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Que los trabajos de investigación desarrollados en la memoria de la tesis doctoral "**Cambio Climático en plantas de alta montaña: una perspectiva genética**", han sido realizados bajo su supervisión y son aptos para ser presentados por el licenciado Alfredo García Fernández ante el tribunal que un día se consigne, para aspirar al Grado de Doctor en Ciencias Ambientales por la Universidad Rey Juan Carlos

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Cambio climático en plantas de alta montaña mediterránea: una perspectiva genética

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Los hombres odian ser mentalmente fuertes y físicamente débiles. El hecho de que debamos destruir este planeta a la vez que a nosotros mismos no nos llena de alegría. En cambio admiramos a los atletas y a las personas que ejercen la violencia física, y *odiamos* a los intelectuales. Un puñado de gilipollas lanza un cohete a la puñetera *luna*, y ¿a quién mandan? A un tipo rubio llamado *Arm*strong, incapaz de decir lo que debía decir

Burlando a la parca – Josh Bazell

Índice

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RESUMEN

Antecedentes

Los motores de todo: cambio global y cambio climático.

El concepto de cambio global incluye todos aquellos impactos de la actividad humana que repercuten en la composición y funcionamiento de la biosfera (Duarte 2006). La Tierra ha estado en permanente cambio desde su formación, no solo en el nivel biológico con las especies que lo han habitado, sino también en los niveles geológicos e incluso físico-químicos. Sin embargo, los cambios que actualmente se están produciendo están íntimamente relacionados con la actividad humana (Duarte 2006; Sala et al. 2000; IPCC 2007) resultan altamente impredecibles (Sala et al. 2000; Thuiller et al. 2008) y su velocidad no es comparable con ninguno de los procesos anteriores (Loarie et al. 2009). Se estima que durante el siglo XX, la temperatura global del planeta se incrementó en 0.74 ± 0.18 ºC (IPCC 2007); mientras que la mayoría de las proyecciones actuales estiman que la temperatura media del planeta aumentará entre 1.1 y 6.4 ºC durante el siglo XXI (IPCC 2007). En líneas generales, estos incrementos pueden jugar papeles decisivos en distintos procesos que afectan a escala global, como el ascenso del nivel del mar, cambios en las corrientes marinas o fusión y deshielo de los polos y glaciares (IPCC 2007; Oerlemans 2005; Rahmstorf 2007; Winton 2003). Sin embargo, estos valores medios pueden verse muy alterados por factores locales, produciendo cambios en las dinámicas de precipitaciones y temperaturas, tanto en la dimensión temporal como espacial (por ejemplo, en la Península Ibérica se estima que en zonas puntuales pueden aumentar los regímenes de lluvia, de Castro et al 2004). Además, más allá de los cambios globales, también se está observando un aumento en la frecuencia de eventos extremos, en forma de sequías intensas, olas de frio/calor etc. los cuales pueden llegar a ser tan críticos para las especies o los sistemas como los cambios o tendencias en los valores medios (Jentsch et al. 2007; De Boeck et al. 2010).

Según el Millenium Ecosystem Assement (2005), el cambio global puede asociarse con cinco grandes factores: cambios en los usos del suelo, introducción de especies invasoras, sobrexplotación de recursos naturales, contaminación (suelos, agua y aire) y cambio climático. Aunque todos los factores son importantes y pueden ver su efecto modificado si interaccionan entre ellos (Opdam & Wascher 2004), los estudios que consideran varios de estos factores simultáneamente son escasos (ver por ejemplo Honnay et al. 2002; Matesanz et al. 2009; Caldwell et al. 2003). No obstante, son los cambios en el clima los que más preocupan, debido a su variabilidad (Bradshaw & Holzapfel 2006) y su amplio rango de impacto sobre todas las regiones (Duarte 2006; IPCC 2007; Jentsch et al. 2007).

Respuestas ante el cambio global: ¿Qué sabemos?

Parece fuera de toda duda que el cambio climático supone una de las amenazas más serias para la biodiversidad a nivel mundial (Araújo & Rahbek 2006; Thomas et al. 2004; Parmesan 2006). Se han documentado una gran variabilidad de respuestas ecológicas y biogeográficas (Walther 2003) por parte de todo tipo de seres vivos: movimientos altitudinales y latitudinales (Parmesan 2006; Peñuelas & Boada 2003), cambios en las interacciones entre especies dentro de sus comunidades (Brooker 2006), fluctuaciones en las áreas de distribución (Wilson et al. 2005), cambios fenológicos o reproductivos (p.e. Peñuelas et al. 2002; Matesanz et al. 2009), etc. El caso de las plantas resulta especialmente paradójico. Debido a su limitada capacidad de movimiento, ven disminuida su capacidad de desplazarse ante los cambios (Thuiller et al. 2005; Engler et al. 2009) y se ven forzadas a establecer otro tipo de reacciones ante las nuevas condiciones (ser plásticas o evolucionar, Jackson & Overpeck 2000). Además, muchas de estas especies se encuentran en una matriz ya de por si desfavorable (especies que habitan sistemas extremos, muy sensibles a condiciones ambientales específicas, poblaciones fragmentadas, bien sea en islas oceánicas, afloraciones edáficas o cimas montañosas, etc.). Todo esto nos lleva a comprender por qué el estudio y funcionamiento de determinadas respuestas como la adaptación local y la plasticidad fenotípica son cada vez más documentadas en las especies de plantas de estos sistemas (Jump & Peñuelas 2005; Byars et al. 2007).

Bradshaw & Thoday (1965) describe la plasticidad fenotípica como las diferencias en la fisiología, morfología y/o desarrollo de un organismo que resultan de una respuesta a cambios en su ambiente. De esta forma, abarca tanto los cambios reversibles como aquellos no reversibles relacionados con los fenotipos. La plasticidad fenotípica permite cambios rápidos en los individuos, donde la respuesta por una vía genética sería insuficiente (Bradshaw & Thoday 1965). Por el otro lado, el concepto de adaptación local se explica mediante la actuación de una fuerza de selección que permite la evolución de un rasgo concreto, proporcionando una ventaja en un hábitat determinado, sin tener en cuenta que consecuencias puede tener ese rasgo en otro hábitat donde aparece esa especie (Wiiliams 1966). Son numerosos los factores que pueden influir, tanto en las respuestas de adaptación local (Kawecki 2008; Kawecki & Ebert 2004) como en las de plasticidad fenotípica (Valladares et al. 2006). Esto incluye, entre otros, las características de las fases del ciclo vital de la planta, el grado de estrés al que está sometida, los niveles de aislamiento respecto a otras poblaciones y las interacciones entre especies. Todos estos factores pueden actuar en la misma dirección o en direcciones opuestas y también pueden ver sus efectos potenciados o aminorados entre sí. Incluso dentro de una misma especie, la respuesta puede ser diferente en función del lugar donde se encuentre la población (Kawecki 2008) o incluso ser insuficiente como para impedir la extinción local de la especie. Muchas de estas reacciones ante los nuevos escenarios se han relacionado con medidas de bienestar, supervivencia o procesos biológicos propios de cada una de las especies. Pero hasta la fecha, estas respuestas y las consecuencias que, desde un

punto de vista genético, puede tener el cambio climático en las plantas han supuesto un reto difícil de abordar (Reusch & Wood 2007).

A su vez, el cambio climático puede tener múltiples consecuencias y efectos (Lovejoy & Hannah 2005) y algunas de ellas podrían repercutir de una u otra manera a nivel genético, tanto en poblaciones de especies puntuales como en el conjunto de la comunidad. La fragmentación y la disminución del tamaño de las poblaciones tienen consecuencias directas sobre varios parámetros genéticos, reduciendo en flujo génico entre poblaciones vecinas y haciéndolas más sensibles a fenómenos de deriva o depresión endogámica (Young et al. 1996). Esto, combinado con cambios en factores demográficos o alteraciones en los polinizadores lleva a una reducción de los parámetros de bienestar en el caso de especies de plantas y en casos extremos, podría incluso provocar la extinción de la especie (Lande 1993; Ellstrand & Elam 1993). El aislamiento de poblaciones es un fenómeno descrito principalmente en especies que habitan islas oceánicas (p.e. García-Verdugo et al. 2010), aunque algunos autores también han propuesto este modelo para describir a poblaciones en las cumbres de las altas montañas ("islas en el cielo", Hewitt 2000 & 2001), donde los movimientos entre fragmentos estarían seriamente restringidos. Otro cambios, no tan agresivos, pero que también estaría relacionados con el cambio climático irían desde modificaciones en las frecuencias de determinados alelos (Caicedo et al. 2004) a cambios en la expresión de determinadas secuencias o compuestos (Sørensen et al. 1999), entre otros (Reusch & Wood 2007).

Montañas y cambio climático: laboratorios naturales.

Si bien las montañas ocupan un pequeño porcentaje sobre la superficie de Tierra emergida (un 5% tiene una altitud superior a 2000 metros, Körner (2007), resultan esenciales para muchas especies (Barthlott et al. 1996), entre ellas la humana (Messerli & Ives 1997). Las montañas, a pesar de su enorme fragilidad (Diaz et al. 2003), proporcionan una gran cantidad de servicios ecosistémicos, actuando como fuente de recursos naturales (agua, madera, ganadería, minerales, etc.) u otros servicios relacionados con el ocio y con gran repercusión económica (Nagy & Grabherr 2009). Además, su impacto no es únicamente local, sino que influye en el clima y las condiciones de las regiones próximas (Nogues-Bravo et al. 2007). Simultáneamente, las montañas tienen gran valor albergando al menos un tercio de la diversidad de plantas terrestres (Barthlott et al. 1996), la cual es debida, en gran medida, a los importantes gradientes ambientales que se producen en las montañas que dan lugar a hábitats muy heterogéneos (Nagy & Grabherr 2009; Körner 2003). La altitud funciona como variable resumen, integrando el impacto de varios factores que se modifican según se asciende en altura, permitiendo grandes alteraciones ambientales (precipitación, temperatura, cantidad de nieve etc.) en pequeñas distancias espaciales. Existe gran consenso sobre el alto impacto que está teniendo el cambio climático en las montañas de todo el mundo, aunque las consecuencias que puedan tener sobre las comunidades y especies que las habitan parecen imprevisibles (Nogues-Bravo et al. 2007). Hasta la fecha, existen evidencias sobre cambios en las

comunidades de alta montaña (p.e. García-Romero et al. 2010; Illán et al. 2010) incluyendo movimientos de ascensión de especies que habitan áreas a menor altitud, hacía nuevos hábitats para mantenerse dentro de su nicho ecológico (Wilson et al. 2005; Sanz-Elorza et al. 2003; Kullman 2010). Todo ello conlleva un aumento en la competencia por los recursos disponibles (entre las nuevas especies y las propiamente autóctonas), lo que en un escenario altamente dinámico, multiplica el peligro de extinción (Pounds & Puschendorf 2004; Engler et al. 2009). Sin embargo, los movimientos de ascensión no son siempre posibles, bien debido a la escasa capacidad dispersiva (Engler et al. 2009), a la existencia de alguna barrera, física o ecológica o a que el hábitat disponible es limitado o incluso inexistente (Körner 2003), por lo que las otras respuestas (plasticidad fenotípica o adaptación local) cobran gran importancia. A pesar de los múltiples impactos negativos que puedan producirse, todos estos cambios suponen un reto para la comunidad científica y una oportunidad única para conocer como están reaccionando las especies que habitan las formaciones montañosas. Todas estas características citadas anteriormente convierten a las montañas en grandes laboratorios naturales, donde pueden evaluarse todo tipo de hipótesis evolutivas o adaptativas en distintas condiciones y ambientes, incluyendo el actual marco de cambio climático (Körner 2007).

Sistemas mediterráneos: fragilidad e importancia. Las peculiaridades de la alta montaña mediterránea.

El área mediterránea ha sido identificada como una de las más vulnerables a los efectos del cambio climático (Sala et al. 2000; Parmesan & Yohe 2003). Los modelos predictivos estiman un incremento medio de entre 3 a 5 grados en la cuenca mediterránea en el siglo XXI, a lo que se añadiría un descenso medio estimado del 20% en las precipitaciones (IPCC 2007). A estos valores predecidos, se les sumaría el impacto del aumento de los efectos extremos, tanto en intensidad como frecuencia (p.e. la ola de calor del verano del 2003 en Europa, Schar et al. 2004), lo que podría resultar fatal para numerosas especies. Los ecosistemas mediterráneos son reconocidos a nivel global como uno de los 25 puntos relevantes de la biodiversidad a nivel mundial (Brooks et al. 2002; Hoekstra et al. 2005), debido a las heterogéneas condiciones ambientales que se dan en estos sistemas, el alto número de endemismos que la habitan (p.e. Médail & Quézel 1999) o la prolongada explotación humana a la que están sometidos (Kosmas et al. 2000; Matesanz et al. 2009), entre otros muchos factores. Todo ello no hace sino aumentar el interés por el estudio de estos sistemas, de las especies que lo habitan y como son sus reacciones ante un sistema cambiante.

Dentro de los sistemas mediterráneos, las altas montañas ocupan un lugar singular. Como formaciones de gran altitud en regiones templadas, se ven sometidas a fríos inviernos que las cubren de un manto nevado que impiden o disminuyen en gran medida la actividad biológica en ellas en la época invernal (Körner 2003). A esto hay que sumarle el periodo de sequía estival, típico de regiones mediterráneas (Larcher 2000) y que no se produce de forma tan acusada en montañas más norteñas, lo

cual hace que las especies que habitan las montañas mediterráneas tengan que soportar dos periodos especialmente adversos para su crecimiento y reproducción (Giménez-Benavides et al. 2007b; Lavorel et al. 1999). Aunque pareciera que las montañas mediterráneas son zonas muy hostiles para la vida, en ellas habitan un gran número de especies. Por un lado, aparecen especies típicas de latitudes superiores que quedaron aisladas en estas montañas en los sucesivos movimientos migratorios asociados a los procesos de glaciación que azotaron Europa durante el cuaternario (Hewitt 2000). Por otro, existe un importante número de endemismos, de especies que han permanecido aisladas y han evolucionado de forma singular (Giménez et al. 2004; Martin et al. 2000). Todo ello no hace sino aumenta el interés por la conservación y estudio de estas zonas, así como de las especies que las habitan y los mecanismos que están gobernando las respuestas que permiten su supervivencia en medios tan hostiles, incluyendo los procesos genéticos.

Objetivos

El objetivo general de esta tesis se centra en evaluar los efectos y las consecuencias genéticas que están produciéndose a raíz de las respuestas de las plantas de alta montaña mediterránea ante los procesos derivados del cambio climático. Para ello, se han seleccionado dos especies representativas de la comunidad: *Silene ciliata* y *Armeria caespitosa,* de las cuales se conocen numerosos aspectos de su biología, estado demográfico de las poblaciones, éxito reproductivo, etc. A su vez, se han empleado distintos tipos de técnicas moleculares y aproximaciones, en función de las hipótesis planteadas en cada uno de los capítulos.

Más concretamente, para alcanzar este objetivo general anteriormente citado, se han planteado una serie de objetivos específicos que se abordan en los cinco capítulos de esta tesis doctoral:

a) Estudiar el posible efecto de la altitud y los factores de estrés asociados sobre las características cariológicas. Empleando una especie con una gran variabilidad en sus niveles de ploidía (*Silene ciliata)*, estudiamos la existencia de posibles modificaciones, tanto en el número de cromosomas, como de alteraciones en los mismos o en su tamaño (Capítulo 1).
b) Evaluar el impacto de la fragmentación y el aislamiento de los pastos psicroxerófilos de alta montaña sobre los parámetros genéticos generales de las poblaciones de plantas que los habitan (diversidad, endogamia, alelos exclusivos, etc.). Resulta indispensable conocer en qué estado se encuentran las poblaciones para evaluar tanto sus respuestas como su potencial. Este objetivo se plantea tanto a gran escala, evaluando un gran número de poblaciones de A*rmería caespitosa* con distinto grado de aislamiento, que cubre prácticamente el rango de distribución de la especie (Capítulo 2); como a una escala local, mediante seis poblaciones de *Silene ciliata* con evidencias de adaptación local dispuestas a lo largo de dos gradientes altitudinales en sendas montañas próximas (Capítulo 3).
c) Estimar los niveles de flujo génico y estructura genética entre las poblaciones de plantas de alta montaña mediterránea. Esto incluye, en la medida de lo posible, considerar movimientos a lo largo del gradiente de altitud, tanto presentes como pasados, pero también movimientos en sentido horizontal, a lo largo de la misma franja altitudinal. Para alcanzar este objetivo, se realizó un estudio con cada una de las especies empleando distintos tipos de marcadores moleculares, debido a las diferentes características y distribución de sus poblaciones (Capítulo 2 y Capítulo 3).
d) Determinar la importancia de la depresión endogámica en plantas de alta montaña mediterránea en ambientes con diferentes grados de estrés y estimar el nivel de afección en cada población. Para este propósito se seleccionaron tres poblaciones de *Silene ciliata*

(por su capacidad para auto-polinizarse) con evidencias de adaptación local, dispuestas en un gradiente de altitud en el que se identificaron distintos niveles de estrés (Capítulo 5).

e) Evaluar, en la medida de lo posible, el posible impacto que las fuerzas de selección están ejerciendo sobre el genoma de las plantas de alta montaña mediterránea. Para ello se emplearon dos estrategias: un rastreo genético sobre la totalidad del genoma de *Armeria caespitosa*, buscando posibles loci sobre selección (Capítulo 2) y el uso de microsatelites EST transferidos en *Silene ciliata*, que, al encontrarse en regiones expresadas del genoma en la especie original, podrían estar más relacionados con determinados procesos de selección o de adaptación local (Capítulo 3).

f) Determinar la posible relación existente entre determinados parámetros genéticos y la adaptación local. Esto incluye buscar evidencias de la adaptación en la estructura genética (Capítulo 2), controlar el impacto de la diversidad genética en la respuesta (Capítulo 4) y por último, evaluar si existe una respuesta diferenciada en los individuos adaptados localmente a nivel de proteico (Capítulo 4).

Metodología general

Área de estudio

La totalidad de los individuos y especies vegetales de esta tesis doctoral forman parte de las comunidades de pastos de alta montaña mediterránea situados en el Sistema Central, más concretamente en la Sierra de Guadarrama y algunas formaciones contiguas. Dichos pastos se extienden desde una altitud de 2000 metros, sobre suelos pobres y poco profundos. Algunas poblaciones seleccionadas para esta tesis fueron elegidas a menor altitud, en pequeños fragmentos aislados, donde la comunidad o algunos de sus componentes pueden aparecer. La comunidad vegetal de estos pastos está formada mayoritariamente por gramíneas duras, entre las que destacan *Festuca curvifolia* y *Nardus stricta*, formando estructuras de bandas o "guirnaldas". También son numerosos los nanocaméfitos almohadillados, como *Jasione crispa* subsp. *Centralis* o *Thymus penyalarensis.* A su vez, es importante el número de especies de origen alpino-pirenáico, como *Agrostis rupestres* o *Jurinea humilis,* que aparecen en el Sistema central como reflejo de su paso por estas montañas durante los procesos glaciares. Más detalles sobre la composión florística (ver ejemplo de la Figura 1) de estas comunidades pueden consultarse en Escudero et al. (2005) or Gutierrez-Girón & Gavilán (2010).

Figura 1. Disposición típica, en bandas o guirnaldas, de un pastizal de alta montaña dominado por *Festuca curvifolia*.

En todos capítulos se incluyeron poblaciones que aparecen dentro del gradiente altitudinal del Pico de Peñalara (2420m), que forman parte del Parque Natural de la Cumbre, Circo y Lagunas de Peñalara. Además, en los Capitulos 2 y 3 se emplearon poblaciones que se encuentran dentro del Parque Regional de la Cuenca Alta del Manzanares. Ambos espacios naturales se encuentran dentro de la red de espacios protegidos de la Comunidad de Madrid.

Geologicamente, la Sierra de Guadarrama es un conjunto de estribaciones montañosas formadas por una serie de elevaciones y depresiones (Graben y Horts respectivamente) de naturaleza granítico-metamórfico. Este alzamiento se produjo en la orogenia alpina (que comenzó hace unos 65 millones de años). Durante el Cuaternario, estas montañas se vieran afectas por los procesos glaciares que han modelado de forma significativa su relieve. La actividad de estos procesos glaciares es especialmente importante, dado que ha limitado el hábitat disponible para las especies vegetales en los últimos miles de años, forzándolas a desplazarse a lo largo del gradiente altitudinal.

La estación meteorológica más representativa de la zona se encuentra en el puerto de Navacerrada (40º46' N, 4º 19' O), a una altitud de 1890 metros. La temperatura media anual (medida entre 1971 y 2000) esta en torno a los 6,4ºC mientras que la precipitación anual media se estima en 1330 mm. La marcada sequía estival se refleja con el descenso de las precipitaciones entre los meses de Junio y Septiembre, que no alcanzan el 10% de las precipitaciones totales. La Figura 2 resume las condiciones climáticas de la zona estudiada.

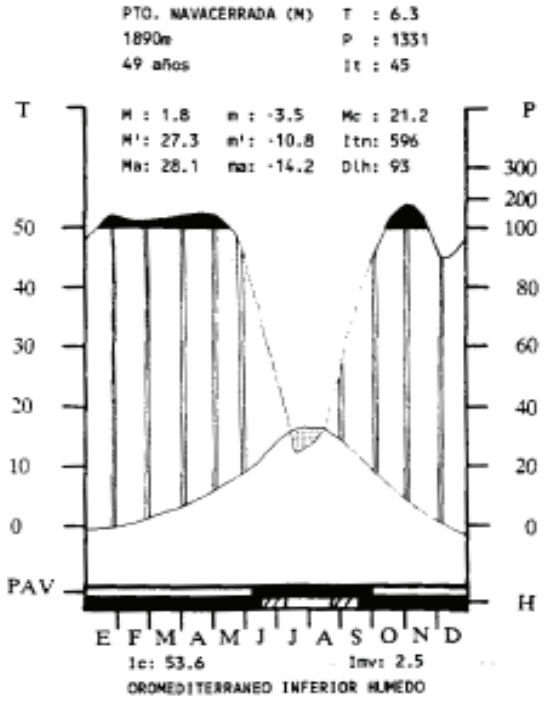

Figura 2. Esquema ombroclimático correspondiente a la estación de Navacerrada. Obtenido de Rivas-Martínez et al (1990)

Silene ciliata y _Armería caespitosa_ como especies modelo. Similitudes y diferencias

Silene ciliata Pourret es una especie perene que pertenece a la familia de las *Caryophyliaceae*. Tiene un porte de pequeño tamaño, alcanzando los 25-30 centímetros de diámetro máximo y poco más de 10 cm de altura, con forma almohadillada o de "cojin". Habita las regiones montañosas del arco mediterráneo, desde el Sistema Central a los Balcanes, incluyendo los Pirineos, Apeninos y Alpes (Tutin et al. 1995). Es una especie geitonogámica (en la planta se aparecen flores hermafroditas y femeninas) con una fuerte protandria (las estructuras masculinas de desarrollan previamente a las femeninas) lo cual limita en cierta medida posibles autocruzamientos, aunque la planta no es autoincompatible. Una de las características principales es su alto nivel de poliploidia, dado que se han descrito desde individuos diploides con 24 cromosomas a tetraploides con más de un centenar (Küpfer 1974; Blackburn 1933). Esta variabilidad en el número de cromosomas puede estar detrás de la gran diversidad fenotípica de la especie, que ha llevado a algunos autores a proponer la existencia de varias subespecies. En nuestra zona de estudio, la especie aparece puntualmente en poblaciones aisladas en torno a los 1900 metros de altitud (lo que supone su límite meridional de distribución) hasta poblaciones más o menos continuas en pastos a partir de 2200 metros. Su floración es relativamente breve y se produce en torno a las últimas semanas de agosto y primeras de septiembre (Giménez-Benavides et al. 2010). Aunque puede ser polinizada por varios tipos de insectos generalistas, *S. ciliata* presenta una relación mutualista con la polilla nocturna *Hadena consparcatoides* (Giménez-Benavides et al. 2007).

Por otro lado, *Armeria caespitosa* (Gómez Ortega) Boiss. *in* DC. es una planta de alta montaña que pertenece a la familia *Plumbaginaceae*. Al igual que *Silene ciliata,* es un planta perene de pequeño tamaño (hasta 20 cm) y con forma almohadillada (Nieto Feliner 1990). *A. caespitosa* es una especie endémica aunque muy abundante de la Sierra del Guadarrama, apareciendo desde los 1600 metros de altitud hasta los 2420. También se han encontrado poblaciones en el entorno de la Sierra de Ayllón y en las proximidades de la Sierra de Gredos, donde debido a las débiles barreras internas de este género, forma híbridos con su congénere *Armeria bigerrensis* subsp. *bigerrensis*, perdiendo sus rasgos caracerísticos (Nieto Feliner 1990). Tiene una extraordinaria capacidad de sobrevivir en todo tipo de hábitats (García-Camacho & Escudero 2009), formando desde poblaciones aisladas en pequeños roquedos o grandes poblaciones continuas durante cientos de metros en los pastos de alta montaña. Tiene una dotación diploide (2n = 18, Moore 1982) y un sistema de autoincompatibilidad estricta que impide autocruzamientos. *A. caespitosa* desarrolla flores hermafroditas, siendo una de las primeras especies que florecen en la comunidad de alta montaña y con una longevidad floral bastante elevada (Giménez-Benavides et al. 2010), lo cual favorece que sea polinizada por varios tipos de insectos (García-Camacho et al. 2009).

Figura 3. Ejemplares de *Silene ciliata* (izquierda) y *Armeria caespitosa* (derecha) en sus poblaciones naturales.

Técnicas moleculares aplicadas al estudio de las consecuencias genéticas del cambio climático en plantas de alta montaña.

Cada vez es mayor el número de herramientas moleculares de las que se dispone para el estudio genético de los organismos, lo que favorece su empleo en todo tipo de estudios. Esto ha permitido obtener una gran cantidad de información, incluso de especies no modelo y a unos costes cada vez menores. Sin embargo, antes de realizar cualquier estudio, conviene reflexionar sobre cual utilizar en función de las preguntas que pretendamos resolver, nuestros recursos y conocimientos sobre la propia especie a estudiar.

Citometría de flujo.

La citometría de flujo es una técnica de análisis celular que implica medir las características de dispersión de luz y/o fluorescencia que posen algunas células o partículas según se las hace pasar a través de un rayo de luz (Roger et al. 2005). Aunque sus primeras aplicaciones fueron en el campo de la biología celular, sus aplicaciones son cada vez más numerosas, dado que permite trabajar con todo tipo de muestras en estado líquido (o sólido en suspensión) y permite el conteo de miles de células u otro tipo de partículas (granos de polen o cromosomas).

Una de las aplicaciones más extendidas del citómetro de flujo es la medida del tamaño del genoma (Bennett & Leitch 2005) y la estimación de los niveles de ploidía u otras variaciones en el número de cromosomas (aneuploidía, cromosomas B etc.). Para ello se emplean distintos compuestos fluorescentes (yoduro de propidio, 4',6-diamidino-2-phenylindole (DAPI) y plantas con ploidía y genomas de tamaño

conocido, también llamadas plantas patrón (entre las mas empleadas destacan *Pisum sativum* "Express Long" y *Petunia hybrida* "PxPc6", Johnston et al. (1999), que al tratarse junto con la especie problema, permite un análisis comparativo de gran fiabilidad.

Western Blots.

El Western Blot es una técnica analítica empleada para la detección de proteínas, normalmente a partir de una muestra compleja formada por un conjunto de ellas. Por lo general, viene precedida de una separación de las proteínas mediante un gel sometido a electroforesis y que posteriormente son transferidas a una membrana adsorbente (Towbin et al. 1979).

Tras este paso, mediante anticuerpos específicos, se analiza la presencia de determinadas proteínas de interés así como su comparación cualitativa con otras. Para ello, es necesario realizar un bloqueo de aquellas zonas de unión que han quedado libres tras la transferencia, mediante una solución de albumina de suero bovino (BSA en sus siglas en ingles) y posteriormente, una detección mediante un anticuerpo específico de la enzima buscada. Para su visualización se emplean anticuerpos secundarios, que reconocen regiones concretas del anticuerpo específico y que en presencia de determinados sustratos, emiten fluorescencia o colorimetría.

Amplified Fragmented Length Polymorphism (AFLP).

Los polimorfismos en la longitud de fragmentos amplificados (o más conocidos por su acrónimo en ingles, AFLP), son marcadores moleculares basados en la restricción del ADN genómico mediante enzimas de restricción y subsecuentes amplificaciones de algunos de estos fragmentos mediante reacciones en cadena de polimerasa (PCR) usando cebadores inespecíficos (ver descripción de la técnica en Vos et al. (1995).

Esta técnica permite trabajar con la totalidad del genoma, lo cual proporciona una gran cantidad de información para su análisis a un coste relativamente bajo. Además, los reactivos y protocolos son independientes de la especie con la que se trabaje (siempre con ciertos matices) por lo que no es necesario ningún conocimiento previo sobre su genoma y pueden utilizarse los reactivos en varias especies simultáneamente. Por el contrario, el gran volumen de información generada dificulta en cierta medida los análisis y favorece los errores por parte del tratamiento posterior de datos. Por otro lado, la información obtenida con estos marcadores es de naturaleza dominante, lo cual limita hasta cierto punto los análisis posteriores. Finalmente, se les consideran marcadores neutrales, puesto que se somete a la totalidad del genoma a la acción de enzimas de restricción no específicas, por lo que en un principio, no es posible relacionar estos fragmentos con fuerzas selectivas (Freeland 2005).

Microsatélites (SSR) y Microsatélites de Expresión Génica (EST-SSR).

Los microsatélites (conocidos por su acrónimo en inglés SSR) son secuencias del ADN distribuidas en el genoma que se caracterizan por presentar un número variable de repeticiones en tándem (generalmente superior a 5) de una secuencia corta de nucleótidos denominada motivo de repetición (Tautz 1989). Estas regiones han sido localizadas en todo tipo de organismos estudiados, tanto eucariotas (Tautz & Renz 1984) como procariotas (Gur-Arie et al. 2000). Los microsatélites se clasifican en función de la longitud del motivo de repetición y la pureza de la secuencia (Weber 1990). Han sido muy utilizados en todo tipo de estudios relacionados con la biología molecular de plantas al ser regiones altamente polimórficas (Schlötterer 2000). Si bien no existen loci caracterizados para todas las especies, el cada vez menor coste de las técnicas de secuenciación masiva generaliza su uso y permite crear una mayor base de datos disponible para todo tipo de organismos. Además, la naturaleza codominante de estos marcadores permiten implementar un gran numero de cálculos y estimadores para una gran variedad de estudios.

El mayor inconveniente de los marcadores microsatélites viene asociado a que se les considera marcadores neutrales, esto es, que no están relacionados con ningún proceso de expresión génica y su localización en el genoma suele ser en lugares no codificantes. Por ello, relacionar cualquier característica de los microsatélites a fuerzas selectivas sería en principio erróneo y fruto del azar. Los microsatélites *EST* (Express Sequence Tag en sus siglas en inglés) solventan este problema, pues no son obtenidos de cualquier parte del genoma, sino a partir de ARN mensajero que se retrotranscribe en ADN complementario y a partir del cual, se obtienen los marcadores. Esto asegura que este tipo de marcadores genéticos están relacionados con algún proceso de expresión y por lo tanto, pueden estar relacionados con algún proceso de selección (Varshney et al. 2007).

Estructura general de la tesis

Todos los capítulos de la presente memoria han sido escritos en inglés para su publicación en revistas científicas de ámbito internacional. Por ello, se presentan los manuscritos originales de dichos artículos. A modo de resumen, se incluye a continuación una traducción de su título, el nombre de los coautores y una breve síntesis de cada uno de ellos, así como de las hipótesis planteadas.

Capítulo 1 - Niveles de ploidía y tamaño del genoma en poblaciones localmente adaptadas de *Silene ciliata* a lo largo de un gradiente altitudinal. García Fernádez, A. Iriondo, J.M.; Vallès, J.; Orellana, J. & Escudero, A. Manuscrito en revisión en *Plant Systematics and Evolution.*

La adaptación local parece ser una de las respuestas más comunes ante el actual escenario de cambio climático. Se cree que este proceso puede llevar involucrados numerosos cambios a nivel genético, entre ellos alteraciones en genes, cambios en complejos génicos, expresión diferenciada etc. En este capítulo, evaluamos si la respuesta de adaptación local detectada en *Silene ciliata* a lo largo de un gradiente altitudinal, pudiera tener alguna relación con el número de cromosomas de los individuos, al ser una especie de la que se tienen documentados grandes variaciones en sus niveles de plodía, o con diferencias en el tamaño de sus cromosomas. Concretamente, nuestras hipótesis de trabajo fueron: i) Las poblaciones de *Silene ciliata* poseen distintos niveles de ploidía en función de su altitud. ii) El tamaño de los cromosomas de los individuos de diferentes poblaciones de *Silene ciliata* se encuentra relacionado con la ubicación de la población en el gradiente de elevación. iii) En algunas poblaciones pueden aparecer procesos de alteración en el numero de cromosomas (aneuploidía, mixoploidía, cromosomas B, etc.) como vestigios de poliploidía en generaciones anteriores. Empleamos para ello dos técnicas diferentes: recuentos de cromosomas empleando técnicas de conteo al microscopio (contraste de fases y fluorescencia), y estimas del tamaño del genoma mediante citometría de flujo, lo que nos permitió trabajar con un gran número de individuos. Los resultados mostraron que los individuos no variaron su dotación cromosomática, siendo la dotación diploide 2n = 24 la única encontrada, pero el tamaño de los cromosomas si fue significativamente diferente. Los mayores tamaños del genoma aparecieron en las poblaciones intemedias, frente a las poblaciones extremas, probablemente asociado al mayor estrés al que están sometidas estas poblaciones, de acuerdo con el modelo unimodal propuesto en relación con la altitud.

Capítulo 2 - Islas en el cielo: un rastreo genético sobre todo el rango de distribución de una planta endémica de alta montaña. García-Fernández, A., Iriondo, J. M., Escudero, A., Fuertes-Aguilar, J. & Nieto-Feliner, G. Manuscrito enviado a *Molecular Ecology*

A la hora del estudio de poblaciones fragmentadas suelen emplearse lugares concretos dentro de la distribución de una especie, normalmente en zonas más o menos aisladas. Es posible que sean las islas, rodeadas por mares u océanos, uno de los ejemplos más claros de este marco de trabajo. En este capítulo realizamos una aproximación similar al estudio de poblaciones fragmentadas en islas, si bien consideramos que los parches están formados por las cumbres de las montañas. La altitud juega un papel fundamental en el análisis espacial, modificando el hábitat disponible, resumiendo el efecto de varios gradientes ambientales, o influyendo en la migración y el flujo genético entre otras muchas cosas. Este estudio lo llevamos a cabo incluyendo todo el rango de distribución de una especie endémica, *Armeria caespitosa*, muy abundante en las formaciones montañosas de la Sierra de Guadarrama, que constituye poblaciones aisladas en montañas a partir de 1600 metros. Empleamos para ello marcadores de Polimorfismos en la Longitud de Fragmentos Amplificados (AFLP en sus siglas en inglés), que nos permiten realizar un rastreo sobre la totalidad del genoma, obteniendo un gran número de marcadores polimórficos que intentamos relacionar con otras variables. Los supuestos que barajamos en este trabajo fueron: i) La diversidad genética de las poblaciones de *Armeria caespitosa* está influenciada por su posición en el gradiente altitudinal. ii) La estructura genética de las poblaciones de *Armeria caespitosa* está relacionada con la distancia espacial y/o altitudinal entre las poblaciones. iii) Algunos de estos marcadores pueden estar relacionado con procesos de selección natural, reflejando el efecto de variables ambientales sobre el genoma de esta especie. Obtuvimos unos valores altos de diversidad genética, similares en todas las poblaciones, lo cual supone altos valores de flujo genético entre poblaciones. Esto soportaría la ausencia de una relación significativa con variables ambientales, aunque los modelos de agrupamiento bayesiano mostraron algunos escenarios con cierto agrupamiento entre poblaciones. Sin embargo, si aparecieron un elevado de loci potenciales bajo selección y algunos de ellos aparecieron relacionados con variables ambientales, lo que demostraría su relación con fuerzas selectivas.

Capítulo 3 - Desenredando el flujo genético en la cima. ¿Islas de montaña o bandas de altitud? García-Fernández, A., Segarra-Moragues, J.G., Widmer, A., Escudero, A. & Iriondo, J. M. Manuscrito Inédito.

El flujo genético, tanto a nivel de movimiento de polen como de dispersión de semillas, se ha considerado como uno de los principales factores limitantes en las respuestas de adaptación local ante el cambio climático, dado que permite la llegada de individuos no adaptados o bien diluye el efecto de aquellos genes que se están seleccionando. Considerando las presentes evidencias de adaptación local en *Silene*

ciliata y sus características biológicas, en este capitulo nos planteamos hasta qué punto se está produciendo flujo genético entre las poblaciones y si éste es suficientemente intenso como para limitar los procesos adaptativos. Por ello, nos planteamos i) Evaluar hasta cuál es la dirección predominante del flujo génico en un entorno de alta montaña: horizontal, dentro de un mismo piso altitudinal o vertical, dentro de una misma montaña, a lo largo del gradiente de altitud y ii) evaluar si la estructura genética de las poblaciones es compatible con las evidencias de adaptación local previamente detectadas. Para ello empleamos dos tipos de marcadores moleculares, microsatélites SSR específicamente diseñados para *Silene ciliata* y microsatélites EST, transferidos de *Silene latifolia* y que podrían reflejar procesos de selección activos. Los resultados mostraron altos valores de flujo genético, especialmente a lo largo del gradiente altitudinal, frente a movimientos horizontales (aunque las diferencias no fueron significativas), lo cual estaría acorde con los valores homogéneos de diversidad genética hallados en cada población. También se detectaron evidencias de la adaptación local, que está teniendo lugar en las poblaciones de *Silene ciliata,* dentro de la estructura genética de las poblaciones, formando un clúster independiente en los modelos de agrupamiento bayesiano.

Capítulo 4 – Respuestas al esters hídrico en una planta de alta montaña a lo largo de un gradient altitudinal ¿Hay evidencias de adaptación local? García-Fernández, A., Iriondo, J. M., Bartels D. & Escudero, A. Manuscrito inédito.

Ante la actual situación de cambio climático, se han hallado distintas evidencias que sugieren la adaptación local en altitud en las poblaciones de especies de alta montaña como una de las respuestas más comunes. Este es el caso de *Silene ciliata,* donde se han encontrado evidencias en la germinación y los aprámetros de crecimiento en las primeras fases de desarrollo, así como una adaptación del proceso de floración a las condiciones de estrés abiótico. Muchos de estos procesos producen una respuesta diferenciada ante el estrés hídrico, aunque ningún trabajo lo ha medido de forma directa. Por ello, nos proponemos medir la resistencia ante un evento de sequía extremo, cada vez más frecuente en los sistemas mediterráneos, y evaluar si existen diferencias entre las poblaciones adaptadas. También es posible que estas respuestas se den en aquellas rutas metabólicas directamente relacionadas con el stress hídrico, y que tienen su máximo exponente en las proteínas que expresan los individuos. Concretamente nos preguntamos: i) ¿Existe una respuesta diferenciada en cuanto la resistencia a los efectos de la sequía en individuos adultos de diferentes poblaciones? De ser así, ¿Qué población está mejor adaptada a condiciones extremas de sequía? ii) ¿Existen diferencias en la expresión proteica global y en la de proteínas directa o indirectamente relacionadas con el estrés hídrico? ¿Podemos identificar las proteínas diferentes? En caso de existir, ¿Las diferencias se producen entre poblaciones o también se detectan entre individuos de una misma población? Encontramos evidencias sobre una mayor resistencia a la sequía en los individuos procedentes de las poblaciones más bajas dentro del gradiente altitudinal, las cuales están sometidas más frecuentemente a este tipo de fenómenos de estrés. Estas diferencias

aparecieron únicamente en las primeras fases de estrés hídrico por lo que tampoco las poblaciones más al sur pueden soportar fenómenos de escasez de agua excesivamente prolongados. También hallamos diferencias en el patrón de proteínas y en la respuesta mediante anticuerpos específicos pero este patrón se repitió en cada una de las poblaciones, por lo que es posible que la repuesta diferencial esté asociada a un patrón cuantitativo de expresión. Finalmente, las técnicas de identificación de proteínas mediante espectrometría de masas nos permitió identificar la degradación de la enzima *Rubisco* como uno de los procesos que tienen lugar cuando las plantas de *Silene ciliata* se ven sometidas a procesos de sequía extrema.

Capítulo 5 - Endogamia en el límite de distribución: ¿Sé incrementa la depresión endogámica bajo condiciones estresantes? García-Fernández, A.; Iriondo, J.M. & Escudero A. Manuscrito enviado a *Journal of Ecology*.

Existe una gran controversia sobre la existencia de algún tipo de relación entre los niveles de estrés ambiental y la incidencia de depresión endogámica en poblaciones naturales. De la misma forma, también se desconoce la influencia de la depresión endogámica en procesos de adaptación local. Por todo ello, hemos realizado una siembra experimental de semillas de *Silene ciliata* en sus poblaciones naturales a lo largo de un gradiente altitudinal (lo que supone un gradiente de estrés) en donde se encuentran poblaciones localmente adaptadas. Simultáneamente, se realizaron experimentos en cámara de germinación para comparar los resultados obtenidos en campo con los obtenidos en condiciones óptimas. Las semillas provienen de distintos tipos de cruzamientos (auto-cruzamientos, cruces entre individuos de la misma población y cruces entre individuos de distintas poblaciones), y se monitorizó tanto la germinación como el crecimiento de las plántulas durante las primeras semanas de vida. Nuestras hipótesis de trabajo fueron: i) La depresión endogámica se ve potenciada en los ambientes más estresantes. ii) La adaptación local puede reducir los efectos negativos de la depresión endogámica. iii) La descendencia procedente de los cruces entre individuos de distintas poblaciones tendrá un mayor fitness tras reducir su endogamia. Todas las poblaciones mostraron evidencias de un menor fitness en semillas procedentes de cruces endogámicos,a si como una menor germinación en las semillas depositadas en cámara. La población de inferior altitud mostró un mayor impacto de la depresión endogámica, frente a semillas procedentes de cruces con individuos de otras poblaciones, por lo que los mayores niveles de estrés a los que está sometida esta población podría influir en el mayor impacto de la endogamia, aunque el hecho de que esta población esté más aislada que las otras también favorece que la endogámia sea más fuerte.

Conclusiones generales

De los trabajos llevados a cabo en esta tesis doctoral se pueden extraer las siguientes conclusiones generales:

1. No encontramos evidencias del impacto de los distintos factores de estrés sobre el los niveles de ploidía, a pesar de emplear para ello una especie con gran variabilidad en su número de cromosomas como es *Silene ciliata*. Sin embargo si hemos encontrado una distribución del tamaño del genoma en relación con la altitud donde fueron recolectados. Desconocemos si estas variaciones se deben a procesos de acumulación de ADN no codificante, transposones o de genes que puedan estar relacionados con algún factor de stress. Por otro lado, no hemos encontrado diferencias en la expresión de proteínas entre las poblaciones situadas a distinta altitud.
2. La depresión endogámica tiene un fuerte impacto en los parámetros de germinación y supervivencia durante las primeras fases del desarrollo de las plántulas en las poblaciones naturales de *Silene ciliata*. El impacto de la depresión fue diferente según las poblaciones estudiadas. El distinto grado de aislamiento de cada población así como los niveles de estrés a los que está sometida cada población estarían detrás de esta diferente respuesta.
3. La llegada de genes de otras poblaciones resulta ser beneficiosa en un primer momento para las poblaciones aisladas de *Silene* ciliata, aumentando la viabilidad de los nuevos individuos en las primeras fases de desarrollo. Sin embargo, desconocemos posibles impactos a largo plazo de estos cruzamientos (p.e. outbreeding-depression).
4. El flujo genético entre poblaciones, de ambas plantas estudiadas, se manifiesta en los resultados obtenidos con los marcadores moleculares. Estos movimientos son posibles a lo largo del gradiente altitudinal y entre poblaciones situadas a la misma altitud en diferentes montañas, aunque el primer supuesto parece más frecuente. Si bien estos movimientos son posibles en las actuales circunstancias, algunas características biológicas de las especies así como las propias condiciones de las montañas parecen dificultarlos. Es posible que los valores que encontramos reflejen, en cierta medida, movimientos de ascensión y descenso en estas montañas, asociados con los procesos glaciares-interglaciares en las montañas que se han producido en el pasado cercano (glaciaciones en el Cuaternario).
5. Encontramos evidencias de fenómenos de adaptación local a la altitud en la estructura genética de las poblaciones de *Silene ciliata* en uno de los gradientes altitudinales estudiados. La presencia de altos valores de flujo génico entre poblaciones tiende a diluir el potencial efecto de la adaptación local, por lo que su efecto sobre el genoma debe ser notable.
6. Un numero elevado de loci potencialmente bajo selección fueron hallados en el genoma de *Armeria caespitosa*. La relación de algunos de ellos con la altitud o variables ambientales

altamente relacionadas (climáticas) probarían la acción estas variables como fuerzas de selección sobre el genoma.

7. No encontramos ningún patrón definido a la hora de relacionar la distribución de la variabilidad genética a lo largo del gradiente de altitud, a pesar de emplear para ello especies con características biológicas diferenciadas y distintos tipos de marcadores. Los movimientos de recolonización, asociados con los procesos glaciares o las actuales presiones existentes debidas al cambio climático podrían estar detrás de que no exista un patrón común en las diferentes montañas.
8. Hemos encontrado nuevas evidencias de adaptación local en los individuos de *Silene ciliata*. En este caso, aparecieron reacciones diferenciadas en individuos adultos según su población de origen frente a un periodo de sequía extrema. Por el contrario, no hemos hallado diferencias en la expresión proteica frente a este proceso de estrés hídrico, tanto en el patrón global como en proteínas específicas involucradas ante fenómenos de escasez de agua. Aun así, no podemos descartar que existan diferencias cuantitativas a nivel proteico entre las poblaciones.

Chapter 1

Ploidy level and genome size of locally adapted populations of *Silene ciliata* across an altitudinal gradient

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Abstract

Silene ciliata Poiret is a small perennial that presents several ploidy levels and inhabits the mountain ranges of the European Mediterranean basin. Recent studies have shown evidence of local adaptation in populations located across an altitudinal gradient in Sierra de Guadarrama (Central Spain) at the species' southernmost distribution limit. In this study, we assessed whether the existence of local adaptation in these populations was related to differences in karyological features (ploidy level or chromosome number modification) or in nuclear DNA amount. Optical microscope (phase contrast and epifluorescence after DAPI staining) and flow cytometry were used to estimate the ploidy level and genome size of several family lines in three populations across the altitude gradient. With a sampling three times higher than usual in genome size assessments, all individuals showed a constant diploid set, so that polyploidy or other chromosome number modifications were discarded. The small genome size found was within the range of those found in other *Silene* species. Significant differences in genome size were found when the three populations of *S. ciliata* were compared. The largest genome size found at the intermediate population may be associated to lower environmental stress at the mid elevation in line with the recent studies in this area.

Introduction

Altitudinal gradients are excellent scenarios for testing hypotheses related to the present global warming framework (Körner 2003). This is because altitudinal gradients encompass several stress levels that operate in the same way and are positively correlated with altitude or, in some cases, operate in the opposite way. Plant response to changes in geophysical variables can also elicit evolutionary and genetic questions that can be approached through the study of altitudinal gradients in mountains systems around the world (Körner 2007). Mountains currently support one third of the biodiversity of terrestrial plants (Barthlott et al. 1996). If we also consider that mountains plants have reduced habitat availability (Körner 2007) and that many of them have limited dispersal ability, an obvious conclusion would be that mountains play an important role in plant diversification and evolution (Ohsawa & Ide 2008).

Even though change in ploidy level is a very frequent phenomenon in angiosperms (Masterson 1994; see also Soltis et al.(2009) and references there in), the effect of stress on ploidy level distribution is still uncertain (Cui et al. 2006; Otto & Whitton 2000). An increase in chromosome number has important physiological costs, such as cell size increase, which has negative effects for tolerance to abiotic stress (Stebbins 1971). However, polyploids are known to have greater ability to colonize new environments simply because they have greater potential genetic variation on which selection can play (Soltis & Soltis 1995; Leitch & Leitch 2008 and references therein). Furthermore, polyploids may be more tolerant to some conditions (Hagerup 1932; Li et al. 1996) and may contribute to local adaptation, increasing phenotypic variability or promoting phenotypic changes that randomly pre-adapt plants to new ecological conditions (Ramsey & Schemske 2002). Contradictory results have been obtained when ploidy levels were analysed in order to know whether they were subjected to different types of stress, like soil moisture (Baldwin 1941; Li et al. 1996) or cold gradients (Hardy et al. 2000; Sharma & Dey 1967). Ploidy levels have also been studied in altitude gradients (Liu et al. 2004). As a general rule, higher rates of polyploidy have been found at higher altitudes than at lower altitudes (Stebbins 1950), although a non negligible number of exceptions to this rule are found (cf., among others, Mráz et al. (2008), Sonnleitner et al. (2011) and references therein). These controversial results on gradients indicate that additional mechanisms or evolutionary processes play a significant role in plant tolerance and responses to stress (Ramsey & Schemske 2002).

The study of genome size has enormous potential in evolutionary research and quantitative genetics (Bennett & Leitch 2005; Bennett & Leitch 2011), including predictive and practical applications. In plants, significant variations in genome size have been reported, including changes of up to 2,400 times in angiosperms (Pellicer et al. 2010). The relationship between this ample variation in genome size and other biological variables has been a matter of prime interest (Greilhuber 2005). At the same time, different cellular processes in organisms have been related to genome size (Gregory & Hebert 1999), including many phenotypic features and physiological processes in plants (Bennett 1998; Vinogradov 2004). Nevertheless, the evolutionary process of genome size change and the forces bringing about these

changes are still incompletely known (Bennett & Leitch 2005). The relationship between genome size and stress and, more specifically, altitude has been discussed by many authors and analyzed in different plant species with different results (see revision in Knight et al. 2005). These contradictory results suggest the lack of a linear relationship between altitude, and probably other stress gradients and genome size (Knight et al. 2005; Ohsawa & Ide 2008).

Recent studies on *S. ciliata* along altitudinal gradients in the mountains of Sistema Central (Spain), at the southernmost limit of its distribution, have shown evidences of local adaptation (Gimenez-Benavides et al. 2007a). Moreover, some critical demographic processes, including reproductive success, recruitment, and seedling survival have been described along these gradients (Gimenez-Benavides et al. 2007a; 2007b), where differences found between altitudes may also be partially explained by phenotypic plasticity. These results make these *S. ciliata* populations a fine study system to evaluate responses to rapid climate change at the rear edge of the species' distribution (Eckert et al. 2008).

Taking into account that several ploidy levels have been described in *S. ciliata* (Blackburn 1933; Küpfer 1974), we hypothesized that local adaptation in *S. ciliata* populations might be associated with changes in ploidy levels (Tutin et al. 1995) or differences in genome size. The different levels of environmental stress, associated with altitudinal gradient might affect genome size and/or ploidy level in each population, or might cause other chromosome pattern modifications, such as B chromosomes and aneuploidy or myxoploidy phenomena. To test this hypothesis, we employed two different approaches: classical chromosome counts based on phase contrast and fluorescence microscopy and estimation of genome size using flow cytometry.

Materials and Methods

Species and study area

Silene ciliata Poiret (Caryophyllaceae) is a perennial cushion plant that typically forms rosettes of up to 2 cm in height and 15 cm in diameter, with high variability in size. Self-pollination experiments (A. García-Fernández, unpublished data) indicate that *S. ciliata* is a self-compatible species, but passive autogamy is not possible due to a pronounced protandry. *S. ciliata* inhabits the main mountain ranges in the northern half of the Iberian Peninsula, the Massif Central in France, the Apennines and the Balkan Peninsula (Tutin et al. 1995). This species reaches its southern latitudinal limit in Central Spain.

The studied populations were located in Sierra de Guadarrama (Peñalara Natural Park) a SW-NE mountain range located 60 km North-West of Madrid, Spain. Three populations were chosen at different altitudes along the gradient to cover the local altitude range. The lowest population (1980 m, hereafter "Low") was located in a moraine deposit at Hoya de Peñalara, a Pleistocene glacial cirque. Vegetation is dominated by a shrub matrix of *Cytisus oromediterraneus* Rivas Mart. et al. and *Juniperus communis* L. subsp. *alpina* (Suter) Celak with stunted pines (*Pinus sylvestris* L.) interspersed in a *Festuca curvifolia* Lag. pasture. The intermediate population (2250 m, hereafter "Intermediate") was located on the summit of Dos Hermanas peak. The community at this location is dominated by *Festuca curvifolia* in xerophytic pasture patches with some individuals of *Cytisus-Juniperus*. The highest population (hereafter "High") was situated on the summit of the Peñalara peak (2420 m). The summit area and crest are covered by patches of *F. curvifolia*, occasionally displaced by *Nardus stricta* L. This community, which supports extreme winds and is characterized by a high diversity of cushion plants, constitutes the main habitat of *S. ciliata* (see Gavilán et al. (2002) or Escudero et al. (2005) for details). These three study populations are the same as those used by Giménez-Benavides et al (2007a) and Giménez-Benavides et al. (2007b) in previous studies on this species. Figure 1 shows the location of the populations along the altitudinal gradient.

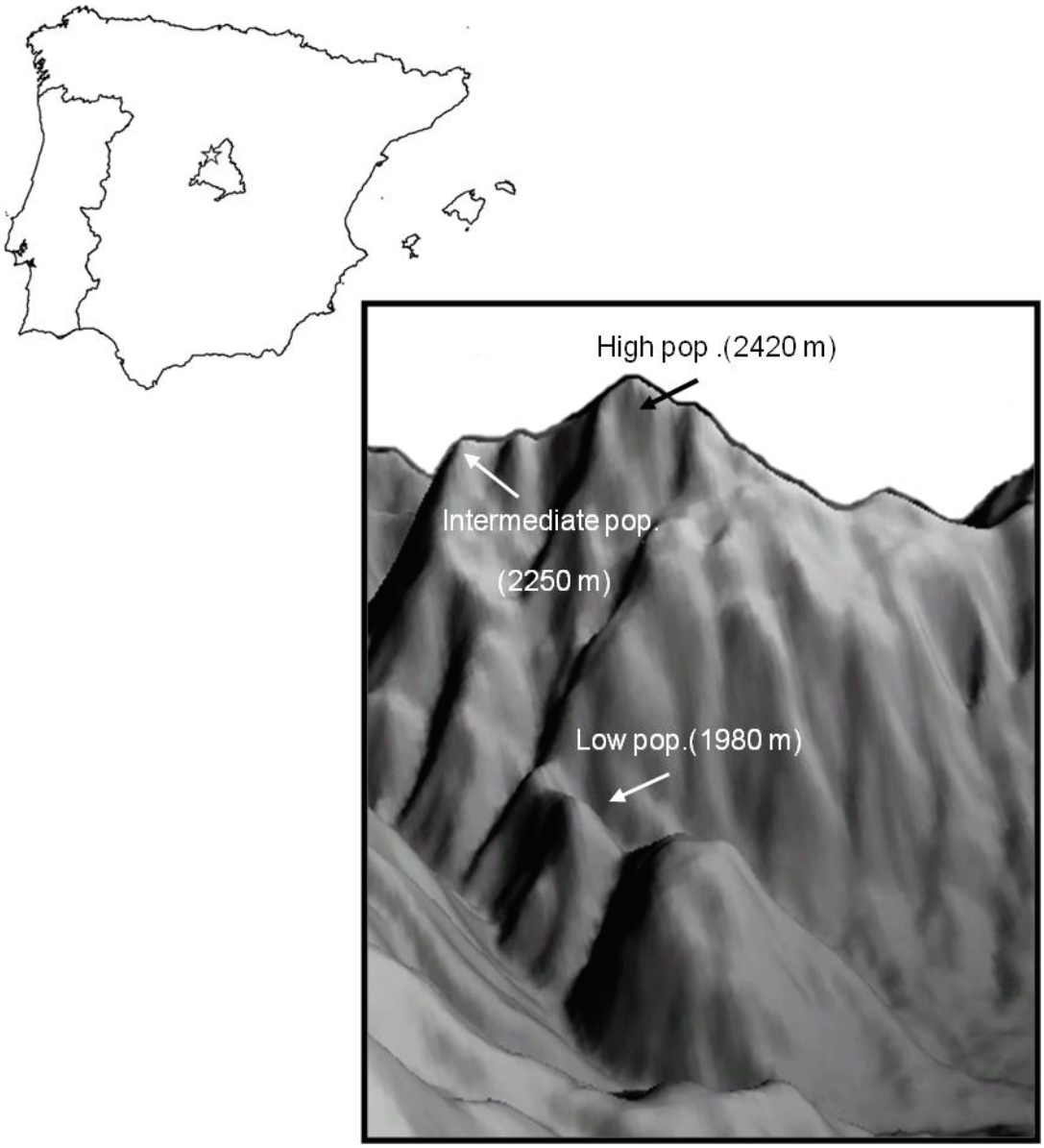

Figure 1 Location of Peñalara Natural Park in the Madrid province (white star in the centre of the Iberian Peninsula) and 3D model map indicating the position of Low, Intermediate and High populations in Peñalara Mountain, where seeds and leaf material were collected.

Chromosome numbers

Seeds of Low, Intermediate and High populations were collected directly from mother plants in September 2007. They were grouped by mother plant origin (hereafter family lines) and cleaned in the laboratory. A cold-wet stratification treatment was carried out to promote germination (Giménez-Benavides et al. 2005). Seeds were placed between two layers of filter paper in 8 cm Petri dishes which were then wrapped in aluminium foil and stored in a refrigerator at 4 ºC for two months. After this stratification process, seeds were placed in new Petri dishes with wet filter paper for germination. The germination chamber was set with a 16 h light/8 h dark photoperiod and a constant temperature regime (15ºC). Filter papers were kept

soaked until the end of the experiment. The location of Petri dishes within the chamber was changed weekly to homogenize environmental conditions. Dishes were checked for radicle emergence (more than 2 cm) twice a week.

A minimum of five family lines were selected from each population. For each family line, radicles were cut and kept in Eppendorf tubes with water during 24 h in the refrigerator. Subsequently, Carnoy´s solution (absolute ethylic alcohol-glacial acetic acid (3:1) was added and radicles were kept in the refrigerator for at least one week. Radicles were then warmed at 37 ºC for acid hydrolysis with 0.2 N HCl for five minutes, and then the root tip meristems were squashed on a drop of 45% acetic acid. We employed a phase contrast Nikon Type 104 microscope for chromosome observation and counting selecting well-spread somatic metaphase cells. In some especially difficult cases, fluorescence microscopy (ZEISS LSM 5 EXCITER) with different set of filters was also used to determine the chromosome numbers. In these cases, the samples were frozen in liquid nitrogen for a few minutes and dyed with 4',6-diamino-2-phenylindole (DAPI). In most family lines, samples were prepared with roots from more than one individual to increase the reliability of results.

Genome size determination

Fifteen individuals were randomly selected in each population to estimate genome size. Fresh young leaves were collected from each individual, kept in moist paper and stored in the refrigerator (4 ºC) for a few days until they were analyzed.

We followed the protocol described by Doležel (2007). *Petunia hybrida* Vilm. 'PxPc6' leaves (2C=2.85 pg; Marie and Brown (1993), obtained from the Institut des Sciences du Végétal (CNRS, Gif-sur-Yvette, France), were used as the internal standard. The *S. ciliata* leaves were chopped using a razor blade along with the leaves of the internal standard and placed in 600 µl of Galbraith´s buffer (Galbraith et al. 1983), supplemented with 100 µg/ml of ribonuclease A (RNase A, Boehringer). We used the same amount of standard and sample tissue (approximately 2 cm^2). In order to ensure peak identification, a sample containing only the standard was first prepared and analyzed. Nuclei were filtered through 30 µm nylon to eliminate cell debris before adding 60 µg ml^{-1} of propidium iodide. Samples were kept on ice for 20 minutes before measurement. Two samples were measured independently for each individual studied. Fluorescence analyses were carried out using an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL, USA). All the measurements were carried out at the Serveis Cientificotècnics Generals, Universitat de Barcelona. The instrument was set up in the standard configuration (see Garnatje et al. (2006) for details). Time was used as a control for the stability of the instrument. Total nuclear DNA content was calculated by multiplying the known DNA content of the plant standard (*Petunia hybrida*) by the quotient between the 2C peak positions of the target species and the standard in the histogram of fluorescence intensities, under the assumption that there is a linear correlation between the fluorescent signals for the stained nuclei of the unknown specimen, the known internal standard and the DNA content.

Statistical analysis

To estimate the genome size, mean and standard deviation values were calculated independently for each population. A one way ANOVA test was carried out to evaluate differences in genome size among populations. The ANOVA was performed using the 2C value as a dependent variable and population as a treatment factor. When the ANOVA models revealed significant differences at the population level, the Tukey post-hoc test was performed. R 2.9.0 (R Team Core Development 2007) was used for all statistical analyses.

Results

Chromosome numbers

All chromosome counts performed on germinated seeds from the 23 analyzed family lines (at least five family lines for each population) corresponded to diploid organisms of 2n = 24 chromosomes. No aneuploid, B chromosomes or myxoploid cases were found. Fluorescence microscopy was used in seven of the 23 family lines. Chromosome counts were the same independently of the technique employed.

Genome size

Mean and standard deviation of the genome size of *S. ciliata* was 2C = 1.76 ± 0.06 picograms (pg) with a coefficient of variation of 3.8%. A histogram of fluorescence intensities is shown in Figure 2. The half peak coefficient of variation was always lower than 5%, which is a good indication of the high quality of the analyses. Table 1 shows the genome sizes corresponding to each of the three populations studied. Genome size values, which ranged between 1.73 and 1.82 pg, discard the presence of polyploids in the populations. Nevertheless, the ANOVA detected significant differences in genome size between populations ($F = 7.64$, $p < 0.01$). The post-hoc Tukey test showed that genome size of Intermediate population was significantly greater than that of the High population. No significant differences were found between Low popualtion and the other two populations (Table 1).

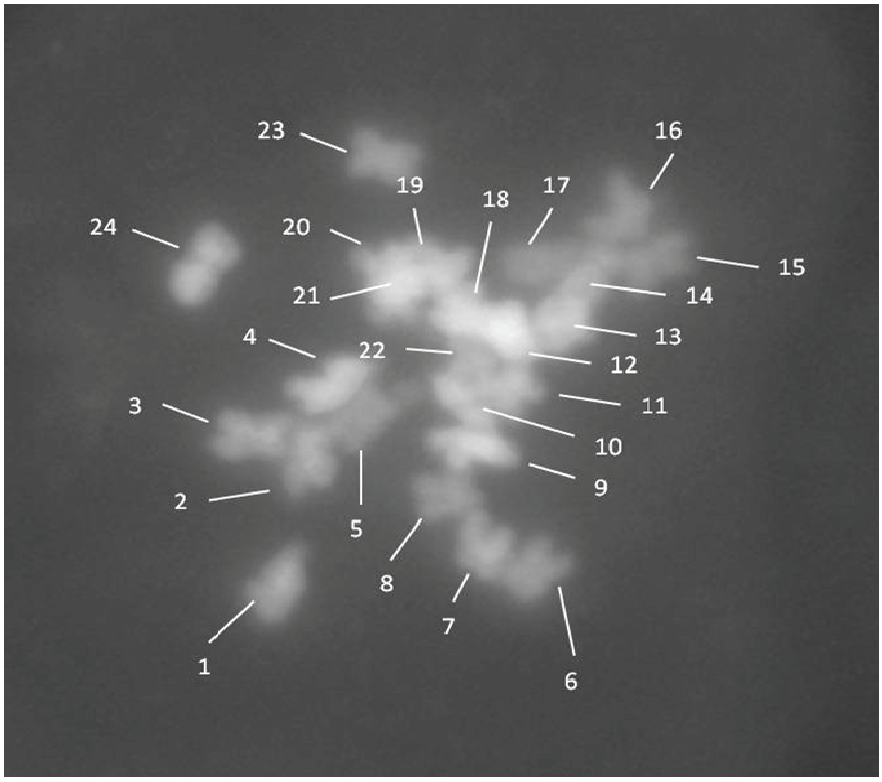

Figure 2 Chromosome count of the Low population of *Silene ciliata* (2n=24) stained with DAPI.

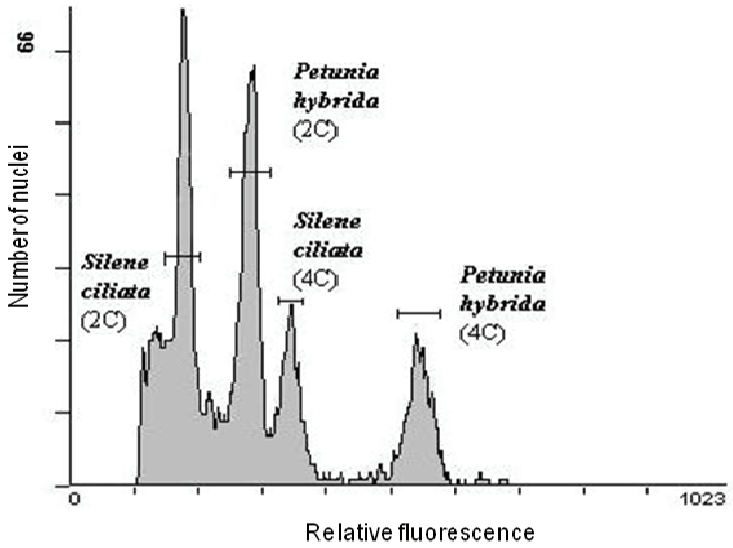

Figure 3 Histogram of relative fluorescence intensity (relative nuclear DNA contents stained with propidium iodide) for *Silene ciliata*. *Petunia hybrid*a was used as internal standard.

Table 1 Chromosome number, genome size (mean **±** standard deviation) in three populations of *Silene ciliata*. Coefficients of variation (CV) are shown in brackets. Post-hoc Tukey comparisons are provided as superscript letters. Values with the same letter do not differ significantly ($p < 0.05$).

Population	Chromosome number	Genome size: mean ± SD (pg), (CV)
Low	2n=2x=24	1.78^{ab} ± 0.04 (3.73%)
Intermediate	2n=2x=24	1.82^{a} ± 0.03 (2.91%)
High	2n=2x=24	1.73^{b} ± 0.05 (3.19%)

Discussion

Both chromosome counts and flow cytometry results indicate that the cytotype of *S. ciliata* is diploid in all the studied populations. These constant values contrast with the high variability in chromosome counts described by Blackburn (1933) in other Iberian populations, such as those in Picos de Europa, where diploids, triploids and tetraploids were found together, or those in the Pyrenees, where several polyploid individuals were described. Similarly Küpfer (1974) found different ploidy levels in the Pyrenees (2n = 24) and in Picos de Europa (2n = 48). The value of 2n = 24 is in agreement with the chromosomal count obtained by Küpfer (1974) in the High population. This is the first time that the nuclear DNA amount is assessed in the studied species, according to the very recently updated DNA C-values database (Bennett & Leitch 2010), which currently contains data on 12 *Silene* species. In spite of meiotic abnormalities or B chromosomes having been described for some species of genus *Silene* (Sheidai et al. 2009), none of them were found in our *S. ciliata* individuals.

Diploid karyotypes in Silene ciliata rear edge populations

Our results showed no polyploid individuals in the southernmost populations of the species, even though several polyploid cytotypes of *S. ciliata* have been documented in various populations of the Iberian Peninsula (Blackburn 1933). The existence of these different ploidy levels in the studied species makes it particularly important to do a large sampling, basic to assure the cytotypes in a population (Sonnleitner et al. 2011). For this purpose, in the present work we sampled 15 individuals per populations instead of the most common sample size of five. Moreover, the fact that all chromosome counts (implying more individuals with respect of those used for nuclear DNA amount assessment) agree with genome size estimations, discarding genome duplication or other chromosomal number modifications, suggests that if polyploids had ever occurred in this area they must have become extinct. Mediterranean mountains are considered a potential refuge for alpine plants that colonized new ice-free habitats during Quaternary glaciations ("*tabula-rasa* hypothesis", e.g. Brochmann et al. 2003). After ice melting, these alpine plants moved to higher latitudes (Gabrielsen et al. 1997). Some of these species survived in the higher Mediterranean mountains and became isolated from northern populations (Hampe & Petit 2005). These habitats may actually be marginal for some of these species since Mediterranean mountains often become stressful environments with short growing seasons truncated by summer droughts (Giménez-Benavides et al. 2007a; Cavieres et al. 2005; Cavieres et al. 2007; Ramírez et al. 2006). A similar scenario in which diploids are confined to harsher environments was also found by Liu et al.(2004) in his study of some species of the genus *Isoetes* in Asia, where diploid individuals survived in a wider range of conditions including altitudinal edges.

During the last few years we have accumulated evidence of local adaptation in the populations of this study (Giménez-Benavides et al. 2007a). At the same time, additional support has been gathered through common garden experiments with plant material from these populations (Luis Giménez-Benavides & José

Margalet, personal observations). It has been postulated that local adaptation may sometimes be related to genome enlargement and duplication (Levin 1983), hybrid stabilization (Kawecki 2008) or at least, to processes that modify genome complement (Ramsey & Schemske 2002). Although these processes have not modified *S. ciliata* karyotypes, our results show the existence of some differences in genome size that could be compatible with differences found in other critical genetic parameters such as genetic diversity and inbreeding in these populations (A Garcia-Fernández unpublished data).

Genome size of Silene ciliata and altitude variation

The mean genome size value obtained for *S. ciliata* (1.76 pg) is the smallest 2C value found in the genus *Silene.* This value is close to those of the genome sizes of *S. coeli-rosa* (l.) Godr (2 pg Miller and Lyndon (1977), *S. vulgaris* (Moench) Garcke (2,25 pg Široký et al. (2001) or *S. pendula* L. Sp. (2.32 pg Široký et al. (2001). These *Silene* species a with small genome size differ greatly from other species of the genus, like *S. latifolia* Poir. or *S. chalcedonica* (L.), with 2C values ranging from 5.11 to 6.59 pg (Široký et al. 2001; Bennett & Leitch 2010; Loureiro et al. 2007). The position of *S. ciliata* in the phylogenetic history of the genus *Silene* is hard to precise (Desfeux & Lejeune 1996; Rautenberg 2009), but his location in the trees was quite near to the species with smaller genome than the bigger ones. The overlap of genome size in forthcoming phylogenetic trees of genus *Silene* would be useful for describing the origin of the numerous species of the genus and clarifying the relationships between them.

Between-population variations in genome size must be interpreted with care (Noirot et al. 2005). Many uncontrolled variables, like temperature of the laboratory where the analysis was conducted, tissue contamination by fungi or insect eggs (Doležel et al. 2007) or cytosolic compounds can alter the results obtained in the flow cytometer (Noirot et al. 2000). In our case, temperature of the lab was controlled but we lack information about possible variation in cytosolic compounds among populations in *S. ciliata.* Concerning other *Silene* species, variation in cytosolic compounds has only been described in *S. vulgaris*, where Kovacik et al. (2009) found variation in polyphenols compounds in some Slovak populations adapted to soils with high copper concentration. In any case, the low values of the coefficient of variation obtained reinforce our idea that the protocol was properly implemented and that our results are realistic. Assuming the presence of this variability in genome size , we still do not know if it plays a role in the local adaptation phenomena described in these populations (Giménez-Benavides et al. 2007a).

The effect of altitude on genome size is currently under discussion (see revision in Knight et al.2005). The genome size of *S. ciliata* at our study site seems to shape an unimodal distribution, where the populations of an intermediate altitude present larger genome size than the populations of the altitudinal edges, similar to the pattern described by Knight et al. (2005) for different genera, and also in agreement with more recent data from the genus *Echinops* (Sánchez-Jiménez et al., unpublished research). Reproducibility of this experiment in other altitudinal gradients and with other cytotypes of *S. ciliata* would support the generality of this pattern relating altitude and genome size. This between-population variability

in the genome size could be, at least partially related, to the activity of transposable elements. It is well known that genome size is highly dependent on the activity of these elements in plants, which are also ubiquitous in the genomes of many other eukaryotic organisms (Sanmiguel & Bennetzen 1998). Transposable elements can contribute to genome evolution (Bennetzen 2000), increases in genome size (Sanmiguel & Bennetzen 1998) and even local adaptation phenomena (Wood et al. 2008). Minder and Widmer (2008) described the high presence of retrotransposon "Retand" in wild populations of *S. latifolia* and S. *dioica*. Also, several transposable elements have been described in some *Silene*'s species (Matsunaga et al. 2002; Cermak et al. 2008). It would be interesting to examine the presence of this transposable element (or others) in the genomes of *S. ciliata* and to test their relation to genome size and other genetic parameters and processes.

Our results complement those obtained by Giménez-Benavides et al. (2007a), in an attempt to explain the local adaptation phenomenon in *S. ciliata* populations across altitudinal gradients in the southernmost range the species' distribution. The hypothesis of the existence of different ploidy levels in this area can now be rejected, but the variation in genome size found among populations might be associated with other variables in explaining the local adaptation processes found across the altitudinal gradient of this species.

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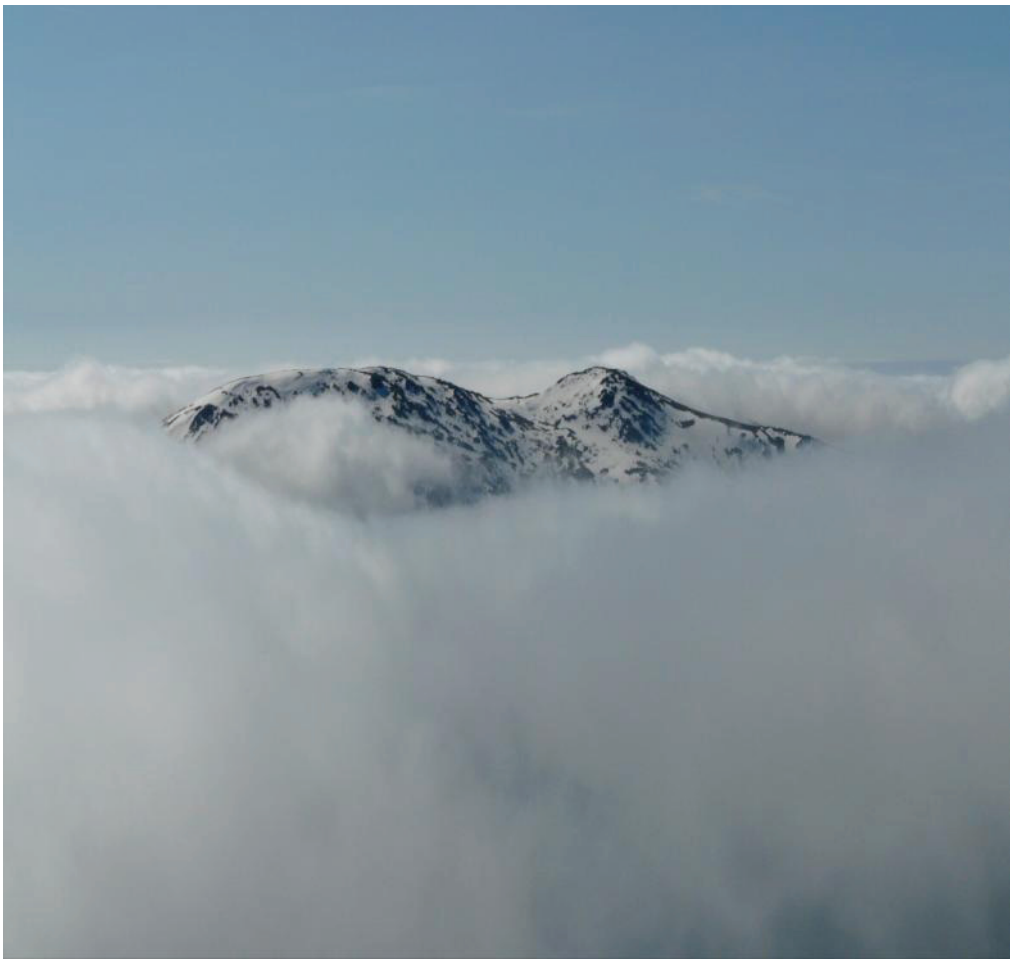
Chapter 2

Islands in the sky: a genome scan over the whole range of a mountain endemic plant.



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Manuscrito enviado a *Molecular Ecology*

Abstract

Genetic approaches allow us to evaluate the structure of isolated populations and how habitat fragments are connected. Stochastic processes and/or selection forces can act on the genome of these isolated populations modulating their genetic structure and diversity. As the effect of these processes may be different across the distribution range of a species, genetic studies should ideally encompass all the distribution range of the species.

In this context, we approached the study of *Armeria caespitosa*, an endemic mountain plant with populations distributed in isolated mountains across the Sierra de Guadarrama, Madrid, Spain. Genetic structure, diversity and possible loci under selection were analyzed in 17 populations located across the species' distribution range with a gene scan using AFLP markers. Altitude was considered in the analyses because it enhances the habitat range available on the mountain island and may play an important role in the selective and stochastic processes which influence the genome.

Results showed similar values of genetic diversity in all populations, which suggests the existence of certain levels of gene flow, at least along the elevation gradient. This hypothesis is also supported by the Bayesian clustering approach, which grouped populations belonging to the same mountain. Spatially explicit analyses only showed a weak relationship when genetic parameters were related to spatial, altitude or climatic distances. However, a large proportion of outlier loci were detected and divergent loci showed different patterns to those found in the analyses for all loci.

Introduction

Current anthropogenic global warming constitutes the greatest world threat to biodiversity (Parmesan 2006). Ongoing novel conditions seem to promote an abrupt increase in the risk of plant extinction (i.e. Thuiller et al. 2005) and enhance of the isolation of marginal populations (Ackerly & Monson 2003). Biological responses to this global trend vary greatly in space and time (Loarie et al. 2009), and include altitudinal and latitudinal shifts of populations and communities (Peñuelas & Boada 2003; Sturm et al. 2001), increases in biological invasions (Walther et al. 2009) and changes in local community composition and structure (Jump & Peñuelas 2005).

Altitudinal or latitudinal shifts are usually coupled with co-occurring processes related to other interacting global change drivers, such as loss of quality and amount of suitable habitat (Travis 2003), increase in competition with invasive plants (Brooker 2006; Godoy et al. 2009) and the pernicious effects related to fragmentation (Aguilar et al. 2006), especially in the case of plants occurring in islands (García-Verdugo et al. 2010) or mountains (Robledo-Arnuncio et al. 2005; Gutierrez Larena et al. 2002). As several drivers could be acting simultaneously in similar or opposite ways (i.e. Jump & Peñuelas 2005; Herrera & Bazaga 2008; Wilson et al. 2005) the study of this complex changing scenario (Nogues-Bravo et al. 2007) requires a multidimensional approach.

Shifts in species distribution are not the only alternative to respond to ongoing changes; for instance, local population persistence can be guaranteed through phenotypic plasticity and local adaptation (Jump & Peñuelas 2005) or even demographic compensation (Doak & Morris 2010). All these processes can have profound implications in the genetic diversity and structure of the species, not only in their marginal populations, but at the whole species range scale (Kawecki 2008; Hughes et al. 2008). However, knowledge on the different factors that could enhance or limit genetic diversity and structure is still scarce. An enhancement of genetic diversity, especially in relation to traits under current natural selection, may increase the probability of persistence through local adaptation (Rehfeldt et al. 1999) or plastic responses (Tsutsui & Neil 2004; Hughes et al. 2008). However, processes like inbreeding, genetic drift or restricted gene flow, which are likely to occur in marginal populations, may inhibit adaptation to new environmental conditions (Savolainen et al. 2004). Finally, the prevalence and efficiency of genetic flux between fragments by dispersal and pollination may also affect genetic diversity and structure (Bridle & Vines 2007). Consequently, it is extremely difficult to predict the genetic diversity and structure of plant populations affected by global change drivers. This is especially true in the case of plants inhabiting isolated habitats for which new insights are required (Reusch & Wood 2007). Thus, the study of population genetic diversity, estimates of gene flow between populations, or the incipient process of local adaptation, are essential to characterize the degree of isolation of populations, their genetic relatedness and how they are responding to global change (Kawecki 2008; Kawecki & Ebert 2004; Leimu & Fischer 2008).

Mountains are especially well-suited scenarios for testing hypotheses related to adaptation and evolution by comparative observations or manipulative experiments with populations along local altitudinal gradients (Körner 2007). In theory, moving along these altitudinal gradients is extremely hard for plant species and establishing new populations is difficult because the available habitat has already been colonized (Körner 2007). When properly chosen, altitudinal gradients in mountains modify environmental conditions in a very predictable fashion (Walther et al. 2005) and put populations under different selective pressures (Gonzalo-Turpin & Hazard 2009). These gradients include both optimal and marginal environments which can be assimilated to central population conditions and the species' edge conditions, respectively (Normand et al. 2009). One of the main focus of study in temperate mountains (Europe and North America, Hewitt, 2000, Hewitt, 2001, Hewitt 2004) has been the reconstruction of the phylogeographic history of species that colonized northern latitudes, which moved to southern regions in the multiple glaciation periods of the Quaternary searching for warmer refuges, and from there, re-colonized the higher latitudes in a migration to the north (Hewitt 2001; Gomez & Lund 2004). However, little is known about the effect of glaciations and subsequent ice-retreatment effects on plant species that remained in the mountains of southern latitudes (Gutierrez Larena et al. 2002; Hewitt 2000). Unraveling the genetic characteristics of the species in these regions is more complicated than assessing the species that expanded to the north (Hewitt 2001). The plants that now inhabit the top of the mountains range were probably forced to descend from the mountains and colonize low altitude lands during ice coverage ("tabula-rasa hypothesis", Schönwetter et al. 2002, Brochmann et al. 2003), forming large, continuous populations, instead of the highly fragmented ones that now appear on the summits and the crests. These processes of lowland colonization and retreat to the highest peaks (up and down the elevation gradient, Hewitt, 2001) probably took place in each glaciation. Furthermore, if we consider the whole range of a high mountain plant, the mountains could be considered isolated islands ("Islands in the sky", Hewitt, 2001) where the arrival of migrants or gene flow from other islands would be difficult or almost impossible (Thuiller et al. 2005). Altitude surely plays a crucial role in these islands, adding a third dimension to the performance and structure of different biological components, essentially similar to the two dimensions of space ordinarily considered in classical models of evolution at geographic ranges (Herrera & Bazaga 2008).

With this in mind, we evaluated the genetic diversity and structure of *Armeria caespitosa,* a high mountain plant specialist fragmented in several populations or islands of different size, across its whole distribution range. This species, is a high-mountain narow endemic that is relatively abundant in its small mountain range and occurs in a relatively large altitude gradient (García-Camacho & Escudero 2009). Several populations can be found on a single mountain as a function of its size and elevation range and each mountain can be considered an island, with limited and shrinking habitat due to global warming and mostly isolated from other mountain islands. This allows for a complete evaluation of its genetic structure and diversity along the several mountains which form its whole range and, more specifically, to assess

how the effect of altitude and environment extend throughout the specie's distribution. Isolated populations could be under typical stochastic processes like mutation, inbreeding and genetic drift, that enhance genetic differentiation but also, genetic selection forces could play a role acting as divergent pressures along the elevation gradient (Körner 2007) leaving specific signatures or patterns in the genome of high altitude organisms (Storz et al. 2007; Fischer et al. 2011). Similar works have been conducted in some local gradients in mountains species (Byars et al. 2009) or in fractions of the distribution area (rear margin of *Lavandula latifolia,* Herrera and Bazaga 2008), but not covering the whole area of geographical and altitude distribution. More specifically we aimed to answer the following questions: A) Is the genetic diversity of the populations influenced by the current island fragmentation? B) What is the role of altitude and the spatial configuration of island-archipelago in genetic structure? C) Are there diverging natural selection processes that leave a signal in the genome of *Armeria caespitosa*? Are these processes related to altitude, climatic variables or fragmented island distribution?

Materials and Methods

Plant features

Armeria caespitosa (Gómez Ortega) Boiss. *in DC*. is a high-mountain dwarf chamaephytic cushion plant with rosettes that grow up to 20 cm in diameter. It is a narrow endemic of the Iberian Central Range and occurs from Ayllón to East Gredos ranges, with most populations located in the Sierra de Guadarrama, at altitudes ranging from 1700 to 2430 m. This species is diploid (2n=18, Moore (1982); Castroviejo & Valdes-Bermejo (1991) and like most *Armeria* species, it is self-incompatible (Baker 1966; García-Camacho & Escudero 2009), although internal barriers between species are weak (Nieto Feliner et al. 1996) In the East Gredos massif, the species comes into contact and hybridizes with high-mountain endemic congener *Armeria bigerrensis* subsp. *bigerrensis* (Nieto Feliner 1991).

Sampling and DNA extraction

We selected 17 populations covering the whole distribution range of the species (Figure 1) except East Gredos (westernmost part of the range) to avoid hybridization interferences. In summer and autumn 2007, ten individuals were randomly sampled in each population with the only constraint of keeping a minimum distance of 10 m between individuals. Green leaves were collected from sampled individuals and dried in silica-gel. Genomic DNA was extracted following standard protocol of DNEasy Plant Minikit (Quiagen) using approximately 10 mg of clean dried tissue. DNA quality and quantity were estimated using 1% agarose gel and Sybr-green staining.

AFLP protocol

To cover the widest genomic region and ensure high-quality, reproducible bands, an initial screening was developed using a subsample of 30 individuals randomly obtained from 10 populations. AFLP analyses have been developed in several species of genus *Armeria* (Baumbach & Hellwig 2007; Piñeiro et al. 2007; Piñeiro et al. 2009) and substantial information on possible primer combinations is available. Forty primer combinations were performed in the initial screening, and checked for band polymorphism, band brightness and reproducibility of band patterns in agarose gel (1%) electrophoresis. Thus, eight primer combinations for AFLP analysis were selected: *Eco*RI+ACT/*Mse*I+CAC, *Eco*RI+ACC/*Mse*I+CACC, *Eco*RI+ACC/*Mse*I+CCT, *Eco*RI+ACG/*Mse*I+CTAC, *Eco*RI+ACT/*Mse*I+CTT, *Eco*RI+AGA/*Mse*I+CTA, *Eco*RI+AGG/*Mse*I+CAC, *Eco*RI+AGG/*Mse*I+CTT.

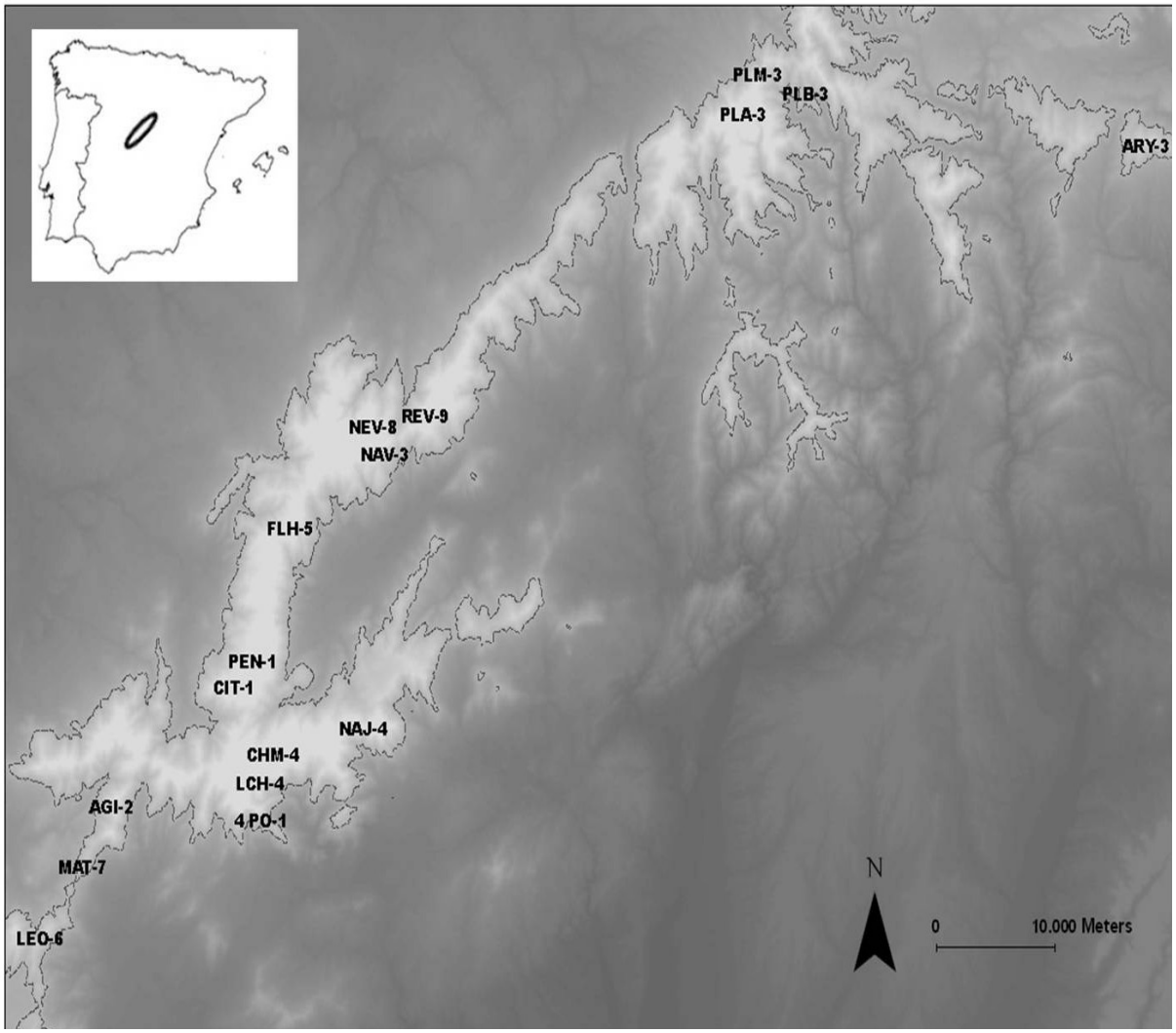

Figure 1 – *Armeria caespitosa* populations in the Central Range in the Iberian Peninsula. The Central Range is indicated on the map of the Iberian Peninsula (black line in the left upper corner). Plant populations are represented by their initials (see Table 1 for details), and the number is associated with the spatial Bayesian cluster analysis BAPS for genetically homogeneous groups. Darker-shaded areas indicate lower altitude, while lighter-shaded areas indicate higher altitude (dashed line denotes the 1500 meter isoline).

Restriction and ligation of genomic DNA with EcoRI and MseI were performed according to Gaudel et al. (2000) with some modifications. We used 5.5 µl of genomic DNA for a final volume of 11 µl. Final product from restriction-ligation was confirmed in agarose gel (1%) and diluted 10 times in purified water. Pre-amplification and selective amplification were performed as described by Gaudel et al.(2000) with some modifications. Final product of selective PCR was labeled by a fluorocrome (6-FAM or VIC) added to the *Eco*RI primer. All reactions were incubated in a Thermocycler Mastercycler Pro (Eppendorf, Hamburg, Germany). All reactions were checked in agarose gel before the next step of the procedure. Fragments were separated using an ABI PRISM 3700 sequencer (Applied Biosystems, Foster City, CA, USA) using 1 µl of selective reaction product and GeneScan-500 ROX as size standard. Fragment electrophoresis was developed at the Parque Científico de Madrid (Madrid, Spain).

Although AFLPs are widely used in molecular ecology (Meudt & Clarke 2007) genotyping errors can be generated at any step of the process (Bonin et al. 2004) potentially causing serious effects on data performance (Pompanon et al. 2005). To mitigate this type of error, a reproducibility test was carried out, using one or two individuals from each population. Replicate samples were analysed throughout the AFLP procedure as well as 17 water samples (one per population).

Amplified fragments were analysed using GENEMAPPER 3.7 (Applied Biosystems, Foster City, CA, USA). Peaks were recorded in a range from 80 to 500 base pairs and peak height threshold was situated in 100 units. Automated binning protocol was developed for genotype as dominant markers (presence/absence) and posterior manual correction was performed. AFLP scorer was employed to estimate error rates (Whitlock et al. 2008). As recommended, error rate was fixed to 5% for each primer combination (Skrede et al. 2006; Bonin et al. 2004). AFLPdat R package (Ehrich 2006) was used to transfer data between the different software used.

Climatic data collection

Rainfall and temperature data for each population were estimated with the ESTCLMA program (http://www2.montes.upm.es/Dptos/DptoSilvopascicultura/edafologia/aplicaciones/Aplicaciones.htm) which uses a multiple regression model based on Spanish meteorological data (Sanchez-Palomares et al. 1999). Mean monthly temperature (ºC), mean annual temperature (ºC), total monthly precipitation (mm) and total annual precipitation (mm) were obtained for each population. Principal Component Analyses (PCA) were used to summarize the complexity of this climatic dataset. Because Mediterranean mountain plants are known to be limited by a summer drought period (Sanz-Elorza et al. 2003; Ruiz-Labourdette et al. 2011), we carried out a second PCA analysis including only the precipitation data for the summer months (June, July and August), annual precipitation and the mean annual temperature. Since both PCAs gave similar results we only used those obtained with the first PCA.

Genetic diversity and population differentiation.

To estimate genetic diversity, three parameters were computed for each population and for the total number of collected individuals: i) allelic richness, in terms of percentage of polymorphic loci; ii) Nei's gene diversity (Nei 1978) and iii) Shannon's index (Shannon 1948). These analyses were implemented with POPGENE version 3.2 (Yeh and Boyle 1977) and ARLEQUIN version 3.5 (Excoffier et al. 2005).

Pairwise F_{ST} values (Weir & Cockerham 1984) were calculated for each population pair using ARLEQUIN 3.5 (Excoffier et al., 2005). Significance was evaluated through 10000 permutations. These results were visualized with a Minimum Spanning Tree implemented in R (R Core Development, Vegan package).

Population structure

Bayesian clustering analyses were done with STRUCTURE 2.3 (Pritchard et al. 2000). User manual recommendations for dominant markers were followed, adding a missing value to each marker, and choosing the ancestry model of no admixture and the correlated allele frequencies model, to consider that allele frequencies are expected to be similar in the different groups. We ran 10 independent simulations for each value of *K* from *K*=1 to 17, using a burn-in period of 10^5 and run lengths of 10^6. We also used the algorithm proposed by Evanno et al. (2005) to estimate the best value of *K* for our data set. Simultaneously, additional Bayesian analyses were attempted with BAPS (Bayesian analysis of Population Structure, Corander & Marttinen 2006) , which uses stochastic optimization instead of Markov Chain Monte Carlo to find optimal partition (with highest estimated probability). Simulations were run from *K*=1 to *K*=17 as the maximum number of groups, with five replicates for each *K*.

AMOVA analyses were carried out using ARLEQUIN 3.5 (Excoffier et al., 2005) to estimate genetic differentiation following an alternative non-Bayesian approach that does not assume Hardy-Weinberg equilibrium or independence of markers. A first AMOVA analysis was implemented without a previous regional genetic structure. We also carried out independent AMOVAs including the regional models proposed by BAPS and STRUCTURE. Pairwise binary genetic distance was used for AMOVA analyses, considering genetic variation within populations, between populations within regions and between regions in each case.

Spatially explicit analyses.

Mantel tests and Partial Mantel tests were performed to evaluate the correlation between genetic, climatic and geographic distance matrices. The Mantel test compares two similarity matrices and the Partial Mantel test is similar to a partial correlation which is able to detect the correlation between two matrices of interest when the effect of a third matrix is partialled out (Legendre & Legendre 1998). Genetic distance matrices were using F_{ST} values between populations. Climatic distance matrices were calculated using the

Euclidean distance of the two first PCA axes of each population. Geographic matrices were calculated in several ways. A first matrix was made by calculating the Euclidean 2D spatial distance (only X and Y coordinates) between populations. Secondly, we estimated a Euclidean 3D spatial distance to include the altitude component in the analyses. Finally, a third distance matrix that only considered differences in altitude. Mantel tests and Partial Mantel tests were calculated using the "Vegan" package implemented in R software version 2.9.2 (R Team Core Development 2007). All tests were carried out with 10000 permutations, including Pearson and Spearman assumptions to evaluate parametric and non-parametric relationships, respectively, and considering a *p*-value = 0.05.

SPAGEDI software (Hardy & Vekemans 2002) was used to estimate the spatial structure of the kinship multilocus coefficient with dominant markers. Inbreeding coefficient (F_{IS}) was set at 0.05 (other values were used in a prelimar stage, but results were very similar), as suggested by Hardy (2003), and the permutation test included 10000 permutations. Spatial distance was included using the matrices described above (Euclidean 2D and 3D spatial distance).

We also computed Mantel correlograms, to evaluate not only the existence of spatial dependences but also the shape of these spatial genetic structures and to test the presence and intensity of spatial, altitudinal and climatic autocorrelation. Genetic, climatic and geographic distance matrices were used in these analyses. Ten distance classes were defined for each analysis, including at least 10 population pairs in each distance class. We used the PASSAGE program (Rosenberg 2001) to estimate the Mantel coefficient (r_M) in each distance class. Each r_M value was tested for significance by permutation tests and correlograms were tested for global significance using Bonferroni's criterion (Oden 1984). Moran's *I* correlograms were also used to evaluate the relationship between genetic diversity, considering the Nei diversity index (Shannon was also used, but results were similar in all cases) and the distance matrices described above (Euclidean 2D and 3D spatial, altitude and climatic distances (Rosenberg 2001). In these last cases, interval lags were evenly spaced climatic distances, thus we computed population pairs at similar climatic distances (see Matesanz et al. 2011). Each value of Moran's *I* was tested for significant deviations from the expected value under the null hypothesis of random spatial distribution (Cliff & Ord 1981). Global significance of the lags was tested using the Bonferroni correction (see Mantel correlogram).

Detecting loci under selection: Outlier loci

Outlier loci scan test procedure is based on F_{ST} comparisons, identifying the loci that present F_{ST} coefficients that are significantly different than expected under neutrality and a given demographic model. To identify possible outlier loci from all AFLP loci detected, we used the approach proposed by Excoffier et al. (2009) based on Beaumont and Nichols (1996). The outlier loci approach was also run using the hierarchical island model (the interchange of migrant seems more plausible between a neighbourhood deme than between demes with different neighbourhoods, Slatkin and Voelm (1991), Excoffier et al. (2009), including 100000 interactions, with 10 groups and five demes in each group. This model is highly

sensitive to certain demographic parameters and species life histories (i.e. Eckert et al. 2010) and can produce an unknown number of false positives (type-I errors). To avoid this, we adopted a conservative approach, only considering the loci outside the estimated 99% confidence interval to be outlier loci (i.e. Bonin et al. 2006; Mäkinen et al. 2008). Outlier loci we calculated using the ARLEQUIN 3.5 application (Excoffier et al. 2005).

Three data sets were created for the detected outlier loci: all outlier loci, loci under divergent selection (positive outliers, above the confidence interval of our null model) and another for loci under balancing selection (negative outliers, below the confidence interval, Mäkinnen et al., (2008), Minder et al., (2008). Genetic diversity (Nei diversity) and F_{ST} between all populations were estimated for each loci dataset as described above. Finally, the previously-described spatial analyses (Mantel test, Partial Mantel test, Mantel correlograms, Moran correlograms and SPAGEDI (Kinship coefficients) were performed on the outlier loci datasets to asses the spatial structure of the outlier loci.

Results

AFLP Profile

A total of 2472 loci were scored in the eight primer combinations for 170 individuals of *Armeria caespitosa*. Manual correction reduced the number of potential useful loci to 766 (30.5%), by eliminating monomorphic loci, unrepeatable fragments, low intensity peaks and great change of intensity bands between samples. After filtering by the AFLP scorer, total available loci were reduced to 659 (26.7%), representing an average of 82.4 loci per primer combination.

Genetic diversity and population differentiation.

Genetic diversity indices for the 17 studied populations are shown in Table 1. Genetic diversity (based in Nei index) ranged from 0.25 (NEV population) to 0.09 (LEO population). All pair-wise differences (F_{ST}, Average ± SD = 0.364 ± 0.116) were significant. The highest differentiation was found between LEO and REV populations (F_{ST} = 0.642), while PLM and PLA had the smallest values (F_{ST} = 0.147). Figure 2 shows the Minimum Spanning Tree for the pair-wise comparisons between all populations.

Population genetic structure

STRUCTURE analysis revealed that a nine-group classification was the most adequate partition for our data (Figure 3). Some populations such as LEO, FLH and REV always appeared in isolated groups, whereas other pairs (PEN & CIT, PLA & PLM, AGI & NEV, CHM & LCH) were always grouped together. Additionally low and higher elevation populations occurring on the same mountain were usually grouped in the same cluster (although in some simulations 4PO, MAT or PLB appeared in isolated clusters. In acconcordance with the results obtained with STRUCTURE, BAPS optimal partition also estimated nine clusters reinforcing the optimality of the partition. Summaries of BAPS and STRUCTURE classifications are shown in Table 1.

We also tested this genetic structure with a classical AMOVA analysis which showed significant genetic differentiation between all groups proposed with the Bayesian models, and was able to explain 20.14% of the total genetic variance ($p < 0.01$), while the proportion of genetic variation among populations within groups was 19.08 % ($p < 0.01$). Finally, 60.8% of the remaining variation was present among individuals within populations.

Table1 – Name, code, altitude, genetic diversity indices, and BAPS and STRUCTURE clustering scenarios of the 17 populations of *Armeria caespitosa* included in this study.

Population name	Population code	Altitude (m)	Percentage useful loci	Polymorphic loci	Nei's gene diversity	Shannon Index	BAPS groups	STRUCTURE groups K = 7	STRUCTURE groups K = 9
4th Porrón	4PO	1689	80.3%	246	0.17	0.24	A	1	1
Peña Águila	AGI	2008	100%	369	0.22	0.29	B	2	2
Alto Rey	ARY	1840	88.8%	306	0.20	0.27	C	3	3
Cabeza Hierro	CHM	2370	89.8%	292	0.18	0.25	D	4	4
Peña Cítores	CIT	2156	65.6%	179	0.19	0.26	A	1	1
Collado Flecha	FLH	2050	88.8%	280	0.16	0.22	E	5	5
Loma de Cabezas	LCH	1980	100%	314	0.17	0.24	D	4	4
Alto de los Leones	LEO	1720	64.3%	143	0.09	0.13	F	6	6
Cerra Matalafuente	MAT	1674	72.4%	267	0.20	0.27	G	2	2
Pico Najarra	NAJ	1980	100%	272	0.15	0.21	D	4	4
Puerto Navafría	NAV	1820	91.6%	325	0.20	0.27	C	3	7
Pico Nevero	NEV	2209	100%	429	0.25	0.33	H	2	2
Peñalara	PEN	2428	90,4%	290	0.20	0.23	A	1	1
Pico Lobo Alto	PLA	2253	100%	333	0.18	0.26	C	3	7
Pico Lobo Bajo	PLB	1680	75.9%	276	0.18	0.24	C	3	8
Pico Lobo Medio	PLM	1995	100%	329	0.19	0.26	C	3	7
Alto Reventón	REV	2070	90.4%	157	0.11	0.14	I	7	9
	Mean	1995	88.1%	282 (42.8%)	0.18	0.24			

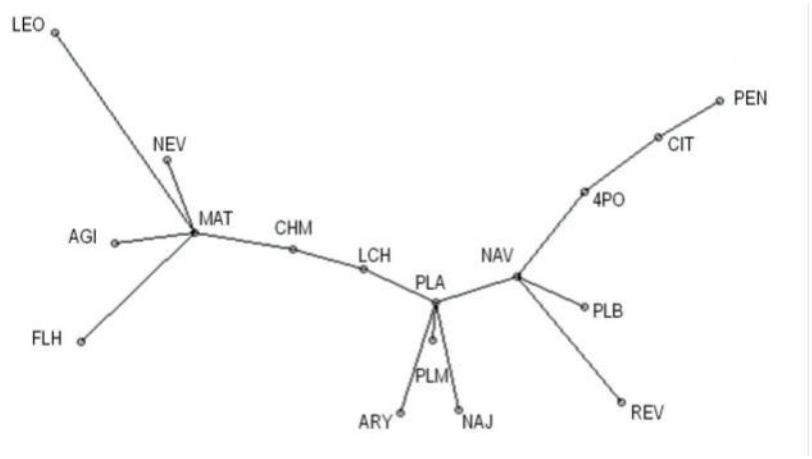

Figure 2. Minimum Spanning Tree based on F_{ST} pairwise distance between the 17 *Armeria caespitosa* populations.

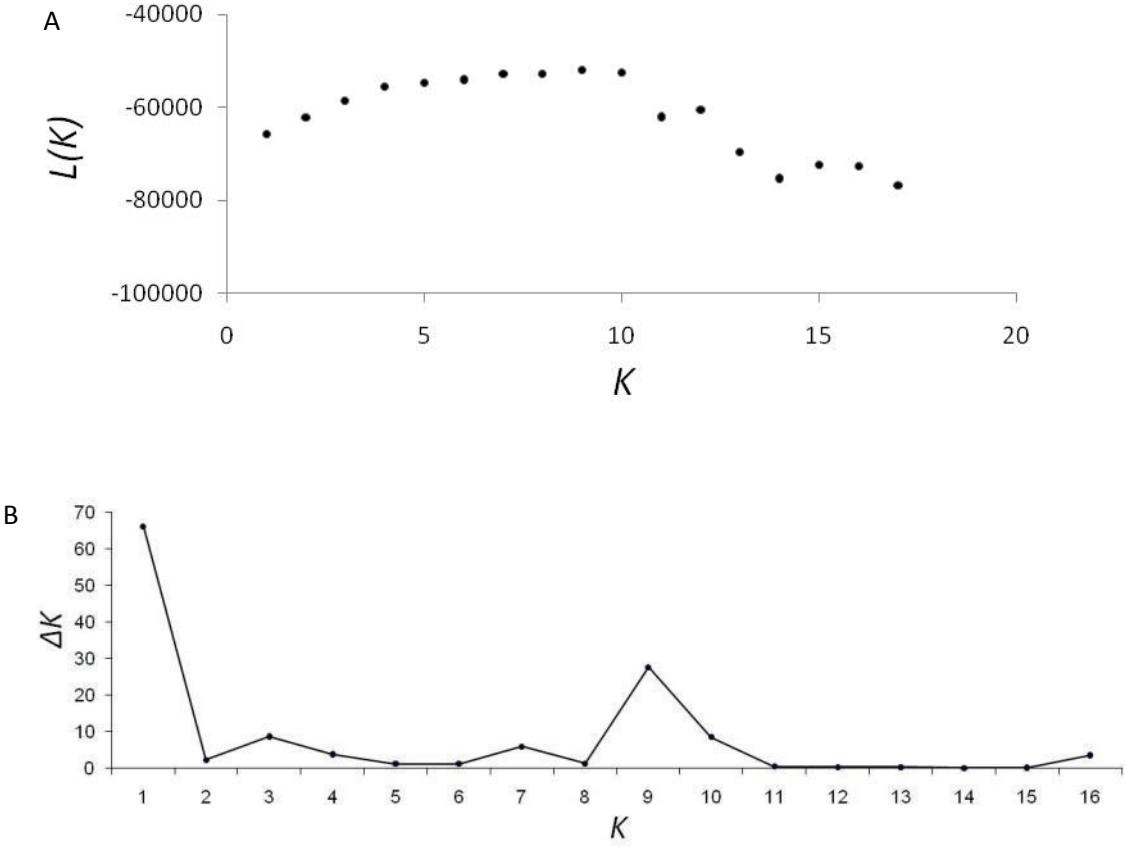

Figure 3. Log probability of data *L(K)* as a function of *K* for 10 STRUCTURE runs at *K* = 1 – 17 (Fig. A.) and Δ*K* rate of change in the probability between successive runs, as a function of *K* (Fig. B. Evanno et al. 2005).

Spatially explicit analyses

None of the Mantel or Partial Mantel tests performed with the distance matrices detected any significant correlations when the whole multivariate dataset comprising the variation in the 659 loci and all individuals was used (data not shown). This suggests that the genetic population structure found is not geographically or climatically organized. Worth to note these approaches are able to detect only linear relationships between these data sets.

However, a more detailed approach such as those conducted with SPAGEDI analysis for kinship coefficient showed significant negative autocorrelation at intermediate distances (12-18 km) and significant positive autocorrelation at longer distances (28-37 km) when both 2D and 3D geographical distances were considered (Figure 4).

When the multivariate F_{ST} distance matrix was analyzed with Mantel correlograms, we also found a relatively similar pattern with significant negative autocorrelation at the shortest distances (0-7 km and 14-20 km) with the Euclidean 2D spatial distance (Figure 5a). Moran's *I* correlograms implemented for Nei´s genetic diversity with 2D geographical distance found significant negative autocorrelation at intermediate distances (6-11 km) and significant positive autocorrelation at longer distances (28-37 km, Figure 5b).

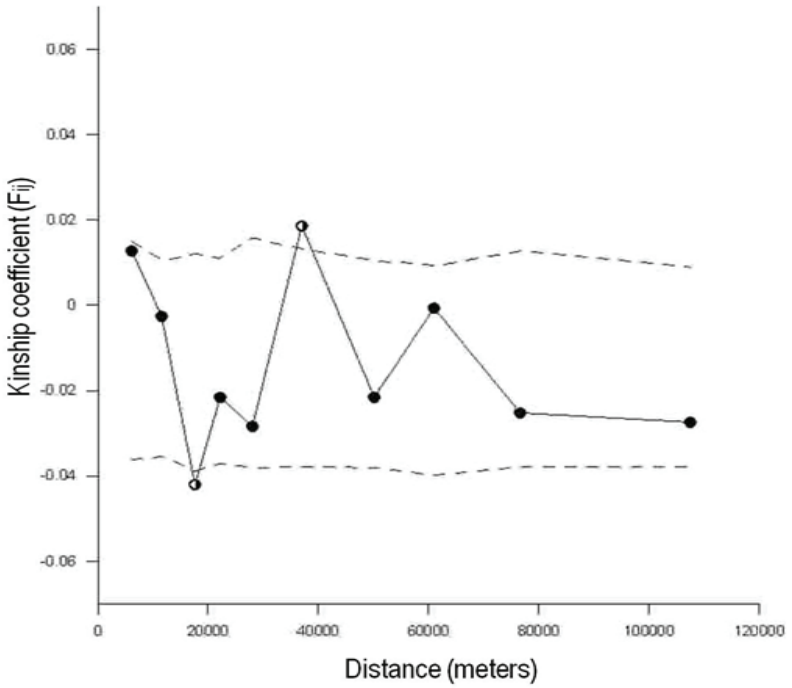

Figure 4. Analysis of spatial genetic structure in *Armeria caespitosa* based on Kinship coefficient (F_{ij}) on AFLP data over the full geographic distance range. Solid lines indicate the mean kinship coefficient per distant class and dashed lines the limits of its 95% confident limit. White squares refer to partially significant intervals (0.05 < *p* value > 0.01), more similar than expected (above dashed lines) or more different than expected (under dashed lines).

A

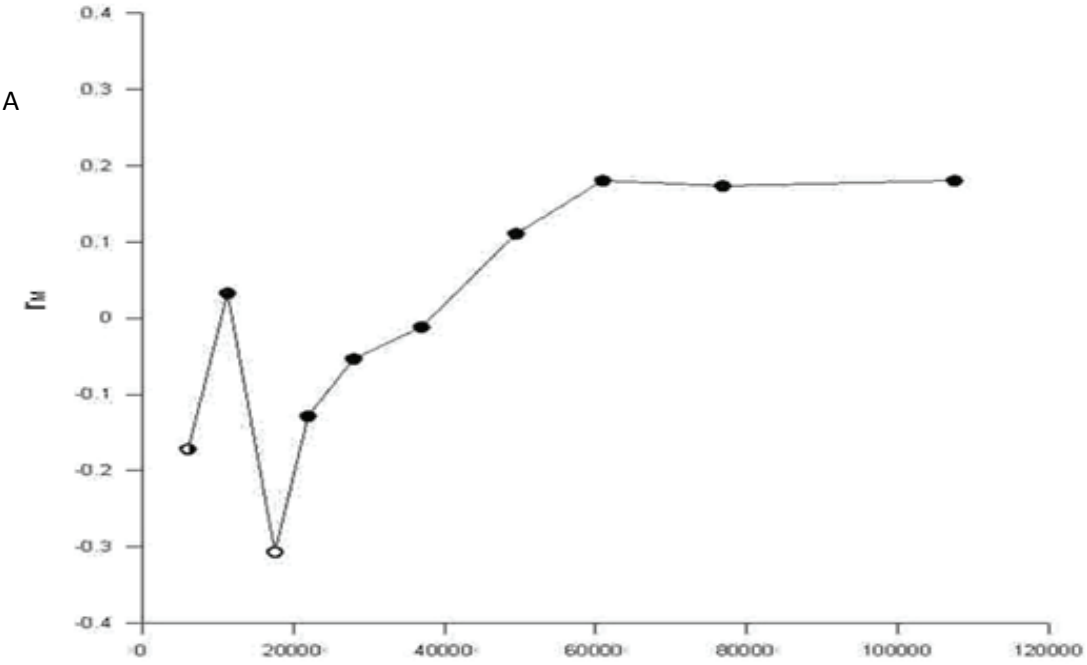

Figure 5. Correlograms carried out with Euclidean 2D spatial distance (X axis in both figures). A - Mantel Correlogram output. Y axis showed r_M values based on F_{ST} coefficients. White circles showed significant values (*p-value* < 0.005 with Bonferroni correction) and half black circles showed partially significant results (*p-value* < 0.01 with Bonferroni correction). B - Moran *I* correlogram. Y axis showed *I* values based on Nei diversity. White circles showed significant values (*p-value* < 0.005 with Bonferroni correction) and half black circles showed partially significant results (*p-value* < 0.01 with Bonferroni correction).

Detecting loci under selection: outlier loci.

Since the spatial structure found was so weak we looked for outlier loci and evaluated their patterning. The outlier test performed using ARLEQUIN detected 52 (7.89%) possible outlier loci using a 99% confidence level (upper and lower interval, Figure 6). The same outlier loci were found if hierarchical model were considered (data not showed). With a dataset of 659 markers, we would expect approximately 7 loci (0.01 x 659) to considered false positive. Since this number was notoriously higher, 28 loci (4.24%) under the lower interval (balanced loci) and 24 loci (3.64%) above the upper limit (divergent loci), we were confident that a significant group of loci had been submitted to selection.

The results of all of our specific spatial approaches, Mantel tests, Partial Mantel tests, SPAGEDI Kinship analyses and Mantel (matrix approach) – Moran (univariate approach) correlograms, were non-significant when all outlier loci were simultaneously included in the dataset (data not shown). However, when the dataset was reduced to just divergent loci, the Mantel correlogram between the F_{ST} pairwise distances and the climate distance matrix showed negative autocorrelation at intermediate climate distances (Fig. 7A). In addition, the Moran's *I* correlogram with the Nei´s genetic distance found significant negative autocorrelations at 160-190m and 260-300m when calculated using differences in altitude (Fig. 8A), and also a significant negative autocorrelation at 5 distances units when calculated using the climate distance metric (Fig. 8C).

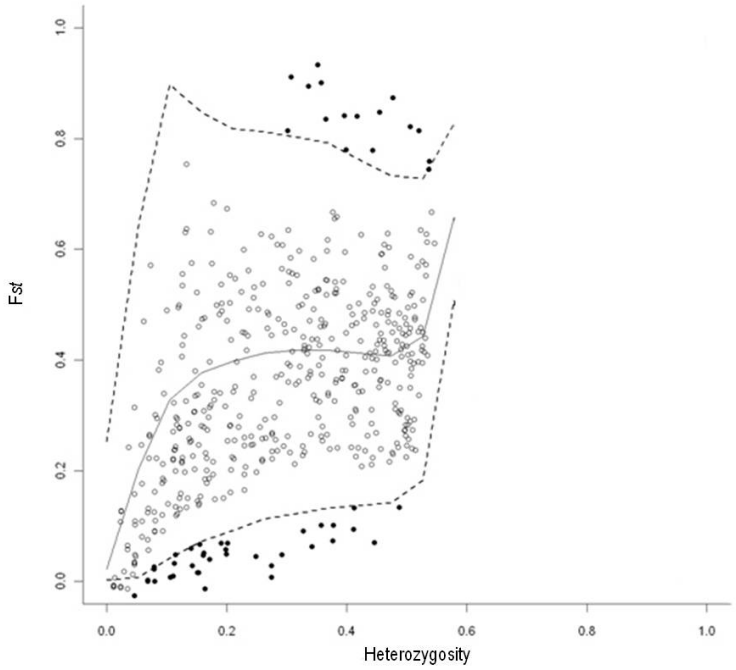

Figure 6.- Distribution of the F_{ST} values as a function of heterozygosity. The solid intermediate line represents the median value, whereas the upper and lower dashed lines represent the limits of the 99% confidence interval. Black solid dots represent loci outside the 99% confidence interval.

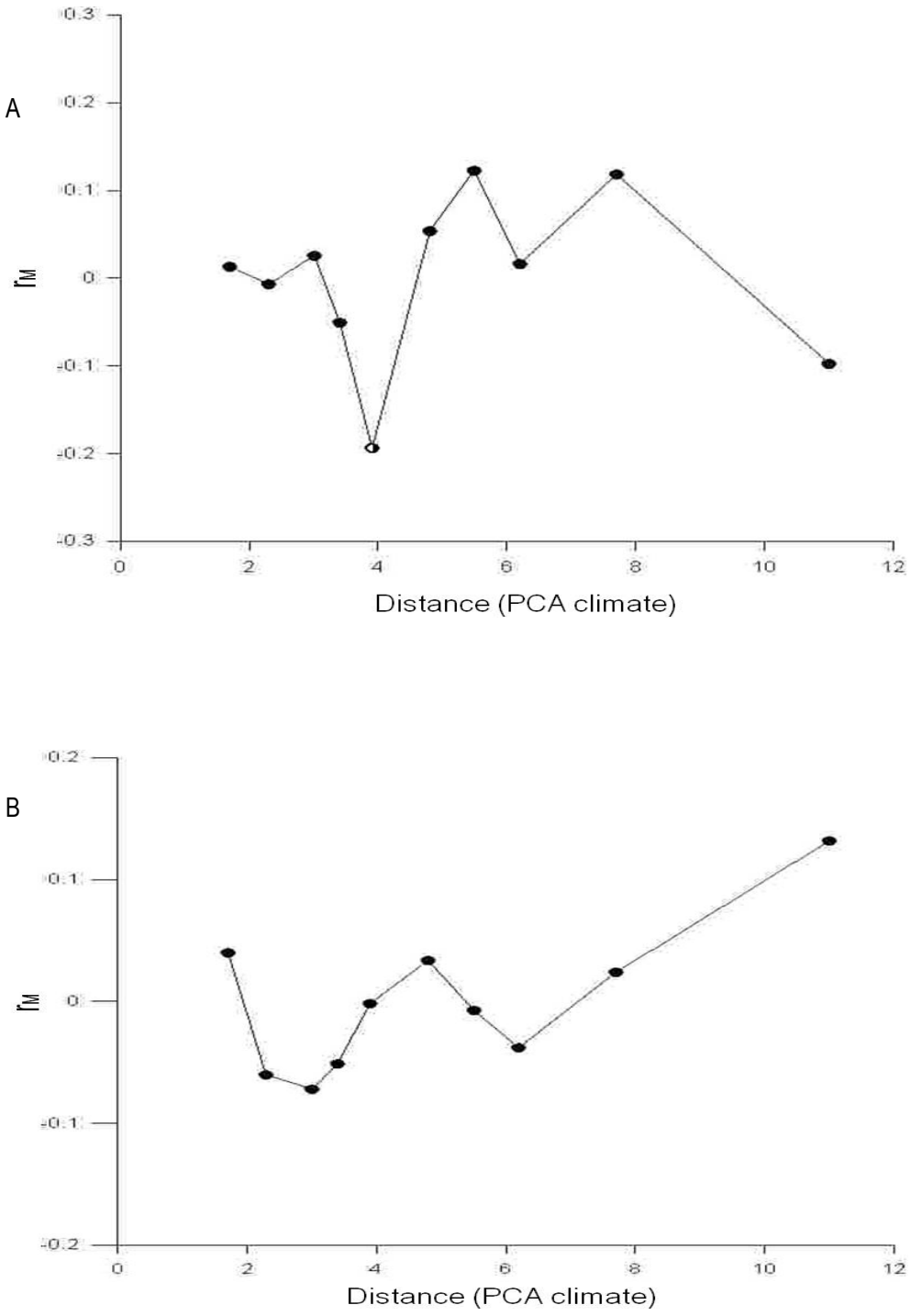

Figure 7. Mantel correlogram outputs. A – Mantel correlogram, r_M values based on F_{ST} coefficient distance between populations (Y axis), calculated with only the divergent loci selected from the outlier scan. X axis distance was calculated with the first two ordination axes of the PCA analyses carried out with the monthly precipitation/temperature ratio. B – Mantel correlogram as described before, but with r_M values based on F_{ST} coefficients distance between populations (Y axis), calculated with all loci. White circles indicate significant values (*p-value* < 0.005 with Bonferroni correction) and half black circles indicate partially significant results (*p-value* < 0.01 with Bonferroni correction). Y axis shows r_M values based on F_{ST} coefficients.

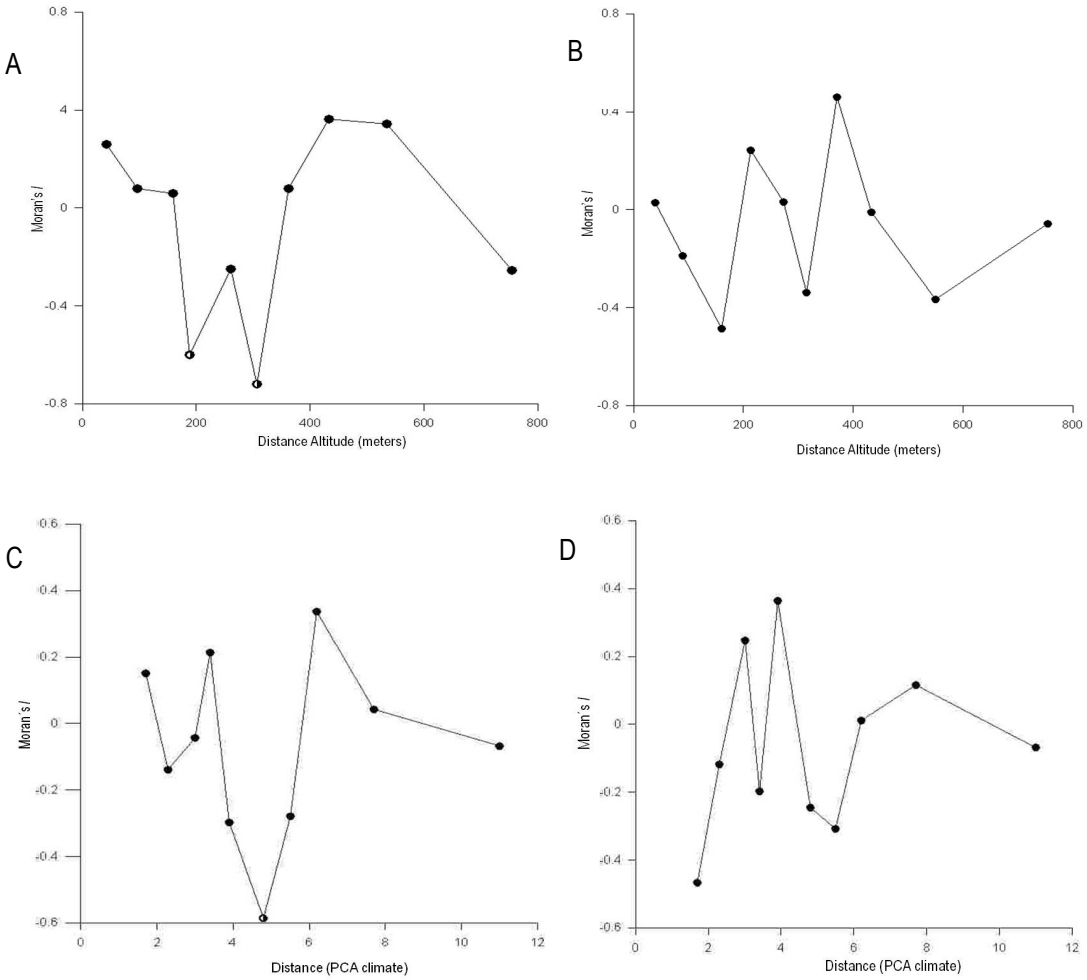

Figure 8. Moran's *I* Correlograms. A - Moran's *I* correlogram, in relation to Nei diversity of divergent outlier loci (Y axis) with differences in altitude (X axis). B –Moran's *I* correlogram as in A, but in relation to Nei diversity of all loci (Y axis). C - Moran's *I* correlogram in relation to Nei diversity of divergent outlier loci (Y axis) and with distance calculated with the first two ordination axes of the PCA analyses developed with the monthly precipitation/temperature ratio (X axis). D –Moran's *I* correlogram as in C, but in relation to Nei diversity of all loci (Y axis). White circles indicate significant values (*p-value* < 0.005 with Bonferroni correction), while half black circles indicate partially significant results (*p-value* < 0.01 with Bonferroni correction).

Discussion

Our results suggest the existence of a weak spatial structure pattern, in spite of certain population differentiation, shown by our Bayesian clustering approaches. This weak spatial patterning and similar genetic diversity values seem to be the norm in other *Armeria* species (Piñeiro et al. 2007; Piñeiro et al. 2009; Nieto Feliner et al. 2002; Baumbach & Hellwig 2007). In addition, the number of outlier loci found was much higher than expected by chance, suggesting that selective forces are acting on the populations. These outlier loci, which did not show a strong pattern according to geographical, altitude or climatic distances, although results were different than those obtained in the analyses of all with all loci. All significant differences were only found with the divergent loci, reflecting some relationship between selective forces and the climate and altitude distance metrics used.

Genetic diversity in Armeria caespitosa populations

We found some differences in the genetic diversity of *A. caespitosa* among populations (Table 1). This variability was not related to any conspicuous source of variation, such as expected differences between marginal and central populations or predictable differences along altitude (see Eckert et al. (2008). In fact, this pattern of relatively small genetic differences among populations and low total-genetic diversity is similar to that of other *Armeria* species, even though they have a wider geographical distribution (Gitzendanner & Soltis 2000). For instance, *A. pungens* populations, which are distributed along the coast of the Iberian Peninsula and on some Mediterranean islands, also showed small differences in genetic diversity (Piñeiro et al. 2007) and the cosmopolitan *A. maritima*, showed similar values to *A. caespitosa* in some genetic parameters (Baumbach & Hellwig 2007). These small genetic differences may be due to some inherent biological characteristics of this genus, such as the existence of a very efficient self-incompatibility system (Baker 1966), but it could also suggest certain levels of gene flow between populations. A more plausible explanation is the possible existence of an ancestral population which covered the whole current distribution area without discontinuities. After several upward and downward shifts along the mountains as a result of ice expansion and retreatment of the Quaternary glaciations (Clayton et al. 2008), *A. caespitosa* would show its current island distribution, with small differences between populations because they were once connected (maybe several times) in the past. These shifts along the elevation gradient have probably modeled the genetic diversity of other *Armeria* species like *A. splendens*, *A. ficaulis* and *A. villosa* (Gutierrez Larena et al. 2002), in the Sierra Nevada massif (southeastern Iberian peninsula) allowing certain levels of horizontal gene flow or even, hybridization between different species.

Structure and spatially explicit analyses of Armeria caespitosa populations.

Although the Bayesian partitions (K= 9) showed weak spatial support they suggested a genetic structure which was also supported by AMOVA analyses. Our findings showed that one of the main structuring

forces was co-occurrence on the same mountain island, as shown by the clusters of populations occurring on the same mountain (Table 1 and Figure 1). These results support the feasibility of a common ancestry distribution. They also reinforce the idea that certain levels of gene flow (through pollen and/or seed dispersal) along the elevation gradient could be the driver that homogenizes this genetic diversity and reduces the likelihood of differentiation along altitude. In this sense, some preliminary results provide conflicting evidence. For instance, we know that *A. caespitosa* seeds may be dispersed within a range of approximately one meter (Carlos Lara & Juan J. Robledo-Arnuncio, personal communication), a value similar than reported for other *Armeria* species (0.68-0.88 meters for *A. maritima* Philipp et al. (1992). This indicates very inefficient dispersal which should prevent genetic homogenization. However, pollen dispersal in *A. caespitosa* is carried out by several generalist pollinators, like bumblebees or syrphid flies (García-Camacho et al. 2009) favoring longer distance connection. *A. caespitosa*'s long flowering period which may also favor successful pollination between contiguous populations for a longer period of timeg (García-Camacho & Escudero 2009; Giménez-Benavides et al. 2010). However, we found that on some isolated mountains, populations are grouped in different genetic clusters even thought they are only a few kilometers apart (e.g. MAT and AGI). In this sense, the MAT population is the lowest population sampled and is surrounded by an important shrub-pine community that might filter the arrival of pollen or seeds from other populations and favor its isolation (Elias & Crocker 2008). An alternative explanation for these isolated clusters could be related to the upward-downward movements associated with ice retreatment and expansion. Under extremely severe ice expansions, high and intermediate altitude *A. caespitosa* populations would shift downward and reach low altitude populations (like LEO or MAT), but in mild ice expansions periods, they would not reach these locations, isolating these populations from the rest. This model encompassing a common range and isolated islands during ice retreatment periods could also be compatible with the existence of a cluster of all the easternmost populations (PLA, PLM, PLB and ARY). This differentiation from other populations could be due to isolation by the long distance to the closest populations (20 kilometers) and the existence of almost impassable topographical or ecological barriers (Widmer et al. 2009) like the forests of the lowest mountain pass of Puerto de Somosierra.

In an attempt to find patterns of spatial or climate-driven explicit genetic structures, we conducted a wide array of analyses. However, we only detected some weak evidence of spatially explicit genetic structure with some correlogram analyses and kinship coefficients related to geographical distances (Figures. 4 and 5), which were also significant when altitude was included. This extremely weak spatially explicit patterning is backed by the existence of a self-incompatibility system that impedes the establishment of new populations from an isolated individual (Baker's rule, Baker (1955, 1966) and deters the typical process of isolation by distance (e.g. stepping stone model Kimura and Weiss (1964). Past upward-downward movements along the mountains associated with ice expansion-retreatment (Hewitt 2001) might have enhanced contact between populations at several times, diluting the genetic differences produced by isolation by distance and fragmentation (e.g. Ezard & Travis 2006). The presence of non-

spatially explicit differentiation could be related to stochastic processes (i.e. genetic drift, inbreeding or mutation, Frankham et al. (2002). These processes are not correlated with spatial or environmental variables and could be strong enough to create genetic differences in the species genome (e.g. Lammi et al. 1999; Balloux & Lugon-Moulin 2002). On the other hand, the weak spatial patterns found in all tests carried out had converging results. For example, negative correlations were found in the kinship coefficient (SPAGEDI, Fig. 4) and F_{ST} distance (Mantel correlogram, Fig. 5) at distances between 12 and 20 kilometres. As these distances are similar to the average size of the mountains in Sierra de Guadarrama, these negative correlations could indicate differences between populations occurring on different mountains, in accordance with the Bayesian cluster analyses results.

Outlier loci: Selection mediated processes

The outlier loci approach has been frequently used in genome scan analyses, to detect specific loci under selection (i.e. Egan et al. 2008; Minder & Widmer 2008; Storz et al. 2004). In spite of having conducted our analyses with very restrictive conditions to avoid type I errors (Mäkinen et al. 2008) a surprisingly high number of outlier loci was found. This suggests that in addition to the effect of stochastic processes selective pressures may play a significant role in the structure of genetic diversity among populations. Genome scans have been described as an efficient marker technology (Reusch & Wood 2007) for better understanding the ecological relevance of genetic processes, including non-model organisms (Karrenberg & Widmer 2008), if results are interpreted cautiously and appropriated genetic models are used to build null models (Narum & Hess 2011).

Most of the analyses of spatial explicit structure developed with the outlier loci showed non-significant results, similarly to the analyses carried out with all loci. However, when the analyses were performed with only the divergent loci, some significant spatial correlations emerged for some distance intervals. Divergent loci appear when the presence of selection forces benefits specific genotypes, enhancing the frequency of certain loci under selection and increasing differences between populations (Nielsen 2005). The Mantel and Moran correlograms with divergent loci showed different patterns from those of the analyses with all loci (mostly neutral), showing correlations to altitude and climatic variables (Figures. 7 A versus B, Figures. 8 A & C versus Figures. 8 B & D). These differences in the structure patterns suggest that selective forces acting on the divergent loci have a climate or altitude signal, but these signals were masked when the analyses were scaled up and carried out with all loci. Other genome scans have also related outlier loci to altitude (i.e. Bonin et al. 2006; Storz et al. 2004) or to environmental gradients directly related to elevation (Temperature, Jump et al. (2007). These studies used analyses and distance matrices similar to ours, but they were carried out in local gradients where differences could be more pronounced than in a whole mountain range. In our case, several stress factors might be playing different roles at the same time, hiding differences. Although little is known about the location and function of loci involved in adaptation to high altitude conditions, the detection of divergent loci in a genome scan

and their correlation with altitude or climatic differences make these outlier loci ideal candidates for further research, on specific genes related to both factors (Fischer et al. 2011).

Concluding remarks

To our knowledge very little is known about the species and plant features that survived the ice-ages and facilitated their permanence in south-temperate mountains (Hewitt 2001). Our results agree with the the island-mountain model proposed by Hewitt (2001) especially if we also consider an ancient common continuous population to be the origin of the present *A. caespitosa* populations and possible upward and downward movements in the mountains, to have facilitated the connection between populations. The Bayesian clustering approach showed certain levels of fragmentation, which may not necessarily be related to spatial distance. However, results support the existence of some gene flow between the .org/10.1111/j.1365-294X.2007.03659.x </url></related-urls></urls></record></Cite></EndNote> ADDIN high number of outlier loci and the correlation between altitude and climate variables with divergent loci, suggest that selective forces are also acting on the genome of *Armeria caespitosa*.

Acknowledgements

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Chapter 3

Unravelling gene flow at the top: mountain islands or isolated belts?

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Manuscrito inédito

Abstract

Gene flow has been recognized as one major limiting factor precluding local adaptation responses. However, recent studies have described important levels of gene flow in locally adapted populations, suggesting that certain levels of gene flow are essential for maintain genetic diversity values that prevent deleterious effects of drift and inbreeding. In mountain plant populations, local adaptation has been described as one of the main responses against climate warming, allowing plant survival under stressful conditions. In this study we used microsatellite markers to unravel the levels of genetic diversity, gene flow and population structure of *Silene ciliata,* a mountain specialist, in two nearby altitudinal gradients. Significant gene flow was found both along altitudinal gradients and, horizontally, within each elevation belt, although it was greater in the former. These geneflow movements may be responsible for the homogeneous levels of genetic diversity between populations. Bayesian cluster analyses also suggested that altitudinal movements are the most plausible scenario. Past population shifts associated to glaciations and interglaciation periods in the temperate mountains may partially explain current distributions of genetic diversity values. The AMOVA and Bayesian cluster analyses carried out to determine the genetic structure were congruent with some local adaptation patterns observed in previous studies, indicating that existing levels of gene flow were insufficient to prevent the occurrence of local adaptation.

Introduction

Possible responses of plants to environmental change include migration, local adaptation and phenotypic plasticity. The ability of species to adopt any of these strategies or a combination of them is largely unknown (Jump & Peñuelas 2005). Rapid and extreme changes (Loarie et al. 2009) in complex scenarios with diverse factors acting together, in the same or opposite directions (Nogues-Bravo et al. 2007) can determine plant population dynamics. Marginal populations, located at the edge (rear or front) of the distribution range have become the focus of several ecological studies in a variety of habitats (Eckert et al. 2008; Sagarin & Gaines 2002 and references therein). Joint features of marginal populations include restricted habitat availability, more stressful conditions than in central populations and usually, geographical isolation of the populations (Lawton 1993).

Adaptation to marginal habitats plays a crucial role in the evolution of populations growing in peripheral areas (Kawecki 2008) making adaptive processes particularly important in species dwelling geographically disconnected areas (Beniston 2003). Several aspects of local adaptation have interested evolutionary biologists (Kawecki & Ebert 2004), including the mechanism allowing for the maintenance of enough levels of genetic variation (essential for maintaining enough evolutionary potential in small populations, Hedrick 1986), the traits affected by natural selection (Hedrick 2006) or even its role in the process of diversification (see review in Schluter 2001). Adaptation is a complex response that involves a variety of factors (Kawecki & Ebert 2004), requiring of the formulation of hypotheses which engage ecological and genetic factors that may promote or hinder such response (Feder & Mitchell-Olds 2003; Mitchell-Olds et al. 2008).

Gene flow has long been recognized as a potentially major factor limiting adaptation to marginal habitats, because high levels of gene flow may swamp locally adapted genotypes (Kirkpatric & Barton 1997). This makes the balance between gene flow and natural selection essential to determine the level of local adaptation of a population (Savolainen et al. 2007). Some basal levels of gene flow seem essential for the maintenance of certain values of genetic diversity within populations where selection could act upon (Kawecki 2008). However, high levels of gene-flow homogenize the populations, thus blurring local adaptation. This process also causes the reduction in the frequency of locally adapted traits, ultimately reducing the fitness of the population (Garcia-Ramos & Kirkpatrick 1997). Nevertheless, some populations may still manage to maintain locally adapted genotypes despite not having interrupted their gene flow from other populations (Sambatti & Rice 2006; Byars et al. 2009; Gonzalo-Turpin & Hazard 2009). Finally, the success of gene flow is not only dependent on the dispersal capabilities, but also on the fitness of the offspring, the performance of the immigrants (Kawecki & Ebert 2004) or the number of genes or traits that are involved in local adaptation (Hedrick 2006).

Inbreeding is another critical processes reducing local adaptation, especially in small populations (Kawecki 2008; Armbruster & Reed 2005). Inbreeding processes can lead to a rapid excess of

homozygous genotypes, reducing dramatically within-population genetic diversity (Pertoldi et al. 2007) and producing an increase in the frequency of deleterious alleles (Ebert et al. 2002; Gautschi et al. 2002) if this have not been previously purged. This is especially true for populations with a short history of inbreeding or that have experienced recent bottlenecks or fragmentation. The incidence of inbreeding depression can make peripheral populations more prone to extinction (Nieminen et al. 2001), despite the benefits of incipient local adaptation processes. However, it is difficult to interpret inbreeding depression responses in stressful and marginal conditions, because the intensity of inbreeding depression could vary depending on the existing environmental conditions (Armbruster & Reed 2005; Cheptou & Donohue 2011).

Inbreeding and gene flow are the main (but not the only) factors that could modify genetic diversity in the populations (Loveless & Hamrick 1984; Hamrick et al. 1992). Under oncoming warming conditions, genetic variability may be the target for natural selection, increasing the frequency of better adapted genotypes or phenotypes to the new environmental conditions (Ennos 2001; McGill et al. 2006). Nonetheless, little is known about the potential ecological effects of genetic variability (Hughes et al. 2008). Genetic variation has been correlated with fitness parameters if both variables run in the same direction (Gautschi et al. 2002; Vellend & Geber 2005). In certain cases they have been correlated with relevant ecological traits (Reed & Frankham 2001) however, there may be many other variables that can mask the potential effects of genetic variation, thus making the local adaptation signals undetectable (Hughes et al. 2008).

High mountain habitats are ideal natural laboratories for testing evolutionary hypotheses related to local adaptation (Körner 2003). Local altitudinal gradients, which allow for great environmental heterogeneity in a small geographical distance permit conducting comparative observations or manipulative experiments in natural populations (Körner 2007). Mountains have been identified as one of the most fragile environments in the world (Nogues-Bravo et al. 2007) and plant populations in mountain systems are especially vulnerable to global change and its consequences (Jump & Peñuelas 2005, see also www.gloria.ac.at). Shifts of plant populations along altitudinal gradients have been inferred for past and present times (see review in Hewitt 2004; Taberlet et al. 1998). Phylogeographical evidences have shown not only that population movements along latitudinal ranges were frequent during the glacial-interglacial periods (Hewitt 2000; Hewitt 2001), but also that upward and downward migrations along the altitudinal range occurred. These movements facilitated gene flow between individuals of distant populations during their rejoining at glacial refuge and even, the occurrence of hybridization zones (Gutierrez Larena et al. 2002). Recent research has also shown that a gene flow and population shift along the altitudinal gradient is a currently ongoing process (Grabherr et al. 1994; Lloyd & Fastie 2003; Gonzalo-Turpin & Hazard 2009). These altitudinal shifts of plant populations in a landscape of interspersed isolated mountains may lead to a scenario where gene flow occurs predominantly along altitudinal gradients and genetic diversity is therefore expected to be structured as “mountain islands” (Hewitt 2001). On the other hand, alternative scenarios where gene flow among populations located at the

same altitude but in neighboring mountain ranges prevails over gene flow along the altitudinal gradient have also been described (Byars et al. 2009; Taberlet et al. 1998) leading to the structure of genetic diversity in "isolated belts" (Figure 1). In both scenarios, gene flow may still not be sufficient to restrict local adaptation (Gonzalo-Turpin & Hazard 2009; Byars et al. 2007), which makes it difficult to elaborate a general predictions about the influence of gene flow along the altitudinal gradient in population genetic variability. All these population shifts may underlie the different observed patterns when genetic diversity is assessed across altitudinal gradients, which foster the study of the implication of genetic diversity and gene flow in the process of local adaptation of plant populations in high altitude mountains (see Ohsawa & Ide 2008 and references therein).

Silene ciliata Pourret (Caryophyllaceae) is a perennial high mountain specialist that inhabits mountain ranges of the Mediterranean basin (Tutin et al. 1995). In its southern range, in the mountain systems of the central part of Iberian Peninsula, recent research has shown evidence of local adaptation across an altitudinal gradient (Giménez-Benavides et al. 2007a). Populations which inhabit in the lower parts of the altitudinal gradient were identified as the most vulnerable (Giménez-Benavides et al. 2010) but also as the ones where local adaptation was more liable (Gimenez-Benavides et al. 2007a). The aim of this study was to characterize the patterns of gene flow in *S. ciliata* populations in two nearby mountains and its effects on genetic diversity, inbreeding, and the genetic structure of the populations along the altitudinal gradient. For this purpose we used EST-SSR markers transferred from the congener *S. latifolia* Poir., and newly developed nuclear SSR loci from *S. ciliata* to characterize populations of *S. ciliata* and addressed the following questions: 1) Do patterns of genetic diversity and other genetic variables (such as inbreeding, Hardy-Weinberg equilibrium) in the populations depend on their location across the elevation gradient? 2) Is gene flow more intense along the elevation gradient of each mountain (mountain islands) or along similar altitudinal stages of nearby mountains (isolated belts)? and 3) Is the spatial genetic structure compatible with the empirical evidences of local adaptation found so far by Giménez-Benavides et al. (2007a) in these populations?

Material and Methods

Study species and population selection

Silene ciliata is a perennial plant forming pulviniform rossetes of up to 2 cm in height and 15 cm in diameter. Self-pollination experiments (A. García-Fernández, unpublished data) indicate that *S. ciliata* is a self-compatible species. However, passive autogamy is partially restricted due to a pronounced protandry. *Silene ciliata* inhabits the main mountain ranges in the northern half of the Iberian Peninsula, the Massif Central in France, the Apennines and the Balkan Peninsula (Tutin et al. 1995). On the Iberian Peninsula, *S. ciliata* reaches its southern latitudinal limit in the Sierra de Guadarrama. These populations are considered as relictual, because of their isolation from populations the remaining northern range in other mountain systems. *S. ciliata* presents variable levels of ploidy in natural populations (Blackburn 1933; Küpfer 1974) however, for Guadarrama populations, all individuals of the studied populations were diploid (2n=24, García-Fernández unpublished data). *S. ciliata* has nocturnal pollination interactions with *Hadena consparcatoides* Schawerda*,* Lepidoptera: Noctuidae, but can also be pollinated by diurnal insects (Giménez-Benavies et al. 2007). *S. ciliata* seeds lack of any specialised structure to promote wind or animal dispersal, so barochorous dispersal seems the most probable mechanism.

For this study, two altitudinal gradients were selected in the Sierra de Guadarrama (Fig. 1). Altitudinal gradients (Gradient 1 and Gradient 2) were located on the two highest peaks of the mountain system (Peñalara at 2420 m.a.s.l. and Cabeza de Hierro at 2380 m.a.s.l., respectively). The two peaks are separated by 6.1 kilometres. The highest populations along each gradient, (hereafter H1 and H2), were located near the summit of each mountain peak. The plant communities on these peaks and crests are dominated by patches of *Festuca curvifolia* Lag. *ex* Lange grassland, occasionally displaced by *Nardus stricta* L. grassland*,* coexisting with a high diversity of pulviniform plants. The intermediate populations were located at at 2200 m. a. s. l. in both gradients. We selected Dos Hermanas summit as the intermediate population of Gradient 1 (hereafter I1) and Bola del Mundo summit as the intermediate population of Gradient 2 (hereafter I2). Population I1 is 1.7 kilometres away from population H1, whereas the distance between the populations I2 and H2 is over 4.4 kilometres. In both sites, *F. curvifolia* grassland is the dominant vegetation, with the occasional presence of *Cytisus oromediterraneus* Rivas Mart. *et al*. or *Juniperus communis* L. subsp. *alpina* (Suter) Čelak. The lowermost populations are located at 1980m altitude in each gradient. The lowermost population in Gradient 1 (hereafter L1) is located in the lateral moraine deposit of the glacial cirque of Laguna de Peñalara and is about 1.9 kilometres away from population I1. The lowermost population in Gradient 2 (L2) is located in the margin of an old abandoned Ski slope and 1.5 km away from population I2. In both populations, the plant community is dominated by a shrub matrix of *C. oromediterraneus* and *J. communis* with scots pines (*Pinus sylvestris* L.) interspersed in a *F. curvifolia* pasture. All the populations from the Gradient 1 are located inside the Peñalara Natural Park, whereas the high and the intermediate populations of Gradient 2 (H2 and M2) are located within the

boundaries of the Cuenca Alta del Manzanares Regional Park. Figure 1 shows the schematic representation of all the populations and the different scenarios about gene flow between populations.

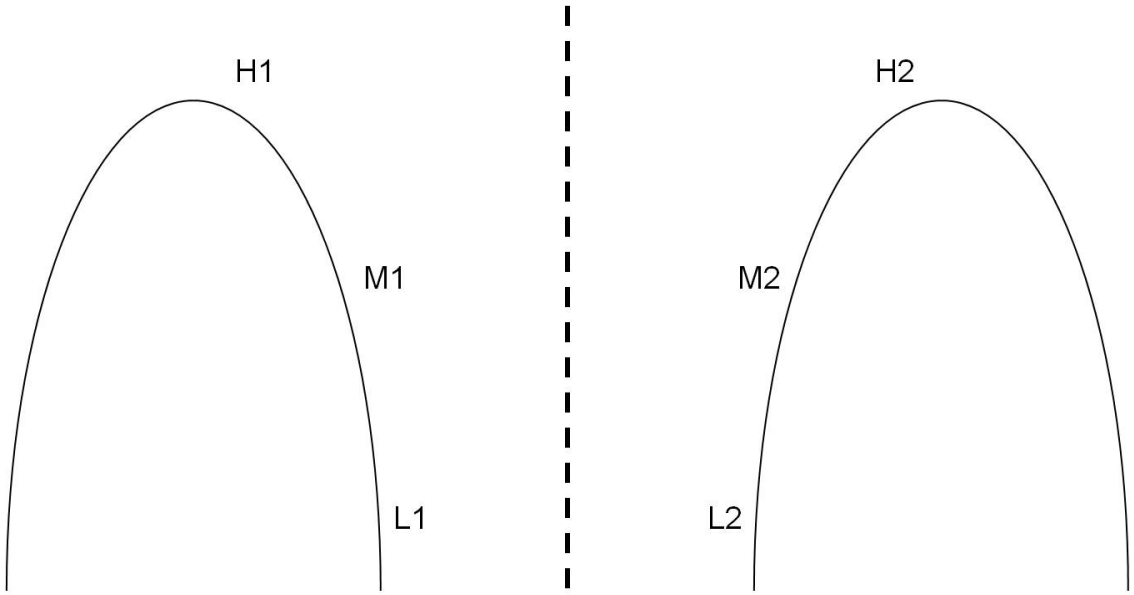
In each population, 30 individuals were haphazardly sampled but separated at least five meters from each other. Plant material consisting of green leaves was collected from all individuals, taking care that it did not show any signs of parasites, fungal infection or drought injuries. The geographical coordinates of each individual plant were recorded using a Garmin GPS 12 receiver. Leaf material was cleaned and dried in silica gel (Chase et al. 1991) for storage until DNA extraction. DNA was extracted following the Elphinstone et al.(2003) protocol in 96-well plates, from 10 to 20 mg of dried tissue of *S. ciliata.* DNA concentration was estimated with Nanodrop 3300 Fluorospectrometer (ThermoScientific) and confirmed in agarose gels. For all samples extracted, DNA concentration was higher than10 ng/µl.

EST-SSR *transfer from S. latifolia to S. ciliata*

Transfer of EST-SSR markers from *S. latifolia* was conducted from an initial screening of the EST-SSR markers developed by Moccia et al. (2009) and by A. Widmer (unpublished data) to identify markers with high reproducibility and reliable interpretation. Some of these markers have been previously assayed in *S. ciliata* (Moccia et al. 2009) to identify whether markers are polymorphic in *S. ciliata*, we used three samples of *S. ciliata* from different populations and one sample of *S. latifolia* as a positive control.

Amplification of EST-SSR followed the procedure of Schuelke (2000). PCR reactions consisted of a three-primer system: The 5' end of the forward primer was extended with a universal M13 tail, the second primer was the reverse locus-specific primer, and the third primer was a fluorescently labelled M13 primer. PCR reactions were conducted in 10 µl reaction volume containing 10 ng of template DNA, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM fluorescently labelled (FAM or VIC) M13 primer, 0.2 µM reverse primer, 0.05 µM forward primer with M13 tail and 0.05 U Promega GoTaq. For this assay The PCR cycling profile was 94 ºC, 5 min for initial melting step followed by 30 cycles each at 94ºC for 30s, 60ºC, 45 sec. for annealing and 72ºC, 45 sec. for extension, followed by 8 additional cycles in which annealing temperature was dropped at 52ºC, and a final extension at 72ºC for 10 min. One µl of PCR products was diluted with 9.1 µl of a loading mixture made up with 9 µl HiDi Formamide and 0.1 µl Genescan 500 LIZ internal size standard (Applied Biosystems). Samples were run on an automated DNA sequencer ABI PRISM 3730 Genetic Analyzer (Applied Biosystem). Fragment sizes were assigned to alleles using GeneMarker V.1.75 (Softgenetics LLC).

Hypothesis A – Mountain islands.



Hypothesis B – Isolated belts.

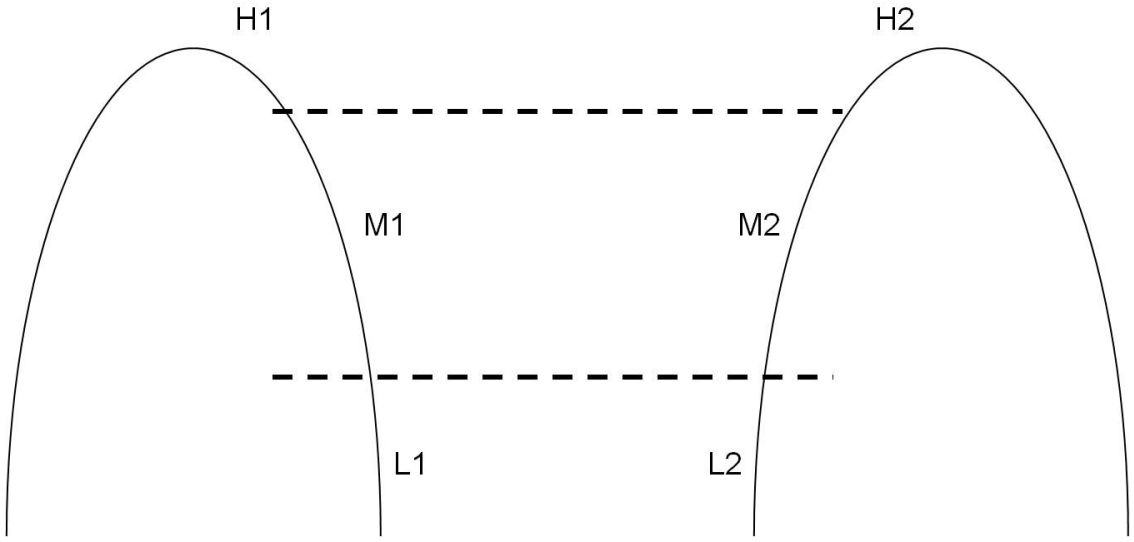


Figure 1. Hypothetical scenarios about predominant gene flow between both mountains. In each mountain (1 and 2), three populations were selected: High (H1 and H2), Intermediate (I1 and I2) and Low (L1 and L2). Discontinuous lines showed the hypothetical barriers for the gene flow in each model.

SSR marker development for S. ciliata

Microsatellite loci were isolated and characterized from three partial genomic libraries enriched for CT/GT/CTT repeats following the FIASCO protocol (Zane et al. 2002). DNA was digested with *Mse*I enzyme (New England BioLabs). Digested products were purified using the Qiaquick Kit (Qiagen) and then ligated to *Mse*I AFLP adaptors (5´- TAC TCA GGA CTC AT-3´/5´-GAC GAT GAG TCC TGA G 3´) using Ligafast (Promega). The mixture was diluted 1/10 and then amplified with the adaptor specific-primers (5´- GAT GAG TCC TGA GTA AN-3´, hereafter *Mse*I-N) using *Taq* Polymerase (Biotools). PCRs were carried out with an Applied Biosystem GeneAmp PCR System 9700. The PCR program consisted of one step of 2 minutes at 72ºC to let the DNA polymerase fill the nicks, 5 minutes at 94ºC, followed by40 cycles of 94ºC, 30 seconds, 53ºC, 1 minute and 72ºC, 1 minute, and a final extension step of 72ºC for seven minutes.

Separated enrichments were carried out using streptavidin-coated M-280 magnetic beads (DYNAL) attached to $(CTT)_8$ GC, $(GT)_8$GC, and $(CT)_8$GC 5´-byotinlated oligonucleotides. The beads were washed three times in Bind and Wash buffer to remove excess oligonucleotides and then resuspended in 100 µl of a solution composed of 3×Sodium Saline Citrate (SSC), 0,1%SDS and 2% PEG. 15 µl of DNA were hybridised to the oligonucleotides at 50ºC for 30 minutes, and then, washed four times each with three decreasing salt-concentration solutions (2×SSC, 1×SSC and 0,5×SSC, respectively, plus 1%SDS and 0,8µM MseI-N) to remove unbound fragments and unspecific hybridisation products. Fragments containing microsatellites were released at 95ºC, 5 minutes in 100 µl of 0,2% of SSC and desalted with Qiaex II Kit (Qiagen).

Twelve PCRs were carried out with 5 µl of enriched DNA in a total PCR volume of 20 µl, using *Mse*I-N primer for amplification and Taq Polymerase (Biotools). PCR products were purified again and cloned using pGEM-T Vector System (Promega) according to the manufacturer´s instructions and transformed into JM109 *E. coli* high-efficiency competent cells (Promega). Transformants were identified by blue/white screening on LB agar plates with ampiciling, X-gal and IPTG. Mainly, white colonies were screened by PCR, employing T7 (5´-TAA TAC GAC TCA CTA TAG GGC -3') and Sp6 (5´- ATT TAG GTG ACA CTA TAG AAT AC - 3´) as primers. DNA sequencing was carried out using BigDye Terminator Kit v. 3.1 (Applied Biosystems), followed by electrophoresis on an Applied Biosystems 3700 DNA sequencer.

One hundred and twenty two clones were sequenced out of which 53% contained microsatellite sequences. Primers were designated for 17 clones using PRIMER3 (Rozen & Skaletsky 2000). Twelve primer-pairs produced clear amplicons of the expected size in 2% agarose gels and were subsequently selected for fluorescent labelling and further analysis. Forward primers were labelled with fluorescent dyes (6-FAM, VIC, PET and NED) for automated electrophoresis. PCRs were performed in a 20 µl mix containing 3 pmol each of the labelled (forward) and unlabelled (reverse) primers, 0.2 mM of each DNTP, 2mM $MgCl_2$ 1×*Taq* Buffer (Biotools), 1 U of *Taq* DNA polymerase (Biotools) and 20 ng of template DNA.

The PCR program consisted of one step of 4 min at 94ºC, followed by 35 cycles of each 94ºC, 1 min; 51 or 56ºC (depending of primer pair), 1 min and 72ºC 1 min, and a final extension step of 7 min at 72ºC. The products were run on an ABI 3700 automated sequencer (Applied Biosystems) and the amplified fragment lengths assigned to allelic size with GENEMARKER V 1.75 (Softgenics LLC) using LIZ500 as the internal lane standard.

Statistical analyses

SSR polymorphism and genetic diversity

Genetic diversity indices, including total number of alleles (*A*), observed (H_O) and expected (H_E) heterozygosities and the inbreeding coefficient (F_{is}) were calculated with Genepop (Rousset 2008). This same software was used to estimate Wright's *F*-statistics according to Weir and Cockerham (1984) and to check for departures from Hardy–Weinberg equilibrium at each locus and population and tested for significance by Fisher's exact tests, and to check for genotypic linkage disequilibrium between pairs of loci within each population using the log-likelihood ratio *G* statistic (based in 5000 permutations). The same parameters were estimated after grouping populations by mountain island origin (Gradient 1 *vs* Gradient 2) or isolated belts (Low *vs.* Intermediate *vs.* High). FSTAT 2.9.3 (Goudet 1995) was used for comparing genetic diversity indices (Allelic Richness, Heterozigosity Expected and Observed in the two hypothesised ecological scenarios (mountain island and isolated belts) and significance was assessed with 10000 permutations. All parameters were calculated for each transferred EST-SSR and each SSR locus. All tests which involved multiple comparisons were corrected at the table-wide significance level with the Dunn–Šidák method (Sokal & Rohlf 1995).

Population structure and differentiation

Pairwise F_{ST} values (Weir & Cockerham 1984) were calculated for each population pair and between population groups considering both hypothesis, mountain islands or isolated belts, grouping populations of the same altitudinal range using FSTAT 2.9.3 (Goudet 1995). The significance of between-site relationship was evaluated with 10000 permutations in each case. Number of migrants between population pairs were estimated with ARLEQUIN 3.5 (Excoffier & Lischer 2010) according to Slarkin (1985).

Mantel and Partial Mantel tests were used to check for the correlation between genetic, geographical and altitudinal distances, searching for the sign of isolation by distance. Slatkin's pairwise linearized F_{ST} values (i.e., F_{ST} / (1 - F_{ST}) (Slatkin 1995) were calculated using ARLEQUIN 3.5 (Excoffier & Lischer 2010) while geographical and altitudinal distances were generated from GPS coordinates in Arc-View ™ (ESRI ®) and subsequently log-transformed. Partial Mantel tests are similar to a partial correlation, being able to detect the correlation between two matrices of interest when the effect of a third matrix is kept constant (Legendre & Legendre 1998). Mantel and Partial Mantel tests were carried out in R 2.9.2 using the Vegan package, with 10000 permutations, using the Kendall and Pearson correlation methods.

Genetic structure was also analysed with the Bayesian clustering method implemented in STRUCTURE 2.3.1 (Falush et al. 2003). Our analyses were based on an admixture ancestral model with correlated allele frequencies (because of high F_{IS} values; Falush et al. 2007), for a range of *K* values starting from 1 to the number of populations considered plus 2 (i.e. 8). The proportion of membership of each individual and population to the inferred *K* clusters were then calculated. We used a burn-in period and a run length of the Monte Carlo Markov Chain (MCMC) of $5x10^5$ and $1x10^6$ iterations, respectively. Ten runs were carried out for each *K* in order to quantify the amount of variation of the likelihood. The number of *K* present in the data set was evaluated according to Evanno et al. (2005). This method uses an *ad hoc* parameter (ΔK) to estimate the rate of change of likelihood values between successive *K* values. Additional Bayesian analyses were attempted with BAPS (Bayesian Analysis of Population Structure; Corander & Marttinen 2006), which uses stochastic optimization instead of MCMC to find the optimal partition (with highest estimated probability). For these approaches, 5 replicates for each possible *K* were run. Finally, we also assessed population structure with the INSTRUCT algorithm (Gao et al. 2007). This software reduces the effect of deviation from HW equilibrium during the estimation of the number of clusters and computes the Deviance Information Criterion (DIC) to provide a more formal means of model section. Settings for INSTRUCT runs were identical to those described in STRUCTURE, except that we allowed INSTRUCT to estimate the population-level inbreeding coefficient (F_{IS}). Following established guidelines, we considered a difference in DIC (ΔDIC) between the best and second best model as indicative of substantial support for the best model (Spiegelhalter et al. 2002). All Bayesian analyses were performed with the combined matrix of all markers.

To estimate genetic structure following an alternative non-Bayesian approach, Analyses of Molecular Variance (AMOVA) were conducted to quantify the proportion of molecular variance within and among populations using ARLEQUIN 3.5. Nested AMOVAs, were conducted to further decompose genetic variance among predefined population groups according to both ecological scenarios considered (mountain islands or isolated belts) but also to the results provided by the Bayesian clustering analyses STRUCTURE, BAPS or INSTRUCT (see below).

Table 1. Characterization of four nuclear microsatellites in 6 populations of *Silene ciliata*. For each locus the, repeat motif, forward (F) and reverse primer (R) sequences, annealing temperature (Ta), size range in base pairs, total number of alleles (A), and GenBank accession numbers (GB), are given.

Locus	Repeat motif	Primer sequences (5'-3')	Ta (ºC)	Size range (bp)	A	GB
Sci-1224	(CT)18	F: NED-ACCTGATTAGAAGACACAGGAGGA R: TTTATGTTGCCGCATCCTTATC	56	140-196	26	JF979125
Sci-1208	(CT)10	F: PET-TGGAAACGATGTATGAGACGA R: TGTGATTGAAGTAGCCAAACCT	56	158-182	11	JF979126
Sci-0106	(CT)7	F: VIC-AAACAAACGAGCGATCATCTAA R: TTCCGATGCTTCTGGTACTTCT	56	111-135	12	JF979127
Sci-1443	(CT)7	F: FAM-GACGCCTTTCTCAAAATCACC R: GGGAATCTAGGGTTTAGCAGTGA	56	126-160	11	JF979128

Results

Transferability analysis of EST-SSR loci from S. latifolia to S. ciliata

While most of the forty-two assayed EST-SSR loci from *S. latifolia* produced amplicons of the expected size in *S. ciliata*, only eight of them were reproducible and consistently interpretable. From these eight loci, two were monomorphic and after their sequencing the absence of a microsatellite motif was confirmed thus, were subsequently discarded. Six EST-SSR loci were finally chosen to analyse the entire set of samples.

Genetic diversity of EST-SSR and SSR loci in S. ciliata.

A total of 88 and 60 different alleles were scored from the six EST-SSR and the four SSR loci in 180 individuals analysed, respectively. Mean number of alleles was 14.8 ± 5.7 (average ± SD) per locus. Summarized data of total number of alleles (*A*), observed (H_O) and expected (H_E) heterozigosities, respectively and inbreeding coefficient (F_{IS}), are shown in Table 2 and detailed in Table S1. The highest mean number of alleles was found in the I1 population, 8.2 ± 4.2 (average ± SD), whereas H1 and H2 had the smallest values (7.1 ± 3.5 and 7.2 ± 3.9, respectively).The L2 and L1 populations had more exclusive alleles (13 and 10, respectively) than the remaining, especially than the two higher altitude ones H2 (2 alleles) and H1 (5 alleles) which had the lowest number of exclusive alleles. Observed heterozigosities ranged from 0.35 ± 0.29 L1 to 0.33 ± 0.26 H1, whereas expected heterozigosities varied from 0.61 ± 0.25 (I1) to 0.55 ± 0.28 (I1) (Table 2).

Inbreeding coefficients (F_{IS}) values ranged from 0.334 ± 0.322 in the H2 population to 0.465 ± 0.309 in the H1. Only in the L1 population, F_{IS} estimated with the EST-SSR loci data set was smaller than that calculated with SSR markers (see Table S1). All populations showed significant departures for HW equilibrium including all loci. Of the 60 loci per site comparisons for HW equilibrium, 27 (45%) were significant after correction for multiple comparisons. All the populations had four or five loci showing significant heterozygote deficiency No consistent linkage disequilibrium was found between any 60 loci across sites comparisons, and none resulted in significant results after correction for multiple comparisons.

No significant differences were found for all genetic diversity estimators, including (number of alleles, observed and expected heterozigosity, and F_{IS}) were found when the populations were grouped according to the “mountain islands” model (Table 3). However, when the populations were grouped by altitude belts, the Low populations had more (but not significant) alleles, higher value of observed heterozigosity and smaller F_{IS} values. Expected heterozigosity values were quite similar between all altitudinal ranges (Table 3).

Table 2. Genetic diversity indices in 6 populations of *S. ciliata* from two altitudinal gradients derived from 6 EST-SSR transferred loci from *S. latifolia* and 4 SSR loci developed in *S. ciliata*. ***N***, sample size; ***A***, total number of alleles; H_O and H_E, average observed and expected heterozygosities, respectively, and F_{IS}, average inbreeding coefficient. Asterisks indicate signification values, (*p-value* < 0.05). See Table S1 for detailed results for each marker and popualtion.

Population	Code	N	A	H_O	H_E	F_{IS}
Morrena	L 1	30	75 (10)	0.352	0.506	0.348* (5)
Dos Hermanas	I 1	30	82 (9)	0.289	0.551	0.394* (5)
Peñalara	H 1	30	71 (5)	0.206	0.502	0.465* (4)
Ski slope	L 2	30	81 (9)	0.283	0.561	0.359* (5)
Bola Mundo	I 2	30	77 (5)	0.303	0.522	0.429* (4)
Cabeza	H 2	30	72 (2)	0.301	0.556	0.334* (4)

Numbers in brackets show the number of exclusive alleles in A column, and the number of loci that are significantly deviated from Hardy-Weinberg equilibrium in the F_{IS} column.

Table 3. Comparison of mean genetic diversity values in the two ecological models tested in *Silene ciliata* based on 6 EST-SSR and 4 SSR loci. ***A*** = allelic richness calculated after the rarefaction method of El Mousadik & Petit (1996) and based on minimum sample size of 30 individuals. H_O, H_E, average observed and expected heterozygosity within populations, respectively. Mountain islands: Gradient 1 and 2, *N*=3 populations each; Isolated belts: Low, Intermediate and High, *N*=2 populations each.

	Mountain islands			Isolated belts			
	Gradient 1	Gradient 2	*p*-value	Low	Intermediate	High	*p*-value
A	7.27	7.29	0.99	7.35	7.59	6.89	0.13
H_O	0.358	0.356	0.899	0.370	0.355	0.349	0.773
H_E	0.597	0.600	0.698	0.593	0.602	0.602	1
F_{IS}	0.400	0.406	0.897	0.377	0.410	0.420	0.672
F_{ST}	0.041	0.031	0.719	0.071	0.132	0.063	0.337

Population genetic structure and spatial analyses.

All pairwise values of differentiation between populations (F_{ST}) were significantly different from zero except for the population pair L2-I2 (Table 4). Significant values ranged between 0.021 (I1-H1) and 0.155 (H1-I2) with an overall value of (F_{ST}, Average ± SD = 0.087 ± 0.044). In all of the cases, F_{ST} values were smaller (or very similar) when the populations of the same mountain gradient were compared (Table 4). When populations were grouped as in the two proposed ecological scenarios ("mountain islands" and "isolated belts"), no significant F_{ST} values were found (Table 3). According with these low levels of F_{ST}, number of migrants between population pairs were higher than one in all populations comparisons. However, the numbers of migrants were higher between populations between populations along the elevation gradient than between populations at the same altitude (Table 4).

We found significant correlation between genetic and log-transformed geographical distances (r = 0.72, $p < 0.01$). However, no significant correlation was found between the genetic distances and the log transformed altitudinal distances (r = 0.18, $p = 0.72$). Partial Mantel test was significant (r = 0.71, $p < 0.01$ according to both Pearson and Kendall methods) when the log-transformed altitudinal distance matrix was included as the covariable, but non-significant results were obtained when the matrix changes its relation (spatial distance matrix used as the covariable).

Results from the Bayesian clustering analyses conducted with BAPS and INSTRUCT considering the whole set of loci converged at *K*=3 as the most likely number of genetic clusters (Table 5). For *K* = 3, L2 – I2 – H2 clustered together with high proportion of membership to one independent cluster, populations I1 and H1 grouped together in another cluster and L1 conformed the third independent genetic cluster (BAPS *K* = 3 cluster probability = 0.99). However, when analyses were developed with STRUCTURE, the clustering scenario that groups together all populations from each altitudinal gradient (two genetic clusters, *K*=2, L1-I1-H1 in one cluster and L2-I2-H2 in another) was also presented as highly plausible (Fig. 2 A and B, Table 5). According to the method of Evanno et al. (2005), for *K* = 2 a modal value of ΔK = 398.53 was obtained, whereas for *K* = 3 the value was lower ΔK = 121.71 (Table 5, Fig. 2B).

AMOVA analyses conducted for *S. ciliata s.l.* attributed 8.83% of the total variation between populations. Hierarchical AMOVA analyses (Table 6) revealed highly congruent patterns of explained variability with the Bayesian clustering analyses. A largest proportion of variation among groups (6.94%) coupled with the lower proportion of variation among populations within groups (i.e. the more homogeneous groups, 4.42%) was found for the hierarchical AMOVA that tested the mountain islands ecological scenario, compared to the AMOVA that tested the isolated belt scenario where 2.4% (not significantly different from zero) and 10.79% where found for these two partitions (Table 6). These results agreed with the primary number of genetic clusters (*K* = 2) detected by STRUCTURE analyses. Further hierarchical AMOVA conforming three population groups (independent L1 vs. I1+H1 vs. complete altitudinal gradient 2) revealed even higher proportion of variation among groups (7.36%) and higher homogeneity among populations within groups (3.26%) as obtained for the secondary number of genetic clusters (K = 3) detected by STRUCTURE, BAPS and INSTRUCT analyses.

Table 4. Matrix of pairwise differentiation: F_{ST} (lower diagonal) values comparisons between populations. Significantly different from zero F_{ST} values ($p < 0.05$) are in bold face. Migration (N_em; upper diagonal) based on Slarkin (1985)

	Low 1	Intermediate 1	High 1	Low 2	Intermediate 2	High 2
Low 1		4.165	2.861	2.472	2.751	1.802
Intermediate 1	**0.057**		11.701	2.705	1.936	1.411
High 1	**0.08**	**0.021**		2.938	1.743	1.228
Low 2	**0.083**	**0.114**	**0.125**		6.328	3.448
Intermediate 2	**0.12**	**0.151**	**0.169**	0.018		13.367.
High 2	**0.092**	**0.085**	**0.078**	**0.038**	**0.068**	

Table 5. Bayesian models-based clustering likelihoods and model selection for the number of demes (*K)* present with 10 SSR markers. ΔK is estimated following Evanno et al. (2005). DIC was estimated following Gao et al. (2007).

	Structure		**Instruct**	
K	**Average ln P(D)**	**ΔK**	**LL**	**DIC**
1	-5619.94		-4583	9166
2	-5319.26	398.53	-4333	8666
3	-5188.91	121.71	-4229	8460
4	-5107.65	71.04		
5	-5067.33	1.09		
6	-5028.92	7.88		

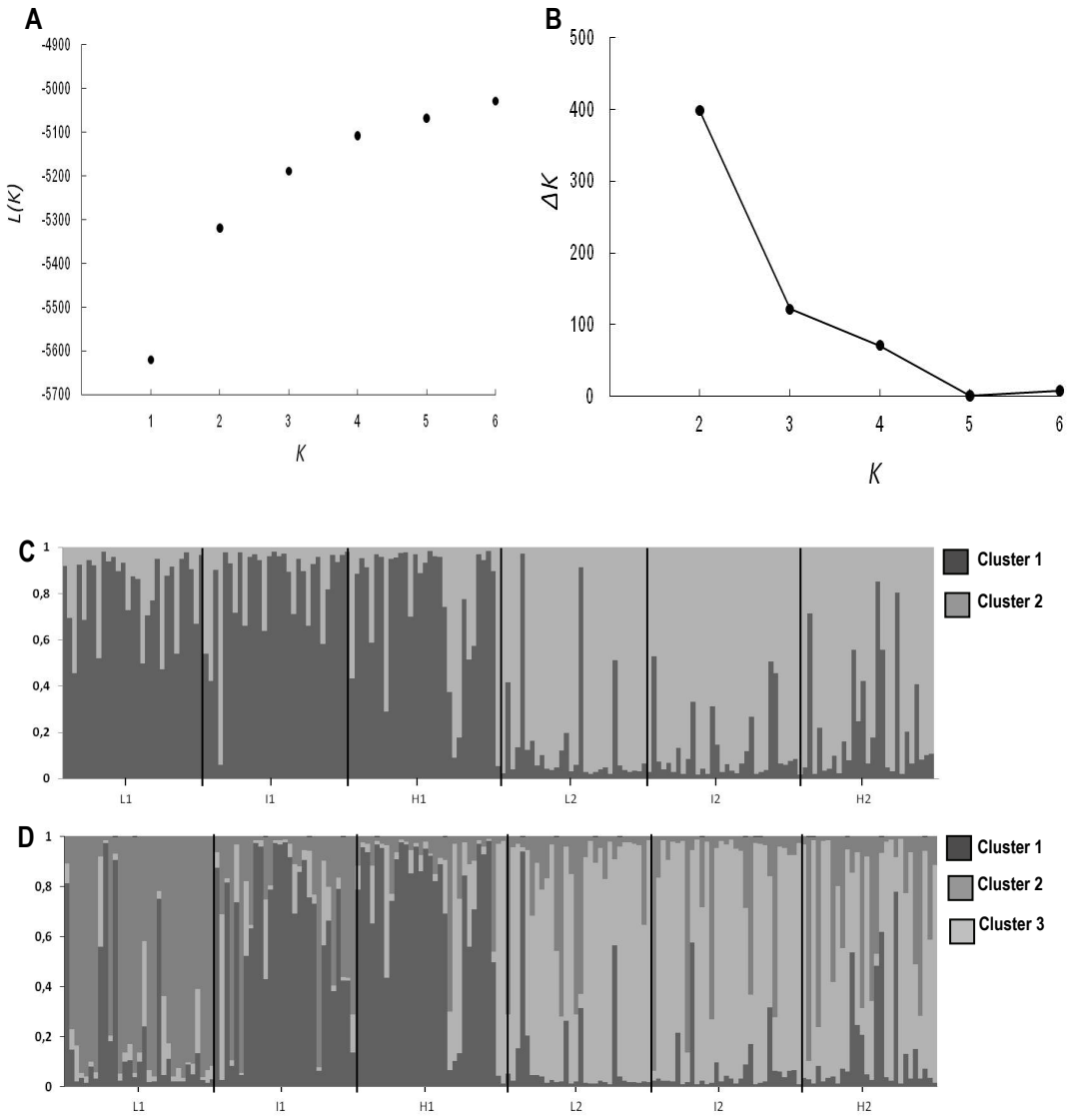


Figure 2. Log probability of data *L(K)* as a function of *K* for 10 STRUCTURE runs at *K* = 1 – 6 (Graphic A) and ΔK rate of change in the probability between successive runs, *K*, as a function of *K* (Evanno et al. 2005) based on the whole data set (six EST-SSR and four SSR characterized in *S. ciliata*). Graphic C and D showed Bayesian analyses of genetic structure of 180 individuals from six populations of *S. ciliata.* The proportion of membership if individuals for *K* = 2 and 3 predefined clusters, respectively.

Table 6. Analyses of molecular variance (AMOVA) of *Silene ciliata* based on 10 microsatellite markers. The four possible scenarios were considered: no populations structure (1), mountain islands (2), mountain island with L1 population isolated (3) and isolated belts (4).

Source of variation	d.f	Sum of sqares	Variance components	Fixation indices	Proportion of variation	p-value
1. Silene ciliata s.l.						
Among populations	5	86.4	0.25	0.09	8.83	< 0.001
Within populations	354	898.3	2.53		91.17	< 0.001
2. Mountain islands: L1+I1+H1 vs. L2+I2+H2, (K=2)						
Among groups	1	45.87	0.19	0.06	6.94	0.1
Among populations within groups	4	40.53	0.13	0.04	4.42	< 0.001
Within populations	354	898.3	2.53	0.11	88.64	< 0.001
3. Mountain islands with L1 isolated: L1 vs. I1+H1 vs. L2+I2+H2, (K=3)						
Among groups	2	62.13	0.21	0.07	7.36	0.01
Among populations within groups	3	24.27	0.09	0.04	3.26	< 0.001
Within populations	354	898.3	2.53	0.11	89.38	< 0.001
4. Isolated belts: L1+L2 vs I1+I2 vs H1+H2 (K=3)						
Among groups	2	25	-0.06	0.08	-2.4	0.73
Among populations within groups	3	61.4	0.3	0.1	10.79	< 0.001
Within populations	354	898.3	2.54	-0.2	91.61	< 0.001

Discussion

Genetic diversity in Silene ciliata in elevation gradients

Genetic diversity within populations is determined by opposed evolutionary forces that may increase or decrease the net levels of variation. While mutation and migration on one hand and drift on the other hand represent the primarily evolutionary forces with opposite effects on the levels of genetic diversity, there exists a variety of species-specific factors contributing to shift the mutation-migration/drift equilibrium in one direction or another. Thus, several life history traits related to longevity (i.e., annuals vs. perennials) and reproductive traits (i.e., the presence of self-incompatibility systems and types of pollination) and seed dispersal mechanisms have been identified among the most relevant in determining the levels and distribution of genetic diversity in plant populations (Hamrick et al. 1979; Hamrick & Godt 1996). Besides, historical factors, especially in alpine populations likely affected by glaciations, cannot be discarded as factors potentially having shaped the genetic diversity of current populations (Hewitt 2000; Segarra-Moragues et al. 2007). Understanding the relative contribution of each of these factors may help to identify the key forces driving population dynamics and evolution.

Because our study is focused on a single species in a narrow geographical scale, we do not expect to find important differences in genetic diversity levels among populations of *S. ciliata* populations. Similar values of genetic diversity (number of alleles or H_E), inbreeding coefficients or number of loci under Hardy Weinberg disequilibrium were described in all populations (Table 2). These parameters were slightly lower than those found in other alpine species studied along altitudinal gradients in alpine mountains (e.g. Byars et al. 2009; Gonzalo-Turpin & Hazard 2009; Ohsawa et al. 2008) although in these cases, populations were separated by larger geographical distances or showed completely different life-history and reproductive traits. Each mountain showed a different pattern of genetic diversity between populations (in mountain 1, the Intermediate population had the greatest genetic diversity values, while in mountain 2, the Low population was the most diverse). However, the when genetic diversity was compared between mountain islands or between isolation belts non-significant differences were found (Table 3). This lack of a clear pattern in the genetic diversity of populations along an elevation gradient has also been observed in other species (see review in Ohsawa & Ide 2008). In these situations, high levels of gene flow have been proposed as the main driver which could homogenize the values of genetic diversity (Truong et al. 2007). This would be facilitated by the existence of intermediate populations along the altitude gradients (Giménez-Benavides et al. 2007). High levels of gene flow would be inconsistent with the levels of inbreeding (F_{IS}) found in this study (Tables 2 and 3). Autogamous and geitonogamous pollinations, low seed dispersal and, a positively skewed and leptokurtyic pollen dispersal shadow would favor that many of the matings take place at short distance, therefore, increasing consanguineous matings or kinship. Other factors, such as ecology or topography barriers which restrict gene flow in specific directions, like a valley

that difficult horizontal movements, could also help maintain the pattern of similar values of genetic diversity in the populations along the altitude gradient (i.e. Ohsawa et al. 2008).

Gene flow in altitude: Mountain islands vs. Horizontal belts

Gene flow along altitudinal gradients has been frequently detected in other alpine systems (e.g. Stöcklin et al. 2009), including plants from other Mediterranean mountains (e.g. Medrano & Herrera 2008). Alternatively, horizontal gene flow between populations located at the same altitude has been also been described as predominant in other mountain species (Byars et al. 2009; Schneller & Liebst 2007). Besides, long distance seed dispersal has been described in some alpine regions, covering hundreds of kilometers (Alsos et al. 2007).

Bayesian clustering and AMOVA analyses (Fig. 2, Tables 5 and 6) showed that the genetic structure of *S. cilata* is most likely the consequence of a mountain island scenario. Populations from the two altitudinal gradients clustered separately into the two main different genetic clusters detected in the Bayesian analysis with the highest ΔK values (Figs. 2B and 2C, Table 5) and F_{ST} values were higher when comparing populations from the same altitude than when comparing populations from the same altitudinal gradient (Table 4). These results suggest that the valley between both mountains, that reaches a low elevation of 1700m probably acts as a topographical barrier that severely hinders gene flow between mountains (Widmer et al. 2009). The lower number of migrants estimated for among altitudinal gradient pairwise comparisons compared to within altitudinal gradient comparisons also supports this idea (Table 4).

Gene flow between *S. ciliata* populations along the altitude gradient (and to a less extent, between populations at the same altitude) must be highly influenced by its life-history and reproductive traits and local ecological conditions in this rear edge distribution area. Gene flow between populations could be highly restricted by several factors, including seed dispersal and pollination. Generally, *Silene* species showed short average seed dispersal distance (Jongejans & Schippers 1999). *S. ciliata* seeds, without any specialized mechanism for long distance moving, have been estimated to disperse at distances of approximately one meter (Carlos Lara & Juan J. Robledo-Arnuncio, personal communication), which would not contribute to significant gene flow among populations.

Besides restricted seed dispersal, geographical location along the altitudinal range can condition the accessibility to larger pollinator guilds and to a more extended flowering phenology at the lower altitude populations compared to the higher altitude populations, thus contributing to differences in gene flow, migration rates and genetic diversity levels along the altitudinal gradient. Yet flowering asynchrony, which is typically associated to the elevation gradients (Körner 2003) is also frequent in rear-edge populations of *S. ciliata* (Giménez-Benavides et al. 2007b). The highly temporarily restricted flowering season of *S. ciliata*, which occurs late in the summer (Giménez-Benavides et al. 2010) can contribute to shorten the

pollen dispersal distances. However, in especially warm years flowering asynchrony is drastically reduced because there seems to be a photoperiodic limit to how much flowering start can be anticipated (Gimenez-Benavides et al. 2007b), allowing for larger levels of gene flow via pollen dispersal among populations along the altitudinal gradient. On the other hand, pollination of *S. ciliata* is carried out not only by diurnal pollinators like syrphid flies, wasps, bees and moths, but also by the nocturnal moth *H. consparcatoides* for which it seems to have developed a specific insect-plant association (Giménez-Benavides et al. 2007) can easily cover the geographical distances separating populations within an altitudinal gradient (Schulke & Waser 2001; Darvill et al. 2004; Pasquet et al. 2008), as in other species of *Silene* (e.g. Young 2002; Barluenga et al. 2011) and as shown in this study (Table 4).

The results found in our study contrast with the expected differences in genetic diversity and genetic structure pattern among populations concerning reproductive traits alone. In this study we found an overall high similarity of all genetic diversity indices among populations of *S. ciliata* (Table 2) irrespectively of their geographical location (Table 2), with no significant differences between both altitudinal gradients or among the three isolated belts (Table 3). An additional factor explaining these similarities may be historical population movements related with ice contraction and expansion during glaciations in the Quaternary (Clayton et al. 2008) which might have contributed significantly to genetically homogenize the populations. These periods of ice expansion or retreatment, which forced the migration of northern species to warmer refuges in the south of Europe, the *tabula-rasa hypothesis* (Hewitt 2000), could also have caused local altitudinal migrations in our mountain system (like those suggested in the southern Sierra Nevada Massif, Gutierrez Larena et al. 2002) , rather than *in situ* survival (nunatak hypothesis, Stehlik et al. 2002; Stehlik et al. 2001). Our strong similarity in genetic diversity indices among populations (Tables 2 and 3) is consistent with population migrations to southern latitudes during the glacial periods followed by subsequent recolonization during the interglacials. Although we did not find significant differences among populations at different altitudinal ranges, a slight decrease on allelic richness was observed from the lowermost populations to the higher most ones (Table 2), this result being consistent with a gradual upward migration with no significant reductions of population sizes. Population migration patterns in the southern European mountains are still not well studied and very little is known about them, including their genetic consequences (Hewitt 2001).

Local adaptation evidences in genetic structure

Local adaptation occurs as a response to highly-contrasted environmental conditions and low levels of gene flow have traditionally been considered as essential for such adaptive differentiation, especially in marginal habitats (Kawecki 2008). Nonetheless, evidences of local adaptation have also been found in other altitudinal gradients using microsatellite markers despite high levels of gene flow (e.g. Byars et al. 2009). On the other hand, increased degrees of isolation and habitat or population fragmentation may increase the frequency of deleterious mutations and levels of inbreeding in the populations (Kawaguchi et

al. 2004), thus reducing their overall fitness and compromising their options of survival and ultimately decreasing the potential for local adaptation.

Our study has revealed evidences of restricted gene flow between the two altitudinal gradients of *S. ciliata*, at least at recent past (Figs. 2C and 2D, Table 4). Previous studies , based in greater germination of seeds in reciprocal transplant and high resistance against drought stress, have provided evidences of local adaptation in the L1 population (Giménez-Benavides et al. 2007a, Garcia-Fernández, A. unpublished results). This population, though highly related to its altitudinal gradient conformed an independent genetic cluster in the Bayesian analysis (Fig. 2D, Table 5) and was also supported by AMOVA results (Table 6), this being congruent with the previously detected local adaptation in this population.

Concluding remarks

Low differences in genetic variability and similar values of inbreeding support the existence of important levels of gene flow between the populations studied. Our results suggest that seed and pollen movements are more plausible along the elevation gradient than between populations situated in the same altitude. If gene flow is difficult nowadays, may be recent movements along the mountain, associated with glaciations periods could explain these lack of genetic differentiation. Furthermore, the existing gene flow seems to be not large enough to dilute the local adaptation response previously found in these populations which also appears in the genetic structure.

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SUPPORTING INFORMATION

Additional supporting information may be found in this appendix:

Table S1 Estimated genetic parameters per locus and population.

Table S1. Per locus and population estimates of genetic diversity. *A*, number of alleles, Observed heterozigosity (H_O), expected heterozygosity (H_E), and inbreeding coefficient (F_{IS}) for the six populations of *Silene ciliata* analysed, calculated with the six EST-SSR markers transferred from *Silene latifolia* and four SSR specifically developed for *Silene ciliata*. Asterisks indicate significant departures from Hardy-Wenger equilibrium after Bonferroni correction; ns, not significant.

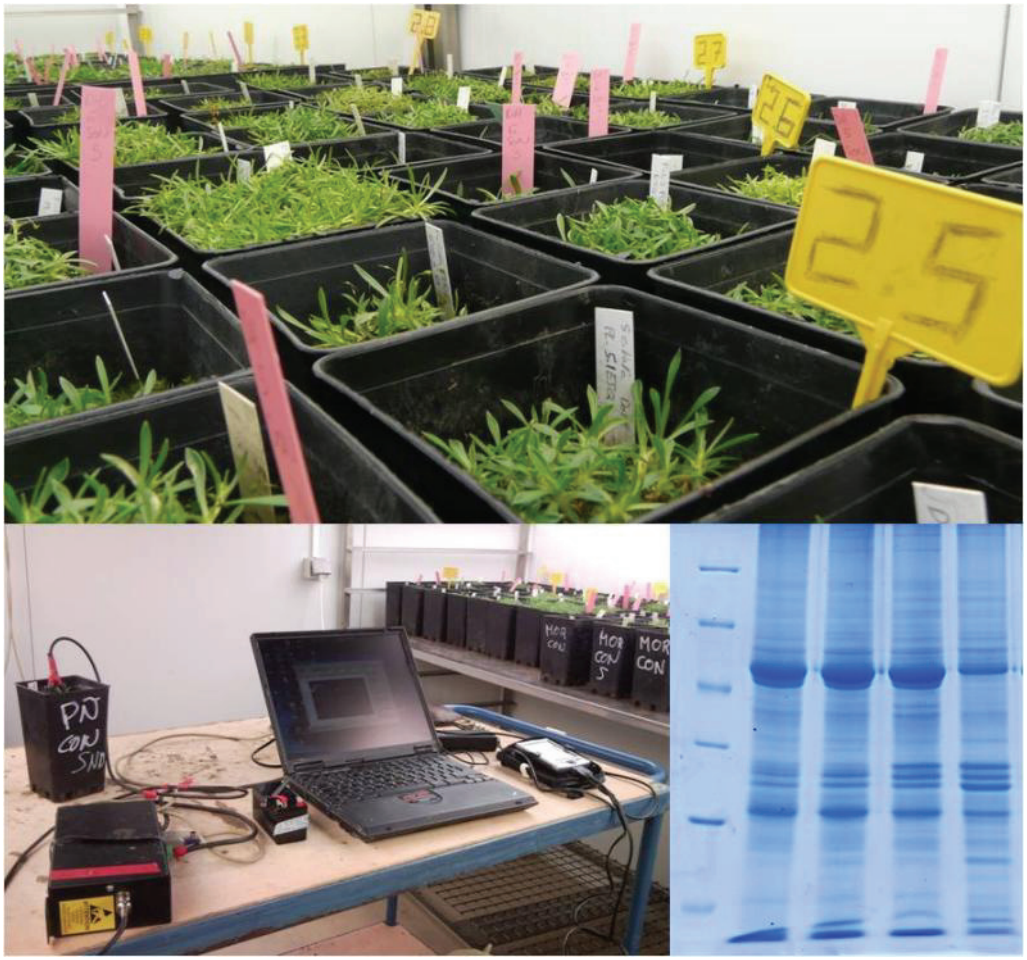
Population	Marker (Type – Name)	*A*	H_O	H_E	F_{IS}	Population	Marker (Type – Name)	*A*	H_O	H_E	F_{IS}
Low 1	EST – 2HTS	9	0.30	0.80	0.631*	Low 2	EST – 2HTS	12	0.23	0.73	0.683*
	EST – 8HTS	4	0.13	0.13	-0.031 ns		EST – 8HTS	12	0.33	0.77	0.579*
	EST – 37 HTS	8	0.40	0.60	0.326 ns		EST – 37 HTS	10	0.30	0.67	0.561*
	EST – X4-3	6	0.27	0.67	0.605*		EST – X4-3	4	0.03	0.40	0.921*
	EST – G34D06	7	0.97	0.80	-0.214 ns		EST – G34D06	8	0.73	0.73	0.022 ns
	EST – G47A02	2	0.03	0.03	0		EST – G47A02	3	0.07	0.07	-0.009
	SSR – 0106	9	0.63	0.77	0.207		SSR – 0106	8	0.80	0.73	-0.069 ns
	SSR – 1208	8	0.67	0.77	0.151*		SSR – 1208	7	0.70	0.70	0.017 ns
	SSR – 1224	15	0.27	0.80	0.672*		SSR – 1224	14	0.23	0.87	0.737*
	SSR - 1443	7	0.17	0.43	0.637*		SSR - 1443	3	0.07	0.07	-0.009 ns
Intermediate 1	EST – 2HTS	6	0.17	0.67	0.754*	Intermediate 2	EST – 2HTS	7	0.20	0.67	0.710*
	EST – 8HTS	6	0.20	0.27	0.279 ns		EST – 8HTS	12	0.30	0.90	0.668*
	EST – 37 HTS	5	0.40	0.67	0.399*		EST – 37 HTS	6	0.20	0.50	0.617*
	EST – X4-3	6	0.10	0.60	0.837*		EST – X4-3	5	0.20	0.27	0.272 ns
	EST – G34D06	18	0.73	0.90	0.191 ns		EST – G34D06	10	0.87	0.77	-0.093 ns
	EST – G47A02	5	0.13	0.20	0.303 ns		EST – G47A02	2	0.03	0.03	0
	SSR – 0106	7	0.63	0.73	0.142ns		SSR – 0106	10	0.77	0.73	-0.041
	SSR – 1208	9	0.80	0.80	0.023		SSR – 1208	7	0.37	0.60	0.417 ns
	SSR – 1224	13	0.37	0.90	0.594*		SSR – 1224	12	0.13	0.77	0.832*
	SSR - 1443	7	0.20	0.37	0.443*		SSR - 1443	6	0.13	0.23	0.469 ns

Table S1. (cont) – Per locus and population estimates of genetic diversity. *A*, number of alleles, Observed heterozigosity (H_O), expected heterozygosity (H_E), and inbreeding coefficient (F_{IS}) for the six populations of *Silene ciliata* analysed, calculated with the six EST-SSR markers transferred from *Silene latifolia* and four SSR specifically developed for *Silene ciliata*. Asterisks indicate significant departures from Hardy-Wenger equilibrium using Bonferroni correction.

Population	Marker (Type – Name)	*A*	H_O	H_E	*Fis*	Population	Marker (Type – Name)	*A*	H_O	H_E	F_{IS}
High 1	EST – 2HTS	6	0.03	0.70	0.954*	High 2	EST – 2HTS	10	0.53	0.70	0.266 ns
	EST – 8HTS	2	0.07	0.13	0.477 ns		EST – 8HTS	10	0.17	0.70	0.764*
	EST – 37 HTS	6	0.40	0.47	0.152 ns		EST – 37 HTS	5	0.27	0.63	0.591*
	EST – X4-3	11	0.23	0.77	0.699*		EST – X4-3	6	0.20	0.60	0.663*
	EST – G34D06	11	0.47	0.90	0.482*		EST – G34D06	7	0.60	0.67	0.123 ns
	EST – G47A02	2	0.03	0.03	0		EST – G47A02	2	0.03	0.03	0
	SSR – 0106	7	0.73	0.77	0.039 ns		SSR – 0106	6	0.73	0.80	0.105 ns
	SSR – 1208	10	0.70	0.77	0.089 ns		SSR – 1208	8	0.63	0.67	0.057 ns
	SSR – 1224	11	0.20	0.83	0.764*		SSR – 1224	12	0.20	0.83	0.771*
	SSR - 1443	5	0.17	0.23	0.331 ns		SSR - 1443	6	0.33	0.33	-0.021 ns

Chapter 4

Water stress responses in a high mountain plant species along an altitudinal gradient: could they back up local adaptation?



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Manuscrito inédito

Abstract

Climate change is imposing more thermic and arid conditions on high mountain Mediterranean pasture plants that occur above the treeline. Severity of oncoming conditions is known to be more intense in lower elevation populations and, in concordance, it has been postulated that persistence at these new conditions may be critical for many species. A question that needs to be posed on the table is: Does local adaptation play a significant role in the response of these populations to climate change? And if so, what are the involved mechanisms involved? Recent works suggested the existence of local adaptation responses in the rear edge populations of *Silene ciliata* in the early stages of development (seed and seedling). In order to know if these adaptations are due to water stress and also involve adult individuals, an experiment were conducted, in which *Silene ciliata* plants (from populations located at different elevations), were subjected to a complete lack of water availability. Results showed that plants from the Low population had in the earliest stages of water shortage, greater tolerance to water stress than plants from the High population, whereas the Intermediate population showed a midway response. Protein expression was evaluated before the drought experiment and after one month of total lack of water. All plants from all populations showed a similar qualitative response. Differential response between the beginning and the end of the drought experiment was related to the large subunit of 1.5 Ribulose-1,5-biphosphate carboxilase oxygenase. The presence of LEA proteins known to play a role in tolerance to water stress were expressed along the drought treatment, although some differences were detected in secondary band. In conclusion, this study detected evidence of local adaptation to water stress in populations distributed along an elevation gradient. The comparative study of protein expression pattern suggests that this adaptation is probably more related to quantitative differences in protein expression than to qualitative changes involving the synthesis of different proteins

Introduction

Under current anthropogenic climate warming, altitudinal and latitudinal shifts appear as the general response in most regions for many species (Klanderud & Birks 2003; Parmesan 2006), when they attempt to respond against the fast changes laying ahead (Loarie et al. 2009). However, for species with little habitat available (Körner 2007) and limited dispersion and moving capabilities like most plants (Thuiller et al. 2005), plastic responses and/or local adaptation may constitute the main responses for their survival in these changing scenarios (Jump & Peñuelas 2005).

There are many stress factors that might be behind these local adaptations (Gomez-Mestre & Tejedo 2003) or plastic responses (Agrawal 2001). Some of these factors can play opposite roles in different environments, increasing their negative influence in certain habitats whereas resulting essential in for others (i.e. Cheptou & Donohue 2011). This implies that all these drivers are involved in an unpredictable way in each evolutive scenario. In this context, water stress appears as one of the most important factors that would determine the responses to global warming and is highly correlated with many other stress factors, like increase of temperature or CO_2 concentration (McDowell et al. 2008). Water availability, especially for organisms with restricted moving capabilities like plants, is a limiting factor that combined with many others, determines their current distribution and could be highly related with their response to climate warming (Thuiller et al. 2005). To know how water stress affect plant performance can be approached following different alternatives (Bartels & Sunkar 2005) since, it seem to affect highly complex metabolic pathways, specific proteins, multi-gene expressions involved and several tissues and organ functions are some of them (De Boeck et al. 2010). Consequently, the understanding of the complete circuits of water stress reactions is really difficult (Bartels & Sunkar 2005). Shifts of proteins against drought stress, in terms of quality and quantity, are good examples of these complex reactions because their presence and abundance is not only activate metabolic pathways to avoid water loss, but also are involved with other process such as cell protection and repair (Zhu 2002).

The response against stress factors is variable, not only between species, but also between individuals of the same species or even, between individuals of the same population (Li et al. 1999). This variability can be associated not only to intrinsic species characteristics or micro-habitat effects, but also to the genetic variability of the population (Huang et al. 2002). This genetic variability is a reservoir which can constitute a ground for evolution to work. Thus under oncoming warming conditions different selection forces could act and select certain genotypes that are better adapted to the new environmental conditions (Hughes et al. 2008). These makes the selection forces as leading agents to select the most adapted genotypes and explain the possible differences in the genetic variability (Ennos 2001). Genetic variability also modulates the responses associated to climate change, because global warming summarizes many types of stress, including precipitation reduction and water stress (IPCC 2007). Genetic variability is highly related to local adaptation, because it allows genes and traits where selection forces can act and select

the most adapted genotypes (Kawecki 2008). But also, genetic variance is crucial in phenotypic plasticity responses (i.e. Balaguer et al. 2001; Grassein et al. 2010), determining the ability of the species to respond with different phenotypes to changing selection regimes (Weber & Declerck 1997). Therefore, genetic variability seems essential for both responses to climate warming and response against stress.

Mountains are suitable natural laboratories for testing hypothesis related to evolution and adaptation (Körner 2007), but also with stress factors, because altitude gradients allow high modifications of the environmental conditions in short geographical distances in a predictable fashion (Walther et al. 2005), which puts the populations under different selective pressures (Gonzalo-Turpin & Hazard 2009). The responses against this gradient variability can be different in each altitude point (Byars et al. 2009; Giménez-Benavides et al. 2007b; Giménez-Benavides et al. 2007a) which allows testing genetic or evolutionary questions by using populations located along altitude. These conditions make mountains also excellent scenarios for evaluating the responses of organisms to climate warming.

With all these considerations, we initiated a study on *Silene ciliata*, a high mountain specialist for which evidences of local adaptation across an altitudinal gradient have been found in different earlier life stages like seed germination and seedling growing and also in demographic dynamics (Giménez-Benavides et al. 2007a; Giménez-Benavides et al. 2007b; Giménez-Benavides et al. 2008). However, we do not know if these responses could be scale up to adult stages and even more, whether or not this adaptive response involved drought stress amelioration mechanism. With this in mind, we produced clonal individuals (see Hughes et al. 2008), in order to manipulating and controlling some genotypes from different populations situated along the elevation gradient. Our working hypothesis is that physiological responses to an extreme drought period are different along altitude being the population located at the lowest and most stressful limit better suited to encompass such climatic perturbation. We postulated that differences may involve different responses along some metabolic pathways, mediated with specific proteins. We combined a monitoring of the plant fitness along a drought stress treatment with total protein scans and also, searching for specific proteins that could be related with drought stress. Specifically, we aimed to answer the following questions a) Are the adults of certain populations more able to survive a period of long drought stress than those of other populations? Is the genetic variability related with this response? B) Are there differences in protein expression before and after the drought treatment? If so, are there differences between populations? C) Are there any differences in specific proteins related to stress? Is this response variable between individuals or populations?

Material and methods

Silene ciliata, population sampling and propagation of cuttings.

Silene ciliata Poiret (Caryophyllaceae) is a perennial plant which grows in cushion rosettes of up to 2 cm in height and 15 cm in diameter. It is a self-compatible species, but passive autogamy is partially restricted due to a pronounced protandry. *S.ciliata* inhabits the main mountain ranges in the northern half of the Iberian Peninsula, the Massif Central in France, the Apennines and the Balkan Peninsula (Tutin et al. 1995). On the Iberian Peninsula, *S. ciliata* reaches its southern latitudinal limit in the Sierra de Guadarrama. These populations are considered as relictual, because of their isolation from the remaining northern populations in other mountain systems.

Three populations were selected along the largest local elevation gradient in Sierra de Guadarrama. The lowest population (1980 m, hereafter "Low") was situated in the lateral moraine deposit of the glacial cirque of Laguna de Peñalara. The intermediate population (2250 m, hereafter "Intermediate") was located on the Dos Hermanas summit, approximately 3 km from the Low population. The highest population (2420 m, "High") was situated on the summit of Peñalara peak, the highest peak of Sierra de Guadarrama, approximately 3 km from the Intermediate population. All three populations are located in the Peñalara Natural Park and had a relatively high number of individuals (more than 200). Vegetation composition in each population is detailed in Escudero et al. (2005) or Gutierrez-Girón & Gavilán (2010).

Differences between these three populations are not only associated to plant community composition. Gimenez-Benavides et al. (2007a) used the same gradient in a reciprocal sowing experiment searching for local adaptation in the populations. In this study, edaphic properties were measured from three soil samples (top 10 cm of bare soil) which were randomly collected in each population. Differences were found in organic matter, nitrogen, acidity and phosphorus content as well as soil texture. Soil water content was also monitored during summer drought (the most important constraint for plant survival in Mediterranean regions, Larcher 2000), showing important differences between sites (Giménez-Benavides et al. 2007a).

In each population, 10 random healthy adults (more than 10 cm of size, with green leaves and no signs of fungus or parasite infection) were selected in spring 2008 and uprooted. Each plant was kept in moist paper and transported to the greenhouse of the CULTIVE infrastructure (Rey Juan Carlos University, Móstoles, Spain). On the same day, each individual was cut in at least 15 small cuttings and each cutting was planted in plastic pots with a commercial substrate enriched in NPK and watered every 48 hours. Cuttings were left to grow for one year and transplanted to larger plots when necessary (plastic pots 10 cm wide and 18cm high).

Growth chamber conditions and plant performance.

After one year of growth, 10 cuttings from each original adult were randomly selected (100 per population, 300 in total) and measured for maximum width as an estimator for plant size. Cuttings were randomly placed in a Phytotron chamber (INKOA Sistemas, Spain) and grown with cycles of 16 hours light/8 hours darkness, at 20-10 ºC of temperature and constant 50% humidity. Light was provided by 12 Phillips lamps 58 W "TL"D standard types, wavelength 400-650 nm, and average intensity 1.35 cd/cm^2. No water was provided to the plants until the end of the experiment. The experiment started on 8 April 2009 and was continued for 40 days until all plants showed unequivocal signals of drought induced death, as suggested by the massive presence of yellow leaves, turgence loss, etc. To ensure that all changes were associated to water stress, five additional individuals from each population were included in the chamber as controls and irrigated every two days with 40 ml of water.

A time-domain reflectometry (TDR, Topp & Davis 1985) was carried out using a Campbell TDR100 system (Campell Scietific Ltd, Loughborough, UK) to measure soil moisture in the pots and to accurately know when no water was available for the plants. In three pots of each original adult (30 pots per population, 90 pots in total) 15 cm long TDR probes were vertically installed. This soil depth was chosen because pots were 18 cm high and we wanted to avoid the last centimetres of soil. Soil moisture was measured every day. TDR probes were also installed in 10 control plants which were regularly irrigated, to verify that control cuttings did not experience water stress.

Non-invasive methods to estimate fitness performance were used to conserve the integrity of the plants and avoid possible plant damage. We analyzed the potential photochemical efficiency of *Silene ciliata* cuttings measuring the maximum quantum yield of PS II (Fv/Fm) of dark adapted leaves (during the 8 hours of darkness) with a pulse modulated flurometer (FMS2, Hansatech Instrument, Norfolk, UK). This parameter has long been utilized for non-invasive surveys of stress perturbations to photosynthesis for several decades (Conroy et al. 1986; Maestre et al. 2003) and has been found to match results with other performance measurements (i.e. Aragón et al. 2008; Aragón et al. 2009). The average of Fv/Fm response was calculated every two days using two measurements taken for each plant.

Protein extraction, screening and transference of antibodies related with drought stress.

We collected some leaves of each individual before the start of the drought experiment **(**hereafter **"before")** and also for each individual that survived after the 40 days of water stress (hereafter **"after"**), to make a survey of the total protein expression before and after the drought stress. Approximately 15 miligrams of leaf tissue from each individual (before and after the treatment) were used for protein extraction. Tissues were ground and mixed with 250 µl of a sample buffer (0.2 M of DTT, 4% SDS, 20% glycerin and 0.8 % Tris 120 mM, 0.01% Bromophenol blue, pH 6.8 and water). The mix was incubated for five minutes at 95ºC, mixed and centrifuged for five minutes at maximum speed (10000 xg). Pellets were discarded and the protein concentration of the supernatant was determined using a Bradford assay (Bio-Rad, Munich, Germany). All protein extracts had a concentration of approximately 1µg/µl. Total proteins

were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SPS-PAGE one dimension gel) 10 µl of each sample was loaded on a 12 % polyacrylamide gel. The gel was stained with Coomasie blue after electrophoresis.

We were also interested in searching for differences between the genotypes or the original population in the expression of specific proteins which are associated with plant responses to water stress. Two abundantly expressed phosphoproteins were chosen to search for these differences: *CDeT11-24*, dehydration- and abscisic acid (ABA) responsive protein (Velasco et al. 1998) and *CDeT6-19* (Schneider et al. 1993). Both proteins are encoded by a small multigene family and accumulate abundantly in response to dehydration. Röhrig et al. (2006) described the presence of these proteins in the resurrection plant *Craterostigma plantagineum* and their involvement in desiccation tolerance.

10 µl protein extracts (described above) were analysed for at least three different genotypes for each population, before and after the drought treatment. Proteins were separated in SDS-PAGE and transferred to a nitrocellulose membrane (Towbin et al. 1979) following a Western-Blot protocol. Proteins were transferred at 80 V. for two hours in a Mini-Trans Blot System (Bio-Rad). After transfer the membranes were rinsed three times with TBST buffer and blocked in a 4% (w/v) bovine serum albumin (BSA) in TBST buffer overnight at four degrees. The membrane was washed three times with TBST and incubated again for 1 hour in a 4% (w/v) BSA dilution in TBST with specific antibodies for each protein. Antibodies were used in a dilution 1:1000. The membrane was rinsed again three times with TBST buffer to remove excess BSA and subsequently incubated with anti-immunoglobulin secondary antibodies, in a 1:500 dilution with 4% (w/v) BSA in TBST for two hours. Finally, the membrane was rinsed with TBST three times and the presence of the antibody protein complex was detected using Amersham ECL Kit, Western Blot detection reagent (RPN 2106), following instructions of the company. A Lumi-Image Reader Fujifilm LAS-1000 was used to detect the presence of luminescence indicating the presence of the protein.

Protein Identification by MALDI-TOF MS or MS/MS

The protein bands of interest were manually excised from SDS-PAGE gel, placed in an Eppendorf tube and washed twice with double distilled water. Proteins for analysis were in-gel reduced, alkylated, and digested with bovine trypsin (12.5 ng/µl, sequencing grade; Roche Applied Science) according to the procedure published by Sechi & Chait (1998). After digestion, the supernatant was collected, and 1 µl was spotted on a MALDI target plate and allowed to air dry for 10 min at room temperature. Subsequently 0.4 µl of matrix (3 mg/ml of α-cyano-4-hydroxy-*trans*-cinnamic acid (Sigma) diluted in 0.1% TFA-ACN/H2O (1:1, v/v)) were added to the dried peptide digest spots and allowed to air dry foranother 5 min at room temperature. The samples were analyzed with a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA). This MALDI-TOF/TOF instrument consists of a MALDI source with a 200-Hz neodynium YAG (yttrium aluminium garnet) laser operating at 355 nm and operated inpositive ion reflector mode with an accelerating voltage of 20,000 V. All MS spectra were

internally calibrated using peptides from the autodigestion of trypsin. The analysis by MALDI-TOF mass spectrometry produced peptide mass fingerprints, and the peptides observed can be collated and represented as a list of monoisotopic molecular weights. For MS analyses, a monoisotopic peak is selected, and all known contaminant ions were excluded during the process. The parameters used to analyze the data were: a signal to noise threshold of 20 and a resolution higher than 10,000 with a mass accuracy of 20 ppm. Proteins ambiguously identified by peptide mass fingerprints were subjected to MS/MS sequencing analyses using the 4700 Proteomics Analyzer (Applied Biosystems). Hence from the MS spectra, suitable precursors were selected for MS/MS analyses with CID (atmospheric gas was used) in 1-kV ion reflector mode and precursor mass windows of ±10 Da. The plate model and default calibration were optimized for the MS/MS spectra processing. The parameters used to analyze the data were: a signal to noise threshold of 10 and a resolution higher than 6000. For the protein identification, both MS and MS/MS spectra were automatically searched using a Local license of Mascot 1.9 from Matrix Science through the Protein Global Server (GPS) from Applied Biosystems. The search parameters for peptide mass fingerprints and tandem MS spectra obtained were set as follows: two sequence databases were used, Swiss-Prot/TrEMBL non-redundant protein database (www.expasy.ch/sprot) and an specific database created only with *Silene* data present in NCBi dataset (http://www.ncbi.nlm.nih.gov/guide/). Fixed and variable modifications were considered (Cys as *S*-carbamidomethyl derivative and Met as oxidized methionine), allowing for one missed cleavage site; precursor tolerance was 50–100 ppm and MS/MS fragment tolerance was 0.3 Da; a restriction was placed on isoelectric point (pI 3–10); and a protein mass range from 10 to 100 kDa was accepted. In all protein identifications, peptide mass fingerprint,or MS/MS, the probability scores were greater than the score fixed as significant with a p value < 0.05. Analyses were carried out in the Proteomics Facility UCM-PCM (Madrid, Spain), a member of ProteoRed network.

Statistical analyses

Fv/Fm signal was model with General Linear Mixed Models (GLMM, Breslow & Clayton 1993). Population origin (Low, Intermediate and High) was included in the analysis as fixed factors, while genotype (adult origin of the cuttings) nested in population was included as random factor. Fv/Fm was included in the analyses as a normal variable, using Identity as link function.

Two additional GLMM analyses were developed, considering the same model but using as target variable, the stress condition computed as binomial variable (1, “stressed” for Fv/Fm values < 0.6 and 0.2 for each binomial model; 0, “not stressed” for Fv/Fm value > 0.6 or 0.2). The logit link-function was used in the GLMM analyses. The 0.6 value was considered as a threshold for drought damage, while the 0.2 value was chosen as a value that reflected drought mortality effects in the plant (threshold for recover, Woo et al. 2008).

Individual size (measured in mm) was included in all GLMM as fixed factor. Plant size has been described as fundamental factor for survive in extreme conditions (Horvitz & Schemske 1995; Giménez-Benavides et al. 2008), but also could be related with the efficiency in the use of the available resources in the Mediterranean environments (Lloret et al. 1999).

Shape of drought damage and mortality curves were also analysed, using the binomial data set estimated before for Fv/Fm binomial analyses (censored data related with the physiological status and considering two thresholds in each case, 0.6 and 0.2, drought damage and drought mortality respectively) using a right accelerated failure-time model, i.e. a linear regression model in which the response variable is the logarithm or a known monotone transformation of a failure time (Fox 2001). This approach allows the use of censored data to estimate parametric regression models using a maximum-likelihood approach. The best failure-time distributions were chosen for the data sets based on the comparison of possible distributions with the likelihood ratio test (Fox 2001). Population origin and plant size were included in both analyses. A log-logistic distribution was therefore used for the binomial rate data. Analyses were performed with SAS 9.0, following the LIFEREG procedure (SAS Institute 1996). Analyses were performed with SAS 9.0, using the GLIMMIX module (SAS module).

Results

Fv/Fm signal, GLMM and survival analyses

Plants were moved to the chamber on 8 April 2010, but water was detected (with TDR signal) in the pots until 16 April 2010. The experiment concluded one month later, on 17 May 2010, when only 6 individuals were still alive (Fv/Fm > 0.6). Average value of Fv/Fm under non-stress conditions for all the populations was 0.86. Figure 1 shows the evolution of the average signal for each population.

Five dates were selected to cover the whole evolution of plant response in the experiment. 19 April 2010 was the first date as it corresponds to the day when the TDR was unable to detect water in the pots. The other four dates were chosen each seven days (26 April and 3, 10 and 17 May). The analyses of the Fv/Fm signal of the first dates (19 April) did not find significant effects of any of the variables considered. In the next date, 26 April, the Low population had a higher Fv/Fm signal than the High population, whereas in the last analysis (17 May 2010), a significant effect of population was also detected. Genotypes were significant in the last three census (3, 10 and 17 May).

In the binomial GLMM developed for drought signals (Fv/Fm value < 0.6, Table 2), genotype were significant in four analyses (26 April, 3 May, 10 May and 17 May) meanwhile plant size and population were significant in the last analysis (17 May). In this census, individuals from Low population had significantly higher tolerance to drought than the High population, while large plant sizes also had positive significant results. In the GLMM developed for drought mortality (Fv/Fm value < 0.2, Table 3), genotype was significant in the 3, 10 and 17 May surveys and plant size was also significant (with a positive effect) in the last date.

The shape of the curves of the right accelerated failure-time model (Table 4) was influenced by the population of origin and the size of the cutting. In the drought signal curve (Fv/Fm < 0.6), plant size had a significant negative effect (the greater plant size had greater rates of drought signals). The Low population had significantly higher resistance to the drought signal than the High population. In the drought mortality analyses (Fv/Fm < 0.2), the size had a significant but opposite effect, i.e. large plants, had greater mortality rates. The Low population also showed lower drought mortality rates than the High population.

Protein screening, identification and CDeT11-24 & CDeT6-19 responses.

Total protein extracts of all populations are shown in Fig. 2. Individuals within a population showed the same protein patterns when individuals were compared before and after the drought treatment. Differences between individuals before and after the drought treatment appeared at 45 kDa (band A, Fig.3), with bands before the drought treatment that nearly disappeared thereafter. It was also noted that bands appeared at 35 kDa (band B, Fig.3) and 18 kDa (band C, Fig.3) after the drought treatment. Protein identification of band B (35 kDa, Fig. 3) showed significant matching with the large subunit of the ribulose-

1,5-bisphosphate carboxylase / oxygenase large subunit of some *Silene* species, like *S. dioica* (17 matches $p < 0,05$) or *S. schafta* (17 matches, $p < 0.05$). Identification of band C (18 kDA, Fig. 3) also showed significant similarities with ribulose-1,5-bisphosphate carboxylase / oxygenase large subunit but of different *Silene* species: *S. armeria* (7 matches, $p < 0.05$) or *S. vulgaris* (6 matches, $p < 0.05$).

S. ciliata showed a positive reaction against antibodies of *CDeT6-19* and *CDeT11-24* proteins (Fig 4 A and B, respectively). However, signals were quite similar before and after the water stress. The only difference appeared in the *CDeT6-19* analysis, where a secondary band was found in the individuals after the drought treatment that was not visible before the stress. (Band A in Figure 4A).

Table 1. GLMM results for the effects of population (Pop L, Low population, Pop I, Intermediate population, Pop H, High population), cutting size and Genotype. Fv/Fm was interpreted as normal distribution using identity as link function. GLMM developed the 19th of April was not included because results were not significant.

Effect	Solution for effects					Deviance change	
	Coefficient	SD	df	t	P	F	P
GLMM 26th of April							
Population						3.63	0.03
Pop L	1.83	0.69	196	2.63	<0.01		
Pop I	0.38	0.63	196	0.6	0.54		
Pop H	-	-	-	-	-		
Size						1.63	0.2
GLMM 3rd of May							
Population						2.12	0.14
Size						3.28	0.07
GLMM 10th of May							
Population						1.42	0.26
Size						0.01	0.92
GLMM 17th of May							
Population						0.94	0.4
Size						0	0.99

* Genotype was included in each analyses as random factor (26th April: $Z = 0$, p – value = 1; 3rd May $Z = 3.04$, p – value = < 0.01, 10th of May $Z = 3.27$ p – value = < 0.01 and 17th of May $Z = 3.21$, p – value = < 0.01).

Table 2. GLMM results for drought signals of *Silene ciliata.* Population (Pop L: Low population, Pop I: Intermediate population and Pop H: High population) and cutting size were considered fixed factors. Seed drought was considered as binomial variable (0 for Fv/Fm signal bigger than 0.6 and 1 signal under 0.6) using logit as link function. GLMM developed the 19th of April was not included because results were no significant.

Effect	Solution for effects					Deviance change	
	Coefficient	SD	df	t	P	F	P
GLMM 26th of April							
Population						0.37	0.69
Size						1.33	0.25
GLMM 3rd of May							
Population						0.97	0.39
Size						0.34	0.56
GLMM 10th of May							
Population						1.49	0.25
Size						0.61	0.44
GLMM 17th of May							
Population						3.29	0.04
Pop L	2.89	1.27	34.7	2.28	0.03		
Pop I	2.17	1.23	32.2	1.76	0.09		
Pop H	-	-	-	-	-		
Size	0.32	0.09	274	3.53	<0.01	12.49	< 0.01

* Genotype was included in each analyses as random factor (26th April: $Z = 0$, p – value = 1,3rd May $Z = 2.8$, p – value < 0.01; Estimator ± Error = 4.98 ± 1.85; 10th of May $Z = 2.66$ p – value < 0.01 Estimator ± Error = 2.77 ± 1.42; and 17th of May $Z = 2.28$, p – value = 0.01; Estimator ± Error = 2.46 ± 1.08).

Table 3. GLMM results for drought mortaltity of *Silene ciliata* cutttings. Population (Pop L: Low population, Pop I: Intermediate population and Pop H: High population) and cutting size were considered fixed factors. Seed drought was considered as binomial variable (0 for Fv/Fm signal bigger than 0.2 and 1 signal under 0.2) using logit as link function. GLMM developed the 19th and the 26th of April was not included because results were no significant.

Effect	Solution for effects					Deviance change	
	Coefficient	SD	df	t	P	F	P
GLMM 3rd of May							
Population						1.3	0.29
Size						2.22	0.14
GLMM 10th of May							
Population						1.78	0.19
Size						0.39	0.53
GLMM 17th of May							
Population						1.56	0.23
Size	0.24	0.07	269	3.36	<0.01	11.26	< 0.01

* Genotype was included in each analyses as random factor (3rd May $Z = 2.7$, p – value < 0,01, Estimator ± Error = 4.53 ± 1.67; 10th of May $Z = 2.66$ p – value < 0.01, Estimator ± Error = 2.08 ± 0.79; and 17th of May $Z = 2.43$, p – value < 0.01, Estimator ± Error = 3.03 ± 1.25).

Figure 1. Evolution of Fv/Fm signal along drought treatment. No water was detected in the pots until the 16 of April. Circle-continuous line showed the evolution for Low population cuttings, square-pointed line for Intermediate population cuttings and triangle-discontinuous line for High population cuttings.

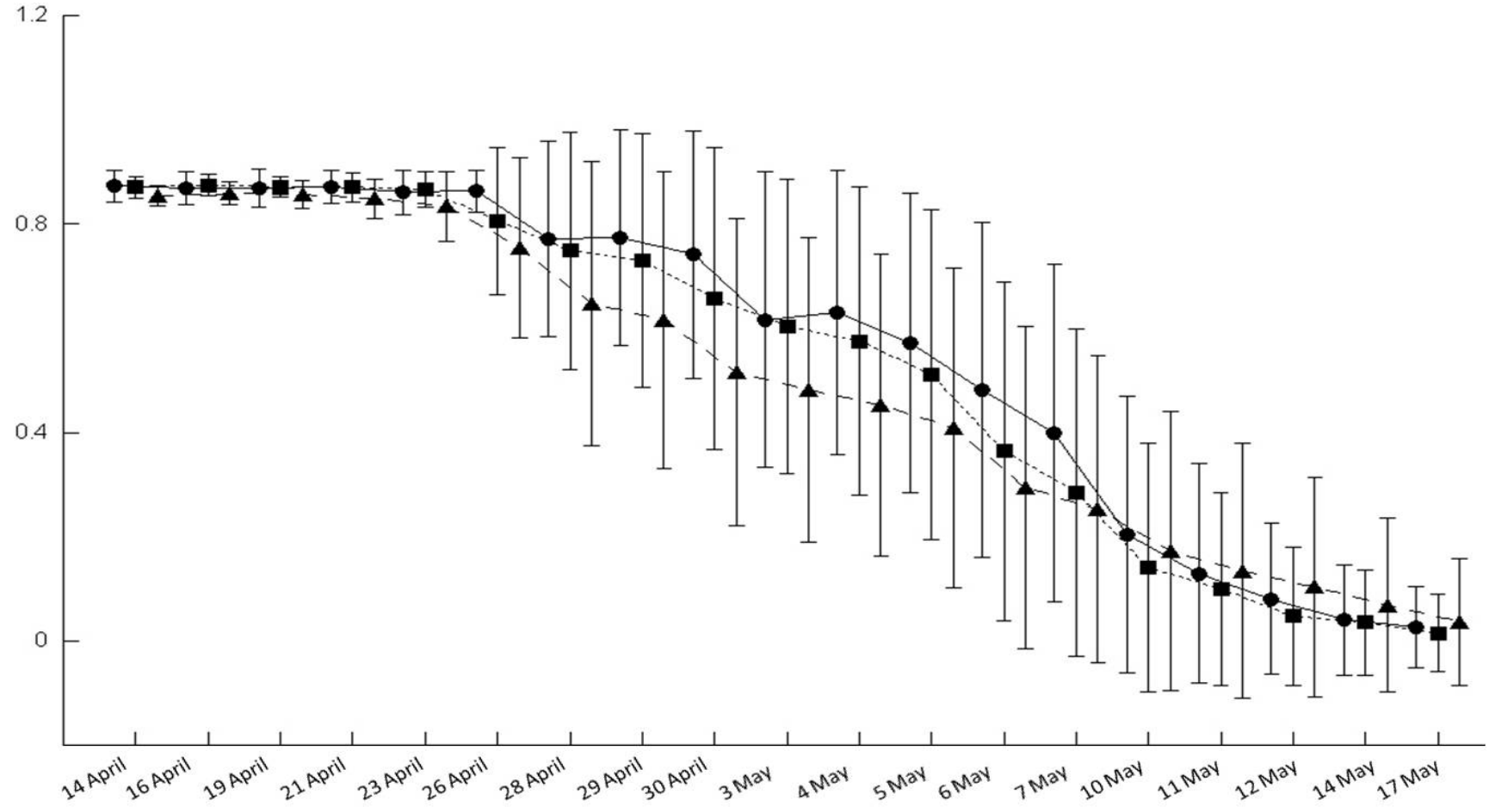


Figure 2. Evolution of the average number of survival individuals, grouped by genotype (adult origin). No water was detected in the pots until the 16 of April. Circle-continuous line showed the evolution for Low population cuttings, square-pointed line for Intermediate population cuttings and triangle-discontinuous line for High population cuttings.

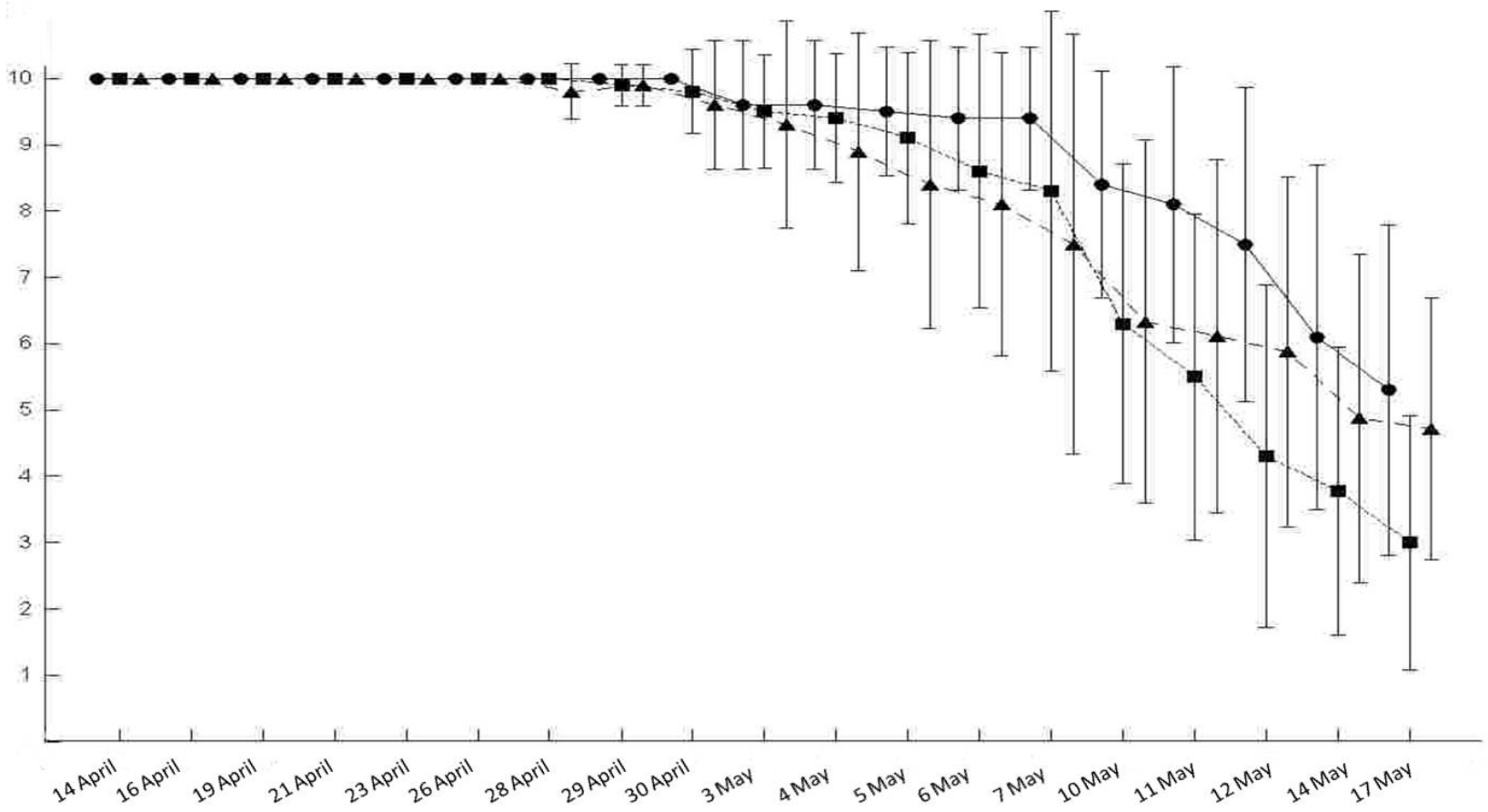


Table 4. Accelerate survival model (Weibull distribution) for Fv/Fm signal, considering a value under 0.6 as drought signals and under 0,2 as drought mortality as a function of population (Pop L, Low population, Pop I, Intermediate population and Pop H, High population) and cutting size.

Survival Model drought signals				
Variable	d.f.	χ^2	*p*	*Estimate*
Intercept		315.94	< 0.01	1.04 ± 0.06
Population	2	17.96	<0.01	-
Pop L	1	7.08	< 0.01	-0.08 ± 0.03
Pop I	1	2.42	0.12	0.04 ± 0.03
Pop H	0	-	-	-
Size	1	107.66	< 0.01	-0.07 ± 0.01
Scale	1			0.21 ± 0.01
Survival Model drought mortality				
Variable	d.f.	χ^2	*p*	*Estimate*
Intercept		0	0.98	-0.01 ± 0.12
Population	2	58.88	<0.01	-
Pop L	1	48.91	< 0.01	-0.46 ± 0.06
Pop I	1	0.37	0.54	-0.04 ± 0.06
Pop H	0	-	-	-
Size	1	99.41	< 0.01	0.14 ± 0.01
Scale	1			0.45 ± 0.02

Figure 3. SDS page gel with protein screening in three individuals of Intermediate population, before and after drought treatment. The left column showed the weight marker in KiloDaltons (kDa). Second and third columns were used for control individuals (before and after drought treatment) which were watered during the experiment. Arrows indicate the different bands found in the gel between before and after treatment.

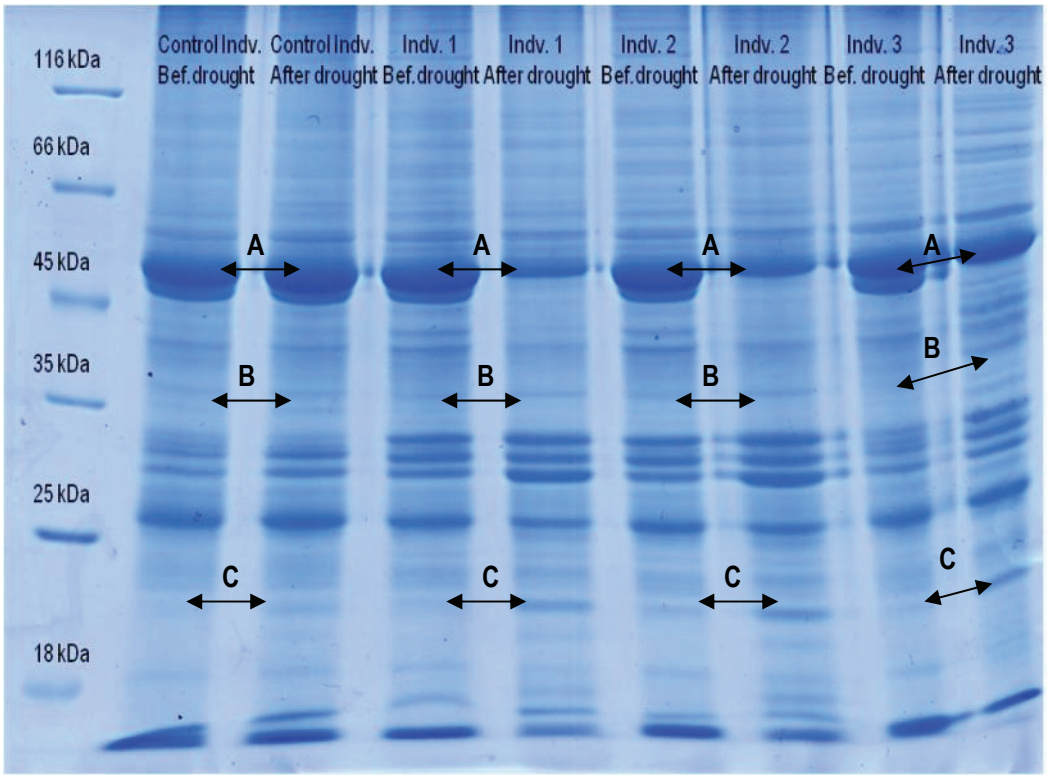
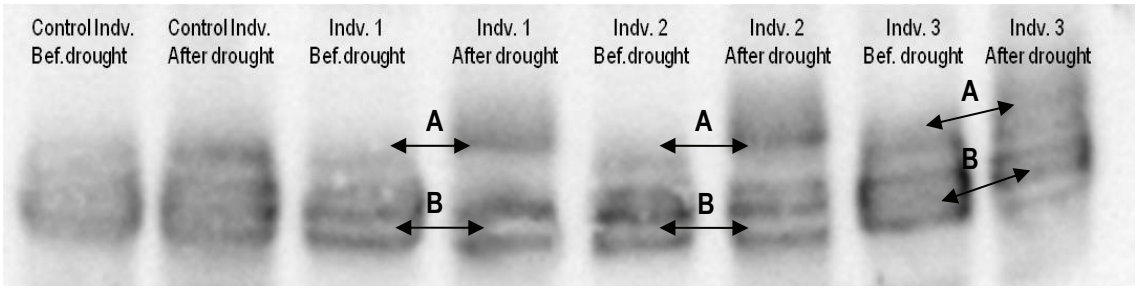
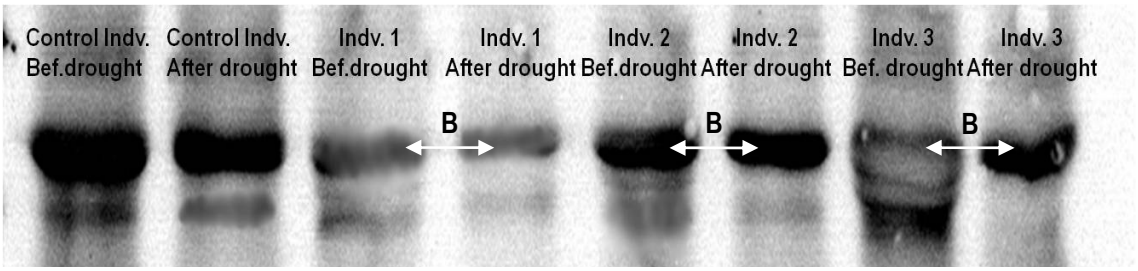


Figure 4. Response of three individuals from the Low population to 6-19 (Fig.A) and 11-24 (Fig.B) antibodies, before and after drought treatment. First and second column showed the response of Control individuals (before and after drought treatment) which were watered during the experiment. Arrow A indicates the different bands found in the membrane between before and after treatment in the same individual meanwhile arrows B showed the expected response of the antibodies.

A



B



Discussion

Summer drought stress in Mediterranean regions involves high temperatures and water scarcity, which are considered limiting factors for plant survival, growth or reproduction (Larcher 2000), and this also includes Mediterranean mountains (Castro et al. 2005). Local adaptation may help plant populations to survive under current scenario of climate warming and limited resources (Parmesan 2006). In past studies, local adaptation has been measured in early stages of plant development (germination and seedling survival, (Giménez-Benavides et al. 2007a) and in important processes for plant fitness like flowering (Giménez-Benavides et al. 2007b) or demography (Giménez-Benavides et al. 2010). Our results also showed evidences of local adaptation in adult individuals of *Silene ciliata* surviving to longer periods of water restrictions in those populations where the water balance is less favorable. Present results suggest that the Low population has greater tolerance to drought stress than the High population when drought levels are not severe. Results also revealed the importance of plant size against drought stress, and a strong genotype effect, which was found in most of the analyses, indicating that the response to drought varied greatly between individuals.

No differences were found in the protein patterns shown by the different individuals in each population, nor between populations. Nevertheless, differential bands were identified in protein patterns before and after the drought period. The degradation of ribulose-1,5-bisphosphate carboxylase oxygenase large subunit might be involved in this response to the drought treatment. Similarly, the expression of *CDeT6-19* and *CDeT11-24* proteins related to water stress was certified before and after the drought treatment, with some slight differences in the expression of a secondary band.

Population and size effects.

After one month of absence of water, approximately one third of the cuttings (107) showed some signal of fluorescence (Fv/Fm). This indicates that plants still has their photosystems available, but only 13 of them (4.3%) had a Fv/Fm signal higher than 0,2 (threshold for viability, Wooetal2008PlantMet). All analyses suggested a higher resistance of the Low population against drought stress, especially at the beginning of the study. After 10 days of water limitation, first significant differences were found between populations in GLMM Fv/Fm signal (Table 1) but also in the survival accelerated model for drought signals and drought mortality (Table 4). This resistance against drought stress was not detected in the next analyses. Higher evidences of local adaptation in the Low population were also found in field conditions at the first steps of plant development (Giménez-Benavides et al. 2007a) but these are the first results obtained for adults, directly related with water stress. Evidences of local adaptation in adults are scarce in populations located along an elevation gradient (Angert & Schemske 2005). Reciprocal transplants with adults individuals are not always viable (Raabová et al. 2007) and sometimes, ethically or logistically impossible. Common garden approaches could be useful in these situations and interesting results have been obtained

reciently, related with responses to altitude and climate warming (Kawecki & Ebert 2004; Montesinos-Navarro et al. 2010; Byars et al. 2007).

Differences between High and Intermediate populations were less significant than between the High and the Low population. No differences were found in accelerated survival model and differences in GLMM for Fv/Fm signal were partially significant in the last survey (p = 0.02 Table 1). This suggests that the drought responses of the three populations follow a pattern that reflects the increase of aridity as we move down along the altitudinal gradient.

Genotype effect were found in all GLMM carried put, allowing differential responses between individuals in the populations. These differences would reflect the influence of the genetic variability on the responses, where each genotype reacts in different way. However, previous analyses developed with microsatellite markers showed small differences in population genetic diversity between the same populations (Garcia-Fernandez, A., unpublished results). The small differences in genetic variability between populations not seem a problem for allow local adaptation to the altitude in other species and develop different strategies for improve water efficiency. Mackay et al., (2001) showed that the populations of *Arabis fecunda,* locally adapted to altitude, had low differences in genetic variability between populations, but showed significant differences in water use, suggesting that these differences could be related with root size in each population. Other specific traits involved in water efficiency have related could local adaptation and genetic effects, like specific leaf area (SLA, Scheepens et al. 2010). Drought tolerance and water efficiency are considered selective traits that allow local adaptation in plants (Montalvoetal1997RestoEco), but it seems necessary enough levels of genetic variability were selection could operate.

Effect of the plant size in the survival is not a surprising result in long-lived perennial species (Horvitz & Schemske 1995). GLMM and survival accelerated models showed an enhanced in the moratility as plant size increase at the end of the study (Tab. 3 & 4, drought mortality). For analyses developed for drought damage, results were contradictory. In the survival accelerated model (Tab. 4, drought signals), size have a positive effect, while in the binomial GLMM for drought signals (Tab.2), size had a negative effect to avoid drought damage in the last census. These results should be interpreted carefully, especially under controlled conditions like common garden experiment. Small plants do not consume large amounts of resources and need more time to show drought damage. However, one month after the water scarcity, only some big individuals could have enough reserves to avoid drought damage. Effects of plant size on *Silene ciliata* survival were also found in field experiments, following different trends according to the population's altitude (Giménez-Benavides et al. 2010). Low population showed a lower value of plant survival for large individuals than Intermediate and High populations. Size dependence has been commonly observed in a great variety of plant and environments but also modify plant process like growth or inflorescence production (Horvitz & Schemske 1995).

Total protein expression, protein identification and specific antibodies signal.

Our results from totally protein expression in SDS-PAGE gel showed differences before and after drought stress in the same individual (Fig. 2). Several proteins could be behind these responses, some of them directly associated with drought stress tolerance; while others could be indirectly associated with processes related to the negative consequences of water deficit (regulatory proteins, transcription factors, kinases protein etc. Shinozaki et al. 2003). These proteins could modulate the response against stress either co-operatively or independently (Shinozaki & Yamaguchi-Shinozaki 2007). In this sense, it has been observed that water stress decreases the level of some plant proteins, maintains others and induces the synthesis of some specific ones (Salekdeh et al. 2002).

All individuals showed the same pattern in protein expression and antibodies membranes pattern after drought treatment, independently of their origin population. This lack of variability between populations is an expectable result, because proteins show less variability than other markers (like DNA markers for instance, Freeland 2005), but also because proteins related with water stress are essential for plant survival (Shinozaki et al. 2003) and the variability in a sensible region should be harder than in other parts of the genome (Freeland 2005). Other analyses, like different proteins expression between populations, are not discarded (but not measurable with these results). Populations under more stressful conditions (Low population) could express more amounts of specific proteins against water stress than others. Finally, we only measured proteins at the beginning and at the end of the experiment, but intermediate stages could also be interesting, searching for differences in the populations when they express any specific protein.

Protein identification using mass spectrometery showed the presence of the large subunit of 1.5 Ribulose-1,5-biphosphate carboxilase oxygenase (Rubisco, arrow B and C in Fig.2) in *Silene ciliata* individuals after drought stress. Both bands have a significant identificaction with similar Rubisco large subunits from another *Silene* species like *Silene schafta* (35 kDa, arrow B) and *Silene vulgaris* (18 kDa, arrow C). Both species are closely related to *SIlene ciliata* in the phylogeny trees obtained for the genus (Rautenberg 2009) which increase the accuracy of the band identification. The results in SDS-PAGE gels also showed a decrease of the quantity of Rubisco complex (50 kDa, band A in Fig.2) in the after drought individuals. Increasing severity and duration of drought stress decreases Rubisco activity (Tezara & Lawlor 1995), but short-term responses of Rubisco to drought stress are not clear, its regulation activity and quantity remains very complex and results are contradictory (Feller et al. 2008). Rubisco is very susceptible to proteolysis if it is not carbamylated (Parry et al. 2002) and the degree of the stress has appeared as relevant for Rubisco proteolysis in other plants like wheat (Demirevska et al. 2009).

Silene ciliata showed a reproducible and consistent signal in the immunoprecipitation assays with *CDeT11-24* & *CDeT6-10* antibodies. The band presence and the different pattern in after drought individuals suggest that these proteins are part of the mechanism that *Silene ciliata* individuals showed

against drought stress, as were detected in *Craterostigma plantagineum* (Röhrig et al. 2006). LEA proteins (as *CDeT6-10*, Schneider et al. 1993) are induced by osmotic stress and comprise the vast majority of stress-responsive proteins (Ingram & Bartels 1996). Their expression has been detected in several angiosperms species (Bartels & Sunkar 2005) and also in some animals (Browne et al. 2002). ABA hormone and proteins which regulate its pathways (*CDeT11-24*, Velasco et al. 1998) have also played a central role in many aspects of stress responses (Rock 2000; Finkelstein et al. 2002), including water deficit. Membranes showed the presence of an exclusive bigger band than expected with *CDeT6-10* antibody (Fig.3), which is absent in the individuals before drought treatment. Finally, the signals of both proteins (*CDeT11-24* & *CDeT6-10*) are observed in the individuals after, but also before beginning the drought stress experiment, which implies a basal expression of both proteins. These early responses to water stress are largely identical among organisms, including several process like a decrease of photosynthesis or hormonal processes (Bartels & Sunkar 2005).

Concluding remarks

As in early stages of development (Giménez-Benavides et al. 2007a), adults of *Silene ciliata* also showed evidence of local adaptation against drought stress, supporting the idea that selection forces that favored the survival in the first days of life may also be selecting the adults were able to tolerate better a severe drought stress episode. These evidences disappear when water scarcity was severe and prolonged in time. All individuals showed similar proteins patterns, which allow them to survive and protect against the pernicious effect of drought. Quantitative mechanisms (e.g. real time PCR, Livak & Schmittgen 2001), that allow the estimation of the amount of protein synthesized or gene expression might be able to show clearer differences in protein expression between populations, especially in the case of water stress (Nicot et al. 2005).

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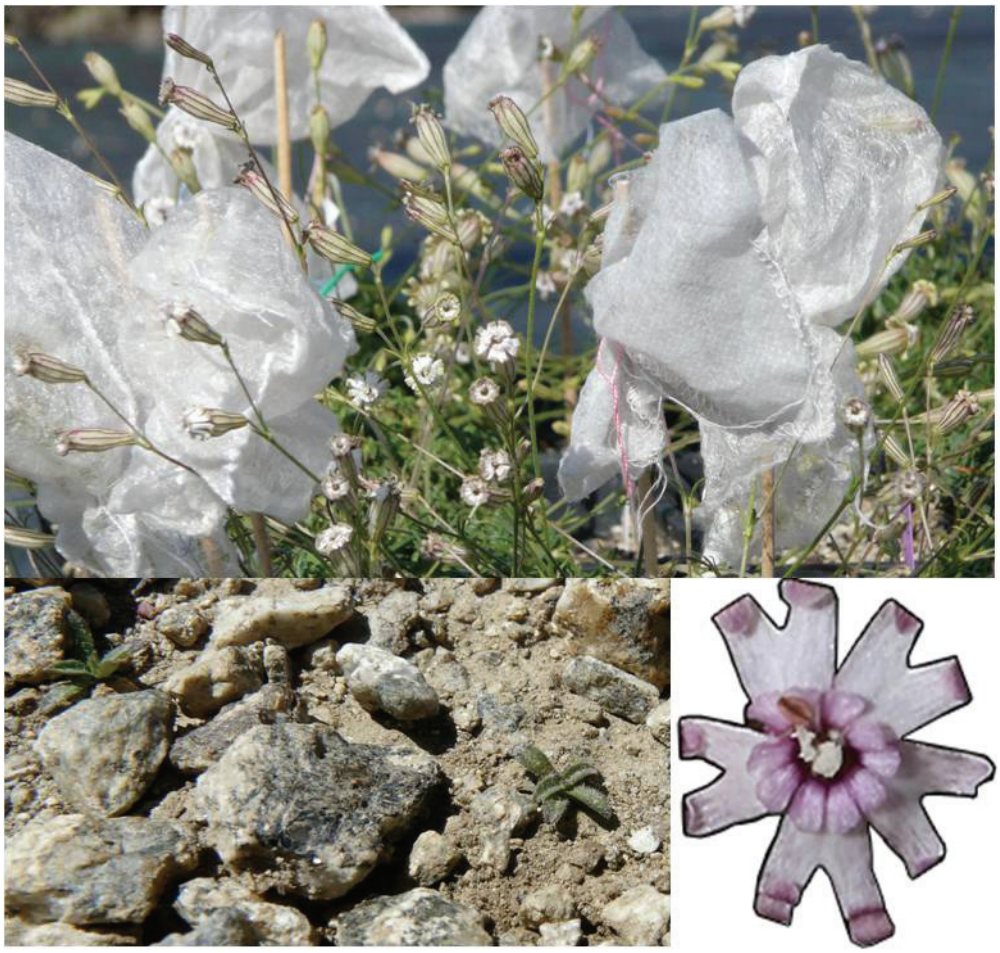
Chapter 5

Inbreeding at the edge: Does inbreeding depression increase under more stressful conditions?



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Abstract

Edge populations are frequently small-sized and subject to stressful conditions that may compromise their viability in the long term. Inbreeding can play an important role in small populations by reducing their genetic diversity, leading to the fixation of pernicious mutations and, finally, carrying populations to an extinction vortex through inbreeding depression. Although stressful conditions may enhance the intensity of inbreeding depression, existing evidence is inconclusive in marginal habitats. Local adaptation and gene flow are two factors that can have different effects on the intensity of inbreeding depression. Three local populations of *Silene ciliata* distributed across an elevation gradient at the southernmost edge of the species distribution were used for this study. Fitness estimates of germination, survival and growth rate of inbred seedlings were compared to those of seedlings from both within- and between-population outcrosses. Fitness surrogates were estimated in field and controlled conditions. In general terms, inbred seedlings had lower fitness values than outcrossed seedlings. For most of the variables analysed, similar inbreeding effects were found in all three populations of the gradient, but, for seed weight and accelerated failure-time analysis of seedling survival, inbreeding depression was only found in the most stressed populations. Similarly, inbreeding depression effects were more evident in the field than in controlled chamber conditions. Gene flow between populations contributed to an increase in most fitness estimates and populations, suggesting that the benefits of reducing inbreeding depression overrode the potentially deleterious effects of disrupting local adaptation. Our results suggest that inbreeding depression plays a determinant role in the fitness of early life stages of *Silene ciliata* in its rear edge populations. In most fitness estimates of early life stages, similar inbreeding effects were detected in the studied populations, regardless of their level of environmental stress across the elevation gradient.

Introduction

Marginal populations, both those located at the eroding edge (rear edge) and at the expanding edge (front edge) tracking climate change, have become the focus of many current studies (Parmesan et al. 2005; Pearson et al. 2009; Herrera & Bazaga 2008; Hampe & Petit 2005). Rapid warming is expected to have a great impact on marginal populations and change species range limits (Sexton et al. 2009) with contrasting responses among species (Jump & Peñuelas 2005). This makes edge populations well-suited for assessing the ability to respond to the new environments that emerge during rapid climate fluctuations (Ackerly & Monson 2003).

It is also well known that marginal populations are usually isolated due to extreme conditions at range edges (Kawecki 2008). Isolation, which is frequently exacerbated due to fragmentation and poor habitat quality, is recognized as one of the major threats for the long-term viability of marginal populations (Vitousek 1994; Gibson et al. 2009). This, together with small population size, reduces the genetic diversity of edge populations and can significantly increase their extinction risk (Sagarin & Gaines 2002). This low demographic viability results from a decrease in the number of reproductive individuals and a parallel increase in mating between close relatives (Lawton 1993; Lande 1993). Processes such as genetic drift and inbreeding depression can act as catalysts for extinction (Ellstrand & Elam 1993; Menges & Dolan 1998).

Inbreeding depression is widely accepted as one of the main causes of fitness reduction in small populations (Charlesworth & Charlesworth 1999). This situation can become especially unfavourable for self-incompatible or partially self-incompatible species, as they are more vulnerable to genetic deterioration (losing specific mating type alleles, Byers 1995), the negative effects of allele loss (Ellstrand & Elam 1993), and particularly to early inbreeding depression processes (Husband & Schemske 1995).

On the other hand, edge populations can have enhanced potential to deal with extreme environmental conditions through adaptation (Kawecki 2008). From a genetic point of view, the appearance of new alleles or the fixation of some mutations that support an adaptive advantage may be especially fruitful in marginal populations (see review in Kawecki & Ebert 2004). However, local adaptation processes require edge populations to have a minimum population size and genetic diversity where adaptive alleles and natural selection can take place (Leimu & Fischer 2008; Kawecki 2008).

Although some studies have found that stressful conditions may favour inbreeding depression in plant populations (Frankham et al. 2002; Hauser & Loeschcke 1996), there is no clear evidence and other studies in other organisms have reported lower inbreeding depression under stress (Henry et al. 2003). Thus, this hypothesis remains controversial (Armbruster & Reed 2005) and still requires detailed examination (Paschke et al. 2005). Several local factors, like lineage effect (inbreeding in past generations e.g. Falconer & Mackay 1996), nature of pollen donor (Paschke et al. 2005), original population size (see Paland & Schmid 2003), different direct phenotypic response and different adaptive values of stable, nonplastic phenotypes (Cheptou & Donohue 2011) may also affect inbreeding depression, and, in some

instances, diminish the pernicious effects of stressful environments. In this context, we hypothesized that discrepancies in relation to this hypothesis may be related to some extent to different degrees of local adaptation success in marginal and stressful conditions.

Mountains are especially well suited for testing hypotheses related to the adaptation and evolution of plants by comparative observations and manipulative experiments with populations along local elevation gradients (Körner 2007). When properly chosen, elevation gradients modify environmental conditions in a very predictable fashion (Walther et al. 2005) and put populations under different selective pressures (Gonzalo-Turpin & Hazard 2009). These gradients include both optimal and marginal environments which can be assimilated to central population conditions and the species' rear and front edge conditions, respectively. Furthermore, some mountains are isolated islands where the arrival of migrants or gene flow from other areas is almost impossible. Low and high altitude limits in such mountains are ideal areas for testing whether inbreeding depression is more intense in marginal populations and whether it can be masked or influenced by local adaptation.

Our model plant, *Silene ciliata* Pourret, a Mediterranean high mountain specialist, is an appropriate species for this study since it shows evidence of local adaptation (greater seed germination and seedling survival of native individuals than seeds from other populations) in the lower populations of its southernmost limit in Central Spain (Giménez-Benavides et al. 2007a). In addition, the *S. ciliata* populations at low altitudes are partially isolated from the upper ones because their flowering periods are significantly asynchronous (Giménez-Benavides et al. 2007b).

The aim of this study was to compare the relevance of inbreeding depression on locally adapted populations of *S. ciliata* across an environmental (elevation) gradient, where the lowest population experiences the most stressful conditions (Giménez-Benavides et al. 2007a). We combined field and germination chamber experiments and sowed seeds obtained from controlled selfings and outcrossings within and between populations. Specifically, we asked: a) Is there inbreeding depression in *S. ciliata* populations distributed across the species` altitudinal range at its southernmost limit? b) If so, is inbreeding depression related to the intensity of environmental stress? c) Does gene flow from other populations increase or reduce seedling fitness (reducing inbreeding or reducing local adaptation, respectively)? d) Do all populations react similarly to gene flow from other populations?

Material and Methods

Study species and population selection

Silene ciliata Poiret is a dwarf cushion plant that inhabits Mediterranean mountain ranges of the northern Mediterranean Basin from the Cordillera Central in the Iberian Peninsula to the Massif Central in France, the Apennines in Italy and the Balkan Mountains (Tutin et al. 1995). *S. ciliata* reaches its southernmost margin in central Spain in the Sierra de Guadarrama where populations are considered remnants because they are isolated from northern populations. *S. ciliata* presents variable levels of ploidy in natural populations (Blackburn 1933) but in Sierra de Guadarrama, individuals in all populations are diploid (2n=24,García-Fernández A., unpublished data). The species is gynomonoecious, but spontaneous autogamy is restricted by pronounced protandry (Giménez-Benavides et al. 2007b).

Three populations of *S. ciliata* were selected along the largest local elevation gradient in Sierra de Guadarrama which mimics a steep global range distribution gradient of the species (see Giménez-Benavides et al. 2007a or Giménez-Benavides et al 2008). The lowest population (1980 m, hereafter "Low") was situated in the lateral moraine deposit of the glacial cirque of Laguna de Peñalara. The intermediate population (2250 m, hereafter "Intermediate") was located on the Dos Hermanas summit approximately 3 km from the Low population. The highest population (2420 m, "High") was situated on the summit of Peñalara peak, the highest peak of Sierra de Guadarrama, approximately 3 km from the Intermediate population. All three populations are located in the Peñalara Natural Park, 50 km northwest of the city of Madrid (Figure 1). All populations occur on gentle south-facing slopes and had a relatively high number of individuals (more than 200). Vegetation composition is detailed in Escudero et al. (2005) and Gutierrez-Girón & Gavilán (2010).

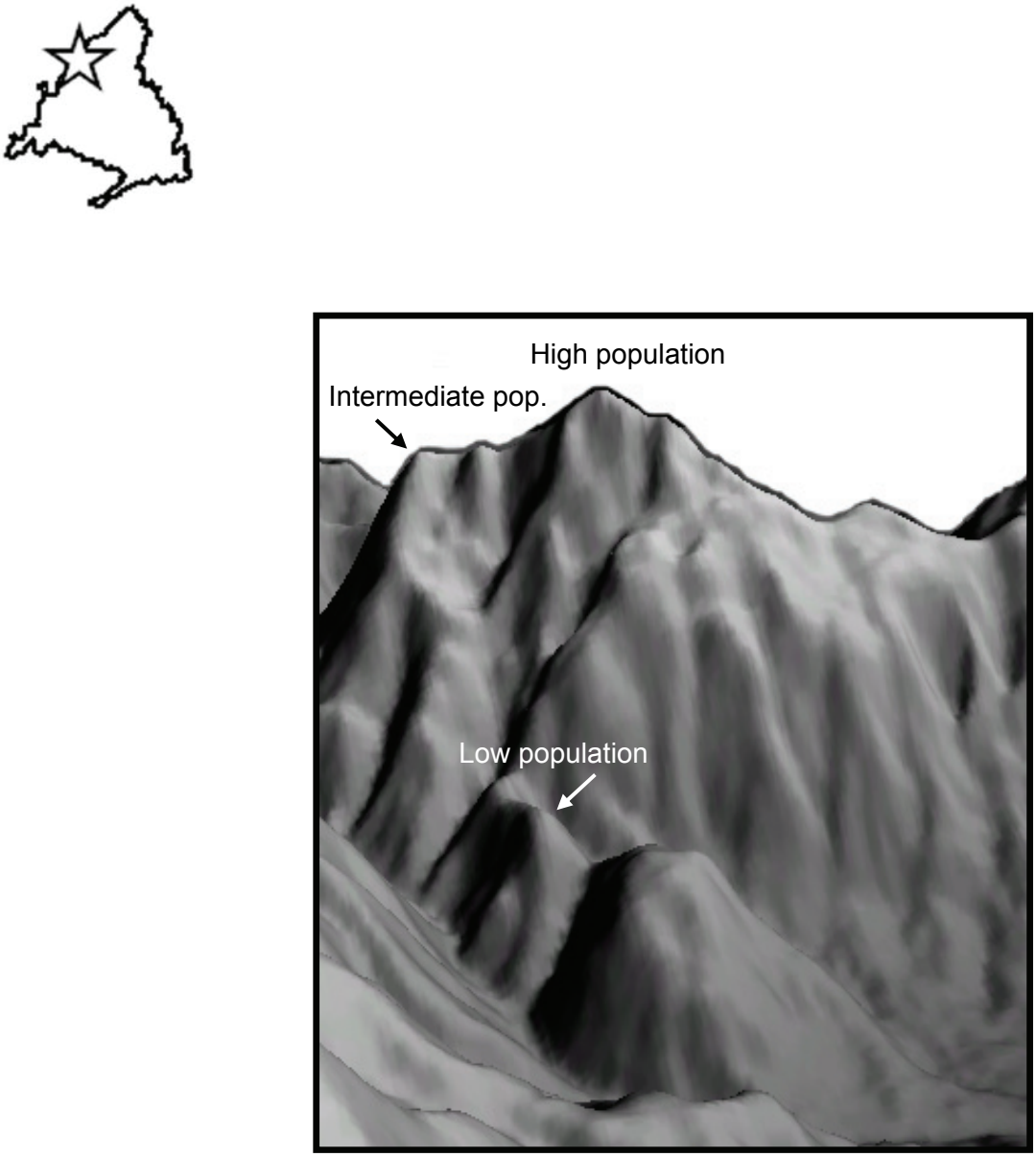

Figure 1. Location of Peñalara Natural Park in the Madrid province in Central Spain (white star) and 3D model map with the position of the studied populations.

Seed collection, cultivation of plants and manual crossings

In autumn 2006, mature fruits were collected from 20-25 healthy plants distanced at least 5 m from each other in the three populations. Fruits were dissected in the laboratory, and seeds were immediately cleaned and placed in Petri dishes with moist filter paper according to population of origin. Petri dishes were wrapped in aluminium foil and stored at 4ºC for humid stratification for 6 months to break seed dormancy (see Giménez-Benavides et al 2005 for details). In spring 2007, the seeds were sown in plastic

pots with a commercial substrate enriched in NPK and watered every 48 hours. Plants were grown in the phytotron of the CULTIVE Laboratory (Rey Juan Carlos University, Móstoles, Spain) until February 2008 when they were moved outside the greenhouse with the same water regime described above.

Plants flowered in May and June. Manual pollinations were carried out using a fine paint brush. Before anthesis, flowers were emasculated to avoid spontaneous self-pollination and covered with a cotton net to avoid accidental pollination. When the flower was unable to receive new pollen because the female structures were fading off, the net was removed. Developed fruits were removed from plants immediately before dispersal. Seeds were collected and placed in paper bags with silica gel.

We selected four flowering individuals from each population (Low, Intermediate and High) grouped in two pairs. On each plant, one flower was self-pollinated (self-pollination treatment) and a second flower was pollinated with pollen from its paired neighbour of the same population (within-population cross pollination treatment). A third flower was pollinated from a randomly selected individual from a different population. Likewise, the third flower of its paired neighbour was crossed with a random individual from the remaining population. These crosses with the third flower were called the between-population cross pollination. Thus, we obtained three sets of seeds for each population: 1- Inbred seeds (four different seed subsets from each population, one from each mother plant); 2- Within-population outcrossed seeds (two seed subsets, one from each reciprocal cross between paired individuals); and 3- Between-population outcrossed seeds. In case 3, we obtained two seed subsets in each population by crossing individuals from different populations (Low-Intermediate, Low-High and Intermediate-High crosses, as each pair of plants in each population received pollen from the other two populations). All plants contributed to each seed subset in the same way and with the same number of seeds. The experimental crossing scheme is shown in Figure 2.

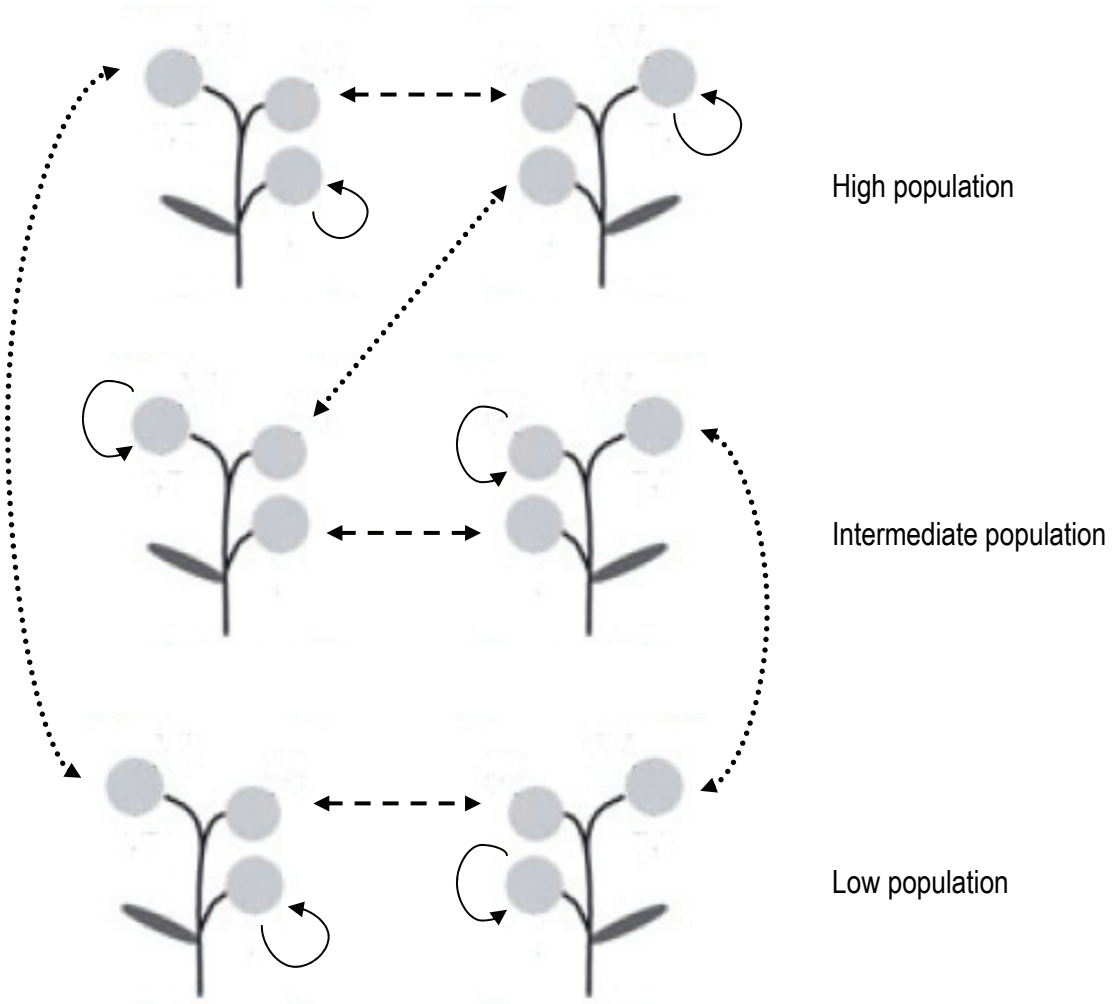

Figure 2. Diagram of the types of crossing performed with plants at the greenhouse. Continuous lines represent self-crossings, discontinuous lines represent within-population outcrossings and pointed lines between-population outcrossings.

Germination tests and seed weight

One hundred seeds were selected from each seed subset (900 seeds total). All seeds were weighed with a precision Micro Balance (MX5 Mettler Toledo). They were then placed in Petri dishes and subjected to a cold humid stratification treatment for three months as described above.

For germination tests, we prepared four replicates of 25 seeds per type of crossing and population origin. Seeds were placed in 8 cm Petri dishes with two layers of filter paper which were kept moistened throughout the germination tests. Germination tests were conducted in a germination chamber (Selecta Hotcold GL), equipped with six cool-white fluorescent light tubes (Philips 18 W standard type). Germination conditions were set at alternating 25/15 ºC temperature with a 16 hour light/8 hour darkness photoperiod. Seeds were surveyed every 2-3 days and removed from the dish after radicle emergence.

Germination tests were maintained until no seeds germinated for two consecutive surveys. We calculated the total number of germinated seeds.

In situ seed germination, seedling growth and survival in field populations

In September 2008, 12 sites were randomly selected in each of the three original populations (Low, Intermediate and High). Each site consisted of a bare soil area where small stones and wooden sticks had been previously removed. At each site, we established a plot with a transparent plastic quadrat of 16 x 16cm. The quadrat contained a 4 row x 5 column grid of cells. Each cell had a 1cm diameter hole in the centre. This plastic sheet was used for accurate seed placement and to facilitate further monitoring. Two seeds were sown in each hole to ensure seed germination. In each population four plots were established for each of the three types of crossings. For each crossing type, only the seeds of the plants native to each population were sown. In selfings, five holes were used per maternal plant. In within-population outcrossings, ten holes were allocated to each crossing. Finally, in between-population outcrossings, ten holes were assigned to each of the two seed subsets derived from the crossings where the maternal population had participated. Thus, a total of 1440 seeds were sown.

Emergence, survival and growth were checked regularly. The first survey was made on 15 May 2009 after snow melt. Three surveys per month were carried out in June, July and August 2009. A last survey was made on 22 September 2009 to census the number of surviving plants at the end of the growing season. In each census, seed germination, seedling survival and seedling size (maximum diameter measured with a digital calliper) were determined. When both seeds in a hole germinated, the last seedling to emerge was clipped to avoid growing interference. This only happened one or two times in each population. Each dead seedling was assigned a most-likely cause of death. Thus, summer drought was assigned to dried-out seedlings without any visible damage, while herbivore attack was assigned to dead seedlings with external signs of predation, cotyledon removal or whole removal. Climate conditions during the monitoring period were similar to average values recorded for the last 30 years. Although climatic data for the specific locations were unavailable, annual rainfall at the closest weather station (Navacerrada Pass, 1890m) is about 1400 mm with a very pronounced summer drought when less than 10% of annual precipitation occurs. Summer drought is a well-known process in Mediterranean systems (Beniston et al. 2007). High mountains in Mediterranean areas are also influenced by this process and summer drought has been considered as an important stress factor (Castro et al. 2005). Giménez-Benavides et al. (2007a) measured the soil water content (SWC) at the study site during the summer of 2004, a mild and wet year. SWC descended to less than 5% even at the highest locality (Peñalara peak 2428 m). SWC was inversely proportional to elevation throughout the growth season (June-September).

Data analysis

Germination chamber test and seed weight

Differences in seed mass and total seed germination were analysed using General Linear Models (GLM) (McCullagh & Nelder 1989). For seed germination, the response variables were assumed to follow a binomial distribution (0 for no germination and 1 for germination), and thus we used the logit as link-function. For seed mass, the response variable was assumed to follow a Gaussian distribution and the identity function was used. GLMs were implemented using the crossing types (selfing, within-population outcrossing and between-population outcrossing) and the maternal populations (Low, Intermediate and High) as fixed factors, and including the interaction between these two factors. GLMs were implemented using SAS 9.0, using the GLIMMIX module (SAS Institute 1996).

Germination rate was also analysed using a right accelerated failure-time model, i.e. a linear regression model in which the response variable is the logarithm or a known monotone transformation of a failure time (Fox 2001). This approach allows the use of censored data to estimate parametric regression models using a maximum-likelihood approach. The best failure-time distributions were chosen for the data sets based on the comparison of possible distributions with the likelihood ratio test (Fox 2001). A log-logistic distribution was therefore used for the germination rate data. Analyses were performed with SAS 9.0, following the LIFEREG procedure (SAS Institute 1996).

Field sowing experiments

Seed germination and seedling survival were analyzed with General Linear Mixed Models (GLMM) (Breslow & Clayton 1993). Population origin (Low, Intermediate and High), type of cross (selfing, within-population outcrossing and between-population outcrossing), and the interaction between maternal population and type of cross were included in the analysis as fixed factors, while plot was treated as a random factor. Seed germination and seedling survival at the end of the growing season were assumed to be binomial variables (0 for no germination or seed death and 1 for seed germination or seed survival) and the logit link-function was used. As summer drought in Mediterranean mountains causes very low seedling survival, another GLMM model was developed using seedling survival at mid-summer (second survey in July) as the response variable. In this GLMM, seedling size in the previous census (first survey in July) was also included as a fixed factor. The response variable (seeding survival) was introduced as described above. Seedling size for the same date was also analyzed with GLMM, including type of cross and population as fixed factors. In this analysis, seedling size was included as a normal variable, using identity link-function. Analyses were performed with SAS 9.0, using the GLIMMIX module (SAS Institute 1996).

Germination and survival rates at mid-summer and at the end of the experiment were also analysed using an accelerated failure-time model as described above (SAS Institute 1996). A log-logistic distribution was used for the seedling emergence data set and Weibull distribution for seedling survival.

When the interaction between factors was found to be significant, independent GLMMs were carried out for each population. In these cases, the analyses followed the same conditions as explained before. The same strategy was followed for the accelerated failure-time models for germination and survival rates.

Reference population and estimations of inbreeding depression

In addition to evaluating the change in deviance in each of our multinomial fixed variables, population and type of cross, we also calculated a coefficient that indicated the change and intensity of this shift in relation to the performance of the High population (fixed population variable) and the between-population outcrossing (type of cross variable). The High population treatment was selected as a reference treatment because it was considered to represent the least stressful conditions for the species. The species is able to grow at altitudes far above our upper limit in other mountains with higher altitudes such as the Pyrenees. This suggests that the species has the potential to extend its upper limit far above the present one in its southernmost limit, but is constrained by the altitude of these mountains (Talavera 1991). Similarly, the between-population outcrosses were used as a reference treatment because they represent a random outcrossing scenario for the species from which to estimate the magnitude of inbreeding depression. For each type of cross, the estimated coefficient was used to estimate inbreeding depression in populations. Positive values of this coefficient for selfing indicate inbreeding depression as a result of the sum of within-population inbreeding and among-population inbreeding *sensu* (Keller & Waller 2002), i.e. inbreeding depression due to relatedness between parents of within-population outcrosses, while the values of this coefficient for within-population outcrossing measure among-population inbreeding depression.

Results

In general terms, between-population outcrossed seeds were heavier, germinated faster and had greater final germination percentages than within-population outcrossed and inbred seeds. Seedling survival and seedling size, two important fitness components, were also slightly greater in between-population outcrossed seeds.

Seed weight and germination in the growth chamber

Seed weight for the three populations and the three types of crossings was 0.38 ± 0.09 mg (Mean ± S.D.). Seed weight was significantly affected by the population of the parent plants, the type of cross and their interaction (Table 1). In the Low and the Intermediate populations, between-population outcrossed seeds were significantly heavier than either within-population outcrossed seeds or inbred seeds. In the High population, however, within-population outcrossed seeds were the heaviest.

Mean germination percentage in the chamber was 67 ± 27%. Seed germination was affected by population origin, type of cross and their interaction (Table S1 in Supporting Information). In the Low population, within-population outcrossed seeds had greater germination percentages than between-population outcrossed seeds, while in the High population, between-population outcrossed seeds had the greatest germination percentages. The results from accelerated failure-time models of germination rates (Table S2) showed significant effects for both factors. Within-population outcrossed seeds germinated faster than between-population outcrossed seeds, which germinated faster than inbred seeds.

Table 1. GLM results for the effects of population, cross type and their interaction (Population x Cross) on *Silene ciliata* seed weight. An independent GLM was performed for each population because the interaction term was significant. Seed germination was considered as binomial variable, using logit as link function.

Effect	Solution for effects					Deviance change	
	Coefficient	SD	df	t	P	F	P
Population	-	-	-	-	-	45.43	<0.01
Cross type	-	-	-	-	-	88.19	<0.01
Population x Cross type	-	-	-	-	-	68.93	<0.01
Low population							
Cross type						208.87	<0.01
Selfing	-0.125	0.011	298	-11.47	<0.01	-	-
Within-pop. outcross	-0.19	0.011	298	-17.49	<0.01	-	-
Between-pop. outcross	-	-	-	-	-	-	-
Intermediate population							
Cross type						35.73	<0.01
Selfing	-0.042	0.011	298	-3.81	<0.01		
Within-pop. outcross	-0.093	0.011	298	-0.844	<0.01		
Between-pop. outcross	-	-	-	-	-	-	-
High population							
Cross type						12.27	<0.01
Selfing	0.014	0.01	298	1.39	0.16		
Within-pop. outcross	0.048	0.01	298	4.8	<0.01		
Between-pop. outcross	-	-	-	-	-	-	-

Field sowing experiments

Although around 50 % of the sown seeds germinated under field conditions, final seedling survival was barely 3% at the end of the experiment. The highest seedling mortality peak was during the month of July. Drought was assigned as the cause of death for more than 90% of seedlings.

Seed germination in field conditions was significantly affected by population and type of crossing and marginally affected by sowing plot (Table 2). Between-population outcrossed seeds had the highest

germination percentages (Figure 3). On the other hand, germination in the Intermediate population was higher than in the Low population. Germination percentages of inbred seeds in the Intermediate and the High populations had lower variance than those of other cross types and inbred seeds in the Low population.

Table 2. GLMM results for seed germination of *Silene ciliata* considering population (Pop L: Low population, Pop I: Intermediate population and Pop H: High population), cross type and the interaction between both factors (Pop x Cross). Seed germination was considered as binomial variable (0 for no germination and 1 for germination) using logit as link function.

Effect*	Solution for effects					Deviance change	
	Coefficient	SD	d.f.	t	P	F	P
Population	-	-	-	-	-	4.61	0.01
Pop L	-0.168	0.465	24.9	0.36	0.72	-	-
Pop I	0.619	0.491	30.1	1.26	0.22	-	-
Pop H	-	-	-	-	-	-	-
Cross type	-	-	-	-	-	16.34	<0.01
Selfing	-1.417	0.466	25.1	-3.04	<0.01	-	-
Within-pop. outcross	-1.371	0.466	25.1	-2.94	<0.01	-	-
Between-pop. outcross	-	-	-	-	-	-	-
Pop. x Cross type						0.24	0.91

* Sowing plot was included as random factor; $Z = 1.72$, p – value = 0.04, Estimator ± Error = 0.21 ± 0.12.

Seeds in the Low and Intermediate populations germinated faster than seeds in the High population, as shown by the accelerated failure-model (Table 3). No significant effects of cross type were detected with respect to between-population outcrossed seeds.

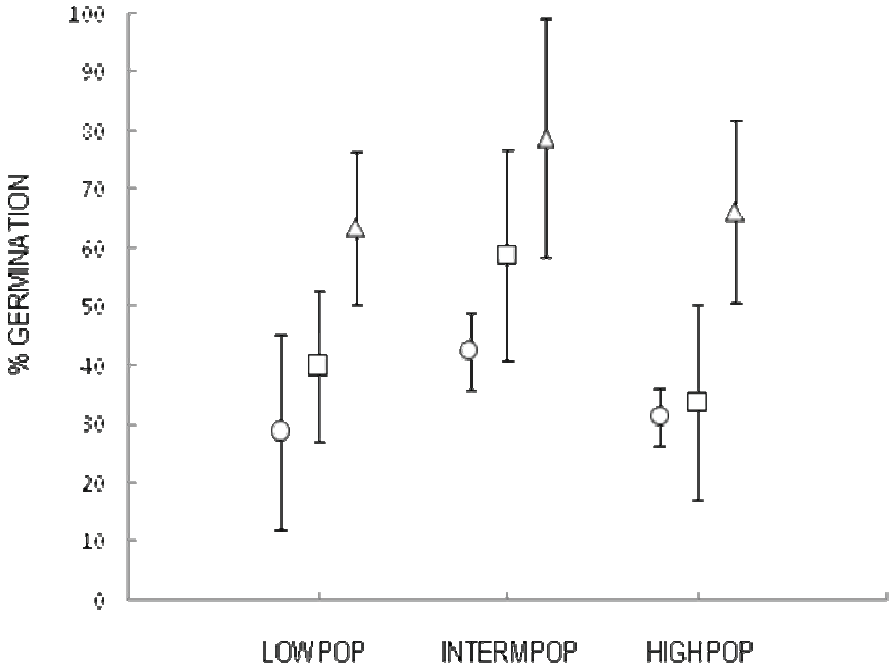

Figure 3. Percentage germination of *Silene ciliata* in each sowing plot (Y axis, Plot average± SD) for the field experiment, grouped by population and cross type (circle: inbred seeds, square: within-population outcrossed seeds and triangle: between-population outcrossed seeds).

The GLMMs showed that seedling survival at the end of the growing season was significantly affected by sowing plot (as a random factor, Z value=2.1 p = 0.017), while seedling survival at mid-July (when drought stress was only at the beginning, Table 4) depended on seedling size at the previous census (size correlated positively with survival). Cross type had only a marginal effect on seedling survival at mid-July, but significantly affected seedling size. Population origin had a marginal effect on seedling size (Table 4) with larger seedlings in the Low population. Figure S1 shows average seedling sizes for each population and cross type. The main differences were found in the Low population where between-population outcrossed seedlings were larger and inbreeding seeds had greater variance.

Table 3. Accelerated failure-time analyses (log-logistic distribution) of total seed germination of *Silene ciliata* seeds considering population (Pop L: Low population, Pop I: Intermediate population and Pop H: High population), cross type and the interaction between both factors (Pop x Cross type).

Variable	d.f.	χ^2	*p*	*Estimate*
Intercept		447.69	< 0.01	0.933 ± 0.044
Population	2	346.24	<0.01	-
Pop L	1	180.11	< 0.01	-0.832 ± 0.062
Pop I	1	170.85	< 0.01	-0.791 ± 0.061
Pop H	0	-	-	-
Cross type	2	7.01	0.03	-
Selfing	1	0.45	0.50	0.052 ± 0.573
Within-pop. outcross	1	0.61	0.44	-0.058 ± 0.073
Between-pop. outcross	0	-	-	-
Pop. x Cross type	4	2.59	0.63	-
Scale	1			0.191 ± 0.008

The shape of the survival curves for seedlings until the mid-July census was not affected by the factors included in the analysis or their interaction (Table 5). On the other hand, the shape of the survival curves until the end of the growing season was significantly affected by population, cross type and their interaction (Figure 4, Table 5). The Low and Intermediate populations had higher survival rates than the High population. In independent survival models for each population (Table S3), Low population inbred seedlings had lower survival rates than between-population outcrossed seedlings, whereas in the Intermediate population, between-population outcrossed seedlings had the highest survival rates.

Table 4. GLMM results of seedling survival and seedling size of *Silene ciliata* at mid-summer (July census) considering as predictor factors population (Pop L: Low population, Pop I: Intermediate population, and Pop H: High population), cross type and the interaction between both factors (Pop x Cross type). Seed survival was analyzed as binomial variable, using Logit as link function while seedling size was considered a gaussian variable (identity as link function).

GLMM seedling survival	Solution for effects					Deviance change	
Effect*	Coefficient	SD	d.f.	t	P	F	P
Population	-	-	-	-	-	0.88	0.43
Cross type	-	-	-	-	-	4.71	0.02
Selfing	-1.559	0.921	23.1	-1.69	0.10	-	-
Within-pop. outcross	-0.503	0.929	21.7	-0.54	0.59	-	-
Between-pop. outcross	-	-	-	-	-	-	-
Size at previous census						7.18	<0.01
Pop. x Cross type						0.53	0.71
GLMM seedling size	Solution for effects					Deviance change	
Effect	Coefficient	SD	d.f.	t	P	F	P
Population	-	-	-	-	-	3.49	0.04
Pop L	3.238	1.029	21.3	2.6	0.02	-	-
Pop I	0.433	1.024	20.4	3.15	<0.01	-	-
Pop H	-	-	-	-	-	-	-
Cross type	-	-	-	-	-	4.62	0.02
Selfing	-0.967	1.102	26.3	-0.88	0.39	-	-
Within-pop. outcross	-0.353	1.149	29.4	-0.31	0.76	-	-
Between-pop. outcross	-	-	-	-	-	-	-
Pop. x Cross type						1.16	0.35

* Sowing plot was included in both analyses as random factor (Seedling survival: $Z = 1.53$, p – value = 0.06; Seedling size: $Z = 2.56$, p – value < 0.01 Estimator ± Error = 1.7 ± 0.66).

Table 5. Accelerated failure-time analyses (Weibull distribution) of total seed survival of *Silene ciliata* at the mid-July survey and at the end of the growing season as a function of population (Pop L, Low population, Pop I, Intermediate population and Pop H, High population), cross type and the interaction between both factors (Pop x Cross type).

Survival Model in Mid July				
Variable	d.f.	χ^2	*p*	*Estimate ± Error*
Intercept	1	4576.1	< 0.01	1.72 ± 0.03
Population	2	0.35	0.84	
Cross type	2	3.24	0.19	
Pop. x Cross type	4	2.32	0.68	
Scale	1			0.18 ± 0.01
Survival Model at the end of the growing season				
Variable	d.f.	χ^2	*p*	*Estimate ± Error*
Intercept		1535.25	< 0.01	1.789 ± 0.046
Population	2	30.96	< 0.01	
Pop L	1	37.68	< 0.01	0.4 ± 0.065
Pop I	1	8.53	< 0.01	0.181 ± 0.062
Pop H	0	-	-	-
Cross type	2	22.96	< 0.01	
Selfing	1	0.41	0.52	-0.051 ± 0.078
Within-pop. outcross	1	0.01	0.92	-0.008 ± 0.079
Between-pop. outcross	0	-	-	-
Pop. x Cross type	4	11.97	0.02	
Scale				0.332 ± 0.127

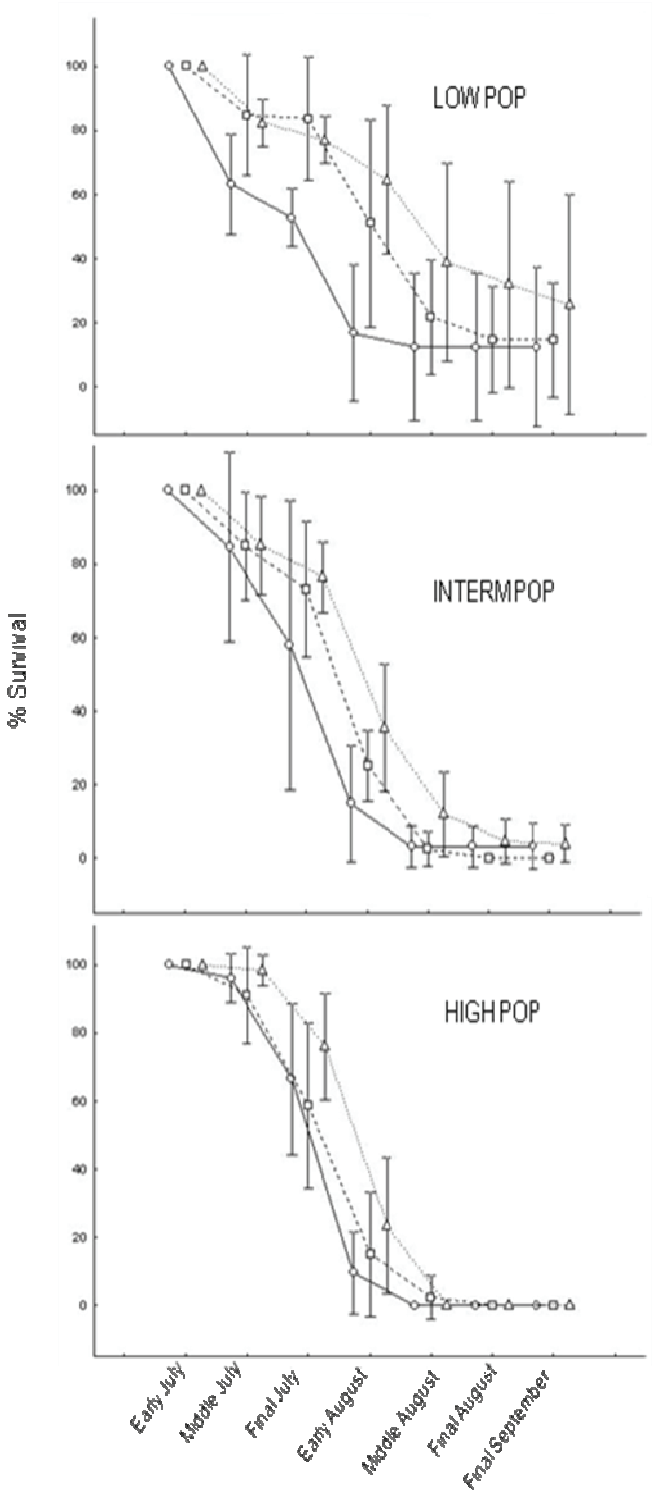

Figure 4. Seedling survival percentage (Y axis, Mean percentage ± SD) of *Silene ciliata* in the field sowing experiment, grouped by cross type (circle-continuous line for inbred seedlings, square-discontinuous line for within-population outcrossed seedlings and triangle-point line for between-population outcrossed seedlings).

Discussion

This study provided information on how inbreeding depression is affecting the rear edge populations of a high mountain Mediterranean specialist at the southernmost limit of its whole distribution range. We found that inbreeding depression is present along the elevation range and that there are no differences in the intensity of inbreeding depression between populations for most fitness surrogates. However, in a few cases, inbreeding depression was greater in populations or situations considered to be under greater environmental stress. Inbreeding depression runs in parallel to evidence of local adaptation in these populations (Giménez-Benavides et al. 2007a). Local adaptation is also supported by the extremely low seedling survival found in the three populations (3% at the end of the study) which provides the necessary selective ground for evolution to operate (Kawecki & Ebert 2004). Decoupling the processes of inbreeding depression and local adaption seems critical for achieving a good understanding of species' responses to stressful conditions.

Inbreeding depression in Silene ciliata populations

The greatest values of several fitness traits considered in this work (i.e., seed weight, germination or survival) were found in between-population outcrossed seeds. Inbreeding depression was not only detected when inbreeding was maximized through selfing, but also when within-population outcrosses were considered. This suggests the presence of biparental inbreeding, or at least, some degree of kinship between the parents in the studied populations. Biparental inbreeding has been documented in populations with low genetic diversity, and has often been found in marginal populations when compared to central ones (see Eckert et al. 2008). Preliminary results obtained with microsatellite markers (SSR) developed for *S.ciliata* (Garcia-Fernandez et al., unpublished data) and with EST-SSR markers transferred from *Silene latifolia* (Moccia et al. 2009) showed significant departure from Hardy-Weinberg equilibrium and similar values of F_{IS} coefficients in the Low (F_{IS} = 0.35), Intermediate (F_{IS} = 0.39) and High populations (F_{IS} = 0.47). Inbreeding depression has also been detected in wild and cultivated populations of other species of the genus *Silene* (Bernasconi et al. 2009), such as *S. regia* (Menges 1991), *S. litorea* (Vilas et al. 2006) and *S. vulgaris* (Glaettli et al. 2006).

Relationship between inbreeding depression and stressful conditions

No interactions were found between population origin and type of crossing in the studied fitness components, except in seed weight and accelerated failure-time analysis of seedling survival at the end of the experiment. As the three populations have comparable amounts of inbreeding, inbreeding depression, was similar for most of these fitness components in all the populations. However, both seed weight and seedling survival curves show inbreeding depression only in the Low and Intermediate populations. Assuming that the High population has the least stressful environmental conditions, this is consistent with the hypothesis that inbreeding depression may be more intense in more stressful habitats (Armbruster &

Reed 2005). The germination experiments also yielded results consistent with this hypothesis if we assume that natural habitats are a more stressful environment for germination than the germination chamber (inbreeding depression was found in the germination of all populations in the natural habitats (Table 2), but only in the High population in the germination chamber experiments). Nevertheless, these results should be interpreted with caution, because these fitness components are concentrated in the early life stages and may not necessarily correspond to individuals' total fitness (Armbruster & Reed 2005). Moreover, the definition of stressful conditions merits careful consideration, because the stressfulness of a habitat may differ between life stages and/or fitness components (Körner 2007).

Discrepancies among studies comparing inbreeding depression under different environmental conditions suggest that other factors, in addition to stress, may affect inbreeding depression (Waller et al. 2008). Local adaptation might help explain some of the apparently contradictory findings, since it could mitigate the effect of inbreeding depression through the benefits of the locally adapted genotypes (Ebert et al. 2002) or, on the contrary, enhance the pernicious effects of inbreeding, reducing quantitative genetic variation (Lande 1993). The same loci associated with inbreeding depression in some environments may even contribute to adaptation in others (Cheptou & Donohue 2011). Furthermore, local adaptation is also associated with other genetic processes and factors, such as the intensity of gene flow or available genetic diversity, which further complicate the interpretation of results (Kawecki & Ebert 2004).

Gene flow, inbreeding depression and local adaptation

Several fitness components were higher in between-population outcrossed plants than in within-population outcrossed plants, suggesting that gene flow between populations contributes to reducing inbreeding depression. These results also imply that the possible negative effects of gene flow reducing local adaptation in some genes were overridden by the reduction of inbreeding depression. However, it is noteworthy that this did not occur in all fitness components or studied populations. Thus, in the High population, seeds were heavier in within-population outcrosses than in between-population outcrosses. These results might be interpreted as a case where the optimal local arrangement of gene complexes is broken by the arrival of foreign gene flow (i.e., outbreeding depression Whitlock et al. 2000). Similarly, greater germination of within-population outcrossed seeds was detected in the Low population and faster germination was obtained in the growth chamber experiments. However, between-population outcrossed seeds had greater germination and marginally faster germination in natural habitats. This contrasting response may indicate the interaction of two processes: the better germination results of within-population outcrossed seeds in the growth chamber may reflect the consequences of breaking the local arrangement of gene complexes by foreign gene flow (between-population crosses) in a favourable environment where the deleterious effects of inbreeding are not expressed, whereas the better germination results of between-population outcrossed seeds in the field may indicate the greater effect of inbreeding depression in the overall balance of this fitness component, when environmental stress unmasks the differential

performance of normal and deleterious alleles (Keller & Waller 2002). Although between-population outcrossing can be positive in the short-term, reversing the effects of inbreeding depression by hybrid vigour (Frankham et al. 2002), this initial effect could be masked by late outbreeding depression in the descendants (Edmands 2007).

Local adaptation is highly influenced by gene flow between core and peripheral populations (Lenormand 2002). In this sense, gene flow is considered the most limiting force against local adaptation in marginal populations because it can carry alleles that counteract the effect of selection on the local gene pool (Kawecki 2008). However, recent studies of gene flow in populations along a geographical gradient have shown that some marginal populations can become locally adapted despite substantial gene flow (Sexton et al. 2009). For example, in mountain populations of *Festuca eskia* gene flow was concurrent with the development of local adaptation (Gonzalo-Turpin & Hazard 2009). On the other hand, Byars et al. (2009) found low levels of gene flow between local adapted populations of *Poa hiemata* across an elevation gradient. These apparently contradictory results suggest that the effect of gene flow on local adaptation might depend on the steepness of the gradient (Sexton et al. 2009; Bridle et al. 2009) or other uncontrolled factors. We currently do not know the amount of gene flow between the studied populations of *Silene ciliata* at the rear edge. Although populations are separated by only a few kilometres, gene flow is limited by differences in flowering phenology between populations (Giménez-Benavides et al. 2007b) and the close flowering altitudinal tracking of the local pollinator assembly, which goes up in response to the flowering shift in the community (Wilson et al. 2005). This flowering asynchrony could limit pollen flow between populations along the elevation gradient (Körner et al. 2007) enabling local adaptation (Byars et al. 2007; Byars et al. 2009), as recently found for the Low population through reciprocal sowings experiments (Giménez-Benavides et al. 2007a). Yet our results suggest that between-population outcrossing gave seeds an important advantage, suggesting that local adaptation and inbreeding depression may be operating at the same time (Cheptou & Donohue 2011)

Our results highlight that although local adaptation could play an important role in marginal populations by increasing the fitness of native genotypes with respect to foreign ones (Bradshaw 1984), inbreeding depression may override the effects of local adaptation, making the population more prone to extinction (Nieminen et al. 2001). They also reinforce the idea that inbreeding depression can limit the impact of local adaptation when populations in marginal habitats lose their contact with the rest of populations, a typical scenario in rear edge conditions (Hereford 2010).

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SUPPORTING INFORMATION

Additional supporting information may be found in this appendix:

Table S1 GLM results for seed germination in growth chamber.

Table S2 Accelerated failure-time analyses results for seed germination in growth chamber.

Table S3 Accelerated failure-time analyses results for seedling survival at the end of the growing season in field conditions.

Table S1 GLM results of *Silene ciliata* for the effects of type of crossing, population and their interactions on seed germination in the growth chamber.

Effect	Solution for effects					Deviance change	
	Coefficient	SD	df	t	P	F	P
Population	-	-	-	-	-	47.05	<0.01
Crossing	-	-	-	-	-	4.87	<0.01
Pop x Crossing	-	-	-	-	-	7.7	<0.01
Low POP							
Crossing						11.08	<0.01
Selfing	0.462	0.308	297	1.5	0.13	-	-
Within-pop out.	2.219	0.472	297	4.71	<0.01	-	-
Between-pop out.	-	-	-	-	-	-	-
Intermediate POP							
Crossing						1.93	0.15
High POP							
Crossing						3.83	0.02
Selfing	-0.729	0.289	297	-2.52	0.01		
Within-pop out.	-0.646	0.288	297	-2.24	0.03		
Between-pop out.	-	-	-	-	-	-	-

Table S2 Results of accelerated failure-time analyses (log-logistic distribution) for seed germination of *S. ciliata* in the growth chamber.

Variable	d.f.	χ^2	*p*	*Estimate*
Intercept		1137.38	< 0.001	1.172 ± 0.035
Population	2	6.565	0.037	-
Pop L	1	2.58	0.108	0.079 ± 0.049
Pop I	1	0.03	0.853	-0.008 ± 0.044
Pop H	0	-	-	-
Crossing	2	19.026	<0.001	-
Selfing	1	5.1	0.024	0.183 ± 0.573
Within-pop out.	1	7.66	0.005	-0.201 ± 0.073
Between-pop out.	0	-	-	-
Population * Cross	4	9.019	0.061	
Scale				0.162 ± 0.006

Table S3 Accelerated failure-time analyses (Weibull distribution) of seedling survival of *S. ciliata* in each population at the end of the growing season in the field experiment.

Low POP	d.f.	χ^2	*p*	*Estimate*
Intercept	1	1212.47	< 0.01	2.15 ± 0.06
Crossing	2	16.01	< 0.01	
Selfing	1	15.5	< 0.01	-0.43 ± 0.02
Within-pop out.	1	0.47	0.49	-0.07 ± 0.09
Between-pop out.	0	-	-	-
Scale	1			0.43 ± 0.03
Intermediate POP	d.f.	χ^2	*p*	*Estimate*
Intercept	1	2009.46	< 0.01	1.97 ± 0.04
Crossing	2	8.14	0.01	
Selfing	1	6.96	0.01	-0.19 ± 0.07
Within-pop out.	1	4.07	0.04	-0.13 ± 0.07
Between-pop out.	0	-	-	-
Scale	1			2.94 ± 0.19
High POP	d.f.	χ^2	*p*	*Estimate*
Intercept	1	3764.53	< 0.01	1.81 ± 0.03
Crossing	2	1.7	0.43	
Scale	1			0.21 ± 0.02

Discusión General: Consecuencias y efectos genéticos del cambio climático en plantas de alta montaña mediterránea.

Se acumulan cada vez más evidencias que manifiestan como el cambio climático está actuando sobre la diversidad biológica del planeta en sus diferentes niveles de organización. La alta montaña mediterránea es uno de los escenarios idóneos para estudiar los efectos del cambio y sus consecuencias, así como las respuestas que se están desencadenando, tanto por sus características ambientales como por la diversidad de especies que la habitan. Saber como el cambio climático puede estar afectando la diversidad y estructura genética es una prioridad en este contexto máxime en un escenario como éste para el que las predicciones de cambios se encuentran entre las mayores esperables a nivel global. Si bien ya se han documentado las bases genéticas para algunas de las respuestas halladas en plantas a lo largo de gradientes altitudinales mediterráneos, éstos son muy fragmentarios, no tienen un marco teórico consistente, si no que son muy descriptivos, y no afectan a plantas de alta montaña. En esta tesis se han empleado varios tipos de aproximaciones genéticas en dos especies distintas, con marcadas diferencias en su biología, lo que nos ha permitido plantearnos la posibilidad de testar hipótesis específicas y completar la descripción de la variabilidad genética en estas especies.

La altitud condiciona los parámetros ambientales, alterando las precipitaciones, temperaturas, horas de luz, vientos, composición y profundidad del suelo, número de días con cobertura de nieve y fecha de deshielo etc. (Körner 2003; Nagy & Grabherr 2009). Todo ello hace que la altitud juegue un papel muy relevante en el valor que toman distintos parámetros y como se producen determinados procesos biológicos (Montesinos-Navarro et al. 2010): por ejemplo puede retrasar o adelantar el proceso de floración de una especie (Giménez-Benavides et al. 2007b), influir en el peso de las semillas (Wirth et al. 2010), cambiar la comunidad de polinizadores (Medan et al. 2002) o de especies vegetales (Walther 2010)… Todos estos cambios tienen que estar gobernados, de una u otra manera, por rutas de genes específicos, que estarían regulando estas reacciones (Reusch & Wood 2007; Ouborg & Vriezen 2007), aunque la fuente de esta adaptación y su forma de activarse sigue siendo desconocida en muchos organismos y procesos (Stapley et al. 2010). De esta forma, podemos afirmar que la altitud es un buen surrogado de lo que cabe esperar en relación con el cambio climático simplemente porque permite ver respuestas a condiciones ambientales muy diferentes que pueden ser relacionadas con los cambios climáticos esperables en el futuro. Obviamente esta aproximación no está exenta de dificultades si tenemos en cuenta que cualquier relación entre variables o parámetros genéticos y la altitud puede ser debido a un conjunto de procesos de gran complejidad y de difícil abordaje.

Aislamiento y fragmentación: flujo y estructura genética.

La fragmentación y el consiguiente aislamiento de las poblaciones es una de las principales consecuencias del cambio climático, que no ocurre solamente en alta montaña (Young et al. 1996). Sin

embargo, debido a las peculiaridades de estos sistemas, con la escasa capacidad de dispersión de algunas especies (Engler et al. 2009), la escasez de polinizadores (Körner 2003) o la reducción de hábitat disponible en altura (Körner 2007), hace que sea una de las principales amenazas a la viabilidad de plantas de alta montaña. Un aumento en la fragmentación y el aislamiento por desplazamiento hacia arriba como consecuencia del cambio de clima puede suponer un aumento de la dificultad para el movimiento de genes o flujo de genético entre poblaciones (Krajick 2004). Este flujo es el principal limitante para alguna de las posibles respuestas ante el cambio global como es la adaptación local (Kawecki 2008), dado que la llegada de genes de otras poblaciones no adaptadas diluye el efecto de genes pre-adaptados que se están seleccionando en poblaciones sometidas a condiciones ambientales extremas y que estarían reaccionando en este nuevo escenario mediante alguna de las estrategias descritas (adaptación, plasticidad…). Este precepto tiene especial relevancia en el caso de las poblaciones periféricas (Eckert et al. 2008) o marginales (para las montañas, las poblaciones en los extremos superior e inferior), sometidas a fuerzas de selección, que suelen recibir flujo génico de poblaciones centrales, limitando su respuesta y capacidad de adaptación (Kirkpatric & Barton 1997). Sin embargo, algunos autores han descrito que ciertos niveles de flujo génico parecen necesarios para mantener unos niveles suficientes de variabilidad genética en la población como base para ese cambio adaptativo e incluso no tendrían que estar influyendo significativamente para que algunas especies no estuvieran reaccionando por esta vía frente al cambio climático (Byars et al. 2007; Byars et al. 2009; Gonzalo-Turpin & Hazard 2009). Tanto en el Capítulo 2 como en el Capítulo 3 de esta tesis doctoral hemos encontrado evidencias de la existencia de un importante flujo genético entre las poblaciones, más o menos fragmentadas, tanto de *Armeria caespitosa* como de *Silene ciliata*. Sin embargo, también hemos observado que algunas poblaciones, situadas en zonas más marginales tienen parcialmente restringido el flujo genético y por lo tanto, presentan importantes diferenciaciones genéticas respecto a otras poblaciones (ver modelos de agrupamiento bayesiano en ambos capítulos).

Ante un importante incremento esperable de la fragmentación y aislamiento, las poblaciones tenderían a reducir su tamaño, dando lugar a unas condiciones que favorecerían la erosión genética. Este empobrecimiento génico estaría asociado a varios procesos, aunque normalmente es debido a fenómenos de deriva genética o depresión endogámica (Frankham et al. 2002). En casos extremos de descenso en la variabilidad genética, la llegada de genes de otras poblaciones puede ser beneficiosa en un primer momento (vigor híbrido, Frankham et al. 2002). Sin embargo, la introducción de genes de otras poblaciones no siempre favorecería el aumento de la persistencia, puesto que en posteriores generaciones, podrían romper complejos de genes coadaptados y supondría un gran descenso en los parámetros de bienestar de los futuros descendientes (fenómeno conocido como depresión exogámica, Edmands 2007). Este proceso podría darse en las poblaciones estudiadas en el capítulo 5, donde claramente los individuos procedentes de cruzamientos entre poblaciones mostraban mejores parámetros de supervivencia y crecimiento en las primeras fases de su ciclo vital, pero desconocemos que efectos

podrían darse en generaciones futuras. Estos resultados concuerdan con los obtenidos por Vilas et al., (2006; 2006) con otra especie del género *Silene* (*S. littorea*), donde también se encontraron evidencias de la depresión endogámica usando semillas procedentes de polinizaciones manuales y realizando un seguimiento en su hábitat original.

Son varios los trabajos que han alertado de la importancia de la dispersión en el caso de las plantas alpinas y de montaña (Thuiller et al. 2005; Engler et al. 2009), debido a que el actual marco de cambio climático estaría alterando las condiciones ambientales y forzaría a las especies a moverse o adaptarse. Para grandes especies de árboles, con mayor capacidad de dispersión (ver referencias en Savolainen et al. 2007) o especies con dispersión zoócora o anemócora de las semillas (p.e. García 2001), no tendría por qué suponer un gran inconveniente ya que podrían desplazarse con relativa facilidad. Por el contrario, en aquellas plantas alpinas que no han desarrollado estructuras específicas para su dispersión alarga distancia, la dispersión apenas alcanzan las decenas de centímetros (Stöcklin & Bäumler 1996). Ninguna de nuestras especies estudiadas muestran estructuras para dispersión a larga distancia y los estudios preliminares desarrollados mediante modelización inversa confirman esta tendencia (distancia media aproximada de dispersión de un metro, Carlos Lara y Juan Jose Robledo-Arnuncio, datos no publicados). En ambos capítulos donde se trata este tema, confirmamos la existencia de flujo genético entre las poblaciones estudiadas de ambas poblaciones, tanto a gran escala con grandes distancias entre poblaciones (Capítulo 2) como a una escala mucho más reducida (Capítulo 3). Si la dispersión de semillas es compleja, la dispersión del polen es el otro mecanismo que permite la dispersión del flujo génico, si bien en los hábitats alpinos se considera que muchas plantas se encuentran limitadas por la cantidad de polen disponible (Kevan 1972). Esta limitación está íntimamente relacionada con el número de polinizadores disponibles (Kevan & Baker 1983) por lo que se considera que la polinización mediada por insectos es un proceso crítico y muy importante en plantas alpinas (Körner 2003). Las especies modelo utilizadas en esta tesis siguen estrategias muy diferentes en cuanto a su floración y relación con los polinizadores (Giménez-Benavides et al. 2010). Por un lado, *Armeria caespitosa* puede ser polinizada por todo tipo de insectos (García-Camacho et al. 2009) aunque limitada por el hecho de ser una de las primeras especies en florecer (Giménez-Benavides et al. 2010) por lo que la comunidad de insectos disponible en ese momento suele ser muy pobre. Por otro, *Silene ciliata* presenta una relación de polinización-depredación con la polilla *Hadena consparcatoides*, aunque puede ser polinizada por otros polinizadores generalistas (Gimenez-Benavides et al. 2007). Su principal limitación viene por ser una de las últimas especies en florecer del sistema (Giménez-Benavides et al. 2010), cuando lo recursos se encuentran muy limitados. En ambos casos, no podemos descartar que la polinización por parte de un insecto pueda mediar en una dispersión a mayor distancia que la obtenida con las semillas.

Cuando se realizan estudios sobre especies alpinas y de montaña, resulta esencial tener en cuenta como han cambiado las condiciones en estas zonas a los largo de los último miles de años (Cuaternario). Los existencia de distintos periodos glaciares e interglaciares tienen como consecuencia la existencia de

periodos altamente dinámicos en cuanto a los cambios en las condiciones ambientales y en la distribución de las especies (p.e. Vargas 2003; Zhang et al. 2001). Existen bastante evidencias sobre como las plantas soportaron y reaccionaron ante estos cambios de condiciones, especialmente por los estudios desarrollados en los Alpes (Schönswetter et al. 2002; Schönswetter et al. 2005) y otras montañas de latitudes medias (Hewitt 2000). Por un lado, algunas especies se vieron forzadas a descender en altitud, buscando refugios más cálidos, huyendo de las frías temperaturas (Tabula-rasa hipótesis, Petit et al. 2003; Demesure 1996). Incluso algunas de ellas realizaron importantes movimientos de descensos latitudinales, lo que convirtió a las penínsulas del sur de Europa (Balcanes, Italiana, Ibérica etc.) en grandes refugios glaciares (Hewitt 1999). Desde ellas, tras la retirada de las masas heladas, iniciaron procesos de recolonización hacia el norte. Este modelo podría ser compatible con la existencia de un patrón de "salvamento" insular en el cual, otras especies pudieron sobrevivir en pequeñas islas rodeadas de hielo, conocidos como Nunataks (Nunatak hipótesis, Stehlik et al. 2002; Stehlik et al. 2001). Una vez se fueron retirando los glaciares, estos puntos sirvieron como punto de partida para la recolonización. Todos estos movimientos han tenido importantes consecuencias genéticas en las poblaciones y explican numerosas peculiaridades encontradas en las especies. Dado que los procesos colonizadores suelen iniciarse con unos pocos individuos, las nuevas poblaciones deberían mostrar bajos niveles de diversidad genética en la actualidad (Taberlet et al. 1998; Van Rossum & Prentice 2004), así como evidencias de la existencia de cuellos de botella genéticos. Estos bajos niveles pueden posteriormente elevarse significativamente si confluyen varios linajes provenientes de refugios independientes (Petit et al. 2003; Hewitt 2000). Por otro lado, las especies que sobrevivieron en nunataks suelen mostrar evidencias de endogamia y deriva génica y muestran patrones de estructura genética y diversidad muy diferentes a poblaciones que formaron los refugios meridionales (Stehlik et al. 2001; Bauert et al. 1998).

A pesar de los numerosos estudios publicados hasta la fecha sobre diversidad genetíca en plantas que sufrieron desplazamientos latitudinales, se desconoce en gran medida que ocurrió con aquellas poblaciones que permanecieron en estos refugios glaciares. Es posible que las especies pudieran ascender o descender montañas, así como remontar cursos de agua buscando condiciones ambientales óptimas (Hewitt 2001). Desenmarañar estos movimientos y aclarar su impacto en las poblaciones actuales es realmente complicado (Hewitt 2001). Un ejemplo claro de esta situación es el descrito por Gutierrrez-Larena et al. (2002), donde los movimientos de ascenso y descenso de varias especies del genero *Armeria* generó una especie híbrida por contacto entre progenitores con requerimientos ecológicos divergentes. En definitiva, todos estos procesos pueden enmascarar las situaciones reales respecto al estado genético de las poblaciones y deben de ser tenidas en cuenta a la hora de interpretar datos de flujo genético y variabilidad genética como los obtenidos en los Capítulos 2 y 3. Si estos movimientos se han producido, sin duda han servido para homogeneizar los valores de diversidad genética y aumentar, por ejemplo, el número de migrantes entre poblaciones, pero que no tendrían reflejo en las presentes condiciones de aislamiento hoy en día.

Diversidad genética

La variabilidad genética resulta esencial para aquellas poblaciones que están sometidas a distintas fuerzas selectivas, proporcionando el verdadero material donde la evolución puede actuar (Fischer 1930). Unos niveles altos de variabilidad significan un reservorio de genes o rasgos que, potencialmente, podrían conferir una ventaja adaptativa para esa especie o población en caso de cambios en las condiciones ambientales. En este sentido no sólo es importante conocer los valores de diversidad en una población, sino como ésta se distribuye entre las poblaciones de una especie y dentro de una población (Eckert et al. 2008) y los potenciales efectos que puedan tener en algunos parámetros de fitness, tanto en el presente (p.e. Leimu et al. 2006) como en el pasado (presencia de cuellos de botella genéticos, Frankham et al. 2002). La aproximación más frecuente para controlar el efecto de la diversidad genética es mediante experimentos que usen organismos que se reproduzcan asexualmente (p.e. clones, Hughes et al. 2008). Esto permite un evaluar el efecto directo del genotipo en cada replica experimental (Johnson et al. 2006; Reusch et al. 2005). Esta última aproximación basada en el uso de clones fue empleada en el capítulo 4, donde incluimos individuos genéticamente iguales y el factor genotipo fue controlado en el análisis. Esto nos permite confirmar que las diferencias que encontramos entre las poblaciones, permitiendo una mayor resistencia a la sequía, no se deben a unos individuos puntuales donde fueron medidos los parámetros de bienestar.

La forma más frecuente de estimar la diversidad genética de una población es mediante el empleo de marcadores moleculares neutrales, tipo microsatelites o AFLP, que aportan frecuencias alélicas discretas (Avise 2004). Esto restringe en cierta medida las posibles conclusiones que pueden obtenerse con su uso, dado que estos marcadores no siempre reflejan los procesos activos de selección (aunque algunos autores opinan que no tiene porque ser así, Thibert-Plante & Hendry 2010). En el caso de poblaciones situadas a lo largo de gradientes de elevación, resulta interesante conocer como se distribuye la diversidad genética a lo largo de este gradiente. Las fuerzas que determinan los niveles de variación genética, como mutaciones, deriva, flujo genético o selección natural (Lowe et al. 2004) pueden verse muy alteradas dentro de los gradientes, al variar los niveles de estrés ambiental en cada una de sus poblaciones. Ohsawa & Ide (2008) realizaron una revisión sobre los distintos patrones que puede seguir la variabilidad genética dentro de los gradientes altitudinales. La principal conclusión de su trabajo es que todas las distribuciones de la diversidad genética en poblaciones situadas a lo largo de un gradiente de elevación son posibles, si bien en latitudes intermedias (el caso de las montañas estudiadas en esta tesis) es más frecuente que poblaciones intermedias muestren una mayor diversidad. Sin embargo, en las montañas aquí estudiadas (Capítulos 2 y 3), no existió ningún patrón constante en las distintas montañas, a pesar que usamos dos especies diferentes y distintos tipos de marcadores. Parece obvio que la distribución genética a lo largo del gradiente de altitud es un fenómeno complejo (Williams & Arnold 2001) y que son muchos y muy variados los factores a nivel local que pueden influir. Además de los citados anteriormente, factores ecológicos o topográficos pueden servir como barreras dificulten la conexión entre

poblaciones (Ohsawa et al. 2008) o por el contrario, altos niveles de flujo genético, que diluyan posibles diferencias entre poblaciones, podrían homogenizar los valores de diversidad genética (Truong et al. 2007).

Cromosomas y tamaño del genoma

Son numerosas los trabajos que han relacionado el impacto del estrés abiótico (de distintos tipos) con el tamaño del genoma (Bennett & Leitch 2005). Los distintos niveles de estrés que se producen en los gradientes altitudinales también han sido recientemente relacionados con el tamaño del genoma, si bien el marco teórico disponible en este momento es bastante embrionario. Reeves et al. (1998) proponen que los genomas más pequeños aparecerán en lugares de mayor altitud, dado que las condiciones ambientales más duras favorecen a los genotipos con un menor tamaño. Como prolongación de este modelo, Knight et al. (2002) y (2005) proponen que el tamaño del genoma seguiría una distribución unimodal, por la cual tamaños mayores aparecerían en poblaciones intermedias mientras que las poblaciones situadas en ambos extremos, al estar sometidas a mayores niveles de estrés, disponen de un tamaño menor. Este modelo se ajusta a lo observado en el capítulo 1 de esta tesis, donde las poblaciones extremas, sometidas a mayor estrés ambiental, mostraron un tamaño del genoma inferior al de los individuos de poblaciones intermedias. La relación de la dotación cromosómica con los factores de estrés (y por consiguiente con la altitud) resulta más controvertida (Cui et al. 2006; Otto & Whitton 2000). Aunque el aumento del nivel de ploidía puede tener importantes costes fisiológicos para las plantas (Stebbins 1971), se considera que los poliploides tienen mayor potencial para adaptarse a nuevas condiciones (Leitch & Leitch 2008). En relación a la altitud, los estudios hasta la fecha son contradictorios (Liu et al. 2004; Mráz et al. 2008) aunque por norma general, se considera que los individuos poliploides son más frecuentes a altitudes mayores (Stebbins 1950). En el Capítulo 1 no encontramos diferencias en la dotación cromosómica de los individuos de *Silene ciliata* a lo largo del gradiente altitudinal. Podría ser interesante repetir este estudio en montañas con poblaciones más norteñas o con otros sustratos, donde si hay documentados fenómenos de poliploidía en *Silene ciliata* (Tutin et al. 1995; Blackburn 1933). Al ser una especie que permaneció de forma remanente en estas montañas, puede ser que únicamente los individuos diploides pudieran permanecer en las latitudes meridionales. Por el contrario, si es conocida la variación en cromosomas B en algunas especies, que habrían seguido sus propios caminos evolutivos dentro del gradiente altitudinal, como en el caso de *Crepis capillaris* (Parker et al. 1991), en el cual aparecen estos cromosomas más frecuentemente en poblaciones intermedias, desapareciendo en individuos que no se desarrollan en condiciones óptimas. Tampoco encontramos evidencias de este u otros fenómenos (aneuplodias, mixoplodias) en los cariotipos de *Silene ciliata*.

Fuerzas de cambio: loci bajo selección.

Aunque en la historia de la tierra se han producido numerosas épocas de cambios, la magnitud y la velocidad a la que se están produciendo los procesos actuales no se habían registrado en los últimos

cuatro millones de años (Overpeck et al. 2005; Loarie et al. 2009). Como lo han venido haciendo hasta la fecha, las especies reaccionan ante este escenario cambiante, evolucionando ante las nuevas fuerzas selectivas. Estos cambios no actúan aisladamente, sino de forma combinada (Drake et al. 2005), lo que dificulta encontrar relaciones directas entre uno o varios genes y una fuerza selectiva. La complejidad aumenta en el caso de no trabajar con especies modelos, de las cuales apenas se conoce información sobre su genoma y procesos internos, aunque no por ello deben de abandonarse las aproximaciones con éstas (Karrenberg & Widmer 2008). Reusch & Wood (2007) sugieren que primero deben de identificarse aquellos genes que están directamente asociados con rasgos que se puedan encontrar bajo selección, buscar la relación con el escenario del cambio climático y posteriormente, establecer la relación hasta el nivel de fenotipo. Un ejemplo de esta situación sería emplear aquellos genes directamente relacionados con la tolerancia ante el stress o procesos relacionados (protección de la célula, mantenimiento de estructuras etc.) que favorecen la supervivencia de fenotipos tolerantes. Los genes que pueden estar involucrados en los procesos de estrés generalmente implican una respuesta complicada (Zhu 2002), aunque cada vez se conocen más complejos de genes que responden de forma coordinada y para los cuales se conoce su forma de actuar, al menos en especies modelo como *Arabidopsis thaliana* (Shinozaki & Yamaguchi-Shinozaki 2007). Las proteínas de shock de calor (Heat-shock), han sido muy estudiadas, tanto en caso de animales (Feder & Hofmann 1999) como en plantas (Boston et al. 1996). Su presencia se ha documentado en situaciones de elevado estrés, protegiendo rutas metabólicas y y degradando proteínas rotas. En el Capítulo 4 de esta tesis intentamos una aproximación de este tipo, viendo respuesta fenotípicas de caracteres probablemente ligados a estrés hídrico con *Silene ciliata,* empleando para ello anticuerpos específicos de dos proteínas obtenidas de *Craterostigma plantagineum* y que están íntimamente relacionadas con el estrés hídrico y la protección de las estructuras de las células ante los distintos cambios que se producen (Röhrig et al. 2006). Si bien encontramos una reacción positiva ante estos anticuerpos (las plantas expresan dichas proteínas cuando están estresadas), no encontramos diferencias entre las poblaciones que estudiamos, aunque no podemos descartar que exista una diferencia en la expresión cuantitativa de dichas proteínas.

Un segundo enfoque para detectar el efecto de las fuerzas de selección sobre el genoma ha sido propuesto mediante la utilización de los loci outliers procedentes de los "Genome scans" (escaneos del genoma), que permite, mediante una aproximación estadística, seleccionar aquellos marcadores genéticos que indicarían grandes diferencias en estudios comparativos entre poblaciones utilizando modelos nulos de características bien conocidas como el de equilibrio H.W, por lo que si detectamos comportamientos desviantes es razonable suponer que estos productos se encuentren bajo algún tipo de selección (Beaumont & Balding 2004). Aunque este enfoque presenta ciertas limitaciones técnicas y resulta ser muy sensible ante determinadas asunciones, estimas demográficas y parámetros genéticos (Eckert et al. 2010), permite una aproximación hacia la existencia de algún proceso selectivo. Esta técnica esta siendo muy empleada buscando todo tipo de fuerzas de selección que puedan estar actuando sobre

el genoma. Esto incluye desde loci que están involucrados en procesos de adaptación a la altitud (Bonin et al. 2006), loci relacionados con la hibridación de especies (Minder & Widmer 2008) o loci relacionados con la adaptación a la salinidad del medio (Mäkinen et al. 2008). Esta aproximación se ve beneficiada cuando el número de marcadores con los que se trabaja es muy alto, como en el caso de los marcadores AFLP (Capitulo2). Nuestro enfoque fue similar al usado por Jump (2006) con *Fagus sylvatica*, donde encontraron una relación entre la presencia de un alelo con el gradiente de temperatura asociado a la altitud. Nosotros también encontramos ciertas relaciones entre los loci divergentes y el gradiente de elevación así como con algunas variables climáticas, aunque debe tomarse con precaución y únicamente como un posible punto de partida ante nuevas búsquedas de loci que pudieran estar relacionados con la altura (como propone Fischer et al. 2011).

Otro horizontes, secuenciación de nueva generación y nuevas aplicaciones.

El uso de las nuevas tecnologías está consiguiendo reducir los costes de secuenciación y el tiempo necesario para ello hasta unos niveles inimaginables hasta hace unos pocos años (Margulies et al. 2005). Esto permitirá no solo ampliar los estudios genéticos a cada vez más organismos a un coste menor (Ungerer et al. 2008), sino secuenciar cada vez un número mayor de genes y por que no, organismos enteros. Si bien esto plantea algunas limitaciones, como el tratamiento de tal volumen de información, las ventanas que se abren no han sido vistas hasta la fecha (se estima que con un único análisis de un secuenciador de última generación se obtendría más información que toda la disponible en el GenBank hasta hace 10 años Stapley et al. 2010). Ejemplos de estos enfoques novedosos son la aplicación de enfoques filogenéticos a nivel de comunidad que cohabitan en un mismo hábitat (Kress et al. 2009). Se considera que las tres preguntas necesarias para comprender los procesos de adaptación y que la genética puede ayudar a resolver son: ¿Son muchos loci los que influyen de forma leve en la adaptación o únicamente son unos pocos loci los que influyen de forma muy significativa? ¿Qué loci son los que permiten la adaptación? y ¿Cuál es la fuente de la variación genética adaptativa?

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