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DIFERENCIACIÓN DE HÁBITATS EN LAS AQUILEGIAS IBÉRICAS: IMPLICACIONES EN LA RADIACIÓN ADAPTATIVA DEL GÉNERO

PRESENTADA POR:
RAFAEL JAIME BUENO

DIRIGIDA POR:
DR. D. PEDRO J. REY ZAMORA
DR. D. JULIO M. ALCÁNTARA GÁMEZ

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Memoria presentada por

D. Rafael Jaime Bueno

para optar al Grado de Doctor por la Universidad de Jaén

Esta Tesis Doctoral ha sido realizada bajo la dirección de:

Dr. Pedro J. Rey Zamora y Dr. Julio M. Alcántara Gámez

Dr. Pedro J. Rey Zamora, Catedrático de Ecología, y Dr. Julio M. Alcántara Gámez Profesor Titular de Ecología, del Departamento de Biología Animal, Biología Vegetal y Ecología de la Universidad de Jaén.

Certifican:

Que el trabajo recogido en la presente Memoria, titulada: "*Diferenciación de hábitats en las Aquilegias Ibéricas: Implicaciones en la Radiación Adaptativa del género*", presentada por D. Rafael Jaime Bueno, ha sido realizada bajo nuestra dirección y autorizamos su presentación y defensa para optar al grado de Doctor por la Universidad de Jaén.

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Fdo. Dr. Pedro J. Rey Zamora Fdo. Dr. Julio M. Alcántara Gámez

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INTRODUCCIÓN GENERAL

Uno de los fenómenos que más ha contribuido históricamente al desarrollo de la teoría evolutiva es la observación de la existencia de conjuntos de especies que difieren solo sutilmente en algún carácter que es, sin embargo, el que les permite ocupar distintos ambientes, alimentarse de distintos recursos o desarrollar un despliegue sexual distinto. La interpretación de este fenómeno es definido en la actualidad como "Radiación Adaptativa". Este término se remonta a los trabajos de Lack (1947), Dobzhansky (1951) y, especialmente, Simpson (1953), los cuales llevaron a la formulación de lo que se conoce como Teoría Ecológica de la Radiación Adaptativa, que ha sido recientemente actualizada por Schluter (2000), que la define como el resultado de procesos de selección natural divergente, causada por diferencias ambientales y competencia por los recursos, que dan lugar a la rápida diversificación de un linaje. Según Schluter (2000), la demostración de que la diversificación taxonómica de un linaje es el resultado de un proceso de radiación adaptativa requiere la constatación de la concurrencia de cuatro hechos: 1) que los taxones involucrados proceden de un único ancestro común, 2) que las diferencias fenotípicas entre los taxones están relacionadas con las diferencias en los ambientes que ocupan, 3) que los caracteres fenotípicos diferenciados son objeto de selección en los ambientes que ocupa cada taxón, y 4) que la diversificación taxonómica (en última instancia, especiación) ha ocurrido de forma relativamente rápida, al menos a mayor velocidad que en otros linajes próximos.

El estudio de los procesos de radiación adaptativa se ha visto enormemente incrementado desde la publicación de la revisión realizada por Schluter (2000). No obstante, son muy escasos los estudios que han explorado la existencia de presiones selectivas divergentes entre ambientes, que explicarían la diferenciación fenotípica observada en la radiación. Esta carencia es especialmente llamativa dado que la juventud y

frecuente endemicidad de los taxones involucrados en una radiación hace que éstos sean sujetos particularmente adecuados para el estudio de procesos de divergencia adaptativa.

Entre los ejemplos mejor estudiados de radiación adaptativa en plantas se encuentra la del género *Aquilegia* (Ranunculaceae) en Norte América (Schluter, 2000, Hodges *et al*. 2004, Bastida 2010). Diversos estudios han mostrado que el linaje norteamericano, compuesto por una veintena de especies, procede de un único ancestro común (Hodges y Arnold, 1994; Ro y McPheron, 1997; Bastida, 2010), cuya tasa de diversificación fue muy superior a la de otros linajes próximos (Hodges, 1997). Las distintas especies difieren en caracteres florales. Esta variabilidad se correlaciona con el uso de distintos polinizadores (Hodges y Arnold, 1994), que pueden ser abejas, abejorros, mariposas colibrí (esfíngidos) o colibríes y, a su vez, determina variaciones en el éxito de polinización (Fulton y Hodges, 1999). La visión general que dan estos estudios es de que la radiación del linaje norteamericano ha tenido lugar mediante procesos de adaptación a distintos polinizadores y aislamiento reproductivo entre taxones también mediado por polinizadores. Una evidencia más a favor de esta visión es el hecho de que el grado de diferenciación en caracteres florales entre los taxones del linaje europeo es mucho menor, lo que está acorde con la menor diversidad de polinizadores en este continente (casi exclusivamente abejorros). Esta última evidencia, sin embargo, plantea un nuevo interrogante en cuanto a los patrones de radiación: dado que el número de taxones del género *Aquilegia* en Europa es semejante al de Norte América, aún cuando la diversidad de polinizadores es muy inferior, los procesos conducentes a la radiación del género en Eurasia (básicamente diferenciación fenotípica y aislamiento reproductivo) deben haber tenido una base distinta a la de la interacción con polinizadores (Bastida *et al*. 2010). Por tanto, el proceso

de radiación adaptativa del género no parece haberse repetido exactamente igual en ambos continentes.

Recientes trabajos realizados en el seno de nuestro grupo de investigación sugieren que la diversidad del género es el resultado de dos eventos de radicación independientes, uno que implica especies asiáticas y norteamericanas y otro que implica especies asiáticas y europeas (Bastida *et al*. 2010). Los resultados de Bastida *et al*. (2010) indican también que las aquilegias europeas, al igual que las norteamericanas, son un linaje monofilético que se ha diversificado rápidamente de un ancestro que probablemente ocupó montañas del centro-sur de Siberia. Sin embargo, los procesos ecológicos que han favorecido la radiación en ambos continentes han sido diferentes, siendo el aislamiento reproductivo y cambios en el uso del hábitat los que han actuado en Europa y el aislamiento reproductivo ligado a especialización en polinizadores en Norte América. Los resultados Bastida *et al*. (2010) para las *Aquilegias* Europeas, y los de Medrano *et al*. (2006), Alcántara *et al*. (2010) y Castellanos *et al*. (2011) para taxones de *Aquilegia* de la Península Ibérica, confirman que la diferenciación entre taxones europeos se basa más en caracteres vegetativos que en caracteres florales. Concretamente los resultados de Alcántara *et al*. (2010) demuestran que variables del medio abiótico como son la cobertura de roca del suelo y la altitud imponen selección divergente sobre caracteres vegetativos, y además estos patrones de selección parecen estar relacionados con su diversificación taxonómica. Más aún la diferenciación entre poblaciones de cada taxón en caracteres vegetativos parece estar relacionada con procesos de adaptación al ambiente abiótico, mientras que en el caso de los caracteres florales tal diferenciación parece responder más bien a procesos de deriva genética (Medrano *et al*. 2006, Castellanos *et al*. 2011).

Trabajos anteriores de Bastida (2009) han explorado algunas dimensiones del nicho ambiental, que parece que juegan un papel importante en la diferenciación fenotípica de las aquilegias ibéricas. Concretamente estas dimensiones son, por un lado, el tipo y profundidad del suelo, que diferencia a las dos principales especies de *Aquilegia* presentes en la península ibérica (*A.vulgaris* y *A. pyrenaica*) por su tolerancia a la variación edáfica, que parece estar relacionada con una distinta plasticidad adaptativa. Y por otro lado, la altitud, demostrándose la existencia de divergencia altitudinal de al menos dos subespecies de *A. vulgaris* (*A. v. vulgaris* y *A. v. nevadensis*) que coexisten en el sur de la península Ibérica. Además, también demostró que tanto la densidad de pubescencia no glandular como la fenología de germinación parecen estar relacionadas, a través de la diferenciación entre subespecies, con fenómenos de adaptación local. Sin embargo, encontró que la subespecie de amplia distribución (*A. v. vulgaris*) tiene plasticidad adaptativa en algunos rasgos, mientras que el taxón endémico (*A. v. nevadensis*) no. Estos resultados concuerdan con la hipótesis especialista versus generalista de divergencia de taxones (Ghalambor *et al*. 2007). Tomados en su conjunto estos hallazgos sugieren que en el proceso de radiación de las aquilegias ibéricas el aislamiento geográfico causado por la compleja historia biogeográfica del sur peninsular pudo provocar que poblaciones de taxones generalistas y plásticos se adaptaran finalmente a condiciones abióticas locales una vez sobrevenido su aislamiento, produciéndose selección divergente conducente a diferenciación ecotípica y diversificación taxonómica.

Tenemos, por tanto, cada vez más claro que el motor de diversificación de nuestras aquilegias habría sido la especialización en el ambiente abiótico, pero no se han explorado todavía todas las múltiples dimensiones (tanto abióticas como bióticas) que podrían conducir a diversificación de este género. Así, otros ejes ambientales que podrían

desempeñar un papel en la diferenciación de nicho y la diversificación de los taxones ibéricos del género *Aquilegia*, como son la dimensión propiamente climática, el estrés hídrico y lumínico y la herbivoría, no han sido aún explorados. La diferenciación de nicho y posible selección divergente impuesta por estos ejes son algunos de los frentes a abordar para seguir tratando de dilucidar los procesos de diferenciación taxonómica y divergencia de hábitats que ocurre en las aquilegias ibéricas. Estos frentes son detallados en el siguiente epígrafe dedicado a establecer nuestra hipótesis de partida y los objetivos a llevar a cabo en esta Tesis doctoral.

HIPÓTESIS DE TRABAJO, OBJETIVOS Y ESTRUCTURA GENERAL DE LA TESIS

El objetivo principal de esta tesis es contribuir a responder a la pregunta de qué ha promovido la diferenciación de nicho y la divergencia de hábitats entre los taxones específicos y subespecíficos de las *Aquilegias* ibéricas, y evaluar en qué medida dicha diferenciación de nicho y caracteres está conectada o es congruente con un proceso de radiación adaptativa. Nuestra hipótesis de partida es que, al contrario de lo que ocurrió en Norte América, en el caso de las *Aquilegias* ibéricas fue la especialización en el hábitat, mediada por caracteres vegetativos y eco-fisiológicos, y no la especialización en polinizadores, el principal motor de la radiación. Por tanto, ello debería manifestarse en claras segregaciones entre los taxones en las dimensiones abióticas del nicho, así como en una diferenciación acorde de caracteres. Para tratar de responder dicha cuestión, desarrollaremos 4 objetivos específicos, que se corresponden con los 4 capítulos centrales de esta memoria.

Objetivo 1. Caracterizar el nicho de los taxones y ejes ambientales que determinan la diferenciación de hábitats. Se trata de aclarar si los taxones de estudio realmente tienen nichos segregados en la actualidad, si lo solapan más o menos o si el nicho de un taxon es un subconjunto del nicho de otro. Este objetivo es cubierto en el capítulo 1 '*Complex patterns of environmental niche evolution in Iberian columbines (Gen. Aquilegia)*'. Para ello con la ayuda del software de modelación ecológica Maxent se construyó un modelo ecológico de nicho de 7 subespecies pertenecientes a las tres especies de *Aquilegia* presentes en la península ibérica. A partir de estos modelos se compararon los nichos ambientales (definidos por variables climáticas y de suelo) de las diferentes subespecies.

Objetivo 2. Explorar la existencia de variación entre taxones en la respuesta fisiológica (eficiencia de uso del agua) ante el estrés hídrico y

lumínico y su relación con la diferenciación de nicho entre taxones. Este objetivo se abordará en el capítulo 2 '*Gas exchange in response to water and light stresses contributes to habitat differentiation in Iberian Columbines*'. Para ello se evaluó el papel del estrés hídrico y lumínico como dimensiones que determinan la diferenciación de nicho entre 4 subespecies de aquilegias ibéricas (dos pertenecientes a *A. vulgaris* y otras dos a *A. pyrenaica*), mediante diferencias en su comportamiento relativo al intercambio gaseoso. Este objetivo implicó experimentos manipulativos en jardín experimental y seguimientos de la variación natural en condiciones de campo en parámetros de intercambio gaseoso por las plantas.

Objetivo 3. Explorar la existencia de variación entre taxones en la respuesta ante la herbivoría y su relación con la diferenciación de nicho entre taxones. Este es el objetivo central analizado en el Capítulo 3 '*Glandular trichomes as an inflorescence defence mechanism against insect herbivores in Iberian columbines*'. Para ello se evaluó, mediante experimentos manipulativos en campo, el papel defensivo de la pubescencia glandular de la inflorescencia contra pequeños insectos herbívoros, y su variación entre poblaciones y entre taxones en relación a la abundancia de herbívoros y la presión selectiva potencial. El experimento se llevó a cabo en 8 poblaciones pertenecientes a 4 subespecies de dos especies de *Aquilegia* ibéricas (*A. vulgaris* and *A. pyrenaica*).

Objetivo 4. Explorar la existencia de varianza genética aditiva y de varianza y covarianza genética en rasgos vegetativos y florales y su relación con la diferenciación taxonómica. Dicha exploración se realizará en el capítulo 4 '*The role of genetic constraints on the diversification of Iberian taxa of the genus Aquilegia*', para lo cual se obtuvieron parámetros de genética cuantitativa (varianza genética aditiva, heredabilidad) para 25 caracteres (vegetativos y florales) de 4 taxones

ibéricos de *Aquilegia*. Además, para dos caracteres vegetativos y uno floral se obtuvieron también las matrices de varianza y covarianza genética aditiva y correlaciones genéticas entre caracteres en cada subespecie.

Finalmente, los resultados obtenidos en esta tesis son integrados en una Discusión general, en la que también se incorpora los hallazgos obtenidos en otros estudios del grupo. En ella se discute el papel jugado por distintas dimensiones del nicho en la diferenciación fenotípica y de hábitats entre los taxones ibéricos de *Aquilegia* así como en qué medida los resultados obtenidos son congruentes con una hipótesis de radiación adaptativa por especialización en hábitat mediada por divergencia en caracteres vegetativos y eco-funcionales.

HISTORIA NATURAL DEL GÉNERO *AQUILEGIA*

Morfología del género. El género *Aquilegia* L., está incluido en la tribu *Isopyreae* dentro de la familia *Ranunculaceae*, formado aproximadamente por unas 80 especies y un alto número de taxones infraespecíficos (Munz, 1946; Nold, 2003). Se trata de herbáceas perennes (Fig. 1), que presentan una roseta de hojas basales con largos pecíolos. Las hojas suelen ser biternadas, pudiendo encontrar especies con hojas ternadas y triternadas. La superficie de las hojas puede ser más o menos pubescente glandular o bien tener un aspecto glauco, e incluso glabro. Según la especie, las inflorescencias miden desde unos 10-20cm hasta unos 150cm, y pueden presentar desde una única flor hasta varias decenas por inflorescencia. El tallo de la inflorescencia, al igual que las hojas, puede presentar pubescencia glandular. Los capullos florales son, al principio del desarrollo, colgantes, incluso en especies con flores erectas. El color predominante en las flores del género es alguna forma entre el azul, violeta y púrpura, siendo menos frecuentes las gradaciones de blanco, amarillo y rojo. Las flores son pentámeras, actinomorfas e hipoginas, y constan de cinco sépalos petaloideos, cinco pétalos alternando con los sépalos, y un grupo de estambres de 40-60 que pueden estar incluidos o no en la corola. Los estambres centrales normalmente están reducidos a estaminodios. Los pétalos constan de lámina y espolón. Los espolones son cónicos, más o menos alargados y curvados según la especie, y poseen una glándula nectarífera en su ápice. El fruto es un folículo con cinco carpelos libres (pudiendo ser en ocasiones mayor, 10 o 15). Los folículos en la madurez aparecen erectos, pueden presentar pubescencia glandular y los estilos pueden ser persistentes o no. El número de semillas es elevado, con un tamaño no superior a 2 mm y de color negro brillante. Véase Munz (1946) o Nold (2003) para más detalles.

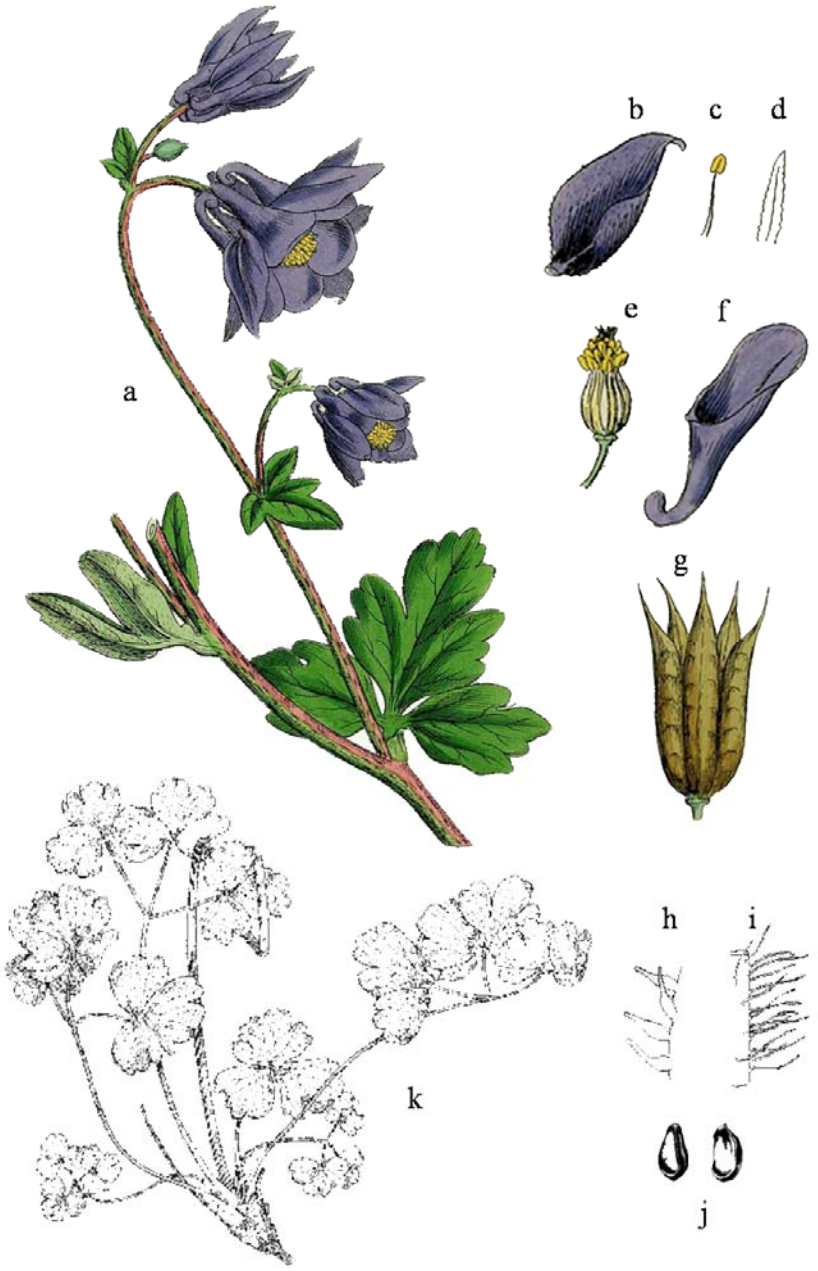

Figura 1. Ilustración de *Aquilegia vulgaris* subsp. *vulgaris* mostrando el aspecto habitual de las especies del género. a) inflorescencia; b) sépalo; c) estambre; d) estaminodio; e) detalle de la flor sin perianto; f) espolón; g) fruto; h) detalle de pubescencia glandular; i) detalle de pubescencia no glandular; j) semillas; k) roseta de hojas basales. (Fuente http://delta-intkey.com/angio/www/ranuncul.htm)

Sistemas de reproducción. Los miembros del género *Aquilegia* son autocompatibles y con un alto grado de interfertilidad interespecífica. Son capaces de producir gran número de semillas generadas por autogamia en ausencia de polinizadores, aunque esto varía a escala geográfica, al igual que el grado de hercogamia (Herlihy *et al*. 2002, 2004, 2005; Mavraganis *et al*. 2001). Estos autores han constatado que existen altos niveles de depresión por endogamia. Dentro del género encontramos tanto especies protoginas (*A. yabeana*) como protándricas (*A. coeruela*) (Huang *et al*. 2004; Brunet *et al.* 1998).

Las aquilegias pueden ser polinizadas por tres grandes grupos de polinizadores, por lo que se puede decir, que presentan tres tipos distintos de síndromes de polinización, ornitofilia, esfingofilia y melitofilia. Este último síndrome sería el más extendido dentro del género (mayoría de especies euroasiáticas y algunas norteamericanas). En este caso los principales polinizadores serían abejas y abejorros. Las flores con este tipo de síndrome suelen ser azules o púrpuras, colgantes, el espolón recto o ganchudo y de tamaño inferior a los espolones de las flores con los otros dos tipos de síndromes, y con sépalos grandes (Hodges *et al*. 2003).

Otro de los síndromes sería la esfingofilia, donde los agentes polinizadores son polillas. Este síndrome es frecuente en especies de Norteamérica, aunque en especies euroasiáticas se han observado visitas ocasionales de este tipo de polinizadores, no por ello son consideradas especies esfingófilas. Las flores esfingófilas presentan coloraciones que van desde el blanco hasta el amarillo, pasando por el azul y colores pálidos. La posición de la flor suele ser erecta, los espolones son rectos, y los más largos que encontramos entre las diferentes especies de *Aquilegia* y el sépalo también es de un tamaño relativamente grande. En este caso de síndrome, la recompensa para el polinizador es el néctar, que suele producirse al amanecer o al anochecer, coincidiendo con el hábito de los diferentes tipos de polillas.

Por último, la ornitofilia que es exclusiva de algunas especies americanas, siendo los colibríes los principales polinizadores. Las flores suelen presentar colores rojos, rojo-amarillos o naranjas, la posición de ésta suele ser colgante, los espolones rectos y cortos y los sépalos de pequeño tamaño (Grant 1993b; Hodges *et al*. 2003).

Distribución geográfica y ecología. El género está presente en las regiones templadas de todo el hemisferio norte (Eurasia y Norteamérica), tiene una distribución holártica (Fig. 2). Según Grant (1952, 1993b) y Bastida *et al*. (2010), el origen del género parece estar en Asia, hace unos 10 millones de años. Hacia el final del Mioceno, el género se dividió en dos linajes que emigraron desde el centro de Asia hacia el este y el oeste, de forma que hacia el Plioceno medio (hace unos 2-3 millones de año) un linaje cruzó el estrecho de Bering hacia Norte América y otro avanzó por los Urales hacia Europa (Bastida *et al*. 2010). Existen tres centros principales de diversificación del género: regiones montañosas del sur de Europa (Alpes, Balcanes, Córcega, Cerdeña y la Península Ibérica), la región montañosa de Tien Shan en el extremo occidental de China y en los sistemas montañosos de las Rocosas, Sierra nevada y los desiertos de Sonora o Mohave en el suroeste de Norteamérica. Debido a que el área de distribución en Asia abarca zonas de accesibilidad complicada, y que en Europa varias especies han sido descritas recientemente, cabe la posibilidad de que el número de especies del género este subestimado.

Las especies de *Aquilegia* ocupan gran variedad de hábitats, como bosques, paredones rocosos, prados de alta montaña, estepas y desiertos (Fig. 3). En lo que se refiere al rango altitudinal, éste varía desde el nivel del mar hasta unos 4000 m de altitud. La mayoría de especies crecen sobre sustratos básicos (generalmente calcáreos), aunque algunos taxones pueden ocupar sustratos ácidos, siempre que estos estén muy lavados. La mayoría de taxones del género ocupan sustratos permanentemente

húmedos (márgenes de ríos y arroyos, fuentes o paredones húmedos), siendo menos frecuentes los taxones que habitan en lugares umbríos y frescos en los que la humedad edáfica no es permanente (grietas de rocas, cantiles, lapiaces). Por lo general se trata de especies alopátricas, es decir, se trata de especies que raramente se pueden encontrar creciendo juntas. Así, las especies y subespecies del género están formadas por pequeñas poblaciones muy aisladas (Strand et al. 1996), que parecen segregarse por diferencias en factores abióticos (Chase y Raven, 1975). Aunque el aislamiento geográfico es lo habitual entre taxones, éstos retienen un alto grado de inter-fertilidad (Prazmo, 1965). Sin embargo, en las ocasiones en que dos taxones solapan su distribución se ha apreciado que existen barreras a la hibridación natural, basadas tanto en diferencias en los agentes polinizadores (Fulton y Hodges, 1999), como en segregación fenológica (Medrano *et al*., 2006; obs. pers.).

En la Península Ibérica existen 3 especies del género *Aquilegia* (Fig. 4). *Aquilegia vulgaris*, con 4 subespecies (*A. v. vulgaris*, *A. v. dichroa*, *A. v. nevadensis* y *A. v. paui*), con rangos de distribución coincidentes con los principales sistemas montañosos de la Península Ibérica. *Aquilegia pyrenaica* tiene una distribución más reducida, con 4 subespecies distribuidas por los Pirineos (*A. p. pyrenaica*), sistemas montañosos Pre-Pirenaicos (*A. p. guarensis*), cordillera Cantábrica (*A. p. discolor*) y el extremo oriental de las cordilleras Béticas (*A. p. cazorlensis*). Y por último, *Aquilegia viscosa*, representada por un único taxón (*A. v. hirsutissima)*, endémica del sur de Francia y de las estribaciones orientales de los Pirineos.

Figura 2. Distribución mundial del género *Aquilegia*.

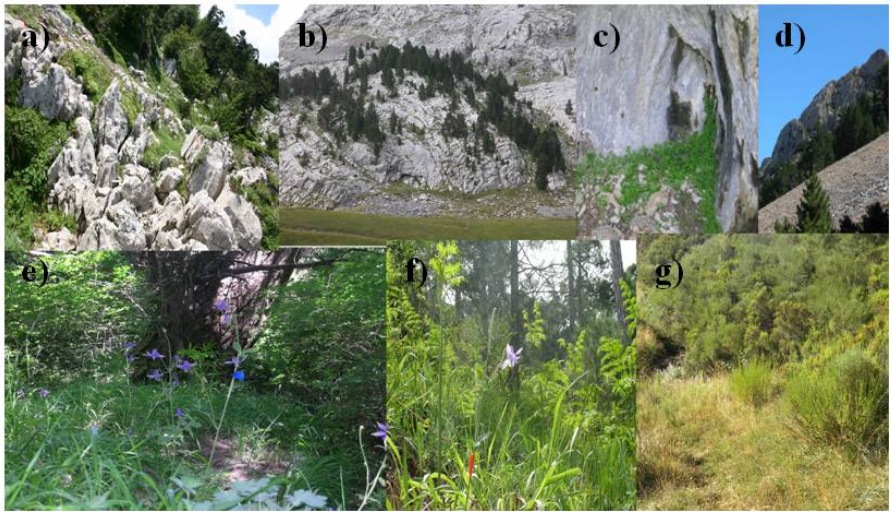

Figura 3. Ejemplos de hábitats ocupados por algunas de las aquilegias ibéricas: a) y b) *A. pyrenaica pyrenaica*. c) *A. pyrenaica. cazorlensis*. d) *A. pyrenaica guarensis*. e) *A. vulgaris vulgaris*. f) *A. vulgaris nevadensis*. e) *A. vulgaris dichroa*.

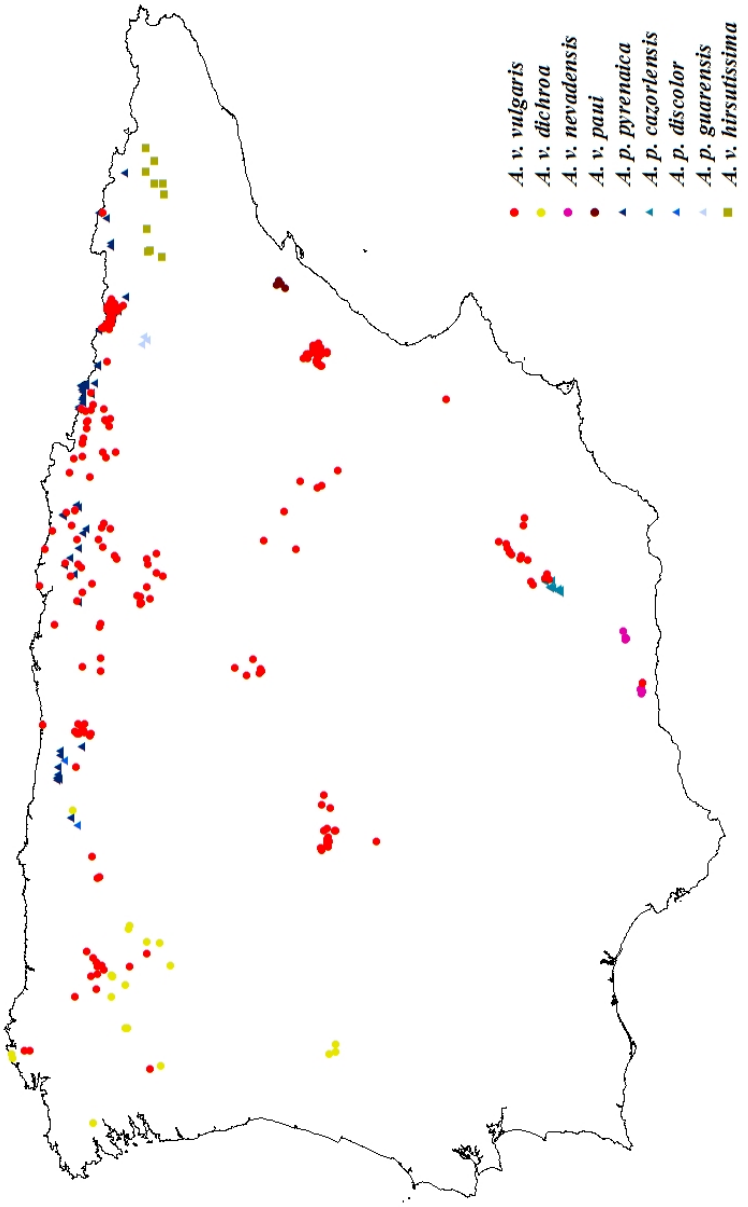

Figura 4. Distribución de los taxones ibéricos del género *Aquilegia*. Citas obtenidos del Proyecto Anthos http://www.anthos.es/, GBIF http://www.gbif.es/ y datos propios.

METODOLOGÍA GENERAL, ÁREA Y ESPECIES DE ESTUDIO

MÉTODOS GENERALES

Aunque distintos aspectos de esta memoria de Tesis son tratados con un número diferente de taxones, en su conjunto, este estudio ha manejado información de 7 subespecies pertenecientes a las 3 especies ibéricas del género *Aquilegia* (anteriormente citadas): *A. vulgaris* subsp. *vulgaris*, *A. vulgaris* subsp. *nevadensis*, *A. vulgaris* subsp. *dichroa*, *A. pyrenaica* subsp. *pyrenaica*, *A. pyrenaica* subsp. *discolor*, *A. pyrenaica* subsp. *cazorlensis* y *A. viscosa* subsp. *hirsutissima*.

Esta Tesis ha recabado una gama considerable de tipos de información recopilada en campo, laboratorio y jardín experimental. El trabajo en el Jardín Experimental de la UJA (JEUJA) ha sido fundamental para 3 de los 4 capítulos de esta Memoria (Capítulos 2-4), habiéndose desarrollado el 2 y el 4 en su mayor parte en tales instalaciones. La amplia gama de tipos de información de esta memoria pueden resumirse en 4 grupos: (1) Datos de campo e información de bases de datos sobre distribución de los taxones (Proyecto Anthos -http://www.anthos.es/- y GBIF -http://www.gbif.es/-) que fue utilizada para la modelación del nicho en la Península Ibérica mediante diferentes software de modelación ecológica (MAXENT, ENMtools) y sistemas de información geográfica (ArcGis). (2) Datos observacionales de campo en poblaciones de distintos taxones, que concierne tanto a la caracterización del ambiente biótico (abundancia e intensidad de herbivoría) y abiótico (caracterización lumínica, humedad del suelo, etc) de poblaciones, como a la caracterización de respuesta ecofisiológica y reproductiva de la planta. (3) Datos de genética cuantitativa a partir de progenies procedentes de cruces entre plantas diseñados a tal efecto, lo que conllevó la cría y generación de familias en jardín experimental de múltiples ejemplares de 5 taxones durante varios años y la obtención posterior de medidas de caracteres florales y vegetativos de estas plantas en laboratorio. (4) Datos

procedentes de manipulación experimental (tanto en campo como en jardín experimental) del ambiente abiótico (estrés lumínico e hídrico) y biótico (herbivoría) en que se desarrollan las plantas y sus consecuencias en el crecimiento, respuesta ecofisiológica y/o éxito reproductivo.

Mientras el modelado de nicho realizado en el Capítulo 1 ha considerado todos los taxones arriba indicados, el estudio experimental y la información ambiental recabada en campo se ha centrado principalmente en las subespecies *nevadensis* y *vulgaris* de *A. vulgaris* y las subespecies *pyrenaica* y *cazorlensis* de *A. pyrenaica* (Capítulos 2 y 3). Para el estudio de la existencia de variación inter-taxa en diferentes caracteres (vegetativos, florales y funcionales) de estos taxones, durante la primavera-verano de 2008, 2009 y 2010 se muestrearon un subgrupo de 13 poblaciones (Tabla 1), pertenecientes a 4 de las subespecies estudiadas, a las que se les tomó información relacionada con el ambiente que ocupan y se les midieron diferentes caracteres, analizados posteriormente mediante métodos estadísticos convencionales.

Además de un estudio "*in situ*" de las poblaciones, se recolectaron semillas procedentes de una única población de cada una de 5 subespecies (*A. vulgaris* subsp. *vulgaris*, *A. vulgaris* subsp. *nevadensis*, *A. vulgaris* subsp. *dichroa*, *A. pyrenaica* subsp. *pyrenaica* y *A. pyrenaica* subsp. *cazorlensis*), para su posterior siembra en las instalaciones del Jardín Experimental de la Universidad de Jaén. A partir de estas semillas se obtuvieron progenitores cuyas progenies crecidas en el jardín fueron la base para la experimentación de los Capítulos 2 y 4.

ÁREAS Y ESPECIES DE ESTUDIO

Aquilegia vulgaris está ampliamente distribuida a lo largo de numerosos bosques de montaña de Europa. En la Península Ibérica, esta especie engloba a cuatro subespecies (*vulgaris*, *dichroa*, *paui* y *nevadensis*) (Díaz González, 1986), de las cuales tres se han empleado en el presente estudio: *A. v. vulgaris*, de amplia distribución por la península, *A. v. dichroa* presente exclusivamente en el cuadrante noroeste de la península *y A. v. nevadensis*, un endemismo de Sierra Nevada, Sierra de Baza y Sierra Tejeda (véase Figura 4). Estas plantas crecen a lo largo de los márgenes de arroyos o en pequeñas surgencias de agua de prados de montaña, situados en orlas de bosques o matorrales umbrosos, entre 0 y 2500 metros de altitud. Su floración tiene lugar desde mayo hasta julio, variando su fenología dependiendo de la subespecie, así las subespecies *vulgaris* y *dichroa* son más tempranas que *nevadensis*.

Otra de las especies objeto de este estudio es *A. pyrenaica*. En la Península Ibérica aparecen las subespecies *pyrenaica*, *discolor*, *cazorlensis* y *guarensis* (Díaz González, 1986), de las cuales en este trabajo se han estudiado las tres primeras. La subespecie *pyrenaica* ocupa los Pirineos y Este de la Cornisa Cantábrica, *discolor* aparece en la zona Oeste de la Cornisa Cantábrica, mientras que *cazorlensis* es un endemismo de las Sierras de Cazorla, Segura y las Villas (Figura 4). Las poblaciones de *A. pyrenaica* ocupan, por lo general, pequeñas grietas de rocas siempre que estén sombreadas, a los pies de cantiles umbrosos y en pastos pedregosos calcáreos, entre 1200 y 2250 metros de altitud. En este caso la floración tiene lugar de junio a julio, con mayor solapamiento fenológico entre subespecies que en el caso de *A. vulgaris*.

Y la última especie objeto de este trabajo es *A. viscosa* compuesta por dos subespecies en la Península Ibérica, *hirsutissima* y *montsicciana*, esta última subespecie no estudiada en este trabajo. *A. viscosa* subsp.

hirsutissima se distribuye por el pirineo catalán sobre gleras y pedregales calcáreos desde los 1900 a los 2350 metros de altitud (Figura 4) y su floración tiene lugar durante el mes de julio.

Tabla 1. Subgrupo de poblaciones muestreadas durante la primavera-verano de 2008-2010, pertenecientes a 4 de las subespecies estudiadas. En negrita se muestran las poblaciones de las cuales se recolectaron las semillas utilizadas para la generación de las progenies, destinadas a los experimentos de genética cuantitativa (Capítulo 4).

Especie	Subespecie	Población	Zona	Hábitat	Altitud	Coordenada UTM
A. vulgaris	*vulgaris*	**Fte. Reina**	S. Cazorla	Bonal en bosques de *Pinus nigra*	1325m	30S5147/ 41995
A. vulgaris	*vulgaris*	Cabrilla	S. Cazorla	Bonal en bosques de *Pinus nigra*	1690m	30S5187/ 41976
A. vulgaris	*vulgaris*	Garrotegordo	S. Segura	Arroyos y narcisales en bosques de *Pinus nigra*	1115m	30S5335/ 42293
A. vulgaris	*vulgaris*	Jabalises	S. Segura	Arroyos y narcisales en bosques de *Pinus nigra*	1390m	30S5363/ 42288
A. vulgaris	*nevadensis*	**Pradollano**	S. Nevada	Arroyos alpinos en claros de bosque	2110m	30S4646/ 41058
A. vulgaris	*nevadensis*	Dúrcal	S. Nevada	Arroyos alpinos	1912m	30S4564/ 41032
A. vulgaris	*nevadensis*	Cortijuela	S. Nevada	Claros de bosque húmedos	1780m	30S4579/ 41032
A. pyrenaica	*pyrenaica*	**C-Tobazo**	P. Aragonés	Prados y roquedos alpinos calizos	1676m	30T7015/ 47397
A. pyrenaica	*pyrenaica*	C-Tortiellas	P. Aragonés	Prados y roquedos alpinos calizos	1685m	30T7009/ 47397
A. pyrenaica	*pyrenaica*	Larra	P. Navarro	Claros de bosque alpinos	1570m	30T6796/ 47588
A. pyrenaica	*cazorlensis*	**Bco. Canal**	S. Cazorla	Grietas umbrías	1405m	30S5034/ 41825
A. pyrenaica	*cazorlensis*	Bco. Charca	S. Cazorla	Paredes y roquedos húmedos	1245m	30S5119/ 41994
A. pyrenaica	*cazorlensis*	Cabañas	S. Cazorla	Paredes y oquedades umbrías	1790m	30S5038/ 41849

Lamina 1. Tomando medidas de intercambio gaseoso con IRGA.

Lamina 2. Progenies sembradas en las instalaciones del Jardín Experimental de la UJA, utilizadas para la realización de los capítulos 2, 3 y 4 de esta memoria.

Lamina 3. Aplicando el tratamiento de remoción de la pubescencia para el desarrollo del capítulo 3 de esta memoria.

Lamina 4. El Dr. Bastida tomando una muestra de suelo.

RESULTADOS

CAPÍTULO 1

Complex patterns of environmental niche evolution in Iberian columbines (Gen. *Aquilegia*).

Introduction

Understanding the process of niche evolution, the series of changes in niche parameters that accompany the process of taxonomic diversification within a lineage, is fundamental to understand the origin and maintenance of diversity (Knouft et al. 2006). The geographical distribution of a species has much to do with its ranges of tolerance to environmental abiotic conditions, especially in the case of plants, so the detailed analysis of the geographic range of a plant species can be used to infer the properties of its environmental niche. Many studies are recently exploiting this relationship in ecological, evolutionary and conservation contexts (Pearson and Dawson 2003, Peterson 2003, Kozak et al. 2008, Peterson 2011), thanks to the availability of georreferenced large-scale datasets of environmental variables (e.g. climate, land use, topography, soil) integrated into ecological niche modelling (ENM) algorithms (Elith et al. 2011).

The theoretical finding that niche evolution under natural selection should be slow led to the hypothesis of phylogenetic niche conservation (Peterson et al. 1999) whereby closely related species should tend to retain similar ecological characteristics over evolutionary time. Niche conservation is a component of non-adaptive radiation as defined by Gittemberger (1991; see also Rundell and Price 2009): the proliferation of species not accompanied by relevant niche differentiation. Evidence is accumulating suggesting that niche conservation is indeed a common pattern of niche evolution (Wiens and Graham 2005, Peterson 2011). However, niche conservation must not be confounded with niche stasis. Niches do evolve, and can do so in complex ways, being this particularly prominent in cases of adaptive radiation (Schluter 2000). For example, Knouft et al. (2006) described, in a small lineage of 11 lizards within the genus *Anolis*, cases of niche conservation, divergence, convergence (i.e. niche similarity between distantly related taxa) and specialization or

nesting (i.e. the niche of one taxon is a subspace of the niche of a sister taxon). Most likely, many radiations contain elements of both adaptive and non-adaptive diversification (Rundell and Price 2009).

One of the best known examples of adaptive radiation in plants is the genus *Aquilegia* (Ranunculaceae) in North America (Schluter, 2000; Hodges et al., 2003). According to Bastida et al. (2010), the diversity of the genus is the result of two recent and independent events of radiation, one involving Asiatic and North American species (North American lineage) and the other involving Asiatic and European species (Euroasiatic lineage). The radiation of the North American lineage has taken place through processes of reproductive isolation between taxa mediated by adaptation to different pollinators (Hodges and Arnold, 1994). This does not seem to have occurred in the Euroasiatic lineage of the genus which shows a much lesser degree of differentiation in floral traits and much lower pollinator diversity (Bastida et al. 2010). These contrasting processes of diversification pose a new question about radiation patterns in the genus. Since the number of taxa in the Euroasiatic and North American lineages is similar, the processes leading to the radiation in Eurasia must have had a basis other than interaction with pollinators. Unlike what happened in North America, where columbines speciation appears to be associated to sympatry, diversification in the Euroasiatic lineage occurred mostly in allopatry, suggesting that processes of geographic isolation have been critical in the diversification of the genus in this continent.

The present study explores the patterns of niche evolution in a group of 7 columbine taxa (three species, two of them represented by 3 subspecies each) from the Iberian Peninsula. These taxa are known to be under divergent patterns of selection across environments (Alcántara et al. 2010), what suggests the hypothesis that their taxonomic differentiation involved processes of adaptation to different local environments, and so,

niche differentiation should be more common than niche conservation in this group. To test this hypothesis we use methods of niche comparison based in ENMs (Warren et al. 2010) and on multivariate environmental gradients (McCormack et al 2009). Our results confirm that the process of diversification in the Iberian columbines involves complex patterns of niche conservation, divergence, convergence and specialization.

Material and Methods

Ecological Niche Modelling

Study species and occurrence data.- This study focused on seven subspecies of the three species of the genus *Aquilegia* present in the Iberian Peninsula: *A. vulgaris* (subspecies *vulgaris*, *nevadensis* and *dichroa*), *A. pyrenaica* (subspecies *pyrenaica*, *discolor* and *cazorlensis*) and *A. viscosa* subsp. *hirsutissima* (Díaz González, 1986). Other two subspecies present in the Iberian Peninsula (*A. v. paui* and *A. p. guarensis*) were excluded for the present study because they have too few occurrence data (<10). Díaz González (1986) tentatively recognized the subspecies *hispanica* of *A. vulgaris*, but due to its uncertain taxonomic status we considered this taxon within *A. v. vulgaris*. With the exception of *A. v. vulgaris,* which occurs through Central and Eastern Europe, all the studied taxa are endemic to the Iberian Peninsula. Occurrence data covered the fullest extent possible of the geographic range of each taxon (Fig. 5), and were obtained by direct observation and from the databases of Anthos project (http://www.anthos.es/) and Global biodiversity information facility in Spain (http://www.gbif.es/). We only included occurrence points with available UTMs with a resolution of at least 1 x 1 km. We obtained a total of 346 presence points for the 7 subspecies (Table 2).

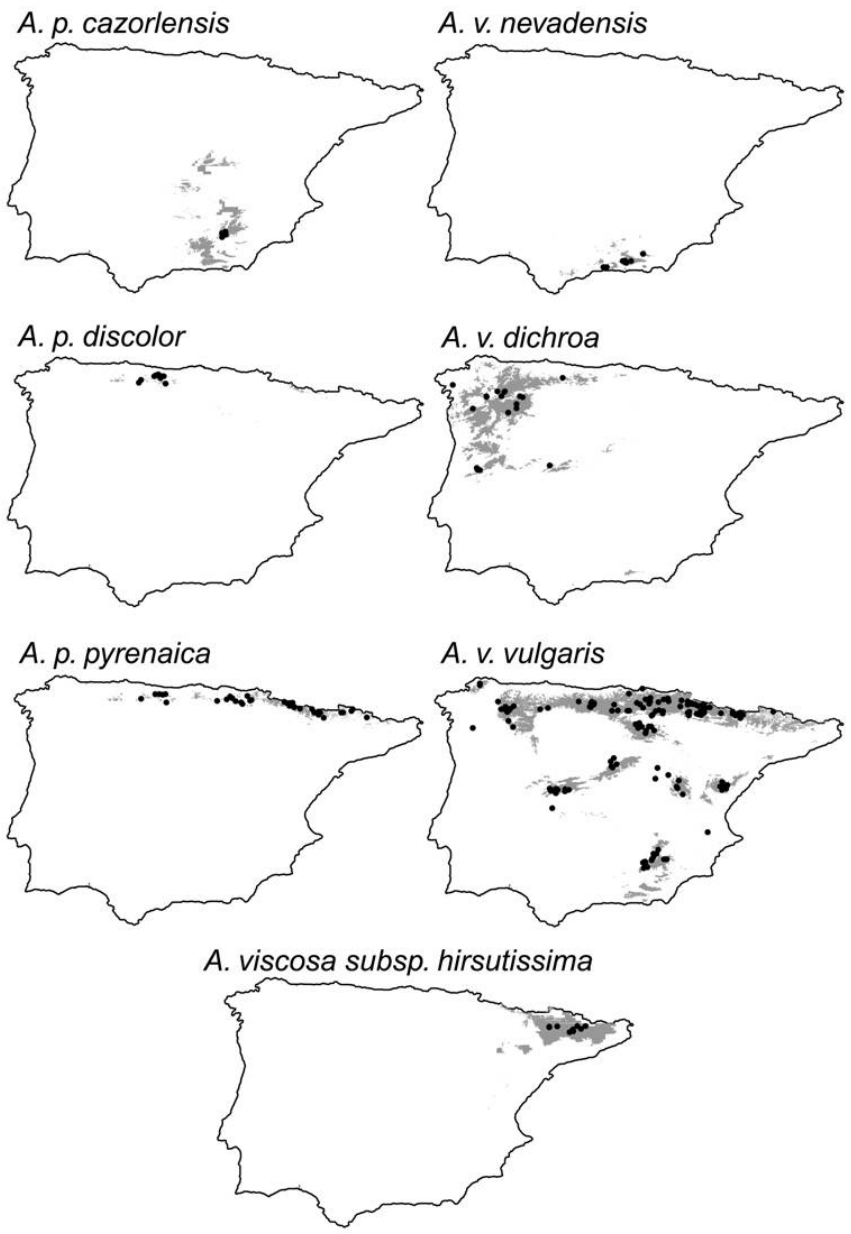

Figure 5. Occurrence points for 7 Iberian taxa of the genus Aquilegia. The shaded areas correspond to the geographic distribution of areas with suitability scores higher than 0.2 according to the Environmental Niche Model of each taxon fitted with Maxent.

Table 2. List of variables and their contribution to the Environmental Niche Model (ENM) of the study taxa generated with Maxent. The number of occurrence points used in fitting the ENM is indicated under each taxon name. The permutation importance of each estimate is indicated in brackets.

Environmental Variable	Code	*A. v. vulgaris* 226	*A. v. dichroa* 20	*A. v. nevadensis* 11	*A. p. pyrenaica* 58	*A. p. discolor* 10	*A. p. cazorlensis* 11	*A. viscosa* 10
Annual Mean T^a	Bio1	30.1 (1.1)	0 (0)	0 (0)	4.5 (0)	6 (0)	0 (0)	0 (0)
Mean Diurnal Range	Bio2	1.7 (2.7)	0 (0)	25.8 (52.4)	0.2 (0)	0 (0)	0 (0)	0 (0)
Isothermality	Bio3	8.7 (2.5)	0 (0)	0 (0)	2.3 (0.9)	17.3 (4.3)	0.1 (0)	0 (0)
T^a Seasonality	Bio4	2.2 (1.5)	0.3 (0.2)	0.8 (2.6)	0.8 (18.3)	0.6 (0)	1.6 (40.8)	1.5 (7)
Max T^a of Warmest Month	Bio5	12.1 (2.4)	1.2 (7)	0 (0)	0 (0)	3.9 (0)	0 (0)	0 (0)
Min T^a of Coldest Month	Bio6	0.8 (0.5)	0.3 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
T^a Annual Range	Bio7	2.1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	41.8 (0)	0 (0)
Mean T^a of Wettest Quarter	Bio8	5 (2.7)	42 (84.8)	5.6 (0)	3 (0)	15 (0)	0 (0)	1.2 (0)
Mean T^a of Driest Quarter	Bio9	1.2 (2.2)	0 (0)	0 (0)	3.1 (0.7)	0.1 (0)	0 (0)	59.3 (1.2)
Mean T^a of Warmest Quarter	Bio10	1.7 (1.2)	7 (0)	0 (0)	0 (0)	41 (95.5)	0 (0)	0 (0)
Mean T^a of Coldest Quarter	Bio11	0.1 (2.5)	0 (0)	0 (0)	0.3 (38.2)	0 (0)	0 (0)	0 (0)
Annual Precipitation	Bio12	1 (4.9)	3.8 (0)	0 (0)	0.1 (0)	0 (0)	0 (0)	0 (0)
Precipitation of Wettest Month	Bio13	0.7 (1)	3.5 (2.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Precipitation of Driest Month	Bio14	1.8 (1.2)	3.2 (0)	0 (0)	57.4 (0)	0 (0)	0.3 (0)	0 (0)
Precipitation Seasonality	Bio15	3.7 (20)	0 (0)	11.2 (0)	11.3 (31.9)	0.4 (0)	0 (0)	0 (0)
Precipitation of Wettest Quarter	Bio16	0.5 (0)	7.7 (0)	0 (0)	0.7 (6.8)	0 (0)	0 (0)	0 (0)
Precipitation of Driest Quarter	Bio17	7 (29.4)	0 (3.3)	5.1 (10.3)	11 (0)	0 (0)	2 (7.3)	0 (0)
Precipitation of Warmest Quarter	Bio18	3.4 (7.9)	0.1 (0.3)	0 (0)	0.2 (1.4)	0 (0)	0.5 (0)	24.6 (53.5)
Precipitation of Coldest Quarter	Bio19	2.3 (4.7)	25.2 (0)	0.5 (0.7)	1.1 (0)	0 (0)	0.5 (1.2)	0 (0)
Altitude	Alt	11.4 (10.6)	0 (0)	51 (34)	2.7 (0)	8.3 (0)	35.8 (39.4)	2 (0.4)
Topsoil pH	PH	1.9 (0.4)	0 (0)	0 (0)	1.1 (1.2)	0.3 (0)	14 (10.7)	7.1 (2.1)
Soil Moisture Storage Capacity	Moist	0.1 (0.2)	5.6 (1.8)	0 (0)	0 (0)	0 (0)	0.1 (0.2)	0 (0)
Effective Soil Depth	Depth	0.4 (0.4)	0 (0)	0 (0)	0.2 (0.4)	6.9 (0.2)	0.3 (0)	2.6 (34.1)
Topsoil Carbon/Nitrogen Ratio	C/N	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (0.4)	1.7 (1.6)

Environmental variables.- Our analyses of the environmental niche included 23 environmental variables (Table 2), which were handled in ArcGIS 9.3 (ESRI, Redlands, California, USA). We collected information on 19 climatic variables and altitude from the Worldclim database (http://worldclim.org), and 4 soil variables from the GeoNetwork database (http://www.fao.org/geonetwork). Climatic variables have 30 × 30 arc-seconds resolution. However, the resolution of the soil variables is 5 x 5 min so we converted these layers to cell size of 30 × 30 arc-seconds. The environmental layers were cut to span the whole Iberian.

Niche Models.- Occurrence data and environmental variables were used to generate ENMs with the program Maxent version 3.3.3e (Phillips et al., 2006). Maxent uses a probability distribution of maximum entropy to predict approximate species´ niche and potential geographic distributions from presence data (Phillips et al., 2006; McCormack et al., 2010; Warren et al., 2008; Glor and Warren, 2011; see in Elith et al., 2011). Maxent was run for each species using the default setting (Phillips et al., 2006; Phillips and Dudik, 2008), except that 25% of the occurrence localities were used for testing the model performance. We focused our ENMs analyses in the Iberian Peninsula.

Testing for niche divergence and conservation

Our main aim was to compare the ecological niches among the studied taxa. To this end, we used two approaches, the first uses ENMtools (Warren et al. 2010), and second is conducted with multivariate methods. Both use data from species occurrence points and other points from within the region inhabited by the species. Using only an ENM-based approach might overlook smaller, but nonetheless important ecological differences, while the multivariate method provides more detailed information on niche divergence, as it is in better keeping with the Hutchinsonian idea of the niche as a multidimensional hypervolume (Hutchinson 1957), in

which some axes will remain conserved while others diverge (McCormack et al., 2010).

Test of niche equivalency using ENMtools.- Following Warren et al. (2008), we estimated niche overlap between two subspecies using Schoener's *D*, a measure of similarity of the potential geographic distribution of two species:

$$D(P_X,P_Y) = 1 - 1/2 \sum_i | P_{X,i} - P_{Y,i} |,$$

where, $P_{X,i}$ (or $P_{Y,i}$) is the probability of occurrence of species X (Y) in cell *i* according to the ENM. Schoener's *D* ranges from zero (i.e. no niche overlap) to one (i.e. identical niches; Warren et al., 2008). This index was originally proposed by Schoener to quantify niche overlap in terms of diet or microhabitat use (Schoener 1968). In its general form this index measures the similarity between two probability vectors of equal size, so it has a long tradition in the ecological literature (Renkonen 1938, Keyfitz 1968, Whittaker 1975).

To assess whether the ENMs of two subspecies are significantly different we use the equivalence test as proposed by Warren et al. (2008). The equivalence test consists in taking *n* occurrence points at random from each of two taxa to construct a pooled dataset. This pooled data are then shuffled randomly and partitioned in two pseudoreplicate sets of *n* cases. Niche models are created from each pseudoreplicate and these are compared using *D*. This process is repeated 100 times to create a null distribution of *D*-values under the hypothesis that the ENMs are equivalent. We can conclude that two ENMs are significantly different (i.e. there is niche differentiation between the compared taxa) when the observed value of *D* is below the lower limit of the 95% confidence interval of this null distribution.

Background similarity test.- The equivalence test may fail when the niche of one taxon is underestimated, which is more likely for narrowly

distributed taxa (Warren et al. 2008, McCormack et al. 2010). A taxon may occupy a small geographic range either because its environmental niche is very narrow or because it cannot disperse to (or has become extinct in) other places with suitable abiotic environmental conditions. In the first case the ENM would be correct and the range of occurrence would be embedded in a geographical context of abiotically unsuitable environments (i.e. unsuitable environmental background). In the second case, there could be suitable environmental backgrounds within the reach of the taxon, but such places are not currently occupied for some reason; in this case the ENM would likely underestimate the true environmental niche of the taxon. Since several of our study taxa have a narrow distribution, we complement the results of equivalence test with a test of niche similarity (Warren et al. 2008) that takes into account the differences between the environmental backgrounds of each taxon.

The background similarity test compares the observed niche overlap (using Schoener's *D*) of two taxa (A and B) to a null distribution of 100 overlap values generated by comparing the ENM of one taxon (e.g. taxon A) to an ENM created from *n* random points drawn from the geographic range of the other taxon (i.e. the background of B), where *n* equals the number of occurrences of taxon B. This process is then repeated for both taxa in the comparison, so two null distributions are generated per analysis (A vs. background B and B vs. background A). One critical decision in this analysis concerns the definition of the background geographic area. Ideally, the background area should include accessible habitats and therefore should reflect information on dispersal ability (Soberón and Peterson, 2005). Given the low dispersal capabilities of *Aquilegia*, we generated narrow background areas of 3 km radius around each known occurrence locality.

The null hypothesis of the background similarity test states that observed niche overlap between taxa is explained by regional similarities

in available habitat (background environments). This hypothesis involves a two-tailed test, so it is rejected if the observed *D* between two taxa falls outside of the 95% confidence limits of the null distribution. Niche conservation is supported when niches are more similar than expected based on their background environments (i.e., species are occupying niches that are as similar as possible given what is available), so the observed value of *D* is larger than the upper 95% confidence limit of the null distribution. Niche divergence is supported when niches are more divergent than expected based on background divergence (the observed value of *D* is smaller than the lower 95% confidence limit of the null distribution).

Interpretation of the background tests can be complicated, since it involves a two-way test that can render contradictory results in each direction (Warren et al. 2010, Nakazato and Warren 2010, Couvreur et al. 2011, Rödder and Engler, 2011). Such contradictory conclusions are not uncommon but most authors just dismiss them as non conclusive. However, such contradictory results may provide insights on niche specialization/generalization. We illustrate this with hypothetical examples in figure 6. First note that the background of a species is likely to include a wider range of environments than its set of occurrence localities, so we can assume that the geographic distribution predicted by the ENM of a species is geographically nested within the prediction based in the ENM generated from its background. Suppose now a widespread generalist species and a specialist one with a narrow environmental niche nested within the niche of the generalist. The projections of the ENMs would form a nested set, with the projected distribution of the specialist (which we denote by S) nested within the projection based on its background (S'), which is nested within the projected distribution of the generalist (G), and all nested within the projection based on the background of the generalist (G'). The overlap between S' and G would

be larger than the overlap between S and G, what would be interpreted as niche divergence (i.e. the similarity between the observed niches is smaller than expected based on their backgrounds). On the other way around, the overlap between G' and S would be smaller than the overlap between S and G, what would be interpreted as niche conservation (i.e. the similarity between the observed niches is larger than expected based on their backgrounds).

Test of niche similarity using multivariate analyses.- Data for the 23 environmental variables were extracted for occurrence points and for a random sample of 50% of background points of each taxon with ArcMap. The 23 variables were reduced with Principal Component Analysis of the correlation matrix, using Statistica 7.0 (StatSoft 2007). We obtained 4 principal components (environmental axes) which explained 86.14% of total variance. We used one-tailed Student-t tests to compare mean factor scores between subspecies (presence data), which indicate whether the observed niche distance between taxa (d_n) along an environmental axis is significantly higher than zero.

To complement the conclusions of the t-test, we conducted the following randomization test. We built 1000 random samples, each formed by 50% of randomly chosen background points, from each taxon to obtain a null distribution of mean distances between the backgrounds of each pair of taxa (d_b). To determine the meaning of d_n (i.e. whether it indicates niche conservation or divergence) we compared its value with the value of d_b. According to McCormack et al. (2010), niche conservation can be concluded when d_n is smaller than d_b. However, this conclusion is only valid if the environmental backgrounds of the compared taxa overlap (Peterson 2011). Under the assumption that the dispersal abilities of a taxon confer it the potential to reach every point within its background area, overlapped environmental backgrounds would

indicate that both taxa have places available with the same environmental niche, implying that niche conservation can be detected. In this case, if d_n is not significantly different from zero, we can conclude that both taxa occupy the same niche (i.e. there is niche conservation) along the environmental axis under analysis (Fig. 7a). On the other hand, if d_n is significantly higher than zero we can conclude the there is niche divergence between the taxa (Figs. 7b to g). When the environmental backgrounds do not overlap, d_n is always significantly different from zero. However, it would not be totally correct to infer in this case that the niches are truly divergent, because the lack of background overlap may prevent the expression of the true environmental niche of one or both taxa. Therefore, in the absence of background overlap, we should conclude: i) that niche divergence is apparent when $d_n < d_b$ (Fig. 7e) because the niches are different, but more similar than what could be expected based on background divergence (McCormack et al. 2010 considered this combination as evidence of niche conservation, but we prefer to call it apparent divergence because the niches are actually different); or ii) that there is niche divergence when $d_n \geq d_b$ (Fig. 7f, g) because the niches are more different than they could be according to the background environments available.

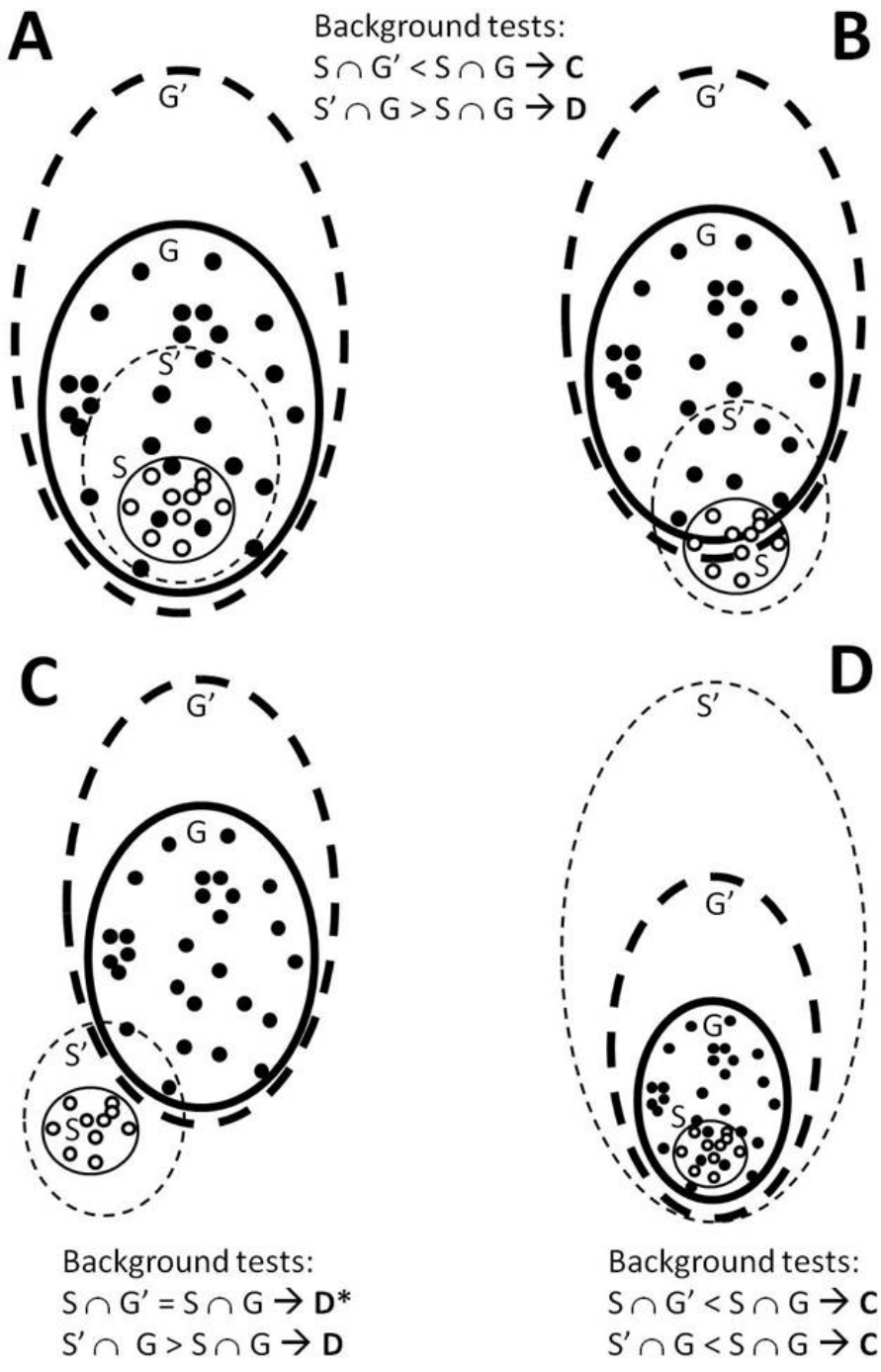
A
Background tests:
S ∩ G' < S ∩ G → C
S' ∩ G > S ∩ G → D
G'
G
S'
S
B
G'
G
S'
S
C
G'
G
S'
S
Background tests:
S ∩ G' = S ∩ G → D*
S' ∩ G > S ∩ G → D
D
S'
G'
G
S
Background tests:
S ∩ G' < S ∩ G → C
S' ∩ G < S ∩ G → C

Figure 6. Idealized results of an environmental niche model illustrating how the background test from MAXENT can be interpreted in the case of nested ENMs. Each figure represents the occurrence locations of a generalist and a specialist taxon (black and white points respectively), their respective predicted distributions (thick and thin solid lines, respectively), and the distribution predicted by an ENM based on their background areas (thick and thin dotted lines respectively). G and S are, respectively, the area covered by the ENM of the generalist and the specialist. G' and S' are, respectively, the area covered by the ENM generated from the background of the generalist and the specialist. For each figure we show what the results of the background test would look like and the inferred conclusion. The results of the background test are indicated as the area of the intersection (indicated with the intersection symbol $\cap$) between the two ENMs (this is largely equivalent to Schoener's *D*, but is easier to interpret). The conclusions of the background test are indicated as C (niche conservation) or D (niche divergence). D***** indicates that the conclusion on divergence would not be based in the background test (because S/G' = 0) but in the absence of overlap between S and G (S/G = 0). The relative size of G, G', S and S' is maintained constant in figures A, B and C. In figure D, the relative size of G, G' and S is the same as in the other figures, but the size of S' is larger, indicating that the specialist is now placed in a more heterogeneous background than the generalist, so S' > G'. When the specialist and its background are totally (A) or partially (B) nested within the generalist, the background tests render contradictory conclusions (niche conservation is inferred in one direction and divergence in the other direction).

a)
b)
c)
d)
ENVIRONMENTAL NICHE AXIS
Sp. 1 - Sp. 2
NICHE CONSERVATION
NICHE DIVERGENCE
e)
f)
g)
NICHE DIVERGENCE IMPOSED OR APARENT)

Figure 7. Criteria to infer niche conservation or differentiation from tests based on observed and background environmental distances between taxa. Each panel represents the observed mean (white point) and range of values for two taxa, and the mean (black point) and range of values of their respective background areas. Means and ranges are represented along a vertical environmental niche axis. Inference on niche divergence or conservation between two taxa depends on whether their environmental backgrounds overlap (panels a to d) or not (panels e to g). When the backgrounds overlap, the niches of two taxa are free to evolve to any range of similarity. In this case we can infer that there is niche conservation when niche distance is not significantly different from zero (a), or that there is niche divergence when the niche distance between two subspecies is significantly different from zero regardless of whether their niche distance is higher than the distance of their backgrounds (b), niche and background distance are the same (c), or even if niche distance is lower than background distance (d). However, when the backgrounds do not overlap the niches of two taxa are not free to evolve (i.e. the niches are necessarily differentiated), so niche conservation cannot be inferred unambiguously because the observed niches are significantly different. A niche distance smaller than the background distance suggests that the niches tend to be more similar than they could (i.e. there would be a trend towards niche conservation) but this trend does not suffice to maintain the niches similar, so we infer that niche divergence is imposed or apparent (e). On the contrary, if niche distance is larger (f) or equal (g) than background distance, then the niches have evolved to be more different than they could, so we can classify these instances as niche divergence.

Results

Distribution from Ecological Niche Modelling

Maxent distributions conformed closely to actual distribution of the study taxa (Fig. 5). Maxent performed reasonably well in generating predicted niches. The area under the receiver operating characteristic curve (AUC) ranged from 0.936 to 0.999, and the threshold of equal sensitivity and specificity was relatively small (average 35.6) for the most of the species. The binomial probabilities for all species at this threshold were significantly better ($< 10^{-4}$) than the random expectation.

The relative contribution of environmental variables in ENM construction is indicated in Table 2. In each taxa's ENM, only 2 or 3 variables contributed more than 10% to the model, and there was scarce agreement between taxa in which variables are the most relevant. Three variables related to temperature regime (Temperature Seasonality, Minimum Temperature of Coldest Month and Mean Temperature of Coldest Quarter), three related to the rainfall regime (annual precipitation, precipitation of the wettest month and precipitation of the wettest quarter), and three soil variables (soil moisture storage capacity, effective soil depth and topsoil carbon/nitrogen ratio) contributed less than 10% to the ENMs.

Testing for niche divergence and conservation using ENMtools

Equivalence test between pairs of subspecies showed niche divergence in all cases but *A. p. pyrenaica* and *A. p. discolor* (Table 3). The mean values of Schoener's *D* were much larger in comparisons between sympatric than between peripatric and allopatric taxa (0.27 ± 0.13, 0.18 ± 0.08 and 0.03 ± 0.03 respectively; means ± SD), so niche similarity decreases as geographic overlap decreases. Within the sympatric taxa the values of *D* were twice as higher in comparisons between conspecific subspecies than between heterospecific subspecies (0.39 ± 0.07 and 0.19 ± 0.09

respectively). On the other hand, in the case of allopatric taxa, comparisons between conspecific and heterospecific subspecies had similarly low values of *D* (0.03 ± 0.05 and 0.03 ± 0.02 respectively) while in the case of peripatric taxa, heterospecific comparisons showed more similarity (0.19 ± 0.08) than the single comparison between conspecific taxa (*D* = 0.09). However, because such divergence may be partly due to latitudinal climatic differences, we carried out background similarity tests (Table 3, Fig. 8). Out of 5 comparisons between sympatric taxa, 3 showed evidence of niche conservation, one suggested niche divergence and the other was not conclusive. In turn, in 10 comparisons between allopatric taxa, 6 showed evidence for niche divergence and 3 for niche conservation (one was not conclusive). In the case of peripatric taxa, two tests suggested niche conservation, another two were not conclusive and two rendered contradictory results. Considering only comparisons within species (i.e. between conspecific subspecies), those background tests that afforded some conclusive results suggest niche conservation among sympatric subspecies and niche divergence among allopatric subspecies. Equivalence and background tests suggest that sympatric conspecific taxa show niche conservation while allopatric conspecific taxa have different niches. On the other hand, heterospecific taxa are equally likely to show similar or different niches regardless their degree of geographic overlap. It is worth mentioning the particular results of the comparison between the two endemic taxa from the southeast of the Iberian peninsula (*A. p. cazorlensis* and *A. v. nevadensis*), that showed one of the highest similarity *D*-values in spite of being heterospecific and peripatric, and also showed support for niche conservation in the background test.

Table 3. Results of analyses testing for the existence of niche convergence or divergence among Iberian columbines. For each pair of taxa we indicate their degree of geographic range overlap and whether the comparison is between subspecies of the same (within) or different (between) species. For each comparison we provide the value of niche equivalency of Schoener's *D* (significantly greater than expected according to randomization tests are in bold), as well as the results of niche similarity tests controlling for background differences. For tests comparing the distribution of pairs of taxa along multivariate axes of environmental variation (PCs) we indicate the distance between subspecies means along each axis, whether such distance was significantly different from zero according to Bonferroni-corrected one-tailed t-tests ($P < 0.0006$; with asterisk), and whether their environmental backgrounds overlap (in bold); the qualitative conclusion of each comparison is indicated as D (significant niche divergence), C (niche conservation), A (apparent divergence) or NC (not conclusive).

Pairwise comparison	Range Overlap	Comparison	Schoener *D*	Background Test	PC1	PC2	PC3	PC4
A. v. vulgaris - *A. v. dichroa*	Sympatric	Within	0.437	C ($P < 0.01$) - C ($P < 0.05$)	**0.05 C**	**2.11* D**	**0.42 C**	**0.73 C**
A. v. nevadensis - *A. v. dichroa*	Allopatric	Within	0.086	NC - D ($P < 0.01$)	**1.10* D**	**2.19* D**	**1.45* D**	**0.67 C**
A. v. nevadensis - *A. v. vulgaris*	Peripatric	Within	0.095	NC – NC	**1.05* D**	**0.08 C**	**1.87* D**	**0.06 C**
A. p. discolor - *A. p. pyrenaica*	Sympatric	Within	**0.335**	NC - C ($P < 0.01$)	**0.14 C**	**0.07 C**	**0.60* D**	**0.16 C**
A. p. cazorlensis - *A. p. discolor*	Allopatric	Within	0.007	NC - NC	2.33* D	0.64* A	**0.22 C**	**0.43 C**
A. p. cazorlensis - *A. p. pyrenaica*	Allopatric	Within	0.00026	D ($P < 0.01$) - NC	2.46* D	**0.57* D**	**0.82* D**	**0.59* D**
A. v. vulgaris - *A. p. discolor*	Sympatric	Between	0.158	D ($P < 0.01$) - D ($P < 0.01$)	**1.02* D**	**0.10 C**	**1.08* D**	**0.34 C**
A. v. vulgaris - *A. p. pyrenaica*	Sympatric	Between	0.297	NC - NC	**1.16* D**	**0.03 C**	**0.48* D**	**0.50 C**

A. p. cazorlensis - *A. v. vulgaris*	Sympatric	Between	0.119	C (P < 0.01) - C (P < 0.01)	**1.30* D**	**0.54* D**	**1.30* D**	**0.09 C**
A. v. dichroa - *A. p. discolor*	Peripatric	Between	0.127	NC - NC	**0.97* D**	**2.01* D**	**0.66* D**	**1.07 C**
A. v. dichroa - *A. p. pyrenaica*	Peripatric	Between	0.14	NC - C (P < 0.01)	**1.11* D**	**2.08* D**	**0.06 C**	**1.23* D**
A. p. cazorlensis - *A. v. nevadensis*	Peripatric	Between	0.338	C (P < 0.01) - NC	**0.25 C**	**0.45 C**	**0.57 C**	**0.03 C**
A. p. discolor - *A. v. nevadensis*	Allopatric	Between	0.01	D (P < 0.01) - D (P < 0.05)	**2.08* D**	**0.18 C**	**0.79* D**	**0.40 C**
A. p. cazorlensis - *A. v. dichroa*	Allopatric	Between	0.045	C (P < 0.01) - NC	**1.35* D**	2.65* D	**0.88* D**	**0.64 C**
A. v. nevadensis - *A. p. pyrenaica*	Allopatric	Between	0.0006	D (P < 0.05) - D (P < 0.01)	**2.21* D**	**0.11 C**	**1.39* D**	**0.56* D**
A. viscosa - *A. p. pirenaica*	Peripatric	Between	0.168	D (P < 0.01) - C (P < 0.01)	**1.13* D**	**0.43* D**	**1.52* D**	**0.02 C**
A. viscosa - *A. p. discolor*	Allopatric	Between	0.067	D (P < 0.01) - D (P < 0.01)	**0.99* D**	0.50* D	**2.12* D**	**0.18 C**
A. viscosa - *A. p. cazorlensis*	Allopatric	Between	0.041	NC - C (P < 0.01)	**1.33* D**	**0.14 C**	**2.34* D**	0.61* A
A. viscosa - *A. v. vulgaris*	Peripatric	Between	0.2	D (P < 0.01) - C (P < 0.01)	**0.02 C**	**0.40* D**	**1.04* D**	**0.52* D**
A. viscosa - *A. v. dichroa*	Allopatric	Between	0.058	NC - C (P < 0.01)	**0.02 C**	2.51* D	**1.46* D**	**1.25* D**
A. viscosa - *A. v. nevadensis*	Allopatric	Between	0.015	D (P < 0.01) - NC	**1.08* D**	**0.32 C**	**2.91* D**	**0.58* D**

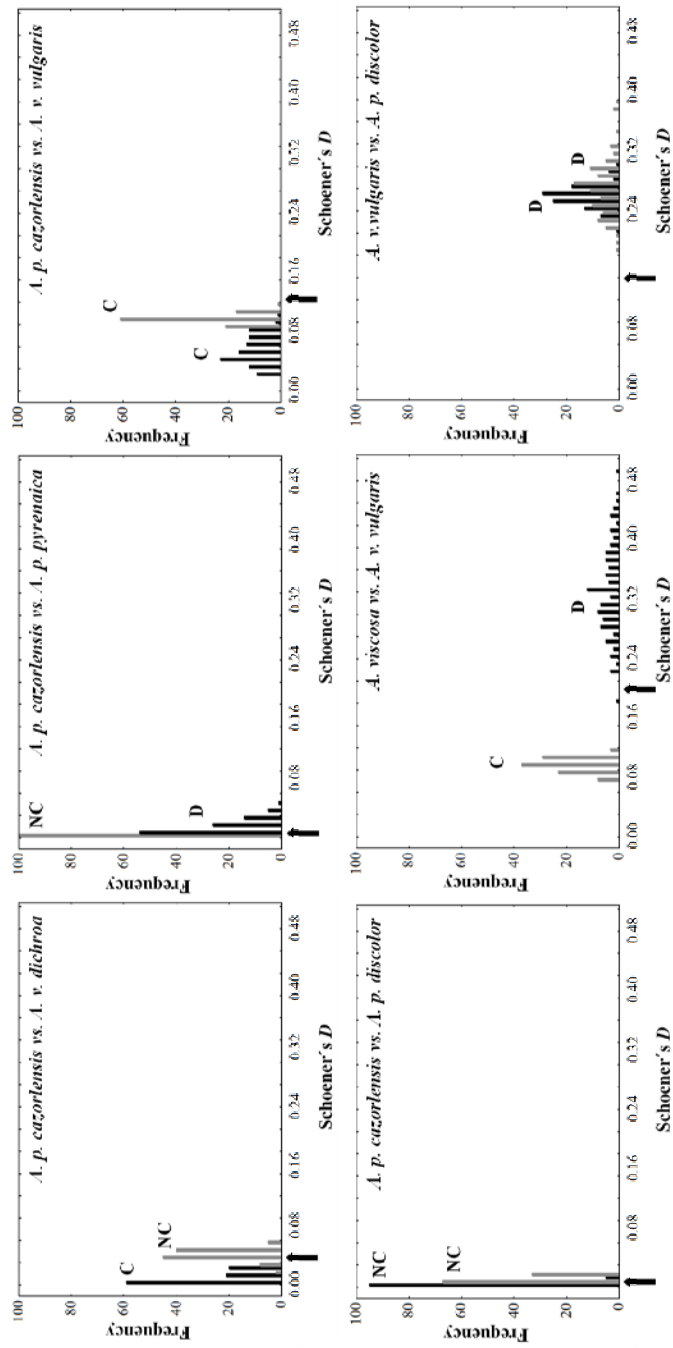

Figure 8. Examples of frequency histograms from tests of niche conservation and divergence from analysis of observed and 'null' niche models using ENMTools. Each histogram represents a pairwise comparison between two listed taxa, where the environmental niche models (ENM) for taxon A is compared with the background points from taxon B and vice versa. Schoener´s *D* values (niche overlap, indicated by an arrow) that are smaller than the null distribution of background support niche divergence (D). Niche conservation (C) is supported when the niche overlap values are larger than the null distribution. When niche overlap value is similar to the null distribution background we cannot conclude (NC).

Testing for niche divergence and conservation using multivariate analyses

To gain a deeper insight on which components of the environmental niches have diverged among taxa, we conducted multivariate tests. We identified four factors in the principal components analysis, which explained 86.14% of the total variation (see Table S1.1 in Appendix). The first axis (PC1) explained most of the variation (46.76%). This axis assigns more positive scores to localities with higher mean annual temperature and hot-dry summer, so it largely describes a geographical gradient from the hot-dry Mediterranean climate in the south to the cool-wet temperate climate in the north of the Iberian Peninsula. PC2 axis explained 17.09% of the variation. This axis assigns more negative scores to localities with colder and dryer winter at higher altitude. This approximately corresponds with a geographical gradient from the rainy lowlands of the north-west to the comparatively dryer climate of the mountains systems of the Iberian Peninsula. PC3 axes explained 11.75% of variation. For localities with similar environments as defined by axes PC1 and PC2, PC3 axis assigns more positive scores to localities at lower altitude and with more stable temperatures along the year. Thus, this gradient is inverse to the typical definition of continentality, with higher values for places with less variable temperatures throughout the year (typical of coastal areas) and lower values for places with wider range of temperatures (typical of the highlands in the interior of the Iberian Peninsula). Finally, PC4 explained 10.54% of variance. Positive scores in this axis indicate areas characterized by shallower and more acidic and xeric soils with lower C/N ratio (higher soil fertility). Thus, this axis sets a gradient of potential soil productivity constrained by soil depth and xericity.

The environmental conditions of the background areas overlapped in 77 out of 84 possible comparisons (4 PC axes times 21 pairwise comparisons, Fig. 9). The observed environmental niches were most

frequently divergent between taxa for PC1 and PC3 (16 and 17 out of 21 tests, see Table 3 and Fig. 9). Evidence for niche divergence or conservation was detected, respectively, in 11 and 9 tests of PC2 axis, while we found apparent divergence in 1 test. Finally, PC4 was the most conserved niche axis, since we detected evidence of niche divergence in just 6 of 21 tests, while niche conservation was detected in 14 tests and one test concluded apparent divergence.

Only one pairs of taxa (*A. p. pyrenaica* vs. *A. p. cazorlensis*) showed evidence for niche divergence in all of the four PC axes, and only one pair showed niche conservation in all the axes (*A. p. cazorlensis* vs. *A. v. nevadensis*). The distances between taxa along the environmental axes did not show any taxonomic pattern, so there is not a phylogenetic signal in the pattern of niche divergence.

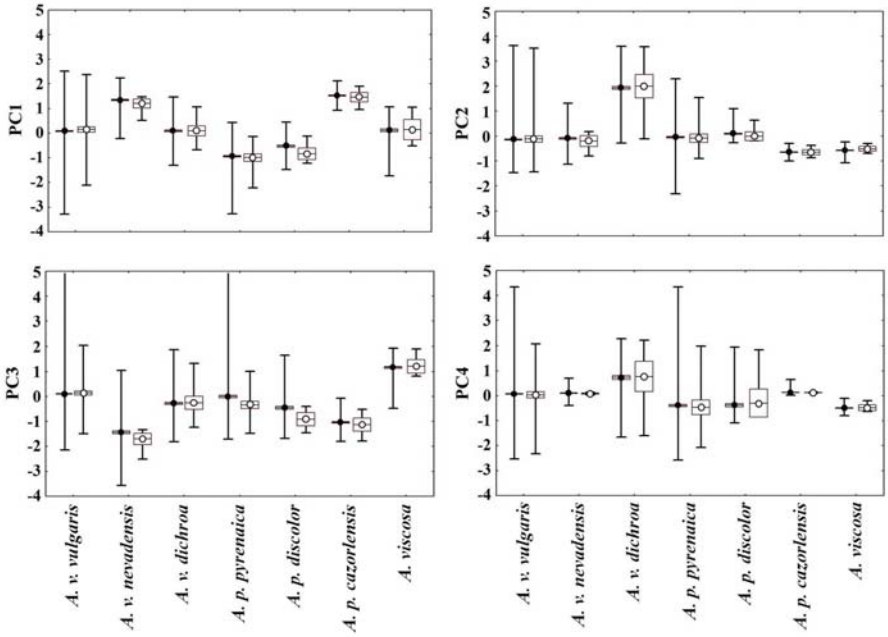

Figure 9. Distribution of the Iberian columbines along the four main axes of environmental variation identified by principal component analysis (see Table S1.1). Black points indicate background means and white points indicate observed means. Whiskers are the range of values and boxes are the 95% confidence interval.

Discusion

Environmental factors determining the distribution ranges.

The environmental niche models generated by Maxent are coherent with the general climatic and soil preferences that characterize the habitats of Columbines (Díaz González 1986; Nold 2003). Evaluation of the environmental variables which contribute most to ENMs suggests that the distribution of the studied taxa at the geographic scale of the Iberian Peninsula is more influenced by climatic variables than by soil variables. Similar results have been found in the case of *Solanum* species from the Andes (Nakazato et al. 2010). This secondary role of soil characteristics could be partly due to the lower spatial resolution of soil variables compared with climatic variables and altitude. Soil variables were studied at 10 km resolution, but most of them can show relevant variation within a few hundreds of meters. This coarser grain for soil variables may decrease the power to detect their influence on the spatial distribution of a given taxon, especially when its geographic range is very small. Thus, conclusions regarding the role of soil properties on geographic distribution must be taken with caution. However, this problem of scale is likely to have a smaller effect on niche comparisons between pairs of taxa, especially if their populations are located more than 10 km away from each other, which is the case in all the comparisons between peripatric and allopatric taxa, and even in the vast majority of comparisons between sympatric taxa (see Fig. 5).

Even though there can be many environmental factors not considered in this study that can help explaining the current distribution of the studied taxa (biotic and abiotic, contemporary or historical), most of the variables considered (15 out of 24) had a significant role in the ENM of at least one taxon. However, none of them had a consistent effect on several taxa, so none of the environmental variables characterizes the geographic distribution of the Iberian columbines as a group. This lack of

general pattern is expected in multispecies studies using ENM, and is likely the result of the colinearity between many of the variables used in the models (see for example Couvreur et al. 2011). On the other hand, there are some common patterns regarding the environmental variables not affecting the studied taxa as a group. Winter temperatures do not play a relevant role in the distribution of any of the studied taxa. This could be expected since the European columbines are typical species adapted to the cold environments occurring in mountain ranges of temperate regions, so the milder winter temperatures of the Iberian Peninsula can be tolerated by these taxa. Rainfall in the wettest periods of the year did not contribute to any of the ENMs. This suggests that rainfall during the wettest season throughout the study area is more than necessary for these plants. Finally, soil variables barely contributed to the ENMs (with the only exception of soil pH in *A. p. cazorlensis*). As explained above, this could be due to the low spatial resolution of soil variables. Still, it is surprising that soil moisture storage capacity did not contribute to any of the ENMs, since columbines are typically linked to moist soils (Nold 2003). It is possible that soil water availability in the habitats occupied by the studied taxa does not depend so much on intrinsic soil properties as it depends on rainfall patterns and subsoil water. Thus, our results suggest that any type of soil within the range of soils present in the study area, can be colonized by columbines as long as rain or subsoil water are not limiting.

Current and potential distribution ranges

The current geographic ranges of Iberian Columbines (Fig. 5) are largely allopatric and peripatric, with exceptional cases of sympatry (loosely defined as the overlap of the current distribution range of two taxa, not implying necessarily their coexistence in the same local community; see Medrano et al. 2006 for an example). The environmental niche models generated by Maxent predicted the potential distribution of the studied

taxa with high confidence. The occurrence of populations of most taxa within the suitable areas identified by the ENMs (except in the case of *A. p. cazorlensis*) suggests that, at the wide geographical scale of this study, the distribution of the studied taxa is largely in equilibrium with the current climate and soil properties of the Iberian Peninsula, and has not been severely modified by human activities. A possible exception to this equilibrium is *A. p. cazorlensis*, which is limited to a small area within its potential distribution range. According to our results, this endemic subspecies should find suitable places in mountain ranges around its current locations. Several reasons may contribute to explain this discrepancy between the current and projected distributions. One possibility is that the small sample size reduced the accuracy of the ENM, resulting in an overpredicted potential distribution. However, other taxa in this study have similar sample size and do not show evidence of overprediction. It is also possible that its current distribution is limited by some environmental factors not included in our analyses or that vary at a spatial scale too small to be detected with the spatial resolution of available environmental datasets. For example, populations of *A. p. cazorlensis* occur in shaded places on large vertical rock outcrops and in deep ravines, so topographic properties like aspect or slope, which can vary within a few hundreds of meters, can contribute to environmental suitability in this taxon. However, there are many places with such topographic properties in nearby areas not occupied by *A. p. cazorlensis*. Historical reasons seem more likely to explain the disequilibrium of the geographic distribution. For example, it is possible that this subspecies originated recently and did not have the opportunity to colonize other suitable areas, or that relatively recent changes in the environment have broadened the range of suitable areas but the taxon has not had the chance or the time necessary to colonize them. Alternatively, it is also possible that populations in most of the suitable areas have become extinct by

human activities (e.g. extensive livestock farming, forest fires, subsoil water extraction). In fact, the Iberian Peninsula has been subject to fast anthropogenic transformation of the landscape over the last centuries, what might have contributed to some local extinctions of this taxon. If population extinctions were aggregated within some geographic regions (e.g. on some river basins or some mountain systems), our ENMs should identify these as unoccupied geographic areas of suitable environments. Distinguishing among these alternative historical explanations can be relevant for the conservation of this endangered taxon, but our data do not allow to discern among them.

Inference on niche nesting.

The two instances of contradictory conclusions from background tests in our study involve peripatric taxa: the narrowly distributed *A. viscosa* compared with the two most widely distributed taxa (*A. v. vulgaris* and *A. p. pyrenaica*). Thus, the background tests suggest that *A. viscosa* is a specialist taxa with an environmental niche largely nested within the niche of the generalist taxa (similar to Fig. 6b). Results of the principal components analysis (Fig. 9) support this interpretation of the background test: *A. viscosa* has very narrow range of values in the four axes (indicative of a high specialization), and these ranges are fully nested within the ranges of *A. v. vulgaris*, and totally (PC2 and PC4) or partially (PC1 and PC3) nested within the ranges of *A. p. pyrenaica*. The species-level phylogeny of *Aquilegia* (Bastida et al. 2010) indicates that *A. viscosa* is closely related to *A. pyrenaica* and *A. vulgaris*, so it seems possible that *A. viscosa* has evolved through a process of specialization in a narrow set of environmental conditions nested within the set of conditions of its ancestors. This result highlights that niche evolution is more complex than the dichotomic conservation/divergence hypotheses that are recently being tested using ENM models (McCormack et al. 2010; but see Knouft et al.

2006), and that the insights on niche evolution offered by the new niche modeling tools can still be expanded.

Niche divergence and conservation

In general, both identity tests as principal components analysis showed that niche differentiation has occurred at the species and subspecies level in Iberian columbines. Niche divergence at species level involves primarily climatic gradients. The environment of *A. pyrenaica* is typically alpine, as it tends to occupy areas at high altitude, with relatively cooler and wetter conditions through the year and a wider range of annual temperatures. This environment is clearly differentiated from the environments occupied by *A. vulgaris* and *A. viscosa*, as indicated by differences among these taxa in PC1 and PC3. Gradients in soil properties seem to contribute to niche differentiation between *A. viscosa* and *A. vulgaris* (they differ in PC4) although, as we have shown above, such differences should be considered a case of niche specialization rather than purely differentiation. Thus, within the range of soils occupied by *A. vulgaris*, *A. viscosa* tends to occupy areas where the predominant soils are more basic and have lower fertility. In fact, *A. viscosa* plants typically grow in screes and limestone cliffs (Lavergne et al. 2005).

Considering niche divergence within species (i.e. between conspecific subspecies), PC1 axis, which represents the strongest gradient in climatic conditions, showed niche conservation among sympatric subspecies (*A. v. vulgaris* vs. *A. v. dichroa* and *A. p. pyrenaica* vs. *A. p. discolor*) and niche divergence between peripatric (*A. v. nevadensis* vs. *A. v. vulgaris*) and allopatric subspecies (*A. v. nevadensis* vs. *A. v. dichroa*, and *A. p. cazorlensis* vs. *A. p. pyrenaica* and *A. p. discolor*). Climatic niche is conserved among conspecific taxa that occur in the north of the Iberian Peninsula but has diverged in those subspecies distributed exclusively in the south (*A. v. nevadensis* and *A. p. cazorlensis*).

Interestingly enough, the two southern subspecies occupy identical environmental niche according to the four axes of the principal components analysis, what strongly suggests that these heterospecific subspecies have undergone a process of niche convergence. This pattern of climatic niche divergence and convergence seems to agree with the patterns of divergent and convergent selection currently experienced by populations of these taxa: populations of *A. v. nevadensis* often experience convergent selection on phenotypic traits (number of leaves per plant and number of flowers per inflorescence) with populations of *A. p. cazorlensis*, but divergent selection with populations of *A. v. vulgaris* (Alcántara et al. 2010).

Patterns of divergent selection derived from environmental differences between populations could lead to local adaptation promoting niche differentiation and geographic isolation between sister taxa. If this were the case, southern subspecies of columbines would be locally adapted to climatic niches different from those of their northern sister taxa, so the suitable areas defined by the ENMs of southern and northern taxa should not overlap. Alternatively, Wiens (2004) proposed the hypothesis that allopatric distributions may originate when a geographic barrier (i.e. an area of unsuitable environmental conditions between two sets of populations) develops faster than adaptation to these new ecological conditions. In this case, populations on each side of the barrier would still have the same niche, so it would be niche conservation what maintained the allopatric distribution. If this were the case, we should find a clear overlap between the suitable areas defined by the ENMs of allopatric sister taxa (Kozak and Wiens 2006). Our results show that niche overlap (Schoener's D) between allopatric sister taxa is very small. Thus, we can conclude that southern taxa have undergone a process of niche differentiation through adaptation to the climatic conditions of the

Mediterranean mountains, with higher and wider range of temperatures and lower precipitations through the year.

In a recent review, Peterson (2011) concluded that short-term events, like those associated to the distributional shifts at the end of the Pleistocene, show a considerable tendency towards niche conservation. Recent studies disagree with this conclusion in the case of plants. Nakazato et al. (2010) found a complex pattern of niche evolution among *Solanum* species separated between 1 and 4 Myr, with cases of niche divergence, conservation and specialization. Loera et al. (2012) found niche conservation and divergence in species of *Ephedra* distant between 1 and 5 Myr. European columbines began to diversify between 1 and 4 Myr ago (Bastida et al. 2010), achieving high diversification rates which, according to the present study, were accompanied by complex patterns of niche evolution. In three species and two lineages of subspecies we have found instances of niche conservation, divergence, convergence and specialization (niche nesting) (see Knouft et al. 2006, Smith and Donoghue 2010 for similar results). This complexity of niche evolution suggests that Columbines have been able to respond adaptively to the fast but profound changes experienced by the environments of the Iberian Peninsula through the glacial cycles of the Pleistocene.

Appendices

Table S1.1. Results of Principal Component Analysis on environmental variables. Data correspond to the pooled set of all the occurrence points and their background areas as defined for the background similarity tests (i.e. 3 km around each occurrence point).

Environmental Variable	Code	**PC1**	**PC2**	**PC3**	**PC4**
Annual Mean T^a	Bio1	**0.86**	0.42	0.15	-0.15
Mean Diurnal Range	Bio2	**0.78**	-0.10	-0.57	0.09
Isothermality	Bio3	0.36	0.42	-0.49	-0.30
T^a Seasonality	Bio4	0.56	-0.45	-0.57	0.16
Max T^a of Warmest Month	Bio5	**0.95**	0.16	-0.22	-0.00
Min T^a of Coldest Month	Bio6	**0.64**	**0.60**	0.41	-0.20
T^a Annual Range	Bio7	**0.69**	-0.29	**-0.62**	0.16
Mean T^a of Wettest Quarter	Bio8	0.50	-0.20	0.41	-0.09
Mean T^a of Driest Quarter	Bio9	0.47	0.49	-0.34	-0.07
Mean T^a of Warmest Quarter	Bio10	**0.93**	0.24	-0.04	-0.08
Mean T^a of Coldest Quarter	Bio11	**0.75**	0.55	0.29	-0.18
Annual Precipitation	Bio12	**-0.83**	0.37	-0.31	-0.25
Precipitation of Wettest Month	Bio13	**-0.68**	0.57	-0.32	-0.20
Precipitation of Driest Month	Bio14	**-0.91**	-0.09	-0.08	-0.27
Precipitation Seasonality	Bio15	**0.70**	0.37	-0.31	0.26
Precipitation of Wettest Quarter	Bio16	**-0.64**	**0.62**	-0.35	-0.18
Precipitation of Driest Quarter	Bio17	**-0.90**	-0.04	-0.08	-0.29
Precipitation of Warmest Quarter	Bio18	**-0.88**	-0.06	-0.02	-0.31
Precipitation of Coldest Quarter	Bio19	-0.52	**0.72**	-0.38	-0.16
Altitude	Alt	-0.49	-0.57	-0.56	0.22
Topsoil pH	PH	0.22	-0.51	-0.02	**-0.69**
Soil Moisture Storage Capacity	Moist	-0.58	0.28	0.24	0.45
Effective Soil Depth	Depth	0.41	-0.32	-0.01	**-0.74**
Topsoil Carbon/Nitrogen Ratio	C/N	0.43	-0.37	-0.03	**-0.71**
Explained Variance		11.22	4.10	2.82	2.53

CAPÍTULO 2

Gas exchange differences contribute to habitat differentiation in Iberian columbines from contrasting light and water environments.

Introduction

Leaves lose water via their stomatal pores as a consequence of the photosynthetic activity of the mesophyll cells (Lambers et al. 1998). Indeed, more than 90% of the water that a plant needs in its lifetime is lost via transpiration (Xu et al. 2009). Thus, water availability may be considered the major limiting environmental factor for terrestrial plants (Iovi et al. 2009), which frequently face a compromise between the maximisation of photosynthesis and the minimisation of transpiration (Lambers et al. 1998).

The importance of the trade-off associated with photosynthesis and transpiration is better appreciated by considering the different combinations of water and light availability in natural habitats and the different adaptations that plants have evolved to specialise in particular combinations (C fixation strategies). This interplay between photosynthesis and transpiration is especially challenging for those taxa that grow in a variety of environments (Mooney et al. 1987; Heschel et al. 2004a) because each particular species rarely displays specific adaptations to each condition. To a large extent, this trade-off is summarised by the concept of water use efficiency (WUE), which is defined as the carbon uptake per unit of water lost through stomatal transpiration (Heschel et al. 2002).

Shifts in the WUE in response to natural variations in water and light availability vary among species. In general, plants living in water limited environments tend to avoid excessive water loss by regulating their stomatal conductance, or at least by maximising their WUE. Thus, the optimum WUE can vary substantially in different environments. Plants can adapt physiologically to drier conditions by decreasing their stomatal conductance of water vapour, thereby increasing their WUE (Zangerl & Bazzaz 1984; Ares et al. 2000; Heschel et al. 2002, 2004a; Heschel & Riginos 2005). However, increasing the WUE involves closing

the stomata partially with a concomitant decrease in photosynthesis due to the reduced carbon dioxide uptake (Larcher 1995).

This trade-off has been investigated frequently in ecophysiological and agricultural contexts (see, for example, Condon et al. 2002, 2004; Xu et al. 2009). The ability of plants to cope with this trade-off by regulating their photosynthetic rate and stomatal conductance may be involved in niche differentiation between species (Ackerly et al. 2000). Thus, the natural variation in WUE among populations of the same species inhabiting different environments (e.g., Heschel et al. 2002, 2004a; Wu et al. 2010), as well as its possible role in habitat differentiation within species and/or among closely-related species, have received recent attention (Givnish et al. 2004, Heschel et al. 2004a, 2004b; Donovan et al. 2007; Savage & Cavender-Bares 2011; Manzaneda et al. 2012).

The genus *Aquilegia* (Columbines) is an example of adaptive radiation in North American and Eurasian continents (Schluter 2000; Hodges et al. 2003; Bastida et al. 2010, 2011). The soil type and other abiotic factors have been suggested to contribute to habitat differentiation among North American columbines (Chase & Raven 1975, Grant 1976) but the radiation of the genus in North America appears to be related to the divergent selection applied by pollinators, so the differentiation of species is based mainly on floral traits (Hodges & Arnold 1994, 1995; Hodges 1997; Fulton & Hodges 1999; Schluter 2000; Hodges et al. 2003). In Eurasia, columbines are also diversified but they have substantially lower pollinator diversity and floral differentiation, which suggests that their process of radiation is not based on pollinator specialisation (Medrano et al. 2006; Bastida et al. 2010). Bastida et al. (2010) hypothesised that geographic isolation and habitat specialisation via vegetative and ecophysiological trait divergence must have been the basic processes driving the radiation of European columbines. However, the

mechanisms and traits underlying their habitat and niche differentiation have been scarcely investigated (but see Jaime et al. 2013). In particular, different columbines found in the Iberian Peninsula occur in habitats with different light and water regimes (Alcántara et al. 2010, Jaime et al. 2013). Thus, Iberian columbines are a good system for study gas exchange and WUE variation and their relationships to drought and irradiance stresses, as well as their connections with habitat differentiation among closely-related species.

The present study is framed around the hypothesis that habitat differentiation in Iberian columbines has been driven by differential gas exchange behaviour in response to light and water environments. More specifically, this study assessed the roles of irradiance and water stress as two important dimensions that may determine niche differentiation among Iberian columbines at the species and subspecies level via distinct gas exchange behaviours. Thus, gas exchange and plant performance measurements were made in individuals from four Iberian columbines in the field and under manipulated common garden conditions.

The hypothesis of habitat differentiation predicts that the effects of specialisation for a specific water and irradiance environment would be as follows. (1) The occupants of permanently flooded soils should have the poorest performance during droughts. This poor performance during droughts should be linked to lower stomatal conductance and photosynthetic rates. By contrast, occupants of open and rocky habitats (drier environments) should have better tolerance of drought conditions, where they would maintain their photosynthetic rate. (2) If high irradiance is a stress factor that is independent of soil moisture, the occupants of shaded environments would have lower performance in full sun, even with appropriate soil moisture. This would be linked to a sharp decline in the CO_2 assimilation rate in full sun conditions, which would be less marked among open-habitat occupants. (3) Both stress factors may

interact so a poorer performance in drought might be aggravated in full sun compared with shaded conditions. Similarly, poor performance in high irradiance might be critical in drought, although it may be mitigated substantially by appropriate soil moisture.

Material and methods

Study system and plant material

Columbines are perennial herbs with a slender rhizomatous stem and one to several basal rosettes with pubescent ternate compound leaves. The mature plants produce glandular-pubescent paniculate inflorescences (Díaz González 1986; Nold 2003). In this study, the two most widespread columbine species in the region (*A. vulgaris* and *A. pyrenaica*) were selected, while further consideration was given to two subspecies within each species. *Aquilegia vulgaris* subsp. *vulgaris* is widespread in Europe. In the study sites, the plants grew in the forest understory near streams and springs. They generally grow in permanently wet places, from elevations of 1300 m to 1900 m and they flower from May to early June. *Aquilegia vulgaris* subsp. *nevadensis* is endemic to the Sierra Nevada, Sierra de Baza and Sierra Tejeda-Almijara, in the southeast of the Iberian Peninsula. In the study populations, the plants grow on permanently moist soils near streams or springs, in forest gaps and alpine meadows, from elevations of 1900 m to 2200 m and they are in bloom during June−July. *Aquilegia pyrenaica* subsp. *pyrenaica* is distributed in the Pyrenees and east of the Cantabrian Mountains (in the north of the Iberian Peninsula) where they occur in alpine meadows, rocky outcrop, and calcareous rocky grasslands from 1600 m to 1780 m, and they flower in July. Finally, *Aquilegia pyrenaica* subsp. *cazorlensis* is endemic to the Sierra de Cazorla and El Pozo in the southeast of the Iberian Peninsula. It grows in the shaded areas of rocky outcrops and cliffs from 1700 m to 2000 m, and

is in bloom from June to early July. Both subspecies of *A. pyrenaica* are typical of xeric soils on rocky substrates and they avoid forest canopies.

Adult reproductive individuals were grown in the facilities of the Experimental Garden of the University of Jaén (JEUJA) to conduct manipulative common garden experiments and to generate photosynthesis vs. irradiance curves (P-I curves hereafter). Selected populations were considered to be representative of the typical climatic and soil environment of each subspecies, as well as of the light and water regimes experienced in the field by each subspecies. Each experimental population was started from seeds collected in the field from 40–120 plants during August, 2004, depending on the population size (see Table 4). Seeds were sown in seed boxes using a mixture of white peat and coconut fibre, gravel and white sand in a ratio of 7:1.5:1.5. Seedlings were transplanted to individual pots where they remained until the beginning of the experiment (5 years) under a shade screen, which reduced the incident PAR by 30%.

Photosynthesis-irradiance curves (P-I curves)

For each taxon, four P-I curves were generated from four different specimens grown in common garden conditions to determine their basic photosynthetic parameters: maximum carbon net assimilation rate (maximum CO_2-AR; μmol CO_2 m^{-2} s^{-1}), photosynthetic efficiency (μmol CO_2 μmol $photon^{-1}$), dark respiration rate (μmol CO_2 m^{-2} s^{-1}), light compensation point (LCP; μmol photon m^{-2} s^{-1}) and light saturation point (LSP; μmol photon m^{-2} s^{-1}) (Table 5, Fig. 10). Thus, a portable IRGA (model LI-6400, LI-COR Biosciences Inc., Nebraska, USA) was used with a leaf chamber for broadleaved plants. To obtain the P-I curves, the incident irradiance was provided by a metal halide lamp coupled to a halogen bulb where the different irradiance levels (0, 100, 250, 500, 750, 1100 and 2000 μmol photon m^{-2} s^{-1}) were obtained by varying the distance

to the light source and interposing neutral grey mesh filters. At each light level, measurements were taken every 5 s during 5 min after rejecting the first 2 min to ensure stable conditions inside the gas chamber. The experimental data were recomputed based on the actual leaf area used for each individual, which was calculated by collecting the leaflets employed and using a portable leaf area meter, (model LI-3000C, LI-COR Biosciences Inc.). Measurements of the P-I curves were made for all taxa between 9.30 am and 12.00 am (GMT +2) during the last week of May in ventilated indoor conditions with a constant CO_2 concentration, 20°C temperature, and 50–60% air humidity.

The photosynthetic parameters were calculated by fitting the data to the equation proposed by Avola et al. (2008) using replicated regressions with Excel PopTools 3.0 (Hood 2008):

$$A_R = R_d + A_{R\max} \times (1 - e^{(-Q_{app} / A_{R\max}) \times PPFD})$$

where A_R represents the instantaneous net photosynthetic rate, R_d is the dark respiration rate, A_{Rmax} is the net photosynthetic rate in saturating irradiance conditions, Q_{app} is the apparent quantum yield (photosynthetic efficiency) and PPFD (photosynthetic photon flux density) is the irradiance of the measurement. After fitting the P-I curves, light compensation point (LCP) was estimated as the value of PPFD when A_R=0, and light saturation point (LSP) as the value of PPFD when A_R = 90% of A_{Rmax} (Rascher et al. 2000; Danner & Knapp 2003; Avola et al. 2008). Given that replication of the P-I curves (four specimens per taxon) was insufficient for statistical testing of differences among species and subspecies in the curves, no tests were conducted. Inferences of differences in these parameters among taxa involved visual inspections of the curves and comparisons of the parameters.

Table 4. Summary of environmental parameters and location of the study populations. In parentheses are indicated the population sizes and the standard errors of global site factor, PAR and soil moisture (%). Asterisks indicate the populations that were used in the common garden.

Species	Subspecies	Population	Zone	GSF and PAR	Water Soil Availability	Altitude	Coordinate UTM
A. vulgaris	*vulgaris*	***Fuente de la Reina (115)**	S. Cazorla	0.41 (0.06) 425 (144)	36.4 (2.36)	1325m	30S514740/ 4199580
A. vulgaris	*vulgaris*	**Cabrilla (138)**	S. Cazorla	0.32 (0.01) 995 (168)	37.2 (1.80)	1690m	30S518770/ 4197610
A. vulgaris	*vulgaris*	**Garrotegordo (27)**	S. Segura	0.61 (0.04) 137 (26)	48.8 (2.37)	1115m	30S533550/ 4229313
A. vulgaris	*vulgaris*	**Jabalises (80)**	S. Segura	0.31 (0.02) 1390 (202)	27.4 (3.12)	1390m	30S536356/ 4228894
A. vulgaris	*nevadensis*	***Pradollano (213)**	S. Nevada	0.36 (0.01) 1162 (218)	57.2 (3.35)	2110m	30S464649/ 4105811
A. vulgaris	*nevadensis*	**Dúrcal (120)**	S. Nevada	0.70 (0.01) 1614 (73)	34.4 (3.79)	1912m	30S456428/ 4103212
A. vulgaris	*nevadensis*	**Cortijuela (71)**	S. Nevada	0.26 (0.04) 319 (119)	38.8 (2.65)	1780m	30S457931/ 4085378
A. pyrenaica	*cazorlensis*	***Barranco la Canal (147)**	S. Cazorla	0.62 (0.03) 18.6 (3.3)	12.0 (1.92)	1405m	30S503431/ 4182541

Table 5. Photosynthetic parameters for each subspecies under common garden conditions. The parameters were obtained by fitting to the equation proposed by Avola et al. (2008) using the data registered/collected for four individuals per subspecies. The photosynthetic efficiency (mol mol^{-1}); dark respiration rate (µmol CO_2 m^{-2} s^{-1}); maximum net photosynthetic rate (µmol CO_2 m^{-2} s^{-1}); LSP: light saturation point (µmol m^{-2} s^{-1}); and LCP: light compensation point (µmol m^{-2} s^{-1}) are shown. The numbers in parentheses indicate the standard errors. R^2 is the coefficient of determination for each replicate regression fit (see Materials and methods).

Subspecies	Photosynthetic efficiency	Dark respiration rate	Maximum net photosynthetic rate	LSP	LCP	R^2
A. p. pyrenaica	0.055 (0.005)	-2.00 (0.68)	18.88 (0.85)	944.56 (68.68)	54.21 (9.20)	0.79
A. p. cazorlensis	0.050 (0.006)	-0.87 (0.63)	17.12 (0.80)	863.39 (20.51)	8.22 (3.43)	0.79
A. v. vulgaris	0.042 (0.008)	-0.99 (0.67)	10.71 (0.78)	700.89 (35.59)	25.78 (4.61)	0.70
A. v. nevadensis	0.055 (0.007)	-1.42 (0.68)	15.60 (0.80)	996.30 (153.93)	45.73 (10.09)	0.85

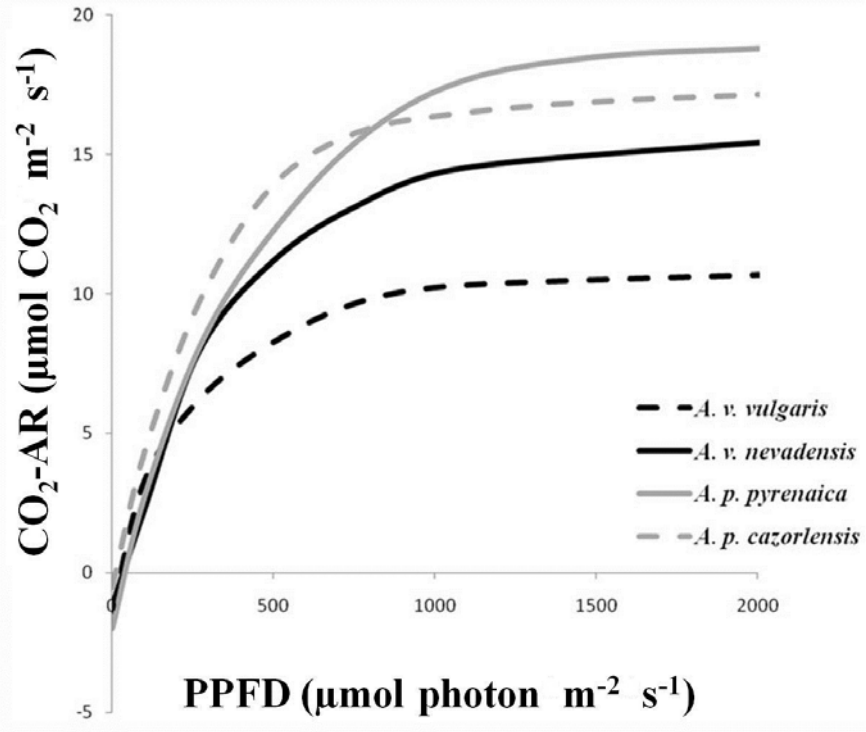

Figure 10. P-I curves fitted using replicate regression for the four study taxa. The curves correspond to the model proposed by Avola et al. (2008) and they show the photosynthetic response to increasing irradiance for individuals grown in a common garden in optimal conditions.

Field data collection

Instantaneous gas exchange measurements were taken in natural populations to compare the gas exchange, photosynthetic response and WUE among taxa. These measurements were carried out between the last week of April and the second week of July, which coincided with the flowering period of each population. Ten flowering individuals were selected randomly and monitored in 12 different localities (2–4 populations per subspecies) (Table 4). Instantaneous determinations of CO_2-AR and stomatal conductance in natural light conditions were performed using a portable IRGA device, as in the common garden experiment.

The openness of the forest canopy was assessed in each locality as the global site factor (GSF), which was obtained from hemispheric photographs (three or four randomly selected photographs per population, depending on the heterogeneity of each population) using a Nikon Coolpix 995 camera with a fish-eye lens. The photographs were taken at sunrise or sunset. The photographs were analysed using Gap Light Analyzer (GLA) 2.0 (Frazer et al. 1999). Water soil availability was assessed as the difference (%) between the wet and dry weights of five soil samples (randomly selected) per population, which were sampled as 15 cm depth cores after the removal of surface litter.

The variations in the GSF, water soil availability, photosynthetic rate, stomatal conductance and WUE in natural populations were analysed using a Type I ANOVA. The dataset was most suited to this analysis because of the hierarchical structures of the populations, subspecies and species. The PAR around each particular plant was used as a covariate in the analyses of the photosynthetic rate, stomatal conductance and WUE. In the analyses of GSF and water soil availability, the order in which the factors were entered into the ANOVA was: species, subspecies and population. In the analyses of the photosynthetic rate, stomatal

conductance and WUE, the covariate (PAR) was the first term in the model. The variables were log-transformed to reduce deviations based on assumptions of homogeneity of variance and normality as necessary. A post-hoc Tukey test was used to identify the levels of factors that differed from each other. All analyses were carried out using Statistica 7.0 (StatSoft 2004).

Irradiance and water stress experiment

A two-factor (irradiance and water supply) common garden experiment was performed with two irradiance levels (full sun and under a shaded screen that reduced incident PAR by 70% where this reduction was within the detected PAR variation limits for all of the populations sampled in the field) and two water availability levels (control and drought). Seven to ten plants, which each had only one inflorescence, were used for each subspecies and treatment combination. The plants were placed in a single block per treatment with one plant per pot and the position in the block of each plant was determined randomly daily. The experiment was performed four times because the flowering time differed among subspecies in the field and in the common garden conditions. The control plants were watered twice each day during all of the experiment. The plants in the drought treatment were deprived of irrigation completely throughout the experiment (17 days), which was a similar time span to typical episodes of summer drought experienced in the Mediterranean climate.

Before the gas exchange measurements, individuals from each treatment were placed in full sun for at least 3 h. These measurements were made randomly approximately from midday to 4 pm (GMT+2) in full sun to ensure saturating light conditions. The measurements were made during the first day of the experiment (day 0) when all the plants were well watered and after 10 and 17 days when plants were subjected to

moderate and severe stress intensities. The determinations of the instantaneous net CO_2 assimilation rate (CO_2-AR) and stomatal conductance in natural light conditions were performed using one leaf per plant with the IRGA. Overall, the gas exchange (CO_2-AR or stomatal conductance) values within each taxon were not correlated with the time of the day of data registration (Spearman's r, $P > 0.1$ for each taxon and gas exchange parameter), with the exception of stomatal conductance in *A. v. nevadensis* (Spearman's r = –0.39 $P < 0.05$, N = 40 plants). In addition to these variables, this device also determined the instantaneous transpiration rate, which was used to calculate the instantaneous WUE as the ratio of the net photosynthesis and transpiration rates. This device was calibrated each day, according to the manufacturer's recommendations. Similar to the P-I curves, these measurements were taken every 5 s during 4 min. After the gas exchange measurements, leaflets were collected to determine the actual leaf area using the leaf area meter to recalculate these measurements. Measurements were also made of the relative growth rate (RGR) for the inflorescence height (RGR = [(Log Inflorescence height$_{t17}$ + 1) - (Log Inflorescence height$_{t0}$ + 1)] / 17) and the number of leaves (RGR = [(Log number of leaves$_{t17}$ + 1) - (Log number of leaves$_{t0}$ + 1)] / 17). These variables were assessed only between days 0 and 17. Furthermore, it was noted whether plants had set fruit by the end of the season. The RGR and fruiting probability were used as plant performance estimators.

To determine the responses of the gas exchange parameters to the experimental treatments, the increment in the gas exchange was calculated as the difference between the measurements taken on day 10 and day 0 (Δ CO_2 AR = [(CO_2AR_{t10}) - (CO_2AR_{t0})] / [CO_2AR_{t0}]; Δ Stomatal Conductance = [(Stomatal Conductance$_{t10}$) - (Stomatal Conductance$_{t0}$)] / [Stomatal Conductance$_{t0}$]), because the mortality at day 17 was almost 100% with certain combinations of species and treatments. These increments were used as the dependent variables in the analyses. The

results of the experiment were analysed using a general linear model that included the effects of treatment, species, subspecies nested within species and the interactions between each taxonomic level and treatment. The two RGR variables were analysed simultaneously by multivariate analyses (MANOVA) while the increments in the gas exchange parameters were analysed by univariate analysis (ANOVA), assuming normal distributions and identity link function. The fruiting probability was analysed using a generalised linear model with a binomial distribution and logit link function using the same effects as the analyses above. Complex experimental designs involving several factors (subspecies, species, light and water) and their interactions were interpreted by considering the significance of higher order statistical interactions. In this case, the statistical interactions between subspecies or species and the environmental factors (light and water) were used to test the ecological hypothesis described in the introduction. Post-hoc Tukey tests were performed as necessary. All analyses were carried out using Statistica 7.0 (StatSoft 2004).

Results

Photosynthesis-irradiance curves (P-I curves)

The P-I curves that described the CO_2-AR as a function of irradiance (PPFD) are shown in Fig. 10. A visual inspection of the P-I curves suggests differences between and within species in the photosynthetic parameters (see also Table 5). The maximum CO_2-AR tended to be higher for both *A. pyrenaica* subspecies than the subspecies of *A. vulgaris*. The LSP and LCP apparently varied more between subspecies than between species, while *A. p. pyrenaica* and *A. v. nevadensis* had higher values than their respective sister taxa. There was a very low LCP in *A. p. cazorlensis*, especially when compared with its sister subspecies *pyrenaica*. Finally, the minimum dark respiration rates were in *A. p. pyrenaica* and *A. v.*

nevadensis but higher (less negative) in *A. p. cazorlensis* and *A. v. vulgaris*.

Environmental variables and gas exchange measurements in natural populations

In wild populations, GSF differed between species, between subspecies and among populations (Table 6), where *A. pyrenaica* inhabited environments with a higher GSF (Fig. 11a). There were differences between subspecies in *A. pyrenaica* (Fig. 11a). *A. p. pyrenaica* populations were exposed to higher GSF values than *A. p. cazorlensis*. This difference was influenced strongly by one population of *A. p. cazorlensis* (Cabañas, see Fig. S2.1a), which had an extremely low GSF which was caused by its location on the north face of a large vertical rock outcrop. The subspecies of *A. vulgaris* did not vary with respect to GSF, while the analysis of PAR showed that it differed between subspecies of *A. vulgaris*. *A. v. nevadensis* was usually exposed to higher PAR values than *A. v. vulgaris* (1032.0 ± 86.8 photon m^{-2} s^{-1}vs. 736.9 ± 75.2 photon m^{-2} s^{-1}, respectively; $F_{1,63} = 6.6$; $P = 0.01$). The variation in GSF between populations was also pronounced in both *A. vulgaris* subspecies (Fig. S2.1a).

In natural populations, the soil moisture varied significantly among species (Table 6). Populations of *A. vulgaris* had higher soil moisture (40.03 ± 1.85) than those of *A. pyrenaica* (19.08 ± 3.02). It also varied among subspecies, although post-hoc tests showed that these differences only occurred among subspecies of different species (Figure 11b). The variation among populations was more pronounced in *A. v. nevadensis* and *A. p. cazorlensis* (Fig. S2.1b).

For CO_2-AR, there were significant differences between species, subspecies and among populations (Table 6) where *A. vulgaris* had a higher CO_2-AR (Fig. 11c). At the subspecies level, there were only

significant differences between subspecies of *A. vulgaris*, i.e., *A. v. nevadensis* had higher values than *A. v. vulgaris* (Fig. 11c). The variation between populations was similar in all subspecies, with the exception of *A. p. pyrenaica,* which did not exhibit differences among populations (Fig. S2.1c).

For stomatal conductance, there were significant differences between species, subspecies and among populations (Table 6). *A. vulgaris* had higher stomatal conductance (Fig. 11d). At the subspecies level, there were only significant differences between subspecies of *A. vulgaris*, i.e., *A. v. nevadensis* had higher values than *A. v. vulgaris* (Fig. 11d). The variation between populations was more pronounced in *A. v. nevadensis* (Fig. S2.1d).

Finally, there were significant differences in the WUE between subspecies and populations but not between species (Table 6). At the subspecies level, WUE varied only in *A. pyrenaica*, i.e., there were higher values in *A. p. pyrenaica* than *A. p. cazorlensis* (Fig. 11e). The variation between populations was only present in both subspecies of *A. pyrenaica* (Fig. S2.1e). In particular, the post-hoc tests (results not shown) showed that a population of *A. p. pyrenaica* and another of *A. p. cazorlensis* departed from the mean WUE in most of the 10 remaining populations. Interestingly, the field values of WUE at subspecies level were similar to those obtained in the common garden conditions when plants were not subjected to stress (compare panel "e" in Fig. 11 and Fig. S2.2).

Table 6. Results of Type I ANOVAs used to test the differences in field conditions for the global site factor (GSF), water soil availability, photosynthetic rate, stomatal conductance and water use efficiency (WUE) at the population, subspecies and species levels. Significant differences ($P < 0.05$) are in bold.

Trait	Effect	df	F	$P <$
GSF	Species	**1**	**635.22**	**0.00001**
	Subspecies(species)	**2**	**313.88**	**0.00001**
	Population(subspecies)	**8**	**288.59**	**0.00001**
Water soil availability	Species	**1**	**176.26**	**0.000001**
	Subspecies(species)	**2**	**6.31**	**0.004**
	Population(subspecies)	**8**	**25.20**	**0.000001**
Net CO_2 assimilation rate	Species	**1**	**34.54**	**0.000001**
	Subspecies(species)	**2**	**5.76**	**0.0005**
	Population(subspecies)	**8**	**6.59**	**0.000001**
	PAR	**1**	**234.02**	**0.000001**
Stomatal conductance	Species	**1**	**35.63**	**0.000001**
	Subspecies(species)	**2**	**27.32**	**0.000001**
	Population(subspecies)	**8**	**14.55**	**0.000001**
	PAR	**1**	**57.92**	**0.000001**
WUE	Species	1	1.42	0.24
	Subspecies(species)	**2**	**10.99**	**0.0001**
	Population(subspecies)	**8**	**2.29**	**0.03**
	PAR	**1**	**25.95**	**0.00001**
	Error	107		

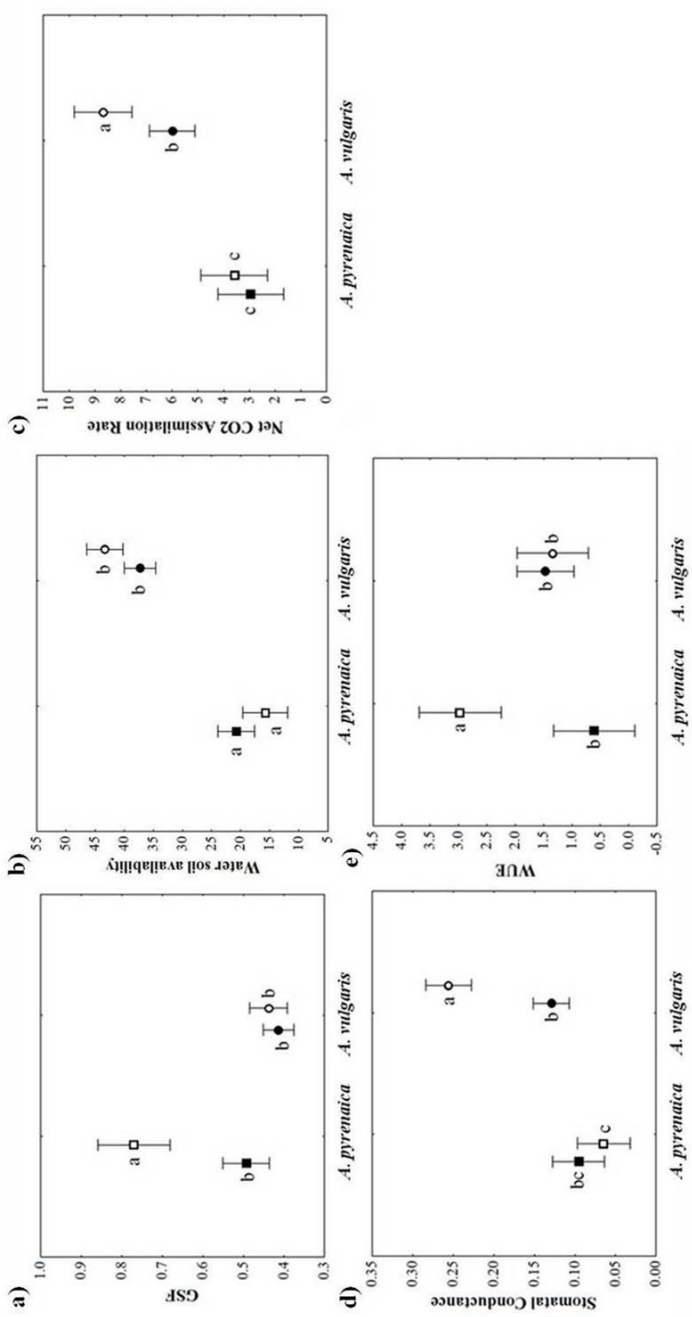

Figure 11. Characteristics of the light environment (GSF) and gas exchange in wild populations of the taxa studied. The values are the subspecies LS means ± 95% confidence limits for: (**a**) GSF (global site factor), (**b**) water soil availability (% weight of water in soil samples), (**c**) net CO_2 assimilation rate (µmol CO_2 m^{-2} s^{-1}), (**d**) stomatal conductance (µmol m^{-2} s^{-1}) and (**e**) WUE (Water Use Efficiency) (µmol CO_2 / mol H_2O). □ *A. pyrenaica pyrenaica*; ■ *A. pyrenaica cazorlensis*; ● *A. vulgaris vulgaris*; ○ *A. vulgaris nevadensis*. Different letters denote post-hoc differences.

Irradiance and water stress experiment

Table 7 summarises the results of the experiment designed to test the gas exchange parameters. There were significant "subspecies (species) × light × water" interactions for CO_2-AR and stomatal conductance (Table 7, Fig. 12), while the "species × light × water" interaction was significant only for stomatal conductance. Thus, the gas exchange parameters responses to the combination of water and light treatments differed only between species and sister subspecies. The response to drought of plants placed in full sun (black triangles in Figs 12a and 12b) were very similar across taxa while no taxa changed their CO_2-AR and stomatal conductance (values around cero) significantly in the watering control treatment in full sun, whereas all of the taxa decreased these parameters significantly to a similarly low level with water stress (Figs 12a and 12b). However, the response of plants in the shade (white triangles in Figs 12a and 12b) differed between taxa, i.e., both subspecies of *A. pyrenaica* maintained their CO_2-AR and stomatal conductance at similar (not significantly different) levels in the watering control and drought treatments, *A. v. nevadensis* reduced their photosynthetic parameters with water stress, while *A. v. vulgaris* increased these parameters significantly in the watering control treatment and reduced them significantly in drought.

In terms of the plant fitness measurements, there was a significant "subspecies × light × water" interaction for RGR (Table 7, Fig. 12c). Moreover, the effects of 'species × light' and 'subspecies × light' were not significant, which indicated that there was not a pure (i.e., independent) effect of light on the RGR differences so the effect of light depended on the water treatment. In agreement with the photosynthetic response, the performance in response to drought for individuals placed in full sun were very similar across taxa, where all maintained similar growth in the watering control treatment and reduced their growth to a similarly low

level with water stress. There were also differences between taxa in response to drought stress with the shade treatment. All of the subspecies had reduced growth with drought and shade but this decrease was more pronounced in the subspecies of *A. vulgaris* and it was only significant in *A. v. vulgaris*.

For the fruiting probability, there was a significant interaction between species and water treatment (Wald Chi-Square = 7.13; P = 0.0283). No significant differences were found between species in the water control conditions, although both species differed in their fruiting probability with drought stress where *A. pyrenaica* performed better than *A. vulgaris* (Fig. 13). Both species performed significantly better with the water control treatment than drought, although this difference was larger for *A. vulgaris*. The fruiting probability at the end of the experiment (day 17) was not simply a consequence of survivorship or mortality. Some of the plants that survived the water stress treatment did not set fruit (subsp. *vulgaris* = 0 fruiting/4 surviving; *nevadensis* 3/11, *pyrenaica* 10/10, *cazorlensis* 8/8) while some plants that survived the water control treatment also failed to set fruit (*vulgaris* 12/17; *nevadensis* 9/20; *pyrenaica* 17/18; *cazorlensis* 14/14).

Table 7. Results of ANOVAs used to test the effects of water and light treatments on the variation between species and subspecies in terms of the Net CO_2-AR (net photosynthetic rate) and stomatal conductance. Results of MANOVA used to test the effects of water and light treatments on the variation between species and subspecies in terms of the relative growth rate (RGR) for height and the number of leaves. Significant differences ($P < 0.05$) are in bold.

		Net CO_2-AR		**Stomatal conductance**		**RGR**	
Effect	df	F	*P*	F	*P*	F	*P*
Light	1	**12.29**	**0.00**	**11.70**	**0.00**	**97.64**	**0.00**
Water	1	**72.97**	**0.00**	**68.45**	**0.00**	**171.30**	**0.00**
Light*Water	1	0.83	0.4	0.69	0.4	**56.34**	**0.00**
Species	1	2.10	0.15	3.24	0.1	0.14	0.71
Subspecies(Species)	2	**5.36**	**0.01**	**4.50**	**0.02**	**15.17**	**0.00**
Species*Light	1	1.89	0.2	1.35	0.3	0.18	0.67
Species*Water	1	**11.88**	**0.00**	**10.30**	**0.01**	**4.11**	**0.04**
Subspecies(Species)*Light	2	0.61	0.6	**3.19**	**0.05**	0.93	0.40
Subspecies(Species)*Water	2	**4.95**	**0.01**	**4.49**	**0.02**	**3.15**	**0.05**
Species*Light*Water	1	1.84	0.2	**7.67**	**0.01**	2.19	0.14
Subspecies(Species)*Light*Water	2	**3.45**	**0.04**	**4.81**	**0.01**	**4.17**	**0.02**
Error	123						

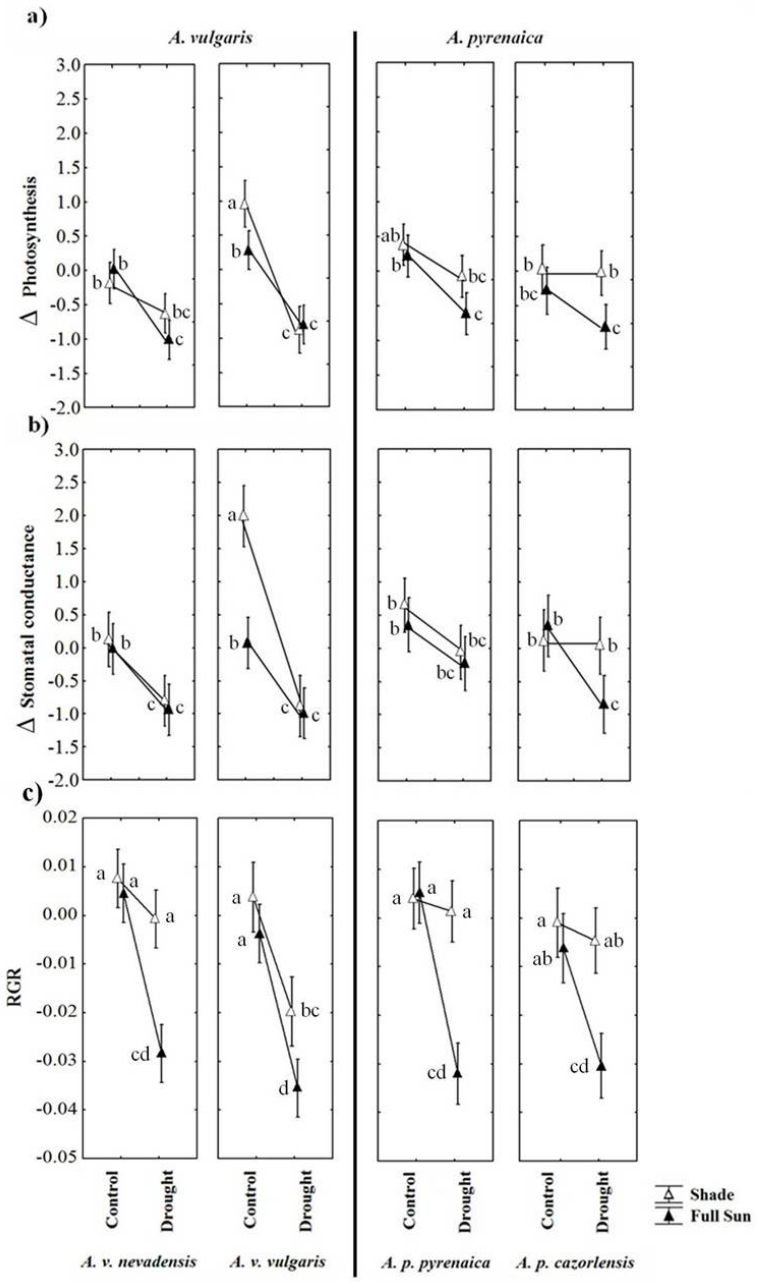

Figure 12. Decomposition of the effects of the experimental treatments on the gas exchange parameters for the taxa studied. The values are the subspecies LS means ± 95% confidence limits for variation, after the application of treatments, in: **a)** Δ CO_2 AR = $[(CO_2AR_{t10}) - (CO_2AR_{t0})] / [CO_2AR_{t0}]$, **b)** Δ Stomatal Conductance = $[(\text{Stomatal Conductance}_{t10}) - (\text{Stomatal Conductance}_{t0})] / [\text{Stomatal Conductance}_{t0}]$ and **c)** RGR (relative growth rate). Different letters denote post-hoc differences.

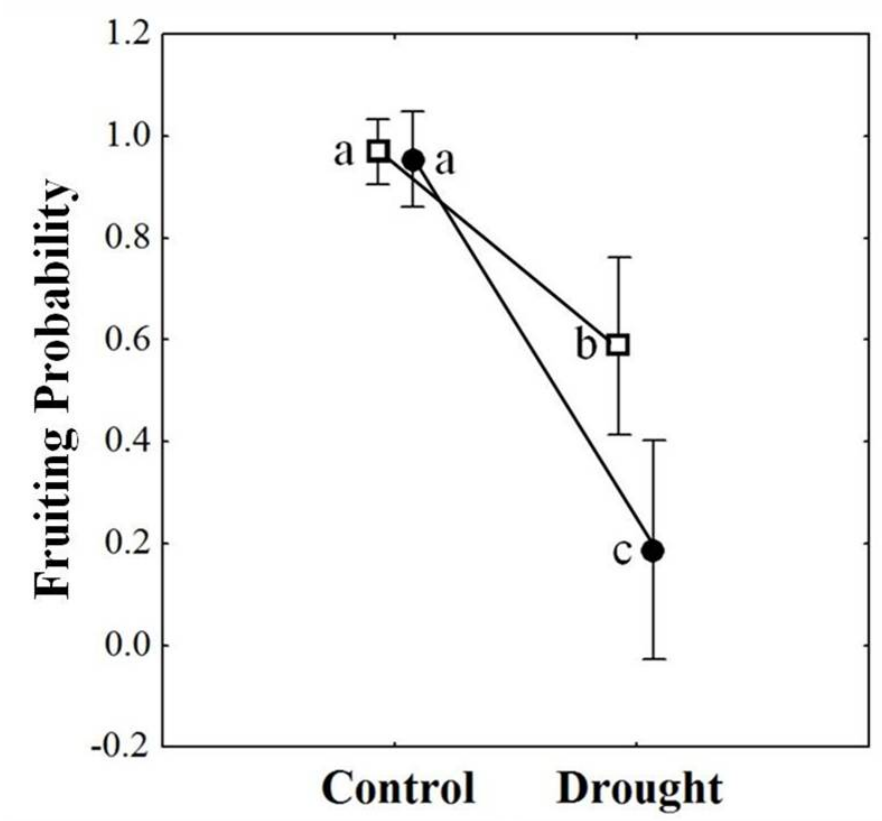

Figure 13. Effects of water stress on the fruiting probability at the species level. The values are the LS means ± 95% confidence limits. Different letters denote post-hoc differences. (□ *A. pyrenaica*; ● *A. vulgaris*).

Discussion

Understanding how plant ecophysiological traits adapt and differentiate among closely-related taxa in response to different environments is a fundamental but still uncertain issue in the study of plant evolution (Ackerly et al. 2000). The gas exchange that occurs during transpiration and photosynthesis is a major ecophysiological trait that limits plant adaptation to different environments. The present study explored the extent to which CO_2 assimilation during photosynthesis, the loss of water by transpiration and the trade-off between both in response to water and irradiance stresses may contribute to niche differentiation among closely-related columbines. Eurasian columbines are believed to have radiated by ecological specialisation to different abiotic environments via vegetative and ecophysiological trait divergence (Bastida et al. 2010, 2011). However, the mechanisms involved in this divergence have not been investigated (but see Alcántara et al. 2010, Jaime et al. 2013). Thus, the present study also helps to explain the mechanisms by which some Iberian columbines have diversified.

Differentiation in P-I curves and gas exchange in field conditions

The variation in the P-I curves suggests the existence of differences in gas the exchange behaviour among taxa (Fig. 10) depending on the environments they typically inhabit. This differentiation appeared to exist at the species and subspecies level. As expected, the species that inhabited full sun environments (*A. pyrenaica*) had a higher maximum CO_2-AR than the species from the shaded forest understory (*A. vulgaris*). Within each species, the photosynthetic efficiency, maximum net photosynthetic rate, LSP and LCP were higher, whereas the dark respiration rate was lower (more negative) in those subspecies exposed to higher irradiance in their natural field conditions (*A. p. pyrenaica* and *A. v. nevadensis*). These results agree with other studies, which showed that plants from full sun

environments had a higher maximum net photosynthetic rate and LCP than those from environments exposed to less sunlight (Toledo-Aceves & Swaine 2008, Liang et al. 2010). Thus, *A. v. vulgaris*, and to some extent *A. p. cazorlensis,* behaved like shade-tolerant plants with a lower carbon respiratory cost in the dark and a lower LCP compared with their sister taxa. *A. p. cazorlensis* was particularly interesting because it does not occur in forest understory and its populations typically thrive in places shaded by the topography (Medrano et al. 2006). It is possible that the intermediate level of shade tolerance displayed by *A. p. cazorlensis* may reflect a compromise so it can withstand the hot and dry Mediterranean summer because it is preadapted to full sun conditions.

Monitoring gas exchange in the field allowed a comparison of the photosynthetic behaviour of each taxon in their natural environments. In field conditions, plants should reflect an interplay between photosynthesis and transpiration, which is optimal (or at least efficient) for each specific combination of water and light availability that they experienced (Larcher 1995). These conditions were not the same as the standardised conditions used to generate the P-I curves in common garden conditions so their gas exchange behaviour in the field was not necessarily the same as that in common garden conditions. *A. vulgaris* had a higher net CO_2 assimilation rate in the field than *A. pyrenaica*. This behaviour in the field is expected because of the existence of an interactive effect of soil water and irradiance on gas exchange parameters, as hypothesised in the introduction (expectation 3). Water is not a limiting factor in the environment of *A. vulgaris* so it can keep its stomata open during the day to enhance carbon uptake. By contrast, populations of *A. pyrenaica* are exposed to drier soil so they must minimise their water loss via transpiration by keeping their stomatal conductance at lower values at the cost of a reduced net photosynthetic rate. The interplay between stomatal conductance and the net photosynthetic rate meant there were non

significant differences in the WUE at the species level (Table 6), which was to some extent exceptional because it has commonly been found that water irradiance environmental differences typically lead to WUE differences within and between species (Heschel et al. 2002, Rosenthal et al. 2005; Knight et al. 2006) where plants exposed to higher stress have a higher WUE. Given the very different irradiance and water soil environments that both species inhabit, they seem to balance their gas exchange to optimise the trade-off between photosynthesis and transpiration.

At the subspecies level, there were variations in the stomatal conductance and the CO_2-assimilation rate in *A. vulgaris*. These differences were apparently unrelated to the soil moisture but they may have been due to the effects of differences in irradiance on the gas exchange behaviour. With an appropriate water supply (which is the rule in the permanently flooded soils of both subspecies of *A. vulgaris*), plants exposed to full sun are expected to exhibit higher CO_2 assimilation rates and higher transpiration (which helps to protect the leaf surface and mitigate photodamage; Larcher 1995, Cai et al. 2007). In agreement, the present study found that *A. v. nevadensis*, which is usually exposed to higher PAR values than *A. v. vulgaris*, had higher stomatal conductance and CO_2 assimilation rates. Despite these differences, the WUE did not differ between them, which was also the cases at the species level.

By contrast, a comparison between *A. pyrenaica* subspecies showed that small differences in gas exchange can lead to very different WUE values (see Heschel et al. 2002, for populations of *Impatiens capensis*). Although it was not statistically significant, *A. p. cazorlensis* had a slightly higher stomatal conductance and a slightly lower CO_2-AR than *A. p. pyrenaica* (Figs 11c and 11d). Thus, the gas exchange strategy of subspecies *cazorlensis* appeared to be more extreme and it led to lower efficiency in the use of water compared with the more conservative

strategy of subspecies *pyrenaica*. This result may be expected because although both suffer similar water stress, they are exposed to different light environments. As is the case for shade-tolerant species, *cazorlensis* attempts to compensate for its low CO_2 uptake caused by a low PAR by keeping its stomata open even at the cost of increased water loss (see Hetherington & Woodward 2003). This demonstrates that the different strategies used to withstand shade or full sun can be adaptive in stressful environments (Donohue et al. 2000; Heschel & Riginos 2005; Knight et al. 2006).

Plant performance and gas exchange in response to experimental stresses

All taxa exhibited decreased plant performance (RGR and fruit set) in stressful drought conditions. This decrease appeared to be related to a decrease in CO_2-AR and stomatal conductance in drought, as shown in other studies (e.g., Heschel & Riginos 2005). However, according to the first prediction (see Introduction), the reduced performance with water stress was more pronounced in the species from permanently flooded soils (*A. vulgaris*) compared with those occupying environments exposed to summer drought (*A. pyrenaica*). This appeared to be linked to the higher capacity for stomatal conductance and photosynthetic rate modulation in *A. pyrenaica* in water stress compared with *A. vulgaris,* at least in shaded conditions. The results are essentially the opposite of those reported by Heschel & Riginos (2005) who found that in water limitation conditions, individuals from wet populations of *Impatiens capensis* maintained higher values of these two parameters compared with individuals from dry populations. The differences between species were not modified at the subspecies level so these results suggest that, as expected, the gas exchange behaviour of *A. pyrenaica* was better suited than that of *A. vulgaris* to environments that experience seasonal drought.

The experimental results did not support an independent effect of light because there was no simple interaction between this environmental factor and the species or subspecies level. Other studies have shown that differences in irradiance may not induce differences in gas exchange per se among populations (see Heschel et al. 2004b, with *Impatiens capensis*). However, the present study found no evidence to support the third prediction regarding the interactive effects of water and light stresses on gas exchange (CO_2-AR and stomatal conductance) and plant performance. As shown in other studies (e.g., Kubiske et al. 1996), it was expected that the poor photosynthetic rate in drought would be aggravated in full sun compared with shade conditions, especially in forest species (*A. vulgaris*). However, this exacerbation occurred in both species, which may have been due to the long, but realistic, period of stress used in the experiment. By contrast, the poor photosynthetic response with high irradiance was critical in drought for the subspecies that are naturally not exposed to full sun (*A. p. cazorlensis* and *A. v. vulgaris*), although it was mitigated substantially with appropriate soil moisture. This effects was more apparent in *A. p. cazorlensis*. *A. p. pyrenaica* was the subspecies with the best CO_2-AR in adverse high radiation and drought conditions, which were expected for a subspecies from typical low soil moisture and high solar radiation environments. A mitigation of the effect of high irradiance on the poor photosynthetic response with increasing soil humidity has also been shown to occur in trees (Kubiske et al. 1996).

Linking the light and water stresses, plant response and habitat differentiation

Overall, the results of the P-I curves, the gas exchange behaviour in field conditions and the plant responses with experimental water and irradiance stresses support the hypothesis that habitat differentiation is, to some extent, associated with differences among taxa (at the species or

subspecies level) with respect to their tolerance of these abiotic stresses, which are mediated by distinct gas exchange responses. Indeed, the present study showed that all of these results were to large extent congruent with the habitat differentiation presently observed for these taxa (see Table 8). As discussed above, the expectations of this hypothesis were fully (expectation 1 was related to water stress) or partially corroborated (expectations 2 and 3 were related to irradiance and the interdependence of irradiance and water stresses, respectively).

Interestingly, and despite differences in the stomatal conductance and CO_2-AR, the taxa did not differ in terms of the instantaneous WUE in field conditions. This was true for comparisons among species and between subspecies of *A. vulgaris*, although there were differences between subspecies of *A. pyrenaica*. Moreover, the pattern of WUE variation across taxa in the field was similar to that obtained in common garden conditions when the plants were not subjected to stress, which may suggest genetic differentiation among subspecies in this trait. The existence of additive genetic variation in WUE has been confirmed in other species (e.g., Dudley 1996a, b; Geber & Dawson 1997; Caruso et al. 2005). Similarly, selection for stomatal conductance and WUE has been reported repeatedly (Heschel et al. 2002, Heschel & Riginos 2005, Caruso et al. 2005, Donovan et al. 2007). The typical lack of differences in WUE among populations of different columbines in the field (10 out of 12 populations had the same value, Fig. S2.1e), despite the very different conditions experienced by these taxa, suggests a similar pattern of stabilising selection for this trait across environments, where some population departures of these patterns are possible cases of local adaptation or plastic responses to very specific environments The similar WUE optima at the taxa and population levels were achieved via comparable modifications of photosynthesis and stomatal conductance depending on the specific environment.

Overall, the results suggest that light and soil moisture are important abiotic axes for physiological trait divergence and habitat differentiation in Iberian columbines. Other factors such as specialisation to unfertile soils (calcareous bedrocks, serpentines, etc.) have been proposed to contribute to plant differentiation in the Mediterranean region (Kruckeberg 1986, Lavergne et al., 2003). Indeed, the soil fertility and rockiness contribute to habitat differentiation in some European columbines (Lavergne et al. 2005; for the taxa used in this study see Bastida 2009, Alcántara et al. 2010). The effects of infertility, low soil humidity and high irradiance (all common features of calcareous bedrocks) on plant responses might be confounded if they are not tested experimentally. The present study showed that the differential response to irradiance and water stresses also contributed to niche differentiation among Iberian columbines. If these conclusions can be generalised, the simple classification of plants into shade-tolerant or -intolerant will be misleading for many taxa because plant adaptations to shaded or light environments cannot be disconnected simply from adaptations to water availability.

Table 8. Links among habitat characteristics, environmental niche differentiation, gas exchange in the field and the experimental responses to water and irradiance stresses. The boxes summarise how the results of this study are congruent with the habitats occupied by each taxon (at the species and subspecies level). Each trait was compared between the taxa in each box, where "+" (or "-") show that the taxon had a greater (lower) value for a given parameter compared with the other taxon, while "0" shows that there was no difference between the taxa. GSF: global site factor; PAR: photosynthetic active radiation; WA: water availability; PE: photosynthetic efficiency; DR: dark respiration; MAX: maximum net photosynthetic rate; LSP: light saturation point; LCP: light compensation point; AR: CO_2 assimilation rate; SC: stomatal conductance; WUE: water use efficiency.

	Typical environment	Environmental differentiation (GSF/PAR/WA)	P-I Curves (PE/DR/MAX/LSP/LCP)	Gas exchange in field (AR/SC/WUE)	Experimental response
Species level †					
A. vulgaris	Permanent flooded soils under closed forest canopies	**-/-/+**	**0/0/-/0/0**	**+/+/0**	Less tolerant to experimental drought
A. pyrenaica	Xeric soils on rocky substrates; It typically avoids forest canopies	**+/+/-**	**0/0/+/0/0**	**-/-/0**	More tolerant to experimental drought
Subspecies level (*A. vulgaris*) ††					
A. v. vulgaris	Forest understory near streams and springs	**0/-/0**	**-/+/-/-/-**	**+/+/0**	In absence of water stress performed better in shade

A. v. nevadensis	Permanently moist soils, near streams or springs in forest gaps and alpine meadows	**0**/+/**0**	+/-/+/+/+	**-**/-/**0**	In absence of water stress was indifferent to light
Subspecies level (*A. pyrenaica*) †††					
A. p. pyrenaica	Alpine meadows, rocky outcrops, and calcareous rocky grasslands	+/+/**0**	+/-/+/+/+	**0**/**0**/+	More tolerant to irradiance stress under water restriction
A. p. cazorlensis	Shaded areas of rocky outcrops and cliffs	**-**/-/**0**	-/+/-/-/-	**0**/**0**/-	Less tolerant to irradiance stress under water restriction

† At the species level, both environmental axes (irradiance and water availability) contribute to the habitat differentiation of each taxon.
†† Within *A. vulgaris*, only irradiance contributed to habitat differentiation between subspecies.
††† Within *A. pyrenaica*, the interdependency of water and irradiance stresses contributed to habitat differentiation between subspecies.

Appendices

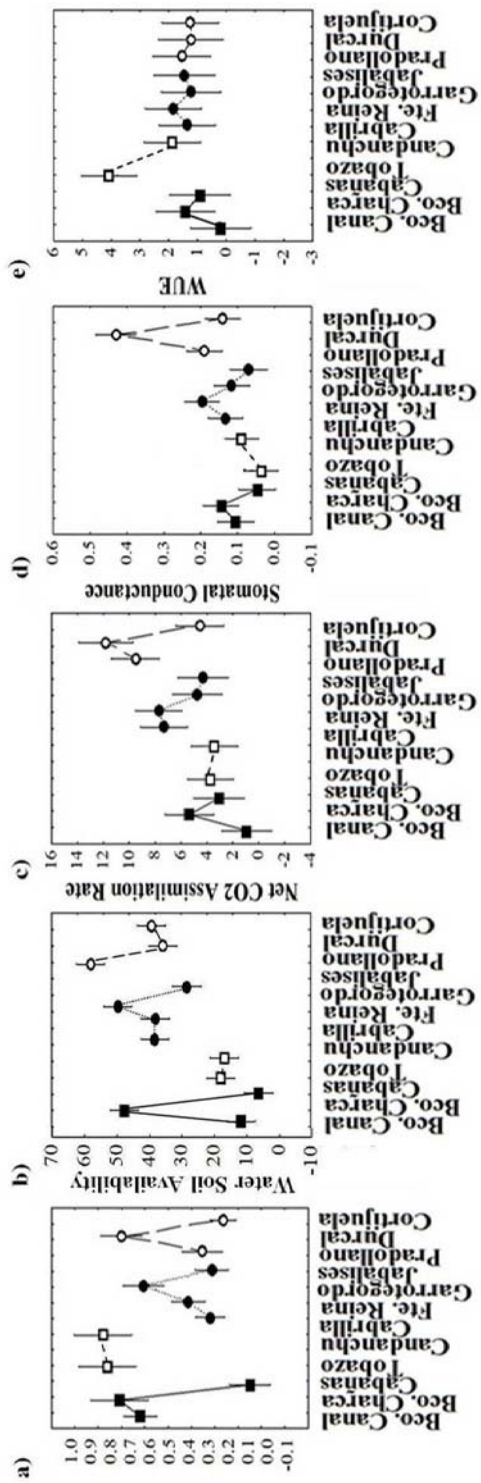

Figure S2.1. Variation among wild populations in the light environment and gas-exchange parameters. Values are LS means ± 95 % confidence limits for the variation in (**a**) GSF, (**b**) water soil availability (% weight of water in soil samples), (**c**) net CO_2 assimilation rate (μmol CO_2 m^{-2} s^{-1}), (**d**) stomatal conductance (μmol m^{-2} s^{-1}), and (**e**) WUE (μmol CO_2 / mol H_2O). □ *A. pyrenaica pyrenaica*; ■ *A. pyrenaica cazorlensis*; ● *A. vulgaris vulgaris*; ○ *A. vulgaris nevadensis*. Lines are represented only for a better identification of populations of the same taxon.

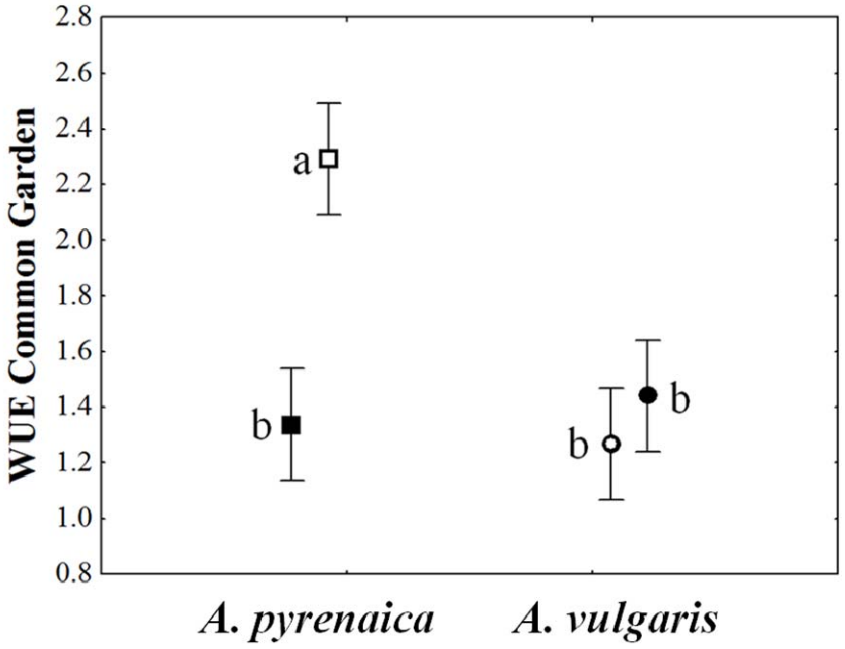

Figure S2.2. Variation in WUE (water use efficiency) among subspecies under common garden conditions. Values are subspecies LS means ± 95 % confidence limits. □ *A. pyrenaica pyrenaica*; ■ *A. pyrenaica cazorlensis*; ● *A. vulgaris vulgaris*; ○ *A. vulgaris nevadensis.* Different letters denote post-hoc differences.

CAPÍTULO 3

Glandular trichomes as an inflorescence defence mechanism against insect herbivores in Iberian columbines.

Jaime R, Rey PJ, Alcántara JM, Bastida JM.
Oecologia 2013 (En prensa).

Introduction

The wide array of defensive adaptations found in plants seems to have evolved to deprive herbivores of their required nutritious and energetic demands, and to reduce their performance and fitness (Strauss and Zangerl 2002). The large number of defensive mechanisms that plants have against herbivores, both chemical and mechanical, can either be constitutive or inducible (Karban and Baldwin 1997). Many forms of physical barriers (e.g., trichomes and spines), toxic secondary compounds, and antifeedants seem to efficiently defend plants (Karban and Baldwin 1997; Cipollini and Bergelson 2002; reviewed in Strauss and Zangerl 2002). It is suggested that they evolved under selection against herbivory.

One such defence mechanism is the presence of trichomes, which are multi- or unicellular epidermal hairs that can be glandular or non-glandular. Trichomes may have different functions besides direct protection against herbivores (Levin 1973). They may protect plants from excess sunlight, enhance water economy and salt secretion (Ehleringer et al. 1976; Vogelmann 1993; Wagner et al. 2004), and attract mutualists for pollination (Martin and Glover 2007) or protection against herbivores (plant bodyguards; e.g., Janzen 1973; Koptur 1984; Heil and McKey 2003, Romero et al. 2008). The defensive role of trichomes against herbivores has been described as one of their most important functions, whether it is direct, or indirect through mutualism with animals (Levin 1973; Agrawal 1998; Traw and Dawson 2002). Although many studies have explored the defensive role of trichomes in the leaves of different plant species (Levin 1973; Treacy et al. 1986, 1987; Buta et al. 1993; Wagner et al. 2004; Hare and Smith 2005), surprisingly few have explored their defensive role in inflorescences, even though inflorescences are expected to have a higher fitness value than leaves. This is important because optimal defence theory (McKey 1974, 1979; Rhoades 1979)

suggests that tissues with a high fitness value should be better defended than less valuable tissues.

Glandular trichomes excrete substances in their tips, and are present in 30% of vascular plants (Wagner 1991). Glandular trichomes may act as barriers, hindering the movement of invertebrate herbivores (Belcher and Thurston 1982; Treacy et al. 1986, 1987; Lovinger et al. 2000). They also secrete sticky compounds that capture insects, or toxic substances that irritate or kill them, or which modify their behaviour (Levin 1973; Buta, Lusby and Neal 1993; Wagner, Wang and Shepherd 2004; Hare and Smith 2005). However, the effectiveness of such defensive mechanisms has frequently been assumed, rather than experimentally demonstrated (but see Wang et al. 2001).

Plant populations of the same species often vary in the frequency and pattern of consumption by herbivores, which vary in local abundance or behaviour (reviewed in Huntly 1991). As herbivores can impose sufficient damage to reduce plant fitness (Marquis 1992), the expression of plant defences may differ among locations varying in herbivore pressure (Hartvigsen and McNaughton 1995; Berenbaum and Zangerl 1998). Thus, herbivore damage can be an important selective pressure, shaping variation in the levels of defensive traits among individuals, populations, and taxa (Berembaum and Zangerl 1998; Valverde, Fornoni and Nuñez-Farfán 2001). However, variation in herbivory intensity, in the function of apparently defensive structures, and in plant fitness has rarely been explored (But see Brenes-Arguedas et al. 2008; Kursar et al. 2009), especially in the context of population and taxa differentiation.

We chose Iberian columbines (genus *Aquilegia*, Ranunculaceae) as a study system to investigate the links between inter-population and inter-taxa variation in herbivore pressure, plant fitness, and differentiation in glandular trichomes density in the inflorescence further demonstrating

the defensive role of this trait against herbivores. The *Aquilegia* genus is particularly suitable for this study, as it is considered a textbook example of radiation in plants (Schluter 2000; Hodges et al. 2003). It is known that the processes leading to the radiation of the American lineage are not the same as those that led to the radiation of the Eurasian lineage (Medrano et al. 2006; Bastida et al. 2010). While the American lineage diversified mainly through pollinator specialisation and floral differentiation, the diversification of the Eurasian lineage, including the Iberian columbines, was not related to the pollination environment (Medrano et al. 2006; Bastida et al. 2010). In the case of Iberian columbines, divergent selection pressures between habitats differing in soil rockiness and altitude have promoted the differentiation of vegetative traits such as inflorescence height, number of flowers per inflorescence, number of basal leaves per inflorescence, and leaf size (Alcántara et al. 2010).

Among the most conspicuously differentiated traits of Eurasian columbines are the density and type of pubescence in the inflorescence (Díaz González 1986; Nold 2003). Such differentiation among congeneric species is common, as seen in the genera *Ononis* (Devesa 2000) or *Delphinium* (Blanché and Molero 1986), among others. However, the factors underlying such differentiation have been largely neglected. This study aimed to ascertain the defensive role of inflorescence glandular trichomes against phytophagous insects in Iberian columbines. Under the premise that variation in glandular trichomes density (GTD, hereafter) among population and taxa has a significant genetic component, we hypothesise that differentiation in GTD in the inflorescence between Iberian columbines is related to different abiotic environments and herbivore pressure. We tested this hypothesis by conducting observational measurements of GTD and trichomes removal experiments in several populations of four Iberian columbines. We predicted that: (i) taxa and

populations from habitats with higher abundance of phytophagous insects have a denser cover of glandular trichomes in the inflorescence; and (ii) experimental removal of glandular trichomes results in an increase in herbivore damage and a decrease of fitness in taxa and populations with denser trichomes.

Material and Methods

Study species and sites

This study focused on the two most widely distributed columbine species from the Iberian Peninsula: *Aquilegia vulgaris* (subsps. *vulgaris* and *nevadensis*) and *A. pyrenaica* (subsps. *pyrenaica* and *cazorlensis*). Columbines are perennial herbs with a slender rhizomatous stem with one to several basal rosettes, each with three to six pubescent ternate compound leaves. Mature plants produce either one or several glandular pubescent paniculate inflorescences, each bearing different numbers of flowers (Díaz González 1986; Nold 2003).

Different types of phytophagous insects consume the reproductive structures of *Aquilegia*. For example, aphids suck the sap from the inflorescence stalks, weevils (Curculionidae) feed on seeds, some Diptera lay eggs in floral buds, resulting in abortion or abnormal flower development, and caterpillars of different species (mostly Noctuidae) feed on flowers and unripe fruits (see also Lavergne et al. 2005).

Aquilegia vulgaris, subsp. *vulgaris*, is widespread in the Iberian Peninsula. In our study sites, plants of this subspecies grow in the forest understory near streams and springs. Generally, they grow in permanently wet places from 1,300 to 1,900 m.a.s.l., flowering from May to early June. *Aquilegia vulgaris* subsp. *nevadensis* is endemic to Sierra Nevada, Sierra Tejeda-Almijara and Sierra de Baza in the southeast of the Iberian Peninsula. It grows in forest gaps and alpine meadows, in permanently

moist soils near streams or springs from 1,900 to 2,200 m.a.s.l., blooming during June and July.

Aquilegia pyrenaica subsp. *pyrenaica* is distributed along the Pyrenees and east of the Cantabrian mountains. It occurs in alpine meadows, rocky outcrops, and calcareous rocky grasslands from 1,600 to 1,780 m.a.s.l., and it flowers in July. Finally, *A. pyrenaica* subsp. *cazorlensis* is endemic to Sierra de Cazorla and El Pozo in the southeast of the Iberian peninsula. It grows in rocky outcrops and shaded cliffs from 1,700 to 2,000 m, and flowers from June to early July.

Experimental design

This study took place between May and August, 2009, and was conducted on two populations of each subspecies (eight populations in total; Table S3.1). Additionally, we used preliminary data (a survey in 2008 from three populations of two subspecies of *A. vulgaris*) to test whether the patterns in abundance of phytophagous insects, herbivory damage, GTD, healthy fruit set, and treatment results are consistent between years. After completion of these analyses, we confirmed that the results were consistent over time (the results of these analyses are shown in Table S3.2). We also measured GTD in 20 plants from one population of each subspecies grown in an experimental garden to evaluate whether differences among wild populations were maintained under common garden conditions (that is, whether GTD is genetically-based rather than environmentally induced). Common garden conditions were the same for all taxa and different to the natural environment of each taxa. Plants were watered daily. In particular, water availability was higher than those naturally experienced by both subspecies of *A. pyrenaica* and, to some extent, lower than that naturally experienced by the edapho-hygrophilous *A. vulgaris*.

To test whether GTD has a defensive function against phytophagous insects, we conducted a trichomes removal experiment. We selected 40 plants per population (except for the population of Cabañas where only 20 plants were available). In each population, half of the plants were used as the control, and the other half were assigned to the trichomes removal treatment. Once the plants were numbered, the first plant was assigned a treatment on the toss of a coin, and subsequent plants received alternate treatments. The removal treatment involved the mechanical removal of the trichomes exudates, through spraying the inflorescence stalks with 50% diluted alcohol. Next, we smoothly rubbed the inflorescences with a piece of filter paper soaked in the same solution. Finally, the inflorescence stalks were sprayed again with distilled water to remove any trace of alcohol. This treatment resulted in the mechanical destruction of trichomes, which thereafter did not produce exudates.

To verify that the treatment application did not induce unperceived damage, which might bias the effect of removing the protection against herbivores, we conducted an experiment on two populations of *A. v. nevadensis*. The experiment was similar to the previous trichomes removal experiment but, in this case, all plants (control and trichomes removed) were also sprayed twice (1 day and 1 week later) with a general contact insecticide. We did not find a significant effect of trichomes removal on healthy fruit set in insecticide-sprayed plants ($F_{1,36} = 0.07$; $P = 0.834$); therefore, we can conclude that trichomes removal treatment was not damaging. It should be noted that the removal treatment might have elicited the emission of plant volatiles, which may have influenced the behaviour of insects in relation to the plants. Unfortunately, little is known about the emission of volatiles by columbines; however, such herbivore-induced responses often act to

reduce further damage (Karban et al. 2000; Kessler and Baldwin 2001; War et al. 2011).

Before treatment application, we estimated GTD and the number of insects stuck in the inflorescence in each plant (Table S3.3). At the end of the season, we estimated the total number of flowers and fruits produced, and the number of flower and/or fruit loss due to phytophagous insects, number of insects stuck in the inflorescence, and inflorescence loss due to vertebrate herbivores. GTD was estimated as the mean number of glandular trichomes per 1 cm^2 in three floral pedicels per plant. Measurements were taken with a 45× magnifying glass. We also counted and classified, to family level, the insects stuck on the upper third of the inflorescence stalk. Damage by phytophagous insects was estimated as the number of fruits and flowers consumed or infected by invertebrate herbivores. Maternal fitness was estimated as healthy fruit set, which is the ratio of final number of healthy fruits to initial number of flowers.

To characterise potential insect herbivore pressure in each population, we used sticky tape flycatcher traps (100 × 8 cm). Early in the flowering season, we placed four traps homogeneously spaced throughout each population. The traps were active for 15 days. We counted and classified, to family level, the insects captured. The phytophagous insects captured in *Aquilegia* stems and flycatcher traps belonged to the families Agromyzidae (Diptera), Curculionidae (Coleoptera), Aleyrodidae, and Aphididae (Homoptera). Other phytophagous insects were occasionally observed feeding on *Aquilegia* but were never stuck in the inflorescence stems or captured in flycatcher traps; these included sawflies (Hymenoptera, Tenthredinidae) and moth caterpillars (Lepidoptera, Noctuidae). Non-phytophagous and mutualistic insects were grouped into a single category (others), which included families of Diptera (Muscidae, Tabanidae, Culicidae, and Tipulidae), Coleoptera (Coccinellidae and

Malachiidae), and Hymenoptera (Vespidae and Apidae). Not all phytophagous insects in a given locality were expected to feed on columbines. Therefore, to correctly characterise the abundance of phytophagous insects relevant for columbines in each population, we compared the abundance of different groups of insects in the inflorescences with their abundance in the traps. Our estimate of insect abundance per population was based only on those groups of insects which occurred significantly more in the inflorescences than was expected, according to their abundance in the locality.

Statistical analysis

Variation in GTD and insect abundance in wild populations was analysed among species, subspecies nested within species, and populations nested within subspecies, using a nested Analysis of Variance (ANOVA). Variation in GTD between field and common garden conditions was analysed with a General Linear Model, with species, subspecies nested within species, and growing conditions as main factors, and including the interactions between each taxonomic level and growing conditions. The effect of trichomes removal on healthy fruit set and insect abundance was analysed through a General Linear Model, which incorporated the effects of treatment, species, subspecies nested within species, population nested within subspecies, and the interactions between each taxonomic level and treatment. In all analyses, effects were considered fixed, except for population that was always considered a random effect. Population means for all variables are given in Table S3.3. When necessary, the variables were log-transformed to reduce deviations from the assumptions of normality and homogeneity of variance. All analyses were conducted using STATISTICA 7.0 (StatSoft 2004).

Results

Variation in GTD and abundance of phytophagous insects

We found significant differences in GTD between species ($F_{1,4.09}$ = 137.01; $P < 0.001$), subspecies ($F_{2,4.07}$ = 11.56; $P = 0.02$), and among populations ($F_{4,264}$ = 7.25; $P < 0.0001$) in the field. Differences between subspecies were more marked in *A. pyrenaica* than in *A. vulgaris* (Fig. 14a). Variation between populations was more pronounced for *A. v. nevadensis* and *A. p. cazorlensis* (Fig. 14a). We detected a significant interaction effect between species/subspecies and growing conditions (Table 9) in GTD. Differences in GTD in the wild plants were reduced in the common garden plants (Fig. 15), although the ranking in mean GTD across populations and between species did not change. No subspecies differed significantly in GTD between the common garden and the wild, except for *A. p. pyrenaica*.

In populations where inflorescences captured more than ten insects (all populations of *A. vulgaris*, one population of *A. p. Cazorlensis*, and no populations of *A. p. pyrenaica*), we used contingency tables to compare the abundance of insects captured in inflorescences with their expected abundance, which was estimated from their relative frequency in flycatcher traps. The contingency table for each population considered up to five categories of insects (Agromyzidae, Curculionidae, Aleyrodidae, Aphididae, and others). In all tested populations, the frequency distribution of insects groups captured in the inflorescences differed from their expected frequency distribution ($P < 0.001$). In all populations, the group of non-phytophagous and mutualistic insects occurred less often in inflorescences than in the traps, while aphids, Aleyrodidae, and Agromyzidae, where present, were captured more often in the inflorescences than in the traps (see figure S3.1 of appendix). Among the phytophagous insects, only Curculionidae occurred in the inflorescences

as often as expected. These results indicate that most of the phytophagous insects stuck in the inflorescences were attracted to the plants, because they were captured more often than expected, based on their relative abundance in the locality. Thus, these groups of phytophagous insects are most likely captured while actively seeking for *Aquilegia* plants.

Regarding the abundance of phytophagous insects stuck on inflorescences, we found significant differences between species ($F_{1,4}$ = 10.53; $P < 0.05$) and populations ($F_{4,24}$ = 24.62; $P < 0.00001$), but not between subspecies of the same species ($F_{2,4}$ = 2.31; P = 0.214). Populations of *A. vulgaris* face a higher abundance of phytophagous insects (Fig. 14b). Finally, there were significant differences in fruit set among populations ($F_{4,264}$ = 4.18; P = 0.002), but not between species ($F_{1,4.15}$ = 0.04; P = 0.84) or subspecies ($F_{2,4.12}$ = 2.35; P = 0.21) (Fig. 14c).

Testing the role of GTD as a defence against insect herbivory

The glandular trichomes removal experiment showed significant interactions between treatment and species on the number of insects stuck on inflorescences, damage on flowers and fruits, and fruit set (Table 10). The treatment was more effective in *A. vulgaris*, significantly reducing the number of insects stuck in the inflorescence, which was not the case for *A. pyrenaica* (Fig. 16a). Accordingly, treated plants suffered significantly higher herbivory and had lower fitness (fruit set), more markedly in the case of *A. vulgaris* (Fig. 16b, c). Taken together, the results of this experiment demonstrate the defensive role of glandular trichomes in *Aquilegia* protecting against the damage of flowers and fruits caused by herbivorous insects.

Table 9. ANOVA to test the effect of growing conditions (field or common garden) on glandular trichomes density.

Effect	DF	F	*P* <
growing conditions	**1**	**61.65**	**0.001**
Species	**1**	**635.15**	**0.001**
species*growing conditions	**1**	**55.36**	**0.001**
subspecies(species)	**2**	**87.25**	**0.001**
subspecies(species)*growing conditions	**2**	**42.44**	**0.001**
Error	152		

Significant values ($P < 0.05$) are in bold.

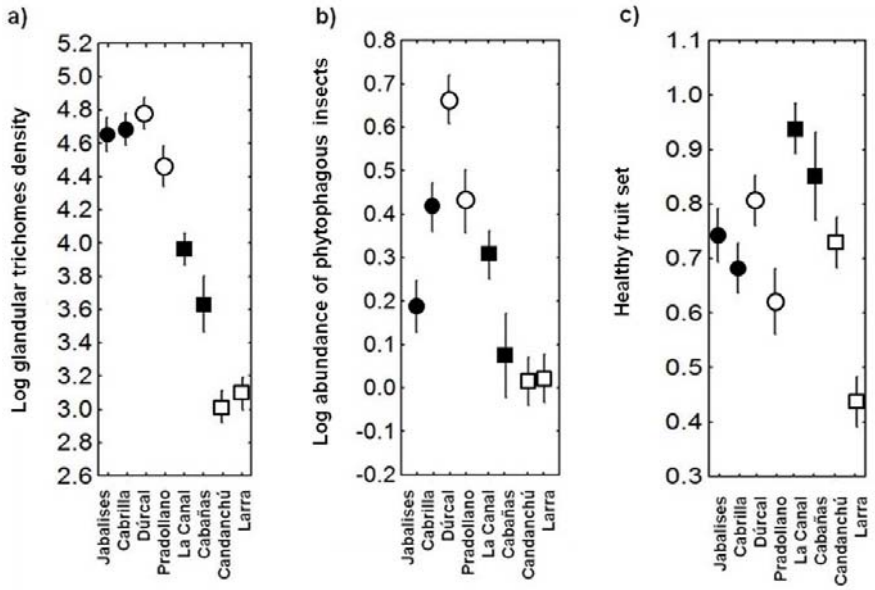

Figure 14. Least square means (± 95 % confidence limits) for the variation in: **a)** density of glandular trichomes, measured as mean number of trichomes per cm^2; **b**) abundance of phytophagous insects, measured as mean number of insects stuck on the upper third of the inflorescence stalk; and **c**) healthy fruit set at population level, measured as ratio of final number of healthy fruits to initial number of flowers. □ *A. pyrenaica pyrenaica*; ■ *A. pyrenaica cazorlensis*; ● *A. vulgaris vulgaris*; ○ *A. vulgaris nevadensis.*

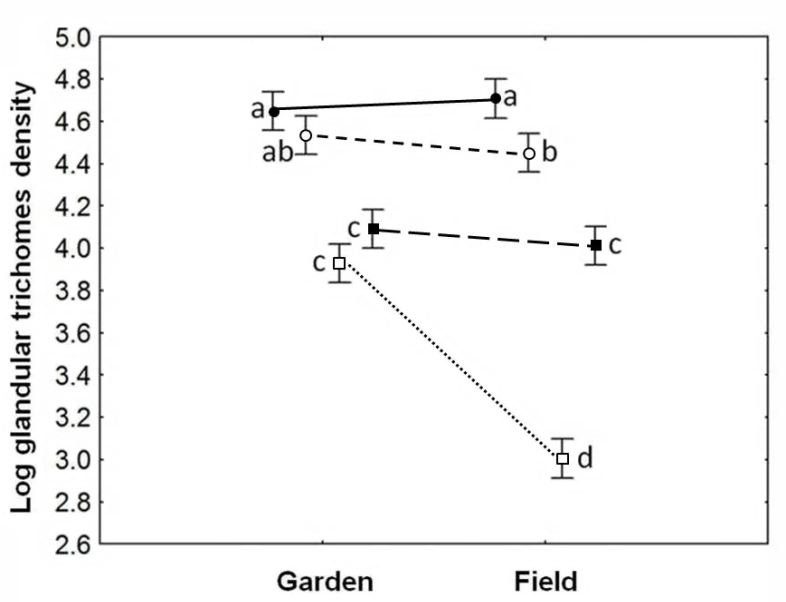

Figure 15. Least square means (± 95 % confidence limits) showing, at subpecies level, the variation between field and common garden plants in glandular trichomes density, measured as mean number of trichomes per cm^2. □ *A. pyrenaica pyrenaica*; ■ *A. pyrenaica cazorlensis*; ● *A. vulgaris vulgaris*; ○ *A. vulgaris nevadensis.* Different letters denote significant differences.

Correlations at population level

We found a significant correlation between phytophagous insect abundance and GTD (r = 0.97; $P < 0.05$; Fig. 17a), with plants in sites with higher abundance of small insects having higher GTD. Accordingly, the increase in herbivore damage following trichomes removal treatment was greater for populations with higher GTD (r = 0.93; $P < 0.05$; Fig. 17b). Additionally, the decrease in fruit set after treatment was greater for populations with a higher GTD (r = -0.76; $P < 0.05$; Fig. 17c).

Table 10. ANOVAs to test the effect of trichomes removal treatment on the variation between species, subspecies and populations in: **a)** number of insects captured on the inflorescence; **b)** herbivory damage; and **c)** healthy fruit set.

		a) Insects captured		**b) Herbivore damage**		**c) Healthy fruit set**	
Effect	DF	F	*P*	F	*P*	F	*P*
Species	**1**	**36.03**	**0.003**	0.12	0.746	0.07	0.802
Subspecies(species)	2	5.73	0.064	0.39	0.699	2.28	0.215
Pop(species*subspecies)	4	3.38	0.133	3.71	0.116	**26.90**	**0.004**
Treatment	**1**	**31.42**	**0.004**	**22.5**	**0.007**	**40.71**	**0.001**
Species*Treatment	**1**	**10.92**	**0.026**	**8.12**	**0.041**	**8.86**	**0.016**
Subspecies(Species* Treatment)	2	1.74	0.279	1.62	0.299	2.94	0.111
Pop(Species*subsps* Treatment)	4	1.74	0.141	1.69	0.153	0.17	0.955
Error	256						

Significant values ($P < 0.05$) are in bold.

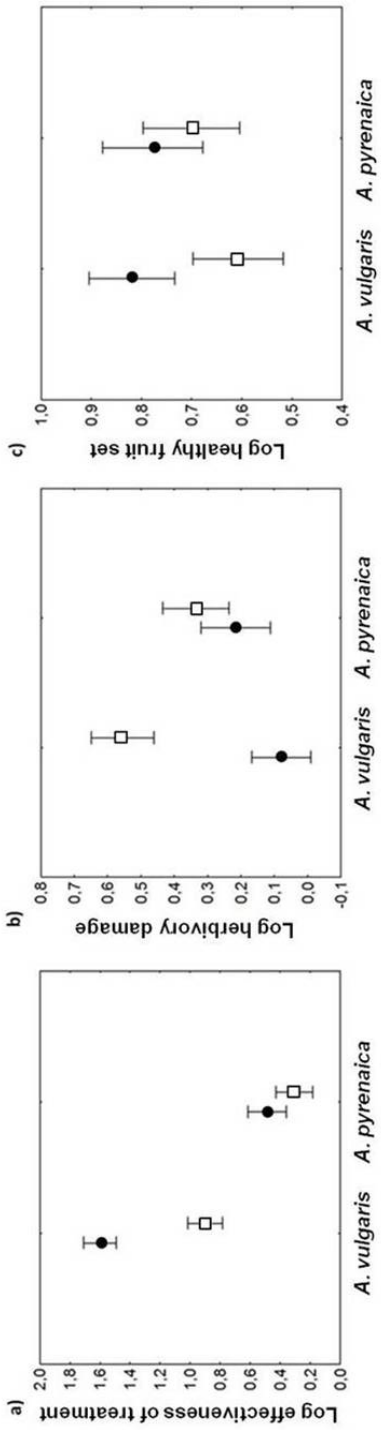

Figure 16. Least square means (± 95 % confidence limits) showing, at species level, the effect of the trichomes removal treatment on: **a)** effectiveness of the treatment, estimated as the total number of insects stuck on the inflorescence; **b)** herbivory damage, measured as the number of fruits and flowers consumed or infected by invertebrate herbivores per plant; and **c)** healthy fruit set, measured as ratio of final number of healthy fruits to initial number of flowers. □ Treatment; ● Control.

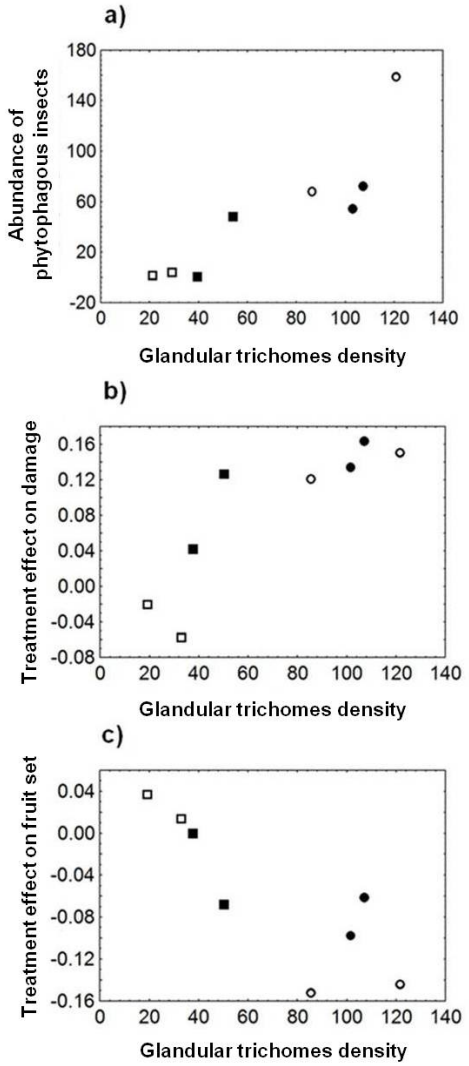

Figure 17. Correlation across populations of mean glandular trichomes density (measured as mean number of trichomes per cm^2) and **a)** abundance of phytophagous insects, measured as mean number of insects stuck on the upper third of the inflorescence stalk; **b)** difference in herbivore damage (measured as the number of fruits and flowers consumed or infected by invertebrate herbivores per plant) between control and treated plants; and **c)** difference in healthy fruit set (measured as ratio of final number of healthy fruits to initial number of flowers) between control and treated plants. □ *A. pyrenaica pyrenaica*; ■ *A. pyrenaica cazorlensis*; ● *A. vulgaris vulgaris*; ○ *A. vulgaris nevadensis.*

Discussion

Our results demonstrate that protection against small herbivorous insects is a major functional role of glandular trichomes in the inflorescences of Iberian columbines. Another commonly cited function of trichomes is protection against UV radiation (Karabourniotis et al. 1992, Yan et al. 2012). However, in the case of glandular trichomes in columbines, our results dispute this function because the subspecies most exposed to UV radiation (*A. p. pyrenaica*) is the taxa with the lowest density of glandular trichomes. This study shows that: (i) the different columbine taxa examined are exposed to differing herbivory pressure in their natural habitats; (ii) there are differences in GTD between species, which are largely genetic rather than environmentally determined; and (iii) geographic (among population) variation in GTD is related to differences in herbivory pressure. Consequently, current scenarios of herbivore abundance in habitats occupied by these taxa can explain the pattern of differentiation in GTD between Iberian columbines.

Variation in the abundance of phytophagous insects and glandular trichomes density

The two columbine species considered here are clearly differentiated in habitat: *A. vulgaris* inhabits deep, permanently moist soils, while *A. pyrenaica* inhabits drier places, such as rocky outcrops and calcareous rocky grasslands (Díaz González 1986; personal observation). We found a higher abundance of phytophagous insects in habitats occupied by *A. vulgaris*. The conditions created by a permanently humid and deep soil may be favourable for insect breeding, protection, and feeding. A similar pattern was found among populations of *Mimulus guttatus* growing in humid or drier habitats in California (Holesky 2007). Despite differences in the abundance of small herbivorous insects, the levels of damage

experienced by control plants did not differ between species. This suggests that defence mechanisms in each species might be adjusted to decrease damage to similarly low levels.

Our results show that *A. vulgaris* and *A. pyrenaica* have significantly different inflorescence GTD. Differences in GTD between taxa were stronger under field conditions than in common garden conditions, suggesting that GTD has an environmental component, particularly in *A. p. pyrenaica*. In this subspecies, differences in GTD between plants in common garden and field conditions clearly emerged, with plants in the field (exposed to drier soil) having substantially lower GTD than plants well-watered under garden conditions. This suggests the existence of phenotypic plasticity to soil humidity, at least in this taxon. Costs of secreting an aqueous solution in soils that become dry during summer (Elle and Hare 2000, Nogueira et al. 2000), may favour the maintenance of such plasticity. However, this environmental component was not strong enough to overcome species-level differences because subspecies of *A. vulgaris* had higher GTD than those of *A. pyrenaica*, both in the field and in the common garden. These results indicate that inter-specific differences in glandular trichomes observed in the field are mostly under genetic control, as found in other species (Lemke and Mutschler 1984, Mauricio 1998, van Dam et al.1999).

GTD as defence against insect herbivory

Optimal defence theory (McKey 1974, 1979; Rhoades 1979) suggests that tissues of high fitness value should be better defended than less valuable tissues. Many studies demonstrate the defensive role of trichomes in the leaves of different species (Treacy et al. 1986, 1987; Levin 1973; Buta et al. 1993; Wagner et al. 2004; Hare and Smith 2005). However, very few studies have explored the defensive role of trichomes in the

inflorescences, even though inflorescences are expected to have a higher fitness value than leaves. Wang et al. (2001) demonstrated the defensive role of glandular trichomes in *Nicotiana tabacum* through modifying the chemical composition of their exudates, and found that such exudates favoured the plant's resistance to aphids. Lambert (2007) proved on tomato plants that glandular trichomes were efficient in the defence against ants. Our experiment demonstrates the defensive role of glandular trichomes against small herbivorous insects in the inflorescences of Iberian columbines. For example, when we removed the glandular trichomes and their exudates from the inflorescences, treated plants captured fewer insects, suffered higher damage, and achieved lower fruit set than control plants. This effect was more pronounced in *A. vulgaris*, which faces a higher abundance of small phytophagous insects, than in *A. pyrenaica*. Thus, when the plants lose their protection, phytophagous insects have better access to flowers and fruits, causing more damage and reducing plant fitness.

Links between herbivory pressure, trait function and taxonomic differentiation

Given the defensive role of GTD in *Aquilegia* and its positive effect on fitness, the existence of genetic differentiation among species could be the result of natural selection acting on GTD, through geographic variation in herbivore pressure. This hypothesis, emerging from our experiments, is in agreement with our field observations (Fig. 16). Firstly, mean GTD was positively correlated with the abundance of phytophagous insects, with more pubescent plants inhabiting environments with a higher abundance of invertebrate herbivores. This correlation across populations also holds across species; populations of *A. vulgaris* had a greater trichomes density and were exposed to a higher abundance of invertebrate herbivores than

populations of *A. pyrenaica*. A similar correlation exists across subspecies of each species (see Fig. 17a). Secondly, populations with a higher mean GTD suffered higher increase in herbivory damage after glandular trichomes removal (see Fig. 17b). Therefore, we would expect that plants in localities facing more small herbivorous insects would be more severely affected if deprived of such a defence; thus, selection for increased GTD should occur in these populations. This expectation is corroborated by a correlation between mean GTD and the decrease in healthy fruit set on treated plants (see Fig. 17c). Several studies show the existence of selection pressures on trichome density in wild plant populations (Valverde et al. 2001). However, Elle and Hare (2000) did not find a net benefit associated with the production of glandular trichomes in *D. wrightii* growing in natural populations. Moreover, they found that the development of this type of trichomes had a high cost, causing plants to produce fewer viable seeds. It is possible that the cost of maintaining secretion of exudates by glandular trichomes would be higher in drier soils occupied by *A. pyrenaica*. Moreover, such a cost for plants in habitats with low abundance of herbivorous insects would result in counter-selection of glandular trichomes, which might explain the very low level of GTD in *A. p. pyrenaica*.

Interestingly, all correlations across localities were clearly structured at the taxonomic level (especially at species level), with *A. vulgaris* populations being exposed to a higher insect abundance, having a higher density of glandular trichomes, and suffering more herbivore damage and a lower healthy fruit set when deprived of its protection, than *A. pyrenaica*. We can therefore conclude that, despite the existence of some phenotypic plasticity (possibly related to soil moisture), glandular trichomes is part of an adaptive response against phytophagous insect damage. Castellanos et al. (2011) showed that vegetative traits of Iberian

columbines have high evolutionary potential. Alcántara et al. (2010) showed evidence of divergence of vegetative traits associated with divergent selection imposed by the abiotic environment in the same taxa of this study. Similarly, the differentiation in GTD among populations and taxa of Iberian columbines seems to be associated with divergent selection imposed on this trait by differences between habitats in the abundance of herbivorous insects.

Appendices

Table S3.1. Location and general characteristics of the populations under study.

Specie	Subspecie	Population	Zone	Habitat	Altitude	Coordinate UTM
A.vulgaris	*vulgaris*	**Cabrilla**	S. Cazorla	Near stream without canopy	1690m	30S518770/ 4197610
A.vulgaris	*vulgaris*	**Jabalises**	S. Segura	Near stream without canopy	1390m	30S536356/ 4228894
A.vulgaris	*nevadensis*	**Dúrcal**	S. Nevada	Near stream without canopy	1912m	30S456428/ 4103212
A.vulgaris	*nevadensis*	**Pradollano**	S. Nevada	Near stream with canopy	2110m	30S464649/ 4105811
A.pyrenaica	*cazorlensis*	**La Canal**	S. Cazorla	Rocky outcrop without canopy	1405m	30S503431/ 4182541
A.pyrenaica	*cazorlensis*	**Cabañas**	S. Cazorla	Rocky outcrop without canopy	1790m	30S503820/ 4184903
A.pyrenaica	*pyrenaica*	**Candanchú**	Pirineo Aragonés	Calcareous rocky grassland without canopy	1685m	30T700972/ 4739703
A.pyrenaica	*pyrenaica*	**Larra**	Pirineo Navarro	Calcareous rocky grassland without canopy	1570m	30T679687/ 4758837

Table S3.2. Two-way ANOVA to test the consistence of trichomes density, abundance of invertebrates herbivores, herbivory damage and healthy fruit set over time (two years) in three populations of *A. vulgaris* (2 populations of *A. v. nevadensis* and 1 population of *A. v. vulgaris*). Note that there was no interaction of population with year, indicating that results were consistent between years. Populations involved in these analyses are Pradollano and Durcal-Fte. Fría (*nevadensis*) and La Cabrilla (*vulgaris*).

Trait	Effect	DF	F	*P* <
Trichomes density	Population	**2**	**27.47**	**0.035**
	Year	1	2.84	0.233
	Population*Year	2	2.68	0.070
Abundance of phytophagous insects	Population	2	4.76	0.174
	Year	1	1.51	0.342
	Population*Year	2	1.37	0.255
Herbivory damage	Population	2	0.06	0.941
	Year	1	7.72	0.107
	Population*Year	2	1.64	0.195
Healthy fruit set	Population	**2**	**42.73**	**0.023**
	Year	1	0.46	0.566
	Population*Year	2	1.24	0.292
	Error	244		

Significant values ($P < 0.05$) are in bold.

Table S3.3. Summary of total insects, and traits and fruit set in plants assigned to control (CP) and trichomes removal (TP). Means are given ± standard error.

Taxon (population)	Insects stuck on inflorescences	Insects captured on traps	Mean glandular trichomes density CP	Mean glandular trichomes density TP*	Mean herbivory damage CP	Mean herbivory damage TP	Mean fruit set CP	Mean fruit set TP
A. v. vulgaris **(Jabalises)**	28	186	104.40 ± 2.43	101.39 ± 3.41	0.21 ± 0.04	0.34 ± 0.04	0.74 ± 0.05	0.64 ± 0.07
A. v. vulgaris **(Cabrilla)**	80	117	108.52 ± 2.34	107.06 ± 3.41	0.18 ± 0.04	0.34 ± 0.04	0.72 ± 0.05	0.66 ± 0.07
A. v. nevadensis **(Dúrcal)**	200	307	120.42 ± 2.34	121.45 ± 3.24	0.20 ± 0.04	0.35 ± 0.04	0.85 ± 0.05	0.70 ± 0.06
A. v. nevadensis **(Pradollano)**	54	230	86.75 ± 2.94	85.60 ± 4.58	0.11 ± 0.04	0.23 ± 0.06	0.64 ± 0.06	0.49 ± 0.09
A. p.cazorlensis **(La Canal)**	69	157	52.77 ± 2.31	50.32 ± 3.32	0.16 ± 0.03	0.29 ± 0.04	0.96 ± 0.05	0.89 ± 0.07
A. p.cazorlensis **(Cabañas)**	5	50	38.50 ± 4.16	37.83 ± 5.91	0.04 ± 0.06	0.08 ± 0.07	0.92 ± 0.09	0.92 ± 0.12
A. p. pyrenaica **(Candanchú)**	5	49	21.41 ± 2.55	19.06 ± 3.62	0.07 ± 0.04	0.05 ± 0.04	0.91 ± 0.05	0.95 ± 0.07
A. p. pyrenaica **(Larra)**	8	68	29.12 ± 2.94	32.91 ± 4.36	0.19 ± 0.04	0.14 ± 0.05	0.73 ± 0.06	0.74 ± 0.09

* Glandular trichomes density measured before treatment application.

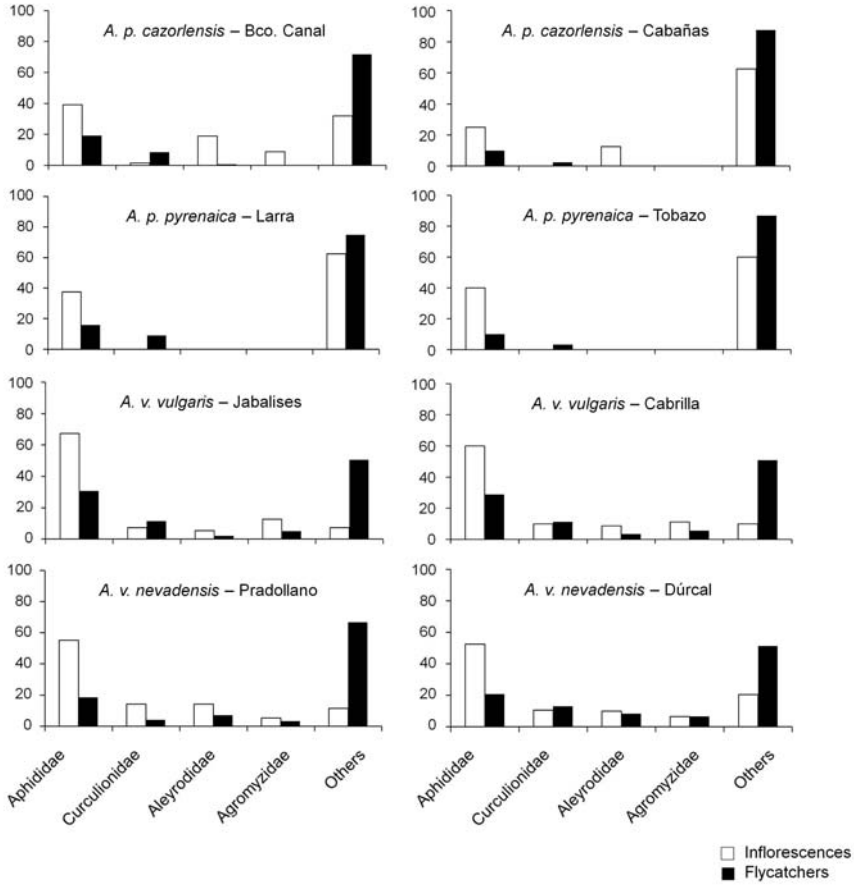

Figure S3.1. Relative abundance of insect stuck in the inflorescences and traps in each population.

CAPÍTULO 4

The role of genetic constraints on the diversification of Iberian taxa of the genus *Aquilegia*.

Introduction

The accumulation of phenotypic variation within a lineage (phenotypic diversification among closely related taxa or populations) is a complex process involving divergent/convergent selection, genetic constraints and history. Understanding the role of each of these components is a difficult task that we are only beginning to undertake (Schluter, 1996; McGuigan, 2006; Chenoweth et al*.,* 2010; Stinchcombe et al*.,* 2010). In the absence of genetic constraints, the patterns of divergent/convergent selection acting across populations of the same species would drive the formation of differentiated sets of populations that could eventually give rise to different ecotypes or taxa (Alcántara et al*.,* 2010). However, the possible adaptive response of populations to natural selection depends on the structure of the genetic variance and covariance matrix (**G**) among their phenotypic traits (Lande & Arnold, 1983). The **G** matrix incorporates fundamental parameters in microevolutionary theory (Lande, 1979; Arnold et al*.,* 2008). Thus, to understand the process of phenotypic diversification we need to assess role of **G**.

There are different approaches to assess the effect of **G** on the pattern of phenotypic divergence. Some studies explore whether the structure of **G** in a population may constrain its immediate response to selection, and so its short-term possibilities of phenotypic differentiation (see Conner, 2012; for a review of methods). For example, eigenanalysis of **G** can be used to determine whether genetic variation concentrates in a few directions, implying that phenotypic divergence would be constrained because if some dimensions lack variance then evolution could only proceed in those dimensions for which variance exists (Kirkpatric & Meyer, 2004; Hine & Blows, 2006). Other studies compare the **G** matrix among populations or taxa to explore its evolution (see review in Roff et al*.,* 2012). In the context of genetic constraint on phenotypic divergence,

the method of Random Skewers (Cheverud & Marroig, 2007) allows assessing whether different **G** matrices would lead to different responses to selection, and so to phenotypic divergence (Stinchcombe et al*.,* 2010). Finally, some studies focus on whether the pattern of phenotypic divergence across populations or closely related taxa shows any signature of genetic constraints as assessed by **G** (Schluter, 1996; McGuigan et al*.,* 2005; Colautti & Barrett, 2011). Typically, these studies measure the angle (θ) between the direction of largest genetic variance (***g*** vectors: the genetically constrained directions) and the direction of phenotypic divergence across populations or closely related taxa (***d*** vectors: the observed pattern of divergence).

In the present study we use some of these approaches to explore the role of **G** in the phenotypic divergence of five closely related Iberian taxa of columbines (the subspecies *vulgaris*, *nevadensis* and *dichroa* of *Aquilegia vulgaris*, and the subspecies *pyrenaica* and *cazorlensis* of *A. pyrenaica*). Columbines provide one of the most important examples of adaptive radiation in plants (Schluter, 2000; Hodges et al*.,* 2003; Bastida et al*.,* 2010). The genus is distributed along temperate regions of North America and Eurasia. The processes that have led to radiation in both continents are different. In North America, radiation has been related to pollinator-mediated divergent selection, being species differentiation based mainly on floral traits (see review in Hodges et al*.,* 2003). On the other hand, radiation in Eurasia is based on habitat specialization (Bastida et al*.,* 2010). The studies conducted in columbines from the Iberian Peninsula suggest that divergent selection pressures between habitats have promoted the differentiation of vegetative traits (Alcántara et al*.,* 2010), but not in the case of flower traits (Castellanos et al*.,* 2011). Since genetic variation in wild populations is similar in vegetative and floral traits, Castellanos et al*.,* (2011) concluded that divergent selection confers

vegetative traits a higher evolutionary potential than floral traits in the Iberian columbines.

Our main objective in this study was to obtain quantitative genetic parameters (additive genetic variances and covariances, and narrow sense heritabilities) for ecologically relevant phenotypic traits in populations of closely related taxa of the genus *Aquilegia* from the Iberian Peninsula, and then determine whether divergence among taxa may be constrained by the amount of genetic variance and by the structure of genetic variance-covariance within populations. Specifically, we address the following questions about genetic constraints on the pattern of phenotypic diversification of the studied taxa:

(1) Does the amount of standing genetic variation constrain the magnitude of phenotypic diversification? At microevolutionary scales, the accumulation of phenotypic differences for a given trait between closely related taxa and populations depends critically on the availability of additive genetic variation. Therefore, we would expect that traits with higher heritability were more likely to diverge through selection or genetic drift, so they should show a higher variability across taxa. One limitation of this expectation is that natural selection may have eroded genetic variation after the divergence, so most traits would be fixed nowadays (showing zero or nearly zero heritability within populations) and we would not be able to detect any relationship between heritability and trait variability among taxa whether or not it existed in the past. Therefore, this expectation can only be addressed under the assumption that the process of phenotypic diversification is an ongoing process, what we can assume since the current patterns of natural selection in wild populations agree with the observed patterns of phenotypic diversification among the studied taxa (Alcántara et al*.,* 2010).

(2) Do **G** matrices impose divergent patterns of phenotypic differentiation between closely related taxa? Theoretically, **G** matrices can change under selection, mutation and drift, but a clear picture of how such changes occur is still under way (Steppan et al., 2002; Arnold et al., 2008). What is clear is that even subtle differences between two **G** matrices may enforce divergent responses to selection. Thus, if the structure of **G** matrices imposes different responses to selection, taxa or populations with more similar **G** matrices can be expected to show less phenotypic differentiation. To assess this expectation we compare the potential response to selection between the studied taxa using the Random Skewers method (Cheverud & Marroig, 2007). If the pattern of phenotypic diversification was constrained by the evolution of **G**, we would expect more similar potential response to selection between conspecific than between heterospecific subspecies.

(3) Does the structure of **G** matrices constrain the pattern of differentiation between taxa? This question goes one step beyond the previous one since it addresses not just whether differences in **G** can potentially bias the responses to selection but whether the observed patterns of phenotypic differentiation agree with those expected from genetic constraints imposed by the structure of **G**. To address this question we use the procedure developed by Schluter (1996) to compare the observed directions of maximum divergence between taxa ($\boldsymbol{d}_{max}$) against the direction expected under genetically constrained divergence ($\boldsymbol{g}_{max}$: the principal eigenvector of **G**). $\boldsymbol{g}_{max}$ represents the linear combination of traits with the greatest potential for evolution (evolvability; Hansen & Houle, 2008), so it captures a significant proportion of the biasing effects of genetic architecture. In the context of phenotypic diversification, the basic hypothesis is that if the pattern of differentiation between closely related taxa is not significantly constrained by **G**, its influence on the

direction of phenotypic divergence should decay over time (Schluter, 1996): the initial pattern of divergence can be biased by **G**, but as taxonomic diversification proceeds, this bias should decay letting more distantly related taxa to evolve towards more different phenotypes. In this case, we would expect that the direction of divergence between conspecific subspecies would be more closely aligned with the direction imposed by their respective $\boldsymbol{g}_{max}$ than the directions of divergence between heterospecific subspecies.

By addressing these questions we seek to add evidence on the general hypothesis that Iberian columbines are undergoing a process of rapid phenotypic diversification that is primarily driven by the selection forces acting on vegetative traits (Bastida et al., 2010; Alcántara et al., 2010; Castellanos et al., 2011).

Material and Methods

Study species

This study focused on the two most widely distributed species of the genus *Aquilegia* in the Iberian Peninsula: *Aquilegia vulgaris* (subsps. *vulgaris*, *dichroa* and *nevadensis*) and *Aquilegia pyrenaica* (subsps. *pyrenaica* and *cazorlensis*). Columbines are perennial herbs, with a slender rhizomatous stem with one to several basal rosettes, each with 3–6 pubescent ternate compound leaves. Mature plants produce one to several glandular pubescent paniculate inflorescences, each bearing different numbers of flowers (Díaz González, 1986; Nold, 2003). Flowers of both species range from pale blue to purple, are pendant and radially symmetrical, with five petaloid sepals alternating with five petals elongated into nectar-producing spurs. The flowers are bisexual, self-

compatible and to some extent self-pollinating in the absence of pollinators (Medrano et al*.,* 2006).

Aquilegia vulgaris subsp. *vulgaris* is widespread throughout Europe. In the Iberian Peninsula, it grows in forest understory, in permanently wet places, from sea level to 2000m elevation, flowering from May to early June. The population used for quantitative genetic analyses was located in Sierra de Cazorla (Jaén province, Spain), in the southern limit of its distribution in the Iberian Peninsula. *Aquilegia vulgaris* subsp. *dichroa* is endemic to the northwest quadrant of the Iberian Peninsula. It grows in shaded areas in forest strips, from 0 to 1300m elevation and flowering takes place during June. The population used for quantitative genetics was located in Sierra del Caurel (Lugo province, Spain), in the north-western limit of its distribution. *Aquilegia vulgaris* subsp. *nevadensis* is endemic from Southeast of Iberian Peninsula (Sierra Nevada, Sierra de Baza and Sierra Tejeda-Almijara). It grows on permanently moist soils near streams or springs, in forest gaps and alpine meadows, from 1100 to 2500m elevation and is in bloom during June-July. The population used for quantitative genetic analyses was located in Sierra Nevada (Granada province, Spain).

Aquilegia pyrenaica subsp. *pyrenaica* is distributed along the Pyrenees and East of Cantabrian mountains (northern Spain) occurring in alpine meadows, rocky outcrops, and calcareous rocky grasslands from 1200 to 2250m. Flowering takes place in July. The population used for quantitative genetic analyses was located in the central Pyrenees (Huesca province, Spain). *Aquilegia pyrenaica* subsp. *cazorlensis* is a narrow endemic from Sierra de Cazorla and El Pozo in the southeast of Iberian Peninsula. It grows in rocky outcrops and shady cliffs from 1200 to 2000m, and is in bloom from June to early July. The population used for

quantitative genetic analyses was located in Sierra de Cazorla (Jaén province, Spain).

Crossing design

During summer of 2004, we collected fruits from 90 to 210 plants (depending on the availability of reproductive individuals) in one wild population of each study subspecies. In fall of 2005, seeds were sown in individual pots with a mix of peat and coconut fibre, sand and gravel (4:1:1), under common garden conditions. We kept only one plant from each field-collected fruit to be used for quantitative genetic analyses. During spring-summer of 2007, when plants reached sexual maturity (depending on the subspecies), we conducted hand pollinations to produce a nested full-sib, half-sib crossing design (Lynch & Walsh, 1998). Each plant only served as a sire (donor pollen) or as a dam (pollen recipient), and each sire was crossed with four randomly selected dams. Plants used as dams were emasculated before anthesis to prevent self-pollination. We also covered each flower´s styles with a small piece of plastic straw to prevent pollen from other flowers. We removed the piece of straw only at the moment of the hand pollination, and kept it place until the stigmas had dried out as part of fruit development. To pollinate, we used a small brush with pollen from dehiscent anthers of the sire and rubbed it on the stigma of the dam. At the end of the season, we collected the mature fruits and stored them in separate paper envelopes at room temperature. In October of 2008, around 20 seeds from each cross were sown in individual pots, under common garden conditions. Some crosses failed to set seeds, and some sowings failed to germinate, what resulted in a lower final number of families available for quantitative genetic analyses (see table S4.1). The progenies were grown in the garden for two seasons until all of them reached sexual maturity, so all the phenotypic traits could be measured.

Phenotypic measurements

In spring of 2010, when plants were in bloom, we measured 25 traits (table 11) per plant (1231 plants in total). Unfortunately, a heavy attack by slugs early in the season killed many plants of *A. p. pyrenaica* (the earliest growing subspecies) so we could not finally obtain genetic parameters for this subspecies. Early in the flowering season of each subspecies we collected two fully opened flowers per plant to measure the following traits: sepal length, width and area, spur length and area, petal blade length, spur aperture (width at its aperture), spur width above the nectary, and the length and diameter of the pedicel at the base of the first flower. The protocol for floral measurements followed Medrano et al. (2006). At the end of the season we measured the number of inflorescences, flowers and leaves, and height and diameter at the base of the largest inflorescence. We collected the largest leaf from each plant to measure leaf length, leaf mass and leaf area. Measures related to area were taken using a portable leaf area meter, model LI-3000C (Li-Cor Biosciences, Nebraska, USA). Leaf and flower dry mass were obtained by oven drying at 70ºC during 48 hours. Finally, we estimated the density of pubescence as the number of hairs per 1 cm^2, using a 45X magnifying glass. We obtained the density of glandular pubescence in leaves and inflorescences and the density of non-glandular pubescence on the leaves (see Jaime et al. 2013 for details).

Quantitative genetic parameters

All traits were log-transformed before analyses. Due to the final imbalance in family sizes for each subspecies, we used restricted maximum likelihood (REML) to obtain quantitative genetic parameters, using the VCE (ver. 4.2.5) package of Neumaier and Groeneveld (1998). Following Lynch & Walsh (1998), we estimated additive genetic

variances as four times the variance among sires. Statistical significance of the sire variance component (and thus significance of additive genetic variance) was assessed using the difference in -2 x log likelihood between the full nested ANOVA model and a model without the sire effect (Conner et al., 2003). This difference is distributed as chi-square with one degree of freedom. Tests are one-tailed because variance components cannot be negative (Littell et al., 1996: p. 44). To calculate genetic covariances and correlations (r_a) we used maternal full-sib family means, transforming the design in a paternal half-sib model as defined by Lynch & Walsh (1998). Since our objectives regarding genetic covariances involve comparisons between taxa, we restricted the estimation of **G** to those traits that showed positive values of additive genetic variance in all the taxa in the REML analyses (inflorescence height, leaf length and spur width above the nectary; see results). A jackknife procedure was implemented with VCE to estimate variances, covariances, genetic correlations and standard errors of these traits. The variances, covariances and genetic correlations were therefore estimated as the mean of all jackknife pseudovalues and their standard error estimated as the standard error of the pseudovalues (Roff & Preziosi, 1994).

To test the hypothesis that traits with larger heritability show higher differentiation between subspecies we calculated mean phenotypic values for each subspecies based on the data published in Alcántara et al. (2010) and Castellanos et al. (2011) which involve several populations per taxa. The phenotypic means of each trait were then divided by the maximum value among subspecies so that all traits were in a common scale (with maximum = 1). The variance among subspecies for each transformed variable was used as an estimate of the amount of phenotypic differentiation accumulated across subspecies for each trait.

G-matrix comparisons using random skewers

We compared the **G** matrices between each pair of taxa trough the Random Skewers method proposed by Cheverud & Marroig (2007). This method is a direct application of the equation for the multivariate response to selection (Lande & Arnold, 1983): $\Delta \mathbf{z} = \mathbf{G}\boldsymbol{\beta}$. This method measures matrix similarity between two taxa by applying the same vectors of random selection gradients ($\boldsymbol{\beta}$) to their respective **G** and comparing their predicted responses to selection ($\Delta \mathbf{z}$). We applied 10000 vectors of random selection gradients to each pair of **G** matrices, and compared their responses using the vector correlation. Elements of the vectors of random selection gradients were drawn from a uniform distribution of values between 0.0 and 1.0 and randomly assigned positive or negative signs with 50% probability. The total length of the vector was then standardized to 1.0 (sum of the squared vector elements equals 1.0). The vector correlation is equal to the cosine of the angle between the vectors, and measures the co-linearity of the selection responses in multivariate morphometric space. If two matrices are equal, the average response to random selection vectors is expected to be co-linear or equals one and contrarily, if two matrices are completely unrelated with no shared structure, the average response is expected to be perpendicular or equal to zero. The statistical significance of a random skewers set was evaluated against the null hypothesis of no shared structure by using the distribution of vector correlations among n-elements random vectors of unit length (where n is the number of characters in the **G** matrix under analysis). If the observed vector correlation exceeds 95% of the vector correlations found among the random vectors, there is significant structural similarity between the **G** matrices.

Calculation of $\boldsymbol{g}_{max}$, $\boldsymbol{d}_{max}$, and the angle between them

The vector $\boldsymbol{g}_{max}$ was obtained for each study population as the principal eigenvector of its **G** matrix through a principal component analysis. The vector $\boldsymbol{d}_{max}$ was calculated as the single mayor axis of variation between the phenotypic means of each pair of subspecies, which is the standardized multivariate direction of maximum phenotypic differentiation between them (Begin & Roff, 2003). The angle θ between $\boldsymbol{g}_{max}$ and $\boldsymbol{d}_{max}$ is calculated as $\theta = \cos^{-1}[(\boldsymbol{g}_{max})^{T} \boldsymbol{d}_{max}]$ (Pimentel, 1979). We estimated θ between each pair of subspecies (e.g. A and B) through the following Jacknife procedure. We calculated **G** for subspecies A with a given paternal family removed, and then estimated a pseudovalue for the angle θ between $\boldsymbol{g}_{max}$ of A and the vector $\boldsymbol{d}_{max}$ between A and B. We estimated θ as the mean of the pseudovalues. To test for significant departures between $\boldsymbol{g}_{max}$ and $\boldsymbol{d}_{max}$ we used the pseudovalues as samples in the Reyleigh test of circular uniformity (Zar, 1999: p. 616).

Table 11. Narrow sense heritabilities (± SE) for vegetative and floral traits in the studied populations. Additive genetic variances were estimated through restricted maximum likelihood, so heritabilities could be obtained only for those traits with estimable component of variance among sires (not estimable heritabilities are indicated with "-"). Asterisks indicate significant sire effect ($P < 0.05$), and values in italics indicate marginally significant sire effects ($0.05 < P < 0.1$). For each group of traits (vegetative and floral) we provide the results of a t-test comparing their heritability within subspecies.

Traits	h^2 ± SE			
	A. p. cazorlensis	***A. v. vulgaris***	***A. v. nevadensis***	***A. v. dichroa***
Number of leaves	0.087 ± 0.044	-	0.257 ± 0.044*	-
Number of inflorescences	-	-	-	-
Number of flowers	-	0.874 ± 0.129*	-	0.498 ± 0.086*
Leaves per inflorescence	-	-	0.102 ± 0.084	0.021 ± 0.080
Flowers per inflorescence	-	0.748 ± 0.121*	-	0.564 ± 0.094*
Inflorescence height	0.338 ± 0.088	0.689 ± 0.127*	0.197 ± 0.117	0.959 ± 0.117*
Diameter of inflorescence stalk	-	0.269 ± 0.138	-	0.741 ± 0.122*
Leaf petiole length	0.561 ± 0.093*	*0.600 ± 0.084*	0.199 ± 0.038*	*0.375 ± 0.080*
Leaf mass	0.312 ± 0.064	0.113 ± 0.049	-	0.221 ± 0.046*
Leaf area	-	-	0.022 ± 0.034	*0.206* ± 0.045
Specific leaf area	0.004 ± 0.053	*0.263 ± 0.054*	-	*0.200 ± 0.0047*
Glandular pubescence in leaves	0.166 ± 0.039	-	-	-

Non-glandular pubescence in leaves	0.129 ± 0.037	-	0.361 ± 0.055*	1.299 ± 0.127*
Glandular pubescence in inflorescence	-	-	-	0.033 ± 0.051
Mean Vegetative ± SD	0.228 ± 0.189	0.508 ± 0.290	0.190 ± 0.118	0.465 ± 0.402
Flower pedicel diameter	0.675 ± 0.120*	0.142 ± 0.056	-	0.120 ± 0.101
Flower pedicel length	0.243 ± 0.104	-	-	-
Sepal length	-	0.195 ± 0.060	-	0.485 ± 0.142
Sepal width	-	0.433 ± 0.089*	0.778 ± 0.156*	-
Sepal area	-	-	0.291 ± 0.084	0.175 ± 0.071
Spur length	-	0.105 ± 0.043	0.208 ± 0.070	-
Spur area	-	-	0.103 ± 0.059	-
Petal blade length	-	-	-	*0.445 ± 0.077*
Spur aperture	-	0.134 ± 0.050	0.087 ± 0.056	-
Spur width above nectary	0.904 ± 0.137*	0.531 ± 0.084*	0.050 ± 0.065	0.427 ± 0.106
Flower mass	-	1.107 ± 0.201*	-	-
Mean Floral ± SD	0.607 ± 0.335	0.378 ± 0.361	0.253 ± 0.272	0.330 ± 0.170
$t_{\text{vegetative vs. floral}}$ **(P)**	3.463 (0.179)	1.088 (0.473)	0.681 (0.619)	1.566 (0.362)

Results

Quantitative genetic parameters

Table 11 summarizes the heritabilities of the studied traits in all subspecies. REML analyses were able to obtain additive genetic variance components (estimable sire effect) for 24 of the 25 traits studied (all but the number of inflorescences per plant) in at least one subspecies (table 11). However, only 11 traits showed significant additive genetic variance in at least one subspecies. The number of traits with significant heritable variation was 7 in the population of *A. v. vulgaris*, 4 traits in *A. v. nevadensis*, 6 in *A. v. dichroa* and 3 in *A. p. cazorlensis*. A *t*-test indicates that heritability was similar in vegetative and floral traits in each subspecies ($t > 0.68$ and $P > 0.17$ in all cases). Moreover, according to two-proportions comparisons tests the relative frequency of vegetative and floral traits with significant heritability was similar within all subspecies, except in the case of *A. v. dichroa* which had a higher frequency of significantly heritable vegetative than floral traits (vegetative vs. floral traits: 4/14 vs. 3/11, $P = 0.94$ in *A. v. vulgaris*; 3/14 vs. 1/11, $P = 0.41$ in *A. v. nevadensis*; 1/14 vs. 2/11, $P = 0.54$ in *A. p. cazorlensis*; 6/14 vs. 0/11, $P < 0.02$ in *A. v. dichroa*).

Castellanos et al. (2011) estimated heritability using molecular markers for 12 traits in wild plants of the same populations we studied for *A. v. nevadensis* and *A. p. cazorlensis*. Our common garden estimates based on controlled crosses largely agree with their results. Considering traits for which we could estimate the sire component of genetic variance, the slope of the regression of field vs. common garden estimates does not depart significantly from one (*A. v. nevadensis*: slope = 0.953 ± 0.761, $t(H_0: \text{slope} = 1) = 0.06$, n = 8, $P = 0.95$; *A. p. cazorlensis*: slope = 0.942 ± 0.356, $t(H_0: \text{slope} = 1) = 0.16$, n = 6, $P = 0.88$; Fig. 18). Moreover, paired

t-tests indicate that the mean heritabilities obtained by both methods do not differ significantly (*A. v. nevadensis*: t = 0.06, n = 8, *P* = 0.95; *A. p. cazorlensis*: t = 0.16, n = 6, *P* = 0.88).

Considering those traits for which we have comparable information from wild populations and quantitative genetics, trait mean heritability was significantly related with trait variance among subspecies for vegetative and floral traits (Fig. 19). The Jacknife estimates of the slope of the relationship were significant for both types of traits (vegetative traits slope ± SE: 0.137 ± 0.025, n = 7, t = 5.52, P < 0.01; floral traits slope ± SE: 0.053 ± 0.014, n = 6, t = 3.92, P < 0.02). Furthermore, these slopes were significantly different (Z = 3.305, P < 0.05; comparison of slopes test based on Jacknife procedure described by Moses & Klockars, 2012).

According to REML analyses, only three traits showed estimable additive genetic variance in the four studied subspecies: inflorescence height, leaf petiole length, and spur width above nectary (table 11). Thus, we only included these traits to obtain **G** matrices. Jacknife tests indicated that all genetic variances and covariances, as well as the genetic correlations between the three traits, were statistically significant in the four studied subspecies (table 12), with the only exception of the relationship between spur width above nectary and inflorescence height in *A. v. vulgaris*, which was marginally significant (r_a = -0.163; *P* = 0.082). The strongest genetic correlation occurred between leaf length and inflorescence height (r_a > 0.97) in all subspecies but *A. v. nevadensis*, for which the strongest genetic correlation occurred between spur width above nectary and leaf length (r_a = -0.91). Most genetic covariances were positive, with the exception of the negative covariance between spur width above nectary and the other two traits in the case of *A. v. vulgaris* and *A. v. nevadensis*. Accordingly, this trait had a negative load on the

g_{max} of these subspecies but a positive load in *A. p. cazorlensis* and *A. v. dichroa* (table 13). The three traits were highly genetically integrated within all subspecies since the largest eigenvalue of the **G** matrices (corresponding to g_{max}), accounted for 85.6% of the variance in the study population of *A. p. cazorlensis*, 87.8% in *A. v. dichroa*, 80.5% in *A. v. nevadensis*, and 91.3% in *A. v. vulgaris.*

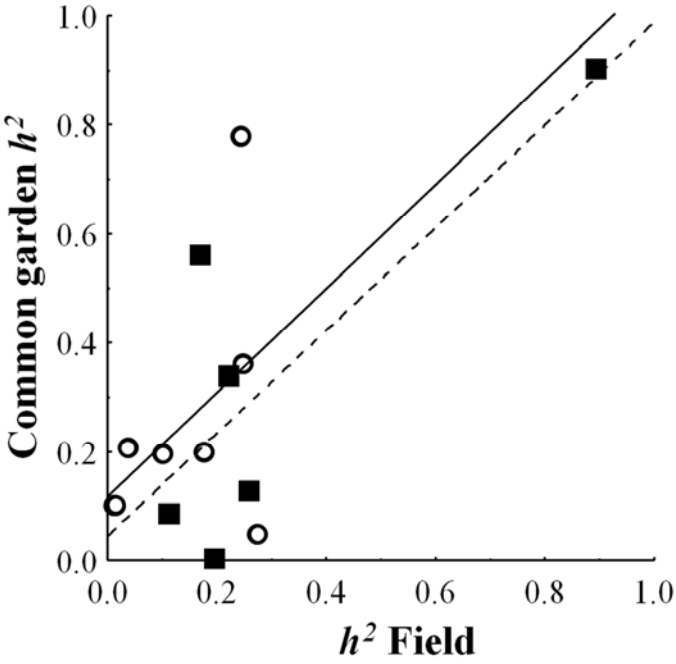

Figure 18. Scatterplot of heritability estimates based on quantitative analyses of paternal half-sib crosses in common garden (y-axis), obtained from this study, and in molecular marker analyses of wild population (x-axis), obtained by Castellanos et al*.,* (2011) in the same populations of *Aquilegia vulgaris* subsp. *nevadensis* (open circles, solid line) and *A. pyrenaica* subsp. *cazorlensis* (filled squares, dashed line). Each point is the estimate for a different phenotypic trait. Regression lines for each subspecies have slopes not significantly different from one.

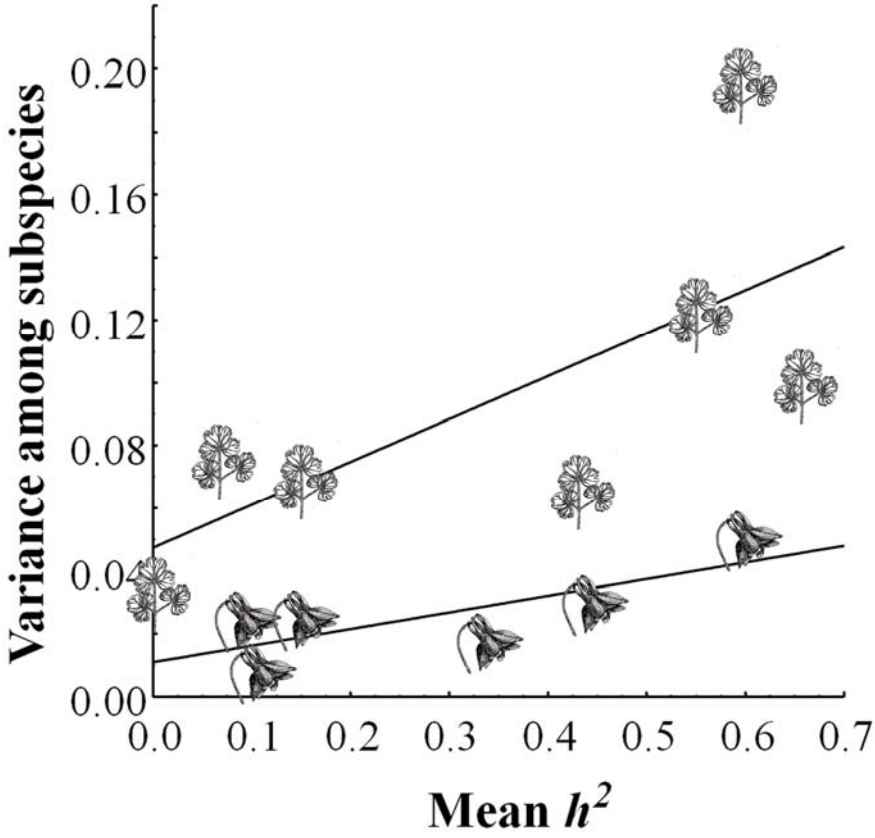

Figure 19. Relationships between mean heritability and the amount of variance among subspecies means for vegetative (leaf symbol) and floral traits (flower symbol). The slopes of the regression lines are significantly different from zero and from each other. The characters included in the analysis are those for which we have comparable information from wild populations and quantitative genetics: number of inflorescences, inflorescence height, number of leaves and flowers per inflorescence, leaf length, SLA, density of non-glandular pubescence in the leaves, sepal length and width, spur width, length and aperture, and petal blade length.

Table 12. Estimates (± SE) for the additive genetic variance (diagonal elements) and covariance (elements above the diagonal), and genetic correlations (elements below the diagonal) for the studied subspecies. Genetic variances and covariances were multiplied by 1000 for clarity. All values differed significantly from zero at $P < 0.01$, except those indicated in italics ($P < 0.1$).

Subspecies	InfH	LeafPetL	SpurDAbNect
	A. p. cazorlensis		
Inflorescence height	0.377 ± 0.035	0.598 ± 0.048	0.634 ± 0.056
Leaf length	0.986 ± 0.004	0.995 ± 0.082	1.204 ± 0.088
Spur width above nectary	0.597 ± 0.060	0.678 ± 0.050	3.326 ± 0.185
	A. v. vulgaris		
Inflorescence height	2.372 ± 0.094	2.397 ± 0.095	*-0.082 ± 0.035*
Leaf length	0.977 ± 0.004	2.550 ± 0.110	-0.292 ± 0.029
Spur width above nectary	*-0.163 ± 0.082*	-0.353 ± 0.063	0.416 ± 0.050
	A. v. nevadensis		
Inflorescence height	0.789 ± 0.126	0.481 ± 0.087	-0.431 ± 0.054
Leaf length	0.616 ± 0.109	1.025 ± 0.137	-0.527 ± 0.059
Spur width above nectary	-0.854 ± 0.056	-0.912 ± 0.037	0.340 ± 0.036
	A. v. dichroa		
Inflorescence height	4.076 ± 0.030	3.159 ± 0.027	0.719 ± 0.022
Leaf length	0.973 ± 0.003	2.602 ± 0.029	0.225 ± 0.018
Spur width above nectary	0.388 ± 0.071	0.181 ± 0.082	0.922 ± 0.019

Table 13. Loadings of traits on the principal eigenvector of **G** ($\boldsymbol{g}_{max}$) of each subspecies.

	A. p. cazorlensis	*A. v. vulgaris*	*A. v. nevadensis*	*A. v. dichroa*
Inflorescence height	0.146	0.511	0.678	0.515
Leaf length	0.264	0.533	0.855	0.405
Spur width above nectary	0.590	-0.044	-0.532	0.08

Analyses involving the **G** *matrices*

Comparisons of **G** matrices between subspecies using random skewers indicated significant differences between all but the pair formed by *A. v. vulgaris* and *A. v. dichroa* (table 14). The potential responses to selection, mediated by **G**, of these two subspecies were very similar (angular correlation close to one). Moreover, the similarity of potential responses to selection was larger in comparisons between conspecific subspecies ($r > 0.59$ in all cases) than between heterospecific subspecies ($r < 0.50$ in all cases), suggesting that differences in **G** matrices increase with taxonomic distance. Figure 20 shows the directions of $\boldsymbol{g}_{max}$ for each subspecies (but *A. p. pyrenaica*) for each pair of traits. Since the average response to random selection should be aligned with $\boldsymbol{g}_{max}$, the angles between the lines portrayed in figure 20 can help to understand the results of the random skewers analysis. The angles between *A. v. vulgaris* and *A. v. dichroa* are the narrowest ones in the three combinations of traits, while the angles between subspecies of *A. vulgaris* and *A. p. cazorlensis* are much broader, particularly in the combinations involving spur diameter above the nectary (figs. 20b, c).

Table 14. Results of random skewers analysis comparing the potential responses to selection between each pair of subspecies based on their **G** matrices. For each comparison we show the mean and variance of the angular correlations between potential response vectors obtained after 10000 Monte Carlo simulations, and the probability of finding a stronger correlation between random vectors than the observed correlation.

Subspecies comparisons	**Mean**	**Variance**	*P* <
A. v. dichroa - *A. v. vulgaris*	0.9185	0.0444	0.043
A. v. nevadensis - *A. v. vulgaris*	0.6957	0.1701	0.164
A. v. dichroa - *A. v. nevadensis*	0.5917	0.1827	0.202
A. p. cazorlensis - *A. v. dichroa*	0.4979	0.1447	0.249
A. p. cazorlensis - *A. v. vulgaris*	0.4386	0.1105	0.281
A. p. cazorlensis - *A. v. nevadensis*	0.2586	0.0667	0.371

The angle θ between $\boldsymbol{g}_{max}$ and $\boldsymbol{d}_{max}$ was significantly different from zero in all pairwise comparisons between subspecies (table 15). The variability of θ was similar in comparisons between conspecific and heterospecific subspecies, ranging between 16.6 and 85.3 and between 15.2 and 73.12, respectively. Figure 20 can help to understand these results by comparing the orientation of $\boldsymbol{g}_{max}$ with the imaginary line connecting subspecies means. The general orientation of $\boldsymbol{g}_{max}$ between inflorescence height and leaf length was consistent with the general direction of divergence at the species level (fig. 20a), but not necessarily at the subspecies level. Moreover, the magnitude (distance) of differentiation at species level was greater along the general direction of $\boldsymbol{g}_{max}$ but much shorter between conspecific subspecies diverging in a nearly perpendicular direction to the general direction of $\boldsymbol{g}_{max}$ (*A. v. vulgaris* vs. *A. v. dichroa*, and *A. p. pyrenaica* vs. *A. p. cazorlensis*). This

pattern did not occur when spur diameter is considered (figs. 20b, c), since the orientations of $\boldsymbol{g}_{max}$ scarcely agree with the directions of divergence at the species level. Moreover, in comparisons involving spur diameter the orientation of most $\boldsymbol{g}_{max}$ is nearly parallel to one of the axes, indicating that one of the traits has much larger genetic variance than the other. More specifically, subspecies of *A. vulgaris* have much larger variance in the direction of inflorescence height (fig. 20b) and leaf length (fig. 20c) than in the direction of spur diameter, while the opposite occurs in *A. p. cazorlensis*.

Table 15. Vectors of phenotypic divergence between pairs of taxa ($\boldsymbol{d}_{max}$) and their angle relative to $\boldsymbol{g}_{max}$ of each taxa (θ). For each pair of taxa A-B, θ_1 is the angle in degrees between $\boldsymbol{g}_{max}$ of A and $\boldsymbol{d}_{max}$; θ_2 is the angle between $\boldsymbol{g}_{max}$ of B and $\boldsymbol{d}_{max}$. The angles were always significantly different from zero ($P < 0.05$) according to Rayleigh´s test.

Taxa comparisons	Inflor. height	Leaf length	Spur width	θ_1	θ_2
Within species		$\boldsymbol{d}_{max}$			
A. v. dichroa - A. v. vulgaris	0.566	-0.707	0.424	85.31	82.23
A. v. dichroa - A. v. nevadensis	-0.686	-0.624	0.374	30.66	16.61
A. v. nevadensis- A. v. vulgaris	-0.932	-0.311	0.186	34.47	28.15
A. p. cazorlensis- A. p. pyrenaica	-0.196	0.588	0.785	27.73	-
Between species		$\boldsymbol{d}_{max}$			
A. p. cazorlensis- A. v. dichroa	-0.808	-0.462	-0.366	46.53	15.21
A. p. cazorlensis- A. v. nevadensis	-0.734	-0.592	-0.332	34.84	67.69
A. p. cazorlensis- A. v. vulgaris	-0.845	-0.349	-0.404	46.70	35.32
A. v. vulgaris- A. p. pyrenaica	0.699	0.455	0.552	38.66	-
A. v. dichroa- A. p. pyrenaica	-0.653	-0.552	-0.519	23.27	-
A. v. nevadensis- A. p. pyrenaica	0.541	0.444	0.714	73.12	-

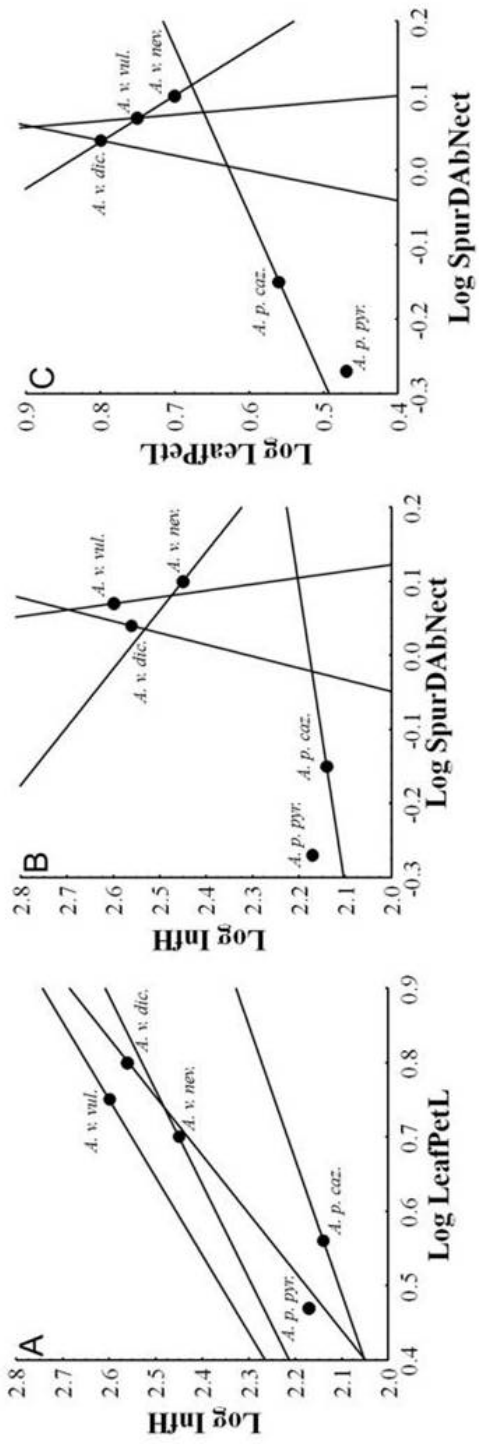

Figure 20. Decomposition of the three dimensional $\boldsymbol{g}_{max}$ vector of each subspecies into bivariate relationships. Within each panel,the phenotypic means of each subspecies and the line corresponding to the slope of the genetic covariance between the two traits in $\boldsymbol{g}_{max}$ are represented. Each line illustrates thus the direction of $\boldsymbol{g}_{max}$ for each subspecies for each pair of traits.

Discussion

Does the amount of standing genetic variation constrain the magnitude of phenotypic diversification?

Quantitative genetic studies require large sample sizes to minimize the error variance and be able to detect even moderate levels of additive genetic variance. In spite of this limitation, quantitative genetic studies accumulated over the last decades suggests that genetic variation is abundant in plant populations for many traits (Geber & Griffen, 2003; Ashman & Majetic, 2006). Our results based on a limited number of paternal families may have low power to detect significant additive genetic variation. Still, our conservative estimates indicate that 11 out of 25 traits measured show significant heritability in at least one population. Our estimates agree largely with the mean heritabilities found in reviews. Ashman & Majetic (2006) found that floral traits have and average h^2 of 0.39, remarkably similar to the mean h^2 of 0.392 ± 0152 in our study. In the case of vegetative traits, Geber & Griffen (2003) found that an average h^2 of 0.23 for vegetative morphology, slightly lower than the mean h^2 of 0.348 ± 0.162 in our study. Estimates of h^2 obtained under greenhouse conditions are expected to be larger than the values of h^2 realized in wild environments. However, our estimates using controlled crosses in a common garden setting largely agree with those obtained directly in the wild through molecular markers by Castellanos et al. (2011). Obviously, there are discrepancies between both methods that may have many causes, like the different amount of genotypes sampled (larger in the case of molecular marker analysis) or the reduced environmental variance in the garden. In any case, we did not find any evident bias for larger or lower estimates using either technique. Thus, our results with the Iberian columbines reproduce the general picture that many traits show heritable variation in wild plant populations and that heritability is of similar

magnitude in vegetative and floral traits. Moreover, these results suggest that the ability to respond to selection is not severely limited by a lack of genetic variation in the Iberian columbines, at least not more limited than in the average angiosperm.

The response to selection of a given trait z (Δz) can be estimated according to the breeders' equation as the product of the selection differential (s) times h^2 ($\Delta z = s\ h^2$). For traits with the same value of h^2, the change in the mean phenotype will increase linearly with the magnitude of selection. Given that vegetative and floral traits tend to show significant and similar heritability within the studied populations, and that vegetative traits are more commonly subject to selection than floral traits in wild populations of Iberian columbines, Castellanos et al. (2011) proposed that differentiation in the studied taxa should be larger for vegetative than for floral traits. In agreement with this hypothesis, we have found that for the same level of heritability, vegetative traits show higher differentiation among taxa than floral traits, and this difference increases with increasing heritability. This result suggests that the availability of additive genetic variation is not responsible for the larger differentiation of vegetative than floral traits in this group of columbines. An alternative explanation for a higher differentiation of vegetative traits could be that they might have larger phenotypic plasticity that would increase their variance among taxa relative to the variance of floral traits. However, if this were the case, the lines in figure 19 would tend to be parallel (since traits with very low heritability would still show a high level of differentiation) unless traits with larger heritability had larger phenotypic plasticity, what seems counterintuitive since phenotypic plasticity involves an environmental effect that increases total phenotypic variation, decreasing the value of h^2. Another possible explanation for the larger phenotypic variability of vegetative than floral traits could be the

existence of stronger phenotypic integration in floral traits (Ashman & Majetic, 2006). Whether phenotypic integration, as measured by **G**, constrains or enhances diversification depends on the patterns of multivariate selection acting on the set of integrated traits, so that diversification would be constrained when the pattern of multivariate selection is in directions other than the directions of the genetic correlations, while diversification would be favored when the directions of genetic correlations agree with the directions of multivariate selection (Merilä & Björklund, 2004; Smith & Rausher, 2008; Agrawal & Stinchcombe, 2009; Conner, 2012). The small number of families we could use in our study prevented a full analysis of genetic integration among the 25 vegetative and floral traits, however the absence of selection on floral traits (Castellanos et al*.,* 2011) suggests that the agreement or disagreement between the patterns of phenotypic integration and multivariate selection has not been a major issue in the diversification of floral traits in the studied taxa. Thus, our findings support the hypothesis that the largest phenotypic diversification in vegetative than in floral traits in the Iberian columbines is more related to differences in the strength of selection acting on these different suites of traits than to the amount of heritable variation for these traits.

Although the larger magnitude of differentiation of vegetative compared to floral traits did not depend on the availability of genetic variance, the positive correlations between trait heritability and variance among taxa actually suggest a constraint on the evolution of some traits caused by their low heritability. This is particularly clear in the case of vegetative traits for which diversification has been much larger in traits with larger h^2. This pattern of diversification can be seen as the univariate equivalent to evolution along genetic lines of least resistance (Schluter,

1996): divergence is larger along the lines corresponding to traits with larger heritability.

*Do **G** matrices impose divergent patterns of phenotypic differentiation between closely related taxa?*

Since **G** matrices can differ between closely related taxa or populations (see Arnold et al*.,* 2008; and references therein), the responses to the same pattern of selection can differ, leading to increased phenotypic differentiation. Although it is not clear how fast these changes may accumulate (Steppan et al*.*, 2002), it seems reasonable to expect that more closely related taxa or populations should show more similar **G** matrices and thus, more similar responses to selection. Our results agree with this expectation since differences in the response to selection increased with taxonomic distance: although only the comparison between *A. v. vulgaris* and *A. v. dichroa* concluded not significant differences in the response to selection, the responses of conspecific subspecies were always more similar than those of heterospecific subspecies. Thus, we can conclude that the process of diversification of the studied taxa has involved changes in the **G** matrix.

The differences in **G** between subspecies were primarily related to differences in the orientation of the genetic covariance between the floral and the two vegetative traits analyzed (fig 20b,c), while the relationships between the two vegetative traits were largely consistent between taxa (fig. 20a). As shown in figures 20b, c, the $\boldsymbol{g}_{max}$ for *A. p. cazorlensis* is largely perpendicular to the $\boldsymbol{g}_{max}$ of the subspecies of *A. vulgaris*, and these correlations also varied between the subspecies of *A. vulgaris*, suggesting that they are labile. In fact, the finding of strong genetic correlations between vegetative and floral traits in some subspecies was unexpected. Ashman & Majetic (2006) surveyed the literature and found

that the average genetic correlation between floral and vegetative traits was 0.15, which is similar to the correlations we found in *A. v. vulgaris* and *A. v. dichroa*. However, the magnitude of the correlations was much higher in *A. v. nevadensis* (-0.854 and -0.912) and *A. p. cazorlensis* (0.597 and 0.678). According to quantitative genetics theory, genetic covariances between traits are caused either by the pleiotropic effects of individual loci on multiple traits or by linkage disequilibrium between loci (Lynch & Walsh, 1998). The fact that floral and vegetative traits were positively genetically correlated in some subspecies and negatively in others suggests that the genetic correlation is not caused by pleiotropic effects of genes (Roff, 1997), since it seems unlikely that the genetic pathways that link two traits could differ so much between closely related taxa to result in opposite effects of the same genes on the expression of a trait. Thus, it seems more likely that the strong genetic correlations we found are the result of linkage disequilibrium. However, the origin of linkage disequilibrium is not clear. Selection may change **G** as a consequence of the generation of linkage disequilibrium (Bulmer, 1980). However, it is difficult to envisage a selection scenario where leaf length or inflorescence height were under correlational selection with spur width, even more since such scenario should be able to change in opposite directions in different environments. Inbreeding can also change the orientation of **G** (Phillips et al*.,* 2001). Moreover, genetic correlations due to linkage disequilibrium are likely to persist in inbred populations because inbreeding reduces the efficiency of recombination. This alternative seems a likely explanation for our results, since we found the strongest correlations in the two narrowly endemic species, which have small populations (Alcántara et al*.,* 2010), and some level of self-fertilization (Castellanos et al*.,* 2011). Moreover, the phylogenetic analyses of Bastida et al. (2010) suggested that geographic isolation played an important role in the process of diversification of the

Euroasiatic lineage of the genus and, accordingly, Garrido et al. (2012) have shown that gene flow is very restricted among populations of columbines located few kilometers away within the island of Sardinia. Thus, small population size, self-compatibility and geographic isolation all could contribute to high levels of inbreeding in narrow endemic columbine populations from southern Iberian Peninsula, what might have contributed to generate and preserve differences in their **G** matrices through linkage disequilibrium.

*Does the structure of **G** matrices constrain the pattern of differentiation between taxa?*

Given that the differences between **G** matrices increased with taxonomic distance, we would expect that the direction of divergence between conspecific subspecies would be more closely aligned with the direction imposed by their $\mathbf{g}_{max}$ than the directions of divergence between heterospecific subspecies. However, the angle θ was significantly different from zero in all pairwise comparisons between subspecies, and the range of values of this angle was similar in conspecific and heterospecific comparisons. This result could be interpreted, in principle, as evidence that natural selection has been strong enough to overrule the constraints dictated by **G** (Merilä & Björklund, 2004). However, not all the traits in **G** may fit unambiguously to this conclusion since the results of the test can be more affected by some traits than by others. Indeed, our results suggest that genetic covariances between vegetative traits constrained the differentiation between taxa, while covariances between floral and vegetative traits do not seem to have constrained the differentiation. Inspection of figure 20a suggests that the general orientation of $\boldsymbol{g}_{max}$ between vegetative traits was consistent with the general direction of divergence at the species level since subspecies belonging to *A. pyrenaica* and *A. vulgaris* diverge along a line of positive

correlation between inflorescence height and leaf length, what largely agrees with the positive genetic correlations between these traits in all subspecies. On the other hand, the magnitude (distance) of differentiation in vegetative traits between the two species, and to a lesser extent between subspecies of *A. vulgaris*, was greater along the general direction of $\boldsymbol{g}_{max}$ and much shorter between conspecific subspecies diverging in the opposite, perpendicular, direction. This pattern could be explained by a process of divergence in vegetative traits through genetic drift constrained by $\boldsymbol{g}_{max}$, but it is also possible that the direction of selection on these traits were in agreement with the direction of $\boldsymbol{g}_{max}$. In fact, Alcántara et al. (2010) found positive selection differentials on inflorescence height and leaf length in populations of four of the studied subspecies (*A. v. dichroa* was not included in their study). Therefore, an agreement between the genetically constrained direction and the direction favored by selection might have maximized the responses to selection, favoring a large differentiation of vegetative traits between the two studied species (Conner, 2012).

The general disagreement between $\boldsymbol{g}_{max}$ and $\boldsymbol{d}_{max}$ in our tests is clearly more related to the patterns of covariation between floral and vegetative traits. Since the $\boldsymbol{g}_{max}$ for spur diameter and vegetative traits are almost perpendicular between *A. p. cazorlensis* and the subspecies of *A. vulgaris*, the differentiation of these traits between taxa can be largely independent. Directional selection on vegetative traits would scarcely affect the evolution of spur diameter since the subspecies with larger variance for vegetative traits have much lower variance for spur diameter and vice versa. Directional selection on spur diameter in subspecies of *A. vulgaris* would result in small change of this trait due to its small genetic variance in these subspecies. However, differentiation in spur diameter is more likely in *A. p. cazorlensis*, which harbours large genetic variance for

this trait. In fact, results of Medrano et al. (2006) suggest that populations of *A. p. cazorlensis* are more phenotypically differentiated in floral traits than populations of *A. v. vulgaris* in the same study region. The general absence of selection on floral traits in the studied taxa (Castellanos et al. 2011) suggests that these differences between populations are more probably attributable to genetic drift than to divergent selection. However, genetic drift does not explain the magnitude of differentiation of spur diameter at species level (i.e. between *A. pyrenaica* and *A. vulgaris*), so we cannot rule out the possibility that divergent selection on this trait occurred in the past.

Conclusions

Our results, combined with previous studies on the Iberian columbines, are shedding light on the factors that have shaped the process of diversification of this group. The amount of genetic variation does not explain the lower magnitude of phenotypic diversification in floral than in vegetative traits in the Iberian columbines, so our results support the prediction that the largest differentiation for vegetative than floral traits in this group is related to the existence of divergent selection acting on vegetative but not on floral traits. Within vegetative traits, a constraint in their differentiation was caused by the low heritability of some traits, so that phenotypic diversification among taxa was larger for traits with larger heritability. On the other hand, the large differentiation of vegetative traits may have been enhanced by the synergistic action of natural selection and genetic correlations between these traits. The **G** matrices of the studied taxa are more different between heterospecific than between conspecific subspecies, due to changes probably caused by linkage disequilibrium between floral and vegetative traits in small isolated populations of narrow endemic taxa. As a consequence of these changes, the pattern of differentiation predicted by the **G** matrices agrees with the observed

patterns of differentiation in the case of vegetative traits but not when vegetative and floral traits are considered together.

Appendices

Table S4.1. Localities of origin and number of families per taxon used in quantitative genetic analyses.

Subspecies	UTM Coordinates	Population	Num. Sires	Num. Dams	Num. sibs	Paternal families used in Gmax
A. v. vulgaris	30S 514740E 4199580N	**Guadalentín**	31	64	377	15
A. v. nevadensis	30S 464649E 4105811N	**Pradollano**	19	41	292	9
A. v. dichroa	29T 065446E 472070N	**Cabaña del Arce**	30	63	257	16
A. p. pyrenaica	30T 701597E 4739703N	**Tobazo**	16	26	72	-
A. p. cazorlensis	30S 503431E 4182541N	**Barranco la Canal**	29	49	233	10

DISCUSIÓN GENERAL

El conjunto de trabajos elaborados en esta memoria van encaminados a dar respuesta a la cuestión ¿que ha promovido la diferenciación de nicho y la divergencia de hábitats entre los taxones específicos y subespecíficos de las aquilegias ibéricas?, y a evaluar también en qué medida dicha diferenciación de nicho y caracteres está conectada con un proceso de radiación adaptativa. Para ello, junto con los trabajos anteriores de miembros de nuestro grupo de investigación, que abordaban distintos ejes ambientales de diferenciación, como son el tipo y profundidad del suelo y la altitud, hemos analizado el papel de otros ejes ambientales que podrían tener un papel en la diferenciación de nicho y la diversificación de los taxones ibéricos del género *Aquilegia*, como son la dimensión propiamente climática, el estrés hídrico y lumínico y la herbivoría.

Entendiendo la diferenciación de nicho mediante la complementación de enfoques macroscópicos y de escala de detalle

La primera aproximación que hemos realizado a la diferenciación de nicho de las aquilegias ibéricas puede considerarse de tipo macroscópico. El modelado de nicho elaborado con MAXENT nos da una idea inicial del nicho ambiental de los taxones y los ejes ambientales que determinan la diferenciación de hábitats en las aquilegias ibéricas en la gran escala. Así, la resolución de este modelado, establecido con variables climáticas y edáficas fue de 1x1 km en el caso de las primeras, mientras que no pudo ser menor de 10x10 km con las variables edáficas. Por tanto, existen limitaciones para la definición de los nichos de las especies, comunes a todas estas aproximaciones, las cuales son especialmente relevantes cuando la diferenciación de nicho obedece a variables que varían en la escala fina, como es esperable entre taxones conespecíficos o congenéricos (Broennimann et al. 2012). En cualquier caso, la modelación

con MAXENT mostró que mientras los taxones conespecíficos que viven en simpatría conservan el nicho ambiental, aquellos que lo hacen en alopatría tienen nichos ambientales diferentes. Este patrón cambia, cuando la comparación se hace entre taxones heteroespecíficos, no encontrándose en este caso un patrón claro, apareciendo tanto conservación como diferenciación de nicho independientemente del solapamiento de su distribución geográfica. En general, por tanto, el nicho ambiental de las aquilegias ibéricas parece muy lábil, y estaría muy determinado por el componente climático. De hecho, la aproximación a través de métodos multivariantes apunta a que la diferenciación de nicho ocurre a lo largo de dos gradientes climáticos, mientras que la conservación ocurre a lo largo de un gradiente de propiedades edáficas. Nakazato et al. (2010) encontraron resultados similares en diferentes especies de *Solanum*. Estos resultados apuntan a que las aquilegias ibéricas no están limitadas por fuerzas de conservación del nicho ambiental, sino que responden adaptativamente a los cambios rápidos y consistentes, especialmente en lo que respecta a los cambios acaecidos en la península ibérica a lo largo de las fluctuaciones glaciales del Pleistoceno. Con más cautela han de tomarse los resultados que atañen a las variables edáficas, más susceptibles de variar en una escala geográfica muy pequeña. Dicho de otro modo la escala de variación ambiental en caracteres edáficos usada con los modelos de MAXENT fue probablemente insuficiente, sobre todo para detectar diferenciación, si la hubiera, en el caso de especies simpátricas.

Una posible solución a esto, es complementar esa información con estudios observacionales o experimentales que exploran la respuesta de las especies a la variación de escala fina. Los capítulos 2 y 3, y otros estudios realizados por nuestro grupo de investigación (Tesis doctoral de J. Bastida 2009; publicaciones en preparación) han explorado la respuesta

a la variación ambiental abiótica (edáfica, altitudinal) y biótica (herbivoría y competencia) de varios taxones de *Aquilegia*. Así para un subgrupo de subespecies ibéricas podemos mejorar y corregir los resultados generados por MAXENT.

En el capítulo 2 comparamos el ambiente lumínico e hídrico de dos subespecies de *A. vulgaris* (*A. v. vulgaris* y *A. v. nevadensis*) y dos subespecies de *A. pyrenaica* (*A. p. pyrenaica* y *A. p. cazorlensis*), y su papel en la diferenciación de estas subespecies. La combinación del estrés hídrico y lumínico, común en el verano de la región Mediterránea, lleva a un trade-off en el intercambio gaseoso durante la fotosíntesis, la respiración y transpiración, que es resuelto de forma diferente por las plantas y que puede resumirse en un rasgo ecofisiológico fundamental, la eficiencia de uso del agua. La variación natural de este trade-off entre poblaciones (pertenecientes a la misma especie) que ocupan ambientes diferentes (e.g. Heschel et al. 2002, 2004a; Wu et al. 2010), y su posible papel en la diferenciación de hábitats dentro de especies y/o entre taxones estrechamente relacionados ha sido recientemente explorado (Heschel et al. 2004a, 2004b; Givnish et al. 2004; Donovan et al. 2007; Savage & Cavender-Bares 2011; Manzaneda et al. 2012), no obstante no hay nada relacionado con diferenciación en caracteres ecofisiológicos entre taxones diferentes taxones de *Aquilegia*. La capacidad para lidiar con este trade-off y regular ambos parámetros (fotosíntesis y conductancia estomática) podría estar evolutivamente involucrada en la diferenciación de nicho entre especies (Ackerly et al. 2000), por lo que su estudio es altamente relevante. Nuestros resultados con *Aquilegia* sugieren claras distinciones a nivel específico en los ambientes lumínicos e hídricos, mientras que a nivel subespecífico solo encontramos diferencias entre las subespecies de *A. pyrenaica* en relación a la disponibilidad lumínica. Además esta diferenciación está asociada a una diferente respuesta funcional

(intercambio gaseoso) a nivel específico, adecuándose cada especie a la singularidad del ambiente que ocupa. Si estos resultados los comparamos con los obtenidos en el PC4 (caracterizado por la xericidad y profundidad del suelo) del análisis multivariante llevado a cabo en el capítulo primero, observamos que para el caso de la disponibilidad hídrica o xericidad, la comparación entre *A. v. vulgaris* y *A. v. nevadensis* y la comparación entre *A. p. pyrenaica* y *A. v. vulgaris* obtenidas mediante la aproximación grosera (MAXENT) coincide con la obtenida en el capítulo 2, en la que para este carácter hay conservación de nicho. Sin embargo, en lo que respecta a la comparación entre las subespecies de *A. pyrenaica* los resultados obtenidos mediante MAXENT no coinciden con los obtenidos directamente en campo para esta comparación, al igual que ocurre cuando comparamos *A. v. vulgaris* con *A. p. pyrenaica*, y *A. p. cazorlensis* con *A. v. vulgaris* y con *A. v. nevadensis*.

En la misma línea de exploración de escala fina, algunos de los trabajos realizados por Bastida (2009) en su tesis doctoral exploraron algunas dimensiones del nicho ambiental, como son la variación edáfica y la variación altitudinal, sugiriendo que juegan un papel importante en la diferenciación fenotípica de las aquilegias ibéricas. Respecto a la variación edáfica, se encontró que *A. vulgaris* (especie de amplia distribución), fue más tolerante que *A. pyrenaica* (especie de distribución más localizada) a la variación en la naturaleza del suelo (silíceo vs. calizo) y profundidad, lo cual se relaciona con que *A vulgaris* sea una especie de amplia distribución (que puede aparecer en suelos calizos y silíceos), mientras que la distribución de *A. pyrenaica* está más restringida y siempre asociada a suelo calizo. A nivel subespecífico, *A. v. vulgaris* (de amplia distribución) también es más tolerante a la variación edáfica que *A. v. nevadensis* (endemismo de Sierra Nevada). Esta diferente tolerancia edáfica quedó patente en los caracteres morfo-funcionales implicados en

la diferenciación entre especies y subespecies, pudiendo además la plasticidad fenotípica adaptativa de los taxones ampliamente distribuidos estar involucrada en esa tolerancia y ocupación de suelos de distinta naturaleza. De hecho, parece ser que en los taxones más ancestrales y de amplia distribución la plasticidad les podría haber permitido ocupar ambientes nuevos, y posteriormente, esa plasticidad podría haberse perdido por especialización en el hábitat dando lugar a especiación y conformación de endemismos.

Los resultados de Bastida (2009) son también sugerentes en lo que respecta a diferenciación de nicho altitudinal entre taxones conespecíficos que son simpátricos regionalmente. Este autor, demostró la existencia de divergencia altitudinal de al menos dos subespecies de *A. vulgaris* (*A. v. vulgaris* y *A. v. nevadensis*) que coexisten en el sur de la península Ibérica, pero que están segregadas altitudinalmente. Esta segregación parece estar basada en procesos de selección natural divergente sobre el número de hojas, algo que también han sugerido Alcántara et al. (2011). No obstante, otros rasgos funcionales y morfológicos no explorados podrían estar involucrados en la segregación altitudinal.

Finalmente, en el capítulo 3, se han explorado experimentalmente la existencia de diferenciación de nicho asociada a un componente biótico del hábitat, la presión de herbivoría. Se demuestra (para estos mismos 4 taxones) que la densidad de pubescencia glandular en la inflorescencia varía entre taxones, y que este tipo de pubescencia juega un papel defensivo importante contra pequeños insectos fitófagos. Si bien, ya hay algunas demostraciones de la funcionalidad de la pubescencia en otros grupos vegetales, y parte de esa funcionalidad ha sido asociada a la resistencia a la herbivoría (Levin 1973; Treacy et al. 1986, 1987; Buta et al. 1993; Wagner et al. 2004; Hare & Smith 2005), su papel funcional en *Aquilegia* no había sido previamente investigado y, en cualquier caso,

raramente ha sido asociado a la diferenciación de nicho entre taxones hermanos. Es reseñable que en *Aquilegia*, la pubescencia glandular es más densa en aquellos taxones sometidos a una mayor presión de herbivoría, que dicha presión parece estar estrechamente relacionada con el ambiente que ocupan dichos taxones y que la pubescencia eficientemente reduce la presión de insectos fitófagos en los ambientes edafohigrófilos, de forma que la remoción de esa defensa disminuye considerablemente la eficacia biológica de las plantas. Así, tenemos, por un lado, las dos subespecies de *A. vulgaris* que ocupan ambientes con una humedad edáfica mayor (especies edafohigrófilas), que favorece la proliferación de insectos fitófagos y, por tanto, desarrollan una mayor pubescencia; por otro lado, tenemos las subespecies de *A. pyrenaica* que ocupan ambientes con una humedad edáfica muy escasa durante primavera y verano, lo que dificulta la proliferación de insectos fitófagos, por lo que la densidad de pubescencia es bastante escasa. Esta diferenciación fenotípica se mantiene en condiciones de cultivo en jardín, por lo que parece escasamente influida por plasticidad fenotípica, y más un producto de respuesta adaptativa ya fijada en relación a la segregación de escala fina en nichos distintos. De forma interesante, en ninguno de estos taxones detectamos varianza genética aditiva (cap. 4) ni gradientes de selección significantes (distintos de cero) en este carácter (resultados no mostrados en esta memoria). Esto sugiere que las poblaciones de los taxones estudiados pueden estar en equilibrio en la actualidad con respecto a la presión herbívora, la cual estaría actuando direccionalmente de forma consistente generación tras generación habiendo provocado la erosión de la varianza genética. Un hecho que reforzaría esta hipótesis es que los tricomas pueden ser heredados de manera monogénica (Aruna et al. 2005). Cuando un solo gen es el responsable de la herencia de un carácter y este está sometido a selección, se puede fijar en la población en pocas generaciones; es decir, la población entera pasaría a poseer el

mismo fenotipo al desaparecer la variación genética para el carácter. Poblaciones que están sometidas a presión por herbivoría lo habrían fijado y ya no muestran varianza genética, mientras que en las poblaciones que no están sometidas a esta presión, el coste de producción de tricomas glandulares y sus exudados daría lugar a una presión selectiva negativa sobre el carácter, por lo que los genotipos que perdurarían serían los que no tienen que desarrollar estos tricomas.

En definitiva, todos estos resultados refrendan la necesidad de complementar los estudios macroscópicos de diferenciación de nicho con investigaciones a la escala de detalle, especialmente cuando la diferenciación puede venir definida por la variación en escala fina, como es esperable en procesos de divergencia taxonómica promovidos por especialización en el hábitat.

Potencial evolutivo en las aquilegias ibéricas

La acumulación de variación fenotípica dentro de un linaje es un proceso complejo que implica a la selección natural (convergente/divergente), a la estructura genética y a eventualidades históricas y/o geográficas. El proceso microevolutivo de diferenciación fenotípica de rasgos cuantitativos entre poblaciones (o taxones estrechamente relacionados) depende de la respuesta de cada población a la acción de la selección natural en su entorno local. Pero esta respuesta puede estar condicionada por la estructura de la matriz de varianza-covarianza genética aditiva entre los rasgos en cada población (matriz G) (Lande & Arnold, 1983).

El capítulo 4 de esta memoria junto con estudios anteriores realizados por miembros del grupo de investigación (Castellanos et al. 2011), aportan información sobre la existencia o no de variación genética

en un gran número de caracteres fenotípicos (tanto vegetativos como florales) en las aquilegias ibéricas. A continuación trataremos de sintetizar los resultados de todos estos trabajos para dar una visión de conjunto sobre el potencial para la evolución fenotípica de los taxones estudiados.

La limitación fundamental a la evolución fenotípica procede de la escasez de variación genética para caracteres sometidos a selección natural en las poblaciones de un taxón. Bajo un mismo régimen de selección natural (o incluso en ausencia de selección natural), los rasgos fenotípicos podrían llegar a diferenciarse entre poblaciones si éstas poseyeran distinta cantidad de variación genética para cada rasgo. Los resultados mostrados en el capítulo 4, combinados con los de Castellanos et al. (2011), permiten concluir que los taxones ibéricos de *Aquilegia* poseen variabilidad genética aditiva para la mayoría de caracteres fenotípicos, ya que se ha detectado varianza genética aditiva en alguna población de alguno de los taxones estudiados en 24 de los 25 caracteres evaluados. Más aún, dentro de cada taxón, más del 50% de los caracteres evaluados presentaban cantidades significativas de varianza genética aditiva (téngase en cuenta, además, que la potencia de los análisis para la estima de la varianza genética aditiva es muy baja, por lo que es probable que estos valores infraestimen la cantidad real de varianza genética aditiva en las poblaciones estudiadas). Por tanto, los taxones ibéricos de *Aquilegia* poseen un gran potencial evolutivo que les permitiría responder adaptativamente a cambios en el ambiente. Este gran potencial puede haber facilitado la radiación del grupo en la península Ibérica, dando lugar a la proliferación de taxones endémicos adaptados incluso a condiciones climáticas poco favorables, como las que tienen lugar bajo el clima mediterráneo del sur de la Península.

Varios estudios sugieren que la diversificación taxonómica del género en la península Ibérica (en general en toda Eurasia) se ha basado

en la diferenciación de caracteres vegetativos más que de los caracteres florales (Alcántara et al. 2010, Bastida et al. 2010, Castellanos et al. 2011; véase también el Capítulo 4). Los resultados de Castellanos et al. (2011) y del Capítulo 4 sugieren que ambos tipos de caracteres pueden responder de forma independiente a la selección natural ya que ambos poseen niveles semejantes de heredabilidad y la covarianza genética entre ellos es escasa. Por tanto, el distinto grado de diferenciación taxonómica entre caracteres vegetativos y florales observado en los taxones ibéricos de *Aquilegia* no se debe a una distinta limitación en la cantidad de varianza genética aditiva sino a la ausencia de covarianza genética entre estos tipos de caracteres y a la actuación de selección natural divergente mucho más intensa y/o continua sobre los caracteres vegetativos que sobre los florales.

Para comprender los procesos microevolutivos que han dirigido la diversificación de las aquilegias ibéricas debemos centrarnos en los factores ambientales y genéticos que inciden en la evolución de caracteres vegetativos. Alcántara et al. (2010) mostraron que la rocosidad del suelo y la altitud pueden haber jugado un papel importante en la diferenciación de caracteres vegetativos, al imponer patrones de selección natural divergente entre poblaciones. Entre los caracteres estudiados por Alcántara et al. (2010), cuatro estaban sometidos a selección natural divergente: número y longitud de las hojas, altura de la inflorescencia y número de flores por inflorescencia. La longitud de las hojas y la altura de la inflorescencia presentan varianza genética aditiva y muestran una fuerte correlación genética positiva en todos los taxones estudiados. Como se ha explicado en el capítulo 4, esta fuerte correlación coincide con la dirección de selección sobre los caracteres, por lo que podría haber catalizado el proceso de diferenciación impuesto por los patrones de selección divergente asociados a diferencias en rocosidad y, en menor

medida, a la altitud. La facilidad para la adaptación local de estos caracteres podría a su vez contribuir a la capacidad de colonización de nuevos lugares.

La diferenciación del número de hojas sí que puede haber estado limitada por la ausencia de varianza genética en algunos taxones. El número de hojas es mayor en taxones situados a mayor altitud, lo cual coincide con la existencia de selección divergente impuesta por la altitud sobre este carácter (Alcántara et al. 2011). Sin embargo, la magnitud de diferenciación entre poblaciones es mucho menor en el caso de *A. v. vulgaris* que en *A. v. nevadensis* o *A. p. cazorlensis* (véase la Fig. 2 en Alcántara et al. 2010), lo que podría ser reflejo de la ausencia de varianza genética aditiva para este carácter en *A. v. vulgaris*.

La pubescencia glandular en la inflorescencia tiene un papel defensivo frente a pequeños insectos que pueden dañar a las flores y semillas, como se ha demostrado experimentalmente en esta memoria. Sin embargo, este tipo de pubescencia solo muestra varianza genética aditiva en uno de los 4 taxones estudiados en el capítulo 4, lo que sugiere que la escasez de variación genética ejerce una importante limitación sobre una posible respuesta adaptativa de este carácter. Como se apuntó en el epígrafe anterior, la ausencia de varianza genética podría explicarse por una combinación de herencia monogénica de la pubescencia, fuertes presiones selectivas y elevados costes de mantenimiento. La existencia de estos costes en *Aquilegia* es un aspecto que merecería un análisis experimental.

De forma general, el conjunto de todos estos trabajos parecen confirmar nuestra hipótesis de partida de que al contrario de lo que ocurrió en Norte América, en el caso de las *Aquilegias* ibéricas fue la especialización en el hábitat, mediada por caracteres vegetativos y eco-

fisiológicos, y no la especialización en polinizadores, el principal motor de la radiación.

CONCLUSIONES

1- La diversificación de las aquilegias ibéricas no parece haber estado limitada por fuerzas de conservación del nicho. Así, los taxones conespecíficos que viven en simpatría muestran conservación de nicho, pero los que viven en alopatría tienen nichos diferentes. Para el caso de taxones heteroespecíficos no existe un patrón claro, y muestran tanto convergencia como diferenciación de nicho independientemente del solapamiento de su distribución.

2- La diferenciación de nicho parece haber ocurrido a lo largo de gradientes climáticos, mientras que la conservación de nicho se habría basado, principalmente, en la conservación de las características edáficas del nicho ambiental.

3- La diferenciación de hábitats en las aquilegias ibéricas está asociada a diferencias entre taxones en su tolerancia al estrés hídrico y lumínico. Esta diferente tolerancia a la combinación de estos tipos de estrés es más manifiesta a nivel específico que subespecífico y está mediada por una diferente respuesta funcional (intercambio gaseoso), adecuándose cada especie a la singularidad del ambiente que ocupa.

4- A pesar de existir diferencias en CO_2-AR y traspiración (conductancia estomática) entre los diferentes taxones, la eficiencia en el uso del agua (WUE) es muy constante entre poblaciones y taxones en condiciones naturales, lo que sugiere que estos taxones consiguen un balance idóneo del uso del agua de acuerdo a las condiciones a las que están sometidos.

5- La pubescencia glandular en la inflorescencia es parte de una respuesta adaptativa contra insectos fitófagos ya que: (1) existe una correlación entre densidad de pubescencia glandular en la inflorescencia y abundancia de insectos fitófagos entre poblaciones y (2) la eliminación mecánica de la pubescencia favorece un mayor daño por herbivoría, y cuanto mayor era

la densidad de pubescencia eliminada mas se incrementaba el daño por herbivoría.

6- La diferenciación en pubescencia glandular entre taxones está vinculada a la diferenciación de nicho de las especies de forma que la especie que vive en ambientes no persistentemente húmedos y con menos abundancia de insectos fitófagos (*A. pyrenaica*) posee menos pubescencia glandular que la que vive en ambientes edafohigrófilos (*A. vulgaris*) con gran abundancia de insectos fitófagos. Estas diferencias se mantienen cuando las plantas son criadas en ambiente común.

7- Las aquilegias ibéricas poseen varianza genética aditiva para la mayoría de caracteres fenotípicos vegetativos y florales. Sin embargo, la baja heredabilidad de algunos caracteres puede haber limitado su respuesta adaptativa, ya que encontramos mayor variabilidad fenotípica entre taxones en aquellos rasgos que mostraban mayor heredabilidad.

8- La diferenciación fenotípica es mayor para los caracteres vegetativos que para los florales. Este patrón no se debería a una menor heredabilidad de los caracteres florales sino a una mayor intensidad o continuidad de presiones selectivas sobre los caracteres vegetativos.

9- La estructura de la matriz **G** es más diferente entre taxones heteroespecíficos que entre taxones conespecíficos, lo que indica que G ha cambiado a lo largo del proceso de diversificación de las aquilegias ibéricas.

10- El patrón de diferenciación predicho por la matriz **G** concuerda con los patrones de diferenciación en el caso de caracteres vegetativos, pero no cuando se consideran caracteres vegetativos y florales conjuntamente. Esta discordancia puede deberse a la existencia en algunos taxones de desequilibrio de ligamiento entre caracteres florales y vegetativos.

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RESUMEN

El género *Aquilegia* constituye un ejemplo de libro de procesos de radiación adaptativa en plantas, siendo la hipótesis canónica el que la diversificación rápida y reciente de este género ha sucedido a través de diferenciación floral mediada por especialización en distintos polinizadores. De hecho este parece ser el caso en Norteamérica. Investigaciones previas realizadas con las aquilegias eurasiáticas sugiere, sin embargo, que en este continente la radiación no ha sucedido a través de este mismo mecanismo. El objetivo principal de esta tesis es contribuir a responder a la pregunta de qué ha promovido la diferenciación de nicho y la divergencia de hábitats entre los diferentes taxones ibéricos de *Aquilegia*, y evaluar en qué medida dicha diferenciación de nicho y caracteres está conectada con un proceso de radiación adaptativa. Nuestra hipótesis de partida es que, al contrario de lo que ocurrió en Norteamérica, en el caso de las aquilegias ibéricas fue la especialización en el hábitat, mediada por caracteres vegetativos y ecofisiológicos, y no la especialización en polinizadores, el principal motor de la radiación. Para tratar de responder dicha cuestión, hemos realizado trabajos de modelación, y experimentación en campo y en condiciones de jardín común que abordan 4 objetivos específicos:

(1) Caracterizar el nicho de los taxones y ejes ambientales que determinan la diferenciación de hábitats. Este objetivo es cubierto en el capítulo 1 'Complex patterns of environmental niche evolution in Iberian columbines (Gen. *Aquilegia*)'. (2) Explorar la existencia de variación entre taxones en la respuesta fisiológica (eficiencia de uso del agua) ante el estrés hídrico y lumínico y su relación con la diferenciación de nicho entre taxones. Este objetivo se abordará en el capítulo 2 'Gas exchange in response to water and light stresses contributes to habitat differentiation in Iberian Columbines'. (3) Explorar la existencia de variación entre taxones en la respuesta ante la herbivoría y su relación con la diferenciación de nicho entre taxones. Este es el objetivo central analizado en el Capítulo 3

'Glandular trichomes as an inflorescence defence mechanism against insect herbivores in Iberian columbines'. (4) Explorar la existencia de varianza genética aditiva y de varianza y covarianza genética en rasgos vegetativos y florales y su relación con la diferenciación taxonómica. Dicha exploración se realizará en el capítulo 4 'The role of genetic constraints on the diversification of Iberian taxa of the genus *Aquilegia*'.

De forma general, el conjunto de todos estos trabajos apoyan la hipótesis de que la diversificación de las aquilegias euroasiáticas, en general, y de las aquilegias ibéricas, en particular, ha sucedido por especialización en el hábitat, mediada por caracteres vegetativos y eco-fisiológicos, como principal motor de la radiación.