], Sonia Mediavilla[4] and M. Montserrat Martínez-Ortega[1,2]

[1]Departamento de Botánica, Universidad de Salamanca, Salamanca, Spain
[2]Banco de ADN vegetal, Banco Nacional de ADN, Salamanca, Spain
[3]Unidad de Conservación Vegetal, Departamento de Botánica, Universidad de Granada, Granada, Spain
[4]Departamento de Biología Animal, Parasitología, Ecología, Edafología y Química Agrícola, Universidad de Salamanca, Spain

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ARTÍCULO 2: **DESIGNING CONSERVATION STRATEGIES TO PRESERVE THE GENETIC DIVERSITY OF *ASTRAGALUS EDULIS* BUNGE, AN ENDANGERED SPECIES FROM WESTERN MEDITERRANEAN REGION**

Julio Peñas[1], Sara Barrios[2], **Javier Bobo-Pinilla**[2,3], Juan Lorite[1] and M. Montserrat Martínez-Ortega[2,3]

[1]Plant Conservation Unit, Department of Botany, University of Granada, Granada, Spain
[2]Department of Botany, University of Salamanca, Salamanca, Spain
[3]Biobanco de ADN Vegetal, Edificio Multiusos I+D+i, Salamanca, Spain

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ARTÍCULO 3: **PHYLOGEOGRAPHY OF *ARENARIA BALEARICA* L. (CARYOPHYLLACEAE): EVOLUTIONARY HISTORY OF A DISJUNCT ENDEMIC FROM THE WESTERN MEDITERRANEAN CONTINENTAL ISLANDS**

Javier Bobo-Pinilla[1,2], Sara B. Barrios de León[1], Jaume Seguí Colomar[3], Giuseppe Fenu[4], Gianluigi Bacchetta[5], Julio Peñas de Giles[6] and María Montserrat Martínez-Ortega[1,2]

[1]Department of Botany, University of Salamanca, Salamanca, Spain
[2]Biobanco de ADN Vegetal, University of Salamanca, Salamanca, Spain
[3]Department of Terrestrial Ecology, (IMEDEA), Esporles, Spain
[4]Dipartimento di Biologia Ambientale, University of Roma ''La Sapienza'', Roma, Italy
[5]Centro Conservazione Biodiversità (CCB), University of Cagliari, Cagliari, Italy
[6]Department of Botany, University of Granada, Granada, Spain

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ARTÍCULO 4: **PHYLOGENY AND PHYLOGEOGRAPHY OF *ARENARIA SECT. PSEUDOMOEHRINGIA***

Javier Bobo-Pinilla[1,2], Julio Peñas de Giles[3], Nélida Padilla-García[1] and María Montserrat Martínez-Ortega[1,2]

[1]Department of Botany, University of Salamanca, Salamanca, Spain
[2]Biobanco de ADN Vegetal, University of Salamanca, Salamanca, Spain
[3]Department of Botany, University of Granada, Granada, Spain

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OTROS ARTÍCULOS REALIZADOS DURANTE EL PERIODO DOCTORAL:

ARTÍCULO 5: **CONSERVATION OF GENETIC DIVERSITY IN MEDITERRANEAN ENDEMIC SPECIES: *ARENARIA BALEARICA* L.**

Javier Bobo-Pinilla[1,2], Noemí López-González[1,2] and Julio Peñas de Giles[3]

[1]Department of Botany, University of Salamanca, Salamanca, Spain
[2]Biobanco de ADN Vegetal, University of Salamanca, Salamanca, Spain
[3]Department of Botany, University of Granada, Granada, Spain

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ARTÍCULO 6: **NUEVOS DATOS SOBRE ORQUÍDEAS SILVESTRES DE LA PROVINCIA DE ZAMORA Y ZONAS LIMÍTROFES**

Patricio Bariego Hernández[1], **Javier Bobo Pinilla[1]** & Fermín Del Egido Mazuelas [2]

[1]Departamento de Botánica y Fisiología Vegetal. Universidad de Salamanca. Salamanca.
[2]Departamento de Biodiversidad y Gestión Ambiental. Facultad de Ciencias Biológicas y Ambientales. Universidad de León. León.

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INTRODUCCIÓN A LA FILOGEOGRAFÍA Y CONSERVACIÓN

Filogeografía y filogenia

La filogeografía, disciplina en la que se apoya esta tesis y las publicaciones que han surgido de la misma, se define como el análisis espacial de los linajes genéticos de las especies (Avise 1998); la filogeografía se considera una subdisciplina de la la biogeografía, como el estudio de la distribución de los seres vivos en relación con los accidentes geográficos y los eventos geológicos, y se fundamenta en la filogenia, o estudio de las relaciones de parentesco entre especies; a su vez la filogenia se basa en la evolución de los caracteres genéticos que nos permiten elaborar árboles genealógicos de los genes (y por extensión de las especies), de forma que podemos inferir ancestros comunes (Hudson 1998) y plantrear hipótesis de distribución en el pasado.

Es por tanto la filogeografía el estudio de la distribución geográfica de los linajes genealógicos, un campo que nos permite describir los eventos históricos asociados a la corología, expansiones o reducciones de distribución de las especies, eventos de fragmentación de hábitats, eventos de migración o/y de vicarianza, o de extinción (Templeton *et al.* 1995; Hardy *et al.* 2002). Aplicándolo a varias especies distribuidas en un plano espacial similar también se pueden plantear hipótesis sobre posibles eventos comunes a las biotas, como por ejemplo eventos de vicarianza o dispersión, existencia de áreas refugio, y así identificar las causas geológicas, ecológicas o etológicas que pudieron promoverlos (Taberlet *et al.* 1998; Hewitt 1999, 2004; Zink 2002; Lanteri & Confalonieri 2003; Arbogast & Kenagy 2008).

En cuanto a la interpretación de los patrones biogeográficos, la dispersión de las plantas también juega un papel esencial; ésta no solo está limitada por la capacidad diferencial de las semillas para ser diseminadas, la geografía es igualmente importante. Las barreras geográficas limitan la dispersión y colonización de áreas; cadenas montañosas, zonas desérticas o, simplemente, áreas cuyas características edáficas no son aptas para la proliferación de las especies, funcionan como barreras que han modelado la estructura genética y poblacional de las especies, promoviendo la diferenciación y especiación por aislamiento. En un marco temporal amplio, las barreras geológicas se crean y destruyen, crecen o se reducen, de modo que el impacto sobre las diferentes especies es extremadamente variable y la interpretación de la imagen estática actual que nos muestra la genética es altamente especulativa (Larmuseau *et al.* 2009).

También las características climáticas son obviamente un factor muy importante en la distribución de las especies, sobre todo en las especies que presentan un rango ecológico de supervivencia estrecho, especies con amplia distribución parecen tener capacidad de adaptación a cambios climáticos, incluso, en ocasiones, a cambios grandes, pero especies de hábitat más reducido o, como es el caso de las especies en las que se focaliza la presente tesis, de "hábitat isla", el impacto que pueden tener cambios relativamente ligeros en el clima puede ser letal (Gaston 1994; Williamson *et al.* 1997; Debussche & Thompson 2003; Lavergne *et al.* 2004; Becker 2010). Esta situación cobra importancia en un marco de cambio climático como al que nos enfrentamos en la actualidad, con las claras implicaciones que éste tendrá para las especies vegetales, sobre su fenología y su distribución (Walther *et al.* 2002; Cleland *et al.* 2007; Doi *et al.* 2008; Sheridan & Bickford 2011; Grimm *et al.* 2013); el conocimiento de las características ambientales de las zonas que ocupan las especies permitirá una mejor adaptación de las actuaciones a realizar para la conservación de las poblaciones.

Filogeografía y conservación

El objetivo principal de la conservación genética de las especies es la preservación de la diversidad genética de forma que las especies puedan adaptarse a los cambios ambientales. En el caso de las especies de "hábitats isla" o distribuciones fragmentadas, el tamaño de las poblaciones hace probable la pérdida de diversidad debido principalmente a la endogamia, esto reduce la reproducción y la supervivencia a corto plazo y disminuyen la capacidad de las poblaciones para evolucionar en respuesta al cambio ambiental a largo plazo. Es ya evidencia empírica el hecho de que descensos en la diversidad genética de poblaciones relativamente pequeñas está íntimamente ivolucrado con la tendencia a la extinción (Frankham *et al.* 2002).

En este contexto, la filogeografía no solo presenta utilidad en relación a conocer los patrones históricos y evolutivos de las poblaciones de especies vegetales, también puede servir para conocer las dinámicas poblacionales (flujo genético, tamaño poblacional, eventos de cuellos de botella, extinción), de forma que podemos establecer prioridades de conservación tratando de preservar no solo las poblaciones más divergentes de las especies, sino también las que reflejan patrones filogeográficos de adaptación o resistencia (Avise *et al.* 1987; Avise 2000; Vázquez-Domínguez 2002, 2007; Freeland 2005; Avise & Robinson 2008; Domínguez-Domínguez & Vázquez-Domínguez 2009).

Se acepta que se ha de priorizar los esfuerzos de conservación en especies consideradas en peligro, y también que la base de la diversidad biológica se sitúa a nivel genético (reconocido

en la Convención Sobre la Diversidad Biológica en Brasil, 1992). Por lo tanto, sería aconsejable no solo plantear medidas de conservación en especies con una distribución reducida y claramente en peligro, sino también en los casos de especies aparentemente no amenazadas, e incluso especies con un rango amplio de distribución que pueden aglutinar variantes genéticas y/o filogeográficas merecedoras de conservación a nivel intraespecífico (Mulcahy 2008).

La conservación basada en patrones genéticos pretende, en base a la información genética, identificar y proponer las especies o poblaciones que engloban la diversidad, rareza y los patrones genéticos que han podido permitir la supervivencia de las especies a largo plazo, favoreciendo la adaptación a los cambios climáticos y geológicos (Crandall *et al.* 2000; Moritz 2002; Pertoldi *et al.* 2012). También es importante mencionar que, aunque la genética ha de ser la base, no debe ser el único factor que nos mueva a proponer estrategias de conservación, el pragmatismo a la hora de aplicar las propuestas y las consideraciones de los datos históricos ecológicos y sociales es también esencial a la hora de plantear propuestas viables (Crandall *et al.* 2000).

Es necesario el uso de un método objetivo para saber cuántas y qué poblaciones merecen la prioridad de conservación para representar la mayor cantidad de diversidad genética de las especies. A lo largo del los últimos años se han propuesto varios estimadores para dar respuesta a esta cuestión: Ryder (1986) propone el concepto de Unidad Significativa Evolutiva (ESU), basado en la estructura genética y la dinámica de las poblaciones; Moritz (1994) define la Unidad de Manejo (MU) para la conservación, basada únicamente en datos alélicos; Riddle & Hafner (1999) describieron las Unidades Geográficas y Evolutivas Fundamentales (FGEU), basándose en valores filogeográfico; y Maes *et al.* (2004) definen las Unidades de Conservación Funcional (FCU) basadas en la distribución y la ecología de las especies. Una de las últimas propuestas es la de Caujapé-Castells & Pedrola-Monfort (2004) que proponen una combinación de dos métodos (establecer el número mínimo de unidades de conservación para la conservación de la de la variabilidad genética total de una especie y la fórmula de selección de las poblaciones que contienen una composición alélica rara o singular) para seleccionar Unidades Genéticas Relevantes para la Conservación (RGUCs) basadas en la posesión común de alelos habituales y raros. Esta propuesta se basa en las premisas de Ciofi & Bruford (1999) y se ha usado para establecer estrategias de conservación en especies como *Boleum asperum* Desv. (Pérez-Collazos *et al.* 2008) y *Borderea pyrenaica* Miégev. (Segarra-Moragues & Catalán 2010). Este tipo de diseño está orientado a diseñar estrategis de conservación, tanto ex situ, como in situ, de manera eficiente desde el putno de vista económico, particualrmente en especies con rango de distribución amplios. Además, tiene en cuenta que

los alelos raros pueden favorecer el establecimiento de las plantas reintroducidas (Bengtsson *et al.* 1995; Pérez-Collazos *et al.* 2008).

PALEO-HISTORIA DEL OESTE MEDITERRÁNEO E ISLAS CANARIAS

La historia geológica del Oeste del Mediterráneo es muy compleja debido a su localización en la convergencia de las placas Africana y Euroasiática (p. ej., Rosenbaum, Lister & Duboz, 2002; y referencias que se incluyen a lo largo del epígrafe). El conocimiento de los diferentes eventos geológicos ocurridos permite explicar los patrones biogeográficos actuales de las especies y la estructuración de su diversidad genética (p. ej., Médail *et al*., 2001; Mansion *et al*., 2008; Ortiz *et al*., 2009). Consideramos que los eventos geológicos que más han influido en las especies objeto de estudio de esta tesis son: Los procesos orogénicos del complejo Atlas-Anti Atlas, la historia geológica del Cinturón Hercínico en el mediterráneo occidental, la formación de las islas del archipiélago canario, la crisis de salinidad del Messiniense y las oscilaciones climáticas del Cuaternario. Toda esta configuración espacio-temporal de la zona oeste del Mediterráneo ha sido, probablemente, una de las causas de la gran diversidad y del gran número de endemismos que esta zona alberga (Medail & Quezel 1997).

Complejo Orogénico Atlas-Anti Atlas

Las cordilleras del Atlas (Alto Atlas, Atlas Medio y Anti Atlas) forman un sistema montañoso que se extiende a lo largo de más de 2.000 km en sentido noreste a lo largo de Marruecos; el Alto Atlas tiene una altitud de 4.167 m, en contraste el Anti-Atlas es un macizo de suave relieve (Teixell *et al.* 2007). La elevación de esta cordillera comenzó aprox. hace uno 65 Ma, a pesar de esto, los indicadores geomorfológicos sugieren que la mayor parte del levantamiento mantélico es relativamente reciente (últimos 5 Ma; Teixell *et al*., 2007), parece ser que esta zona estuvo constituida por zonas altas de estepa y praderas ricas en especies (Terrab *et al.* 2009). Este sistema montañoso, unido al desierto del Sahara y al valle del río Muluya (al norte de Marruecos) han establecido una barrera muy importante para la dispersión de los seres vivos. Muchos estudios se focalizan en el efecto barrera y en las rupturas genéticas de ambos lados de la cordillera (Médail *et al*., 2001; Terrab *et al*., 2008; entre muchos otros), incluso sobre los procesos de especiación que el aislamiento ha provocado (Habel *et al.* 2008); también se ha investigado sobre las características de esta zona como área refugio para especies y como punto de inicio de recolonización (Terral *et al.* 2004; Terrab *et al.* 2008). Aunque muchas especies tienen una distribución de alguna forma relacionada con esta zona, muy poco se sabe de su filogeografía.

Una de las consecuencias de la conjunción de todas estas características geológicas y temporales, así como la gran diversidad de hábitats (Thompson 2005), es que la zona es uno de los *hotspots* de biodiversidad más importantes dentro de la zona mediterránea (Medail &

Quezel 1997), y como algunos estudios indican, una de las zonas más amenazadas respecto de la pérdida de biodiversidad por el cambio climático (IPCC 2001).

El Cinturón Hercínico en el Mediterráneo occidental

En el Oligoceno (25-30 Ma), los movimientos de deriva continental disgregaron parte del Cinturón Hercínico de la región mediterránea occidental en fragmentos continentales. Estos fragmentos se corresponden en la actualidad con las cordilleras Béticas y del Rif, el archipiélago de las islas Baleares, la Cabilia (tierras montañosas del norte de Argelia), Córcega, Cerdeña, el archipiélago Toscano y la región de Calabria; estas zonas se encontraban originalmente unidas al este de la península ibérica y a la zona sur de Francia (Rosenbaum, Lister & Duboz, 2002; Bidegaray-Batista & Arnedo, 2011; entre otros).

Esta zona pre-oligocénica continua, permitía el flujo genético activo entre las poblaciones de plantas que albergaba. Muestra de ello son los estudios de Mansion *et al*. (2008, 2009) sobre la familia Araceae y sobre el orden Boraginales, en estos estudios se relaciona las distancias genéticas de las especies con los eventos de fragmentación oligocénica de la zona. Sin embargo, la mayoría de los estudios concluyen que la distribución actual de las especies que ocupan esta área tiene como punto de origen un momento posterior al oligoceno (p. ej., Salvo *et al*., 2010).

Las reconstrucciones temporales realizadas (recopiladas en Rosenbaum, Lister & Duboz, 2002; Figura 1) indican como a partir de la fragmentación de la zona hace 25 Ma se produjo un desplazamiento dirección sur de los fragmentos continentales provocando la apertura del Canal de Valencia; posteriormente, en el Mioceno medio, las mismas fuerzas de deriva empujaron Córcega, Cerdeña, Calabria y el archipiélago Toscano en dirección Este, abriendo la gran distancia que actualmente hay entre estas regiones y el archipiélago Balear; Calabria y el archipiélago Toscano acabarían situándose junto a la Península Itálica. No hay consenso acerca de la separación entre Córcega y Cerdeña, pero parece que los estudios la datan hace 15-21 Ma (Gattacceca *et al.* 2007). Durante la crisis de salinidad del Messiniense y las oscilaciones climáticas pleistocénicas, es probable que puentes de tierra unieran ambas islas (Salvo *et al.* 2010). En cuanto a la región de la Cabilia, inicialmente junto a al archipiélago Balear, fue empujada en dirección sur (aprox. hace 21 Ma), lo que la llevó a ocupar en el Mioceno medio su posición actual al norte de África en Argelia. El último complejo geológico, formado por las cordilleras Béticas y por la cordillera del Rif, se desplazó dirección suroeste en

el Oligoceno tardío; posteriormente, en el Mioceno, las cordilleras Béticas se situaron al sur de la Península Ibérica y la cordillera del Rif al norte de Marruecos, dando lugar al estrecho de Gibraltar.

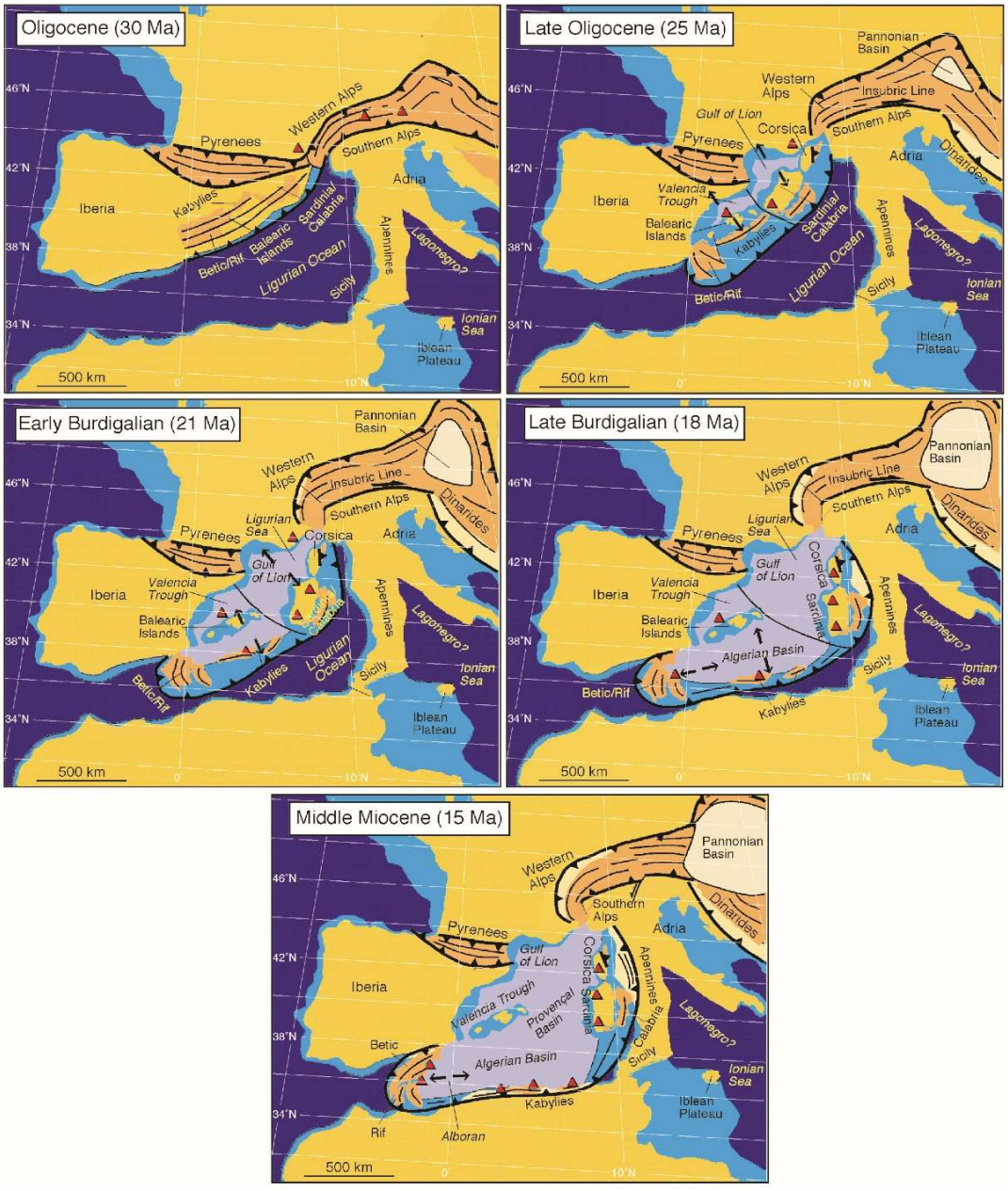

Figura 1: Reconstrucción del oeste mediterráneo desde el Oligoceno, tomado de Rosenbaum et al. 2002.

La formación de las islas del Archipiélago Canario

Como núcleo de la región Macaronésica (Stearn 1973; Whittaker & Fernández-Palacios 1998), y debido a su posición y estructura, las Islas Canarias tienen una gran importancia para el estudio de los eventos filogeográficos. Muchos estudios han utilizado este archipiélago para investigar sobre especiación, extinción, migración, área refugio y post colonización de la zona continental (muchos ejemplos son dados en Bramwell & Caujapé-Castells, 2011) y estudios de modelos de vicarianza *vs*. dispersión (p. ej., Sanmartín, van der Mark & Ronquist, 2008; entre otros).

Actualmente el archipiélago canario se compone de las islas de Tenerife, Fuerteventura, Gran Canaria, Lanzarote, La Palma La Gomera, El Hierro y La Graciosa (que pasó de ser reconocida como islote a isla en 2018), de los islotes de Montaña Clara, Alegranza, Roque del Este, Roque del Oeste y Lobos, y de varios roques menores o peñones. Como todo archipiélago volcánico, las Islas Canarias se formaron a partir de un punto caliente en la corteza oceánica. Se considera que éste se creó hace aproximadamente 60 millones de años, dando origen a la hoy sumergida isla de Lars (Geldmacher *et al*., 2001); de las islas actuales, la más antigua es Salvaje Grande con 27 millones de años (Myr) en el actual archipiélago de las Islas Salvajes (165 km al norte del archipiélago canario); el desplazamiento de este punto caliente dirección sur provocó la aparición del resto de islas que conocemos en la actualidad (Figura 2: Reconstrucción de la región Paleo-Macaronésica y datación de comienzo de la actividad volcánica de cada isla (entre paréntesis), tomada de Caujapé-Castells (2011) con ligeras modificaciones.; Bramwell & Caujapé-Castells, 2011).

La antigüedad del archipiélago canario aumenta en sentido este, siendo Fuerteventura y Lanzarote las más antiguas con aproximadamente 25 millones de años. Merece la pena comentar que originalmente emergieron como una única isla, denominada Mahan, y se han mantenido unidas hasta el pleistoceno (Fernández-Palacios *et al.* 2011). Estas islas se encuentran en un estadío avanzado dentro de la sucesión que se da en las islas oceánicas (Fernández-Palacios & Whittaker 2010), lo que significa que los procesos de vulcanismo formador han cesado (casi por completo) y que la erosión ha eliminado prácticamente todo el relieve que tuvieron en el pasado. Por ejemplo, Fuerteventura tiene hoy una altitud máxima de 800 metros cuando se considera que en el pasado pudo llegar a los 3300 m. (Stillman 1999).

Es importante considerar la situación del archipiélago canario y su proximidad al continente africano (95 km en la actualidad desde Fuerteventura). El archipiélago comparte un zócalo común con el continente africano (Macau Vilar 1963; Anguita & Hernán 1999; Machado

2002), hecho que parece haber detenido el hundimiento que se suele producir en este tipo de islas volcánicas, una vez ha cesado el vulcanismo (Carracedo 2002; Price & Clague 2002).

Además, gran cantidad de "islas sumergidas" mantienen un relieve bastante importante. La combinación de las islas actuales y de las islas que se considera estuvieron emergidas durante los periodos glaciares (al menos en los últimos) determinan la región Paleo-Macaronésica (Fernández-Palacios *et al.* 2011). Esta región ha jugado un papel esencial para la migración de especies, tanto vegetales, como animales, y estas islas sumergidas pudieron funcionar como corredor de tierra intermitente (*stepping stones*) que facilitaba el intercambio biótico entre el continente y la región Macaronésica (García-Talavera, 1999; Carine *et al*., 2004; Bramwell & Caujapé-Castells, 2011; entre otros muchos) en ambos sentidos. (Caujapé-Castells 2011)

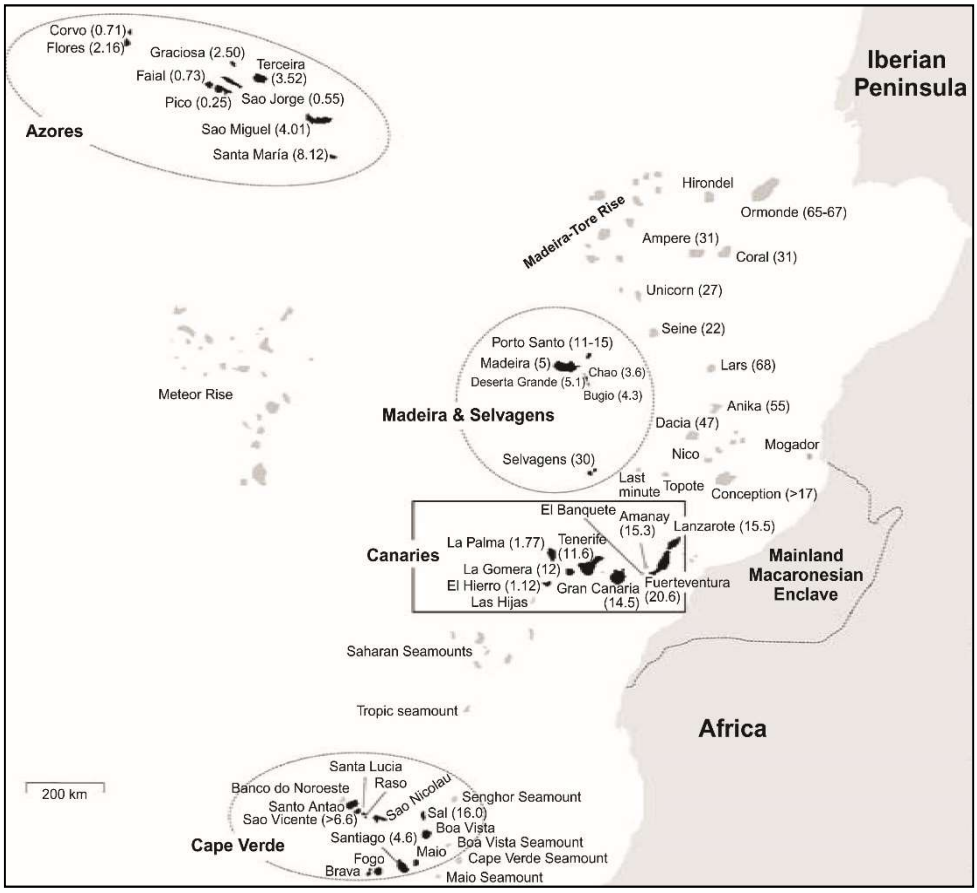

Figura 2: Reconstrucción de la región Paleo-Macaronésica y datación de comienzo de la actividad volcánica de cada isla (entre paréntesis), tomada de Caujapé-Castells (2011) con ligeras modificaciones.

Crisis de salinidad del Messiniense

Se calcula el origen del mar mediterráneo hace 14 o 15 Ma, cuando los aportes de agua desde el este cesaron, cerrándose las conexiones con el antiguo mar de Paratethys; se considera que en este momento, dos estrechos (estrecho Bético y estrecho del Rif; Figura 3) mantenían el mar Mediterráneo abierto por el oeste (Simon & Meijer 2015), estos permitían la entrada de agua desde el actual océano Atlántico. Al final del Mioceno, cambios drásticos en la corteza terrestre, probablemente unidos a un máximo glacial (Hallam 1973), hicieron descender el nivel del mar provocando el cese de aportes al Mediterráneo desde el oeste, cerrándose ambos estrechos y convirtiendo al Mediterráneo en un mar deficitario. Esto desencadenó la Crisis de salinidad del Messiniense (5.5 Ma; Krijgsman *et al*., 1996). Esta desecación masiva tuvo gran influencia en el paisaje, en el clima (no solo a nivel local sino también a nivel global) y en la distribución de flora y fauna. El Mar Mediterráneo se convirtió en un escenario de grandes lagos salados y marjales (Figura 4), que pronto fueron colonizados por especies xerófitas y halófilas con origen en las zonas sahariana e irano-turaniana, como pueden ser los géneros *Gypsophila* L., *Suaeda* Forssk. ex J. F. Gmel., *Eurotia* Adans., *Astragalus* L., Stipa L., *Microcnemum* Ung. Sternb. y *Salsola* L., entre otros (Arroyo *et al.* 2004). Este escenario permitió también contactos entre los fragmentos insulares antes aislados y las zonas continentales que seguramente modificaron la diversidad y estructura de las poblaciones de especies (Krijgsman *et al.* 1999; Silva *et al.* 2015).

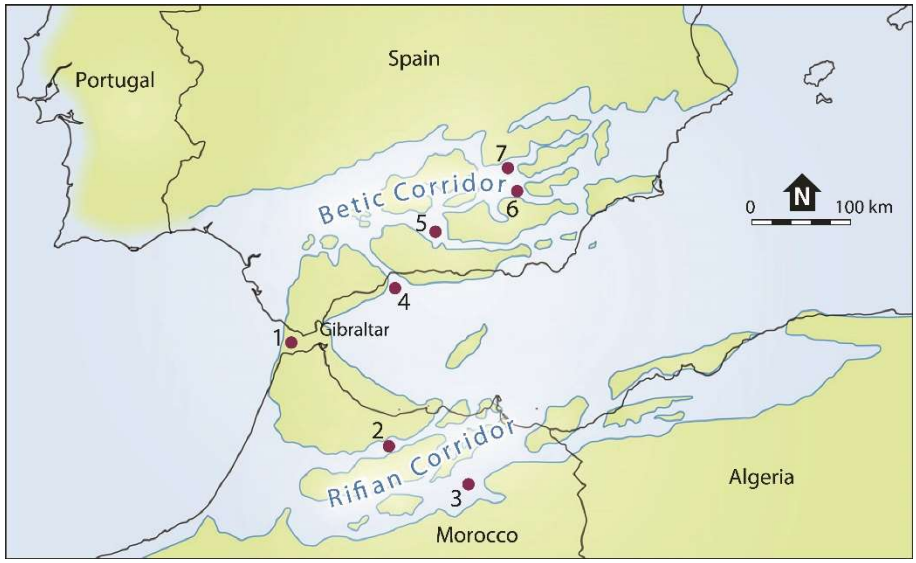

Figura 3: Reconstrucción del oeste Mediterráneo en el Mioceno tardío realizada por Simon & Meijer (2015) y basada en Santisteban and Taberner (1983). Corredores principales (Rif y Bético) y secundarios (numerados).

Al comienzo del Plioceno (5,33 Ma) la reapertura del estrecho de Gibraltar acabó con la crisis salina del Messiniense (Krijgsman *et al.* 1996; Vargas *et al.* 1999; Terrab *et al.* 2008; Rodríguez-Sánchez *et al.* 2008) y la cuenca mediterránea se rellenó con los aportes del Océano Atlántico, eliminando (o al menos, reduciendo) los contactos entre las islas mediterráneas y las zonas continentales. Por ejemplo el estrecho de Gibraltar ha variado de anchura dependiendo del nivel del mar (oscilaciones pleistocénicas debidas al clima; Hewitt, 2000) y esto ha facilitado su actuación como barrera o como zona de contacto dependiendo de las especies (Fernández-Mazuecos & Vargas 2010; Hewitt 2011).

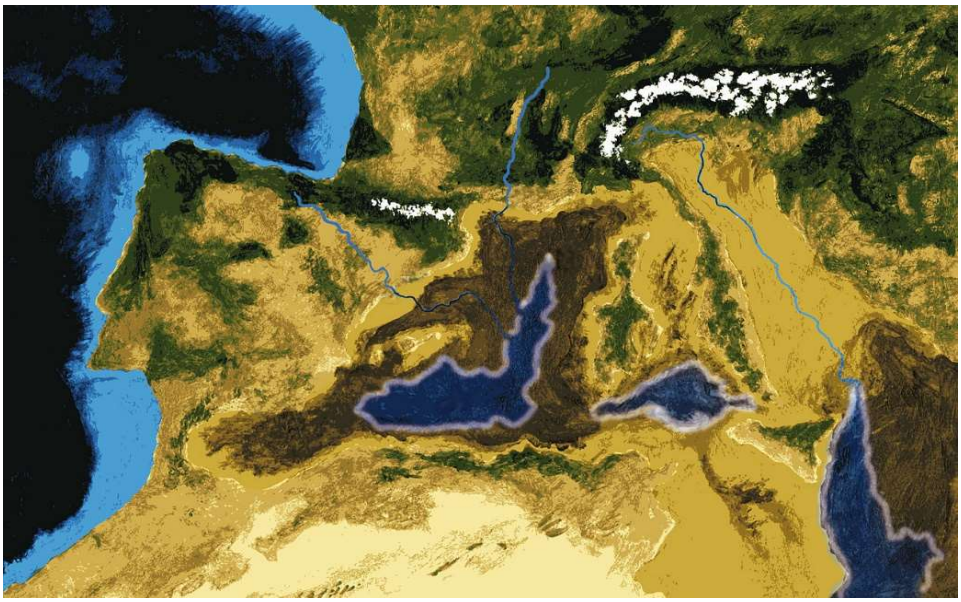

Figura 4: Reconstrucción del Mediterráneo durante la Crisis de Salinidad del Messiniense. Realizada por autor desconocido (2012).

Oscilaciones climáticas en el Cuaternario

A lo largo de la historia del planeta se han sucedido 6 épocas con glaciaciones; las glaciaciones conllevan grandes acumulaciones de hielo (algunas pudieron llegar a cubrir todo el planeta) que hacía descender el nivel del mar y dejaban al descubierto zonas de otra forma sumergidas (Yokoyama *et al.* 2000), esto permitía, entre otras cosas, el intercambio de fauna y flora entre zonas que en periodos interglaciares no tienen contacto directo, con todos los impactos sobre dispersión y aislamiento que se pueden ocasionar (Hewitt 2000; Silva *et al.*, 2015).

En el Cuaternario se dan 10 sucesiones de periodos glaciares e interglaciares (Willis 1996), en estos últimos, las temperaturas pudieron ser muy parecidas a las que encontramos en la actualidad (Bell & Walker 1992); a su vez otros autores calculan que pudieron darse hasta 100 oscilaciones climáticas (Uriarte Cantolla 2003). Hay que tener en cuenta que la aparición del clima mediterráneo ,típico de la Cuenca mediterránea, se da hace unos 3,2 Ma (Suc 1984). Se acepta que los rangos corológicos de las especies se han contraído y ampliado en relación a los ciclos glaciales (y a los cambios climáticos asociados) del Cuaternario (Hewitt 1996; Bennett & Provan 2008), y no solo la distribución de las especies se ha visto modificada por estos ciclos, muchos estudios han demostrado la clara influencia que han tenido sobre los patrones de riqueza y diversidad (Taberlet *et al.* 1998; Willis & Whittaker 2000; Petit *et al.* 2002; Jansson 2003; Schönswetter *et al.* 2005; Bhagwat & Willis 2008; Ortiz *et al.* 2009). En principio, estos patrones se explican por la existencia de refugios durante los periodos más fríos donde las especies pudieron resistir las condiciones adversas y tras las cuales pudieron recolonizar otras zonas (Petit *et al.* 2003; Hampe & Petit 2005; Cheddadi *et al.* 2005; Tzedakis 2009).

Por su localización, las penínsulas mediterráneas han actuado como zonas refugio, se ha observado como estas zonas no solo han permitido la supervivencia de las especies sino que también han funcionado como motores de especiación (Hewitt 2000, 2011). Además, la teoría de refugios dentro de refugios (Gómez & Lunt 2006) parece explicar las distancias genéticas entre muchos de los taxones de plantas de la península ibérica (Heredia *et al.*; Rodríguez-Sánchez *et al.* 2010).

ESPECIES OBJETO DE ESTUDIO

Género *Astragalus* L.

Astragalus L. es un gran género con cerca de 3.000 especies (2.500 en el Viejo Mundo y otras 500 en el Nuevo Mundo, muchas de ellas aún con dudas taxonómicas) y más de 250 secciones (Lock & Simpson 1991; Maassoumi 1998; Podlech 1998; Lewis *et al.* 2005; Mabberley 2008). El género fue descrito por Carlos Linneo y publicado en Species Plantarum en 1753; la especie tipo es *Astragalus christianus* L. *Astragalus* pertenece a la familia Fabaceae (Fabáceas o Leguminosas) y a la subfamilia Faboideae, y es, con diferencia, el género más grande de la familia y de todas las angiospermas. El género está presente en casi todas las partes del hemisferio norte y representa un espectacular ejemplo de radiación adaptativa. El probable origen del género se sitúa en las zonas sur-oeste de Asia y en la región Sino-Himalaya (Wojciechowski 2005), en esta zona se ha descrito más de 1500 spp.

Es un género de gran complejidad y amplitud, por lo que se han realizado muchos estudios, tanto morfológicos como genéticos; estos trabajos trataban de resolver las relaciones filogenéticas entre las especies del género y sus relaciones con las especies más cercanas. El último estudio genético realizado por Azani *et al*. (2017) parece dar solución al grupo y mejora las anteriores aproximaciones (Osaloo *et al.* 2003; Osaloo *et al*. 2005; Wojciechowski 2005), en éste se añaden especies (sobre todo del grupo de *Astragalus* del "Viejo Mundo", y se resuelven los clados que durante tiempo fueron conflictivos, como *Oxitropis* DC., *Biserrula* L., *Phaca* L., *Colutea* L., *Podlechiella* Maassoumi & Kaz. Osaloo y *Phyllolobium* Fisch. (Wojciechowski *et al.* 1999; Osaloo *et al.* 2003; Osaloo *et al*. 2005). Estos géneros han sido incluidos y sacados del género *Astragalus* en varias ocasiones (Figura 5). Las 20 secciones dentro de *Astragalus s. str.* descritas por Podlech & Zarre (2013) para las especies de *Astragalus* del "Viejo Mundo" y basadas en características morfológicas y genéticas no obtienen apoyo y, de hecho, la mayoría se recuperan como parafiléticas o polifiléticas en Azani *et al*. (2017). Este estudio establece 8 clados en los que las especies anuales proceden de ancestros perennes en cada clado (los autores dudan del número total de clados debido a la escasa adición de especies de *Astragalus* del "Nuevo Mundo" que parecen haber dado también forma a varios clados). En esencia, al grupo tradicional de *Astragalus s. str.* (Osaloo *et al.* 2003) se une el clado denominado "Glottis" (Azani *et al.* 2017) [compuesto por *Biserrula pelecinus* L. (=*Astragalus pelecinus* (L.) Barneby) y por *Astragalus epiglottis* L., entre otros] y formando el grupo Eu-Astragalus. Los géneros *Oxitropis*, *Podlechiella* y *Phyllolobium* se recuperan como grupos monofiléticos externos.

Astragalus edulis Bunge

Descrita por Alexander von Bunge y publicada en *Mémoires de l'Académie Impériale des Sciences de Saint Pétersbourg, Septième Série* en 1868, *Astragalus edulis* se ha incluido al subgénero *Trimeniaeus*, subgénero compuesto de especies anuales pero sin utilidad filogenética (Osaloo *et al.* 2003; Azani *et al.* 2017), y dentro de éste a la sect. *Edodimus*, aunque algunos autores lo consideran en la sect. *Bucerates* DC. (Podlech & Zarre 2013). Los resultados de Azani *et al*. (2017) agrupan *A. edulis* en el Clado "*Hamosa*" junto con otros componentes tradicionales de las secciones *Bucaretes* y *Cyamodes,* además de otras especies de secciones perennes (Figura 6).

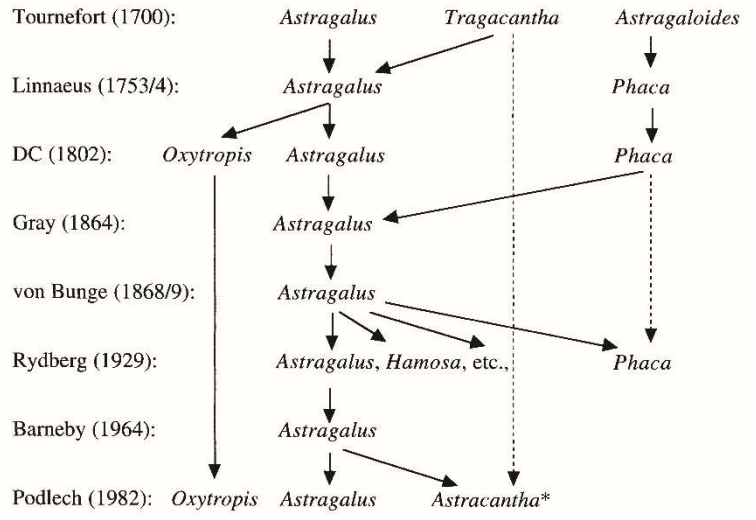

Figura 5: Historia taxonómica de los géneros afines al género Astragalus, tomado de Wojciechowski, Sanderson & Hu (1999).

Siguiendo la descripción realizada por Dietrich Podlech (1999) en *Flora iberica* y por Peñas de Giles & Morales Torres (2009) en Flora Vascular de Andalucía Oriental, la especie es una hierba anual, ramificada en la base, esparcidamente pilosa; indumento formado por pelos mediifijos de 0,4 a 1,2 mm. Tallos de hasta 30 cm, esparcidamente pelosos, con todos los pelos blancos. Hojas de 5 a 13 cm, pecioladas, imparipinnadas, con 7 a 10 pares de folíolos; estípulas de 5 a 10 mm, no soldadas al pecíolo y adnadas entre sí, que abrazan al tallo, con el extremo libre corto y estrecho, con pelos blancos y a veces mezclados con negros; pecíolo de 1 a 2 cm, del grosor del raquis, con indumento esparcido o denso; folíolos de 8 a 15 por 2 a 6 mm, desde estrechamente ovados hasta estrechamente obovados, con ápice redondeado, glabros por el haz, desde esparcidamente pelosos hasta densamente pelosos por el envés. Inflorescencias en racimos, éstos pedunculados, bracteados, densos cuando son jóvenes, laxos en la madurez, con 3 a 10 flores; pedúnculos de 3 a 8 cm, pelosos; brácteas de 1,5 a 3 mm, ciliadas, blanquecinas.

Cáliz de 4 a 5 mm, anchamente tubular, densamente peloso, con pelos negros y a veces mezclados con blancos; dientes de 1,5 a 2 mm, iguales. Corola azulada, con el estandarte más largo que las alas y la quilla; estandarte de 7 a 9 mm; alas de 6 a 7 mm; quilla c. 5 mm. Androceo con 10 estambres. Fruto de 10 a 18 por 4 a 6 mm, péndulo, sentado, de contorno semielíptico, triangular en sección transversal, con una quilla bien desarrollada en la línea de sutura del carpelo o ventral, muy ancho y aplanado en el dorso, con pico corto; valvas papiráceas, con rugosidades transversales, glabras, frecuentemente rojo-parduscas. Semillas c. de 4 por 4 mm, cúbicas (Figura 7).

Astragalus edulis se distribuye por España (SE Ibérico, Lanzarote y Fuerteventura), Argelia y Marruecos (Podlech 1999; Peñas 2004; Reyes Betancort *et al.* 2005). Presenta un ciclo de vida corto, dependiente del régimen de lluvias. Las legumbres no presentan especialización para la dispersión, pero son comestibles, lo que podría indicar endozoocoria (en el supuesto de que las semillas preserven su capacidad germinativa, tras el paso por el sistema digestivo). Habita en zonas áridas moderadamente alteradas por el pastoreo con sustratos poco desarrollados y arenosos. La especie presenta explosiones demográficas dependientes de las lluvias primaverales lo que provoca fluctuaciones poblaciones interanuales que varían entre la germinación abundante y casi nula, en este caso, la especie mantiene un importante banco de semillas en el suelo (Peñas, 2004).

Astragalus edulis se incluye en la categoría "En Peligro" en la Lista roja de la flora vascular española y en la Lista roja de la flora vascular de Andalucía, y sin clasificar a nivel mundial; las principales amenazas para la especie son los factores estocásticos como incendios o sequías debido al escaso número de individuos en algunas poblaciones, también los procesos de sucesión vegetal tras el abandono del pastoreo (la especie presenta escasa plasticidad ecológica) (Peñas de Giles 2004).

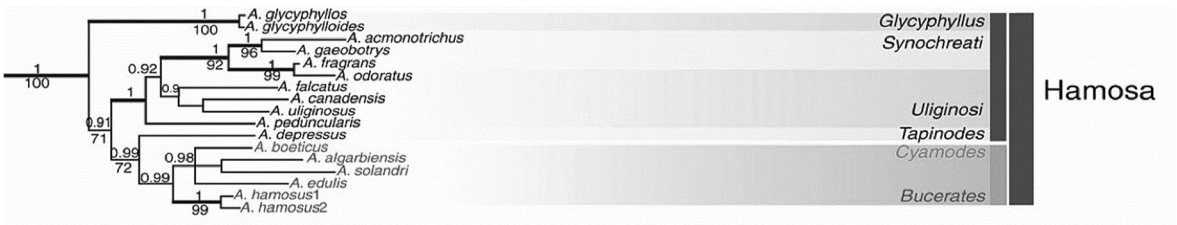

Figura 6: Filogenia obtenida por Azani et al. (2017).

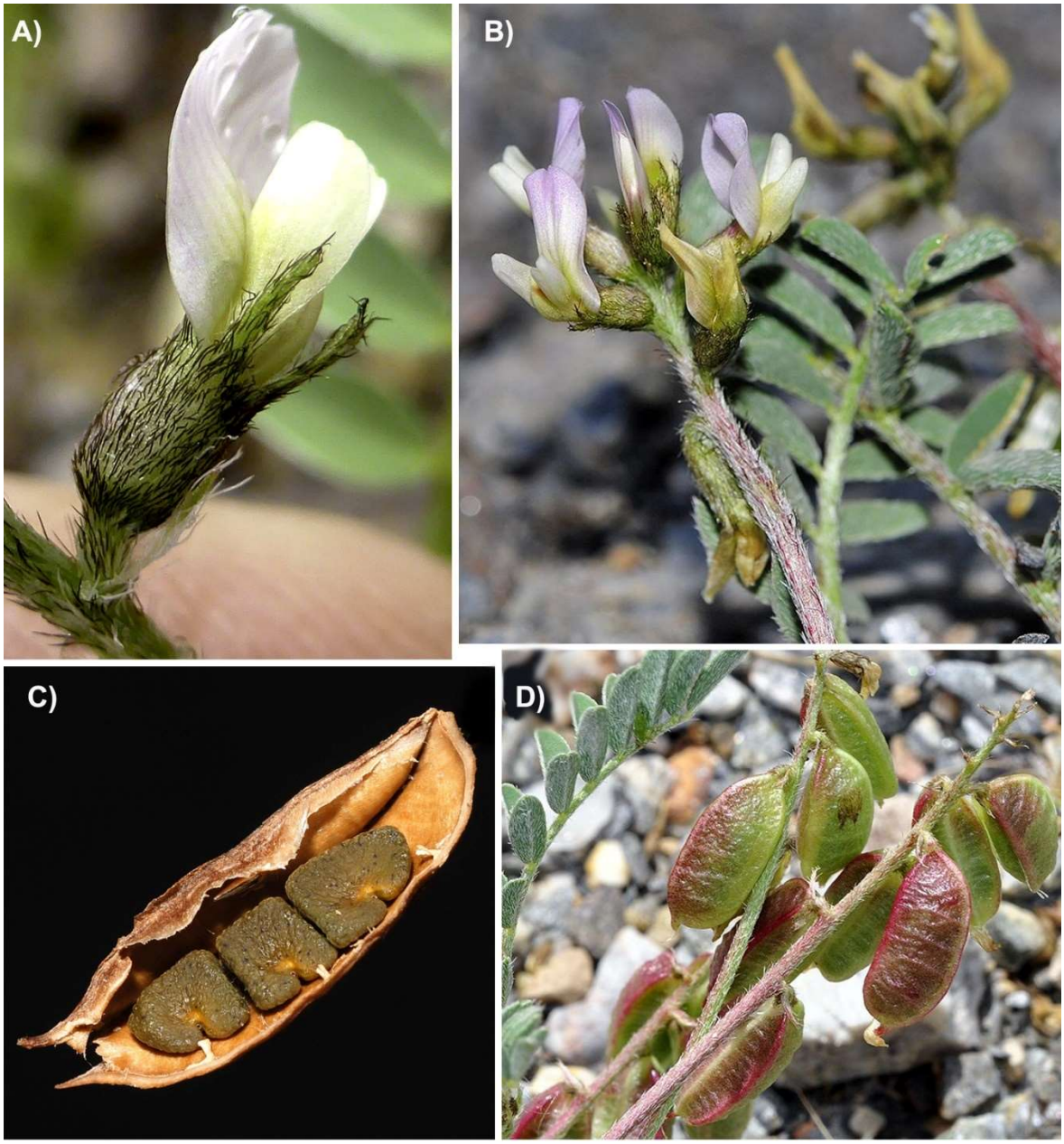

Figura 7: Fotos de *Astragalus edulis*; A) Detalle de la flor, B) aspecto general de la especie, C) detalle del fruto y de las semillas y D) detalle de los racimos.

Género *Arenaria* L.

El género *Arenaria* pertenece a la subfamilia Alsinoideae y a la tribu Alsineae, está distribuido por Eurasia, América y norte de África (Williams 1898; Zhou 1995) con aproximadamente 306 spp., de las cuales 38 están presentes en España (López González 1990), 40 siguiendo los últimos estudios (Fior *et al.* 2006; Sadeghian *et al.* 2015), de las que 33 son endémicas.

Según los estudios filogenéticos el género *Arenaria* es polifilético (Fior *et al.* 2006; Harbaugh *et al.* 2010; Greenberg & Donoghue 2011). Se reconocen 4 géneros dentro de *Arenaria sensu lato*, *Eremogone* Fenzl, *Odontostemma* (Bentham ex G. Don) F. N. Williams, *Dicranilla* (Fenzl) F. N. Williams y *Leiosperma* McNeill (Harbaugh *et al.* 2010), pero las asignaciones de las especies a estos géneros es todavía poco estable (Montesinos & Kool 2015). Además los problemas de homoplasia en la morfología que ya se observaban en la familia se acrecientan (Valcárcel *et al.* 2006; Lorite *et al.* 2018).

***Arenaria balearica* L.**

Teniendo en cuenta la distribución de *A. balearica*, su consideración como paleoendemismo (Favarger & Contandriopoulos 1961; Greuter 1995; Quézel 1995), o endemismo disyunto (Thompson 2005) y su hábitat reducido, suponen que la especie sea un magnífico ejemplo de estudio para filogeografía. *Arenaria balearica* L. pertenece a la sección *Rotundifoliae* McNeil, y está íntimamente relacionada con *A. bertoloni* Fiori & Paol. (Fior & Karis 2007); la especie está ligada al clima marítimo, habita taludes terrosos, en la base de los roquedos y en laderas frescas, en un rango altitudinal de entre 280 y 1440 m.; su distribución es Tirreno-Baleárica, pudiéndola encontrar de forma natural en Mallorca, Córcega, Cerdeña, Capraia, Montecristo y Tavolara, además de estar naturalizada en Gran Bretaña y Francia (López González 1990). Esta distribución se supone asociada a los procesos de deriva continental de la región del Cinturón Hercínico (Mansion *et al.* 2008).

En cuanto a la morfología de *A. balearica* (Figura 8) y según la descripción de López González (2000) en *Flora iberica*, ésta es una planta postrada, de carácter cespitoso, pelosa o glabrescente, de indumento eglanduloso o glanduloso, de pelos patentes más o menos flexuosos; las hojas tienen unas dimensiones de 2 a 4 por 0,9 a 1,5 mm, de forma ovada u ovo-lanceoladas, con peciolo, pelosas o glabras, su disposición es densa en la parte inferior de los tallos, en cuanto a las superiores son sésiles y bractiformes; las flores son solitarias, en ocasiones cimas paucifloras largamente pediceladas de aprox. 30 mm, pelosos o glabros; el

cáliz es de color purpura de 2 a 3 mm con los sépalos ovado-lanceolados o elípticos, obtusos, mucronados, los internos con reborde membranáceos, casi siempre denticulado; pétalos de 3,5 a 5 mm; las anteras miden de 0,4 a 0,5 mm; cápsula ovoidea, membranácea, pudiendo ser exerta (Walters 1964), de 2,5 a 3-5 mm; semillas orbículo-reniformes, con las células de la testa diminutas, redondeadas o con prominencias mamiformes. No presenta ninguna especialización en cuanto a dispersión de semillas y no se conoce tampoco ninguna asociación ni especialización en cuanto a la polinización. *Arenaria balearica* presenta un número cromosómico de 2n=18 (Dahlgren *et al.* 1971; Cardona & Contandriopoulos 1980; Diana Corrias 1981).

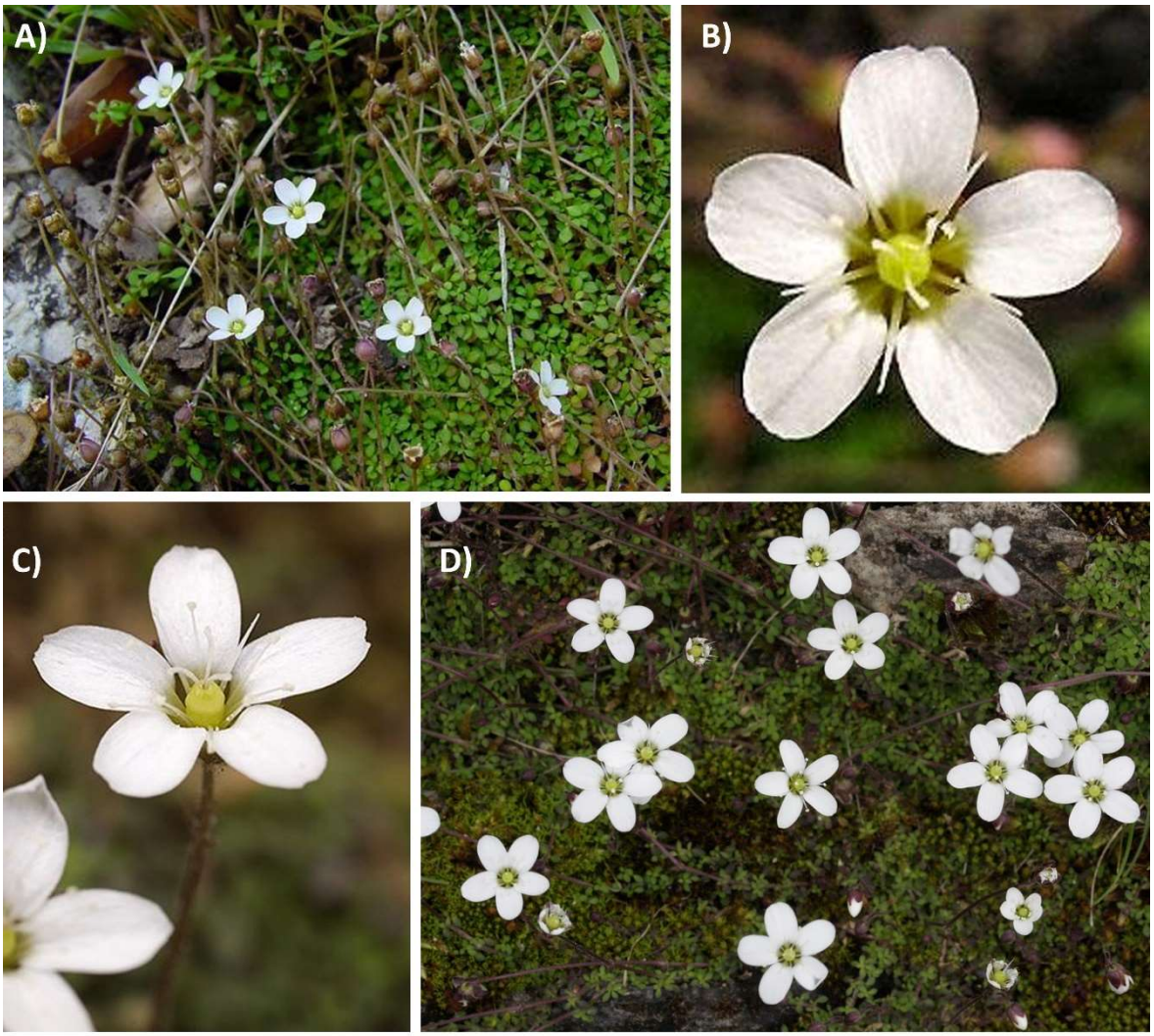

Figura 8: Fotos de *Arenaria balearica;* A y D) Aspecto general de la especie, B y C) detalle de la flor.

Arenaria balearica se encuentra catalogada como especie de preocupación menor en la clasificación de la UICN. A pesar de esto, sus poblaciones reducidas y el hábitat específico que

ocupa hacen que sea necesario conocer la estructura genética de la especie para poder llevar a cabo controles periódicos y plantear medidas de conservación que engloben la singularidad genética de la especie.

Arenaria section *Pseudomoehringia* (McNeill) Rabeler & Zarre

Este complejo de especies anteriormente reconocido como *Moehringia* sect *Pseudomoehringia* McNeill, presentó desde el comienzo problemas de clasificación (McNeill 1962) y ha sido transferido al género *Arenaria* (Fior & Karis 2007). La presencia de estrofíolo parecía apoyar su pertenencia al género *Moehringia*, pero tras los estudios de Minuto *et al*. (2006) y Fior & Karis (2007) se vio que el origen ontogenético del estrofíolo en este grupo era diferente al que presentan el resto de especies del género *Moehringia* [probablemente de origen secundario, formándose no solo desde el funículo sino también de forma papilar desde el hilo y como adaptación a los suelos secos (Minuto *et al*., 2006)].

Taxonómicamente este grupo ha sufrido varias modificaciones, no solo referidas a la asignación a género, sino también, a los límites taxonómicos de las especies y subespecies que lo componen; Propuesto inicialmente por McNeill (1962) incluía a *M. intricata* Willk., *M. tejedensis* Willk. y *M. fontqueri* Pau y, como caracter diferencial, proponía la morfología de las semillas, que el autor describe como "*tipo Arenaria*" (de ahí el nombre de la sección).

Montserrat-Martí (1985) describe *M. glochidisperma* J. M. Mont. en el norte de Marruecos y, poco después (Montserrat Martí 1986), considera solo dos especies para la península ibérica (*M. intricata* y *M fontqueri*) y tres subespecies para *M. intricata* (*M. intricata* subsp*. tejedensis* (Willk.) J. M. Mont., *M. i*ntricata subsp. *intricata* Willk., *M. intricata* subsp. *castellana* J. M. Mont.).

Diaz de la Guardia, Mota & Valle (1991) mediante estudios morfológicos, proponen, aún dentro del género *Moehringia*, la existencia de cuatro subespecies de *M. intricata* (*M. intricata* subsp*. tejedensis* (Willk.) J. M. Montserrat, *M. i*ntricata subsp. *intricata* Willk., *M. intricata* subsp. *castellana* J. M. Montserrat y *M. intricata* subsp. *giennensis* C. Díaz, Mota & F. Valle)

Posteriormente, tras un análisis morfológico exhaustivo, además de análisis de *ITS* y de la región plastidial *MatK*, Fior & Karis (2007) reconocen cuatro especies en la sección y su pertenencia al género *Arenaria*, *A. suffruticosa* Fior et P.O. Karis (con tres subespecies, aunque los autores expresan claras dudas taxonómicas, *A. suffruticosa* subsp. *giennensis, A. suffruticosa*

subsp. *intricata* y *A. suffruticosa* subsp. *castellana*), *A. tejedensis* (Willk.) Fior et P.O. Karis, *A. glochidisperma* (J.M. Mont.) Fior et P.O. Karis y *A. funiculata* Fior et P.O. Karis (Figura 9).

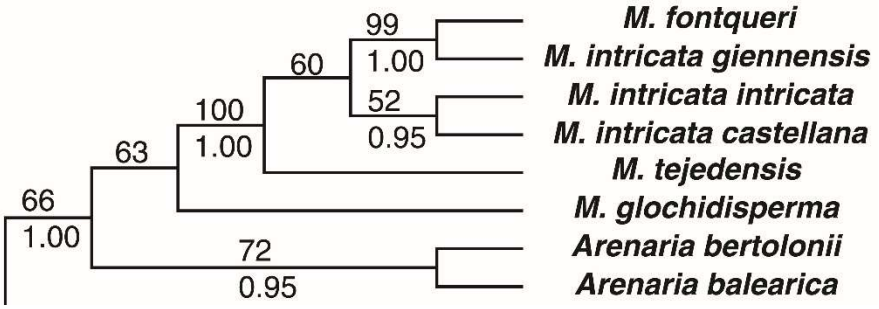

Figura 9: Filogenia parcial del grupo de estudio usando parsimonia con los marcadores *ITS* y *matK*, tomada de Fior y Karis (2007).

Sadeghian *et al*. (2015), tras un análisis parcial de toda la familia, proponen el parafiletismo de *Arenaria* sect. *Pseudomoehringia*. Estos autores incluyen a *Arenaria modesta* Dufour como parte de la sección *Pseudomoehringia*, mientras que los mismos resultados plantean que *A. glochidisperma* no forme parte de la sección (Figura 10). Este estudio ha de tomarse con precaución ya que los analisis filogenéticos realizados obtienen muy poco apoyo en la mayoría de los clados, los valores de "*missing data*" son elevados, no se especifican las subespecies dentro de *A. suffruticosa e* incluso, se obtienen diferentes resultados dependiendo del marcador usado (hay grandes discrepancias entre ITS y marcadores plastidiales).

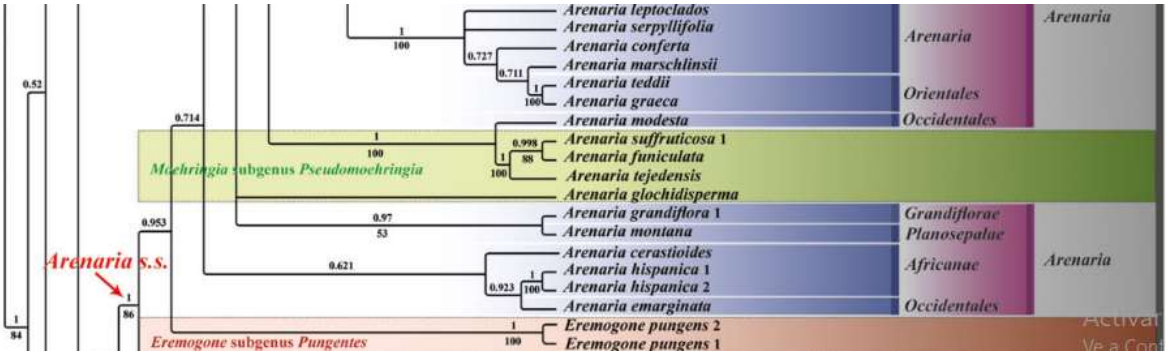

Figura 10: Filogenia de *Arenaria* basada en *ITS* (Sadeghian et al. 2015)

Recientemente, Lorite *et al*. (2018) realizaron un profundo estudio morfológico de la sección según Fior & Karis (2007), los autores concluyen que la morfología no es señal suficiente como para conservar las subespecies (sólo *A. suffruticosa* subsp. *castellana* presenta diferencias morfológicas relevantes).

Arenaria suffruticosa Fior & P.O.Karis

La descripción morfológica de *A. suffruticosa* (Montserrat Martí 2000) puede aplicarse a las subespecies de *A. suffruticosa* (*A. suffruticosa* subsp. *giennensis, A. sufruticosa* subsp. *intricata* y *A. suffruticosa* subsp. *castellana*) y a *A. tejedensis*, ya que en el compendio de *Flora iberica* se consideran las subespecies *Moehringia intricata* subsp. *intricata*, *Moehringia intricata* subsp. *tejedensis*, *Moehringia intricata* subsp. c*astellana* [no se considera en este caso la subespecie *giennensis* pero su morfología es similar (Diaz de la Guardia *et al.* 1991)].

Planta perenne, de 10 a 20 cm, glabra, matas más o menos laxas, frecuentemente colgantes. Tallos muy ramificados, frágiles, leñosos en la base. Hojas pequeñas [de 6 a 10 por 2 a 3 mm], de linear-espatuladas a obovadas o suborbiculares, raramente lineares, agudas u obtusas, brevemente mucronadas, cuneadas o atenuadas en pecíolo corto, a veces sésiles, uninerves, en general glaucas. Flores pentámeras, en dicasios terminales muy laxos, de 2 a 7 flores. Sépalos de 3 a 4 mm, oblongo-lanceolados, agudos o subobtusos, mucronados, de margen escarioso más o menos evidente y 1 a 3 nervios. Pétalos de 6 a 8 mm, espatulados u obovados. Semillas de 0,9 a 1,2 por c. 0,7 mm, reniformes, negras, mates; células dorsales de la testa provistas o no de tricomas o tubérculos agudos u obtusos, a veces ramificados; estrofíolo no laciniado, compuesto de tricomas más o menos gruesos. Número cromosómico de 2*n* = 26.

La teórica distribución de las especies (o subespecies) se describe en Diaz de la Guardia, Mota & Valle (1991) (Figura 11). *A. suffruticosa* subsp. *castellana* se localiza en el sector Celtibérico-Alcarreño, *A. sufruticosa* subsp. *giennensis* en el sector Subbético, *A. suffruticosa* subsp. *intricata* en el sector Guadiciano-Bacense y *A. tejedensis* en el sector Malacitano-Almijarense. En 2015 se publicó una localidad para *A. suffruticosa* en la zona de Debdou al este de Marruecos (Chambouleyron *et al.* 2015), pero la cita no ha podido ser confirmada presencialmente ni se ha tenido acceso al pliego de herbario.

Figura 11: Distribución de las subespecies de *A. suffruticosa* según Diaz de la Guardia, Mota & Valle (1991); ▲ subsp. *castellana*, ★ subsp, *tejedensis*, ● subsp, *giennensis*, ✻ subsp, *intricata*.

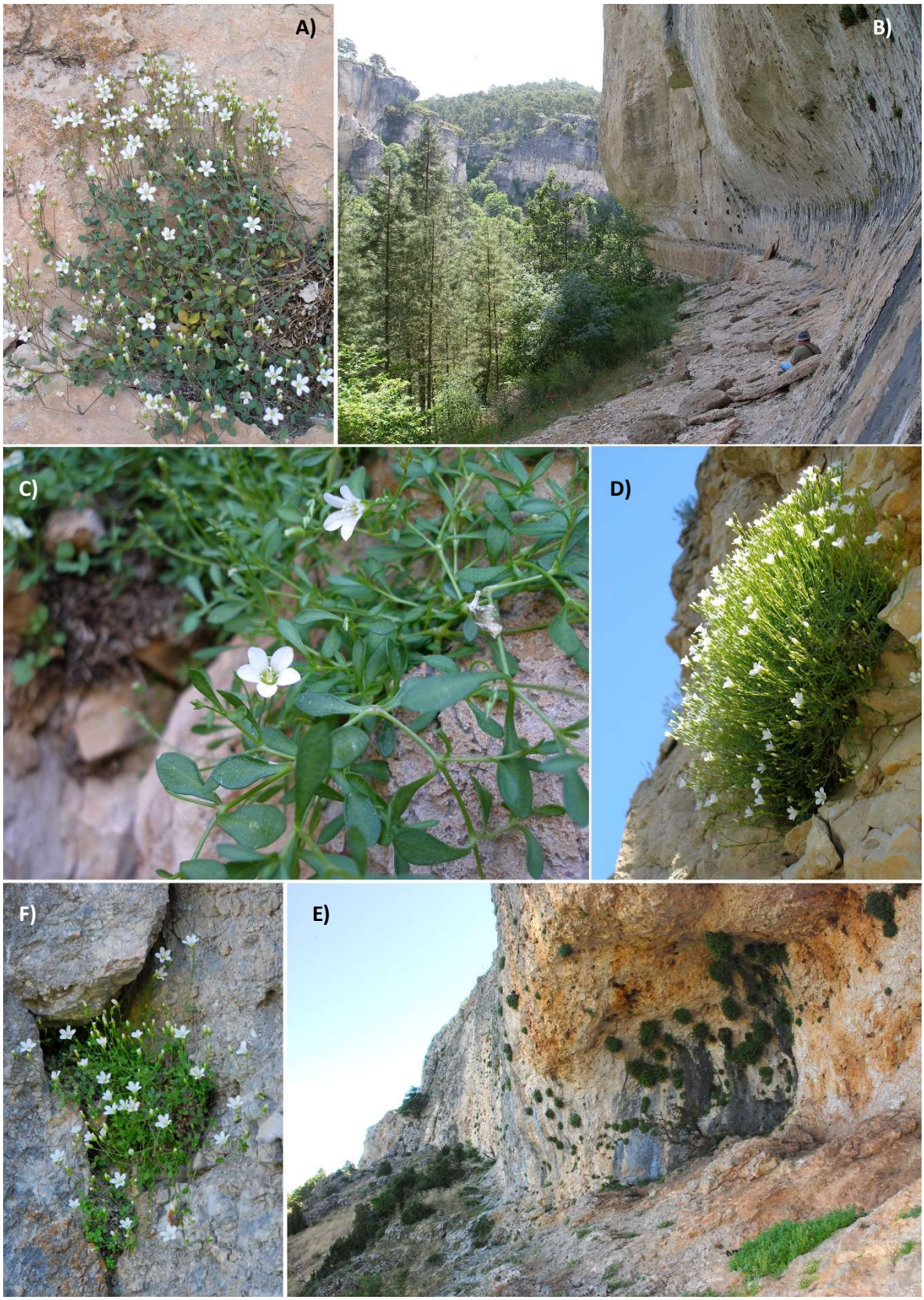

Figura 12: Fotos del complejo *Arenaria suffruticosa*; A) Aspecto general de *A. suffruticosa* subsp *castellana*, B) hábitat de *A. suffruticosa* subsp *castellana, C) detalle de A. suffruticosa subsp. Intricata*, D) aspecto general de A. suffruticosa, E) hábitat de *A. suffruticosa* subsp. *intricata* y F) *A. tejedensis*.

Arenaria funiculata (Pau) Fior & P.O. Karis

Descrita por Pau en 1930, la especie ha mantenido su estatus hasta la actualidad, se incluye en la sección *Pseudomoehringia* por McNeill (1962) y pasa del género *Moehringia* a *Arenaria* (de *M. fontqueri* a *A. funiculata*) a partir de los estudios de Fior *et al*. (2006), Minuto *et al*. (2006) y Fior & Karis (2007).

En *Flora iberica* (Montserrat Martí 2000) la describe como planta perenne, de 8 a 15 cm, cespitosa, pubescente, glandulosa. Tallos rastreros, muy frágiles. Hojas 2 a 5 por 1 a 4 mm, suborbiculares u ovadas; las basales, menores, sésiles; las superiores, de pecíolo muy corto, no ciliado. Flores pentámeras, en dicasios terminales, de 2 a 3 flores. Sépalos de 3 a 3,5 mm, ovados, obtusos, no escariosos en el margen, con 5 nervios poco evidentes. Pétalos de 6 a 8 mm, obovados. Semillas de 0,8 por c. 0,7 mm, subreniformes, negras; células dorsales con prominencias rectas, uncinadas o gloquidiadas, a veces reducidas a un pequeño mucrón; estrofíolo cónico, pequeño, compacto, de superficie alveolada. Numero cromosómico de n = 12.

Su única metapoblación, extensa y fragmentada en aproximadamente 71 subpoblaciones (Lorite 2001), se encuentra en la vertiente norte de la zona almeriense de Sierra Nevada, con escasas excepciones (Pau 1930), la especie habita las fisuras y grietas de los roquedos silíceos verticales.

Como ya se ha comentado, la especie está catalogada como "*En peligro*", a pesar de esto, se considera que no hay amenazas directas a la especie por la aparente estabilidad e inaccesibilidad del hábitat y por estar localizada en zona protegida (Parque Natural de Sierra Nevada)(Peñas & Lorite, 2004).

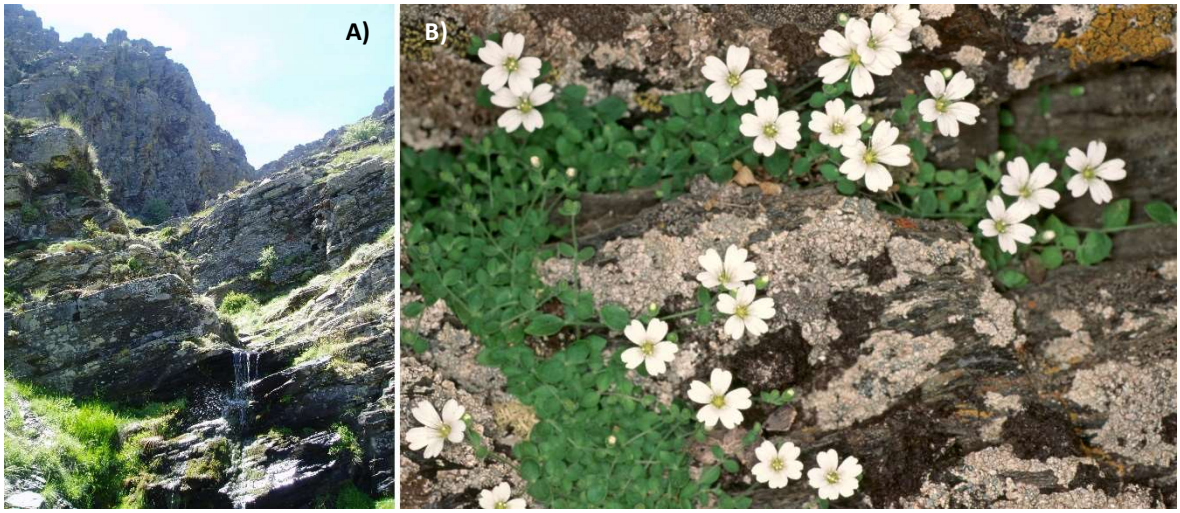

Figura 13: Fotos de *Arenaria funiculata;* A) Hábitat y B) aspecto general de la planta.

Arenaria glochidisperma J. M. Monts.

Descrita por J. M. Montserrat Martí en 1985, como planta perenne, cespitosa, con tallos numerosos, generalmente procumbentes; hojas glaucas, obovadas o redondeadas, más raramente espatuladas o lanceoladas, con el ápice obtuso; hojas suculentas, subcoriáceas, hasta 9 mm de longitud por 5 mm de ancho, ciliadas en la base de la hoja y del pecíolo, que es tres veces más corto que la lámina. Inflorescencia pauciflora, con 1 a 3 flores; pedicelos de 15 a 25 mm de longitud, brácteas lanceoladas, subobtusas, con margen ciliado de hasta 2 mm de longitud. Flores siempre pentámeras; sépalos obovado-lanceolados, obtusos, de hasta 4,5 mm de longitud por 2 de ancho, con 5 a 7 nervios dorsales y margen escaso, siempre ciliados en la base. Pétalos obovado-elípticos, cuneados en la base, de 8 mm de longitud, 10 estambres. Cápsula subglobosa. Semillas subreniforme de 1,3 mm de longitud por 1 mm de ancho; hilo dispuesto transversalmente en el margen ventral de la semilla. Las células de la testa de la semilla están provistas de un tubérculo prominente con el ápice gloquidiado (Minuto *et al.* 2006).

Solo se conocen dos núcleos poblacionales en Marruecos, localizados en Parque Nacional de Talassemtane, el cual de extiende sobre una dorsal calcárea en el Rif centro-occidental.

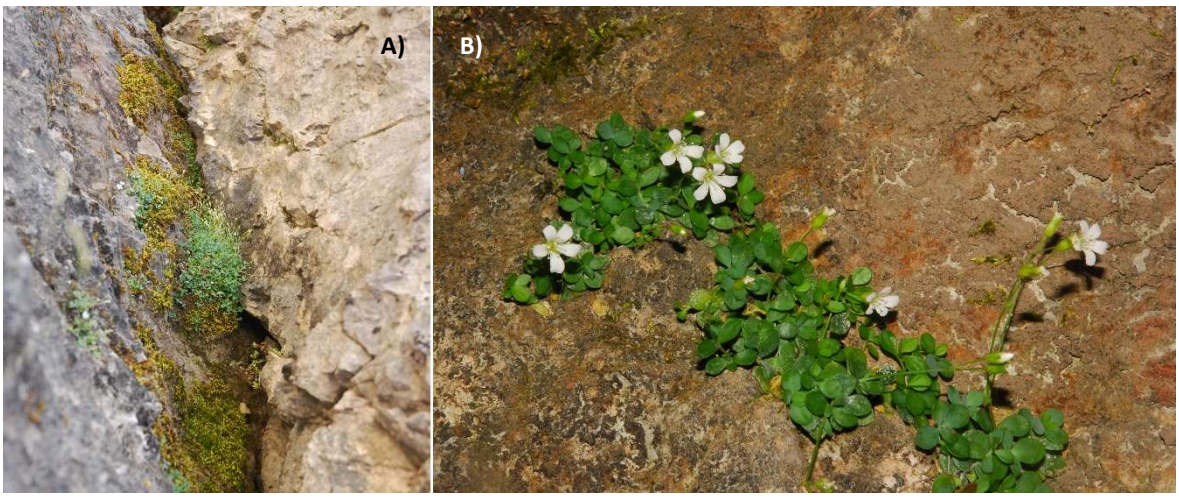

Figura 14: Fotos de *Arenaria glochidisperma;* A) Hábitat y B) aspecto general de la planta.

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OBJETIVOS

En esta tesis se pretende:

A) Reconstruir los patrones filogeográficos de *Astragalus edulis*, particularmente:
- Inferir el área ancestral de la especie y las posibles rutas de colonización que han llevado a la distribución actual.
- Tratar de dar respuesta a cómo se pudo producir la colonización de las Islas Canarias por parte de *A. edulis.*
- Evaluar el papel que las principales barreras geográficas presentes en el área de distribución de la especie han podido jugar a la hora de estructurar la variación genética intraespecífica e identificar zonas refugio.

B) Diseñar estrategias para la conservación de la diversidad genética de *Astragalus edulis*:
- Evaluar la distribución de la diversidad y rareza genéticas de las poblaciones y de las zonas geográficas.
- Identificar y proponer para su conservación las poblaciones que deberían ser el objetivo prioritario por su singularidad genética, incluyendo la singularidad filogeográfica.

C) Reconstruir los patrones filogeográficos de *Arenaria balearica*:
- Evaluar si la baja variación morfológica observada entre individuos que crecen en las diferentes islas en que se encuentra la especie se corresponde con bajas tasas de diversidad genética.
- Probar si su distribución actual guarda relación con la historia paleogeológica de la zona del cinturón Hercínico y conocer los patrones de colonización en su rango actual de distribución.

D) Resolver las relaciones filogenéticas y filogeográficas del complejo de especies que forman la sección *Pseudomoehringia* de *Arenaria*:
- Comprobar la posición filogenética y posible monofilia de las especies y subespecies del grupo.
- Identificar los patrones filogeográficos de la sección en relación a los procesos biogeográficos.

CAPÍTULO I:

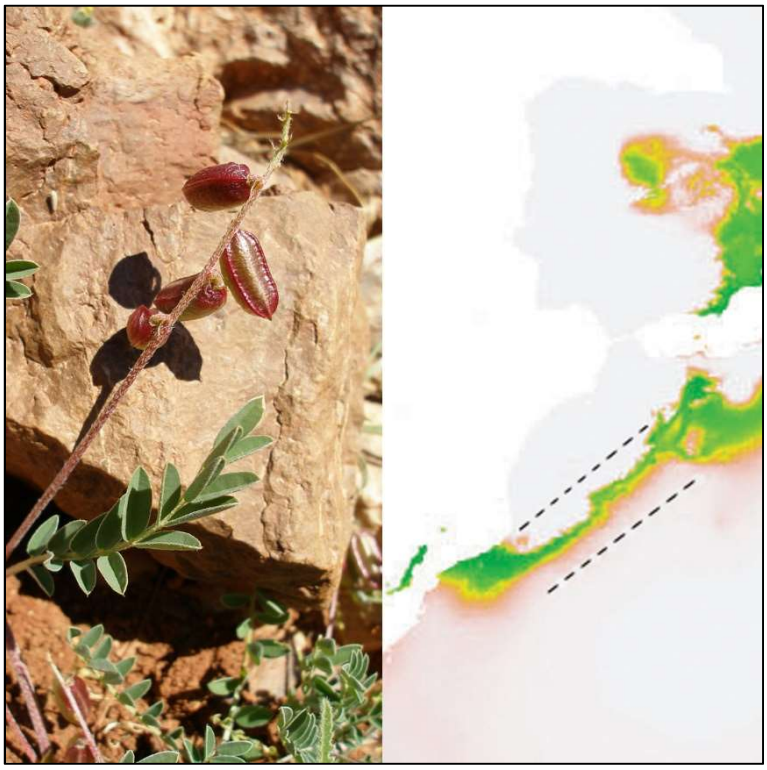
Estudios sobre *Astragalus edulis*

Filogeografía de *A. edulis*

Phylogeography of an endangered disjunct herb: long-distance dispersal, refugia and colonization routes

Conservación de *A. edulis*

Designing conservation strategies to preserve the genetic diversity of *Astragalus edulis* Bunge, an endangered species from western Mediterranean region



ARTÍCULO 1: Filogeografía de una herbácea amenazada de distribución disyunta: Dispersión a larga distancia, zonas refugio y rutas de colonización.

Javier Bobo-Pinilla[1, 3], Julio Peñas de Giles[2], Noemí López-González[1, 3], Sonia Mediavilla[4] and M. Montserrat Martínez-Ortega[1, 3]

[1]Departamento de Botánica, Universidad de Salamanca, España
[2]Unidad de Conservación Vegetal, Departamento de Botánica, Universidad de Granada, España
[3]Banco de ADN vegetal, Banco Nacional de ADN, Salamanca, España
[4]Departamento de Biología Animal, Parasitología, Ecología, Edafología y Química Agrícola, Universidad de Salamanca, España

Resumen

A pesar de la poco común combinación de características de la zona que comprende el oeste mediterráneo y la Macaronesia, no hay estudios específicos que investiguen los efectos paleoclimáticos y paleogeográficos en la estructura genética de las plantas anuales. *Astragalus edulis* es un endemismo con distribución disyunta que ocupa hábitats semiáridos de las islas canarias (Lanzarote y Fuerteventura), el noroeste de África (Marruecos y Argelia) y el sureste de la península ibérica. A pesar de la distribución, la especie no presenta ninguna adaptación concreta a la dispersión a larga distancia. *A. edulis* está catalogada como *en peligro*. Se han analizado datos de AFLP y secuencias de ADN plastidial de un total de 360 individuos a lo largo del rango de distribución de la especie. Se calculó el modelo de distribución actual para la especie y se proyectó sobre las condiciones climáticas del Último Máximo Glacial y del último período interglaciar para obtener las áreas potenciales en el pasado. Los resultados de los AFLP muestran una estructura clara de 4 grupos genéticos. Los análisis filogeográficos, basados en el ADN plastidial, indican un evento de dispersión a larga distancia desde las poblaciones en el norte del Atlas hasta el archipiélago canario. Los modelos proponen la zona al norte de la vertiente norte del suroeste de la cordillera del Atlas como área ancestral, con una posterior colonización del noroeste de Marruecos y de la Península Ibérica. Los resultados también indican posibles zonas de refugio en los alrededores de la cordillera del Atlas. Por último, los modelos de distribución muestran una ruta de colonización sur-norte a través de Marruecos en el Último Máximo Glaciar.

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*Corresponding author's e-mail address: javicastronuevo@usal.es

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Phylogeography of an endangered disjunct herb: long-distance dispersal, refugia and colonization routes

Javier Bobo-Pinilla[1,2]*, Julio Peñas de Giles[3], Noemí López-González[1,2], Sonia Mediavilla[4] and M. Montserrat Martínez-Ortega[1,2]

[1]Departamento de Botánica, Universidad de Salamanca, Salamanca, Spain

[2]Banco de ADN vegetal, Banco Nacional de ADN, Salamanca, Spain

[3]Unidad de Conservación Vegetal, Departamento de Botánica, Universidad de Granada, Spain

[4]Departamento de Biología Animal, Parasitología, Ecología, Edafología y Química Agrícola, Universidad de Salamanca, Spain

Abstract. Quaternary glacial cycles appear to have had a consistent role in shaping the genetic diversity and structure of plant species. Despite the unusual combination of the characteristics of the western Mediterranean– Macaronesian area, there are no studies that have specifically examined the effects of palaeoclimatic and palaeogeographic factors on the genetic composition and structure of annual herbs. *Astragalus edulis* is a disjunct endemic found in the easternmost Canary Islands and the semi-arid areas of north-eastern Africa and south-eastern Iberian Peninsula. This endangered species shows no evident adaptations to long-distance dispersal. Amplified fragment length polymorphism (AFLP) data and plastid DNA sequences were analysed from a total of 360 individuals distributed throughout the range of this species. The modelled potential distribution of *A. edulis* under current conditions was projected over the climatic conditions of the Last Interglacial (130 ka BP) and Last Glacial Maximum (21 ka BP) to analyse changes in habitat suitability and to look for associations between the modelling and genetic results. Amplified fragment length polymorphism analysis showed clear phylogeographic structure with four distinct genetic clusters. Approximate Bayesian computation (ABC) models based on plastid DNA sequences indicated a Middle Pleistocene long-distance dispersal event as the origin of the populations of the Canary Islands. The models also suggested south-western Morocco as the ancestral area for the species, as well as subsequent colonization of north-eastern

Morocco and the Iberian Peninsula. The data compiled indicated the possibility of the presence of refuge areas at favourable locations around the High Atlas and Anti-Atlas mountain ranges. Moreover, palaeodistribution models strongly support the events inferred by ABC modelling and show the potential distribution of the species in the past, suggesting a putative colonization route.

Keywords: AFLP; *Astragalus edulis*; LDD; palaeogeographical models; phylogeography; plastid DNA; western Mediterranean–Macaronesian area.

Introduction

Current diversity patterns are influenced by both historic and recent environmental conditions. Northern Hemisphere phylogeography relies on the idea that Quaternary glacial/interglacial cycles affected the distribution of plant communities and species (Weiss and Ferrand 2007). As a result, the nature of colonization and settlement patterns after the last glacial period is of particular interest to conservation (Soliani *et al.* 2015). Investigating the possible historical dispersal routes of endangered species, with relatively wide and fragmented distribution areas, may provide useful information for the effective implementation of affordable conservation measures.

The Mediterranean basin represents a crossroad for plant migration, being a centre of active speciation and a major Pleistocene refugium (Terrab *et al*. 2008b; Médail and Diadema 2009; and references therein). The western Mediterranean–Macaronesian transition area bears an unusual combination of characteristics, which includes a geographical closeness between continents and between oceanic islands and mainland areas, as well as a broad range of geological ages, palaeoclimatic events and palaeogeographic features. A pre-eminent characteristic of oceanic islands is that they furnish clear-cut spatial and temporal limits and therefore act as living laboratories for studies on the effects of historical colonization, dispersal, geographical isolation and other evolutionary patterns of plants (e.g. Fernández-Mazuecos and Vargas 2011; Lo Presti and Oberprieler 2011; and references therein).

Several authors have proposed that the Mediterranean region has been the main floristic source for dispersal and diversification of new evolutionary lineages in Macaronesian islands (Marrero 2004; Vargas 2007). Numerous molecular studies on Canary Island flora suggest that geographic isolation and colonization between islands, with similar ecologic characteristics, have been strong driving forces for the diversity found within the Canary archipelago (Francisco-Ortega *et al.* 1996; Marrero 2004; Fernández-Mazuecos and Vargas 2011, among others). Moreover, most of the vascular plant clades on the islands have a Mediterranean or North African origin (Francisco-Ortega *et al.* 1996; Carine *et al.* 2004; Marrero 2004; Kim *et al.* 2008). Although the colonization mechanisms and routes probably vary depending on the biological characteristics of each organism, the present and historical relative closeness of the Canary Islands to the potential source areas on the continent (e.g. Fuerteventura is currently ca. 116 km from Cape Juby-Tarfaya on the coast of Morocco, while 21,000 years BP they were separated by only ca. 65 km; Fig. 1) makes both recent and ancient long-distance dispersal (LDD) plausible, especially in plants with long-distance dispersal vectors. Even though the Canaries are oceanic (volcanic) islands, whose colonization is typically explained by long-distance dispersal

events, the disjunct presence of Mediterranean elements in Morocco and the easternmost Canaries (i.e. Lanzarote and Fuerteventura, which are also the oldest extant islands) could be alternatively explained by other hypotheses considering the geographic closeness between the two areas (e.g. 'stepping stones' *sensu* Fernández-Palacios *et al.* 2011). Also, the currently separate islands of Lanzarote and Fuerteventura emerged initially as a single proto-island called Mahan, and the two islands were still joined as recently as the late Pleistocene (Fernández-Palacios *et al.* 2011).

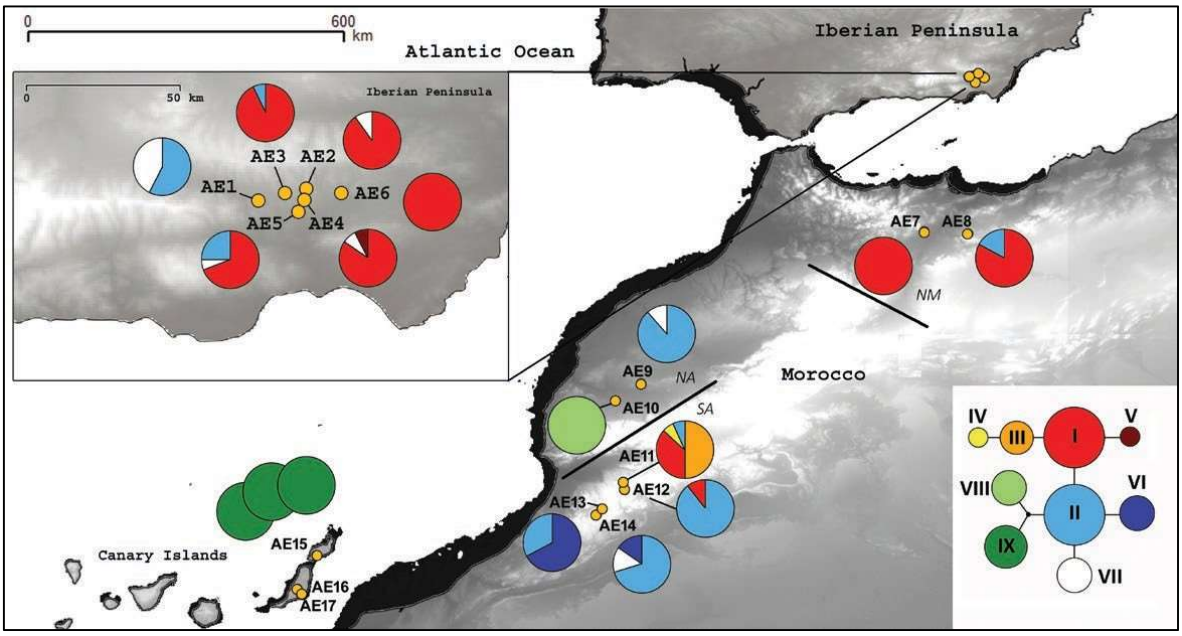


Figure 1. Sampling locations covering the present distribution of *Astragalus edulis*. Coast lines during the LGM (black shadow). Plastid haplotype distribution of the species; plastid haplotype network (circle size is proportional to the number of individuals for each haplotype). Clustering for the DIYABC analysis labelled (Iberian Peninsula; northern Morocco, **NM**; northern Atlas, **NA**; southern Atlas, **SA**; Canary Islands).

The Alboran Sea in the westernmost Mediterranean to the east of the Strait of Gibraltar is a narrow basin (ca. 150 km wide and 350 km long at present) bordered on the north by the Baetic System (southern Spain). On the south it is bordered by the Rif (northern Morocco) mountain belts and by the South Balearic Basin to the east (Comas *et al.* 1992). In this area, the Quaternary climatic oscillations have partially moulded the genetic structure and spatial distribution of the biota and have led to speciation (Hewitt 1999). During the Last Glacial Maximum (LGM) the sea level was ~120–150 m lower than at present (Yokohama *et al.* 2000; Church *et al.* 2001; Clark and Mix 2002) and the Iberian and North African coast lines were closer. At that time, some of the submerged seamounts in the Alboran Sea could have emerged (Comas

et al. 1992), thus facilitating the exchange of species between continents (Fig. 1). The genetic structure and diversity of several plant species has been heavily influenced by these dramatic geomorphological and environmental changes (e.g. Ortiz *et al.* 2007; Terrab *et al.* 2008a; Ortiz *et al.* 2009). However, currently only a few phylogeographic studies have focused specifically on herbs growing on both sides of the Alboran Sea (Silva *et al.* 2015).

Additionally, the Atlas Mountains may represent a formidable barrier for the migration of lowland xerophytic species, but its relative role in preventing such migrations has not yet been directly tested. This could be because North Africa is often under-represented in the surveys of Mediterranean taxa (Terrab *et al.* 2008a). An additional barrier for plant migration in Morocco is the Riffian Corridor, which today is occupied by the Loukos and Sebou river valleys that separate the Rif Mountains to the north from the Atlas ranges to the south. This corridor connected the pre-Mediterranean Sea with the Atlantic Ocean just before the Messinian Salinity Crisis (5.3 million years ago), and represented a strong barrier for the migration of plants both before and after the Messinian (Ortiz *et al.* 2009).

The focal species in this study is *Astragalus edulis*, an herbaceous annual Fabaceae (listed as Endangered in Spain) that lacks evident adaptations to LDD (Peñas 2004). It is restricted to grasslands on poor sandy soils that result from the erosion of volcanic or schistose rocks. This plant species grows in semi-arid ecosystems and currently occupies a highly disjunct distribution area (Peñas *et al.* 2016). It occurs in the semi-desert habitats of the south-eastern Iberian Peninsula, in the islands of Lanzarote and Fuerteventura, and in scattered locations in western North Africa (Morocco and Algeria), where it is distributed in three, disjunct population cores (Fig. 1). These population cores include one in north-eastern Morocco and north-western Algeria and two cores in south-western Morocco, the first one to the north of the High Atlas Mountains (steppes of El Haouz; Jahandiez and Maire 1931–34; Gómiz-García 2001) and the second one to the south of this mountain range (Sous plains and lowlands near the Anti-Atlas range; Gómiz-García 2001). The north-eastern and south-western population cores are roughly separated by the Rif and Middle Atlas Mountains.

Kay *et al.* (2006) have proposed that the genus *Astragalus* dates back 35 Ma, and M. F. Wojciechowski (pers. comm.) found that *A. edulis* diverged from its sister species *Astragalus boeticus* (Wojciechowski *et al.* 1999) significantly later, around 450–500 ka BP (based on ITS mutation rates). This suggests that the Messinian Salinity Crisis does not explain the present distribution of the study species. The strikingly disjunct distribution of *A. edulis* in the Iberian Peninsula, Morocco, and the Canary Islands therefore provides an ideal system to explore the postglacial evolutionary dynamics of a western Mediterranean endemic species present on

both sides of the Alboran Sea and Atlas Mountains, which has also colonized the easternmost islands of the Canary archipelago.

This study seeks to reconstruct the phylogeographic patterns of intraspecific lineages within *A. edulis*, with the general aim of contributing to the understanding of the biogeographic history of the western Mediterranean– Macaronesian area. To do so, we will carry out the following: (i) address how the Mediterranean lineage *A. edulis* colonized the Canary Islands; (ii) infer the ancestral area of the species and explore possible colonization routes; and (iii) assess the role the Atlas Mountains have had as refuge areas for this species.

Materials and Methods

Amplified fragment length polymorphism data and analysis

An amplified fragment length polymorphism (AFLP) matrix corresponding to 360 individuals of *A. edulis* from Peñas *et al.* (2016) was used for this study.

The population genetic structure was examined using a Bayesian clustering method implemented in STRUCTURE v. 2.3.4 (Pritchard *et al.* 2000), following the approach described by Falush *et al.* (2007) for dominant markers. This method uses a Markov chain Monte Carlo simulation approach to group samples into an optimal number of *K* genetic clusters and does not assume the *a priori* assignment of individuals to populations or clusters. Analyses were based on an admixture ancestral model with correlated allele frequencies among populations (Falush *et al.* 2003). The proportion of membership of each individual and population to the *K* clusters was calculated performing 20 runs for each *K* value between 2 and 10 with a run length of the Markov chain Monte Carlo of 1×10^6 iterations after a burn-in period of 1×10^6 iterations. The optimal number of *K* clusters was estimated using the *ad hoc* parameter (ΔK statistic) of Evanno *et al.* (2005), as implemented in the online application of Structure Harvester software (v0.63; Earl and VonHoldt 2012).

Plastid DNA sequencing and analysis

The plastid regions *trn*G–*trn*S, *trn*C–*rpo*B (Shaw *et al.* 2005) and *tab*F–*tab*C (Taberlet *et al.* 1991) were sequenced from 165 individuals from 17 species populations (Table 1). Haplotype variation was also explored using the information available for 61 individuals previously analysed by Peñas *et al.* (2016), using the same PCR conditions and primers for DNA amplification. PCR products were visualized on 1 % agarose gel and purified using the ExoSAP-IT PCR Clean-Up Kit (AFFIMETRIX, Santa Clara, CA, USA), following the manufacturer's instructions. The cleaned

amplicons were analysed using a 3730 DNA Genetic Analyser capillary sequencer (Applied Biosystems), and all sequences were deposited in GenBank. The total plastid DNA data set obtained from the 226 individuals was used (Table 1). Three samples of *A. boeticus* were used as the outgroup, based on the results of Wojciechowski *et al.* (1999).

Table 1. Locations, details and haplotypes for *Astragalus edulis.*

Pop. code	*Locality*	*Altitude*	*Longitude*	*Latitude*	*Nº New individuals*	*Total*	*Haplotypes*
AE1	Spain; Almería, Alcubillas	735	-2,6025	37,0987	8	12	II & VII
AE2	Spain; Almería, Tabernas	915	-2,4643	37,1306	7	13	I & VII
AE3	Spain; Almería, Gérgal	720	-2,5254	37,1209	8	16	I & II
AE4	Spain; Almería, Gérgal, Arroyo Verdelecho	648	-2,4704	37,1002	8	14	I, V & VII
AE5	Spain; Almería, Tabernas, Desierto de Tabernas	621	-2,4863	37,0668	7	13	I, II & VII
AE6	Spain; Almería, Filabres, Rambla del Saltador	541	-2,3610	37,1206	8	15	I
AE7	Morocco; La Oriental, between El-Aïoun and Tanarchefi	919	-2,6016	34,4174	12	14	I
AE8	Morocco ; Taza, Jebel Guilliz	425	-3,3496	34,4669	12	14	I & II
AE9	Morocco; Marrakech, Chemaia, prox. Kettara	480	-8,1875	31,8729	10	12	II & VII
AE10	Morocco; Marrakech, between Marrakech and Chichaoua	380	-8,6185	31,5720	12	14	VIII
AE11	Morocco; Taroudant, between Tasgount and Ighil	1437	-8,4832	30,1831	12	14	I, II, III& IV
AE12	Morocco; Taroudant, between Irherm and Tata	1710	-8,4478	30,0467	13	15	I & II
AE13	Morocco; Taroudant, Tafraoute, Tizi-n-Tarakatine, prox. El Jebar	1484	-8,8587	29,7376	12	14	II & VI
AE14	Morocco; Taroudant, between Tafraoute and Tleta-Tasrite	1620	-8,9385	29,6354	3	6	II, VI & VII
AE15	Spain; Canary Islands; Lanzarote, Vega de Temuime	159	-13,7280	28,9337	14	16	IX
AE16	Spain; Canary Islands; Fuerteventura, Tiscamanita	234	-14,0330	28,3576	7	9	IX
AE17	Spain; Canary Islands; Fuerteventura, Barranco de Majada Blanca	181	-13,9860	28,2673	13	15	IX

The cpDNA sequences were assembled, edited and aligned using $G_{eneious\ pro}^{™}$ 5.4 (Drummond *et al.* 2012), and further adjustments and optimizations of the alignments were carried out manually. Since no incongruence among regions was found (branches with high support were compared among the regions), the sequences from the three regions were concatenated into a single matrix based on the assumption that the plastid forms a single linkage group. Gaps (insertions/deletions) longer than 1 bp (i.e. 10 and 3 pb in *trn*G–*trn*S) were coded as single-step mutations (one binary character added to represent the presence/absence of the gap). In addition, no inversion was found in the regions analysed. Mononucleotide repeats of different sizes were excluded given that they seem to be prone to homoplasy at large geographic scales (Ingvarsson *et al.* 2003).

An unrooted haplotype network was constructed to infer the genealogical relationships among haplotypes using the statistical parsimony algorithm (Templeton *et al.* 1992) as

implemented in TCS 1.21 (Clement *et al.* 2000).

Approximate Bayesian computation analyses with DIYABC

An approximate Bayesian computation (ABC) statistical approach was employed to analyse the plastid DNA using the software DIYABC v2.1 (Cornuet *et al.* 2014). The aim of this approach was to compare the different phylogeographic hypotheses that could be used to explain the present distribution of *A. edulis*. DIYABC allows the posterior probabilities of alternative scenarios to be tested by simulating a large number of data sets in each case. The logistic regression procedure (Fagundes *et al.* 2007) estimates the occurrence of each scenario among the simulated data sets that are closest to the observed data.

Based on the results from a previous study (Peñas *et al.* 2016), as well as the geographical distribution of the species, the five most likely metapopulations (Canary Islands, CI; Iberian Peninsula, IP; northern Morocco, NM; northern Atlas, NA; southern Atlas, SA; Table 1) were previously considered as a working basis for the DIYABC. A set of 34 plausible alternative scenarios was constructed in order to test all possible phylogeographical hypotheses with respect to the following items: (i) what is/are the ancestral metapopulation(s); (ii) what is the origin of the Canary Island populations; and (iii) to test for putative LGM refugial areas.

Prior distributions of the parameters were chosen as an initial approach with a large interval, due to the lack of ancestral information. Parameters were corrected after the first test (a list of all parameters and prior distributions used to model scenarios is summarized in Table 2). Population sizes were set equally in all cases except for founder events. Divergence times were unrestricted to allow the program to set the most likely value. The JC69 model of nucleotide evolution (Jukes and Cantor 1969) was chosen, and the uniform mutation rate was set to (10^{-9}–10^{-7}).

One million data sets were simulated for each scenario (Cornuet *et al.* 2008, 2010). The best scenario was chosen by calculating the posterior probabilities of each one by performing a polychotomous weighted logistic regression on the 1 % of simulated data sets closest to the observed data set (Cornuet *et al.* 2008, 2010). Scenarios under 20% posterior probability (logistic regression procedure) were discarded. In the next step, the different probable scenarios were combined under each hypothesis, at which time 90 % of the scenarios were discarded and those receiving the greatest weights (five, plus null scenario) were selected. Subsequent distributions of parameters were evaluated under the best scenario using a local linear

regression on the 1% closest simulated data sets with a logit transformation (Table 2). Confidence in the choice of scenario was tested by evaluating Type I and Type II error rates (Cornuet *et al.* 2010). Similarity between real data and simulated data sets was assessed for the best scenario to test the model adequacy using the posterior distribution of the parameter values.

Table 2: DIYABC estimated parameters and codes.

Parameter	*Parameter code*	*Prior Distribution*			*Estimated Parameters*
		Type	*Initial Interval*	*Final Interval*	*Mean*
Population effective sizes of the IP group	NIp	Uniform	{10 - 100.000}	{10 - 160.000}	3,13E+04
Population effective sizes of the NM group	NNm	Uniform	{10 - 100.000}	{10 - 40.000}	2,46E+04
Population effective sizes of the NA group	NNa	Uniform	{10 - 100.000}	{10 - 160.000}	1,08E+05
Population effective sizes of the SA group	NSa	Uniform	{10 - 100.000}	{10 - 120.000}	8,73E+04
Population effective sizes of the CI group	NCi	Uniform	{10 - 100.000}	{10 - 40.000}	1,45E+04
Founder event for CI group	NCib	Uniform	{10 - 500}	{10 - 300}	6,79E+01
Time of founder event for CI group	t1	Uniform	{10 - 1.000.000}	{10 - 200.000}	1,50E+05
Isolation time for NA	t2	Uniform	{10 - 1.000.000}	{10 - 30.000}	2,46E+04
Divergence time among the Moroccan populations	t8	Uniform	{10 - 1.000.000}	{10 - 200.000}	
Divergence time among the IP+NM+SA groups	t3	Uniform	{10 - 1.000.000}	{10 - 200.000}	4,11E+03
Divergence time among the IP+NM+NA+SA groups	t4	Uniform	{10 - 1.000.000}	{10 - 200.000}	
Divergence time between CI and NA	t6	Uniform	{10 - 1.000.000}	{10 - 200.000}	
Divergence time between [CI+NA] and [IP+NM+SA] complex	t5	Uniform	{10 - 1.000.000}	{10 - 200.000}	
Divergence time among all groups	t0	Uniform	{10 - 1.000.000}	{10 - 200.000}	
Mean mutation rate	Mμ	Uniform	$\{10^{-9}$- $10^{-7}\}$	$\{10^{-9}$- $10^{-7}\}$	3,44E-09

Distribution modelling and LGM bathymetry

To model the current climatic suitability of *A. edulis* and project it into the LIG (130 ka BP) and LGM (21 ka BP), the Bioclim climatic layers available at www.worldclim. com were

downloaded (Hijmans *et al.* 2005). All known localities of the species (Podlech 1988) were visited to confirm the presence of the plant and the plant was not found in Algeria. Correlation analysis among bioclimatic variables was performed. Afterwards, a hierarchical cluster analysis of these variables was carried out to identify groupings of correlated variables, and a threshold of 0.8 was set to avoid redundancy. One variable from each group was selected and the variance inflation factor (VIF) values (Marquardt 1970) were used to test multicollinearity through the 'vif' function of the 'HH' R package (Heiberger 2015). One variable was excluded from the ones with the highest VIF values, and this procedure was repeated until no variables remained with a VIF value greater than five. This information was combined with theoretical considerations to select the appropriate climatic variables for the modelling; three variables were finally selected. The climatic features that are suspected to have an influence on the ecology and range limits of *A. edulis* are temperature seasonality (bio4), precipitation of wettest quarter (bio16) and precipitation of driest quarter (bio17). All the climatic variables were rescaled to a grid cell resolution of 2.5 arc-minutes (the spatial resolution of the LGM data set) within the function 'resample' implemented in package 'raster' (Hijmans 2015). A non-metric multidimensional scaling was performed for visualizing the relative position of *A. edulis* populations within the ecological space and for checking for climatic differences between populations. This was achieved using the 'metaMDS' and 'ordisurf' functions of the R library 'vegan' (R Core Team 2012; Oksanen *et al.* 2013).

Systematic sampling was implemented to avoid sampling bias, as described in Fourcade *et al.* (2014). Afterwards, multiple scenarios were evaluated using the package ENMeval (Muscarella *et al.* 2014), which implements the maximum entropy algorithm (Phillips *et al.* 2006). These models were run with the L, LQ, H and LQH feature combinations used by Muscarella *et al.* (2014) and a regularization multiple from 0.5 to 4.0 by 0.5. The selected method was the leave-one-out strategy (jackknife) to compensate for the low number of presence records (Pearson *et al.* 2007). The area under the curve (AUC) and the Akaike information criterion (AIC) were used to evaluate the models; models with AUC above 0.75 are considered potentially useful, 0.80–0.90 good and 0.90–1.0 excellent (Elith 2002). The best model was selected using these criteria. The palaeodistributions (LGM and LIG) were generated by projecting the best model onto past scenarios using the package 'raster' (Hijmans 2015).

At the LGM, the Earth's ocean levels were at their lowest point and extensive reaches of dry land were exposed along the continental coasts. Some analyses have substantially narrowed the uncertainties regarding total changes in ice sheets and sea level and their proxies, suggesting a net decrease in eustatic sea level at the LGM ranging from 120 to 135 m (Church *et al.* 2001; Clark

and Mix 2002; Lambeck *et al.* 2014).

The present-day topographic and bathymetric data covering the area were extracted from the ETOPO1 to map in detail the past and current shorelines. This model was built from numerous global and regional data sets, and is available in 'Bedrock' (base of the ice sheets) versions (NOAA 2009).

Results

Population structure based on AFLP

Bayesian clustering conducted using STRUCTURE resulted in a best partition of four clusters with a maximum modal value of ΔK = 249.02 [see Supporting Information— Fig. S1]. Placement of the individuals within the different clusters is shown in Fig. 2. Individuals within Cluster A (orange) were found to be prevalent in the large metapopulation from south-eastern Spain and present in some of the Moroccan populations; individuals within Cluster B (pink) were dominant in all the Moroccan populations and displayed a significant presence in some populations from the Iberian Peninsula (i.e. AE1, AE2 and AE6); individuals within Cluster C (blue) were dominant in the Canary Islands and residual in the other groups; and individuals within Cluster D (yellow) were present (although never dominant) in almost all species populations.

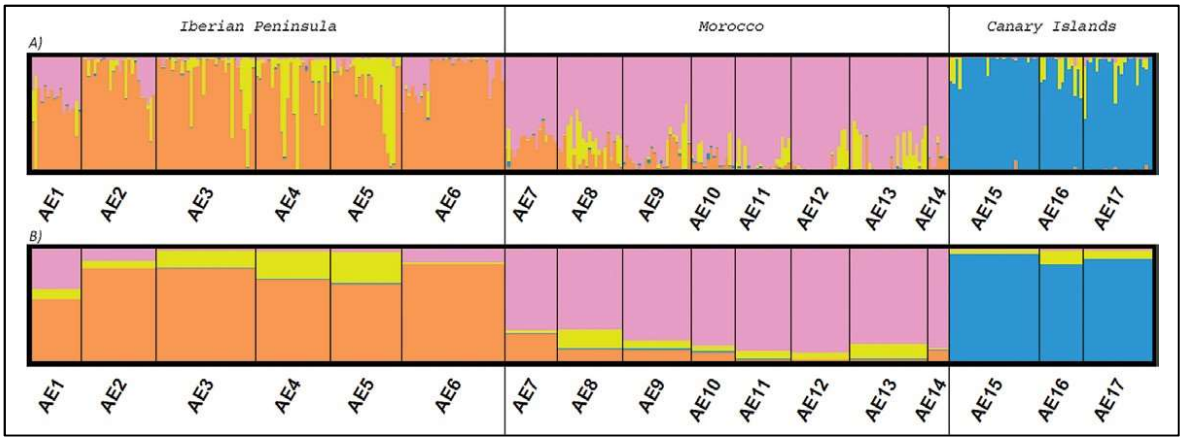


Figure 2. Results from the analysis of AFLP markers for K = 4. Histograms show the Bayesian clustering of individuals within populations, (A) admixture analysis, (B) population genetic structure.

Chloroplast variation and geographical distribution of haplotypes

The length of the three cpDNA regions in the recently collected 165 individuals, plus the 61 taken from Peñas *et al.* (2016), ranged from 630 and 772 bp and resulted in a final alignment of

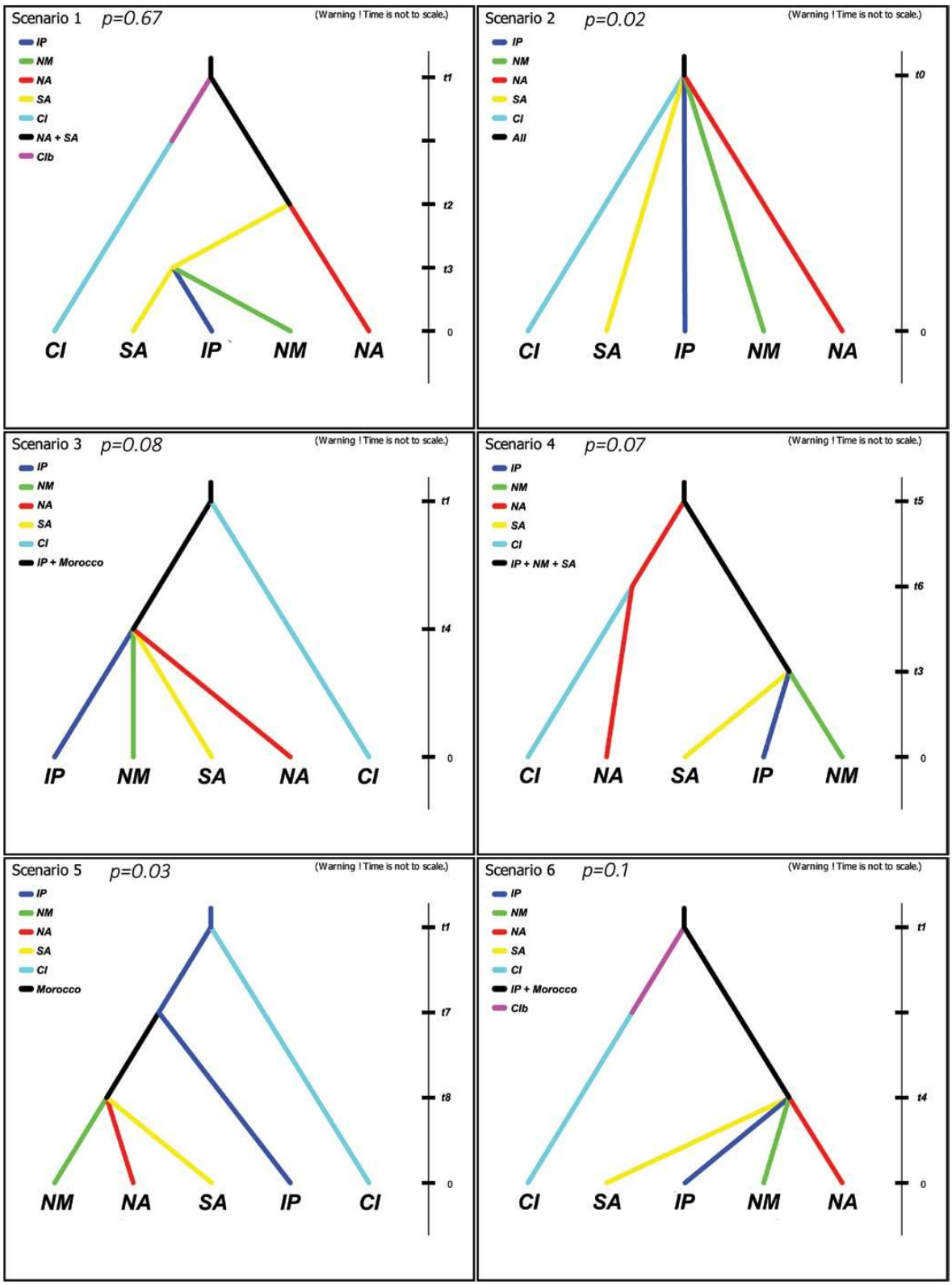
2092 bp. In the *trn*G–*trn*S region, three polymorphisms (two indels/one substitution) were detected across the whole data set, while four substitutions and one substitution for *trn*C–*rpo*B and *tab*C–*tab*F were found, respectively. All mutations together defined a total of nine haplotypes (Table 1). TCS inferred a 95 % parsimony network with a maximum limit of five steps (Fig. 1). Intrapopulational haplotype variation was detected in 11 sampling sites (AE1, AE2, AE3, AE4, AE5, AE8, AE9, AE11, AE12, AE13 and AE14; Table 1; Fig. 1). The most frequent haplotype (I) was found in five sampling sites from the Iberian metapopulation, in the north-eastern Moroccan populations and in AE11 and AE12 from south-western Morocco. The second most frequent haplotype (II) was represented in five populations from south-western Morocco, in one from north-eastern Morocco and in three sampling sites from the Iberian Peninsula. The large Iberian metapopulation contained one endemic haplotype (V) and the south-western Moroccan populations contained four endemic haplotypes (III, IV, VI and VIII). A single endemic haplotype (IX) was found in Fuerteventura and Lanzarote.

Modelling of plausible demographic scenarios and estimated times of divergence

Here, only the six most plausible scenarios are shown (Fig. 3). The scenario with the highest posterior probability was Scenario 1 (P = 0.6799 [0.6703–0.6849]) followed by Scenario 6 (P = 0.1074 [0.1014–0.1134]), Scenario 3 (P = 0.0878 [0.0826–0.0929]), Scenario 4 (P = 0.0733 [0.0680–0.0786]), Scenario 5 (P = 0.0303 [0.0282–0.0325]) and Scenario 2 (P = 0.0214 [0.0194–0.0234]). The best scenario consisted of an early founder event from Morocco mainland to the Canary Islands, which occurred ca. 150000 (127000–173000) generations ago, before the end of the Riss glaciation and when Lanzarote and Fuerteventura were still joined together (Fernández-Palacios *et al.* 2011). This lead to the establishment of an initial population followed by an expansion and colonization of the area, with increasing population sizes (Table 2). According to this scenario, the next evolutionary event would have been the isolation of the NA metapopulation (ca. 24 600 generations ago), while the groups of populations from IP and NM would have diverged from those in SA ca. 2400 generations ago. These data support SA + NA as the original ancestral area.

The Type II error rate, which is the probability that data sets simulated under other scenarios were assigned to the best scenario, was 20 %. The Type I error rate, the probability that data sets simulated under the best scenario were assigned to other scenarios, was 44 %, which may be due to high similarities among scenarios. The similarity between real data and simulated data sets for the best scenario was calculated [see Supporting Information—Fig. S2], and it was found that from a total of 13 summary statistics only one case of statistics diverged from the simulated ones (P-value < 0.05).

Figure 3. Approximate Bayesian computation analysis of *Astragalus edulis*. Most likely DIYABC scenarios (posterior probability is shown); Time is not to scale; Areas (southern Atlas, **SA**; northern Atlas, **NA**; northern Morocco, **NM**; Iberian Peninsula, **IP**; Canary Islands, **CI**; Canary Islands founder event, **CIb**).



Distribution modelling

The model corresponding to the potential present distribution of the species (Fig. 4A) showed high predictive accuracy (AUC = 0.98). The currently known distribution of the species mostly coincided with that predicted by the model (Fig. 4A). From the three bioclimatic variables used in the analyses, bio16 showed the highest explanative power (relative variable contribution 67 %). The past suitable areas for the species in the LGM and LIG are shown in Fig. 4B and C, respectively. They included a continuous corridor (Fig. 4B) that extended along the south of the Atlas Mountains to the north-eastern part of Morocco (with high suitability values in the area of the Moulouya river valley) during the LGM. During this period, an area of high suitability was also found to the east of the Iberian Peninsula (south of the Pyrenees). The model projected to the LIG period showed only two suitable areas for the species: a mainland area along the Atlantic coast at the westernmost edge of the Atlas Mountains, corresponding to the westernmost extremes of population groups SA and NA; and the eastern Canary Islands. Lastly, the extent of the potential area suitable for the species *A. edulis* appears to have been at its largest during the LGM. The estimations of emerged land area at LGM, with respect to the present-day, are the result of raising the values of the digital elevation model 120 m (Fig. 1).

Discussion

Ancient colonization of the Canary Islands by Astragalus edulis

It is generally accepted that all lineages on oceanic islands originated from mainland lineages through long-distance dispersal events (MacArthur and Wilson 1967; Ganders and Nagata 1984; Baldwin and Robichaux 1995; Poulakakis *et al.* 2012). The establishment of *A. edulis* in the Canaries could be a result of either a recent or ancient long-distance dispersal event or a combination of the two, as a consequence of multiple dispersal events that occurred at different times.

As a general pattern, due to founder events and restricted gene flow, lower levels of genetic variation are expected to be found on islands than in their mainland counterparts (Frankham 1997; Baldwin *et al*. 2008), although at least one exception is known (Fernández-Mazuecos and Vargas 2011). Additionally, in long-term isolated populations the rarity value (frequency downweighted marker value, DW) is expected to be high, because rare markers should accumulate due to mutations. Newly established populations, on the other hand, are expected to exhibit low rarity values, and thus help in distinguishing old vicariance from recent dispersal (Schönswetter and Tribsch 2005). Putative refuge areas are typically characterized by

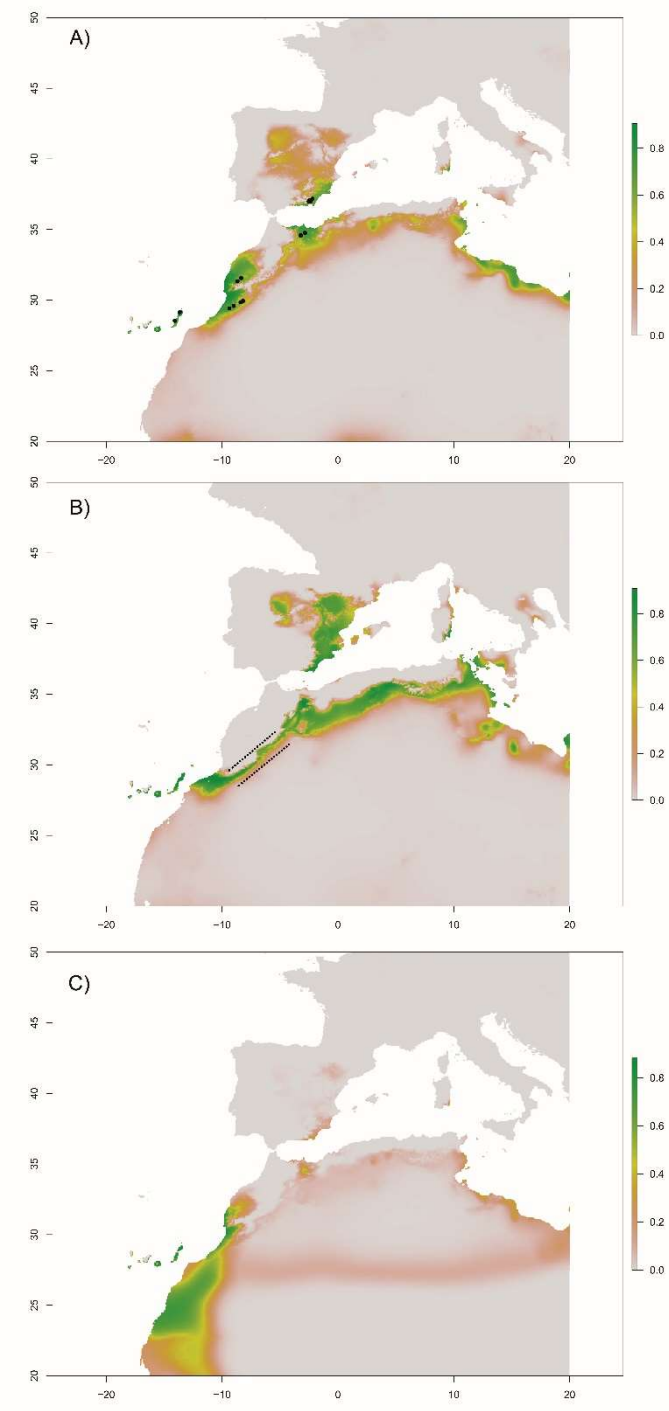


Figure 4. Distribution models; habitat suitability is represented by green-yellow to red (red-yellow = medium, green = high). (A) Present; (B) LGM; (C) LIG. Dotted line represents the south to north colonization route along Morocco. Black dots represent current localities of the species.

high genetic distinctiveness (DW), as well as by high genetic diversity, while long-distance dispersal events can be recognized by comparatively low values of rarity and genetic diversity. The AFLP data suggest that limited gene flow exists among the populations from the Canary Island and the Moroccan or Iberian populations (Fig. 2). This is corroborated by the neighbor joining (NJ) and principal coordinate analysis (PCoA) analyses performed, based on AFLP data (Peñas *et al.* 2016), and suggests long-term isolation of the Canarian populations. Additionally, the genetic diversity and rarity values found do not support recent long-distance dispersal events from Morocco (mean Nei's diversity index 0.1013 in the Canaries vs. 0.1331 in the remaining distribution area of the species; mean DW 4.199 in the Canaries vs. 2.888 in the remaining distribution area of the species). These data would support long-term *in situ* survival of *A. edulis* in the Canaries or simple ancient LDD followed by isolation, which is consistent with the haplotypic diversity pattern (Fig. 1). The hypothesis of multiple LDD events or recurrent contact between the Moroccan and Canarian populations was not supported by our data, since high levels of gene flow between Morocco and the Canary Islands were

not found. These results are further supported by the almost negligible admixture degree detected between the populations of the Canary Islands and any of those from the mainland areas (Fig. 2A).

Regarding plastid DNA, the well-supported close relationship between haplotypes VIII (exclusive to population AE10) and IX (endemic to the Canary Islands; Fig. 1) indicates connections between Morocco and the Canary Islands. Western Morocco, particularly to the north of the High Atlas range, appears to be the primary source area for the initial colonization of the islands. Moreover, the best-supported phylogeographic scenario, as detected by the DIYABC, involves a single ancient LDD founder event (150 ka BP) from the Atlas (NA + SA) area to Mahan, followed by colonization of the area. The age of the inferred LDD event is in concordance with the diversity and DW values obtained. Although *A. edulis* lacks evident adaptations to long-distance dispersal, the Moroccan coast and the eastern Canary Islands are relatively close. Also, the falling sea level during the Riss glaciation would have promoted the emergence of previously submerged seamounts that could have acted as stepping stones to facilitate floristic interchanges between these regions (Fernández-Palacios *et al.* 2011). The AFLP data indicate that Fuerteventura was probably colonized first, given the high levels of diversity and rarity (Peñas *et al.* 2016). This would be consistent with the present and historical (particularly during the glacial maxima) proximity between populations AE16 and AE17 and Cape Juby-Tarfaya in Morocco.

The phylogeographic relationships of *A. edulis* indicate that the inter-island colonization between similar ecological zones found for other plant species (e.g. Francisco-Ortega *et al.* 1996; Fernández-Mazuecos and Vargas 2011) is not, in this case, the mechanism for establishing populations on different islands. Postglacial colonization between Fuerteventura and Lanzarote is not supported by our results, since the populations collected on the two islands share the same Canarian endemic haplotype. Additionally, the overall genetic composition, as revealed by AFLP data, is highly homogeneous, which is congruent with the fact that the currently separate islands emerged as a single proto-island (Mahan) and remained joined together as recently as the late Pleistocene (Fernández-Palacios *et al.* 2011).

South-western Morocco as ancestral area for Astragalus edulis and subsequent migration to the north-east

Palaeodistribution models (Fig. 4C) showed the existence of an area, located to the north and south of the westernmost edge of the High Atlas mountain range, which was highly suitable for the species during the LIG. The coalescent-based ABC method, as implemented by the DIYABC software, also identified this area (metapopulations northern Atlas and southern Atlas) as

ancestral for the species. A similar ancestral area has been found for other annual herbs (e.g. *Hypochaeris arachnoidea*, Ortiz *et al.* 2009). This is also consistent with the haplotype network, which shows haplotype II in a central, probably ancestral,position.

DIYABC also identified an isolation of metapopulation northern Atlas around 24600 generations ago (probably near the LGM) and a subsequent colonization to the north-east from southern Atlas to northern Morocco and Iberian Peninsula. Accordingly, the palaeogeographic models show a corridor in terms of suitable habitat for the species during the LGM along the southern slopes of the Anti-Atlas, High Atlas, and Tell Atlas (Hamada desert habitat) connecting to the north with the Moulouya river valley (Fig. 4B).

The Bayesian modelling of demographic scenarios supports the contention that the divergence between northern Morocco and Iberian Peninsula took place very recently (2400 generations ago). Although this is not supported by the AFLP data, as populations on both sides of the Alboran Sea form distinctive AFLP clusters in the NJ and PCoA analyses (Peñas *et al.* 2016), some degree of admixture has been identified by the STRUCTURE analysis (Fig. 2). Additionally, the central haplotypes I and II, plus haplotype VIII are shared among many populations from the Iberian Peninsula and Morocco and only haplotype V is endemic to the Iberian Peninsula. Notably, both the present and the palaeodistribution models consistently suggest that the area of the Strait of Gibraltar, which was involved in the exchange of species between North Africa and the Iberian Peninsula (e.g. Rodríguez- Sánchez *et al.* 2008; Lavergne *et al.* 2013), presents no appropriate habitat for *A. edulis*. However, both sides of the Alboran Sea have historically presented conditions that are suitable for the species (Silva *et al.* 2015). It also bears noting that many plants, such as *Caralluma munbyana (*Asclepiadaceae), *Launaea arborescens* (Asteraceae), *Logfia clementei* (Asteraceae), *Lycium intricatum* (Solanaceae), *Maytenus senegalensis* (Celastraceae), *Notoceras bicorne* (Brassicaceae), could have also followed this colonization route from southeast Morocco to the Iberian Peninsula through the area of the Alboran Sea.

The role of the High Atlas mountain range in shaping the genetic diversity of Astragalus edulis

Few studies have focused on the role of the High Atlas as a barrier to gene flow for annual and perennial herbs (e.g. Ortiz *et al.* 2009). Moreover, to date, very little is known about the Quaternary range dynamics of plant species in the area and precise locations of refugia frequently remain unknown (Terral *et al.* 2004; Rubio *et al.* 2006). Regarding annual herbs, the existence of refuge areas at low altitudes around the Atlas Mountains has been proposed for plants such as *H. arachnoidea* (Ortiz *et al.* 2009), *Hypochaeris angustifolia* (Terrab *et al.* 2009) and *Arabidopsis*

thaliana (Brennan *et al.* 2014).

In the case of *A. edulis*, the AFLP data analysed (Fig. 2) showed no evidence of the High Atlas Mountains acting as a barrier to gene flow, but these results may underestimate the importance of this mountain range. The maintenance of endemic haplotypes (haplotype VIII to the north of the High Atlas and haplotypes III, IV and VI, to the south of this mountain range) suggests long-term isolation of populations at low altitudes. This idea is also supported by the early isolation of the northern Atlas metapopulation group as detected by the DIYABC analysis. Thus, our data appear to confirm the presence of low altitude refuge areas for annual species at favourable locations around the area of the High Atlas and Anti-Atlas mountain ranges. These locations could represent additional 'phylogeographical hotspots' (Médail and Diadema 2009), which are 'significant reservoirs of unique genetic diversity favourable to the evolutionary processes of Mediterranean plant species'.

Conclusions

Our results suggest that the populations of *A. edulis* on the Canary Islands are the consequence of an ancient LDD event, probably from the western Moroccan populations during the Riss glacial stage. Moreover, our results indicate that the original area for the species is located in the western part of the High Atlas Mountains. A colonization route is proposed that connects the southern Atlas region with the region that is currently occupied by the northern Moroccan populations of *A. edulis*, which finally reaches the Iberian Peninsula. This route may have also been followed by other plant species, some of which are also endangered and with fragmented distributions.

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Conflict of Interest

None declared.

Contributions by the Authors

J.B.-P. performed the experiments, analysed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper. J.P.d.G. conceived and designed the experiments, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper. N.L.-G. analysed the data, prepared figures, reviewed drafts of the paper. S.M. contributed reagents/materials/analysis tools, reviewed drafts of the paper. M.M.M.-O. conceived and designed the experiments, analysed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

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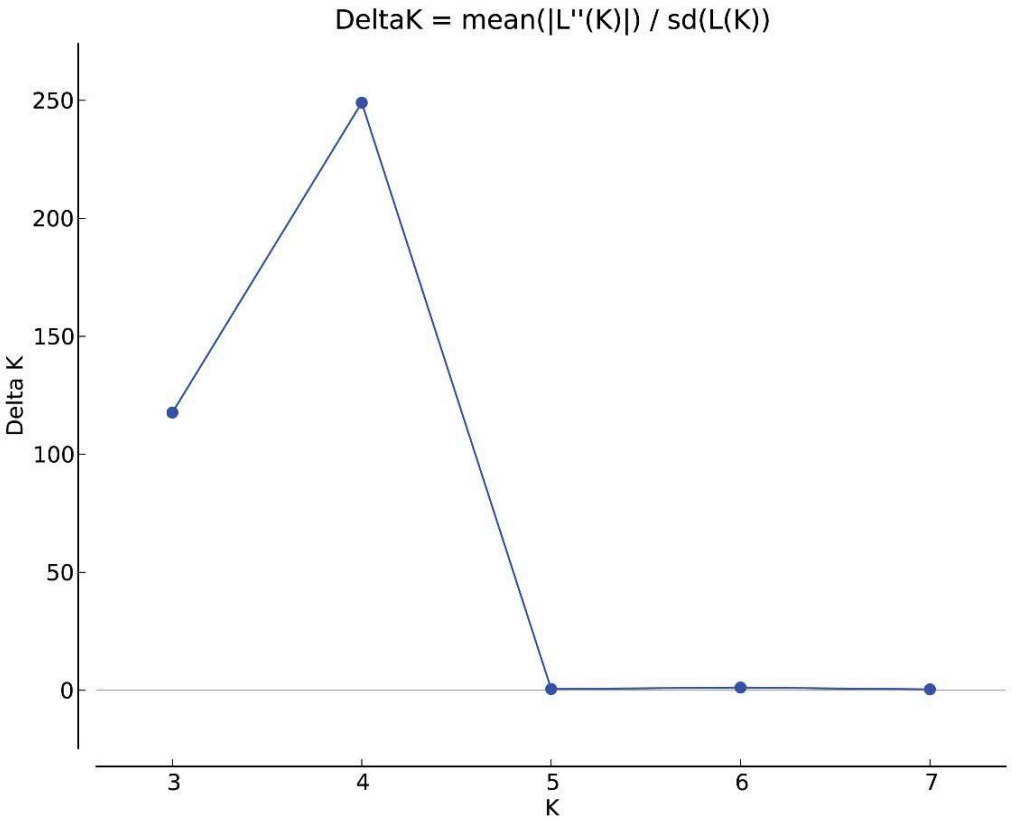
DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:
GenBank sequences have been provided as a Supplemental File (Annex 4).

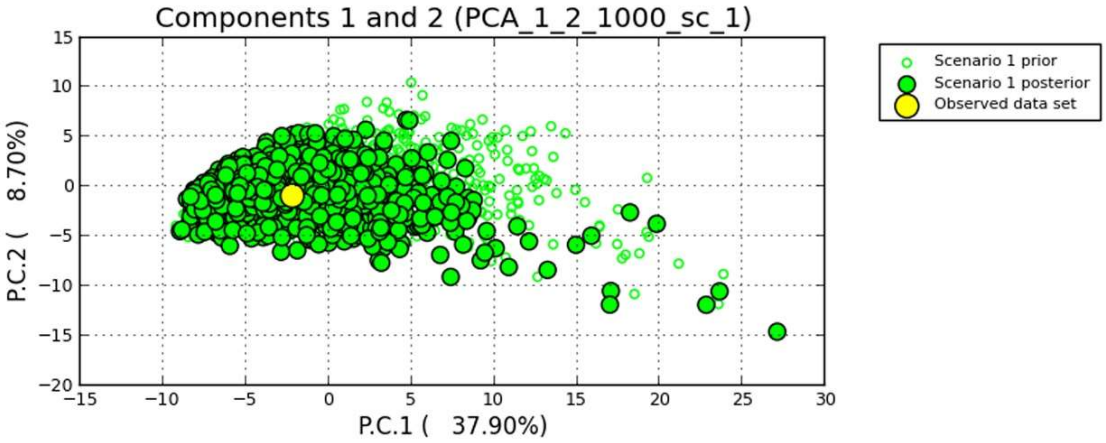
Supporting Information

Full size figures are provided as supporting information (Annex 2 and Annex 3).

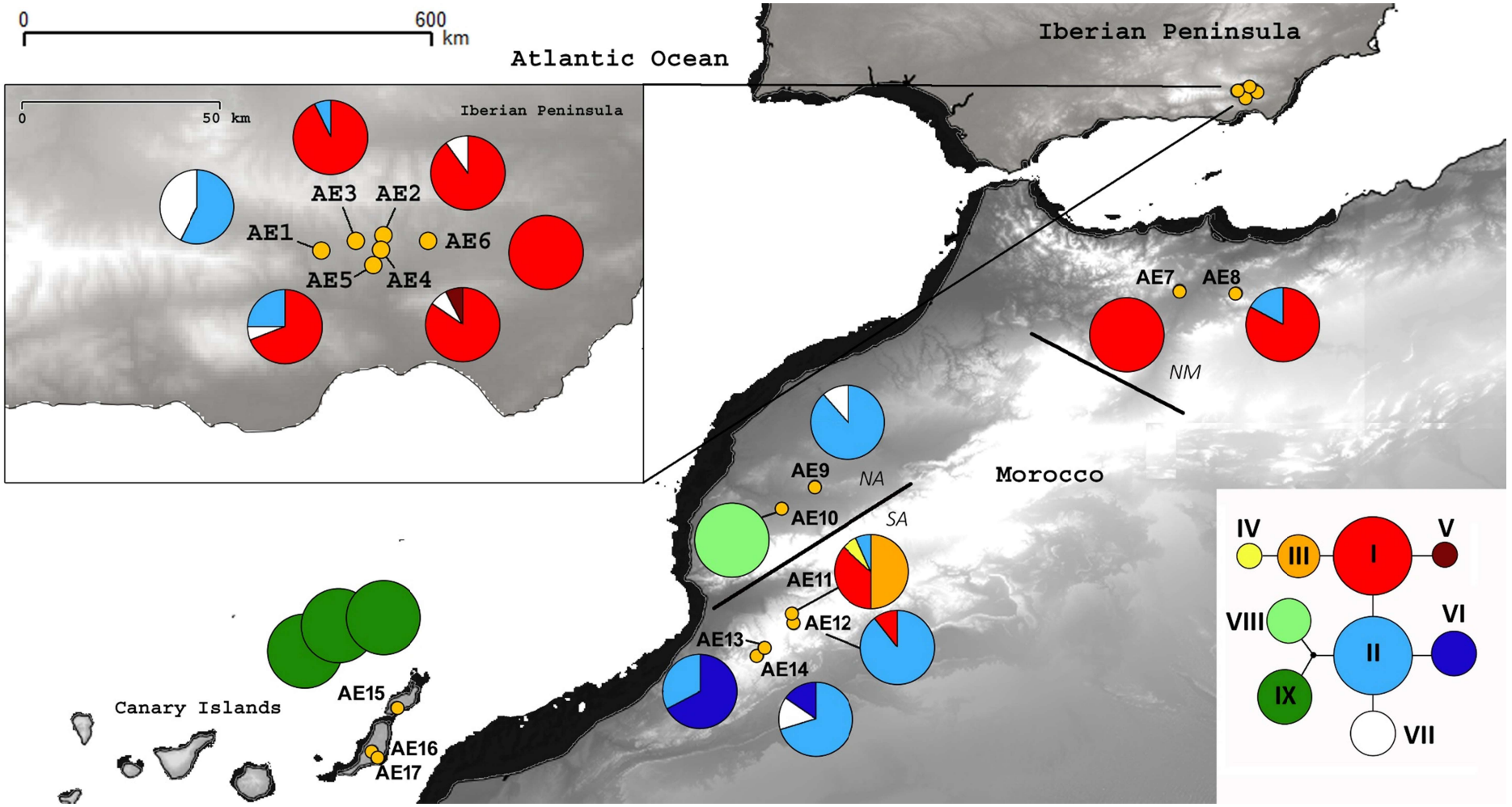
Supporting Information



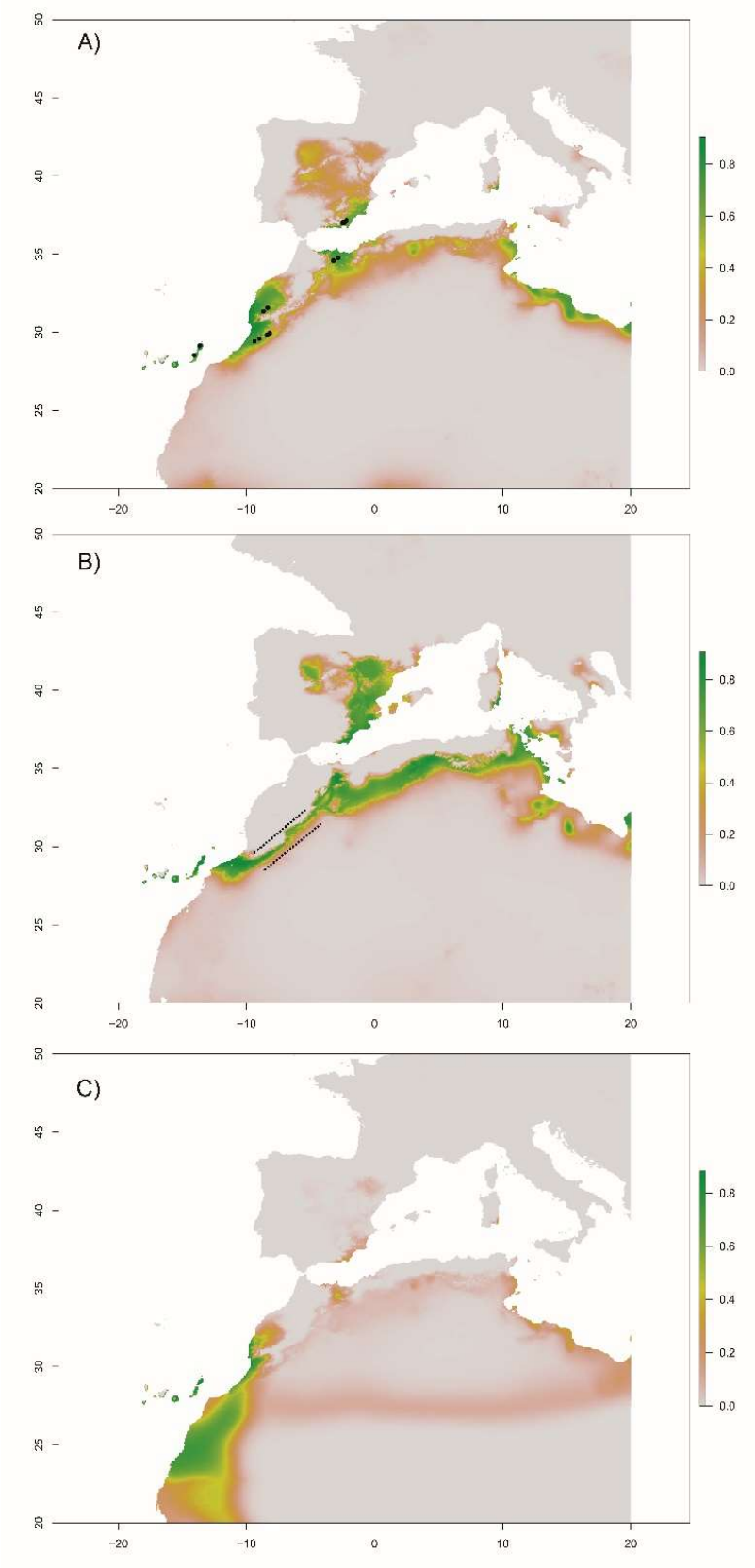
Annex 1: Figure S1. Graph of delta *K* values to determine the ideal number of groups.



Annex 1: Figure S2. Principal coordinates analysis showing the accuracy of the best scenario, as determined by DIYABC.



Annex 2: Full Size Figure 1. Sampling locations covering the present distribution of *Astragalus edulis*. Coast lines during the LGM (black shadow). Plastid haplotype distribution of the species; plastid haplotype network (circle size is proportional to the number of individuals for each haplotype). Clustering for the DIYABC analysis labelled (Iberian Peninsula; northern Morocco, NM; northern Atlas, NA; southern Atlas, SA; Canary Islands)



Annex 3: Full Size Figure 4. Distribution models; habitat suitability is represented green-yellow to red (red-yellow = medium, green = high). (A) Present; (B) LGM; (C) LIG. Dotted line represents the south to north colonization route along Morocco. Black dots represent current localities of the species.

Annex 4: GenBank accession numbers

Samples	Accessions		
	trnC-rpoB	**trnS-trnG**	**trnL-trnF**
AE01_I16_01	*MG570809*	*MG570479*	*MG570644*
AE01_I16_02	*MG570810*	*MG570480*	*MG570645*
AE01_I16_06	*MG570811*	*MG570481*	*MG570646*
AE01_I16_07	*MG570812*	*MG570482*	*MG570647*
AE01_I16_08	*MG570813*	*MG570483*	*MG570648*
AE01_I17_04	*MG570814*	*MG570484*	*MG570649*
AE01_I17_06	*MG570815*	*MG570485*	*MG570650*
AE01_I17_09	*MG570816*	*MG570486*	*MG570651*
AE02_I03_04	*MG570817*	*MG570487*	*MG570652*
AE02_I03_07	*MG570818*	*MG570488*	*MG570653*
AE02_I05_02	*MG570819*	*MG570489*	*MG570654*
AE02_I05_07	*MG570820*	*MG570490*	*MG570655*
AE02_I05_08	*MG570821*	*MG570491*	*MG570656*
AE02_I15_05	*MG570822*	*MG570492*	*MG570657*
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Samples	Accessions		
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AE17_C01_18	*MG570973*	*MG570643*	*MG570808*

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ARTÍCULO 2: Diseño de estrategias de conservación para preservar la diversidad genética de *Astragalus edulis* Bunge, especie amenazada del Oeste mediterráneo

Julio Peñas de Giles[1], Sara Barrios[2], **Javier Bobo-Pinilla**[2,3], Juan Lorite[1] and M. Montserrat Martínez-Ortega[2,3]

[1]Departamento de Botánica, Universidad de Granada, Granada, España
[2]Departamento de Botánica, Universidad de Salamanca, Salamanca, España
[3]Biobanco de ADN Vegetal, Banco Nacional de ADN, Universidad de Salamanca, Salamanca, España

Resumen

Astragalus edulis (Fabaceae) es una herbácea en peligro de extinción de la región del Mediterráneo occidental que está presente en el SE de la Península Ibérica, NE y SW de Marruecos, y en las Islas Canarias más orientales (Lanzarote y Fuerteventura). Aunque en España se han adoptado algunas medidas de conservación para la especie, todavía es necesario desarrollar un plan de gestión adecuado para preservar la diversidad genética en toda el área de distribución. Nuestro principal objetivo fue utilizar la genética de poblaciones, así como los datos ecológicos y filogeográficos, para seleccionar Unidades Genéticas Relevantes para la Conservación (RGUC) como el primer paso en el diseño de planes de conservación para *A. edulis*. En base a las estimaciones de la estructura genética de las poblaciones y las probabilidades de pérdida de alelos raros, identificamos seis RGUCs para la conservación *in situ*. Además, con el fin de establecer otros parámetros complementarios para determinar la prioridad de conservación, se consideraron; el área de ocupación, el tamaño de la población, la vulnerabilidad, el estado legal de conservación y la distribución de haplotipos. Tres poblaciones de la Península Ibérica, dos de Marruecos y una de las Islas Canarias representan la diversidad genética total de la especie y la variación alélica más rara. Se recomienda la conservación *ex situ* para complementar la conservación de *A. edulis*, dado que la protección efectiva *in situ* de las poblaciones no es factible en todos los casos. La consideración de datos complementarios, filogeográficos y ecológicos, es útil para maximizar los esfuerzos de gestión y para preservar el potencial evolutivo de la especie

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Corresponding author
Julio Peñas, jgiles@ugr.es

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Designing conservation strategies to preserve the genetic diversity of *Astragalus edulis* Bunge, an endangered species from western Mediterranean region

Julio Peñas[1], Sara Barrios[2], **Javier Bobo-Pinilla**[2,3], Juan Lorite[1] and M. Montserrat Martínez-Ortega[2,3]

[1]Plant Conservation Unit, Department of Botany, University of Granada, Granada, Spain

[2]Department of Botany, University of Salamanca, Salamanca, Spain

[3]Biobanco de ADN Vegetal, Edificio Multiusos I+D+i, Calle Espejo s/n, Salamanca, Spain

ABSTRACT

Astragalus edulis (Fabaceae) is an endangered annual species from the western Mediterranean region that colonized the SE Iberian Peninsula, NE and SW Morocco, and the easternmost Macaronesian islands (Lanzarote and Fuerteventura). Although in Spain some conservation measures have been adopted, it is still necessary to develop an appropriate management plan to preserve genetic diversity across the entire distribution area of the species. Our main objective was to use population genetics as well as ecological and phylogeographic data to select Relevant Genetic Units for Conservation (RGUCs) as the first step in designing conservation plans for *A. edulis*. We identified six RGUCs for in situ conservation, based on estimations of population genetic structure and probabilities of loss of rare alleles. Additionally, further population parameters, i.e. occupation area, population size, vulnerability, legal status of the population areas, and the historical haplotype distribution, were considered in order to establish which populations deserve conservation priority. Three populations from the Iberian Peninsula, two from Morocco, and one from the Canary Islands represent the total genetic diversity of the species and the rarest allelic variation. Ex situ conservation is recommended to complement the preservation of *A. edulis*, given that effective in situ population protection is not feasible in all cases. The consideration of complementary phylogeographic and ecological data is useful for management efforts to preserve the evolutionary potential of the species.

Subjects: Biodiversity, Biogeography, Conservation biology, Genetics, Plant science

Keywords: Conservation priorities, Relevant genetic units for conservation, Phylogeography, Threatened species, cpDNA sequencing, AFLPs

INTRODUCTION

Although one of the central concepts in biodiversity conservation is that genetic diversity is crucial to ensure the survival of species, until now the conservation of plant genetic resources has received less attention than it deserves. Plant-conservation strategies have been commonly based on general premises, leading to more or less standardized systems for evaluating the extinction risks of the species (Moraes et al., 2014). However, plant species differ enormously in biological traits and environmental requirements, making itunrealistic to apply a single system to all species. Recent years have seen increasing efforts to improve both in situ and ex situ conservation methods, which in theory would foster dynamic conservation of plant species and populations (Volis & Blecher, 2010; Heywood, 2014). Plant genetic diversity is spatially structured at different scales (e.g. geographical areas, populations, or among neighbouring individuals) (Engelhardt, Lloyd & Neel, 2014) as a result of environmental influences, life-history traits, and the demographic past history of the species. Therefore, management schemes for conservation often require an understanding of population dynamics and knowledge of relative levels of genetic diversity, within species genetic structure, as well as within- and among-population genetic differentiation in order to focus efforts on specific populations needing recovery (Haig, 1998; Pérez-Collazos, Segarra-Moragues & Catalán, 2008).

Several estimators have been assayed to answer the question of which and how many populations deserve conservation priority, such as: Evolutionary Significant Units (ESUs; Ryder, 1986); Management Units (MUs; Moritz, 1994); Operational Conservation Units (OCUs; Doadrio, Perdices & Machordom, 1996); Fundamental Geographic and Evolutionary Units (FGEUs; Riddler & Hafner, 1999); Functional Conservation Units (FCUs; Maes et al., 2004), among others (see also Pérez-Collazos, Segarra-Moragues & Catalán, 2008; Domínguez-Domínguez & Vázquez-Domínguez, 2009). Fraser & Bernatchez (2001) reviewed the different concepts of ESUs (the most prominent estimator among those previously mentioned), concluding that differing criteria would work more dynamically than others and can be used alone or in combination depending on the situation. Pérez-Collazos, Segarra-Moragues & Catalán (2008), partially based on Caujapé-Castells & Pedrola-Monfort (2004), as well as on the premises established by Ciofi et al. (1999), introduced the concept of Relevant Genetic Units for Conservation (RGUCs), which was subsequently used to propose sampling strategies for species such as *Boleum asperum* Desv. (Pérez-Collazos, Segarra-Moragues & Catalán, 2008) and *Borderea pyrenaica* Miégev. (Segarra-Moragues & Catalán, 2010). This approach combines two methods that use genetic data (considering both usual and rare alleles) to estimate the minimum number of conservation units (often corresponding to populations) that should be targeted for an adequate representation of the total (or partial) genetic variability of a

threatened species, as well as a way to select among all units (i.e. populations) which contain a singular or rare allelic composition. A list of preferred sampling areas (PSA) indicating the geographical ranges with higher probabilities of capturing a particular rare allele is finally established, helping to identify RGUCs and therefore prioritize particular populations, as well as sampling for ex situ conservation. This method helps identify the most singular populations, based on the idea that rare alleles are essential in conservation because they represent unique evolutionary products that could provide the species with advantageous properties to cope with eventual environmental shifts. Thus, collection designs oriented to sampling rare alleles reinforce declining populations and may aid the survival of reintroduced plants (Bengtsson, Weibull & Ghatnekar, 1995; Pérez-Collazos, Segarra-Moragues & Catalán, 2008). One of the main advantages of this genetic conservation approach is that it objectively prioritizes particular plant populations in low-extinction-risk categories (Segarra-Moragues & Catalán, 2010), particularly in taxa that have many populations and individuals, making active protection and monitoring of the entire distribution area of the species difficult or unaffordable.

The species selected for this study *Astragalus edulis* Bunge (Fabaceae), is an annual plant that inhabits semidesertic areas of south-eastern Spain, western North Africa, and the Canary Islands (Fuerteventura and Lanzarote) (Peñas, 2004; Reyes-Betancort et al., 2005). It is a threatened species evaluated as Endangered (EN) in Spain. Despite its relatively wide distribution area, only a few populations remain, these being highly fragmented. Habitat alteration has been cited as a major threat to this species (Peñas, 2004). Specifically, the abandonment of traditional agricultural practices, overgrazing, and the habitat depletion, caused by the spread of greenhouses, may have had severely negative consequences for species survival (Benito et al., 2009). This species represents an ideal model to test the utility of RGUC identification as an affordable way to conserve taxa that have highly fragmented populations, some of them with many individuals, but they are under extinction-risk categories.

Our specific aims are: (1) to evaluate the distribution of the genetic diversity among the different populations, and/or geographical areas; (2) to assess the number of populations that should be sampled or preserved in order to establish a representative percentage of the total genetic variation of *A. edulis*; (3) to identify which populations should be prioritized to better represent the genetic singularity and geographic variability for both ex situ and in situ conservation.

MATERIALS AND METHODS

Studied species

Astragalus edulis Bunge (Fabaceae) is a short-lived therophytic, hermaphroditic plant. Until now, no information has been available on population sizes, except for the rough estimates by Peñas (2004), indicating that ca. 226,000 individuals were present in SE Spain in 2003. This estimate also indicated a noticeable inter-annual fluctuation in population sizes (number of individuals) and reproductive success (Peñas, 2004; Reyes-Betancort et al., 2005). The reproductive biology of the species is poorly known; it shows an entomophilous pollination syndrome, lacking asexual reproduction as well as evident adaptations to long-distance dispersal, but there is no information available on its pollination biology or dispersal agents. Its habitat is restricted to grasslands on poor sandy soils, resulting from erosion or deposition of volcanic or schistose rocks in semiarid areas of the western Mediterranean region (Peñas, 2004; Reyes-Betancort et al., 2005) (Fig. 1).

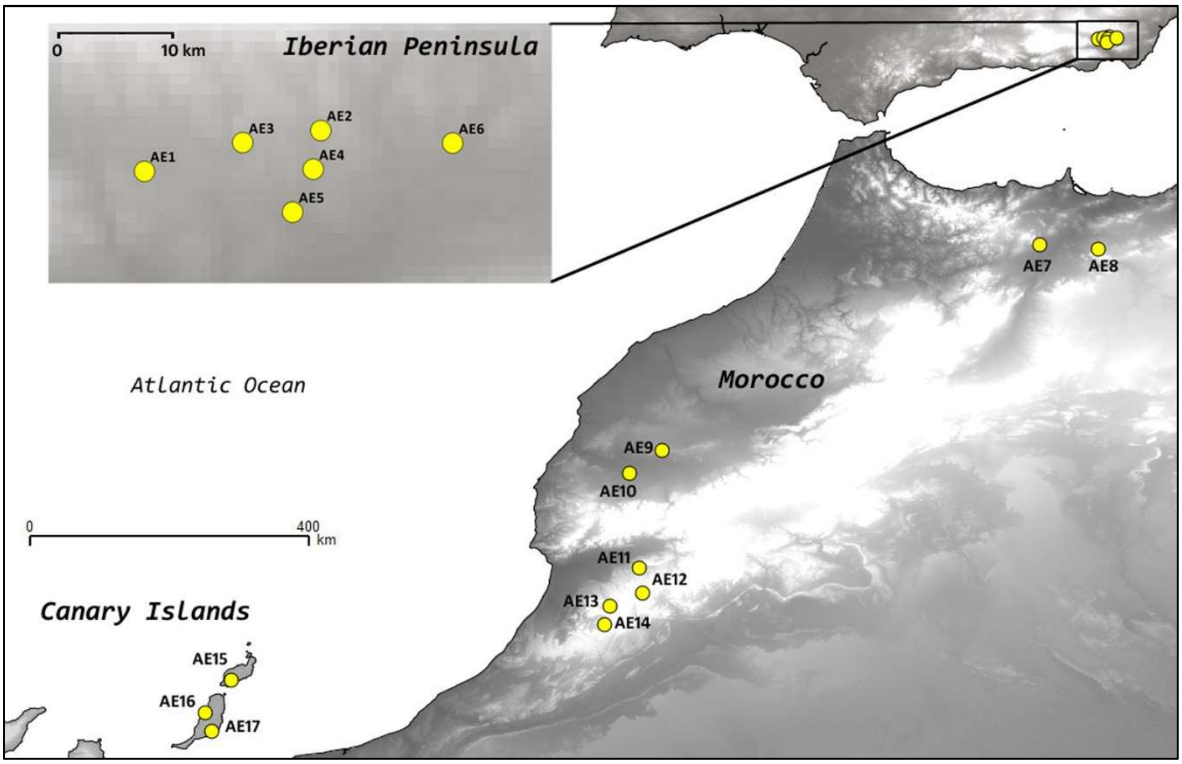

Figure 1: Location of the populations of *Astragalus edulis* sampled for this study.

Astragalus edulis is rare (i.e. constantly sparse in a specific habitat but over a large range; according to Rabinowitz, 1981) and threatened species evaluated as Endangered (EN) in Spain, and consequently included in the Spanish national and regional red lists (Bañares et al., 2004), as well as in the Andalusian (southern Spain) red list (Cabezudo et al., 2005). Also, some populations in Spain are included in Natura 2000 network (Special Areas of Conservation,

Council Directive 92/43/EEC) and in Regional Network of Natural Protected areas of Andalusia (southern Spain), while the areas occupied by the species in Canary Islands and Morocco lack legal protection.

Plant material for DNA study

We collected fresh leaf tissue from 360 individuals belonging to 17 populations; 6 from the Iberian Peninsula (AE1 to AE6), 8 from Morocco (AE7 to AE14) and 3 from the Canary Islands (AE15 to AE17), spanning the entire distribution range of the species (Table 1; Fig. 1). We considered different populations when individuals are more than 1 km apart. We aimed to collect 25 individuals per population whenever possible but due to small population sizes in some cases the final number of individuals sampled per population ranged from 7 to 33. Within a particular population the samples were collected at distances greater than 5 m apart to avoid sampling closely related individuals. All sampling sites were geo-referenced with a GPS (GARMIN GPSMAP 60) and vouchers of the sampled localities were included in the herbaria of the Universities of Salamanca (SALA) and Granada (GDA). Plant material from each individual was dried and preserved in silica gel until DNA extraction.

Table 1: Geographic features of the populations sampled in the study. **(N)** Number of individuals used for the AFLP analyses.

Population	*Locality*	*Altitude*	*Longitude*	*Latitude*	*N*
AE1	Spain; Almería, Alcubillas	735	-2,6025	37,0987	16
AE2	Spain; Almería, Tabernas	915	-2,4643	37,1306	24
AE3	Spain; Almería, Gérgal	720	-2,5254	37,1209	32
AE4	Spain; Almería, Gérgal, Arroyo Verdelecho	648	-2,4704	37,1002	24
AE5	Spain; Almería, Tabernas, Desierto de Tabernas	621	-2,4863	37,0668	23
AE6	Spain; Almería, Filabres, Rambla del Saltador	541	-2,361	37,1206	33
AE7	Morocco; La Oriental, between El-Aı¨oun and Tanarchefi	919	-2,6016	34,4174	17
AE8	Morocco; Taza, Jebel Guilliz	425	-3,3496	34,4669	21
AE9	Morocco; Marrakech, Chemaia, prox. Kettara	480	-8,1875	31,8729	22
AE10	Morocco; Marrakech, between Marrakech and Chichaoua	380	-8,6185	31,572	14
AE11	Morocco; Taroudant, between Tasgount and Ighil	1,437	-8,4832	30,1831	18
AE12	Morocco; Taroudant, between Irherm and Tata	1,71	-8,4478	30,0467	19
AE13	Morocco; Taroudant, Tafraoute, Tizi-n-Tarakatine, prox. El Jebar	1,484	-8,8587	29,7376	25
AE14	Morocco; Taroudant, between Tafraoute and Tleta-Tasrite	1,62	-8,9385	29,6354	7
AE15	Spain; Canary Islands; Lanzarote, Vega de Temuime	159	-13,728	28,9337	29
AE16	Spain; Canary Islands; Fuerteventura, Tiscamanita	234	-14,033	28,3576	14
AE17	Spain; Canary Islands; Fuerteventura, Barranco de Majada Blanca	181	-13,986	28,2673	22

DNA isolation, AFLP protocol and cpDNA sequencing

Total DNA was isolated following the 2x CTAB protocol (Doyle & Doyle, 1987) with minor modifications. AFLP profiles were drawn following established protocols (Vos et al., 1995) with

modifications. A negative control sample was consistently included to test for contamination, and five samples taken at random were replicated to test for reproducibility. Selective primers were initially screened using 24 primer combinations for the selective PCR and three were finally selected (fluorescent dye in brackets): EcoRI-AGA (6-FAM)/MseI-CTG, EcoRI-AAG(VIC)/MseI-CAG and EcoRI-ACC(NED)/MseI-CTG, because they generated a relatively high number (a high number of alleles per individual is desirable in conservation genetic studies given that AFLP are dominant markers; Lowe, Harris & Ashton, 2004) of clearly reproducible bands, for which homology was easy to ensure. The fluorescence-labelled selective amplification products were separated in a capillary electrophoresis sequencer (ABI 3730 DNA Analyzer; Applied Biosystems, Foster City, CA, USA), with GenScan ROX (Applied Biosystems, Foster City, CA, USA) as the internal size standard, at the Genomic Department of Universidad Politécnica de Madrid. Raw data with amplified fragments were scored and exported as a presence/absence matrix.

To complement the information of the mainly nuclear AFLPs, the plastid regions trnG-trnS, trnC-rpoB, and tabF-tabC (Taberlet et al., 1991; Shaw et al., 2005) were explored (see Table 2 for details). These regions showed the highest variability of 23 surveyed cpDNA regions in the preliminary studies using 10 individuals and were therefore used to analyse a total of 61 individuals (i.e., 3–4 individuals per population, due to amplification failure in 7 cases) of *A. edulis*: 38 from Iberian Peninsula (IP), 17 from Morocco (M) and, 6 from Canary Islands (CI). PCR products were purified using PCR Clean-Up with ExoSAP-IT Kit (AFFIMETRIX, Santa Clara, CA, USA) following the manufacturer's instructions. The cleaned amplification products were analysed with a 3,730 DNA Genetic Analyzer capillary sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were deposited in GenBank (see Supplemental Information).

Table 2: PCR primers and conditions used to obtain cpDNA sequence data for *Astragalus edulis*.

Region	*Forward primer*	*Reverse primer*	*Denaturation Temperature/ Time*	*Annealing Temperature/ Time*	*Extension Temperature/ Time*	*Cycles*
trnG-trnS	3'trnG^{UUC}	trnS^{GCU}	95°C/30"	62°C/30"	72°C/1'30"	35
trnC-rpoB	trnC^{GCA}R	rpoB	95°C/30"	55°C/30"	72°C/1'30"	35
tabC-tabF	trnL^{UAA}5'	trnF^{GAA}	95°C/30"	52°C/30"	72°C/2'30"	35

Molecular data analysis

An unrooted phylogram based on Nei and Li's genetic distances (Nei & Li, 1979) and AFLP data was calculated using the Neighbour-Joining (NJ) clustering method, with 1000 bootstrap pseudoreplicates (BS), in order to evaluate genetic structure within *A. edulis*. This was

conducted with the software PAUP v4.0b10 (Swofford, 1998). As an additional estimate of the population genetic structure and based on Dice's similarity coefficient (Dice, 1945; Lowe, Harris & Ashton, 2004), a Principal Coordinate Analysis (PCoA) was performed with NTSYS-pc 2.02 (Rohlf, 2009) as an additional approach to the overall genetic relationships among the individuals analysed.

An analysis of molecular variance (AMOVA) was performed with the software ARLEQUIN 3.5.1.2 (Excoffier, Laval & Schneider, 2005). The analysis was first conducted considering all populations belonging to the same group and, second, partitioning genetic variation into portions assignable to differences among three predefined groups (the three main geographic groups derived from the NJ phylogram, i.e. (IP: AE1–AE6), (M: AE7–AE14), and (CI: AE15–AE17)) in order to test for identifiable genetic structures among geographical divisions. Significance levels of the variance components were estimated for each case using non-parametric permutations with 1023 replicates.

The proportion on polymorphic alleles measured by Nei's gene-diversity index (Nei, 1987) was calculated for each population using the R package AFLPDAT for R (Ehrich, 2006). This package was also used to calculate the frequency down-weighted marker values per population or sampling site (DW; Schönswetter & Tribsch, 2005), which estimates genetic rarity of a population as equivalent to range down-weighted species values in historical biogeographical research (Crisp et al., 2001). Finally, the number of rare alleles (N_r), (i.e. bands that showed an overall frequency lower than 10%, and that are present in less than 20% of the populations (Pérez-Collazos, Segarra-Moragues & Catalán, 2008), was calculated as an additional measure of rarity.

The completeness of haplotype sampling across the range of *A. edulis* was estimated using the Stirling probability distribution. It provides a way to evaluate the assumption that all haplotypes have been sampled (Dixon, 2006). Plastid-DNA sequences were assembled and edited using GENEIOUS PRO™ 5.4 (Drummond et al., 2012) and aligned with CLUSTAL W2 2.0.11 (Larkin et al., 2007), and further adjustments were made by visual inspection. The resulting sequences were concatenated; the gaps longer than one base pair were coded as single-step mutations and treated as a fifth character state. An unrooted haplotype network was constructed using the statistical parsimony algorithm (Templeton, Crandall & Sing, 1992) as implemented in TCS 1.21 (Clement, Posada & Crandall, 2000), and used to infer the existing genealogical relationships.

Selection of relevant genetic units for conservation (RGUCs)

The selection of RGUCs is based on AFLP data and relies on the combination of two methods based on population structure and probabilities of loss of rare alleles. In summary, the values of the probability of rare-allele loss are compared with those of the degree of inter-population subdivision (Caujapé-Castells & Pedrola-Monfort, 2004; Pérez-Collazos, Segarra-Moragues & Catalán, 2008). First, the population-differentiation coefficient (F_{ST}) obtained with ARLEQUIN was used to estimate the total number of populations that should be targeted, according to the Ceska, Affolter & Hamrick (1997) equation modified $\mathbf{P = 1\ F^{n}_{ST}}$ (Segarra-Moragues & Catalán, 2010; but not Pérez-Collazos, Segarra-Moragues & Catalán, 2008) where n is the number of populations to be sampled to represent a given proportion (P) of the among population genetic diversity. For *A. edulis*, a P value of 99.9% of the total genetic diversity was established, to cope properly with high conservation standards. Second, using the mean frequencies of rare bands (i.e. with an overall frequency lower than 10% and present in less than 20% of the populations) and their associated probabilities of loss, the probability that a sample size on N populations fails to include an allele with population frequency p was calculated (Caujapé-Castells & Pedrola-Monfort, 2004; Pérez-Collazos, Segarra-Moragues & Catalán, 2008). For this, the expression $\boldsymbol{L = (1 - p)^{2N}}$ (Bengtsson, Weibull & Ghatnekar, 1995) was used, where p represents the allele frequency and N the number of populations in which a rare allele is present (Pérez-Collazos, Segarra-Moragues & Catalán, 2008). For each rare allele, the observed (Lo) and expected (Le) probabilities of loss were calculated. The negative natural logarithms (−Log Lo and −Log Le) of those values were plotted (*y*-axis) against the mean frequency of each rare allele (x-axis) and used to calculate the respective linear regressions. The representative R value (which indicates the proportion of rare alleles captured by sampling only one population) was calculated as the quotient between the slope of the expected regression line and the slope of the observed regression line, i.e. $\boldsymbol{R = m(-Log\ L_e)/m(-LogL_o)}$ (Bengtsson, Weibull & Ghatnekar, 1995; Caujapé-Castells & Pedrola-Monfort, 2004; PérezCollazos, Segarra-Moragues & Catalán, 2008; Segarra-Moragues & Catalán, 2010).

Several qualitative features of the populations and habitat disturbances were recorded during the field work in order to combine them with the measures of genetic diversity. For this, we selected population variables that were accounted as follows (adapted from IUCN, 2001): i) Occupation area: small <1 km^2 vs. large >1 km^2, ii) population size: high >1,000 individuals vs. low <1,000 individuals), iii) vulnerability: stable = with no disturbances or with minor disturbances/declining = with clear disturbance of both individuals and habitat/critically declining = major disturbances, with major disturbance of individuals and habitat; and iv) conservation status of the area: protected vs. unprotected.

Generalized linear models were used to test whether the main genetic diversity and rarity parameters (i.e. h_{Nei}, DW, and Nr) show associations with qualitative population and conservation features. Beforehand, to enhance the robustness of the models, we resampled the cases 10,000 times by bootstrapping using the R boot package (Canty & Ripley, 2013). Nei's diversity index and the frequency of down-weighted marker values were fitted to Gaussian distributions, whereas the number of rare alleles was fitted to a Poisson distribution. To test significant level differences of a given variable, we used the glht function of the R multcomp package, indicated for multiple comparisons in generalized linear models (Hothorn, Bretz & Westfall, 2008).

RESULTS

Genetic variability and structure

A total of 1134 reliable polymorphic bands (averaging ca. 45 per individual per primer combination) were found from the three primer pairs selected for the 360 individuals studied. The final error rate was insignificant (1.67%). The number of rare alleles, DW values and Nei's genetic diversity values corresponding to each population are given in Table 3. AFLPs detected low levels of intrapopulation genetic diversity for *A. edulis*. Nei's gene diversity index ranged from a minimum value of 0.066 (AE7; in the easternmost population of Morocco) to a maximum of 0.155 (AE5; in the central part of the Iberian distribution of the species) and the diversity values were similar across all other populations studied. The total species diversity was 0.108. Regarding rarity, the genetically most distinctive population (DW=5.713) appeared to be AE16 in Fuerteventura, while the lowest DW values were found in the easternmost part of the Iberian core (AE6; DW = 1.507).

Both the unrooted NJ tree and the PCoA based on the entire data set (Fig. 2) revealed well-defined genetic structure of populations in correspondence to geographic groups. The first group (Fig. 2A) includes all populations from the Iberian Peninsula (85% BS), a second cluster those from Morocco (74% BS) and the third those from the Canary Islands (100% BS), plus some individuals from Morocco (two samples from AE9), although the relationship between these latter two groups is weak (62% BS) and the Moroccan part of this cluster seems to be closely related to the remaining Moroccan individuals. The same geographical groups are revealed by the PCoA (Fig. 2B), but in this case the apparently close relationship between some of the Moroccan and all the Canarian samples suggested by NJ does not seem to be supported, while an affinity between the Moroccan and the Iberian individuals is suggested. The first three axes account for 13.2, 6.4, and 4.7% of the total variance, respectively.

Table 3: Population, geographical groups, AFLP derived diversity and rarity descriptors, rarity assessment through qualitative variables (see text) and cpDNA haplotypes for the studied population of *A. edulis*. Geographical groups: **IP**, Iberian Peninsula**; M**, Morocco; **CI**, Canary Islands; $\mathbf{h_{Nei}}$, Nei's diversity index (Nei 1987); **DW**, frequency down-weighted marker values; $\mathbf{N_r}$, number of rare alleles; **H**, haplotype.

Population	*Geographical area*	h_{Nei}	*DW*	*Nr*	*Size*	*Occupation*	*Vulnerability*	*Legal Status*	*H*
AE1	IP	0.101	3.505	31	small	reduced	critical	unprotected	IV,V
AE2	IP	0.103	2.226	25	large	high	moderate	protected	I,V
AE3	IP	0.125	3.298	45	large	high	moderate	protected	I,IV
AE4	IP	0.151	4.038	38	large	high	acceptable	protected	I,III
AE5	IP	0.155	4.644	47	large	high	acceptable	protected	I,IV,V
AE6	IP	0.076	1.507	16	large	reduced	moderate	unprotected	I
AE7	M	0.066	1.754	14	small	reduced	critical	unprotected	I
AE8	M	0.119	3.2	33	large	high	moderate	unprotected	I
AE9	M	0.114	3.218	51	small	reduced	critical	unprotected	IV
AE10	M	0.082	1.728	8	small	reduced	moderate	unprotected	VI
AE11	M	0.104	2.924	27	large	reduced	moderate	unprotected	II
AE12	M	0.097	2.834	30	small	reduced	critical	unprotected	IV
AE13	M	0.103	2.815	33	large	high	moderate	unprotected	IV
AE14	M	0.076	2.08	12	small	reduced	critical	unprotected	IV
AE15	CI	0.074	2.862	14	small	high	moderate	unprotected	VII
AE16	CI	0.127	5.713	37	small	reduced	moderate	unprotected	VII
AE17	CI	0.110	4.996	55	large	reduced	acceptable	unprotected	VII

AMOVA analysis of the entire data set as a single group (Table 4) revealed that the genetic variation among individuals (71.06%) is meaningfully higher than the variation among populations (28.94%, F_{ST}=0.289, $p < 0.001$). The results of a hierarchical AMOVA confirm that a population division into the three geographic groups defined by NJ and PCoA analyses reveals 24.44% of the variance attributed to differences among these geographical areas (F_{ST} = 0.346, $p < 0.001$), while only 10.14% of the variance is attributed to differences among populations within these three geographic groups.

Table 4: Comparison of analyses of molecular variance (AMOVA), based on AFLP data, of Astragalus edulis across the main geographical groups (**IP**, Iberian Peninsula; **M**, Morocco; **CI**, Canary Islands), and populations (are shown in brackets) (see **Table 1** and **Fig. 1**).

Source of variation	*MS*	*d.f.*	*Absolute var.*	*% of var.*	*FST*	*95% conf.*
One group (A1–A17)					0.289	26.2–30.8
Among populations	9.268.217	16	24.641	28.94		
Within populations	20.755.722	343	60.512	71.06		
Three groups: IP(A1–A6); M(A7–A14) and C(A15–A17)					0.346	21.1–26.8
Among groups	5.694.211	2	22.611	24.44		
Among populations	3.574.006	14	9.383	10.14		
Within populations	20.755.722	343	60.512	65.41		

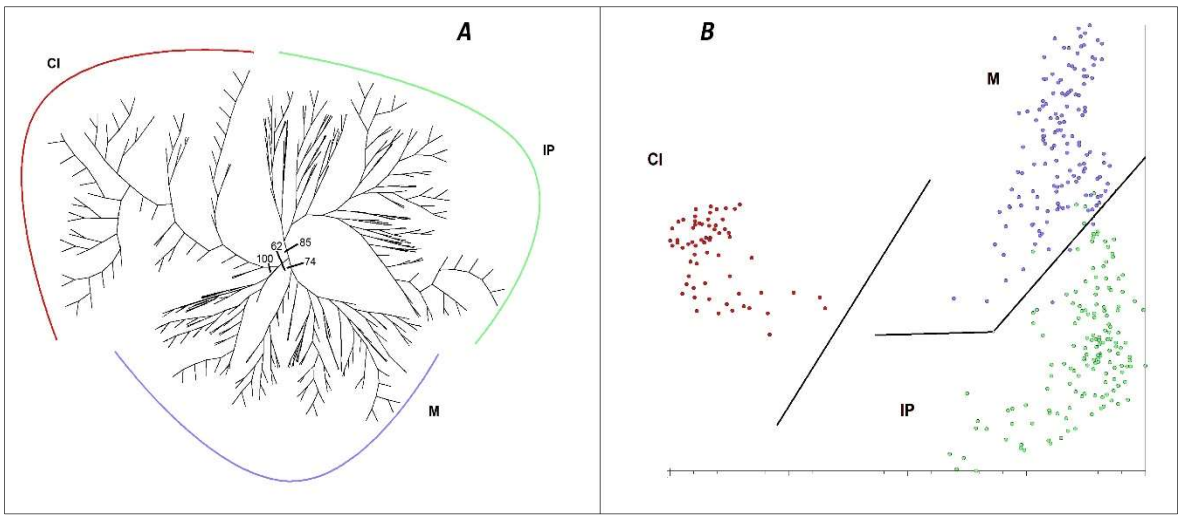

Figure 2: Cluster analysis of genetic diversity, using AFLPs, in *Astragalus edulis*. (A) Neighbour-Joining analysis, BS values are indicated; (B) PCoA. Geographical groups: **IP**, Iberian Peninsula: **M**, Morocco: **CI**, Canary Islands. Full size figure in Supplemental Information II.

The length of the three cpDNA regions for 61 individuals was 712 to 926 bp, and resulted in an alignment of 2545 bp (2549 characters with indels coded). The genetic variability within *A. edulis* was remarkably low (26 cpDNA regions initially tested, 3 of them used to analize a total of 61 individuals), and all the mutations together defined a total of 7 haplotypes. The completeness of haplotype sampling estimated using Dixon's (2006) method was 0.95 (most likely value of haplotypes = 7.002), suggesting that all haplotypes present in the species were sampled. TCS implied a 95% parsimony network with a maximum limit of five steps (Fig. 3). The most frequent haplotype (I) was found in five populations from the Iberian Peninsula and in the north-eastern Moroccan populations, while the second most frequent haplotype (IV) was represented in four western Moroccan populations and also in two Iberian populations. Within the Iberian Peninsula, two endemic haplotypes (III and V) were found and the western Moroccan populations also showed two endemic haplotypes (II and VI). A single endemic haplotype (VII) was found in Fuerteventura and Lanzarote (Fig. 3; Table 3).

Identification of RGUCs

According to our results, 99.9% of the overall genetic diversity through the entire distribution range of *A. edulis* would be represented by just 6 populations (N = 5.69). This should be the minimum number of populations to be targeted for suitable conservation. Of the total 1134 alleles detected by the AFLP analysis, 273 complied with the established rarity criteria (Table 3; Appendix 1). Of these rare alleles, 66 were exclusive to the Iberian Peninsula),

78 to Morocco and 57 to the Canary Islands; the remaining rare bands were distributed among different populations of the three geographical regions (detailed data available upon request). The representative R-value (i.e. proportion of rare alleles determined by sampling only one population) considering *A. edulis* as one group was R = 0.354. This means that the sampling of a single population of the entire distribution area of the species would represent the 35.4% of the whole set of rare alleles of the species. This value, calculated independently for each geographic area, showed slight variations (i.e. IP: R = 0.407, M: R = 0.355 and CI: R = 0.293). Based on the mean frequencies of the rare alleles, as well as on their distribution among populations, the areas where each of these alleles had the highest probability of being found by randomly sampling one population were: IP (124), M (92), and CI (57). Thus, the optimal proportion of populations to be sampled for conservation purposes from each geographical group can be expressed as 0.45 (IP): 0.34 (M): 0.21 (CI).

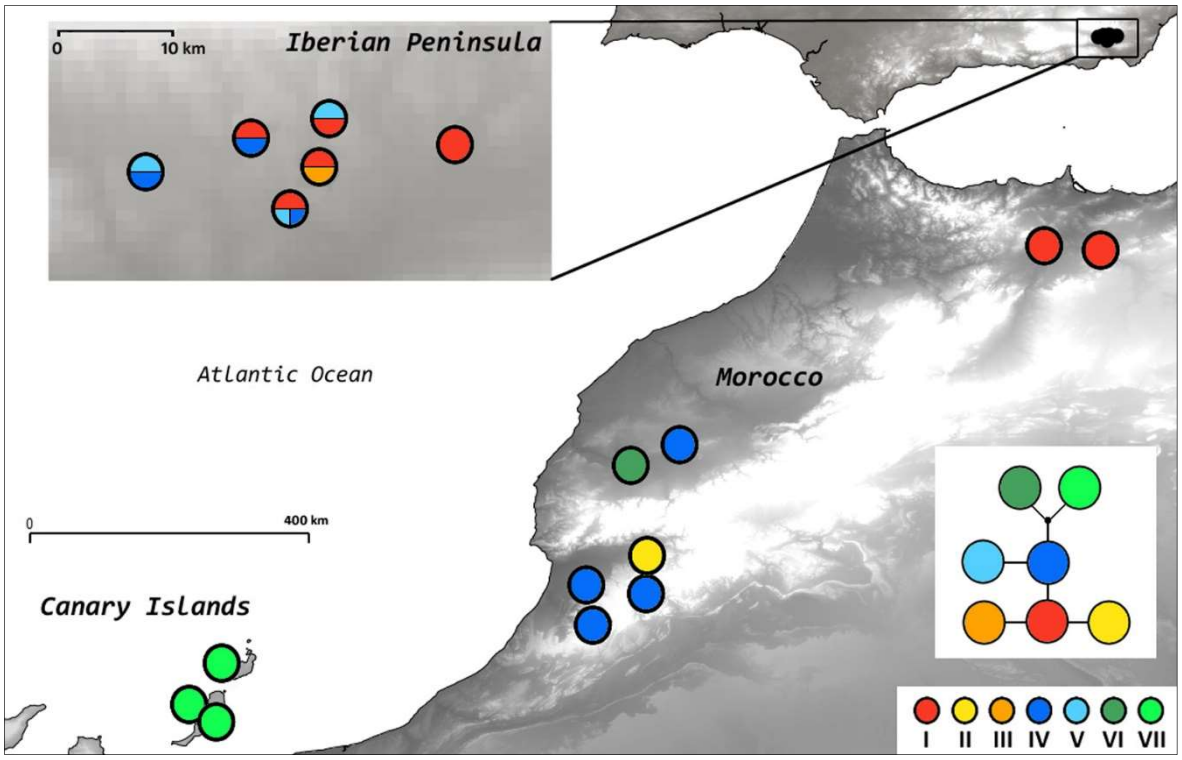

Figure 3: Statistical parsimony network and geographical distribution of plastid DNA haplotypes. The insert shows populations within the Iberian Peninsula. The small black dot represents a missing intermediate haplotype. Sectors within circles in the map indicate the presence of different haplotypes in different individuals of the same population.

Approximately half of the *A. edulis* populations (9/17) occupy large areas (>1 km^2), but only 7 populations exceed 1000 individuals (Table 3). Most of the Iberian populations show large

occupation areas, population sizes, and stable or moderate habitat decline. By contrast, the Moroccan populations present smaller occupation areas, population sizes, and usually severe habitat decline. Only four populations from the Iberian Peninsula occupy protected areas, e.g. within Special Areas of Conservation of the Natura 2000 network or Andalusia regional system of protected areas (RENPA Network), while the areas occupied by the remaining populations lack legal protection.

The generalized linear model (Table 5) revealed significant influence for most of the geographic and population variables on the main genetic diversity and rarity parameters. Geographically, the Iberian Peninsula and Canary Islands accounted for higher genetic diversity than did Moroccan populations. Also, as expected, a significantly higher genetic diversity and rarity (Nei's diversity index, frequency down-weighted marker values, and number of rare alleles) was found in populations occupying larger areas, with higher numbers of individuals, stable populations, and locations in protected areas.

Table 5: Associations between geographical and qualitative population variables (factors) and genetic diversity and rarity (**h_{Nei}**, Nei's diversity index; Nei, 1987; **DW**, frequency down-weighted marker values; **N_r**, number of rare alleles), as tested using the generalized linear model (GLM). Geographical groups: **IP**, Iberian Peninsula; **M**, Morocco; **CI**, Canary Islands. All the values are indicated as **mean ±SE**. Different letters indicate significant differences in the multiple comparison test at **$P < 0.05$**, performed after the bootstrapped GLM.

Factor	Level	h_{Nei}	DW	N_r
Geographical group	IP	0.12 ± 0.01a	3.20 ± 0.47ab	33.66 ± 4.89a
	M	0.10 ± 0.01a	2.57 ± 0.22b	26.00 ± 5.00b
	CI	0.10 ± 0.03a	4.52 ± 0.86a	35.33 ± 11.86a
Occupation area	large	0.12 ± 0.01a	3.30 ± 0.37a	35.44 ± 4.06a
	small	0.09 ± 0.01b	2.96 ± 0.46a	24.62 ± 5.31b
Population size	large	0.12 ± 0.01a	3.29 ± 0.31a	33.57 ± 4.33a
	small	0.09 ± 0.01b	3.03 ± 0.45a	28.10 ± 5.11b
Vulnerability	stable	0.14 ± 0.01a	4.56 ± 0.28a	46.66 ± 4.91a
	declining	0.10 ± 0.01b	2.91 ± 0.41b	26.44 ± 3.99b
	critically declining	0.09 ± 0.01b	2.68 ± 0.33b	27.60 ± 7.05b
Legal status	protected	0.13 ± 0.02a	3.55 ± 0.52a	38.75 ± 4.97a
	unprotected	0.09 ± 0.02b	3.01 ± 0.34a	27.77 ± 4.01b

DISCUSSION

Genetic variability and structure

Although we are aware that AFLP-based estimates of the level of genetic variation are difficult to compare across studies (Nybom, 2004), the genetic-variation levels when standardizing sample size by population (i.e. indicating that relative differences in population diversity are not an artefact of the sampling effort) in *A. edulis* appear to approach those found in another annual species, *Hypochaeris salzmanniana* (Ortiz et al., 2007), which has a comparable distribution area (south-western Spain and Atlantic coast of Morocco). The diversity levels found are also comparable to those of other Mediterranean perennial herbs (*Edraianthus serpyllifolius* and *E. pumilio*; Surina, Schönswetter & Schneeweiss, 2011) belonging to *Astragalus* (*A. cremnophylax*; Travis, Manchinski & Keim, 1996), or even long-lived western Mediterranean trees (*Juniperus thurifera*, Terrab et al., 2008). Nevertheless, AFLPs have relatively low genetic diversity in *A. edulis* populations, compared to that of the Iberian narrow endemic steppe shrubs *Boleum asperum* (Pérez-Collazos, Segarra-Moragues & Catalán, 2008) and *Vella pseudocytisus* subsp. *paui* (Pérez-Collazos & Catalán, 2006).

Diversity as well as rarity values are particularly useful when used to compare populations or geographic areas occupied by the study species. In *A. edulis* the maximum diversity and rarity values within the Iberian distribution range correspond to the most central populations (AE4 and AE5), and within Morocco the AE8 and AE9 populations (Table 3; Fig. 1). Contrarily, on the easternmost edge of the distribution area of the species some of the lowest diversity and rarity values were found, i.e. AE6 (IP) and AE7 (M). The central parts of the Iberian distribution of this species may represent a long-term in situ survival area. By contrast, the easternmost Iberian population AE6 could be the result of a single dispersal event, the extremely low genetic-diversity and rarity values indicating a genetic bottleneck. Within Morocco AE8 is a large population (several hundred individuals) and could have acted as a source area, as confirmed also by the NJ analysis (Fig. 2A). Meanwhile, AE7, with less than 20 individuals, could also have resulted from a single dispersal event. This hypothetical fine-scale west to east colonization pattern described for the Iberian Peninsula parallels that observed in Morocco and the low diversity and rarity values found in the easternmost Iberian and Moroccan sampling sites (AE6–AE7) may indicate that the eastward colonization history of the species in these areas might have been affected by founder effects and genetic bottleneck. This mode of peripheral founder events in small populations may be key in the future genetic differentiation of populations, as described for other plant species (e.g. Tremetsberger et al., 2003; Pérez-Collazos, Segarra-Moragues & Catalán, 2008). In both the Iberian Peninsula and Morocco, aridity is higher eastwards, which on one hand may hamper future survival of these

easternmost populations but, on the other hand, may promote new genetic variants as a response to environmental selection pressure.

In the Canary Islands, diversity and rarity reached their highest levels in AE16 (Fuerteventura), and their lowest levels in AE15 (Lanzarote). Considering that both islands emerged as a single proto-island and remained together as recently as the late Pleistocene (Fernández-Palacios et al., 2011), the current *A. edulis* distribution could be the product of an ancient long-distance dispersal event, a recent long-distance dispersal event, or the result of range fragmentation. The observed diversity and rarity values seem to favour the hypothesis of a rather recent long-distance dispersal event from Fuerteventura to Lanzarote. In any case, AE15, as well as AE7 and AE6, had been affected by founder effects and genetic bottlenecks probably related to genetic drift.

The overall AMOVA analysis led to the conclusion that most of the overall genetic variation of the species could be attributed to intrapopulational (inter-individual) variability, while a smaller percentage of the total variation appeared among populations (Table 4). Comparing our findings with those resulting with AFLPs for other species from the western Mediterranean, either with similar distribution areas (Ortiz et al., 2007; Terrab et al., 2008), or Iberian narrow endemic steppe plants (Pérez-Collazos & Catalán, 2006; Pérez-Collazos, Segarra-Moragues & Catalán, 2008), we detected similar patterns and divergence levels. Also similar patterns were found for the tree *J. thurifera*, which shows a wider distribution area, and surprisingly they also parallel those shown by the perennial shrubs *B. asperum* and *V. pseudocytisus* ssp. *paui*, which are very narrow endemics from NE Spain. It is well known that long-lived and outcrossing species retain most of their genetic variability within populations and, by contrast, annual and/or selfing taxa allocate most of the genetic variability among populations (Nybom, 2004). Nevertheless, we found similar high levels of within-population diversity for the annual *A. edulis* than for the perennials *J. thurifera*, *B. asperum*, and *V. pseucocytisus* ssp. *paui*, while for the annual herb *H. salzmanniana* the levels of inter-individual (within population) genetic variability are significantly lower (Ortiz et al., 2007). These data support the idea that the levels of intrapopulation genetic diversity are relatively high for an annual species, perhaps facilitating the preservation of the gene pool of the species and, therefore, of the evolutionary processes that generate and maintain it.

Designing conservation strategies: selection of RGUCs

Astragalus edulis has a relatively high number of populations and number of individuals (at least in the large Spanish core), hampering the protection in situ of the entire distribution range

of the species, and thus populations need to be identified to apply conservation measures. To select the populations deserving protection, by means of RGUCs, we propose the consideration of factors that could have influenced the evolutionary history of the species lineages (Frankham, Ballou & Briscoe, 2009). The selection of RGUCs has enabled the estimation of the number of populations that should be targeted to sample 99.9% of the total genetic diversity of *A. edulis*. This approach helps to select particular populations that should be prioritized because they have a singular allelic composition. The probabilities of rare-allele loss indicate that the proportions that should be preserved from each geographical group should be 0.45(IP):0.34(M):0.21(CI). Considering the diversity and rarity values found for each population based on AFLP data and also this optimal proportion of populations to be sampled for conservation purposes from each geographical group, we would initially recommend the priority selection of populations AE1, AE4 and AE5 (IP), AE8 and AE9 (M) and AE16 (CI). Nevertheless, linking genetic diversity and rarity with qualitative population and conservation features, we have found that *Astragalus edulis* exhibit a significantly higher genetic diversity and rarity in populations occupying larger areas, with higher numbers of individuals, stable populations, and locations in protected areas. That is the case of populations AE4, AE5 but not of populations AE1, AE9 and AE16.

This selection of RGCUs based on AFLP data and population parameters could be complemented with the available information on haplotypes. The presence of endemic haplotypes in the three main geographical groups suggests an impact of the biogeographic barriers in the study area (Atlantic Ocean, Atlas Mountains, Alboran Sea) in shaping *A. edulis* genetic diversity and divergence. Haplotypes endemic to restricted areas represent singular genetic variants that may have evolved separately from each other and, therefore, they deserve particular conservation efforts. Within the Iberian distribution range of the species, populations AE4 and AE5 show maximum diversity and rarity values and their sampling may warrant conservation of the Iberian endemic haplotypes III and V, apart from the widely distributed haplotypes I and IV (Table 3; Fig. 3). The selection of AE1, the Iberian population with the next highest singularity value, would additionally contribute to the conservation of the endemic haplotype V. Within the Canary Islands, population AE16 registers comparatively the highest values of singularity and diversity; moreover, the selection of AE16 for conservation purposes would warrant the conservation of haplotype VII, which is endemic to these islands. Within Morocco, populations AE8 and AE9 have comparatively the highest values of singularity and diversity, but haplotypes endemic to N Africa –II and VI, which are present in populations AE11 and AE10, respectively–would not be represented by the selection of AE8 and AE9. The protection of populations AE11 and AE10 would also be highly desirable, because

in this case the evolutionary history based on the cpDNA of *A. edulis* in this geographic area would also be taken into account. Given that the Moroccan populations of this species show medium levels of genetic diversity and rarity (considering the overall values of *A. edulis*), our final decision on which particular populations from N Africa deserve priority for conservation would probably be more accurate if based on the consideration of these rare or restricted haplotypes. From this perspective, AE10 and AE11 could be prioritized over AE8 or AE9, although this decision should be taken with care given that our sampling may be low despite the results obtained from Dixon's test. The protection of large populations and smaller dispersed patches usually help preserve genetic integrity and diversity (Alexander, Liston & Popovich, 2004), but some selected RGUCs for *A. edulis* have small occupation areas and population sizes, and are critically vulnerable.

Several conservation measures could be implemented for the populations selected, e.g. studies to gather data on spatial distribution, population-size fluctuations, habitat quality, and fitness trends (Morris & Doak, 2002), reinforcement of the smallest populations, and ex situ conservation in seed banks (Peñas, 2004). Indeed, in order to preserve *Astragalus edulis* at long-term, including the evolutionary potential of its populations, are needed ex situ collections (e.g. botanical gardens and seed banks; Guerrant, Havens & Maunder, 2004) combined with any real in situ conservation value (Cavender et al., 2015).

The identification of highly representative populations based on genetic data is essential to design appropriate conservation guidelines, especially because this species is listed in a threat UICN category. In biological conservation it is useful to combine molecular data with additional environmental, ecological, and biological data sets in multidisciplinary approaches (Habel et al., 2015). The method followed here to choose RGUCs draws not only on the approach of other authors (Ciofi et al., 1999; Pérez-Collazos, Segarra-Moragues & Catalán, 2008; Segarra-Moragues & Catalán, 2010), but also on complementary phylogeographic, population, and ecological data. Therefore, could be more comprehensive and also perhaps more useful for management efforts that should prioritize populations to preserve the evolutionary potential of the species (Rumeu et al., 2014).

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Julio Peñas conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, reviewed drafts of the paper.
- Sara Barrios performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables.
- Javier Bobo-Pinilla performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, reviewed drafts of the paper.
- Juan Lorite analyzed the data, reviewed drafts of the paper.
- M. Montserrat Martínez-Ortega conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, reviewed drafts of the paper.

Supplemental Information

Supplemental information for this article can be found online at:
http://dx.doi.org/10.7717/peerj.1474#supplemental-information.

SUPPLEMENTAL INFORMATION I

Appendix 1. Probabilities of loss of 273 rare alleles when all populations of *Astragalus edulis* are considered as one single management unit and preferred sampling area. **N**, number of populations where each allele was found; **AN** allele number; Geographical groups: **IP**=Iberian Peninsula; **M**=Morocco; **CI**=Canary Islands; **Lo**, observed probability of loss of the allele; **Le**, expected probability of loss of the allele; **PSA**, preferred sampling area.

A_N	N	IP	M	CI	Lo	Le	PSA
B3	3	1	2	0	0,9510	0,9835	IP
B8	3	1	0	2	0,9352	0,9780	IP
B12	1	1	0	0	0,9889	0,9889	IP
B13	3	2	1	0	0,9510	0,9835	IP
B16	3	3	0	0	0,9510	0,9835	IP
B18	3	0	3	0	0,9352	0,9780	M
B21	2	1	1	0	0,9780	0,9889	IP
B31	3	1	2	0	0,7351	0,9047	IP
B33	1	0	1	0	0,9945	0,9945	M
B37	2	1	1	0	0,9780	0,9889	IP
B40	1	0	0	1	0,9889	0,9889	CI
B44	2	0	1	1	0,9671	0,9835	M
B46	1	1	0	0	0,9945	0,9945	IP
B47	1	0	0	1	0,9945	0,9945	CI
B53	1	0	0	1	0,9889	0,9889	CI
B55	3	2	1	0	0,9195	0,9726	IP
B56	1	0	0	1	0,9945	0,9945	CI
B58	1	0	1	0	0,9945	0,9945	M
B66	2	1	1	0	0,9780	0,9889	IP
B67	3	0	0	3	0,9041	0,9672	CI
B68	1	0	1	0	0,9945	0,9945	M
B70	1	0	1	0	0,9945	0,9945	M
B72	3	0	3	0	0,9510	0,9835	M
B73	2	0	0	2	0,9563	0,9780	CI
B74	2	0	0	2	0,9780	0,9889	CI
B81	1	0	0	1	0,9889	0,9889	CI
B82	1	0	1	0	0,9945	0,9945	M
B83	1	0	0	1	0,9945	0,9945	CI
B85	1	0	1	0	0,9945	0,9945	M
B87	1	0	0	1	0,9945	0,9945	CI
B88	3	1	2	0	0,9510	0,9835	IP
B89	2	2	0	0	0,9780	0,9889	IP
B90	3	0	0	3	0,9510	0,9835	CI
B91	1	1	0	0	0,9945	0,9945	IP
B93	1	1	0	0	0,9889	0,9889	IP
B94	1	0	0	1	0,9945	0,9945	CI

A_N	N	IP	M	CI	Lo	Le	PSA
B99	3	1	1	1	0,9352	0,9780	IP
B100	1	0	0	1	0,9945	0,9945	CI
B105	2	0	2	0	0,9780	0,9889	M
B107	1	1	0	0	0,9945	0,9945	IP
B108	1	0	0	1	0,9945	0,9945	CI
B109	2	1	1	0	0,9671	0,9835	IP
B110	1	1	0	0	0,9945	0,9945	IP
B111	2	2	0	0	0,9780	0,9889	IP
B115	1	1	0	0	0,9945	0,9945	IP
B119	2	2	0	0	0,9780	0,9889	IP
B120	1	0	0	1	0,9945	0,9945	CI
B125	3	0	3	0	0,8445	0,9459	M
B129	3	0	3	0	0,9352	0,9780	M
B130	1	0	0	1	0,9889	0,9889	CI
B136	3	1	2	0	0,9195	0,9726	IP
B139	2	0	1	1	0,9671	0,9835	M
B142	3	0	3	0	0,9352	0,9780	M
B146	1	0	1	0	0,9945	0,9945	M
B147	2	0	0	2	0,9780	0,9889	CI
B150	3	1	2	0	0,9195	0,9726	IP
B152	2	1	1	0	0,9780	0,9889	IP
B157	1	0	1	0	0,9945	0,9945	M
B162	2	1	1	0	0,9780	0,9889	IP
B166	2	0	2	0	0,9780	0,9889	M
B169	1	0	1	0	0,9889	0,9889	M
B171	3	1	2	0	0,7613	0,9148	IP
B173	3	1	1	1	0,9352	0,9780	IP
B174	2	1	1	0	0,9780	0,9889	IP
B175	3	2	1	0	0,9510	0,9835	IP
B176	1	1	0	0	0,9945	0,9945	IP
B178	1	0	0	1	0,9945	0,9945	CI
B179	1	0	1	0	0,9945	0,9945	M
B186	2	2	0	0	0,9780	0,9889	IP
B187	2	0	1	1	0,9671	0,9835	M
B189	3	1	2	0	0,9352	0,9780	IP
B190	3	1	2	0	0,9510	0,9835	IP

A_N	N	IP	M	CI	Lo	Le	PSA
B192	3	1	2	0	0,9195	0,9726	IP
B193	2	0	2	0	0,9671	0,9835	M
B194	1	1	0	0	0,9452	0,9459	IP
B196	2	1	1	0	0,9780	0,9889	IP
B197	1	1	0	0	0,9889	0,9889	IP
B198	1	1	0	0	0,9945	0,9945	IP
B199	1	0	1	0	0,9889	0,9889	M
B200	2	0	2	0	0,9780	0,9889	M
B201	3	0	3	0	0,9510	0,9835	M
B202	1	0	1	0	0,9945	0,9945	M
B203	2	0	2	0	0,9671	0,9835	M
B205	2	0	2	0	0,9780	0,9889	M
B207	1	0	0	1	0,9945	0,9945	CI
B208	1	0	1	0	0,9945	0,9945	M
B209	3	1	1	1	0,9352	0,9780	IP
B210	1	0	1	0	0,9615	0,9618	M
B211	1	0	1	0	0,9945	0,9945	M
B212	3	3	0	0	0,7882	0,9251	IP
B213	3	3	0	0	0,6729	0,8798	IP
B214	1	0	0	1	0,9945	0,9945	CI
B215	1	0	1	0	0,9945	0,9945	M
B217	2	0	2	0	0,9780	0,9889	M
B220	1	0	1	0	0,9889	0,9889	M
B221	2	0	2	0	0,8833	0,9407	M
B222	3	0	0	3	0,6729	0,8798	CI
B223	2	0	2	0	0,9780	0,9889	M
B224	1	0	1	0	0,9945	0,9945	M
B225	3	0	2	1	0,9041	0,9672	M
B227	1	0	0	1	0,9945	0,9945	CI
B228	1	0	1	0	0,9945	0,9945	M
B229	1	0	0	1	0,9945	0,9945	CI
B230	2	0	2	0	0,8833	0,9407	M
B231	1	0	0	1	0,9945	0,9945	CI
B232	2	0	2	0	0,8435	0,9200	M
B234	3	0	3	0	0,6042	0,8509	M
B236	1	0	1	0	0,9889	0,9889	M
B238	1	0	1	0	0,9945	0,9945	M
B239	1	1	0	0	0,9237	0,9251	IP
B240	2	2	0	0	0,8435	0,9200	IP
B243	1	0	0	1	0,9945	0,9945	CI
B245	1	0	0	1	0,9945	0,9945	CI
B251	3	0	0	3	0,9041	0,9672	CI

A_N	N	IP	M	CI	Lo	Le	PSA
B252	2	0	0	2	0,9780	0,9889	CI
G5	2	1	1	0	0,9780	0,9889	IP
G15	3	2	1	0	0,9352	0,9780	IP
G17	2	2	0	0	0,9563	0,9780	IP
G22	2	0	2	0	0,9671	0,9835	M
G24	1	0	0	1	0,9945	0,9945	CI
G38	1	1	0	0	0,9945	0,9945	IP
G41	1	0	1	0	0,9945	0,9945	M
G46	1	0	1	0	0,9889	0,9889	M
G53	2	1	1	0	0,8050	0,8997	IP
G54	3	2	1	0	0,8739	0,9565	IP
G55	1	0	1	0	0,9560	0,9565	M
G59	1	0	0	1	0,9945	0,9945	CI
G65	1	0	1	0	0,9889	0,9889	M
G67	2	0	2	0	0,9671	0,9835	M
G69	3	0	0	3	0,7351	0,9047	CI
G70	2	0	2	0	0,9780	0,9889	M
G71	3	0	3	0	0,7097	0,8947	M
G72	2	0	1	1	0,9563	0,9780	M
G73	3	2	0	1	0,8889	0,9618	IP
G74	1	1	0	0	0,9945	0,9945	IP
G76	2	0	2	0	0,7498	0,8701	M
G77	3	2	1	0	0,8739	0,9565	IP
G78	1	0	0	1	0,9945	0,9945	CI
G79	1	1	0	0	0,9945	0,9945	IP
G80	2	1	1	0	0,9780	0,9889	IP
G84	3	0	2	1	0,8739	0,9565	M
G87	2	1	1	0	0,9780	0,9889	IP
G91	3	0	3	0	0,9195	0,9726	M
G92	2	1	1	0	0,9780	0,9889	IP
G93	2	0	2	0	0,9671	0,9835	M
G94	3	0	3	0	0,8445	0,9459	M
G95	3	1	2	0	0,9352	0,9780	IP
G108	2	0	2	0	0,9780	0,9889	M
G115	1	0	0	1	0,9945	0,9945	CI
G116	2	0	2	0	0,9780	0,9889	M
G117	2	2	0	0	0,9780	0,9889	IP
G127	1	0	1	0	0,9945	0,9945	M
G128	3	0	1	2	0,9352	0,9780	M
G129	3	0	2	1	0,9352	0,9780	M
G130	3	0	3	0	0,6972	0,8897	M
G135	1	0	1	0	0,9945	0,9945	M

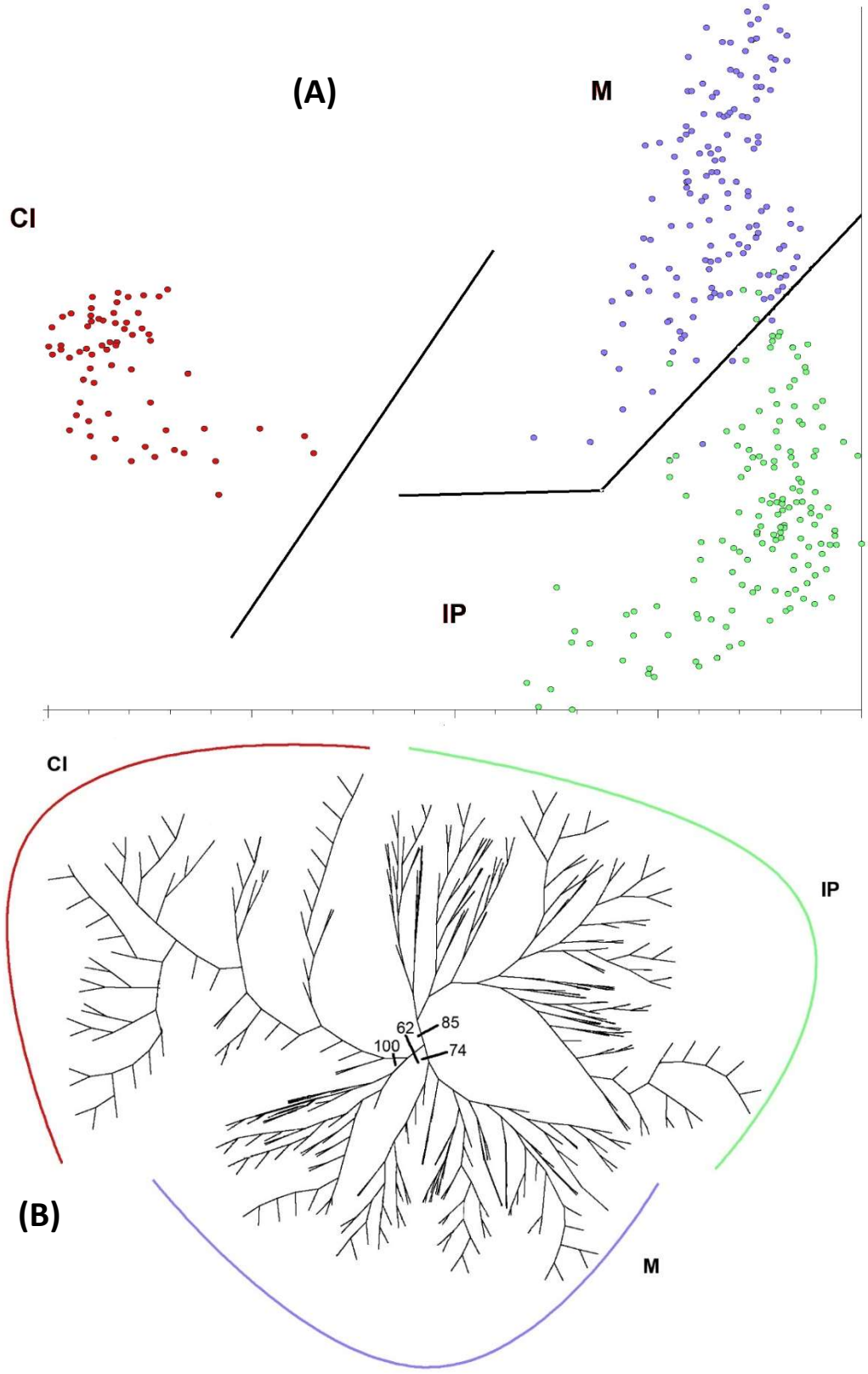
A_N	N	IP	M	CI	Lo	Le	PSA
G137	1	0	0	1	0,9945	0,9945	CI
G138	1	0	1	0	0,9945	0,9945	M
G141	1	0	0	1	0,9889	0,9889	CI
G143	1	1	0	0	0,9945	0,9945	IP
G144	3	2	1	0	0,9195	0,9726	IP
G147	1	0	0	1	0,9889	0,9889	CI
G153	1	0	1	0	0,9779	0,9780	M
G154	2	0	1	1	0,9780	0,9889	M
G155	1	0	0	1	0,9945	0,9945	CI
G157	3	0	3	0	0,7613	0,9148	M
G159	3	0	2	1	0,8889	0,9618	M
G160	1	0	1	0	0,9889	0,9889	M
G163	1	0	1	0	0,9889	0,9889	M
G164	3	0	1	2	0,9041	0,9672	M
G165	3	1	1	1	0,9510	0,9835	IP
G175	2	1	1	0	0,9780	0,9889	IP
G176	1	0	1	0	0,9945	0,9945	M
G177	2	0	0	2	0,9780	0,9889	CI
G180	1	0	0	1	0,9615	0,9618	CI
G184	3	0	2	1	0,9510	0,9835	M
G185	1	0	0	1	0,9889	0,9889	CI
G187	1	0	0	1	0,9945	0,9945	CI
G188	1	1	0	0	0,9945	0,9945	IP
G191	1	1	0	0	0,9945	0,9945	IP
G192	1	1	0	0	0,9945	0,9945	IP
G193	1	0	0	1	0,9889	0,9889	CI
G198	1	1	0	0	0,9945	0,9945	IP
G199	2	0	2	0	0,9671	0,9835	M
G212	3	2	0	1	0,9510	0,9835	IP
G214	3	3	0	0	0,9352	0,9780	IP
G217	1	1	0	0	0,9945	0,9945	IP
G218	1	1	0	0	0,9779	0,9780	IP
G219	1	1	0	0	0,9945	0,9945	IP
G220	1	0	0	1	0,9889	0,9889	CI
G221	1	1	0	0	0,9945	0,9945	IP
G222	1	0	0	1	0,9945	0,9945	CI
G223	1	1	0	0	0,9945	0,9945	IP
G224	1	0	0	1	0,9945	0,9945	CI
G225	1	1	0	0	0,9945	0,9945	IP
G226	1	0	1	0	0,9889	0,9889	M
G227	1	0	0	1	0,9945	0,9945	CI
G228	3	1	2	0	0,9510	0,9835	IP

A_N	N	IP	M	CI	Lo	Le	PSA
G231	1	0	1	0	0,9945	0,9945	M
G233	2	0	2	0	0,9563	0,9780	M
G235	2	0	0	2	0,9456	0,9726	CI
G236	2	0	2	0	0,9671	0,9835	M
G237	2	0	0	2	0,9780	0,9889	CI
G240	1	0	1	0	0,9889	0,9889	M
G241	1	0	1	0	0,9889	0,9889	M
G244	2	0	2	0	0,8732	0,9354	M
G245	1	0	1	0	0,9945	0,9945	M
G247	1	0	1	0	0,9945	0,9945	M
G250	1	0	0	1	0,9945	0,9945	CI
G252	2	0	1	1	0,9671	0,9835	M
G254	1	0	1	0	0,9889	0,9889	M
G255	1	0	0	1	0,9945	0,9945	CI
G256	2	0	2	0	0,9245	0,9618	M
G259	2	1	1	0	0,9780	0,9889	IP
G262	1	0	0	1	0,9945	0,9945	CI
G263	1	0	0	1	0,9945	0,9945	CI
G266	1	0	0	1	0,9945	0,9945	CI
G269	1	0	0	1	0,9945	0,9945	CI
G271	1	0	1	0	0,9945	0,9945	M
G272	1	0	1	0	0,9889	0,9889	M
Y19	2	1	1	0	0,9780	0,9889	M
Y110	1	0	1	0	0,9889	0,9889	M
Y143	1	1	0	0	0,9945	0,9945	IP
Y217	3	3	0	0	0,9510	0,9835	IP
Y221	3	3	0	0	0,9195	0,9726	IP
Y246	3	0	0	3	0,9352	0,9780	CI
Y275	2	0	0	2	0,9780	0,9889	CI
Y302	3	3	0	0	0,9352	0,9780	IP
Y314	3	1	2	0	0,9510	0,9835	IP
Y328	2	1	1	0	0,9671	0,9835	IP
Y349	2	2	0	0	0,9456	0,9726	IP
Y362	3	3	0	0	0,9352	0,9780	IP
Y368	2	2	0	0	0,9456	0,9726	IP
Y372	3	2	1	0	0,9510	0,9835	IP
Y390	3	1	2	0	0,9195	0,9726	IP
Y391	3	3	0	0	0,9352	0,9780	IP
Y392	1	1	0	0	0,9945	0,9945	IP
Y400	3	3	0	0	0,9352	0,9780	IP
Y404	3	1	0	2	0,9352	0,9780	IP
Y409	1	1	0	0	0,9834	0,9835	IP

A_N	N	IP	M	CI	Lo	Le	PSA
Y419	3	3	0	0	0,9195	0,9726	IP
Y425	1	0	0	1	0,9779	0,9780	CI
Y431	3	2	1	0	0,9352	0,9780	IP
Y441	3	3	0	0	0,9510	0,9835	IP
Y444	2	2	0	0	0,9563	0,9780	IP
Y453	3	2	1	0	0,9352	0,9780	IP
Y455	3	3	0	0	0,8889	0,9618	IP
Y458	3	3	0	0	0,9510	0,9835	IP
Y465	2	2	0	0	0,9671	0,9835	IP
Y466	3	3	0	0	0,9510	0,9835	IP
Y467	3	2	1	0	0,9352	0,9780	IP
Y477	3	2	1	0	0,9041	0,9672	IP
Y495	1	1	0	0	0,9945	0,9945	IP
Y496	1	0	1	0	0,9945	0,9945	M
Y507	2	2	0	0	0,9671	0,9835	IP
Y517	3	2	1	0	0,9352	0,9780	IP
Y518	3	2	1	0	0,9352	0,9780	IP
Y527	3	3	0	0	0,9352	0,9780	IP
Y531	3	2	0	1	0,9041	0,9672	IP
Y537	2	2	0	0	0,9456	0,9726	IP
Y539	2	2	0	0	0,9780	0,9889	IP
Y542	3	1	1	1	0,9352	0,9780	IP
Y545	3	2	1	0	0,9352	0,9780	IP
Y547	3	2	1	0	0,9352	0,9780	IP
Y551	2	2	0	0	0,9780	0,9889	IP
Y562	3	2	0	1	0,9510	0,9835	IP
Y568	3	3	0	0	0,9510	0,9835	IP
Y579	3	2	1	0	0,9041	0,9672	IP
Y592	2	2	0	0	0,9245	0,9618	IP
Y601	3	3	0	0	0,9041	0,9672	IP
Y603	3	2	0	1	0,9510	0,9835	IP
Y617	2	2	0	0	0,9563	0,9780	IP
Y619	3	3	0	0	0,9195	0,9726	IP

SUPPLEMENTAL INFORMATION II

Full Size Figure 2: Cluster analysis of genetic diversity, using AFLPs, in *Astragalus edulis*. (A) Neighbour-Joining analysis, **BS** values are indicated; (B) PCoA. Geographical groups: **IP**, Iberian Peninsula: **M**, Morocco: **CI**, Canary Islands.



SUPPLEMENTAL INFORMATION III

GenBank accession numbers

Sample	Accession numbers		
	trnC-rpoB	trnS-trnG	trnL-trnF
AE01_1	*KU297105*	*KU297034*	*KU297081*
AE01_2	*KU297106*	*KU297035*	*KU297082*
AE01_3	*KU297107*	*KU297036*	*KU297083*
AE01_4	*KU297108*	*KU297037*	*KU297084*
AE02_1	*KU297109*	*KU296994*	*KU297085*
AE02_2	*KU297110*	*KU296995*	*KU297086*
AE02_3	*KU297111*	*KU296996*	*KU297089*
AE02_4	*KU297112*	*KU296997*	*KU297090*
AE02_5	*KU297113*	*KU296998*	*KU297079*
AE02_6	*KU297114*	*KU296999*	*KU297080*
AE03_1	*KU297115*	*KU297000*	*KU297087*
AE03_2	*KU297116*	*KU297001*	*KU297088*
AE03_3	*KU297117*	*KU297002*	*KU297091*
AE03_4	*KU297118*	*KU297003*	*KU297092*
AE03_5	*KU297119*	*KU297004*	*KU297095*
AE03_6	*KU297120*	*KU297005*	*KU297096*
AE03_7	*KU297121*	*KU297006*	*KU297075*
AE03_8	*KU297122*	*KU297007*	*KU297076*
AE04_1	*KU297123*	*KU297008*	*KU297093*
AE04_2	*KU297124*	*KU297009*	*KU297094*
AE04_3	*KU297125*	*KU297010*	*KU297073*
AE04_4	*KU297126*	*KU297011*	*KU297074*
AE04_5	*KU297127*	*KU297012*	*KU297077*
AE04_6	*KU297128*	*KU297013*	*KU297078*
AE05_1	*KU297129*	*KU297014*	*KU297097*
AE05_2	*KU297130*	*KU297015*	*KU297098*
AE05_3	*KU297131*	*KU297016*	*KU297069*
AE05_4	*KU297132*	*KU297017*	*KU297070*
AE05_5	*KU297133*	*KU297018*	*KU297071*
AE05_6	*KU297134*	*KU297019*	*KU297072*
AE06_1	*KU297135*	*KU297020*	*KU297055*
AE06_2	*KU297136*	*KU297021*	*KU297056*
AE06_3	*KU297137*	*KU297022*	*KU297057*
AE06_4	*KU297138*	*KU297023*	*KU297058*
AE06_5	*KU297139*	*KU297024*	*KU297059*
AE06_6	*KU297140*	*KU297025*	*KU297060*
AE06_7	*KU297141*	*KU297026*	*KU297061*
AE06_8	*KU297142*	*KU297027*	*KU297062*
AE07_1	*KU297143*	*KU297028*	*KU297063*
AE07_2	*KU297144*	*KU297029*	*KU297064*
AE08_1	*KU297145*	*KU297030*	*KU297065*
AE08_2	*KU297146*	*KU297031*	*KU297066*
AE09_1	*KU297147*	*KU297032*	*KU297067*
AE09_2	*KU297148*	*KU297033*	*KU297068*
AE10_1	*KU297149*	*KU296977*	*KU297038*
AE10_2	*KU297150*	*KU296978*	*KU297039*
AE11_1	*KU297151*	*KU296979*	*KU297040*
AE11_2	*KU297152*	*KU296980*	*KU297041*
AE12_1	*KU297153*	*KU296981*	*KU297042*
AE12_2	*KU297154*	*KU296982*	*KU297043*
AE13_1	*KU297155*	*KU296983*	*KU297044*
AE13_2	*KU297156*	*KU296984*	*KU297045*
AE14_1	*KU297157*	*KU296985*	*KU297046*
AE14_2	*KU297158*	*KU296986*	*KU297047*
AE14_3	*KU297159*	*KU296987*	*KU297048*
AE15_1	*KU297099*	*KU296988*	*KU297049*
AE15_2	*KU297100*	*KU296989*	*KU297050*
AE16_1	*KU297101*	*KU296990*	*KU297051*
AE16_2	*KU297102*	*KU296991*	*KU297052*
AE17_1	*KU297103*	*KU296992*	*KU297053*
AE17_2	*KU297104*	*KU296993*	*KU297054*

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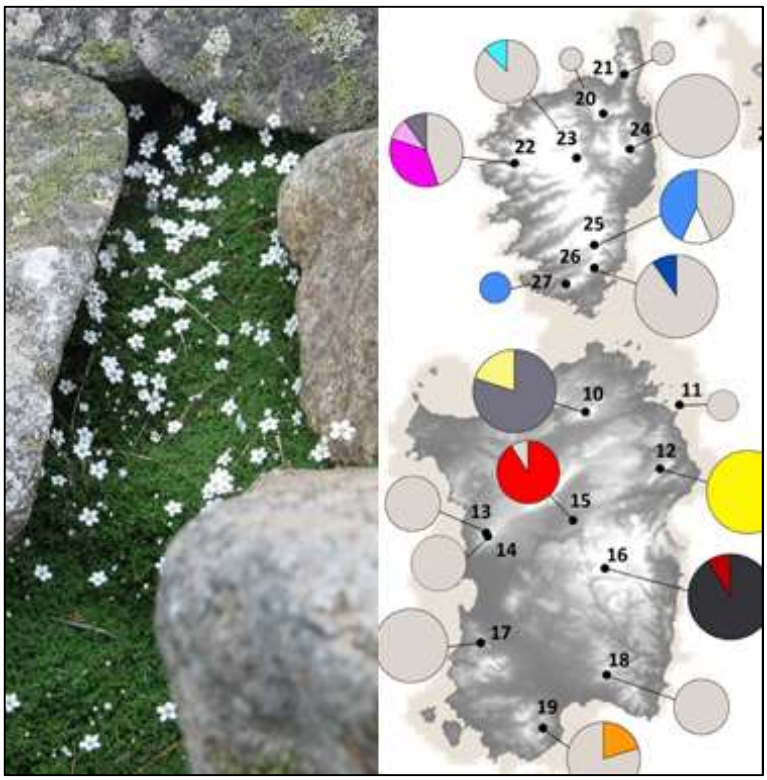
CAPÍTULO II:

Estudios sobre *Arenaria balearica*

Filogeografía de *A. balearica*

Phylogeography of *Arenaria balearica* L. (Caryophyllaceae): evolutionary history of a disjunct endemic from the Western Mediterranean continental islands



ARTÍCULO 3: Filogeografía de *Arenaria balearica* L. (Caryophyllaceae): Historia evolutiva de un endemismo con distribución fragmentada en las islas continentales del Oeste mediterráneo

Javier Bobo-Pinilla[1,2], Sara B. Barrios de León[1], Jaume Seguí Colomar[3], Giuseppe Fenu[4], Gianluigi Bacchetta[5], Julio Peñas de Giles[6] and María Montserrat Martínez-Ortega[1,2]

[1]Departamento de Botánica, Universidad de Salamanca, Salamanca, España

[2]Biobanco de ADN Vegetal, Banco Nacional de ADN, Universidad de Salamanca, Salamanca, España

[3]Departamento de Ecología Terrestre, Instituto Mediterráneo de Estudios Avanzados (IMEDEA), Esporles, España

[4]Departamento de Biología Ambiental, Universidad de Roma ''La Sapienza'', Roma, Italia

[5]Centro de Conservación de la Biodiversidad (CCB), Departamento de Ciencias de la Vida y del Medio Ambiente, Universidad de Cagliari, Cagliari, Italia

[6]Departamento de Botánica, Universidad de Granada, Granada, España

Resumen

Aunque se ha aceptado tradicionalmente que *Arenaria balearica* (Caryophyllaceae) podría ser un relicto terciario, esto nunca ha sido comprobado experimentalmente; tampoco se han investigado las razones paleohistóricas que subyacen a la distribución altamente fragmentada de la especie. Se han utilizado un total de 250 plantas, muestreadas de 29 poblaciones en todo el rango de distribución de *A. balearica* en Mallorca, Córcega, Cerdeña y el Archipiélago Toscano. Se han analizado datos de AFLP (213 individuos) y secuencias de ADN plastidial (226 individuos). Los análisis de AFLP indican una estructura geográfica y una diferenciación interpoblacional muy bajas. En base a los resultados del ADN plastidial, se probaron seis hipótesis filogeográficas alternativas utilizando Approximate Bayesian Computation (ABC). Estos análisis revelaron como escenario más probable una fragmentación ancestral de área, esto concuerda con la topología radial encontrada en la red de haplotipos, que sugiere un patrón de supervivencia a largo plazo y posterior diferenciación *in situ*. En general, se encontraron bajos niveles de diversidad genética (tanto en los AFLPs, como en diversidad haplotípica), lo que refleja el estatidsmo evolutivo a largo plazo de una especie preservada en hábitats estables.

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Corresponding author
Javier Bobo-Pinilla, javicastronuevo@usal.es

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Phylogeography of *Arenaria balearica* L. (Caryophyllaceae): evolutionary history of a disjunct endemic from the Western Mediterranean continental islands

Javier Bobo-Pinilla[1,2], Sara B. Barrios de León[1], Jaume Seguí Colomar[3], Giuseppe Fenu[4], Gianluigi Bacchetta[5], Julio Peñas de Giles[6] and María Montserrat Martínez-Ortega[1,2]

[1]Department of Botany, University of Salamanca, Salamanca, Spain
[2]Biobanco de ADN Vegetal, University of Salamanca, Salamanca, Spain
[3]Department of Terrestrial Ecology, (IMEDEA), Esporles, Spain
[4]Dipartimento di Biologia Ambientale, University of Roma ''La Sapienza'', Roma, Italy
[5]Centro Conservazione Biodiversità (CCB), University of Cagliari, Cagliari, Italy
[6]Department of Botany, University of Granada, Granada, Spain

ABSTRACT

Although it has **been** traditionally accepted that *Arenaria balearica* (Caryophyllaceae) could be a relict Tertiary plant species, this has never been experimentally tested. Nor have the palaeohistorical reasons underlying the highly fragmented distribution of the species in the Western Mediterranean region been investigated. We have analysed AFLP data (213) and plastid DNA sequences (226) from a total of 250 plants from 29 populations sampled throughout the entire distribution range of the species in Majorca, Corsica, Sardinia, and the Tuscan Archipelago. The AFLP data analyses indicate very low geographic structure and population differentiation. Based on plastid DNA data, six alternative phylogeographic hypotheses were tested using Approximate Bayesian Computation (ABC). These analyses revealed ancient area fragmentation as the most probable scenario, which is in accordance with the star-like topology of the parsimony network that suggests a pattern of long term survival and subsequent *in situ* differentiation. Overall low levels of genetic diversity and plastid DNA variation were found, reflecting evolutionary stasis of a species preserved in locally long-term stable habitats.

Subjects: Biodiversity, Biogeography, Evolutionary Studies, Genetics, Plant Science

Keywords: AFLP, *Arenaria*, Island evolution, Phylogeography, Mediterranean, Stasis, Hercynian, Plastid DNA

INTRODUCTION

Within the Mediterranean global biodiversity hotspot, the Tyrrhenian Islands represent ca. 22% of the total surface, and lodge a high percentage of endemic taxa (ca. 10-20%; *Contandriopoulos, 1990*; *Médail & Quézel, 1997*; *Bacchetta & Pontecorvo, 2005*; *Cañadas et al., 2014*). Some of these endemic plant species show narrow distributions (*Médail & Quézel, 1999*; *Thompson, 2005*; *Fenu et al., 2010*; *Bacchetta, Fenu & Mattana, 2012*), but others are distributed in the major Western Mediterranean islands. Some endemic plant species shared by Corsica, Sardinia, and the Balearic Islands have been designated ''Hercynian endemics'' (*Mansion et al., 2008*) and are often considered palaeoendemic in the broad sense of the term (i.e., ancient or relict taxa often systematically isolated, *Favarger & Contandriopoulos, 1961*; *Greuter, 1995*; *Quézel, 1995*). The present distribution of such Hercynian endemic species has been attributed to the Oligocenic connections among the Western Mediterranean islands (*Greuter, 1995*; *Quézel, 1995*; *Thompson, 2005*), but this has not been tested in all cases. Additionally, the term ''palaeoendemic'' has been restricted in concept (*Thompson, 2005*) to include only clearly ancient isolated species in large genera (or monotypic genera) that usually show little variability. There are some endemic species showing distribution patterns that seem to be concordant with the geological history of the Western Mediterranean continental fragments, which have been commonly considered palaeoendemics. But, as it has not been yet demonstrated that they are of ancient origin and do not seem to be highly isolated within large genera, these do not fit into the restrictive concept of palaeoendemism proposed by *Thompson (2005)*. These species are referred to as disjunct endemics and *Arenaria balearica* L. from the family Caryophyllaceae is a good example.

The Mediterranean region has been affected by dramatic palaeogeographical events and by formidable bioclimatic changes during the Late Tertiary and Quaternary (*Kadereit & Comes, 2005*), which have influenced the structure and composition of the flora, have contributed to shape plant species distributions, and have modelled intraspecific genetic variability of species over the past million years (*Thompson, 2005*; *Médail & Diadema, 2009*).

Like most Western Mediterranean islands, Corsica, Sardinia, and Majorca are of the continental type and have been separated from each other by tectonic and glacio-eustatic processes (*Alvarez, 1972*; *Alvarez, Cocozza & Wezel, 1974*; *Rosenbaum, Lister & Duboz, 2002*; *Mansion et al., 2008*; *Mayol et al., 2012*). The post-Oligocene [which started ca. 30 Ma (million years ago)] progressive fragmentation of land masses previously constituting part of the Hercynian belt has been described elsewhere (*Alvarez, 1972*; *Alvarez, Cocozza & Wezel, 1974*;

Rosenbaum, Lister & Duboz, 2002; *Speranza et al., 2002*; *Meulenkamp & Sissingh, 2003*; *Mansion et al., 2008*; *Salvo et al., 2010*).

The Tuscan Archipelago consists of seven small islands and several islets of different geological origins, which are also tectonic fragments that were once integrated within the Hercynian massif (*Salvo et al., 2010*). The granitic basement of Montecristo appears also to be partly a result of the volcanic activity displayed in the area over the past 10 Ma, giving rise as well to other volcanic islands in the region, such as Capraia (*Carmignani & Lazzarotto, 2004*).

With the closure of the Strait of Gibraltar (ca. 5.59 Ma; *Hsü, 1972*; *Garcia-Castellanos et al., 2009*) the Messinian Salinity Crisis of the Late Miocene started and some connections were established between North Africa, Corsica, Sardinia, and continental Europe, as well as between the Balearic Islands and Iberia; but no evidence of direct terrestrial corridors between Corsica or Sardinia and Balearic Islands have been documented (*Alvarez, 1972*; *Alvarez, Cocozza & Wezel, 1974*; *Rosenbaum, Lister & Duboz, 2002*; *Mansion et al., 2008*; *Salvo et al., 2010*). During the Messinian, the Tuscan Archipelago may have connected Corsica, Sardinia, and the Italian Peninsula. The cycles of desiccation and transgression of the Mediterranean Sea in this period enabled interchanges of lineages of biota that predated the Messinian Salinity Crisis in all these territories (e.g., *Salvo et al., 2010*; *Molins et al., 2011*). The subsequent reopening of the Strait of Gibraltar (ca. 5.33 Ma; *Krijgsman et al., 1999*; *Garcia-Castellanos et al., 2009*) caused partial extinction and isolation of previously connected populations and seems to have promoted vicariant speciation and population divergence at least in some documented cases (e.g., *Quercus ilex* L. in *Lumaret et al., 2002*; *Anchusa crispa* Viv. in *Quilichini, Debussche & Thompson, 2004*; *Borago* L. in *Selvi, Coppi & Bigazzi, 2006*; *Abies* spp. in *Terrab et al., 2007*; *Anchusa* L. in *Bacchetta et al., 2008*; *Anchusa* L. in *Coppi, Mengoni & Selvi, 2008*; *Rodríguez-Sánchez et al., 2008*; *Salvo et al., 2008*; *Cephalaria* gr. *squamiflora* (Sieber) Greuter in *Rosselló et al., 2009*; *Bacchetta et al., 2012*; *Aquilegia* L. in *Garrido et al., 2012*).

The subsequent establishment of the Mediterranean climate (ca. 3–2 Ma) promoted the expansion of xerophytic elements and typically Mediterranean taxa (*Suc, 1984*; *Thompson, 2005*). Later, the cyclical climatic oscillations of the Quaternary Pleistocene (ca. 1.8– 0.01 Ma) also significantly shaped the genetic structure and spatial distribution of the biota, leading to population differentiation and eventually to speciation (*Hewitt, 1999*). Particularly, during the Pleistocene glacial maxima the sea level was approximately 120–150 m lower than at present (*Yokohama et al., 2000*; *Church et al., 2001*; *Clark & Mix, 2002*; *Lambeck & Purcell, 2005*) and the Corsican and Sardinian coastlines were directly connected by land bridges (*Salvo et al., 2010*).

These connections facilitated exchanges of plant species and have alternatively limited or favoured gene flow between populations of species distributed in both islands and probably also among them and the Tuscan islets (Fig. 1).

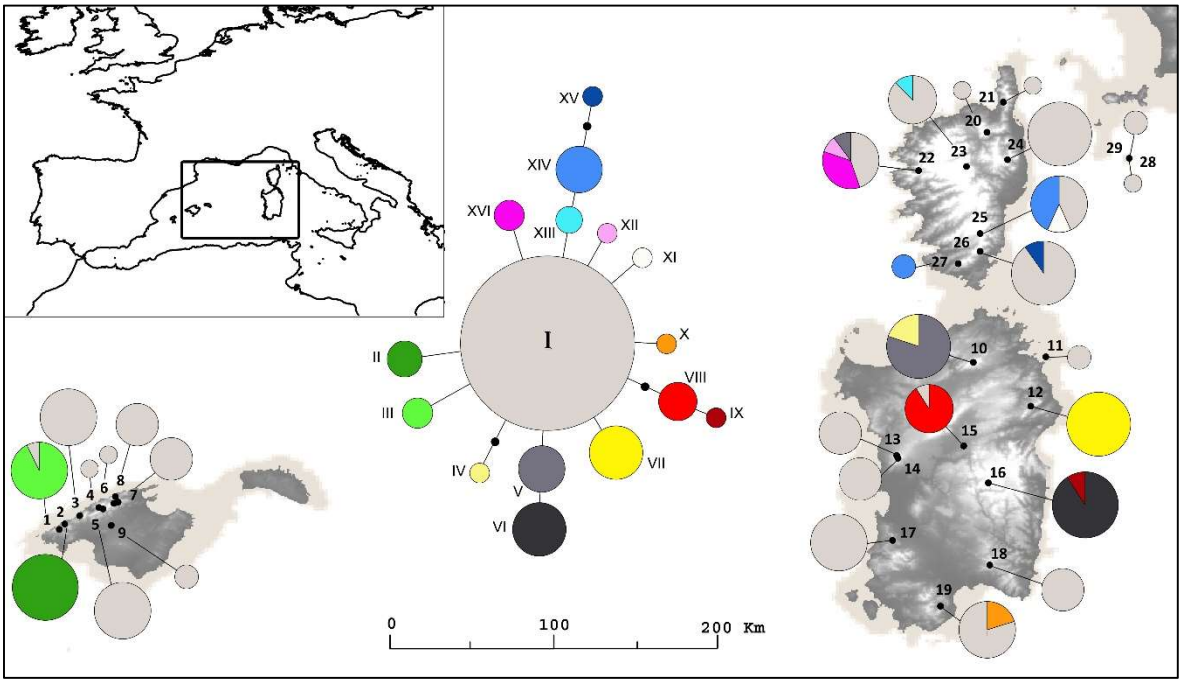


Figure 1: Sampling localities and Haplotypes. Sampling localities of *Arenaria balearica*, reconstruction of the coast line during the Last Glacial Maximum in the study area, spatial distribution of plastid DNA haplotypes and statistical parsimony network for 226 individuals. The small black circles represent missing intermediate haplotypes. Sectors within circles in the map indicate the presence of different haplotypes in different individuals of the same population.

Several Mediterranean disjunct endemic species show high levels of morphological stability despite long-term isolation among populations distributed in different continental fragment islands (*Molins et al., 2011*, 3.2 Ma). The constancy of morphological characters over long time periods has frequently been related to low molecular evolutionary rates, although this may not be completely clear in all cases (*Casane & Laurenti, 2013*) and, recently, high levels of plastid DNA (cpDNA) diversity have been reported for the Tyrrhenian endemic *Thymus herba-barona* Loisel. (*Molins et al., 2011*). Also the apparent inconsistency between the fact that the Mediterranean region has undergone dramatic geological as well as climatic changes and the long persistence of Mediterranean endemic species has been explained as the result of reduced and isolated, but particularly stable, habitats (e.g., rocky habitats) suitable for species survival,

within a sea of unsuitable landscapes (*Hampe & Petit, 2005*; *Thompson, 2005*; *Youssef et al., 2010*; *Molins et al., 2011*; *Mayol et al., 2012*). Although *A. balearica* has been cited (*Molins et al., 2011*) as an example of evolutionary stasis (low levels of morphological variation paralleled with low sequence variation), this has never been demonstrated.

Arenaria balearica is naturally distributed in Tyrrhenian islands of Majorca, Corsica, and Sardinia, including the surrounding minor islands of Tavolara, La Maddalena, Caprera, and Asinara, and in two of the main Tuscan Islands, Montecristo and Capraia (*Diana Corrias, 1981*). Most of the populations known from Majorca, Corsica and Sardinia are placed on the Hercynian basement of the corresponding island (*Alvarez, Cocozza & Wezel, 1974*; *Rosenbaum, Lister & Duboz, 2002*). The species is an alien plant in some European countries, where it is used as an ornamental. Due to its distribution pattern and to the fact that the plant usually inhabits plant communities having a notable relict character (*Bolòs & Molinier, 1958*), *A. balearica* has been traditionally considered to be a Mediterranean paleoendemic in the broad sense of the term (*Favarger & Contandriopoulos, 1961*), and a disjunct endemism by *Thompson (2005)*. The plant produces small seeds (0.5–0.6 mm) and although it lacks any evident adaptation to long-distance dispersal (LDD), such events due to stochastic mechanisms, even human mediated (*López González, 1990*), cannot be *a priori* ruled out to explain its current distribution pattern.

Previous studies on phylogeographic patterns of Mediterranean disjunct endemic species have focused on examples from the Eastern Mediterranean region (e.g., *Affre & Thompson, 1997*; *Widén, 2002*; *Bittkau & Comes, 2005*; *Edh, Widén & Ceplitis, 2007*), as well as from the Western Mediterranean region, including species distributed in Majorca and Menorca (e.g., *Sales et al., 2001*; *Molins, Mayol & Rosselló, 2009*) and Corsica and Sardinia (e.g., (*Falchi et al., 2009*). *Molins et al. (2011)* have studied *T. herba-barona*, a disjunct endemic that shows a distribution similar to that of *A. balearica* except for the facts that the former is not as widespread neither in Majorca (only one population) nor in Sardinia as *A balearica* and that it is absent from the islets of the Tuscan Archipelago.

Using both sequencing of plastid DNA regions and amplified fragment length polymorphism (AFLP) fingerprinting, this study aims to reconstruct the phylogeographic patterns and differentiation of intraspecific lineages within the disjunct endemic plant *A. balearica.* More specifically our objectives are: (1) to test to which extent the observed distribution of *A. balearica* is concordant with the geological history of the continental fragment islands from the Western Mediterranean region; (2) to give a satisfactory answer to the question on how the colonization of the different islands and islets took place; and (3) to evaluate whether the

low morphological variation observed among populations of *A. balearica* located in different islands is in correspondence with overall low levels of genetic diversity.

MATERIALS AND METHODS

Reconstruction of the coastline during the Last Glacial Maximum in the study area

During the Last Glacial Maximum (LGM), ice sheets covered large areas in northern latitudes, and global temperatures were significantly lower than today (*Yokohama et al., 2000*). At the LGM, the Earth's ocean levels were at their lowest point and extensive reaches of dry land were exposed along the continents' coasts. Several analyses have substantially narrowed the uncertainties regarding total changes in ice sheets and sea level and their proxies, suggesting a net decrease in the eustatic sea level at the LGM ranging from 120 to 135 m a.s.l. (*Church et al., 2001*; *Clark & Mix, 2002*). The reconstruction of coastlines at 21 Ka (kiloyears before present) for the study area presented here (Fig. 1) is derived from these references.

To map the past and current shorelines in detail, the present-day topographic and bathymetric data covering the area were taken from the ETOPO1, which is a 1 arc-minute global relief model of the Earth's surface that integrates land topography and ocean bathymetry. This model was built from numerous global and regional data sets, and is available in "Bedrock" (base of the ice sheets) versions (*NOAA, 2009*). Estimates of exposed land area at LGM with respect to the present-day are the result of the values of the Digital Elevation Model being raised by 120 m.

Study species

Arenaria balearica is an herbaceous perennial delicate plant whose filiform, branched stems and small leaves form low, compact ever-green moss-like dense mats, preferentially on cool, moist soils in shaded rocky places (comophyte), although it can be secondarily found also on shady moist slopes, between 0 and 1,800 m a.s.l. (*Diana Corrias, 1981*; *López González, 1990*). Although there are no available data on the reproductive biology of the species, its slender, short, upright stems that bear white, actinomorphic flowers suggest that it is probably partly wind, and partly insect pollinated. Its chromosome number is $2n = 18$ (*Diana Corrias, 1981*; *López González, 1990*). Generation times are not known for the species. The available phylogenetic data based on the analysis of DNA sequences (*Fior & Karis, 2007*) indicate that this species is closely related to *Arenaria bertolonii* Fiori, which is distributed primarily in mainland Italy (*Iamonico, 2013*) and Sardinia (*Conti et al., 2005*). The most recent phylogeny published for the genus *Arenaria* L. (*Sadeghian et al., 2015*) concluded that. *A. balearica* should be excluded from *A.*sect. *Rotundifoliae* McNeill, where the species was traditionally included.

Unfortunately, these authors did not include *A. bertolonii* in the phylogeny and recovered *A. balearica* in a largely unresolved position (very low levels of statistical support).

Sampling strategy, outgroup selection and monophyly test

Leaf material from a total of 250 plants from 29 sampling sites including the islands of Majorca (9), Corsica (8), Sardinia (9), Tavolara (1), and Montecristo (2), representing the entire distribution range of *A. balearica*, was collected and dried in silica gel (Table 1 and Fig. 1). Each sampling site was geo-referenced with a GPS GARMIN GPSMAP 60, and voucher specimens were deposited at the herbaria of the University of Salamanca (SALA), of the University of Granada (GDA) in Spain and/or of the University of Cagliari (CAG) in Sardinia, Italy.

The intent was to include a minimum of 10–12 plants per population in the analysis, but sometimes the population sizes were small and it was not possible to collect such a quantity of well separated (>5–10 m) individuals. Also, further problems were encountered in some cases in the DNA extraction and amplification processes (the leaves are only 2–4 mm and it was many times difficult to get an adequate quantity of DNA). In this situation, a variable number of 1–16 plants per sampling site were finally used (Table 1).

Three additional samples from *A. bertolonii* were selected to be used as outgroup in the plastid DNA haplotype analyses. Given the uncertain phylogenetic position of *A. balearica* within the genus according to the most recent data (*Sadeghian et al., 2015*), the selection of this outgroup was based on the results by *Fior & Karis (2007)*. Furthermore, the monophyly of the study group was assessed in a parallel study (J Bobo-Pinilla, J Peñas de Giles & MM Martínez-Ortega, 2013, unpublished data) through the phylogenetic analysis of nucleotide sequences of the nuclear ribosomal internal transcribed spacer (ITS) using 28 samples belonging to *A. balearica* and several other samples from the related species *A. funiculata* (Pau) Fior & P.O. Karis, *A. tejedensis* (Willk.) Fior & P.O. Karis and *A. suffruticosa* Fior & P.O. Karis. These data further support the sister group relationship between *A. balearica* and *A. bertolonii* already proposed by *Fior & Karis (2007)*.

DNA isolation, AFLP amplification, and data analysis

Total genomic DNA was isolated from crushed dried leaf material (ca. 25 mg) following the 2× CTAB (cetyl trimethyl ammonium bromide) protocol (*Doyle & Doyle, 1987*) with minor modifications. The quality of the extracted DNA was checked in 1% TAE-agarose gel. A negative

control sample was consistently included to test for contamination, and five randomly chosen samples were replicated to test for reproducibility.

Sampling locality	*DIYABC Assign.*	*Elevation (m.a.s.l.)*	*Long./Lat.*	*NAFLP*	*Nei's GD*	*DW*	*Hcp*
1: SP; Majorca, Estellencs,Puig de Galatzó	MAJ	962	2.48°/39.63°	11	0.096	5.872	I (1); III (11)
2: SP; Majorca, Banyalbufar, Mola de Planicia	MAJ	726	2.52°/39.67°	10	0.098	4.491	II (9)
3: SP; Majorca, Valldemossa, Puig des Teix	MAJ	906	2.63°/39.73°	10	0.119	6.775	I (9)
4: SP; Majorca, Escorca, Puig Major	MAJ	847	2.77°/39.79°	–	–	–	I (2)
5: SP; Majorca, Escorca, Tossals	MAJ	972	2.80°/39.78°	10	0.110	5.625	I (9)
6: SP; Majorca, Escorca, Clot d'Albarca	MAJ	468	2.88°/39.82°	–	–	–	I (1)
7: SP; Majorca, Escorca, Puig Tomir	MAJ	882	2.91°/39.83°	10	0.189	14.83	I (7)
8: SP; Majorca, Escorca, Puig Caragoler	MAJ	753	2.89°/39.87°	8	0.095	7.083	I (7)
9: SP; Majorca, Escorca, Puig d'en Galileu	MAJ	879	2.85°/39.81°	9	0.119	5.653	I (4)
10: IT; Sardinia, Madonna del Limbara	NSA	1,23	9.16°/40.85°	10	0.167	8.518	IV (2); V (8)
11: IT; Sardinia, Olbia, Tavolara	-	470	9.69°/40.89°	5	0.179	13.208	I (5)
12: IT; Sardinia, Lula, Punta Turuddò	NSA	1,094	9.58°/40.53°	9	0.161	8.894	VII (10)
13: IT; Sardinia, Cuglieri, La Madonnina	SSA	802	8.60°/40.17°	8	0.135	7.506	I (6)
14: IT; Sardinia, Santu Lussurgiu, Zorzia	SSA	978	8.61°/40.15°	9	0.151	6.808	I (7)
15: IT; Sardinia, Oliena, Monte Corrasi	NSA	980	9.09°/40.24°	9	0.136	6.936	I (1); VIII (7)
16: IT; Sardinia, Desulo, Taccu di Girgini	NSA	120	9.27°/39.97°	16	0.103	5.664	VI (10); IX (1)
17: IT; Sardinia, Guspini, Montevecchio	SSA	276	8.57°/39.55°	8	0.179	9.914	I (9)
18: IT; Sardinia, Burcei, Monte Serpeddi	NSA	856	9.28°/39.37°	10	0.129	6.700	I (7)
19: IT; Sardinia, Villa S. Pietro, Monte Nieddu	SSA	183	8.92°/39.07°	10	0.123	6.744	I (7); X (2)
20: FR; Corsica, Cap Corse, Commune d'Olmeta	COR	800	9.69°/42.75°	–	–	–	I (1)
21: FR; Corsica, Massif de Monte Astu	COR	1025	9.26°/42.53°	–	–	–	I (1)
22: FR; Corsica, Gorges deSpelunca	COR	233	8.76°/42.25°	10	0.187	10.881	I (4); V (1); XII (1); XVI (3)
23: FR; Corsica, Valle de la Restonica	COR	492	9.11°/42.28°	8	0.182	13.227	I (7); XIII (1)
24: FR; Corsica, Valle de'Alesani	COR	677	9.41°/42.33°	10	0.163	9.794	I (10)
25: FR; Corsica, Col de Bavella	COR	1,317	9.21°/41.79°	9	0.169	8.681	I (4); XI (1); XIV (4)
26: FR; Corsica, Piscia di Ghjadu	COR	209	9.21°/41.66°	10	0.194	13.182	I (9); XV (1)
27: FR; Corsica, Gianuccio	COR	537	9.05°/41.57°	4	0.200	12.906	XIV (3)
28: FR; Montecristo, Collo a fundo	–	460	10.31°/42.32°	–	–	–	I (1)
29: FR; Montecristo, Grotta del Santo	-	251	10.30°/42.34°	–	–	–	I (2)

Table 1: Sampling **localities and genetic data.** Population names and sampling localities, AFLP descriptors and plastid DNA haplotypes for the studied populations of *Arenaria balearica*. **SP**, Spain; **IT**, Italy; **FR**, France; **NAFLP**, number of individuals investigated with AFLP**; Nei's GD**, *Nei's (1987*) gene diversity; **DW**, frequency down-weighted marker values; $\mathbf{H_{CP}}$, plastid DNA (cpDNA) haplotypes derived from concatenated sequences, the number of individuals per haplotype per population is given in parentheses.

Given the very small leaf size of *A. balearica*, it was not always possible to extract enough DNA to provide clear and reliable AFLP profiles. Therefore, five populations among the 29 initially sampled had to be excluded from the AFLP analysis (Table 1). AFLP profiles were finally drawn for 213 individuals following established protocols (*Vos et al., 1995*). An initial screening of selective primers was performed using 26 primer combinations. The four finally selected primer combinations (fluorescent dye in brackets), (6-FAM) *Eco*RI-ACT/*Mse*I-CAT, (6-FAM) *Eco*RI-AGA/*Mse*I-CTG, (VIC) *Eco*RIAAG/*Mse*I-CAT, (VIC) *Eco*RI-AGG/*Mse*I-CC, were used for the selective polymerase chain reaction. These combinations were selected because they generated a relatively high number of clearly reproducible bands. A relatively high number of alleles per individual is desirable, given that AFLP are dominant markers (*Lowe, Harris & Ashton, 2004*). Samples (3 µl) of the fluorescence-labelled selective amplification products were combined and separated on a capillary electrophoresis sequencer (ABI 3730 DNA Analyser; Applied Biosystems, Foster City, CA, USA), with GenScan ROX (Applied Biosystems) as an internal size standard.

Raw AFLP data with amplified fragments from 150 to 500 base pairs (bp) were scored and exported as a presence/absence matrix using the software GeneMapper 4.0 (Applied Biosystems). As an initial approach to the global genetic relationships among the individuals analysed and possible structure of the data, a Neighbour-Joining (NJ) analysis including 1,000 bootstrap pseudoreplicates based on a matrix of Nei-Li (*Nei & Li, 1979*) distances was conducted with the software Paup 4.0b10 (*Swofford, 2003*). An unrooted NeighbourNet was also produced using the program SplitsTree 4.12.3. *Huson & Bryant (2006)* and based on Dice's coefficient, which is suitable for multilocus dominant genetic data (*Dice, 1945*; *Lowe, Harris & Ashton, 2004*). Additionally, a Principal Coordinate Analysis (PCoA) based on a matrix of Dice's coefficient among individuals was performed with NTSYS-pc 2.02 (*Rohlf, 2009*).

Population genetic structure was additionally investigated using a Bayesian clustering method implemented in STRUCTURE v. 2.3.4 (*Pritchard, Stephens & Donnelly, 2000*) following the approach described by *Falush, Stephens & Pritchard (2007)* for dominant markers. This method uses a Markov chain Monte Carlo simulation approach to group samples into an optimal number of *K* genetic clusters and does not assume an a *priori assignment* of individuals to populations, nor to clusters. Analyses were based on an ancestral admixture model with correlated allele frequencies among populations.

The proportion of membership of each individual and population to the *K* clusters was calculated by performing 20 runs for each *K* value between 2 and 9 with a run length of the

Markov chain Monte Carlo of 1×10^6 iterations after a burn-in period of 1×10^6 iterations, with λ adjusted at 0.4523. The optimal number of *K* clusters was estimated using the *ad hoc* parameter (*1K* statistic) of *Evanno, Regnatus & Goudet (2005)*, as implemented in the online application of Structure Harvester software (v0.63; *Earl & Vonholdt, 2012*).

Although aware that AFLP-based estimates of the level of genetic variation could be biased in this case by low sampling sizes and relative differences in sampling effort, *Nei*'s (*1987*) gene diversity index was calculated for each population (or sampling site) using the R package AFLPdat (*Ehrich, 2006*). This package was also used to calculate the frequency down-weighted marker values per population or sampling site (DW; *Schönswetter & Tribsch, 2005*), which is an estimation of the genetic rarity of a population.

To test the comparative historical effects of the main biogeographical barriers, a hierarchical analysis of molecular variance (AMOVA) was performed with the software ARLEQUIN 3.5.1.2 (*Excoffier & Lischer, 2010*). For this, genetic variation was distributed into portions assignable to differences among predefined geographical groups (F_{CT}), among populations within these groups (F_{SC}), and among populations across the entire study area (F_{ST}) (*Turner et al., 2000*; *Ortiz et al., 2009*). Additionally, four alternative groupings were tested using AMOVA analysis: the first two tested the groups derived from PCoA and NJ analyses, respectively, while the third and fourth ones tested two additional geographical groupings (i.e., (Majorca) (Corsica) (Sardinia + Tavolara) and (Majorca) (Corsica + Sardinia + Tavolara), respectively).

Plastid DNA sequencing and data analysis

Three regions of the plastid DNA were sequenced and haplotype variation was explored to complement the information given by the mainly nuclear AFLPs. The plastid regions *trn*L^{UAA}-*trn*F^{GAA} (*Taberlet et al., 1991*), *psbA-3*^{0} *trnK-matK* and *rpS16* (*Shaw et al., 2005*) showed the highest variability among seven surveyed regions [*trnQ (UUG)-rps16x1, trnLrpl32F*, *atpI-atpH, Shaw et al., 2007*; *rpoB-trnC*, *trnH-psbA, Shaw et al., 2005*] and were used to analyse a total of 226 plants from 29 populations (Table 1) of *A*. *balearica*. PCR conditions and primers for DNA amplification are detailed in Table 2. PCR products were visualized on 1% agarose gel and purified using PCR Clean-Up with ExoSAP-IT Kit (AFFIMETRIX, Santa Clara, CA, USA) following the manufacturer's instructions. The cleaned amplification products were analysed with a 3730 DNA Genetic Analyser capillary sequencer (Applied Biosystems). All sequences can be found in the Supplemental Information (Data S2 and S3).

Congruence in the phylogenetic signal of the different plastid DNA regions was tested with the partition homogeneity test (ILD; *Farris et al., 1995a*; *Farris et al., 1995b*). ILD significance values were calculated in TNT v.1.1 (*Goloboff, Farris & Nixon, 2003*) with the INCTST script—kindly provided by the authors of the program—with 1,000 replicates. The plastid DNA sequences were assembled and edited using Geneious pro™ 5.4 (*Drummond et al., 2012*) and aligned with ClustalW2 2.0.11 (*Larkin et al., 2007*); further adjustments and optimisations were made by visual inspection. Sequences from the three regions were concatenated based on the assumption that the plastid forms a single linkage group into a single matrix to be analysed, considering also that the ILD test did not report significant incongruities among DNA regions. Gaps (insertions/deletions) were coded as single-step mutations and treated as a fifth character state. Mononucleotide repeats of different sizes were excluded given that they seem to be prone to homoplasy at large geographic scales (*Ingvarsson, Ribstein & Taylor, 2003*).

cpDNA region	*Forward primer*	*Reverse primer*	*Denaturation Temp./Time*	*Annealing Temp./Time*	*Extension Temp./Time*	*Cycles*	*S*	*I*
$trnL^{UAA}$ $trnF^{GAA}$ [a]	tabC	tabF	95 °C/30''	57 °C/30''	72 °C/1'30''	35	3	11
psbA-'trnKmatK [b]	matK8F	psbA5'R	95 °C/30''	52 °C/30''	72 °C/1'30''	35	3	3
rpS16[b]	rpS16F	rpS16R	95 °C/30''	55 °C/30''	72 °C/1'30''	35	5	8

Table 2: PCR values. PCR primers and conditions used to obtain plastid DNA sequence data for *A. balearica*; number of substitutions (*S*) and number of indels (*I*). Notes: [a]*Taberlet et al. (1991);* [b]*Shaw et al. (2005).*

The completeness of haplotype sampling across the range of *A. balearica* was estimated using the Stirling probability distribution. It provides a way to evaluate the assumption that all haplotypes have been sampled (*Dixon, 2006*).

As an approach to infer the genealogical relationships among haplotypes, an unrooted haplotype network was constructed using the statistical parsimony algorithm (*Templeton, Crandall & Sing, 1992*) as implemented in TCS 1.21 (*Clement, Posada & Crandall, 2000*).

Six competing phylogeographic hypotheses were compared using a coalescent based approximate Bayesian computation method (ABC approach), as implemented in DIYABC v2.1 software (*Cornuet et al., 2014*). DIYABC allows testing the posterior probabilities of alternative scenarios involving complex population histories (i.e., any combination of population divergences and multifurcations, admixture events, population size changes, bottlenecks, etc., even with population samples potentially collected at different times and/or with unsampled

populations, *Cornuet et al., 2014*).The logistic regression procedure (*Fagundes et al., 2007*) gives an estimate of the occurrence of each scenario among simulated data sets that are closest to the observed data. In our case, four different metapopulations (i.e., Majorca, Corsica, NE Sardinia and SW Sardinia, correspondingly MAJ, COR, NSA and SSA in Table 1) were considered. Due to low sample sizes and considering that only the most widely represented haplotype was present, populations 11, 28 and 29 were excluded from this analysis in order to avoid increasing exponentially computation times.

The distinction between NE Sardinia and SW Sardinia (Table 1) was made considering relevant geological aspects, particularly the fact that the populations of *A. balearica* present in the island are located exclusively on two different geological units both located on the ancient Hercynian basement of the island and mainly separated by Oligocene and Miocene rift basins and Plio-Pleistocene basalts (*Rosenbaum, Lister & Duboz, 2002*). After some initial analysis and taking into account the haplotype network, the geographical distribution of the species and these geological aspects, six competing phylogeographic scenarios were designed. A list of all parameters and prior distributions used to model scenarios is summarized in Table 3. Prior distributions of the parameters were chosen as a first approach with a large interval due to the lack of ancestral information.

Parameters were subsequently corrected according to values obtained after first tests. Population sizes were set equally in all cases; divergence times were taken unrestricted to allow the program to set the most likeable value. Uniform Mutation rate was set to (10^{-9}–10^{-7}). One million data sets were simulated for each scenario (*Cornuet et al., 2008*; *Cornuet, Ravigné & Estoup, 2010*).The posterior probabilities of each one were calculated by performing a polychotomous weighted logistic regression on the 1% of simulated data sets closest to the observed data set (*Cornuet et al., 2008*; *Cornuet, Ravigné & Estoup, 2010*). The posterior distributions of parameters were evaluated under the best scenario using a local linear regression on the 1% closest simulated data sets with a logit transformation (Table 3). Bias and precision for the parameters estimations were also calculated. Divergence time between groups must be taken carefully, due to the lack of information about generation times for the species. Confidence in scenario choice has been tested by evaluating Type I and Type II error rates (*Cornuet, Ravigné & Estoup, 2010*).

				Prior Distribution		Estimated Parameters	
Parameter	Scenario	Code	Type	Initial Interval	Final Interval	Mean	Median
Population effective sizes of the MAJ group	All	Nmaj	Uniform	{10–100.000}	{10–6.000}	4.500	4.490
Population effective sizes of the COR group	All	Ncor	Uniform	{10–100.000}	{10–30.000}	24.700	26.100
Population effective sizes of the NSA group	All	Nnsa	Uniform	{10–100.000}	{10–5.000}	1.790	1.940
Population effective sizes of the SSA group	All	Nssa	Uniform	{10–100.000}	{10–18.000}	16.000	16.600
Founder event for MAJ group		NFmaj	Uniform	{10–500}	{10–500}		
Founder event for COR group		NFcor	Uniform	{10–500}	{10–500}		
Founder event for NSA group		NFnsa	Uniform	{10–500}	{10–500}		
Founder event for SSA group		NFssa	Uniform	{10–500}	{10–500}		
Divergence time corresponding to ancestral area fragmentation	1	T1	Uniform	{10–1.000.000}	{10–10.000}	4.640	4.730
Divergence time between NSA and SSA	2 & 5	T2 & T9	Uniform	{10–1.000.000}	{10–20.000}		
Divergence time corresponding to differentiation into three main islands	2	T3	Uniform	{10–1.000.000}	{10–20.000}		
Divergence time between COR and NSA	3	T4	Uniform	{10–1.000.000}	{10–30.000}		
Divergence time between SSA and MAJ	3	T5	Uniform	{10–1.000.000}	{10–15.000}		
Divergence time between [SSA+MAJ] and [COR+NSA]	3	T6	Uniform	{10–1.000.000}	{10–40.000}		
Divergence time between COR and MAJ	4	T7	Uniform	{10–1.000.000}	{10–10.000}		
Divergence time among [COR+MAJ], SSA and NSA	4	T8	Uniform	{10–1.000.000}	{10–20.000}		
Divergence time between COR and Sardinia	5	T10	Uniform	{10–1.000.000}	{10–10.000}		
Divergence time between MAJ and [NSA, SSA and COR]	5	T11	Uniform	{10–1.000.000}	{10–20.000}		
Divergence time among groups in Corsica and Sardinia	6	T12	Uniform	{10–1.000.000}	{10–15.000}		
Divergence time for initial isolation of MAJ	6	T13	Uniform	{10–1.000.000}	{10–20.000}		
Mean mutation rate	All	Mμ	Uniform	$\{10^{-9}–10^{-7}\}$	$\{10^{-9}–10^{-7}\}$	6,48E – 08	6,44E – 08

Table 3: Parameters used in DIYABC analyses.

RESULTS

Population structure based on AFLP

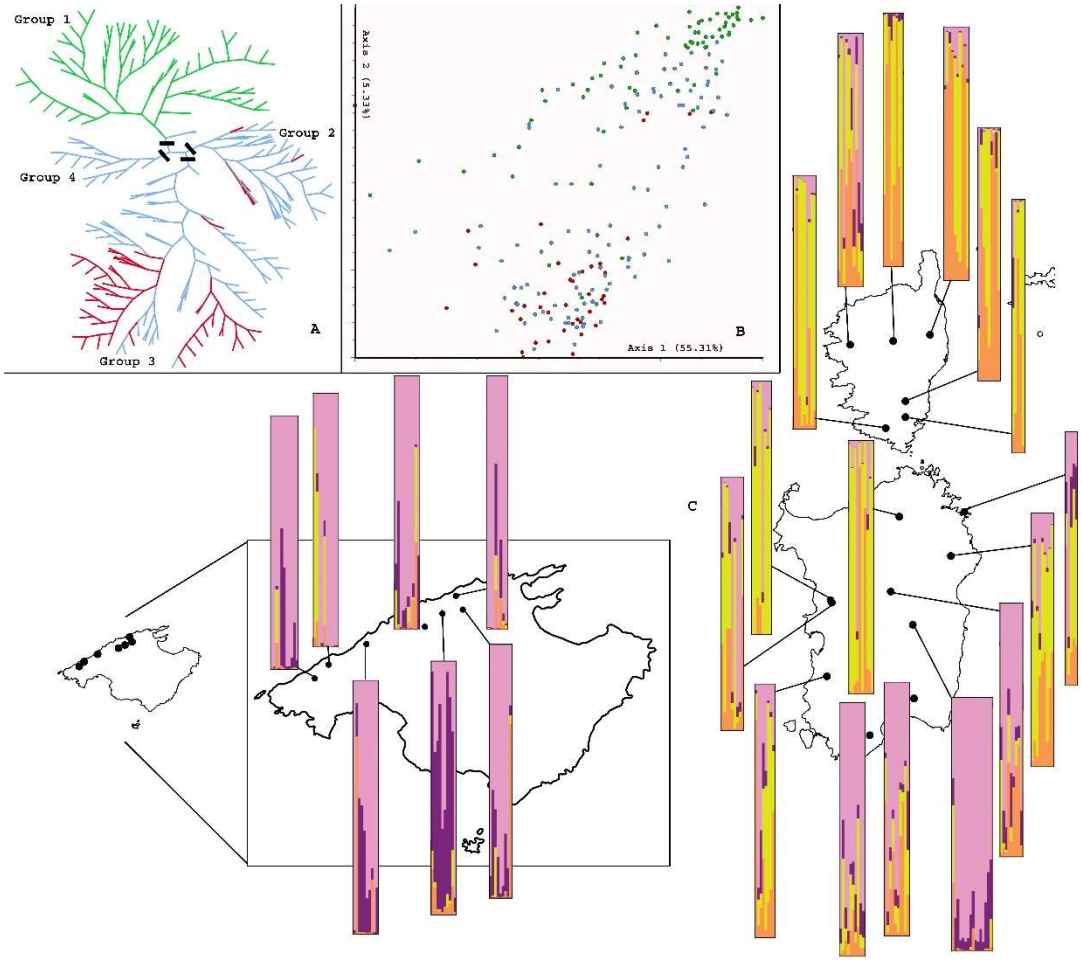
The four primer combinations applied to 213 plants representative of the variation of the species *A. balearica* produced a total of 792 reproducible fragments.

Both the NJ and NeighbourNet diagrams conducted on all individuals revealed a relatively weak overall structure of the genetic variation into two main groups: one comprised the samples collected in Majorca (''group 1'', represented in green in Fig. 2A; populations 1–3, 5, 7–9; with not significant bootstrap support, BS < 75%) and a second poorly supported group (BS < 75%), which clustered together individuals from the remaining populations included in this study. Within the second group, three further subgroups were found: first, ''group 2'', which included samples collected mostly in C and S Sardinia (populations 14, 15, 18 and 19); second, ''group 3'', which grouped populations 10–13, plus 17 from W and NE Sardinia and Tavolara, together with populations 23–27 mostly from S Corsica; and third, ''group 4'', which included all the individuals from population 16 in C Sardinia. None registered significant BS values (BS < 75%).

Apparently, a higher level of overall genetic structure was revealed by the PCoA (Fig. 2B); in this case, the first two axes accounted for 55.31% and 5.33%, respectively, of the total variance, although no evident geographic structure was found. Two groups were roughly distinguished in the PCoA: the first one grouped populations 1–3, 5, 7–9 from Majorca with 10, 12, 15, 16, and 19 from Sardinia, while the second contained populations 11, 13, 14, 17, and 18 from Sardinia and Tavolara, with 22–27 from Corsica. This analysis indicated differentiation to a certain degree of the populations from Majorca and Corsica, but not of those from Sardinia or Tavolara. The genetic structure revealed by NJ and PCoA did not coincide except for the fact that the populations from Majorca were slightly differentiated from the Corso-Sardinian ones.

Nei's gene diversity index (Table 1) ranged from 0.09 (populations 8, 1, and 2, all from Majorca) to 0.20 (population 27 from Corsica, although this result may be biased due to the small sampling size) and DW varied between 4.49 in population 2 and 14.83 in population 7, both from Majorca. Overall, the genetically most distinctive and diverse populations were found in Corsica, while the populations from Majorca displayed generally low diversity and singularity values.

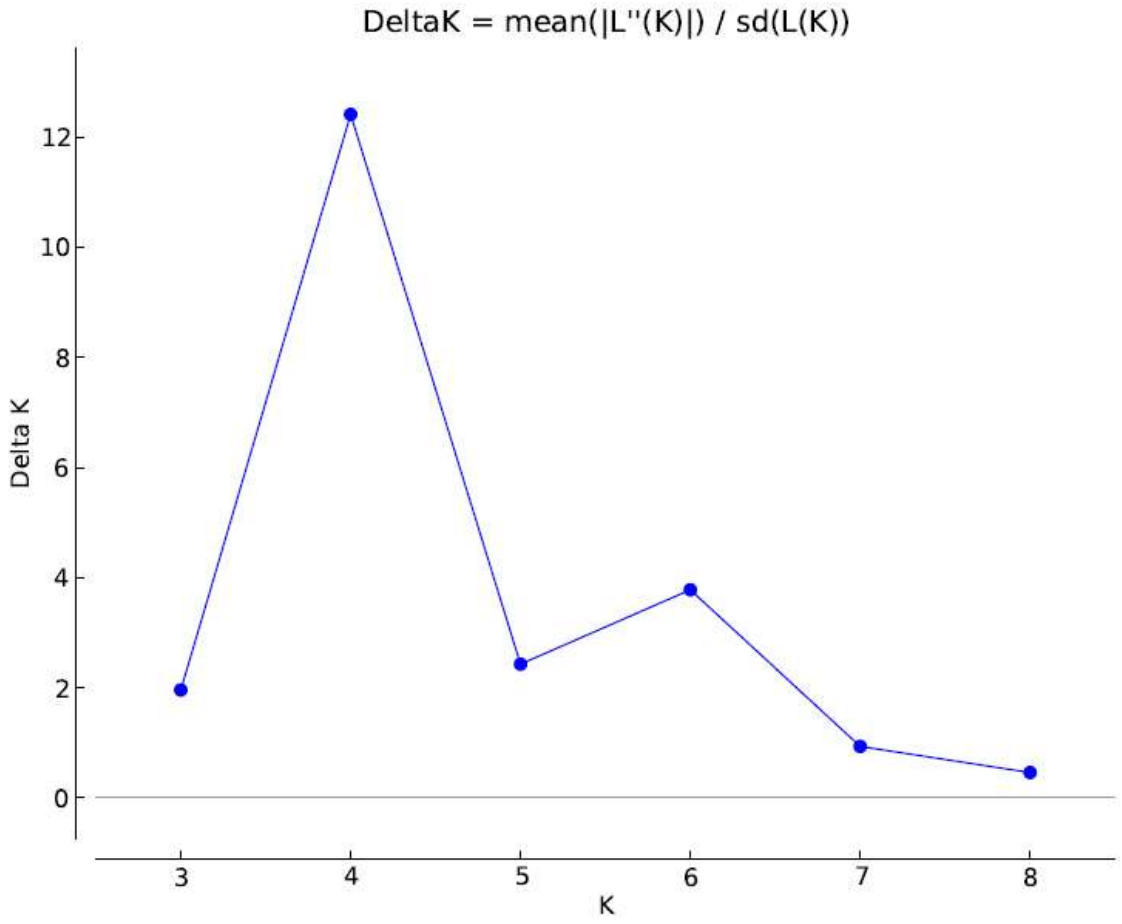
Figure 2: AFLP results. Genetic population structure based on AFLP analysis of 213 individuals of *Arenaria balearica*: (A) Unrooted neighbour-joining analysis; colours correspond to islands: branches in green lead to individuals from Majorca, in red to individuals from Corsica, in blue to individuals from Sardinia; the four groups commented in the text are indicated with a black line. (B) Ordination of AFLP data according to a Principal Coordinates Analysis; colours corresponding to islands as in (A). (C) Admixture analysis conducted with the software Structure: the graphs next to each population projected in the map indicate the proportional assignment of individuals to the genetic clusters A (pink), B (purple), C (yellow) and D (orange).



Bayesian clustering conducted using STRUCTURE estimated $K = 4$ as the most likely number of genetic clusters in *A. balearica*, with a maximum modal value of $\Delta K = 12.414075$ (Fig. 3). This clustering (Fig. 2) showed that all four of these groups were represented in the three main islands and also in Tavolara. In summary, Cluster A (pink) was dominant in the populations from Majorca and S Sardinia (particularly in population 16), was well represented in Tavolara, but its representation was poor in the remaining populations, particularly in populations 23,

25, and 26 from Corsica; Cluster B (purple) was also well represented—but consistently in a lower proportion than Cluster A—in Majorca (especially in population 5), southern Sardinia (particularly in population 16) and Tavolara, but it was present in a very low proportion in the remaining populations included in this study; Cluster C (yellow) was very well represented in all populations from Corsica, northern Sardinia, and Tavolara, but was almost absent from Majorca (completely absent from population 3); and Cluster D (orange) was best represented in Corsica, was present also in Tavolara and Sardinia (in an almost insignificant proportion in population 16), and had also a low representation in Majorca.

Figure 3: Delta *K* values from the method by *Evanno, Regnatus & Goudet (2005)*.



The hierarchical AMOVA (Table 4) showed that the genetic structure in four groups detected by NJ (i.e., (populations 1, 2, 3, 5, 7, 8, 9) (populations 14, 15, 18, 19, 22) (populations 10–13, 17, 23–27) (population 16)) accounted for a comparatively higher amount of the total genetic variance (10.71%), among these groups. This amount was similar, although slightly lower, than that accounted for among populations within groups (11.41%). In the AMOVA

analyses that evaluated other groupings the levels of genetic divergence were remarkably low among all groups considered and most of the variation was consistently found among populations within groups instead of among pre-established groups.

Plastid DNA variation in *Arenaria balearica* and geographical distribution of haplotypes

The length of the three plastid DNA regions for 226 individuals ranged between 846 and 704 bp, and resulted in an alignment of 2291 bp, 17 polymorphisms (12 substitutions/five indels) were detected across the whole dataset, five (four substitutions/1 indels), eight (four substitutions/four indels) and four substitutions were detected for the *trn*L^{UAA}-*trn*F^{GAA}, *psbA-3'trnK-matK* and *rpS16*, respectively. All mutations together defined a total of 16 haplotypes (Table 1). The results of the ILD test did not reveal significant inconsistencies among the plastid-DNA regions studied. The completeness of haplotype sampling estimated using *Dixon*'s (*2006*) method was 0.97 (the most likely value of haplotypes = 16), suggesting that all haplotypes present in the species had been sampled.

Source of variation	*d.f.*	*Sum of squares*	*Variance comp.*	*Variance %*	*F-values*	*95% conf. Int.*
Arenaria balearica						
Populations	22	9274.91	31.72	19.80	F_{ST}: 0.198	
Individuals	190	24415.52	128.50	80.20		
Grouping 1 (PCoA derived): [1,2,3,5,7,8,9,10,12,15,16,19] [11,13,14,17,18,22–27]						
Groups	1	1670.77	12.49	7.51	F_{CT}: 0.075	0.064–0.083
Populations	21	7604.15	25.32	15.22	F_{SC}: 0.165	
Individuals	190	24,415.52	128-50	77.27	F_{ST}: 0.227	
Grouping 2 (NJ derived): [1,2,3,5,7,8,9] [14,15,18,19,22] [10-13,17,23–27] [16]						
Groups	3	3652.31	17.66	10.71	F_{CT}: 0.107	0.096–0.117
Populations	19	5622.61	18.82	11.41	F_{SC}: 0.128	
Individuals	190	24,415.52	128.50	77.89	F_{ST}: 0.221	
Grouping 3 (main islands, Sardinia includes Tavolara): [1,2,3,5,7,8,9] [10–19] [22–27]						
Groups	2	2805.75	15.57	9.42	F_{CT}: 0.094	0.084–0.104
Populations	20	6469.17	21.19	12.82	F_{SC}: 0.141	
Individuals	190	24,415.52	128.50	77.76	F_{ST}: 0.222	
Grouping 4: 2 groups, Majorca vs. Corsica+Sardinia+ Tavolara [1,2,3,5,7,8,9] [10–27]						
Groups	1	1897.31	16.55	9.78	F_{CT}: 0.098	0.081–0.110
Populations	21	7377.61	24.19	14.29	F_{SC}: 0.158	
Individuals	190	24,415.52	128.50	75.93	F_{ST}: 0.240	

Table 4: AMOVA analysis. Comparison of analyses of molecular variance (AMOVA) based on AFLP data. Groupings of populations are shown in brackets (see text).

The statistical parsimony algorithm implemented in TCS inferred a 95% parsimony network with a maximum limit of four steps and star-like topology (Fig. 1). As inferred from the networking analysis, *A. balearica* showed a single major haplotype (present in 24 from the 29 populations studied), probably ancestral (haplotype I), which occurred in all islands (including Tavolara and Montecristo). In addition, there were 15 haplotypes, nine haplotypes (II, III, V, VII, X, XI, XII, XIII and XVI) separated one step from the ancestral one, haplotypes VI and XIV derived one step from haplotypes V and XIII respectively and haplotype XV derived two steps from XIV, two haplotypes derived two steps from haplotype I (IV and VIII) and IX derived one step from VIII. The most derived haplotypes were endemic to one individual island and usually were restricted to single populations (except for haplotype XIV, which was found in two populations from Corsica). Apart from haplotype I, only haplotype V was shared by populations located in different islands (Corsica and Sardinia). *Arenaria bertolonii* is separated 50 steps from the *A. balearica* central haplotype. The levels of haplotypic variation found in Corsica and Sardinia seems to be in accordance with the high levels of overall genetic diversity revealed by AFLP markers.

DIYABC analysis

Scenario 1 (ancestral area fragmentation) was revealed as the most probable. The posterior probability of the logistic regression was 75%, while the alternative hypotheses (Fig. 4) received less than 7%. Type I and type II errors corresponding to Scenario 1 resulted to be 21% and 17% respectively. DIYABC software places the fragmentation of the four areas 4730 generations ago.

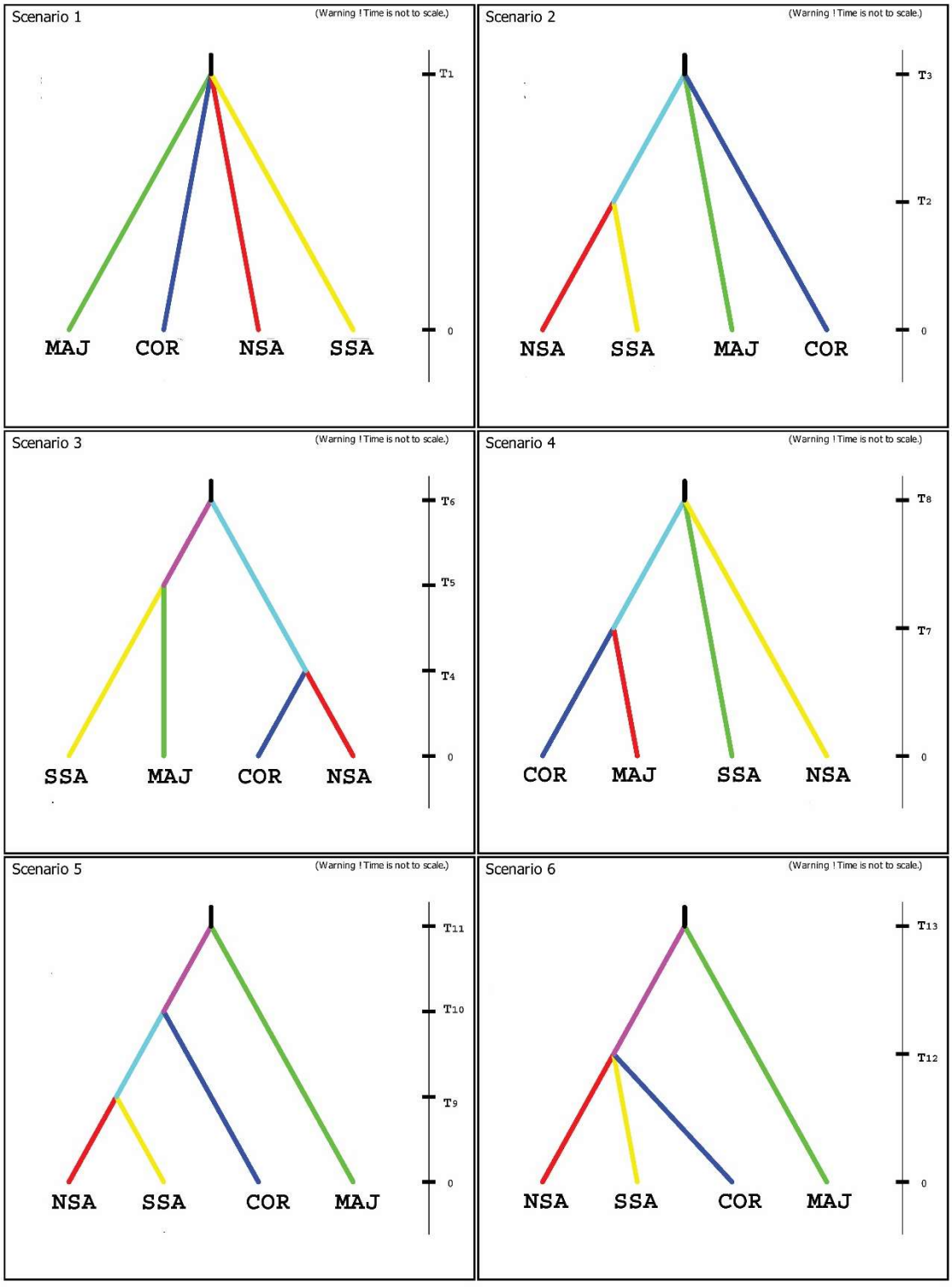
DISCUSSION

Phylogeography of the relict *Arenaria balearica*

Solid analysis in phylogeography should be based on the choice of appropriate study organisms and focal areas. Several requirements for reliable phylogeographic inference should be met, among them a sound phylogenetic framework and the absence of obvious adaptations for LDD from the organism side, and the availability of good historical climatic and geographic data from the focal-area side (*Salvo et al., 2010*). *Arenaria balearica* and the Western Mediterranean region satisfy these prerequisites. One of the most basic questions related with Mediterranean plant populations that still remains open is what part of their present genetic diversity is, as generally assumed, due to isolation in refugia during the Pleistocene glaciations and what part can be traced back to the Tertiary history of taxa (*Magri et al., 2007*; *Médail & Diadema, 2009*). Several authors (*Thompson, 2005*; *Donoghue, 2008*; *Ackerly, 2009*) have suggested that the filtering of elements from the ancient Tertiary geofloras that spread across

the Northern Hemisphere during the Tertiary (*Wolfe, 1975*; *Wolfe, 1978*) played a crucial role in the assembly of the Mediterranean floristic diversity.

Figure 4: Scenarios used in DIYABC. Graphic representation of the 6 scenarios used in DIYABC.



Thus, traditionally, botanists have classified the floristic elements of the Mediterranean region into two main groups, depending on whether these were believed to have arisen before or after the development of Mediterranean-like climates (*Thompson, 2005*; *Salvo et al., 2010*). *Arenaria balearica* was traditionally considered a Tertiary relict palaeoendemic species (*Contandriopoulos, 1962*) and has been particularly mentioned as a ''Hercynian palaeoendemic'' (*Molins et al., 2011*).

Unfortunately, considering that the plant is perennial and that there is no information available on generation times, although we have obtained here an estimated divergence time for T1 (Table 3; Fig. 4), our results are not conclusive regarding the question on the age and hypothetic ancient origin of the species.

Several hypotheses may explain the presence of *A. balearica* in Majorca, Corsica, and Sardinia, plus minor Tyrrhenian continental fragment islands. This striking distribution may suggest that it could be a non-monophyletic lineage, but the phylogenetic analysis of ITS (nrDNA) and plastid DNA sequences, which included samples from all the Tyrrhenian islands where the species is represented, indicated that the study group is clearly monophyletic (J Bobo-Pinilla, J Peñas de Giles & MM Martínez-Ortega, 2013, unpublished data). Additionally, both the careful review of herbarium materials prior to the sampling performed within this study, as well as the field observations, indicate very low morphological variation among populations (J. Lorite, 2014, unpublished data).

Both plastid and nuclear markers show the lack of a phylogeographic break among populations from different islands. Low levels of genetic structure are repeatedly found by the data analyses derived from the anonymous, mostly nuclear, DNA fingerprints (i.e., AFLP data; NJ, NNet and PCoA analyses; Fig. 2) and by the plastid-DNA data. The AMOVA analyses also indicated moderate levels of divergence among populations of *A. balearica* considered as a unique group, which are even lower among the different groups tested with AMOVA. These results contrast with the expectation of high population or geographical group divergence in species that occur in spatially isolated territories, particularly when the species shows limited dispersal abilities (in these situations gene flow tends to be low and, especially when population sizes are small, the effect of genetic drift is usually high).

In the case of *A. balearica*, the moderate levels of divergence found may represent remnants of Messinian contacts among the Tyrrhenian territories and long-term genetic stasis followed by recent differentiation in different stable habitats. Furthermore, the star-like arrangement

of plastid DNA haplotypes (Fig. 1) and DIYABC models suggest a pattern of long term survival and *in situ* differentiation. These results strongly agree with the idea of an ancient haplotype (I) widespread throughout the Tyrrhenian islands where the plant is present today, with different geographically scattered younger *in situ* derived haplotypes. In most cases, they represent endemic local variants that originated in isolation from each other, probably due to insularity or geography, on the one hand, and to the scattered availability of rupicolous habitats, on the other.

The Messinian Salinity Crisis, which has been cited to explain the distribution of many plant species in the Western Mediterranean (e.g., *Molins et al., 2011*) may also be invoked in this case, although the existence of Messinian terrestrial connections between the Corsica-Sardinia block and the Balearic Islands have never been documented (*Alvarez, 1972*; *Alvarez, Cocozza & Wezel, 1974*; *Rosenbaum, Lister & Duboz, 2002*). Also, although there is no evidence for further post-Messinian terrestrial connections between the major Tyrrhenian islands (*Alvarez, 1972*; *Alvarez, Cocozza & Wezel, 1974*; *Rosenbaum, Lister & Duboz, 2002*), direct land bridges existed during the Pleistocene glacial maxima between Corsica and Sardinia that allowed floristic exchanges (*Salvo et al., 2010*). This is also confirmed by the reconstruction of coastline during the LGM performed in this study (Fig. 1). The slightly exerted small capsules, and very small seeds (*López González, 1990*), and the plant's preference for shaded rocky sites (comophyte) are features that probably favoured short-distance dispersal. LDD of *A. balearica*, appears to be unfeasible during the Messinian when the Mediterranean Basin was a saline desert (*Hsü, 1972*). The fact that the plant lacks adaptations for over-water dispersal suggests also that LDD events between Majorca and the other Tyrrhenian islands (Corsica and/or Sardinia) were unlikely even during the Quaternary glacial maxima. No random LDD event was identified in the analyses performed in this study. Additionally, the star-like parsimony network inferred from plastid DNA data compiled (Fig. 1) is not consistent with a range-expansion model after LDD events, and no evidence was found for the existence of such events, either recent or ancient, between Majorca and the other Tyrrhenian islands derived from the almost nuclear AFLPs.

Historical gene flow seems to have existed between Corsican and Sardinian populations, as suggested by AFLPs. Both the NJ and PCoA analyses (Fig. 2) revealed no structuring of the overall genetic variability on a geographical basis. These results are also confirmed by the AMOVA analyses, which show that the genetic structure in four groups detected by NJ accounts for the comparatively highest amount of the total genetic variance, thus supporting the idea that only those populations from Majorca are to some extent genetically differentiated from

the rest. The Bayesian analysis of population structure reveals active historical gene flow and secondary contacts between Corsican and Sardinian populations (Fig. 2C). Particularly, clusters B and D are well represented on both islands but almost absent from Majorca (Fig. 2C) and the levels of admixture of these clusters tend to be higher among the populations located in southern Corsica and northern Sardinia (Fig. 2C). All these facts agree with the hypothesis of recurrent connections between Corsica and Sardinia in Miocene and Plio-Pleistocene times [Messinian Salinity Crisis (*Gover, Meijer & Krijgsman, 2009*); Pleistocene glaciations (*Lambeck et al., 2004*; *Lambeck & Purcell, 2005*)], which facilitated active exchanges of biota, as demonstrated for other organisms (*Zachos et al., 2003*; *Salvi et al., 2010*; *Fritz, Corti & Päckert, 2012*). By contrast, the plastid DNA data do not indicate significant post-Messinian floristic exchanges among Corsica, Sardinia, and the Tuscan Archipelago (only one haplotype is shared between Corsica and Sardinia), as proposed for other plant groups (e.g., *Quilichini, Debussche & Thompson, 2004*; *Salvo et al., 2008*; *Zecca et al., 2011*), a conclusion which may be biased by the fact that we were not able to establish good AFLP profiles for the plants collected in Montecristo and further highlights the importance of including anonymous hypervariable nuclear markers in phylogeographic studies.

Evolutionary stasis and habitat stability in Mediterranean disjunct endemic taxa

The low levels of genetic variation found in the maternally inherited plastid DNA (i.e., low number both of detected and of missing haplotypes, low variation common to all the plastid DNA regions tested, and a maximum limit of four steps from the inferred ancestral haplotype were detected in the haplotype network) are consistent with some of the criteria that usually characterized palaeoendemic species (at least in the traditional broad concept of *Favarger & Contandriopoulos (1961)*. This low variation is usually interpreted as a consequence of long processes of adaptation in relative isolation to the intrinsic characteristics of the local refuge area (*Mansion et al., 2008*).

Molins et al. (2011) have emphasized that several relict endemic species show little or no morphological differentiation despite a long history of isolation on small continental fragments. Even though *A. balearica* was specifically cited in that work as an example of evolutionary stasis, this had never been demonstrated until now. The low mutation rates associated with the plastid genome in *A. balearica* probably correspond to low levels of genetic diversity detected also with AFLPs, thus revealing that stasis in this case agrees with generally low levels of genetic variation. A remarkable lack of variation in all plastid DNA markers scored (including intron regions, intergenic spacers, and plastid microsatellites) was detected for the Tertiary relict *Ramonda myconi* (L.) Rchb. (*Dubreuil, Riba & Mayol, 2008*), which was found to

be in accord with previous results for other relict species (e.g., *Zelkova abelicea* (Lam.) Boiss. and *Z. sicula* Di Pasq., Garfì & Quézel by *Fineschi et al., 2002*; *Quercus suber* L. by *Magri et al., 2007*; *Cephalaria squamiflora* (Sieber) Greuter by *Rosselló et al., 2009*). According to *Dubreuil, Riba & Mayol (2008)*, the absence or low variation in the plastid genome could be a consequence of strong bottlenecks or genetic drift associated with small effective population sizes for maternally inherited markers (*Birky, Fuerst & Maruyama, 1989*), of slow population dynamics (*Dubreuil, Riba & Mayol, 2008*) and/or of slowed sequence evolution (*Dubreuil, Riba & Mayol, 2008*; *Molins et al., 2011*). The latter has been repeatedly associated with morphological stasis (*Barraclough & Savolainen, 2001*; *Soltis et al., 2002*; *Molins et al., 2011*). Nevertheless, *Casane & Laurenti (2013)* have recently suggested that, although a causal link between low molecular evolutionary rates and morphological stasis has been generally assumed, it seems that low intra-specific molecular diversity does not imply a low mutation rate, and also those intraspecific levels of molecular diversity and morphological divergence rates are under different constraints and are not necessarily correlated. As for *A. balearica*, independent markers suggest low levels of intraspecific molecular diversity (i.e., low plastid DNA variation, that seems to parallel the low overall genetic variability as revealed by a technique (AFLP) that covers the whole genome and also with low ITS sequence variation (J Bobo-Pinilla, J Peñas de Giles & MM Martínez-Ortega, 2013, unpublished data) that covers a small proportion of the nuclear DNA), but an explicit correlation between these data and either long-term morphological constancy or slowed mutation rates cannot be established with the available data.

Tertiary relict species have been forced to survive in refugia for long periods of time and their present genetic structure may therefore reflect the impact of a combination of ancient climatic and geographic changes. The ability to persist and resist overall adverse climatic conditions is probably coupled with the availability of relatively stable habitats, where intrinsic local properties have buffered the impact of historical climatic changes, thus allowing long-time persistence of particular species (*Thompson, 2005*; *Médail & Diadema, 2009*). The importance of local properties of refugia for survival of Tertiary relict taxa has previously been highlighted for other Mediterranean species, such as the rupicolous herb *R. myconi* (*Dubreuil, Riba & Mayol, 2008*). Furthermore, several authors (e.g., *Thompson, 2005*; *Peñas, Pérez-García & Mota, 2005*; *Rosselló et al., 2009*; *Youssef et al., 2010*; *Mayol et al., 2012*) have commented on the long-term stability of rupicolous habitats in the Mediterranean region and their role at warranting species survival based on the relatively low incidence of disturbances and interspecific competition and the fact that it is probably not fortuitous that many Mediterranean endemic species occur in rocky habitats (e.g., *Cymbalaria aequitriloba* (Viv.) A.

Chev., *Nananthea perpusilla* DC., *Naufraga balearica* Constance & Cannon, *Soleirolia soleirolii* (Req.) Dandy, etc). *Arenaria balearica* represents a further example of the importance of rocky sites as conservation habitats and as long-term reservoirs of plant diversity within the Mediterranean region.

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Author Contributions

- Javier Bobo-Pinilla performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Sara B. Barrios de León performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, reviewed drafts of the paper.
- Jaume Seguí Colomar, Giuseppe Fenu and Gianluigi Bacchetta contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Julio Peñas de Giles conceived and designed the experiments, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.
- María Montserrat Martínez-Ortega conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

GenBank sequences have been provided as a Supplemental File http://dx.doi.org/10.7717/peerj.2618/supplemental-information (Annex 1).

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.2618#supplemental-information.

Full size figures are provided as supplemental information (Annex 2 and Annex 3).

SUPPLEMENTAL INFORMATION

Annex 1: GenBank accession numbers

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01_04	*KX988419*	*KX988627*	*KX988966*
01_05	*KX988420*	*KX988628*	*KX988967*
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01_07	*KX988422*	*KX988630*	*KX988969*
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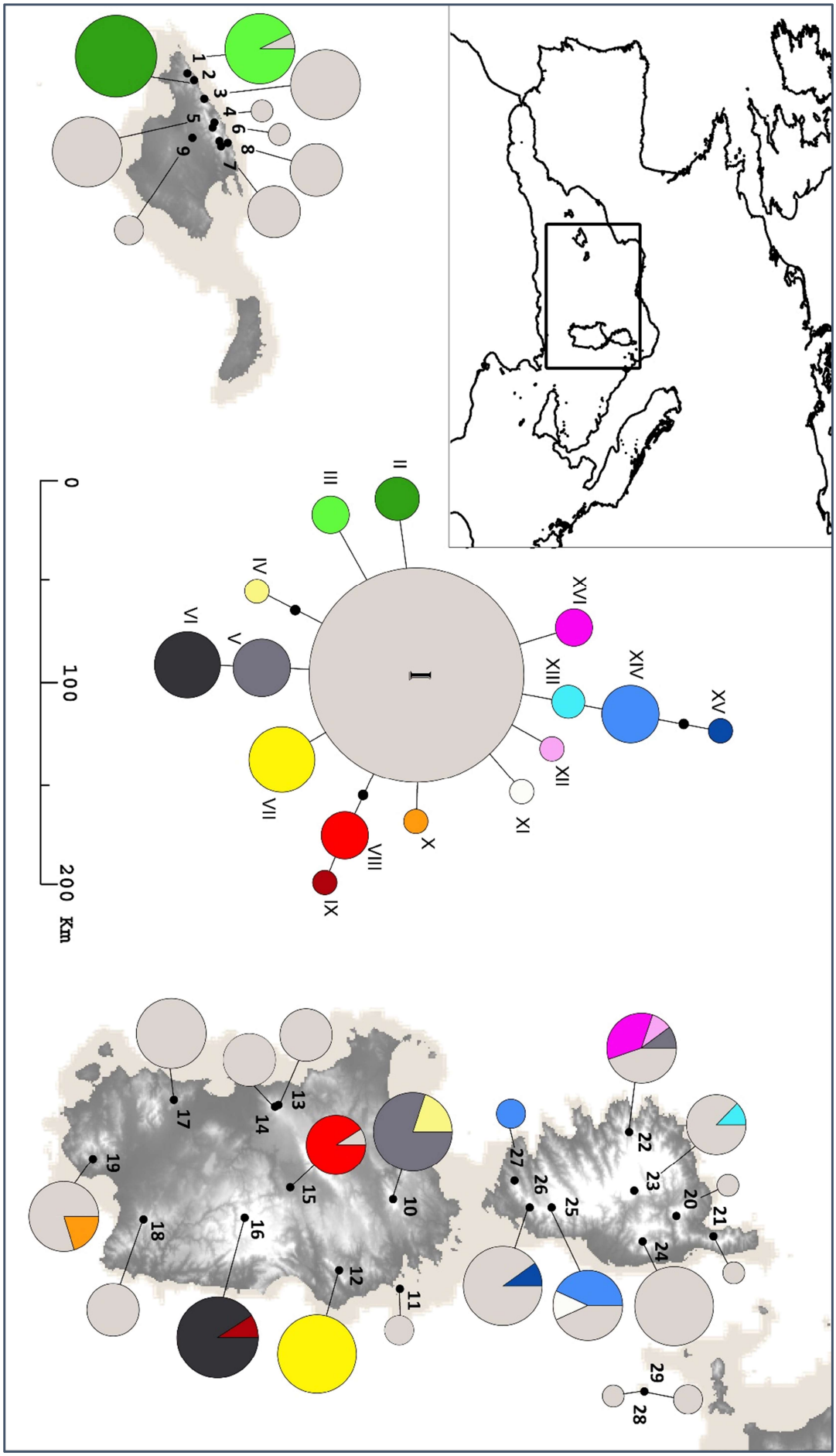
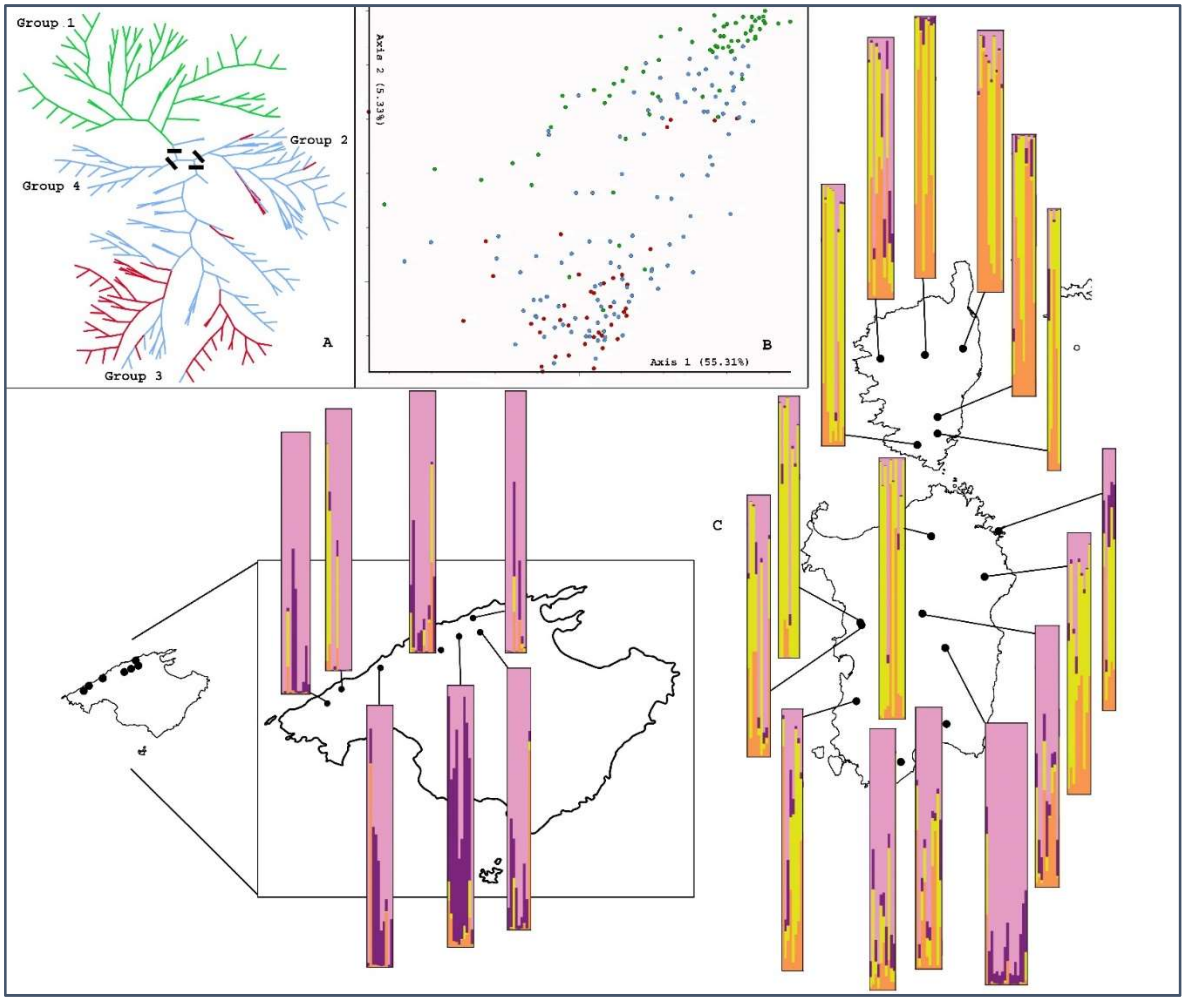


Annex 2 -Full Size Figure 1: Sampling localities and Haplotypes. Sampling localities of *Arenaria balearica*, reconstruction of the coast line during the Last Glacial Maximum in the study area, spatial distribution of plastid DNA haplotypes and statistical parsimony network for 226 individuals. The small black circles represent missing intermediate haplotypes. Sectors within circles in the map indicate the presence of different haplotypes in different individuals of the same population.

Annex 3 - Full Size Figure 2: AFLP results. Genetic *population* structure based on AFLP analysis of 213 individuals of *Arenaria balearica*: (A) Unrooted neighbour-joining analysis; colours correspond to islands: branches in green lead to individuals from Majorca, in red to individuals from Corsica, in blue to individuals from Sardinia; the four groups commented in the text are indicated with a black line. (B) Ordination of AFLP data according to a Principal Coordinates Analysis; colours corresponding to islands as in (A). (C) Admixture analysis conducted with the software Structure: the graphs next to each population projected in the map indicate the proportional assignment of individuals to the genetic clusters A (pink), B (purple), C (yellow) and D (orange).



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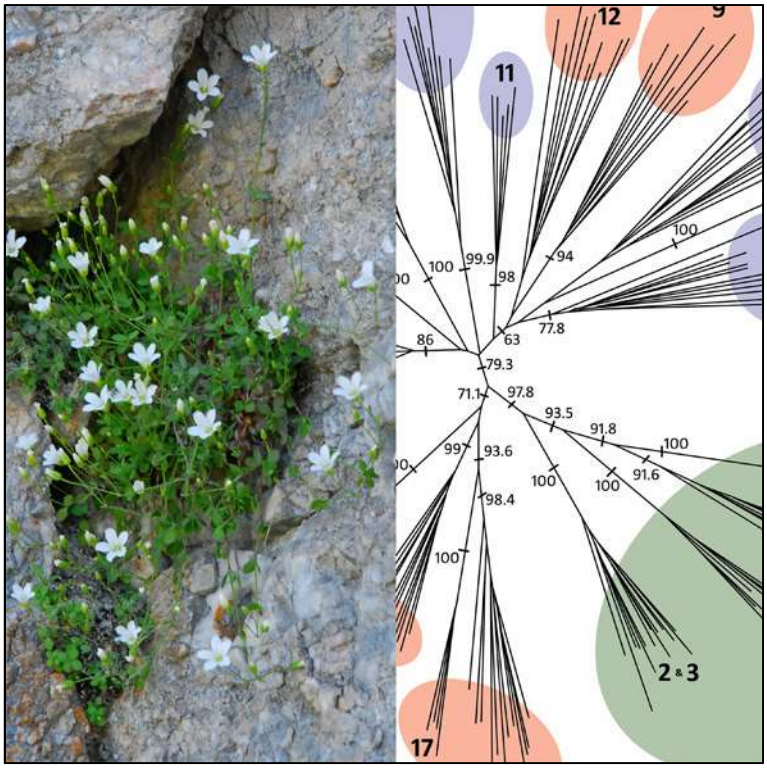
CAPÍTULO III:

Estudios sobre *Arenaria* Sect. *Pseudomoehringia*

Filogenia y filogeografía de *Arenaria* sect. *Pseudomoehringia*

Phylogeny and phylogeography of *Arenaria* section *Pseudomoehringia*



CONCLUSIONES

Sobre los patrones filogeográficos de *Astragalus edulis:*

- Se considera que el área original de *A. edulis* la constituyen las zonas esteparias semiáridas al norte de la vertiente norte del suroeste de la cordillera del Atlas.

- Las poblaciones de *A. edulis* de las Islas Canarias (Lanzarote y Fuerteventura) son el resultado de una dispersión a larga distancia desde las poblaciones del oeste de Marruecos, aproximadamente durante el periodo glaciar Riss.

- Las poblaciones de la especie situadas más al norte de su rango de distribución actúal, parecen resultado de una colonización a través de una ruta hipotética que conectaría la vertiente sur de Anti-Atlas, Alto Atlas y Tell Atlas con el norte de Marruecos y la península ibérica, siguiendo el valle del río Muluya. Esta ruta pudo tener importancia para algunas otras especies de hábitats semiáridos que habitan esta zona.

- Las barreras más potentes para el flujo genético actual son el estrecho de Gibraltar y la separación entre las Islas Canarias y Marruecos.

Sobre la conservación genética de *Astragalus edulis*

Variabilidad genética y estructura poblacional

- Los niveles de diversidad genética y rareza de *A. edulis* son similares a los de otras herbáceas anuales y perennes con distribución geográfica similar.

- Los niveles de diversidad genética y rareza son mayores en las poblaciones centrales de la península ibérica y del norte de Marruecos. Además, se detectan eventos de colonización a pequeña escala.

Diseño de estrategias de conservación

- Según la metodología para la detección de unidades genéticas relevantes para la conservación, seis poblaciones han de ser preservadas para conservar la diversidad genética de la especie. Estas poblaciones son AE1, AE4 y AE5 (península ibérica), AE8 y AE9 (Marruecos) y AE16 (Islas Canarias).

- Los valores de diversidad genética y rareza de la especie son mayores en las poblaciones que ocupan áreas amplias, con alto número de individuos e incluidas en espacios protegidos.

- La presencia de haplotipos endémicos subraya el impacto de las barreras geográficas en la estructura poblacional de *A. edulis*. Se propone incorporar esta información genética al diseño de la estrategia de conservación.

- Se aconsejan medidas adicionales de conservación como, por ejemplo, el refuerzo de las poblaciones más pequeñas y la conservación en bancos de semillas, así como llevar a cabo estudios sobre fluctuaciones poblacionales y de calidad de hábitat.

Sobre los patrones filogeográficos de *Arenaria balearica*

- A pesar de su distribución altamente fragmentada, se demuestra la monofilia *A. balearica.*

- Pese a que *A. balearica* presenta una distribución correspondiente con el macizo Hercínico, en su historia evolutiva no se aprecian las huellas de su fragmentación oligocénica. Los bajos niveles de variación del ADN plastidial confirman el estatus de *A. balearica* como paleoendemismo e implican un patrón de supervivencia a largo plazo en zonas estables.

- La diferenciación genética entre islas es mucho menor de lo esperable en un escenario tan disyunto, lo que nos permite afirmar que los altos niveles de estatismo morfológico de la especie se corresponden también con altos niveles de estaticidad genética.

- Se descartan eventos de dispersión a larga distancia entre Mallorca y las islas tirrénicas. Se detectan posibles contactos entre Córcega y Cerdeña durante la crisis de salinidad del Messiniense y durante las glaciaciones pleistocénicas.

- El aislamiento actual de las poblaciones parece ser el motivo de la presencia de variantes genéticas locales.
- *Arenaria balearica* representa un ejemplo más de la importancia de los hábitats rocosos como refugios estables a largo plazo y pone de manifiesto la necesidad de conservarlos.

Sobre las relaciones filogenéticas y filogeográficas de la sección *Pseudomoehringia* de *Arenaria*

- Esta sección es claramente un grupo monofilético y se compone de tres especies: *A. suffruticosa, A. funiculata* y *A. glochidisperma,* distribuidas al sur de la península ibérica y al norte de Marruecos. *Arenaria tejedensis* no es genéticamente independiente de *A. suffruticosa*, aunque *los marcadores de ADN plasti*dial *apoyan ciertos n*iveles de diferenciación, posiblemente debidos a aislamiento geográfico.

- Los taxones anteriormente reconocidos como *M. intricata* subsp. *giennensis* y *M. intricata* subsp. *castellana* no se recuperan como monofiléticos por lo que deben incluirse dentro de la variación de *A. suffruticosa.* Se propone desestimar su reconocimiento con categoría de subespecie.

- Teniendo en cuenta los cambios taxonómicos recién señalados, se considera que es necesario reevaluar el estado de conservación de las especies haciendo uso de las directrices de la Unión Internacional para la Conservación de la Naturaleza.

- El área conformada por las actuales Sierras de Cazorla y Segura, y Sierra Mágina ha sido identificada como posible origen geográfico de la sección.

- Nuestros análisis apoyan un patrón filogeográfico general para el complejo de *A. suffruticosa* consistente en sucesos de expansión desde un área ancestral y un posterior aislamiento de las poblaciones.

- Las evidencias permiten hipotetizar que *Arenaria funiculata* podría ser producto de adaptación edáfica a suelos ácidos provocada por exclusión competitiva o por presión climática.

- **Artículos:**

Conservation of genetic diversity in Mediterranean endemic species:

***Arenaria balearica* L.**

Nuevos Datos Sobre Orquídeas Silvestres De La Provincia De Zamora Y Zonas Limítrofes

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NUEVOS DATOS SOBRE ORQUÍDEAS SILVESTRES DE LA PROVINCIA DE ZAMORA Y ZONAS LIMÍTROFES

Patricio BARIEGO HERNÁNDEZ[1], Javier BOBO PINILLA[1] & Fermín del EGIDO MAZUELAS [2]

[1] Departamento de Botánica y Fisiología Vegetal. Universidad de Salamanca. Salamanca; pbariego@hotmail.es; javicastronuevo@usal.es

[2] Departamento de Biodiversidad y Gestión Ambiental. Área de Botánica. Facultad de Ciencias Biológicas y Ambientales. Universidad de León. León; fegim@unileon.es

RESUMEN: Se señalan y comentan 15 táxones silvestres de la familia *Orchidaceae* que presentan interés corológico, biogeográfico o conservacionista en la provincia de Zamora y otras provincias limítrofes (España). Todos ellos están escasamente citados en el NW ibérico y algunos son novedades o aportaciones interesantes para las provincias de Zamora, Orense y Valladolid.

Palabras clave: Corología, Plantas vasculares, Orchidaceae, Zamora, Valladolid, Orense, España.

ABSTRACT: New data on wild orchids of Zamora province and surrounding areas (Spain). 15 wild *Orchidaceae* taxa of chorological, biogeographical or conservation interest in the province of Zamora and his surrounding areas (Spain) are remarked and commented. These taxa have been poorly referenced in the NW region of Iberian Peninsula and some of them are interesesting quoted or cited in the province of Zamora or Valladolid for the first time.

Keywords: Chorology, vascular flora, Orchidaceae, Zamora, Valladolid, Orense, Spain

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INTRODUCCIÓN

En los últimos años se ha venido realizando un continuo trabajo de prospección botánica de la provincia de Zamora y zonas próximas cuyos resultados han servido para iniciar recientemente una serie de publicaciones con datos sobre la flora vascular zamorana (BARIEGO & SANTOS 2011; BARIEGO & al., 2015).

En particular, respecto a las orquídeas silvestres se realizó una revisión conjunta de la familia poniendo de manifiesto la presencia en la provincia de 27 táxones silvestres englobados en 13 géneros en el territorio provincial (BARIEGO & GASTÓN, 2005). Posteriormente, se han añadido al catálogo provincial otras especies como *Dactylorhiza insularis* (Sommier) Ó. Sánchez & Herrero (BARIEGO, 2011) y *Cephalanthera rubra* (L.) Rich. (BARIEGO & al., 2015), y se han aportado nuevos datos para alguno de los taxones más escasos como *Neotinea maculata* (Desf.) Stearn y *Spiranthes spiralis* (L.) Chevall. (BARIEGO & al., 2015).

Continuando con el objetivo de mejorar el conocimiento de la situación de la familia *Orchidaceae* en la provincia y su entorno, en la presente nota se recoge información sobre varios táxones que se citan por primera vez (se señalan con un asterisco) y se aportan nuevos datos corológicos para otros táxones cuya presencia en el territorio provincial y otras zonas cercanas es interesante o poco conocida.

Para cada taxon, ordenado con criterio alfabético, se indican los datos referentes a su localización geográfica (municipio, toponimia, UTM 1×1 km y altitud), el hábitat donde ha sido observado, la fecha y autor/es de la recolección y, en su caso, un número de identificación del testigo (referencias personales de cada colector y/o referencia de herbario) o una indicación sobre la existencia de fotografía en la colección personal de los autores. Además, se comentan brevemente algunos datos referidos a su distribución geográfica, con especial atención a las plantas que tienen interés biogeográfico o para la conservación.

RESULTADOS

Barlia robertiana (Loisel) Greuter

ZAMORA: 30TTL8187, Sanzoles, El Monte, jaral en encinar claro, 750 m, 17-V-2014*, P. Bariego,* vd. (fotog. en fruto); 30TTL7974, El Maderal, Gavia Honda, tomillar basófilo, 880 m, 17-IV-2015*, P. Bariego,* PB 4438.

Orquídea de amplia distribución por el sur de Europa y el norte de África, muy escasa en el noroeste de la Península Ibérica. Aportamos dos nuevas localidades zamoranas a añadir a las dos previamente conocidas en el extremo suroeste (Fermoselle) y el centro (Matilla La Seca) de la provincia (BARIEGO & GASTÓN, 2005; GARCÍA RÍO & NAVARRO, 1988). En todos los casos se trata de poblaciones con escaso número de ejemplares (generalmente menos de 10 floridos y algunos más en estado vegetativo), de floración temprana (marzo-abril) y localizadas en claros de matorral, bosques abiertos y pastos en suelos algo nitrificados, tanto silíceos como básicos. Es una planta incluida en el Inventario de Especies de Atención Preferente de Castilla y León (ANÓNIMO, 2007; 2015) cuyas poblaciones en Zamora son vulnerables por su rareza y reducido tamaño poblacional.

Dactylorhiza insularis (Sommier) Ó. Sánchez & Herrero

ZAMORA: 29TPG9462, Galende, El Puente de Sanabria, Prado Coso, pr. campo de fútbol, claros de rebollar, 960 m, 26-V-2016, *P. Bariego*, vd (fotog.).

ORENSE: 29TPG6666, Viana do Bolo, Pradorramisquedo, pr. Salto de San Sebastián, herbazales en suelos rezumantes, 1060 m, 12-V-2017, *P. Bariego*, vd (fotografía).

Orquídea escasa en las zonas de montaña silíceas del NW peninsular. Recientemente la citábamos como novedad para las montañas de Sanabria (BARIEGO, 2011) en una localidad cercana a la que citamos ahora y previamente CORTIZO & SAHUQUILLO (1999) aportaban las primeras citas para Orense y Galicia, señalando algunas localidades de los afloramientos calizos de la zona baja del Bibey. Aportamos ahora una nueva población en Sanabria que cuenta con apenas media docena de ejemplares y otro nuevo emplazamiento en el alto Bibey orensano, en un enclave de interés florístico en el que convive con otras orquídeas poco frecuentes en la zona como *Orchis ustulata* L. y *O. provincialis* Balbis.

Epipactis fageticola (C.E. Hermos.) Devillers-Tersch. & Devillers

***ZAMORA**: 30TTM6866, Maire de Castroponce, pr. puente a Coomonte, ribera del Órbigo, choperas, 725 m, 06-VII-2014, *P. Bariego*, PB 4431; 30TTM7065, Maire de Castroponce, Vega de Arriba, ribera del Órbigo, choperas, 725 m, 06-VII-2014, *P. Bariego*, PB 4432; 30TTM7163, Pobladura del Valle, La Vega, río Órbigo, choperas, 720 m, 21-VI-2015, *P. Bariego*, PB 5012.

Orquídea del sur de Europa (España, Francia y Suiza), relativamente rara en el noroeste de la Península Ibérica, aunque progresivamente se van localizando más poblaciones en el entorno de los principales ríos. De acuerdo a algunos autores estas plantas corresponderían a *E. phyllanthes* G.E. Sm., aunque en el tratamiento de *Flora iberica* se llevan estas plantas de las choperas del interior peninsular a *E. fageticola* (CRESPO, 2005). Novedad para la orquidoflora provincial (BARIEGO & GASTÓN, 2005). Las localidades más cercanas corresponden a la provincia de León, donde se ha señalado recientemente en el río Esla (EGIDO & al., 2017). En las poblaciones zamoranas crece en choperas riparias y convive con otra orquídea próxima y con la que comparte hábitats como es *E. rhodanensis* Gévaudan & Robatsch.

En las zonas en la que se ha localizado forma pequeñas poblaciones, pudiendo ser localmente frecuente en las choperas menos transformadas sin llegar a ser una especie abundante. Es una planta incluida en el *Inventario de Especies de Atención Preferente de Castilla y León* (ANÓNIMO, 2007; 2015).

Epipactis helleborine (L.) Crantz subsp. ***helleborine***

Aunque BARIEGO & GASTÓN (2005) indicaban su presencia en la provincia sobre la base de

distintas citas bibliográficas (SÁNCHEZ RODRÍGUEZ, 1986; GARCÍA RÍO & NAVARRO, 1990; GARCÍA RÍO & NAVARRO, 1994) y ya se ponía en duda su presencia en ella, con posterioridad se han podido revisar los materiales testigo y comprobar que todas estas referencias corresponden en realidad a confusiones con otras especies del género como *E. tremolsii* Pau y *E. rhodanensis* Gévaudan & Robatsch por lo que descartamos por el momento su presencia en la provincia.

Epipactis rhodanensis Gévaudan & Robatsch

ZAMORA: 30TTL7399, Zamora, Aldehuela, pr. confluencia del Valderaduey y el Duero, ribera con chopos, 630 m, 21-VI-2005, *P. Bariego*, vd. (fotog.); 30TTM7241, Bretocino, Las Alamedas, ribera del Esla, chopera próxima al río, 675 m, 1-VIII-2015, *P. Bariego*, PB 5129; 30TTM7163, Pobladura del Valle, La Vega, río Órbigo, choperas, 720 m, 21-VI-2015, *P. Bariego*, PB 5011.

Orquídea del suroeste de Europa, relativamente rara en el noroeste de la Península Ibérica. Al igual que *E. fageticola* es una planta rara en la provincia, aunque poco a poco se van conociendo nuevas poblaciones que expanden su área por el entorno de los principales ríos. En BARIEGO & GASTÓN (2005) ya se incluía una referencia a este taxon en las orillas del Duero. Aportamos ahora otra localidad en el Duero en las proximidades de la ciudad de Zamora y un par de localidades al norte de la provincia en las riberas del Esla y del Órbigo, más interesantes en lo corológico y donde llega a convivir con *E. fageticola*. En todos los casos crece en los bosques de ribera (choperas y alamedas, principalmente) sin llegar a ser abundante.

Himantoglossum hircinum (L.) Spreng.

***ZAMORA:** 30TTM8629, Villafáfila, La Cruz de Maroto, herbazales, 710 m, 16-V-2015, *P. Bariego*, PB 4816.

Orquídea de notable tamaño y amplia distribución por el centro y sur de Europa y el entorno mediterráneo. En la Península Ibérica se extiende principalmente por el norte, centro y sureste, aunque es mucho más frecuente en terrenos calizos de media montaña. En Castilla y león es frecuente en la Cordillera Cantábrica y su entorno y es muy rara en el resto, restringiéndose en la depresión del Duero a estas localidades zamoranas y las salmantinas de La Rinconada (BERNARDOS & al., 2006). En la provincia, donde se cita por primera vez, únicamente se ha localizado un núcleo poblacional reducido (apenas una docena de ejemplares en flor más alguna otra roseta) en pastizales de la zona de Villafáfila.

Ophrys fusca Link subsp. ***fusca***

Tal y como se indicaba en BARIEGO & GASTÓN (2005) todo parecía indicar que las citas bibliográficas referidas a esta especie (NAVARRO & GARCÍA RÍO, 1992; GARCÍA RÍO & NAVARRO, 1994) correspondían a la *O. fusca* subsp. *dyris* (Maire) Soó. Tras estudiar el material de herbario se ha comprobado que tal suposición era cierta por lo que descartamos la presencia de la subespecie tipo en la provincia.

Ophrys lutea (Gouan) Cav.

***ZAMORA**: 30TTM8936, Tapioles, camino de las Praderonas, tomillar, 685 m, 5-VI-2013, *P. Bariego & F. del Egido*, LEB 113155; Madridanos, Bamba, Teso del Viso, 30TTL83 92, 745 m, pastos basófilos, 11-V-2013*, P. Bariego,* PB 4028; 30TTM8704, Coreses, pr. Casa del Montico, orlas herbáceas en pinar de repoblación, 690 m, 05-VII-2013*, P. Bariego,* PB 4029; 30TUM1101, Morales de Toro, Las Minas, tomillares basófilos, 705 m, 28-IV-2014*, P. Bariego,* PB 4434.

Orquídea de amplia distribución por la Región Mediterránea, dispersa por la Península aunque rara en el noroeste. Aprovechando una primavera excepcionalmente húmeda y tardía en el año 2013, hemos colectado en herbazales subnitrófilos de varias localidades este taxon, que había pasado desapercibido y no había sido citado previamente en Zamora, y que se incorpora al catálogo de la orquidoflora provincial (BARIEGO & GASTÓN, 2005). Estas localidades confirman, junto a las recientes primeras menciones leonesas (DÍEZ FERNÁNDEZ, 2014), su presencia esporádica en el NO de Castilla y León.

Ophrys scolopax Cav.

***ZAMORA**: 29TQF1576, Fermoselle, Las Escaleras, Casa de los Carabineros, encinarquejigar abierto, 623 m, 29-IV-2015, *P. Bariego*, PB 4899.

Orquídea de amplia distribución por el entorno mediterráneo y bastante extendida por la Península Ibérica. En BARIEGO & GASTÓN (2005) ya señalábamos su probable presencia en la provincia de Zamora ya que se conocía en zonas próximas de la provincia de Salamanca en los Arribes del Duero (BERNARDOS & AMICH, 2000; BERNARDOS & al., 2003). Confirmamos ahora su presencia en territorio provincial con esta población de los Arribes zamoranos, donde es muy escasa y

crece en pastos en claros de bosque y antiguos cultivos abandonados en bancales. Las plantas de Zamora pueden encuadrarse en las formas típicas de la especie, al igual que el resto de las poblaciones de los Arribes del Duero (BERNARDOS & al., 2003), con cierta variabilidad en la anchura y forma de los pétalos que entrarían dentro del amplio rango de variación de la especie.

Ophrys speculum Link. subsp. ***speculum***

***ZAMORA:** 30TTM8920, Pobladura de Valderaduey, arroyo de Renedo, tomillar, 680 m, 10-IV-2017, *J. Bobo-Pinilla* (fotog.); íbidem, 18-04-2017, *P. Bariego & J. Bobo-Pinilla* (fotog.).

Orquídea de amplia distribución por el entorno mediterráneo, bastante extendida por la mitad E y S de la Península Ibérica y muy rara en el NW peninsular y en la cuenca del Duero. La única población zamorana conocida por el momento crece en un tomillar sobre sustrato arcilloso y pedregoso, aparentemente ácido en superficie, pero sobre una matriz profunda rica en bases, y sólo contaba con 6 ejemplares floridos.

Orchis palustris Jacq.

VALLADOLID: 30TUL5898, Boecillo, El Raso de Portillo, pradera-juncal encharcada, 710 m, 28-VI-2008, *P. Bariego, F. Díez & A. Puente*, PB3612.

Solamente se sabía de esta escasa orquídea en Valladolid por un pliego de las inmediaciones de Olmedo (GUTIÉRREZ, 1908), confirmado por C. Aedo para la revisión de *Flora iberica* (AEDO, 2015). Se da a conocer ahora una nueva población, muy reducida en número de ejemplares y localizada en el Raso de Portillo, sin duda uno de los enclaves de mayor interés botánico en la provincia de Valladolid. Se trata de una orquídea muy rara en Castilla y León por lo que quizá debiera estar incluida en el Inventario de Especies de Atención Preferente de Castilla y León.

Orchis papilionacea L.

***ZAMORA**: 30TTM8836, Revellinos, El Salado, pastizales subhalófilos, 680 m, 16-V-2015, *P. Bariego*, vd (fotog., un sólo individuo en flor); 30TTM9158, San Miguel de la Ribera, El Gatinal, camino del río, herbazales basófilos, 735 m, 18-V-2015, *P. Bariego & F. del Egido*, PB 4826; 30TTM9162, Matilla de Arzón, Monte de la Mata (enclavado), pastizal-tomillar en ladera margosa en claros de encinar-quejigar, 755 m, 18-V-2015, *P. Bariego & F. del Egido*, PB 4829 (LEB 116820).

***VALLADOLID**: 30TTM9262, Roales de Campos, Monte Roales, pastizal-tomillar en claros de encinar, ladera margosa, 755 m, 19-V-2015, *P. Bariego & F. del Egido* (LEB 116821). Ibíd, 30TTN9362, pastizal entre un barbecho y un encinar, 770 m (obs. y fotog.).

Orquídea de amplia distribución por el entorno mediterráneo que en la Península Ibérica parece estar más extendida y ser más frecuente en la mitad meridional y es muy rara en el cuadrante noroeste. En la provincia de Zamora se han localizado dos núcleos poblacionales, uno extremadamente pequeño (un sólo ejemplar en flor) en el entorno de Villafáfila y el otro más extendido y con poblaciones verdaderamente sobresalientes por el elevado número de ejemplares (varios cientos en flor) en las laderas y montes del entorno del río Órbigo, que se extiende también por el entorno de Valderas en la provincia de León (EGIDO & al., 2017) y por el enclavado vallisoletano de Roales de Campos, donde también la señalamos aquí como novedad provincial ya que no se conocen referencias previas (SANTOS & al., 2008). Incluida en el Inventario de Especies de Atención Preferente de Castilla y León (ANÓNIMO, 2007; 2015), las poblaciones que mencionamos del entorno del Órbigo (Za-Le-Va) merecerían una especial protección por su gran tamaño y buen estado de conservación.

Orchis provincialis Balbis

ORENSE: 29TPG6666, Viana do Bolo, Pradorramisquedo, pr. Salto de San Sebastián, herbazales en suelos rezumantes, 1060 m, 12-V-2017, *P. Bariego*, vd (fotog.).

Orquídea poco frecuente en las zonas de montaña silíceas del NW peninsular, ya conocida en la cuenca del Bibey aunque en las zonas más termófilas (CORTIZO & SAHUQUILLO, 1999). Añadimos una nueva referencia en la provincia de Orense, aunque muy cercana a la provincia de Zamora donde no ha sido localizada por el momento.

Orchis ustulata L.

ORENSE: 29TPG6666, Viana do Bolo, Pradorramisquedo, pr. Salto de San Sebastián, herbazales en suelos rezumantes, 1060 m, 12-V-2017, P. Bariego, vd (fotog.).

Orquídea muy rara en la zona de las montañas del macizo Galaico-Leonés de la que tan sólo conocemos la mención zamorana de Ribadelago (LOSA, 1950), donde no se ha vuelto a localizar en

los últimos años. En Orense debe ser también una planta rara, ya que tan sólo se ha citado en las calizas de Rubiana, al noreste de la provincia (LAÍNZ, 1967). En este emplazamiento convive con otras especies del género como *O. morio* L. y *O. provincialis* Balbis.

CONCLUSIONES

Se aportan nuevos datos de la familia *Orchidaceae* en el CW y NW de la Península Ibérica, ampliando el conocimiento de algunas de las especies menos frecuentes en este ámbito como *Barlia robertiana*, *Epipactis fageticola u Orchis palustris*. En particular, para la provincia de Zamora se realizan nuevas aportaciones de información y aclaraciones que permiten actualizar el catálogo de la orquidoflora provincial (BARIEGO & GASTÓN, 2005; BARIEGO, 2011; BARIEGO & al., 2015). En definitiva, la presente nota supone un avance en el conocimiento de la situación de las orquídeas silvestres de la provincia de Zamora y zonas limítrofes, con un listado que muy previsiblemente podrá ampliarse con una mayor prospección del territorio en los próximos años.

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