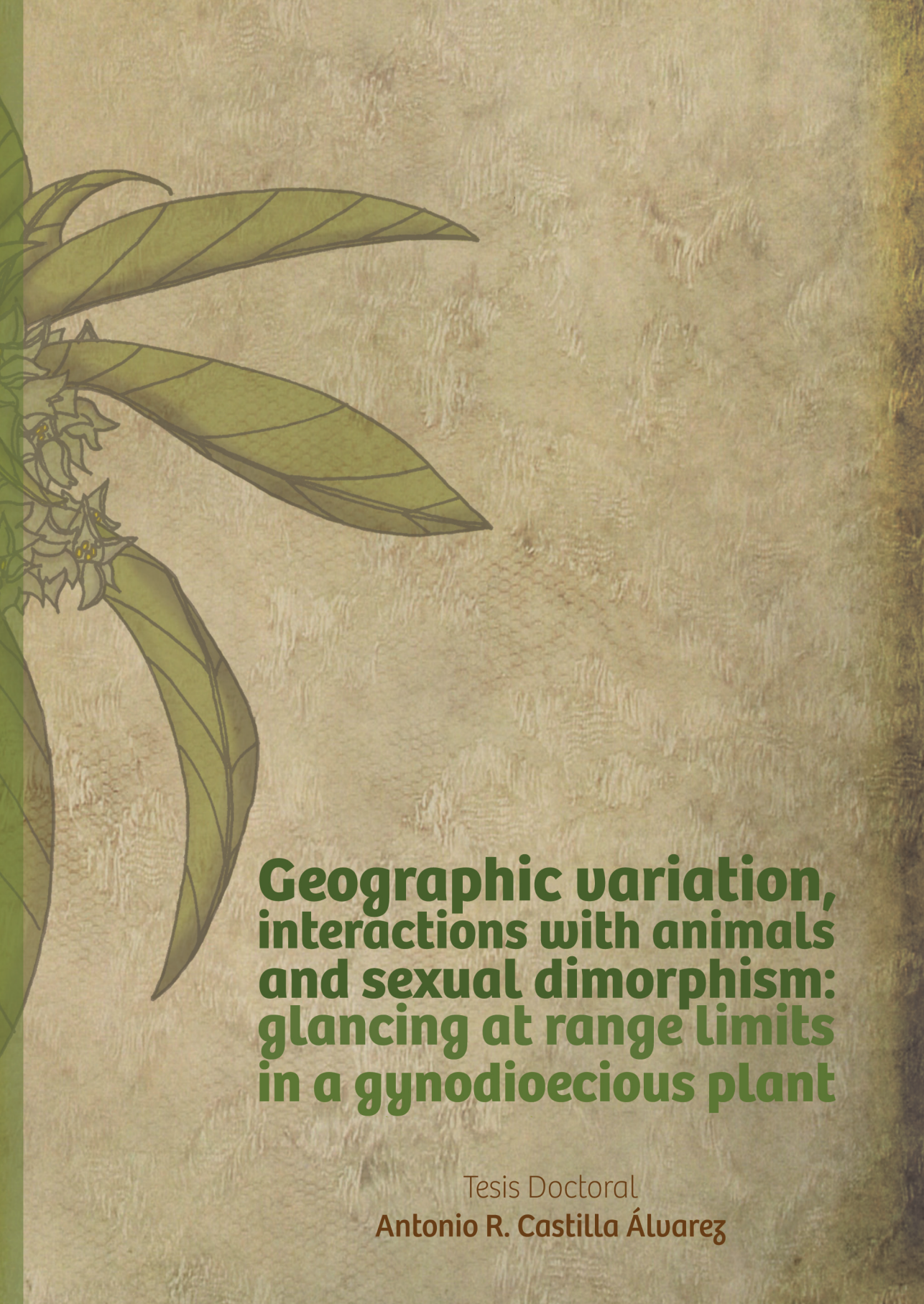

Geographic variation, interactions with animals and sexual dimorphism: glancing at range limits in a gynodioecious plant

Tesis Doctoral

Antonio R. Castilla Álvarez

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Variación geográfica, interacciones con animales y dimorfismo sexual: una mirada a los bordes de distribución de una planta ginodioica

Tesis Doctoral

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A mis padres y a Mercedes

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Sinopsis y resumen de resultados

Según la teoría del mosaico geográfico de coevolución, las variaciones espaciales en la magnitud del flujo génico y en el signo e intensidad de la selección ejercida por animales sobre los rasgos de las plantas, son los dos elementos esenciales que dan lugar a la aparición de mosaicos de selección y determinan el grado de adaptación entre las especies de plantas y animales. Hay ciertas situaciones naturales donde cabe esperar que se produzcan, de manera predecible y a lo largo de extensiones geográficas relativamente pequeñas, gradientes en la magnitud del flujo génico, así como en la forma e intensidad de selección por probables variaciones en la composición y/o abundancia de los polinizadores y herbívoros. Uno de estos lugares dotados de una singularidad ecológica propia son los márgenes de distribución de las especies vegetales. Además de sus bien conocidas peculiaridades ecológicas, los márgenes de distribución también presentan peculiaridades genéticas que los convierten en lugares ideales para verificar las predicciones de la teoría del mosaico geográfico de coevolución.

El trabajo de esta tesis doctoral se diseñó para verificar la siguiente predicción general, basada en los postulados de la teoría del mosaico geográfico de coevolución: gradientes en la magnitud del flujo génico que sean consistentes con gradientes de selección por animales (polinizadores y herbívoros) sobre algunos caracteres vegetales, darán lugar a diferenciación intraespecífica en los caracteres sujetos a selección. Esta predicción se abordó mediante el estudio de patrones de selección por animales (polinizadores y herbívoros), variación fenotípica y flujo génico replicado en grupos de poblaciones centrales y marginales del arbusto ginodioico Daphne laureola. A continuación se presenta un resumen de cada uno de los capítulos incluidos en esta tesis doctoral, señalando los resultados principales de cada uno de ellos.

En el Capítulo 1 se comparan poblaciones centrales y marginales de *D. laureola* en las Sierras Béticas con respecto a su aislamiento espacial, tamaño poblacional, diversidad y diferenciación genética. Se comprobó que las poblaciones marginales no tienen un tamaño menor que las poblaciones centrales pero sí están más aisladas espacialmente. Sin embargo, el grado de aislamiento espacial de las poblaciones del margen occidental fue mucho mayor. Estas diferencias en la estructura geográfica de las poblaciones dieron lugar a un patrón diferente de estructuración genética en los dos márgenes de distribución estudiados y definieron dos grupos genéticos dentro de la especie en el área de estudio.

En el Capítulo 2 aplicamos un enfoque genómico para detectar marcadores (AFLP loci) cuyas frecuencias mostrasen un patrón alejado del patrón neutral (outliers), una evidencia de selección natural sobre tales loci o loci ligados a los mismos. Encontramos que existe selección diferencial en poblaciones centrales y marginales de *D. laureola*. Una vez detectados los loci outliers, los retiramos del análisis de la estructura genética de las poblaciones para observar cuánto de la diferenciación genética entre los grupos disjuntos de poblaciones se podía atribuir a selección diferencial. Sin embargo, la fuerte diferenciación genética entre las poblaciones disjuntas marginales y el resto de poblaciones persistió. Este resultado demostró que factores selectivos y no selectivos son relevantes en la fuerte diferenciación genética de las poblaciones de *D. laureola*. Además, individuos hembra y hermafroditas mostraron patrones de selección diferentes, con hermafroditas presentando siempre un mayor número de loci bajo selección. Esta diferencia entre sexos fue más marcada en las poblaciones disjuntas marginales y sugirió una mayor presión selectiva sobre individuos hermafroditas que podría relacionarse con el ambiente de polinización.

El Capítulo 3 analiza si los individuos de poblaciones disjuntas marginales y centrales continuas se diferencian en el tamaño y crecimiento de los individuos, su éxito reproductivo y ambiente de polinización. Los individuos de poblaciones centrales continuas presentaron tamaños mayores y produjeron más flores que dieron lugar a cosechas de frutos mayores. Sin embargo, la proporción de flores que dieron frutos ("fruit set") fue mayor en las plantas de las poblaciones marginales,

sugiriendo un servicio de polinización más eficiente en estas poblaciones. Además no detectamos diferencias en la densidad de individuos reproductivos en poblaciones centrales y marginales. Nuestros resultados no apoyaron por tanto fuertes diferencias de estrés ecológico entre poblaciones centrales continuas y marginales disjuntas, pero indicaron ciertos cambios en las interacciones planta-polinizador que podrían tener una especial relevancia para el polimorfismo sexual de esta especie ginodioica. Entre ellas cabe destacar que el escarabajo *Meligethes elongatus*, el principal polinizador de la especie en las poblaciones centrales continuas, no está presente en las poblaciones marginales disjuntas.

En el Capítulo 4 comparamos el éxito de polinización en individuos de distinto sexo en poblaciones centrales y marginales, diferenciando sus componentes cuantitativo y cualitativo. Encontramos una reducción en la divergencia entre sexos en las poblaciones marginales disjuntas en la cantidad y calidad del polen recibido. Además, analizamos la variación fenotípica en algunos rasgos florales así como los patrones de selección por polinizadores sobre dichos rasgos. Encontramos una reducción en el dimorfismo sexual más evidente, la longitud del tubo de la corola, por reducción del tamaño de los hermafroditas. Además el único rasgo fenotípico estudiado que presentó un patrón de selección por polinizadores concordante con la variación geográfica observada fue la longitud del tubo de la corola: individuos hermafroditas con flores más largas tuvieron mayor éxito reproductivo únicamente en poblaciones centrales. Por tanto, selección diferencial por polinizadores junto con mecanismos de selección post-polinización parecen estar contribuyendo a las diferencias en el nivel de dimorfismo sexual floral entre poblaciones centrales continuas y marginales disjuntas de *D. laureola*.

Por último, el Capítulo 5 se centra en el papel de los herbívoros sobre la diferenciación intraespecífica de los rasgos vegetativos de *D. laureola*. Los resultados de este capítulo demostraron que los herbívoros no parecen jugar un papel decisivo en este sentido. No encontramos diferencias entre poblaciones centrales continuas y marginales disjuntas en el nivel medio de herbivoría, descartando una mayor presión por herbívoros en los márgenes dedistribución. Además las diferencias

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en los niveles de herbivoría entre individuos no se correspondieron con las distancias genéticas entre ellos. Por tanto, estas diferencias parecen ser fruto de la variación espacial en el consumo por herbívoros debido a variación (micro)ambiental dentro de las poblaciones. Rasgos vegetativos como el área específica foliar o el contenido en fenoles de las hojas fueron diferentes en poblaciones centrales continuas y marginales disjuntas de *D. laureola*, pero esta diferenciación regional no se correspondió con una selección diferencial por herbívoros y podría tener relación con cambios regionales en la composición del dosel arbóreo y el ambiente lumínico del sotobosque.

Introducción

Variación geográfica en las interacciones planta-animal

Lejos de ser entes aislados dentro de los ecosistemas, las plantas constituyen elementos dinámicos que continuamente interaccionan con otros organismos vivos. La naturaleza sésil de las plantas limita decisivamente dos fases críticas en su ciclo reproductivo como son la reproducción sexual y la dispersión de las semillas (Herrera 2002). Estos dos procesos requieren el movimiento en el espacio de estructuras reproductivas. Durante la reproducción sexual, los granos de polen deben ser transportados desde el individuo distancias variables hasta alcanzar los gametofitos femeninos de otras plantas, en la población o fuera de ella. Del mismo modo, la dispersión de semillas implica el movimiento de éstas lejos de la planta madre. Ya que la capacidad de movimiento es una cualidad ampliamente extendida dentro del reino animal, no es de extrañar que las plantas hayan desarrollado una enorme variedad de mecanismos destinados a usar a los animales como vectores que permitan el movimiento de su polen y semillas. De hecho, actualmente está ampliamente aceptado que esta enorme explotación del movimiento animal para la dispersión del polen y de las semillas constituye uno de los factores decisivos en la tremenda diversificación y éxito ecológico de las angiospermas (Price 2002; Kay & Sargent 2009). También las interacciones de tipo antagonista han contribuido a la diversificación dentro de las angiospermas. La herbivoría o consumo de las plantas por animales engloba tipos muy diferentes de interacción que van desde las de tipo depredación (p.ej. consumo de semillas o plántulas) a las que no implican la muerte de individuo (p.ej. consumo de hojas). Debido a su naturaleza sésil, las plantas no pueden escapar del consumo por herbívoros y por tanto han tenido que desarrollar una enorme variedad de mecanismos destinados a evitar y/o tolerar el daño producido por los herbívoros. Por lo tanto, interacciones mutualistas y antagonistas entre plantas y animales han contribuido y continúan contribuyendo a la enorme diversificación dentro de las angiospermas (Labandeira 2002).

Geographic variation, interactions with animals and sexual dimorphism: glancing at range limits in a gynodioecious plant.

Una de la ideas centrales del pensamiento evolucionista actual es que los cambios macroevolutivos son el resultado acumulado de los procesos microevolutivos que tienen lugar a nivel intraespecífico (Simpson 1953, Bock 1970). Por este motivo, una de las maneras tradicionales de abordar el estudio de la adaptación ha sido investigar la variación geográfica en los caracteres de los organismos y su relación con los posibles factores selectivos que pudieran ser responsables de esa variación (Endler 1986). Esta idea la expresan de forma sintética Gould & Johnston (1972, p. 457) cuando afirman: *"Since we view transpecific evolution as an extension of events at the species level, the foundation of most evolutionary theory rests upon inferences drawn from geographic variation or upon the verification of predictions made about it"*. En el caso concreto de las plantas, se ha prestado especial atención a la variación geográfica en los rasgos relevantes para sus interacciones con animales (p.ej. polinizadores, herbívoros, dispersantes de semillas, etc) dada la importancia ya comentada previamente de este tipo interacciones en los procesos de diferenciación intraespecífica. En este sentido, la comunidad animal con la que una determinada especie vegetal interactúa se espera que varíe geográficamente. Por tanto, el estudio de la variación geográfica intraespecífica en rasgos relevantes para las interacciones planta-animal y su posible relación con una selección divergente efectuada por animales, es crucial para entender el vínculo entre micro y macroevolución en estos caracteres y el papel de las interacciones planta-animal en la evolución de los sistemas sexuales en plantas (Barrett et al. 2001; Zangerl & Berenbaum 2003; Herrera et al. 2006). Sin embargo, la mayoría de estas investigaciones sobre las relaciones entre plantas y animales se han llevado a cabo sin atender a su contexto geográfico, a pesar de su importancia para entender los procesos de evolución adaptativa (Herrera et al. 2006).

La teoría del mosaico geográfico de coevolución (TMGC en adelante) propuesta por Thompson (1994, 2005) y los modelos genéticos asociados a ella (Gomulkiewicz et al. 2000; Alleaume-Benharira et al. 2006) proporcionan un modelo teórico que permite estudiar el papel causal de los animales en la aparición y mantenimiento de diferencias intraespecíficas en los rasgos fenotípicos importantes para las interacciones planta-animal. Según la TMGC, las dinámicas coevolutivas globales de las interacciones planta-animal vienen determinadas por tres componentes de la es-

tructura geográfica de las poblaciones de las especies interactuantes: los mosaicos de selección, los puntos calientes coevolutivos y la remezcla de caracteres (*"trait remixing";* Thompson 1994; 2005). Estos tres conceptos se describen brevemente a continuación:

"Mosaicos de selección": el resultado de las interacciones a menudo difiere entre poblaciones de las especies que interaccionan. Esto es debido principalmente a variaciones locales en el ambiente abiótico, en la estructura demográfica y genética y en el contexto de la comunidad ecológica donde se desarrolla la interacción. Estas diferencias entre poblaciones en el resultado de la interacción crean un mosaico geográfico en la interacción que es el material básico para la dinámica de la coevolución.

"Puntos coevolutivos calientes y fríos": consecuencia de la variación geográfica en el resultado de las interacciones, una interacción puede coevolucionar en algunas poblaciones (puntos calientes coevolutivos), mientras que en otras puede afectar a sólo una de las especies participantes o no tener ningún efecto (puntos fríos coevolutivos). Incluso pueden existir poblaciones de una especie participante en la interacción que se localicen fuera del área de distribución geográfica de la otra especie participante.

"Remezcla de caracteres": el mosaico geográfico se reajusta continuamente debido a los efectos combinados del flujo génico entre poblaciones, a la deriva genética y a las dinámicas de extinción/colonización local.

Especies sexualmente polimórficas: importancia de las variaciones geográficas en las interacciones planta-animal

Las condiciones ecológicas en las que se desarrolla una especie varían tanto espacialmente como temporalmente. Poblaciones en diferentes regiones dentro del rango de una especie a menudo se enfrentan a escenarios ecológicos diferentes que dan lugar a cambios en la demografía,

la estructura genética y las características fenotípicas de las poblaciones (Thompson 1994, 2005; Herrera et al. 2006). Tal diferenciación geográfica puede tener importantes consecuencias micro y macroevolutivas que en última instancia pueden llevar al aislamiento reproductivo y diversificación de linajes de plantas (Baker 1959; Lloyd 1965; Strid 1969; Grant 1971; McNeill & Jain 1983; Wyatt 1988; Olmstead 1990; Barrett 1995). Esta variación geográfica tanto en las condiciones abióticas como bióticas se piensa que ha contribuido a diversificar los sistemas reproductivos y sexuales de las Angiospermas (Barrett et al. 2001; Barrett 2002, 2010).

Dentro de la impresionante diversidad de sistemas sexuales en plantas podemos encontrar desde especies monomórficas donde cada individuo contribuye reproductivamente a través de sus funciones sexuales masculina y femenina, hasta especies polimórficas donde los individuos están agrupados en dos o más morfotipos sexuales distintos que contribuyen a la siguiente generación principalmente a través de la función masculina o femenina (Lloyd 1980). Dentro de los sistemas monomórficos tenemos el hermafroditismo, donde los individuos portan flores con la función masculina y femenina desarrolladas y la monoecia donde las funciones masculinas y femeninas están separadas en flores distintas pero dentro del mismo individuo. En el caso de las especies dimórficas podemos encontrar la dioecia donde las funciones sexuales masculina y femenina aparecen en flores distintas que además son portadas por individuos distintos, la ginodioecia con individuos hembras y hermafroditas coexistiendo en sus poblaciones o la androdioecia con individuos machos y hermafroditas. Existen además sistemas hermafroditas pero polimórficos, como la distilia, a los que no nos vamos a referir.

La dioecia no es muy frecuente en angiospermas a diferencia del reino animal donde es la condición más abundante (6 % de las especies de angiospermas; Renner & Ricklefs 1995). No obstante, la dioecia ha evolucionado desde el hermafroditismo en multitud de ocasiones, al menos hay 100 transiciones descritas (Barrett 2002; Charlesworth 2002). En concreto, la ruta evolutiva desde el hermafroditismo a la dioecia vía ginodioecia está apoyada teóricamente y empíricamente (Charlesworth 1999; Webb 1999; Weiblen et al. 2000). Esta ruta evolutiva implica dos fases con-

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secutivas, primero la invasión y expansión de mutantes estériles masculinos (i.e. hembras) dentro de poblaciones hermafroditas y, segundo, la acción de la selección natural para incrementar la función masculina en los hermafroditas. Sin embargo, apenas hay información sobre los mecanismos ecológicos que promueven estas dos transiciones, un aspecto clave para determinar si la ginodioecia puede ser un sistema evolutivamente estable (Ashman 2006).

Darwin (1877) fue el primero que estableció una asociación entre dimorfismo sexual y estrés abiótico, señalando *"a very dry station apparently favours the presence of the female form"* (p. 301). Esta asociación entre dimorfismo sexual y estrés abiótico ha sido documentado en varios estudios filogenéticos (p.e. Hart 1985; Weller et al. 1995) y también por numerosos estudios de variación entre poblaciones (Weller et al. 1998; Asikainen & Mutikainen 2003; Vaughton & Ramsey 2004; Case & Barrett 2004). Sin embargo, aunque se piensa que los factores bióticos tienen un papel relevante en la aparición de sistemas polimórficos sexuales, apenas hay estudios que hayan examinado su papel causal. Por ejemplo se piensa que una reducción en la calidad de la polinización promueve diversas estrategias reproductivas como la transición desde la fecundación cruzada a la autofecundación (Schoen et al. 1996; Herlihy & Eckert 2002; Moeller & Geber 2005) o la evolución desde el monomorfismo al dimorfismo sexual (Sakai & Weller 1999; Barrett et al. 2001; Ashman 2006). En el caso concreto de la ginodioecia, se supone que los polinizadores pueden afectar a la evolución del sistema sexual de dos maneras contrapuestas (Ashman 2006). Por un lado, reducciones o cambios en la composición de la fauna de polinizadores podrían llevar a insuficiente o menor polinización en los individuos, respectivamente (ver Harder & Barrett 1996). En especies ginodioicas con hermafroditas autocompatibles, este peor ambiente de polinización puede llevar a un aumento de la autofecundación en los hermafroditas con el consiguiente incremento de la endogamia en su descendencia. El aumento de la endogamia en la descendencia de los hermafroditas puede suponer una desventaja a la hora de competir con la descendencia de los individuos hembra, producida exclusivamente por fecundación cruzada, promoviendo así la expansión de individuos hembra en las poblaciones (Charlesworth & Charlesworth 1978). Por otro lado, la propagación de los individuos hembras dentro de las poblaciones puede ser impedida por

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limitación por polen si los polinizadores son poco atraídos por los individuos hembra (p.ej. cuando el polen es la única recompensa). En esta situación, el menor número de visitas de polinizadores a las flores de individuos hembra podría reducir su cuajado de frutos y en última instancia evitar su expansión dentro las poblaciones. A pesar de su potencial importancia, muy pocos estudios han relacionado las características de las comunidades de polinizadores en distintas regiones con el polen recibido y los patrones de selección fenotípica sobre los rasgos florales de las plantas de ambos sexos con el objetivo de clarificar el papel de los polinizadores en la evolución de la ginodioecia hacia la dioecia. Además diversos estudios han mostrado que no sólo los organismos mutualistas afectan a la evolución de los sistemas reproductivos sexuales en plantas, sino que también las relaciones antagonistas pueden jugar un papel importante (Collin et al. 2002; Ashman 2006). Por tanto, estudios que tengan en cuenta interacciones mutualistas y antagonistas con animales sobre distintos morfotipos sexuales serán de máxima importancia para una completa comprensión del papel del contexto ecológico en la evolución de los sistemas sexuales.

Márgenes de distribución y la oportunidad de estudiar el cambio

Restricciones en el flujo génico combinadas con cambios en los patrones de selección por animales pueden llevar a diferenciación intraespecífica (Thompson 1994; 2005). Hay ciertas situaciones naturales donde cabe esperar que se produzcan, de forma predecible y a lo largo de extensiones geográficas relativamente pequeñas, gradientes en la magnitud del flujo génico, así como en la forma e intensidad de selección por probables variaciones en la composición y/o abundancia de polinizadores y herbívoros. Uno de estos lugares son las poblaciones marginales en el área de distribución de las especies. Las peculiaridades ecológicas y genéticas de las poblaciones marginales las convierten en lugares idóneos para comprobar las predicciones de la TMGC (Kawecki 2008; Sexton et al. 2009; Gaston 2009).

En comparación con las zonas centrales de distribución, las poblaciones marginales se encuentran en situaciones más estresantes y ecológicamente desfavorables, por lo que tienden a ser más pequeñas y a estar más aisladas entre sí (Brown 1984; Brussard 1984; Vucetich and Waite 2003). Como consecuencia, se espera que las poblaciones marginales tengan menos flujo génico entre sí y con las situadas en zonas centrales, y por tanto deben estar más diferenciadas genéticamente (García-Ramos & Kirkpatrick 1997; Alleaume-Benharira et al. 2006; Eckert et al. 2008). Además las poblaciones marginales deberían presentar menor diversidad genética y estar más sujetas a la influencia de la deriva genética debido a su menor tamaño y a presentar un flujo génico más restringido (García-Ramos & Kirkpatrick 1997; Alleaume-Benharira et al. 2006; Eckert et al. 2008). Por otro lado, cambios en la abundancia y/o identidad en la comunidad de animales con los que interactúa la planta en las poblaciones marginales de las especies vegetales han sido documentados frecuentemente en la literatura (p.ej., Lewinsohn et al. 2005; Kay & Sargent 2009).

Hay que tener en cuenta que, frecuentemente, en el área de distribución global de las especies vegetales, además de los bordes geográficos, existen límites de distribución internos debido a gradientes ecológicos locales. Sin que ello suponga una contradicción con la teoría clásica centro-borde, recientes revisiones de la materia sugieren que similares factores selectivos podrían estar actuando en bordes externos e internos de la distribución de las especies vegetales y por tanto, señalan la importancia del estudio de la adaptación en estos márgenes internos de distribución (Sexton et al. 2009). En estos márgenes internos de distribución las tasas de flujo génico entre poblaciones son probablemente mayores que entre poblaciones centrales y marginales a la escala del área de distribución global de la especie (Sexton et al. 2009). Por tanto, diversos factores tanto bióticos como abióticos que potencialmente podrían limitar la distribución geográfica global de la especie podrían ser examinados en estos gradientes locales en combinación con los efectos antagonistas del flujo génico para la adaptación local de las poblaciones marginales (Bridle & Vines 2007; Sexton et al. 2009; Bridle et al. 2010). Además este tipo de estudios en gradientes locales adquieren especial relevancia en especies que presentan distribuciones disjuntas, donde centro geográfico y óptimo ecológico pueden no coincidir (p.ej. el centro geográfico de la especie se localiza

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en una región donde la especie está ausente; Sagarin et al. 2006). Sin embargo, es llamativo que el valor de estos límites de distribución internos a la hora de comprender los factores que limitan las áreas de distribución de las especies ha sido escasamente explorado (Gaston 2003).

Por todo lo anterior, las poblaciones marginales de las especies vegetales constituyen lugares idóneos para examinar diferenciación intraespecífica en rasgos relevantes para las interacciones planta-animal ligados a selección divergente efectuada por animales. Sin embargo, el número de estudios que han investigado esta cuestión en áreas marginales es muy escaso (Herrera et al. 2006; Pérez-Barrales et al. 2007). Además, dicha escasez de investigaciones es aún más marcada cuando restringimos la búsqueda a estudios que hayan analizado la base genética de las adaptaciones naturales (Orr 2005; Stinchcombe & Hoekstra 2008).

Objetivos de la tesis

Especie de Estudio

Daphne laureola L. (Thymelaeaceae) es una especie arbustiva que crece en bosques de montaña. Presenta una distribución Paleártica disjunta asociada a las principales cordilleras montañosas de sustrato calcáreo de Europa y del norte de África (Fig. 1; Alonso et al. 2007). Dentro de la Península Ibérica, la especie muestra dos parches de distribución disjuntos En el norte peninsular la especie es frecuente en la Cordillera Cantábrica y en los Pirineos. En el sur peninsular la especie está presente en las Cordilleras Béticas. Esta porción de distribución de la especie en el sur peninsular constituyó el área de estudio en el que se desarrollaron todos los trabajos que componen esta tesis doctoral.

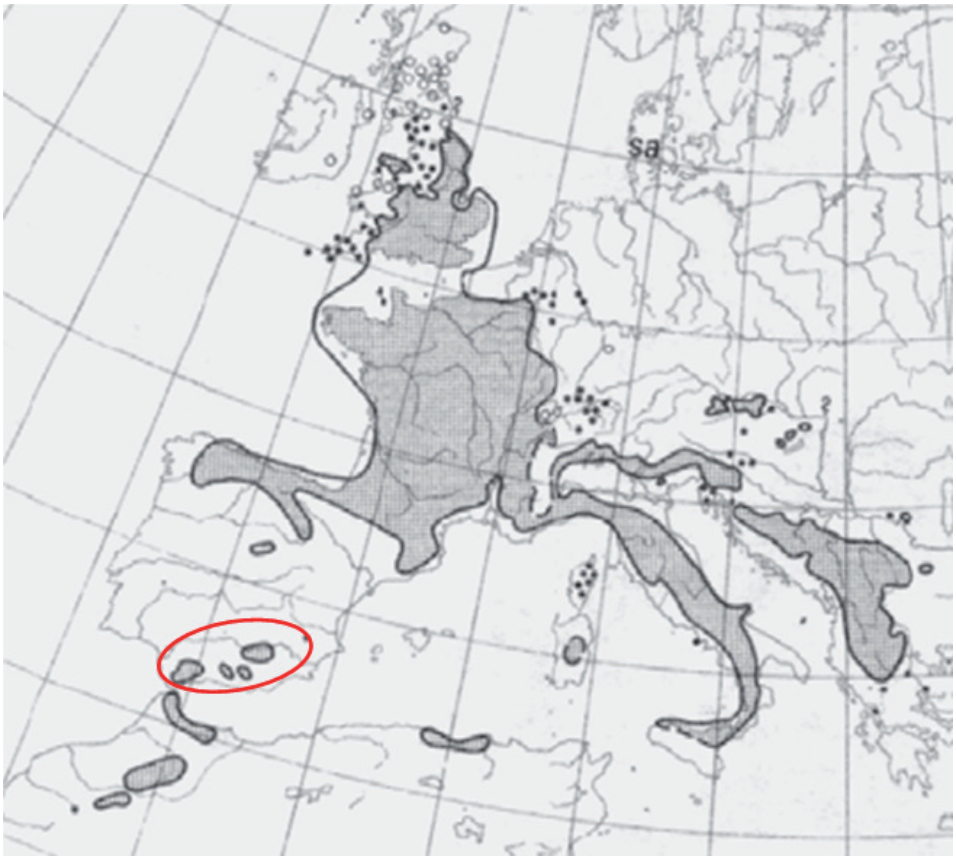

Figura 1. Area de distribución global de *Daphne laureola* L. de acuerdo a Meusel et al. (1978). La zona marcada indica la región de estudio.

Las plantas de *D. laureola* forman matas con un número variable de tallos que sólo presentan hojas en la parte superior, formando rosetas. *Daphne laureola* es una especie caracterizada por presentar un sistema sexual polimórfico. Sus poblaciones frecuentemente son dimórficas, coexistiendo individuos hembra y hermafroditas, pero también existen poblaciones monomórficas compuestas exclusivamente por plantas hermafroditas (Alonso et al. 2007; Fig. 2a-b). La especie presenta un extenso período de floración invernal, durante el cual cada mata produce un gran número de flores pequeñas, tubulares y verde-amarillentas en racimos muy condensados (Alonso 2004). En las poblaciones del sureste peninsular, cuatro especie de lepidópteros pertenecientes a la familia *Noctuidae* (*Trigonophora flammea Esper., Noctua janthe Borkhausen, Noctua fimbriata*

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Schreber, Pseudenargia ulicis Staudinger) se alimentan de las hojas en desarrollo de *D. laureola* durante su período larvario (Alonso & Herrera 1996). Estas orugas también consumen las flores y los frutos. En las poblaciones de la especie presentes en el sureste de la Península Ibérica el principal polinizador es *Meligethes elongatus Rosenhauer*, un pequeño escarabajo perteneciente a la familia *Nitidulidae* (Alonso 2004; Fig. 2c). En el grupo disjunto de poblaciones localizado en el suroeste peninsular se desconoce la identidad de los herbívoros y polinizadores de la especie. La fructificación tiene lugar en Mayo-Junio. Los frutos son de tipo drupa y su coloración cambia del verde al negro durante la maduración. Las semillas son dispersadas por diversas especies de aves (Obeso 1985; Hulme 1992).

Figura 2. Detalle de las flores hermafroditas (a) y hembras (b)de Daphne laureola. (c) Meligethes elongatus, principal polinizador de D. laureola en las poblaciones del sureste peninsular.

Área y localidades de estudio

Todos los trabajos que componen esta tesis doctoral fueron realizados en el área de distribución de *Daphne laureola* localizado en el sur de la Península Ibérica, concretamente en las Cordilleras Béticas (Fig. 1). Esta región biogeográfica constituye un punto caliente de biodiversidad a escala mundial. Además esta región representa el sector de distribución de la especie más meridional en el continente europeo y las poblaciones de la especie son abundantes. Por todo lo anterior, la región seleccionada para este estudio representa un lugar idóneo donde examinar las predicciones planteadas en esta tesis doctoral. Tanto el análisis de estructura genética de las poblaciones como la búsqueda de marcadores genéticos cuyas frecuencias alélicas muestren signos de selección natural (capítulos 1 y 2) fueron llevados a cabo usando 15 poblaciones de la especie de estudio. El resto de capítulos se centraron en la comparación de tres poblaciones en el centro ecológico de la

especie en el área de estudio con tres poblaciones localizadas en el margen disjunto suroccidental (Capítulos 3, 4 y 5).

En el sureste peninsular la especie es frecuente en el macizo formado por las sierras Cazorla-Segura-Castril-Alcaraz donde ocupa un amplio gradiente altitudinal (700-1900 m.s.n.m.). En Cazorla-Segura, en la mayoría de las localidades, *D. laureola* forma parte del sotobosque de pinares de *Pinus nigra subsp salzmanii*, donde también se encuentran otras especies leñosas como *Quercus faginea, Prunus mahaleb, Taxus baccata, Acer monspessulanum, Juniperus communis o Crataegus monogyna*. Mención especial merecen las poblaciones de *D. laureola* localizadas en la sierra de Segura, en el extremo nororiental de las Cordilleras Béticas, donde frecuentemente aparece asociada a acebedas. En estas poblaciones la vegetación se caracteriza por un dosel arbóreo de *Pinus nigra subsp. salzmanii* bajo el que se desarrollan abundantes los acebos (*Ilex aquifolium*), y otras especies tales como *Corylus avellana, Quercus pyrenaica* o *Acer opalus subsp. granatense*.

En el suroeste peninsular *D. laureola* presenta un grupo más reducido de poblaciones localizadas en distintos macizos montañosos. En varias de estas poblaciones la especie que caracteriza el estrato arbóreo es *Abies pinsapo*, que da lugar a una de las formaciones boscosas más singulares de la Península Ibérica (Blanco et al. 1998). Bajo la cobertura de *A. pinsapo* crece una comunidad bastante pobre en términos de diversidad en la que destacan especies como *Hedera helix, Ruscus aculeatus, Rubia peregrina* o *Iris foetidissima* (Arista 1995). En zonas donde la cobertura de *A. pinsapo* es menor se establecen especies como *Quercus faginea, Ulex baeticus, Crataegus monogyna* o *Rosa canina*.

Cada una de las poblaciones de estudio fue clasificada siguiendo el sistema de clasificación climático propuesto por Köppen en su última versión de 1936, conocida también como clasificación de Köppen-Geiger (Köppen 1931, 1936). A pesar de que esta clasificación se definió hace unos 100 años, sigue siendo una de las clasificaciones más utilizadas en estudios climatológicos a nivel mundial (Agencia Estatal de Meteorología & Instituto de Meteorología 2011). La clasificación de

Köppen define distintos tipos de clima a partir de los valores medios mensuales de precipitación y temperatura.

Los valores de precipitación y temperaturas para cada una de las poblaciones de estudio fueron estimados a partir de modelos de interpolación basados en registros meteorológicos de 30 años (Bustamante 2003; Tabla 1). Basándonos en los datos obtenidos, todas las poblaciones de estudio se encuandran en la categoría climática Csb propuesta por Köppen. Es decir, las poblaciones de *D. laureola* del sur de la Península ocupan áreas donde la temperatura media del mes más frío está comprendida entre 0 y 18 ºC, observándose un período estival marcadamente seco pero templado (temperatura media del mes más cálido ≤ 22 ºC y con cuatro meses o más con una temperatura media superior a 10 ºC).

Tabla 1. Caracterización de las poblaciones de estudio. Se indican las coordenadas geográficas (X,Y), la altitud, la precipitación media anual P, la temperatura media anual T, la temperatura media del mes más frío m y la temperatura media del mes más cálido M. Los valores de temperatura y precipitación para cada localidad fueron estimados a partir de modelos de interpolación basados en registros meteorológicos de 30 años (Bustamante 2003).

Poblacion	X	Y	Altitud (m.s.n.m.)	P (mm)	T(ºC)	m(ºC)	M(ºC)
Margen nororiental							
Acebeas	537013	4241386	1320	1067	11.5	3.9	22.0
Navalasna	539809	4240677	1202	969	11.5	3.8	21.9
Río Madera	534676	4236195	1220	1009	11.5	3.8	21.9
La Cumbre	528903	4225256	1480	912	11.7	4.1	22.0
Acebeas de los Jabalises	536391	4229352	1430	907	11.9	4.3	22.3
Región central							
Espinarea	503818	4192104	1173	870	11.2	3.8	21.5
Cañada del Espino	510134	4197123	1575	1068	11.0	3.7	21.3
Fuente Bermejo	514050	4198162	1513	1007	10.2	2.9	20.6
Valdecuevas	511627	4196202	1380	1051	10.7	3.4	21.1
Roblehondo	511209	4200234	1235	1036	11.1	3.6	21.3
Margen suroccidental							
Almijara	424124	4079200	1453	655	12.0	4.4	20.5
Algeciras	274886	3996149	506	887	16.9	8.3	22.8
Cañada de las Ánimas	319005	4063911	1333	1030	11.6	4.7	20.9
Fuente Molina	318354	4063324	1380	1046	11.5	4.6	20.8
Grazalema	283308	4072648	1229	1270	13.2	6.2	22.3
Sierra del Reloj	288021	4067469	1126	1665	13.4	6.4	22.5

objetivos

Estructura de la Tesis Doctoral

Esta Tesis doctoral consta de cinco capítulos cuyo contenido y finalidad se detallan a continuación. Todos los capítulos de esta tesis corresponden a trabajos originales, algunos de ellos publicados, otros en vías de publicación.

En el **Capítulo 1** (*"Genetic structure of the shrub* Daphne laureola *across the Baetic Ranges, a Mediterranean glacial refugium and biodiversity hotspot"*) se examina en primer lugar si en el área de estudio hay una reducción del tamaño poblacional y un incremento del aislamiento geográfico de las poblaciones hacia los márgenes de distribución de *D. laureola*. En un segundo paso, se analiza la estructura genética de las poblaciones en el área de estudio y concretamente se evalúa si hay una reducción de la diversidad genética y un aumento de la diferenciación genética en poblaciones marginales. Publicado en Plant Biology (en prensa).

En el **Capítulo 2** (*"Differential selection between sexes and regions in a gynodioecious shrub revealed by genemic scanning"*) se adopta un enfoque basado en el escaneo genómico (*"genomic scanning"*) para detectar evidencia de selección natural a partir de las frecuencias alélicas de un elevado número de marcadores genéticos (*AFLP loci*). Concretamente se examina si individuos de poblaciones centrales continuas y marginales disjuntas presentan patrones de selección diferencial, y si dentro de cada uno de estos grupos poblacionales, individuos con distinto sexo muestran también evidencia de selección diferencial. En revisión.

El **Capítulo 3** (*"Exploring local borders of distribution in the shrub* Daphne laureola*: individual and population traits"*) está enfocado en la comparación entre poblaciones centrales continuas y marginales disjuntas en relación al tamaño y éxito reproductivo de sus individuos. Tanto la identidad del polinizador principal como la abundancia de otras especies en floración como potenciales competidoras por los polinizadores son tenidas en cuenta en dicha comparación. Publicado en 2011, Acta Oecologica 37: 269-276.

En el **Capítulo 4** (*"Reduced gender divergence in floral traits and pollination success at disjunct marginal populations of a gynodioecious shrub"*) se investiga cómo un cambio en la identidad del polinizador principal afecta al éxito de polinización y a los patrones de selección floral en poblaciones centrales continuas y marginales disjuntas de *D. laureola*. En revisión.

Por último, el **Capítulo 5** (*"Loose herbivory interactions in a long-lived understory plant: non-genetic basis for adult defoliation"*) investiga las diferencias en los niveles de herbivoría en poblaciones centrales continuas y marginales disjuntas de *D. laureola*. También se exploran la existencia de diferencias entre estos grupos poblacionales en el efecto de la herbivoría sobre la fitness así como los patrones de selección fenotípica de los herbívoros sobre rasgos vegetativos. En revisión.

1

Chapter 1.-

Genetic structure of the shrub *Daphne laureola* across the Baetic Ranges, a Mediterranean glacial refugium and biodiversity hotspot

Abstract: Distribution margins constitute areas particularly prone to random and/or adaptive intraspecific differentiation in plants. This trend may be particularly marked in species discontinuously distributed across mountainous ranges, where sharp geographic isolation gradients and habitat boundaries will enhance genetic isolation among populations. In this study we analysed the level of neutral genetic differentiation among populations of the long-lived shrub ***Daphne laureola (Thymelaeaceae)*** across the Baetic Ranges, a glacial refugium and biodiversity hotspot in the western Mediterranean Basin. Within area, core and marginal populations of ***D. laureola*** were compared with regard to their spatial isolation, size, genetic diversity and differentiation. A spatially explicit analysis conducted on the vast majority of the species' known populations in the study area (N = 111) showed that marginal populations (western and eastern) present larger spatial isolation than core populations, but are not smaller. We compared genetic diversity and differentiation between core and marginal populations using a subsample of fifteen populations and 225 amplified fragment length polymorphism (AFLP) markers. Core and marginal populations did not differ in genetic diversity, probably because of the occurrence of large populations on the local margins. Western populations were strongly differentiated from the other populations. In addition, spatial and genetic differentiation among populations was larger on the western margin. Eastern populations constituted a genetically homogeneous group closely related to core populations, despite their greater spatial isolation. Results suggest that studies on phenotypic differentiation between core and marginal populations of ***D. laureola***, and presumably other species likewise having discontinuous distributions across the Baetic ranges, should take into account geographical differences in level of genetic differentiation between the different distribution borders.

Castilla, A.R., Alonso, C. and Herrera, C.M. In press. Genetic structure of the shrub *Daphne laureola* across the Baetic Ranges, a Mediterranean refugium and biodiversity hotspot. Plant Biology.

Resumen

Los márgenes en la distribución de las especies vegetales constituyen áreas donde es particularmente factible encontrar diferenciación intraespecífica neutral y/o adaptativa. Esta tendencia debería ser especialmente marcada en especies distribuidas discontinuamente en distintas cordilleras montañosas, donde fuertes gradientes de aislamiento geográfico y heterogeneidad de hábitat aumentarán el aislamiento genético de las poblaciones. En este capítulo analizamos el nivel de diferenciación genética neutral entre poblaciones del arbusto ***Daphne laureola (Thymelaeaceae)*** en las Cordilleras Béticas, un refugio glaciar y un punto caliente de biodiversidad en la cuenca occidental del Mediterráneo. Poblaciones centrales y marginales de ***D. laureola*** en el área de estudio fueron comparadas con respecto a su aislamiento espacial, tamaño poblacional, diversidad y diferenciación genética. Un análisis espacialmente explícito llevado a cabo usando la mayor parte de las poblaciones conocidas de la especie en el área de estudio (N = 111) mostró que las poblaciones marginales (occidentales y orientales) presentaron un mayor aislamiento espacial que las poblaciones centrales, pero no presentaron un menor tamaño poblacional. Usando una submuestra de 15 poblaciones y 225 marcadores de polimorfismo en la longitud de fragmentos amplificados (AFLPs) comparamos a las poblaciones centrales y marginales en relación a su diversidad y diferenciación genética. Poblaciones centrales y marginales presentaron similares niveles de diversidad genética, probablemente como consecuencia de la existencia de poblaciones de gran tamaño en los márgenes locales de distribución. Las poblaciones marginales occidentales estuvieron genéticamente muy diferenciadas del resto de poblaciones. Además, tanto el aislamiento espacial como genético fue mayor en el borde occidental. Las poblaciones orientales constituyeron un grupo genéticamente homogéneo muy próximo a las poblaciones centrales, a pesar de su mayor aislamiento espacial. Los resultados de este estudio sugieren que los estudios sobre diferenciación fenotípica entre poblaciones centrales y marginales de ***D. laureola***, y probablemente de otras especies con distribuciones disjuntas en sistemas montañosos, deberían tener en cuenta diferencias en el nivel de diferenciación genética entre los distintos bordes de distribución de la especie.

Introduction

High mountains are continental islands from a biogeographic perspective, each one surrounded by low-altitude environments characterized by unsuitable present-day climatic conditions for cold-adapted mountain plants (although see Birks & Willis 2008). Consequently many mountain plants often show disjunct geographical distributions. Within each distribution patch the species colonises a geographical gradient of environmental conditions and becomes more abundant where individual survival, reproduction and hence population growth are highest, and increasingly less abundant as conditions depart from this optimum (Hengeveld & Haeck 1982; Brown 1984). Therefore, within each distribution patch, a species is expected to exhibit a local center of abundance or core region, with populations becoming progressively smaller and more spatially isolated towards the local range limits (Brussard 1984; Vucetich & Waite 2003). Such a combination of geographic variation in population size and spatial isolation is expected to have important consequences for the genetic structure of plant populations (Vucetich & Waite 2003; Alleaume-Benharira et al. 2006). Genetic models of ecological margins pertain to local range margins rather than species global distribution margins because they consider a continuous spatial area that is not much larger than the dispersal distance (Alleaume-Benharira et al. 2006; Bridle & Vines 2007; Bridle et al. 2010). These genetic models predict, within an ecological gradient, (i) reduced genetic diversity of marginal populations because of founder effects, bottlenecks, inbreeding, genetic drift or directional selection; and (ii) increased genetic differentiation among marginal populations through reduced gene flow (Young et al. 1996; Lammi et al. 1999; Lowe et al. 2005). Furthermore, marginal and isolated populations could be genetically distinct as a result of the reduced arrival of maladapted genes from core populations (*"gene swamping"*; García-Ramos & Kirkpatrick 1997; Alleaume-Benharira et al. 2006), favouring adaptation to local environments and thus becoming particularly valuable for species maintenance at the global scale (Lesica & Allendorf 1995). Therefore, determining the level of spatial and genetic isolation of marginal populations with regard to core populations, as well as genetic diversity within populations, genetic differentiation among populations and its relationship with spatial isolation is necessary to evaluate the distinctiveness

and adaptive potential of marginal populations, particularly in plants associated with mountainous environments.

Recently Eckert et al. (2008) reviewed 84 studies involving 67 plant species that tested for declines in within-population genetic diversity and /or increases in among-population differentiation towards range margins using nuclear molecular genetic markers. They found that 64.2 % and 70.3 % of studies detected a decline of genetic diversity and an increase of genetic differentiation towards range margins, respectively, the two trends being positively associated. However, the generality of both trends is questionable given the substantial biogeographic and taxonomic biases in the available studies. First, most studies assume that marginal populations are smaller and more spatially isolated than core populations, although only a few investigations quantified the spatial isolation of populations and its relationship with genetic differentiation (but see Eckstein et al. 2006; Medrano & Herrera 2008). Furthermore, most studies have focused on northern borders of a species yet different selective pressures could operate in contracting versus expanding margins (Hampe & Petit 2005; Cassel-Lundhagen 2010). For most temperate woody species, the Mediterranean mountains constitute the southernmost distribution limits. These areas, in contrast to northernmost expanding distribution limits, represent long-term stable margins which have acted as glacial refugia during the last glacial stage (Benett et al. 1991; Carrión 2002; Carrión et al. 2003; Médail & Diadema 2009) . However, the number of molecular analyses of plant populations in these areas is still limited compared with more northern European mountain ranges such as the Alps, Pyrenees or Carpathians (Kropf et al. 2008; but see Kropf et al. 2006; Herrera & Bazaga 2008 a,b; Medrano & Herrera 2008). In addition, Mediterranean mountains constitute one of the World biodiversity hotspots because of their high complexity in terms of geology, climate and history (Thompson 2005; Blondel et al. 2010). Therefore, analysing the current genetic structure of plant populations in Mediterranean mountains may be useful to understand how the orography of heterogeneous landscapes contributes to genetic isolation of populations promoting intraspecific differentiation (Médail & Diadema 2009; Thompson 2005; pp: 77-80).

In this study, we tested for differences in population size, spatial isolation and genetic diversity and differentiation of marginal and core populations of the temperate evergreen shrub Daphne laureola in the Baetic Ranges, a glacial refugium and one of the most biologically diverse regions in the Mediterranean Basin, located in the southern Iberian Peninsula. This area constitutes the southernmost distribution patch of the species in Europe, encompassing > 100 populations with a maximum distance among them ca. 350 km, and largely isolated from other distribution patches (Alonso et al. 2007). Specifically, we addressed the following questions: (i) **Are marginal populations smaller and more spatially isolated than core populations?** (ii) **Do core populations have larger genetic diversity than marginal ones?** (iii) **Are marginal populations more genetically differentiated than core populations?** (iv) **Do marginal populations form distinct genetic groups to core populations at this southern distribution patch?**

Material and Methods

Study Species

Daphne laureola L. (Thymelaeaceae) has a Palaearctic distribution, extending from the Atlas Mountains in Morocco to the UK and Hungary northwards (Meusel et al. 1978; Fig. 1a). The species grows in the understory of coniferous and mixed forests, associated with the main calcareous ranges of Europe and North Africa. In the Iberian Peninsula, it shows a disjunct distribution, being abundant in the northern Cantabrian Range and the Pyrenees, and also in the southern Baetic Ranges, but absent in central areas (Alonso et al. 2007 and references therein).

The species flowers in winter, and the pollen beetle *Meligethes elongatus Rosenhauer* and, to a much smaller extent, small solitary bees and noctuid moths are its main pollinators in southeast Spanish populations (Alonso 2004). However, a recent study has shown that *M. elongatus* does not visit *D. laureola* flowers in populations on the southwest edge (Castilla et al. 2011). Single-seeded black drupes ripen in June and are dispersed by several bird species (Obeso 1985; Hulme 1992).

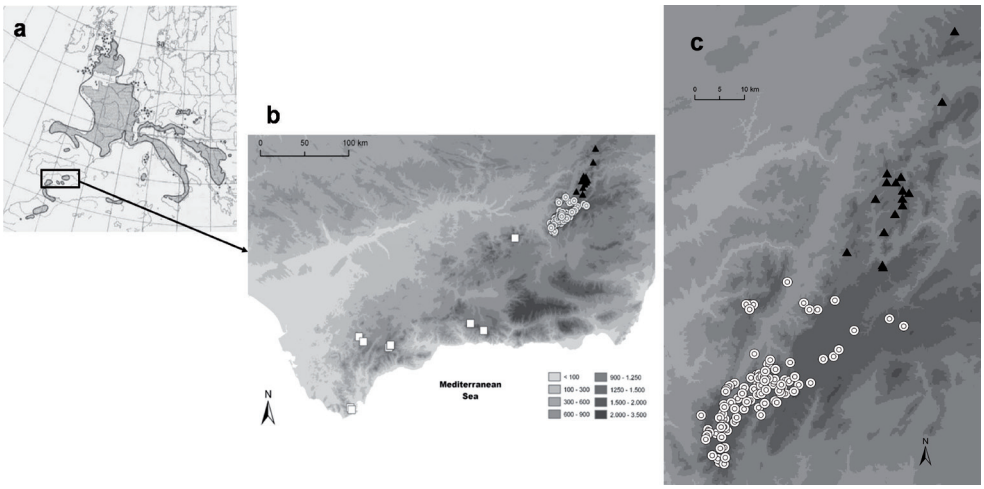

Figure 1. (a) Global distribution range of Daphne laureola indicated with grey shading. (b) Distribution of D. laureola populations in the Baetic Ranges (Southern Spain), and location of the eastern, core and western regions defined in this study, here denoted by black triangles, filled circles and white squares, respectively. The grey scale corresponds to different intervals of altitude in the region (m.a.s.l.). (c) Detailed map encompassing core and eastern populations of D. laureola in the study area (filled circles and black triangles respectively).

Spatial Isolation and Size of *D. laureola* Populations in a Southern Distribution Patch

A heterogeneous spatial distribution of *D. laureola* populations in the study area was evident a priori, with a few isolated and scattered populations located in the west, and a large cluster of core populations that become progressively sparser towards the east (**Fig. 1b and c**). For this study, we located 111 populations of *D. laureola* across the Baetic Ranges through an extensive search over several years (**Fig. 1b**). The wide network of roads and tracks across these mountainous ranges allowed a thorough exploration of the study region. We feel confident that the vast majority of populations of the species in the study region were included and the presence of *D. laureola* populations in other massifs could not be confirmed, despite intensive searches.

We considered a population as a discrete group of individuals distant from other groups of individuals by at least 1 km. Each population was visited at flowering season. Population size was categorised according to the abundance of reproductive individuals: (i) ≤ 30 (small populations), (ii) 31-99 (intermediate populations) and (iii) ≥ 100 (large populations). The geographic coordinates

were recorded with a portable GPS GARMIN eTrex Vista (Garmin, Southampton, UK) in each population. Spatial isolation of populations was quantified through a connectivity index per population (connectivity, hereafter). Connectivity of a given population i was described by a simple neighbourhood index Ci(r) computed as the number of further populations within a given distance r of the focal population i. We used a distance r of 25 km that potentially covers the maximum travel distances of D. laureola's pollinators and seed dispersers. Note that this index is proportional to the individual K-function of population i, e.g., Ci(r) = Ki(r) where is the density of populations in the study area (Illian et al. 2008: pp 256). The common K-function (Ripley 1981) is the average value of the individual K-function taken over all populations.

Genetic Structure of Representative Populations.

For the purpose of determining the genetic structure of populations in this southern distribution patch, we chose a subsample of fifteen populations representing the three regions within the study area (eastern, core and western). We intensively studied the genetic characteristics of five populations per region (**Table 1**) through analysis of amplified fragment length polymorphism (AFLP) markers. Distances between the two closest (CDE-VCV) and farthest (NVL-ALG) populations studied were 1.7 and 360 km, respectively. In each population we counted all reproductive individuals to obtain a more precise estimate of population size (**Table 1**).

Table 1. Population features and genetic diversity of *Daphne laureola* populations studied in the three defined regions. Population size refers to total number of individuals per population; connectivity refers to the number of neighbour populations in a radius of 25 km (see text for details). N: number of plants used in genetic analyses; Hj: within-population gene diversity; PLP: percent of polymorphic loci (out of a total of 225 loci scored); DW: within-population rarity of markers (see text for details).

Population (code)	Altitude (m.a.s.l.)	Population size	Connectivity	N	Hj
Eastern					
Acebeas (ACB)	1320	1436	14	35	0.130 ± 0.011
Acebeas de los Jabalises (ACJ)	1430	354	22	42	0.126 ± 0.010
Las Cumbres (CUM)	1480	56	29	30	0.135 ± 0.011
Navalasna (NVL)	1202	55	14	38	0.105 ± 0.010
Río Madera (RMD)	1220	932	17	30	0.133 ± 0.011
					0.126 ± 0.005
Core					
Cañada del Espino (CDE)	1575	178	89	40	0.113 ± 0.011
Espinarea (ESP)	1173	86	81	29	0.119 ± 0.010
Fuente Bermejo (FBJ)	1513	2115	89	40	0.158 ± 0.011
Roblehondo (RBH)	1235	55	89	26	0.125 ± 0.011
Valdecuevas (VCV)	1380	2787	89	39	0.185 ± 0.012
					0.140 ± 0.014
Western					
Almijara (ALM)	1453	1000	1	31	0.068 ± 0.009
Algeciras (ALG)	506	100	1	39	0.080 ± 0.010
Fuente Molina (FML)	1380	621	2	46	0.195 ± 0.012
Grazalema (GRZ)	1229	1580	2	46	0.100 ± 0.010
Sierra del Reloj (SRJ)	1126	28	2	22	0.124 ± 0.011
					0.113 ± 0.022

Fresh leaf material was collected from 25-40 reproductive individuals per population chosen randomly, placed in small paper envelopes and dried immediately at ambient temperature in sealed containers with abundant silica gel. Dried leaves were homogenised to a fine powder using a Retsch MM 200 mill (Retsch; Haan, Germany). Total genomic DNA was extracted from approximately 15 mg of ground leaf material using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and following the manufacturer's protocol. DNA concentration of extracts was estimated by running electrophoreses of 5-µl aliquots on 0.8 % agarose gels. The AFLP analysis was performed essentially as originally described in Vos et al. (1995), with modifications involving the use of fluorescent dye-labelled selective primers following Applied Biosystems (2005) instructions. Restriction-ligation was conducted using EcoRI / MseI and PstI / MseI endonuclease mixtures and double-stranded adaptors. A total of 32 EcoRI + 3 MseI + 3 and 32 PstI + 3 MseI + 3 primer pairs were first screened for selective amplification in a pilot study conducted with a random subsample of eight individuals from four widely scattered populations. We finally selected four EcoRI + 3 / MseI + 3 and four PstI / MseI + 3 primer combinations because of their larger polymorphism and easier interpretable band patterns (**Table 2**).

Each plant was fingerprinted using the eight combinations chosen. Fragment separation and detection was made using an ABI PRISM 3130xl DNA sequencer (Applied Biosystem, Foster City, USA). The presence or absence of each marker in each individual plant was scored manually by visualising electrophoregrams with GeneMapper 3.7 software (Applied Biosystem, Foster City, USA). All scoring was done by the same person (A.R.C.). Prior to statistical analyses, we excluded markers present in < 1 or > 99 % of individuals (N = 100). Furthermore, only fragments ≥ 150 bp in size were considered, as a way of reducing the potential impact of size homoplasy (Vekemans et al. 2002; Caballero et al. 2008). In addition, five plants that produced noisy sequencer electrophoregrams for some primer combinations, after running the analyses several times, were also excluded from the sample. The combination of 225 polymorphic markers, each one scored for presence / absence, and 25-40 individuals per population (N = 533) used in this study was sufficient

to produce reasonably narrow standard errors and adequate statistical power to detect genetic differentiation among populations (Medina et al. 2006; Bonin et al. 2007).

Genotyping error rates were determined for each primer combination by running repeated, independent analyses for a total of 32 randomly chosen individual plants, and estimated as the ratio of the total number of loci with discordant scores (all individuals combined) to a product of the number of individuals by the total number of scored loci (Bonin et al. 2004; Pompanon et al. 2005). Error rates varied among primer combinations, being larger in individual EcoRI combinations than in PstI combinations (**Table 2**). The mean value (± SE) for the whole set of eight combinations was 0.9 ± 0.4 %.

Table 2. Primer combinations used, number of markers (loci) obtained in the size range 150-500 base pairs, observed percentage of polymorphism level (PLP) and error rates in the amplified fragment length polymorphism (AFLP) analysis of the 15 *Daphne laureola* populations studied.

Combination	Number of markers	% PLP	Scoring error rate (%)
1. EcoRI-AGC / MseI-CTT	27	21.1	0.9
2. EcoRI-AGA / MseI-CGA	17	23.5	3
3. EcoRI-AGG / MseI-CAC	23	43.5	1.4
4. EcoRI-ACA / MseI-CCC	29	36.3	1.7
5. PstI-AA / MseI-CAC	38	46.7	0.2
6. PstI-AC / MseI-CAT	10	38.7	0.1
7. PstI-AG / MseI-CTC	39	43.2	0.1
8. PstI-AT / MseI-CTT	42	28.9	0.2

Data Analyses

Size and spatial isolation of populations

Unless otherwise stated, all statistical analyses were conducted with SAS 9.1 (SAS Institute 2002). Differences between regions in connectivity were tested using a generalized linear model including connectivity as dependent variable and region as the only fixed explanatory factor (Procedure GENMOD). Connectivity was modelled using the Poisson distribution. Population size was analysed with the Kruskal-Wallis Test, considering population size categories as ranks of population size and region as categorical explanatory variable. To test for regional differences in connectivity and population size, we explored differences between core versus marginal populations using post-hoc Wilcoxon tests.

Genetic structure

Gene diversity (Hj), percentage of polymorphic loci (i.e. loci with allelic frequencies within the range 0.05-0.95; PLP hereafter) and population pair-wise FST values were calculated with AFLP-surv 1.0 (Vekemans et al. 2002), computing allelic frequencies with a Bayesian method with non-uniform prior distribution and 10,000 permutations. These parameters were estimated assuming both Hardy-Weinberg equilibrium (Fis = 0) and complete selfing (Fis = 1), with very little effect on the results that did not change the general conclusions. Additionally, the Rarity 1 index (equivalent to the frequency of down-weighted marker values; i.e., DW sensu Schönswetter & Tribsch 2005) was calculated using AFLPdat (Ehrich 2006). Group-based DW estimates were obtained by non-parametric bootstrapping of plant individual values (not population averages) for each population. The number of private bands was also calculated using GenAlEx 6.3 (Peakall & Smouse 2006). The significance of differences between core and marginal populations in genetic diversity and differentiation was tested using non-parametric methods (procedure NPAR1WAY). Gene diversity, PLP and DW presented normal distributions, however we used Wilcoxon exact tests to analyse them because of the reduced sample size in the paired tests (N = 10). Population FST was calculated averaging the FST values of each population with all other four populations within the same region. This variable was not normally distributed; consequently, the differences between core and

marginal populations were also explored with Wilcoxon exact tests. The influence of population size and connectivity on genetic diversity (PLP and Hj) and differentiation (DW and Fst) was analysed using Spearman correlations.

A three-level hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) was calculated with the program GenAlEx 6.3. We analyzed the following levels: (i) region (eastern, core and western); (ii) populations within region; and (iii) individuals within populations. Additionally, separate two level AMOVAs were calculated for each region to analyse the partitioning of molecular variance among and within populations. A standard test for genetic isolation by geographic distance was ruled out for two reasons. On the one hand, the low number of populations per region (N = 5) did not allow testing for genetic isolation by distance in each region. On the other hand, western populations were so distant from the remaining populations (> 138 km) that it introduces a large discontinuity better described by the categorical variable "region" than by the continuous variable "geographic distance".

Relationships among populations were visualized through neighbour-joining clustering of pairwise FST, and the significance of the branches was assessed through 10,000 bootstrap replicates (Felsenstein 1985). A second approach based on statistical inference with Bayesian clustering methods using STRUCTURE 2.2.3. was also used to further elucidate the uppermost level of genetic structure in the study region (Pritchard et al. 2000; Falush et al. 2007). This program probabilistically assigns genotyped individuals into genetic groups in order to minimise departures from Hardy-Weinberg equilibrium and linkage equilibrium. The number of genetic groups was explored by performing 20 replicates of each simulation from K = 1 to K = 17, with a burn-in of 50,000 and MCMC of 100,000, assuming admixture and correlated allele frequencies as recommended in Pritchard et al. (2000). We applied Evanno et al.'s (2005) modal ΔK parameter as the choice criterion to detect the true number of genetic groups in the set of N = 533 individuals assayed.

Results

Are marginal populations more spatially isolated and smaller than core populations?

We found differences between regions in connectivity (2 = 1745.08, df = 2, *P* < 0.0001; Kruskal-Wallis test), with density of neighbour populations being largest in the core region (**Table 3**). Core populations had more than four times the number of neighbour populations within 25 km than eastern populations (W = 43.29, *P* < 0.0001; **Table 3**). The difference with western populations was even larger. Core populations had more than 80 times the number of populations within 25 km than western populations (W = 17.20, *P* < 0.0001; **Table 3**).

Table 3. Average values of connectivity in eastern, central and western populations of *D. laureola* in the south of Iberian Peninsula. N indicates the number of populations in each region. Connectivity refers to the number of neighbour populations in a distance radius of 25 km. Population size was categorized according to the abundance of reproductive individuals (1, ≤30; 2, 31-100; 3, ≥ 100). The table shows the percentage of populations of each category per region.

	N	Connectivity	Population Size Category (%)		
			1	2	3
Eastern	17	20.8 ± 2.5	12	47	41
Central	88	82.6 ± 0.8	8	75	17
Western	6	1.7 ± 0.2	17	0	83

There was a significant difference in population size among regions (2 = 8.52, df = 2, *P* < 0.05; Kruskal-Wallis test). Unexpectedly, the core region had the lowest population sizes, with only 17 % of populations having more than 100 reproductive individuals, whereas the more isolated populations in the western region had the largest average size, with 83 % of populations having more than 100 reproductive individuals (**Table 3**). Eastern and core populations did not differ in population size (W = 605.5, *P* = 0.12) but western populations had larger average population size than core populations (W = 122, *P* < 0.01). Connectivity was not correlated with population size (rs = - 0.15, *P* = 0.11; N = 111).

Do Core Populations Have Larger Genetic Diversity Than Marginal Ones?

Average gene diversity of core populations did not differ significantly from eastern (W = 27, df = 8, *P* = 0.5) or western populations (W = 33, df = 8, *P* = 0.1), although mean Hj tended to be slightly larger in core (0.140) than in marginal populations (0.126 and 0.113 in eastern and western populations, respectively; Table 1). Western populations exhibited the broadest variation range of gene diversity, presenting the minimum and maximum values (0.068 and 0.195) of all studied populations. Similarly, core populations did not show statistically significant differences in the percentage of polymorphic loci (PLP) relative to eastern (W = 27, df = 8, *P* = 0.5) or western populations (W = 33.5, df = 8, *P* = 0.1), although mean PLP tended to be larger in core than in marginal populations (33.9 %, 32.0 % and 26.7 % in core, eastern and western populations, respectively; **Table 1**). Again, the maximum and minimum PLP values (48.9 % and 13.3 %) were found in western populations. The FML population had the largest average values of both gene diversity and PLP in contrast to the rest of western populations with low values for both estimates of genetic diversity. In fact, excluding FML from analysis led to an important decrease in genetic diversity of western region (Hj = 0.093 and PLP = 21.1 %). Population size and PLP showed a positive relationship, although it was only marginally significant (rs = 0.50, *P* = 0.06, N = 15). No significant relationship was found between population size and gene diversity (rs= 0.29, *P* = 0.30, N = 15). PLP and gene diversity were both positively related to connectivity, although the relationships were only marginally significant (rs = 0.47, *P* = 0.07, N = 15 in both cases).

Are Marginal Populations More Genetically Differentiated Than Core Ones?

On average, western populations had more private fragments (2.0) than either eastern or core populations (0.6 and 0.2 private fragments respectively; **Table 1**). The westernmost population (ALG) had the highest number of private fragments despite its low values for mean gene diversity and percentage of polymorphic loci (**Table 1**). Mean pairwise FST was larger in western than in core populations (W = 55, df = 8, *P* < 0.0001; **Fig. 2**), which were in turn more genetically differentiated than eastern ones (W = 64, df = 8, *P* < 0.001; **Fig. 2**).

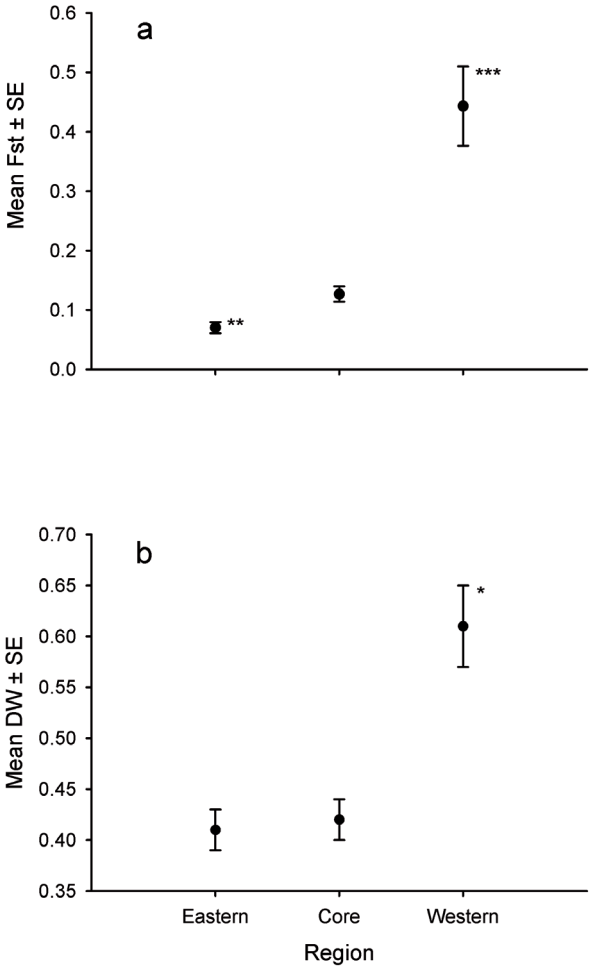

Figure 2. Genetic differentiation of the populations in core and marginal regions. Population pairwise FST and genetic rarity (DW) per region (eastern, core and western) are represented by means (±SE). Statistically significant variation between marginal and core populations is indicated with asterisks (* P <0.01; ** P <0.001; *** P <0.0001).

Furthermore, the genetic rarity index DW was significantly larger in western populations than in core populations (W = 16, df = 8, P < 0.01; **Fig. 2**). In contrast, there were not differences in DW between core and eastern populations (W = 26.5, df = 8, P = 0.4; **Fig. 2**). Interestingly, genetic rarity were negatively related to connectivity (rs = -0.59, P < 0.05, N = 15). Population pair-wise FST also was negatively related to connectivity of populations, although the relationship was only marginally significant (rs = -0.47, P = 0.08, N = 15). Population size was unrelated to genetic rarity and population pair-wise FST (rs = 0.03, P = 0.90 and rs = 0.08, P = 0.79 respectively; N = 15).

The three-level AMOVA revealed a strong genetic differentiation among regions (ST = 0.45). Most molecular variance was accounted for differences among regions and within populations (46 and 40 % respectively), whereas differences among populations within a region accounted for a reduced amount of molecular diversity (16 %). Results of the AMOVA analysis also showed that western populations were more genetically differentiated among them, with larger values of molecular variance explained among populations, and an average ST higher than eastern and core populations (**Table 4**). Eastern populations presented the lowest values of molecular variance explained among populations and ST (**Table 4**).

Table 4. Genetic differentiation (ST) and percentage of molecular variance distributed among regions, among populations and within populations in the three regions defined in this study. 9999 permutations were used in the analysis.

Source of variation	d.f.	% Variance	Φ_{ST}	P-value
1. Three-level AMOVA				
Among regions	2	45	0.455	0.0001
Among populations	12	14		
Within populations	518	40		
2. Eastern				
Among populations	4	9	0.090	0.0001
EWithin population	170	91		
3. Core				
Among populations	4	13	0.125	0.0001
Within population	169	87		
4. Western				
Among populations	4	47	0.472	0.0001
Within population	179	53		

Do Marginal Populations Form Genetically Distinct Groups?

Two distinct genetic clusters, one represented by the western populations and the other grouping together core and eastern populations, were supported by both neighbour-joining and Bayesian approaches (**Fig. 3 and 4**). The dendrogram based on neighbour-joining approach showed a split between western and the other populations with a support of 100 % (**Fig. 3**). Within the largest group, two populations, VCV and FBJ, both located in a particular watershed (Guadalentín Valley; **Fig. 4b**), appeared differentiated from each other and from the rest of the core and eastern populations, with a support of 97 % in both cases (**Fig. 3**). In addition, the Bayesian analysis showed a distinct modal maximum of ΔK at K = 2 genetic groups. Membership assignments to the two genetic groups exhibited a distinct geographic pattern coincident with the results of the neighbour-joining approach (**Fig. 4a**), supporting that western populations formed a genetic group differentiated from the rest of populations analysed.

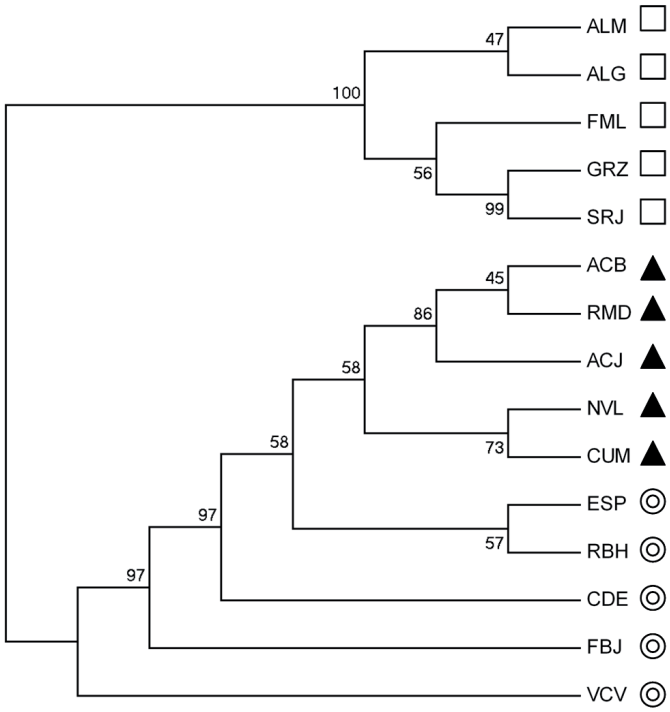

Figure 3. Neighbour-joining clustering based on FST, showing the relationship among the 15 sampled populations of *D. laureola*. Bootstrap percentage values are indicated above the branches. Populations coded as in Table 2 and regions as in Fig. 1.

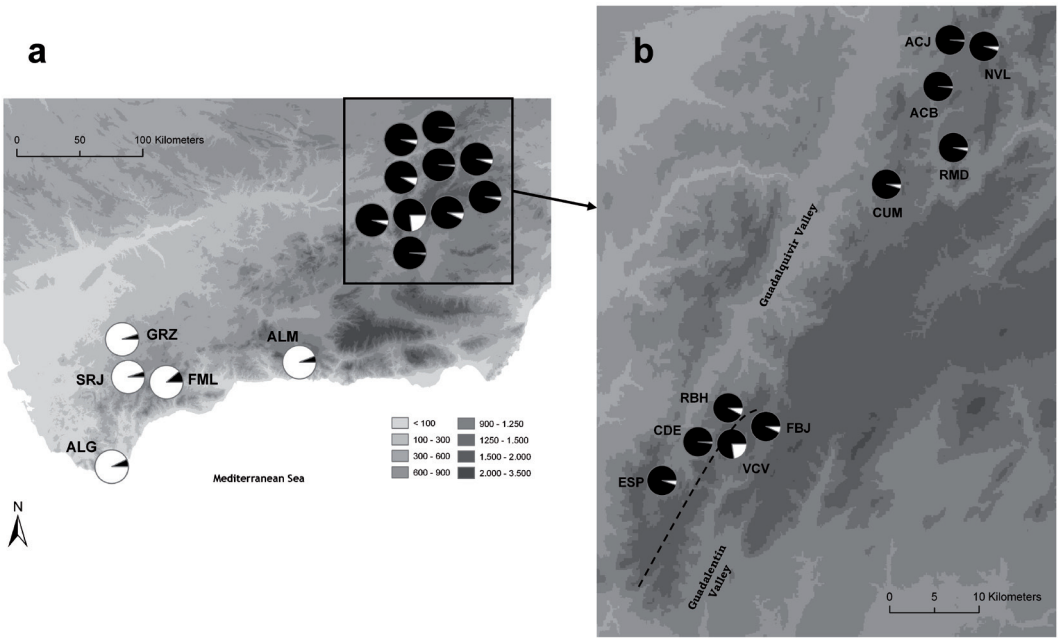

Figure 4. (a) Genetic population structure of *Daphne laureola* in the Baetic Ranges inferred using the model-based Bayesian clustering implemented in STRUCTURE which does not require a priori categories. The individual pie charts indicate the mean proportion of membership of each local population for the inferred number of K = 2 genetic groups. **(b)** Detailed population structure within the largest genetic group that combines core and eastern populations. Dashed line indicates the highest elevation of the mountainous range. Populations coded as in Table.

Discussion

Genetic characteristics of populations may be strongly influenced by the size and spatial distribution of populations through their relationships with genetic drift and gene flow. Thus, these characteristics are expected to vary across species' geographic ranges (Eckert et al. 2008). In the following paragraphs, we will discuss our results supporting, first, the increased spatial isolation of marginal populations and, second, the genetic differentiation of the most spatially isolated local margin of the shrub *D. laureola* in the Mediterranean Baetic Ranges, an area close to the species southern distribution range.

Spatial Isolation and Size of *D. laureola* Populations in a Southern Distribution Patch

Our results, based on an extensive sampling across the Baetic Ranges, showed that populations of this temperate shrub were fewer and more spatially isolated towards local eastern and western margins, supporting the hypothesis of increased isolation at local range margins (Brown 1984; Vucetich & Waite 2003; Bridle et al. 2010), and highlighting the suitability of the spatially explicit analysis adopted here to identify meaningful geographic discontinuities (**Table 2**). The western region comprised a few highly isolated populations located in different mountain ranges (**Fig. 1b**), the core region with abundant and very connected populations at all distances comprised populations mainly located in the Sierras de Cazorla and Las Villas, and the eastern region associated with those populations in Sierras de Segura and Alcaraz, where the species is still common but populations exhibit a reduction in connectivity among them (**Fig. 1c; Table 2**).

The predicted decline in population size towards local borders of distribution was not confirmed in *D. laureola* populations across the Baetic Ranges (see also Jump & Woodward 2003; Herlihy & Eckert 2005). In fact, core populations were on average smaller than marginal populations, although the trend was only significant in the comparison between core and western populations. The wider altitudinal range of core populations could likely contribute to variability in population size in this region through altitudinal gradients in ecological conditions (Herrera and Bazaga 2008b). The presence of large marginal populations with more than 100 reproductive individuals suggests the existence of some suitable locations without evidence of ecological stress for *D. laureola* towards the local borders of distribution (Castilla et al. 2011).

Genetic Diversity, Differentiation and Structure, and their Relationships with the Spatial Isolation of Populations

Core and marginal populations of *D. laureola* in the Baetic Ranges exhibited similar levels of genetic diversity, and regions did not differ significantly in either gene diversity or percentage of polymorphic loci within a population. These results contrast with those found for other plants (e.g., Lammi et al. 1999; Lönn & Prentice 2002; Arnaud-Haond et al. 2006; Eckert et al. 2008). The absence of clear differences between core and marginal populations in genetic diversity could be

related to the influence of population size (Lammi et al. 1999; Leimu et al. 2006) because marginal populations, contrary to expectations, were not smaller than core populations. Further, our results supported that PLP was marginally related to population size. Therefore, the existence of large populations on *D. laureola*'s local borders of distribution could prevent genetic erosion through mechanisms of genetic rescue (Young et al. 1996; Garant et al. 2007). Interestingly, the western margin was internally very heterogeneous with regard to the genetic diversity of the populations. With the exception of FML population that exhibited the highest genetic diversity among all study populations, the other four tended to have a much more reduced genetic diversity than core and eastern populations, mostly supporting the expectation of a reduced genetic diversity in the most spatially isolated populations of the western margin, with the single exception already mentioned.

Strong genetic differentiation among D. laureola populations across the Baetic Ranges was indicated in our results using AFLP markers (ST = 0.45). These results are consistent with observed genetic differentiation in DNA sequence in a much reduced number of sampled populations and individuals (Alonso and Herrera 2011). In addition, the two estimates of genetic differentiation (FST and DW) were positively related to the spatial isolation of populations. In particular, results showed that the highly isolated western populations constitute a genetic group strongly differentiated from core and eastern populations, supporting a positive relationship between geographic isolation and genetic differentiation in our study system (Eckstein et al. 2006; Medrano & Herrera 2008). Also, our results suggest the existence of two types of local borders of distribution in the study area from a genetic viewpoint: a western margin with populations being both more spatially and genetically isolated among them, as usually happens in the rear edge of species distributions (Hampe & Petit 2005); and an eastern margin comprising a group of more spatially isolated but genetically homogeneous populations, closely related to core populations and therefore more typical of a expanding edge (Schönswetter et al. 2002; Csërgo et al. 2009). The genetic similarity among core and eastern populations suggests extensive gene flow between the two regions. Eastern populations are distant by only about 20-30 km from core populations, all located along an extensive mountainous range, which could facilitate local gene flow through natural corridors

for the dispersal vectors of the species (see e.g. Gaudeul et al. 2000; Barrett et al. 2004). However, western populations are located far away from the core populations (130 km approximately), limiting the gene flow among western and core populations to long-distance infrequent dispersal events (Kropf et al. 2006; Kropf et al. 2008). In addition, western populations are located in different mountainous ranges separated by dozens to hundreds of kilometres of unsuitable habitats for the species, which could also reduce gene flow among populations on the western edge, thus promoting their genetic differentiation. Supporting this notion, western populations also had the highest values of genetic rarity estimates and recent studies have also revealed some ecological particularities of these populations. For instance, the female frequency decreases in southwest populations, the ALG population being constituted exclusively by hermaphrodite plants in the most extreme case (Alonso et al. 2007). In addition, individuals of western populations present lower floral displays than those of core populations, coincident with a change in the pollinator fauna (Castilla et al. 2011). The ecological particularities of western populations and their strong genetic isolation highlight the potential role that local adaptation events could have in these marginal disjunct populations (Lesica & Allendorf 1995; García-Ramos & Kirkpatrick 1997; Arnaud-Haond et al. 2006; Cassel-Lundhagen 2010).

Concluding Remarks

Among-population phenotypic divergence is relatively common feature in plant species across Mediterranean mountain ranges, geographic margins of species' ranges being particularly prone to the evolution of this random or adaptive differentiation. Background information on patterns of neutral differentiation is particularly interesting for disentangling the role of natural selection on this phenotypic divergence. This approach is especially useful in plant species of Mediterranean mountain ranges because of the strong relevance of historical processes on the genetic structure of their populations. In this sense, genetic structure analyses comparing core and marginal populations based on neutral markers emerge as a useful tool to estimate the level of neutral differentiation in populations where among-population phenotypic differentiation is expected. Results of our study show that neutral differentiation varies on different local margins of distribution. In

addition, our results are consistent with the historical divergence between western and the remaining populations of *D. laureola* in the study area, as detected through analysis of markers with a much reduced polymorphism as DNA sequences (Alonso and Herrera 2011). Therefore, a phenotypic differentiation at the same level in both borders should be evaluated differentially. For instance, if further studies show that eastern populations of *D. laureola* are phenotypically differentiated from core populations despite their similarity in neutral genetic variation, this phenotypic differentiation could likely represent adaptive differentiation. However, phenotypic differentiation on the western margin should be assesed with more caution because it could be the result of random differentiation via long-term isolation between both groups of populations. Therefore, results of our work highlight the relevance of genetic structure analyses focused on distribution margins as a first step forward to understanding the adaptive basis of intraspecific differentiation.

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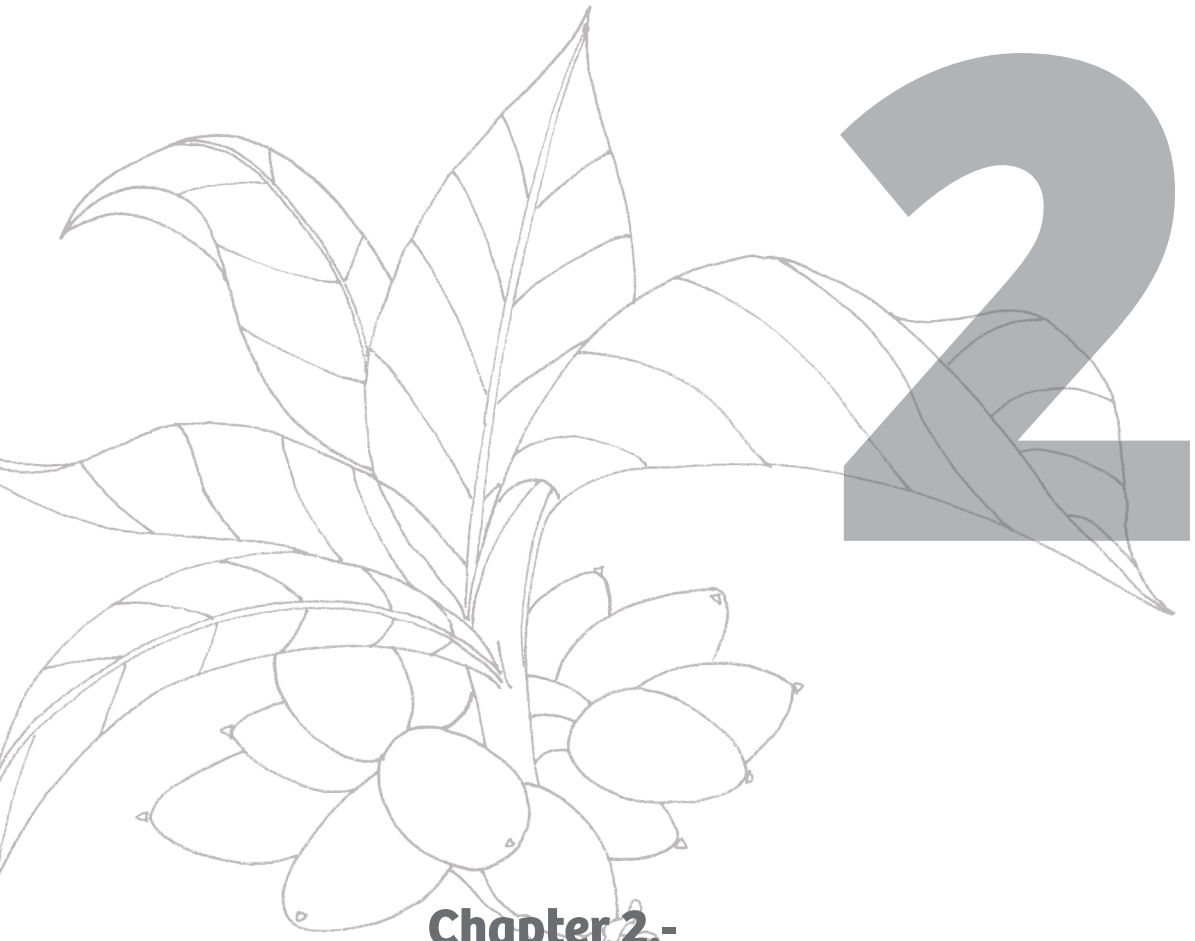

Chapter 2.-
Differential selection between sexes and regions in a gynodioecious shrub revealed by genomic scanning

Abstract: Gynodioecious plants frequently exhibit pronounced sexual dimorphism in reproductive traits, yet the extent to which sexual dimorphism reflects sex-specific selective regimes is unclear. Since genomic scan-based methods allow looking for signatures of natural selection in wild populations, we adopted an AFLP-based population genomics approach to compare selective regimes on hermaphrodite and female individuals of the gynodioecious shrub *Daphne laureola (Thymelaeaceae)* in two regions with genetically distinct populations and differing also in overall female frequency and pollinator composition. Results supported selection at different loci, but similar intensity of selection, in the two regions. Judging from the number of loci involved, hermaphrodites seemed subject to stronger selection than females, the difference being most pronounced in the region where pollen beetles were absent. Variation across regions in sex-specific patterns of selection associated to changes in pollinators highlights pollinators's role as selective agents contributing to floral dimorphism in sexually dimorphic plants.

Castilla, A.R., Alonso, C. and Herrera, C.M. In review. Differential selection between sexes and regions in a gynodioecious shrub revealed by genomic scanning.

Resumen

Las especies vegetales ginodioicas a menudo exhiben un pronunciado dimorfismo sexual en los rasgos reproductivos. Sin embargo, aún no está claro si este dimorfismo sexual es o no debido a regímenes de selección diferenciales entre plantas de distinto sexo. Aprovechando que los métodos basados en el escaneo genómico ("genomic scanning") permiten detectar huellas de la selección natural en las poblaciones naturales, usamos un enfoque genómico poblacional basado en el uso de AFLPs para comparar los regímenes selectivos en individuos hermafroditas y hembras del arbusto ginodioico ***Daphne laureola (Thymelaeaceae)***, en dos regiones genéticamente diferenciadas, con distinta frecuencia media de plantas hembra y con diferente composición de fauna de polinizadores. Los resultados de este estudio apoyaron similar nivel de selección en ambas regiones pero sobre loci diferentes. A juzgar por el número de loci implicados, las plantas hermafroditas parecen estar sujetas a selección más intensa que las plantas hembra siendo esta tendencia más acusada en la región marginal donde el principal polinizador de la especie en el área de estudio está ausente. La variación regional en los patrones selectivos específicos de cada sexo junto con los cambios en la comunidad de polinizadores apunta a los polinizadores como un posible agente selectivo que podría contribuir al desarrollo de dimorfismo floral en especies vegetales con polimorfismo sexual.

Introduction

The application of population genomics in the field of evolutionary ecology has opened a promising window to the study of the natural selection in non-model organisms in the wild (Vermeer et al., 2010). The two main principles of population genomics are (i) **that neutral loci across the genome will be similarly affected by demography and evolutionary history of populations**; and (ii) **that loci under selection will often behave differently and thus they reveal "outlier" patterns of variation** (Black et al., 2001; Luikart et al., 2003; Allendorf et al., 2010). Most studies focusing on the role of natural selection on intraspecific differentiation in plant species rely on artificially breeding individuals of known pedigree obtained through controlled crosses between field-collected progenitors and subsequently exposed to controlled experimental environments (Lynch & Walsh, 1998; Leinonen et al., 2008). Population genomics using a large number of polymorphic markers offers several advantages compared to the more traditional approach. First, it does not restrict the analysis to a few potential selective factors artificially manipulated, but it considers all selective factors which are acting on individuals in wild populations. In addition, no prior knowledge is needed regarding the phenotypic traits of interest because the genome is thoroughly scanned for outlier loci by using anonymous markers (Vasemägi & Primmer, 2005; Stinchcombe & Hoekstra, 2008). Finally, it does not require knowing the relatedness between study individuals and thus, it can be applied to naturally occurring non-model organisms, as perennial plant species (Herrera & Bazaga, 2008; Vermeer et al., 2010; Herrera, in press).

Differential selection on individuals with different sex has been considered a relevant mechanism helping to maintain sexual polymorphism in plant species (Geber, 1999; Delph & Ashman, 2006). However, relatively few studies have so far studied sex-specific selective regimes in sexually polymorphic plants, although increasing support to differential selective regimes on plants with different sex has been found for dioecious and gynodioecious species (Ashman, 2003; Steven et al., 2007; Sakai et al., 2007; but see Caruso & Yakobowski, 2008). Gynodioecious species, a state where hermaphrodite and female individuals coexist in populations, exhibit pronounced sexual dimor-

phism in reproductive traits contrasting with the modest sexual dimorphism in vegetative traits (Ashman, 2005). Therefore, differential selective regimes on reproductive traits (e.g. floral traits) of hermaphrodite and female individuals are expected to occur. Pollinators constitute important selective agents on reproductive traits and thus, they may have a relevant role on determining the extent of sexual dimorphism in these traits, particularly because pollinator fauna often vary geographically (Herrera et al., 2006; Ashman, 2000; Ashman & Diefenderfer, 2001; Ashman, 2006 and references therein).

Daphne laureola L. (Thymelaeaceae) is a sexually polymorphic shrub that presents gynodioecious populations with hermaphrodite and female individuals, and monomorphic populations made up exclusively of hermaphrodites (Alonso et al., 2007). In gynodioecious populations, *D. laureola* shows sexual dimorphism in traits related to its floral morphology and chemical defences. Hermaphrodites have larger flowers than females (Alonso, 2005), a widespread pattern in gynodioecious species (Eckhart, 1999; Shykoff et al., 2003). Furthermore, females present larger concentration of specific chemical defences (coumarins) than hermaphrodites (Alonso et al., 2005). We studied patterns of selection in populations of *D. laureola* located across the Baetic Ranges in southern Iberian Peninsula. Genetic structure analyses have shown that *D. laureola* presents two genetically differentiated population groups (SE and SW populations hereafter; Alonso & Herrera, 2011; Castilla et al., unpublished data). However, the inclusion of only a few outlier loci under selection could greatly bias the estimates of population evolutionary history as determinant of genetic population structure (Luikart et al., 2003; Herrera & Bazaga, 2008). Therefore, the analysis of the same polymorphic markers discarding the outlier loci may reveal how much of the genetic divergence between SE and SW populations of *D. laureola* across the Baetic Ranges is accounted by differences in the selective regimes. Furthermore, SE and SW populations differ also in the pollinator fauna and their overall female frequency (Alonso et al., 2007; Castilla et al., 2011). Differences in reproductive success and phenotypic traits (e.g. floral display) suggest the existence of local adaptation in the most isolated populations of the SW region (Castilla et al., 2011). In this study, we adopted a genomic scan approach to detect signatures of differential selection between

female and hermaphrodite individuals of D. laureola, and to asses whether patterns of sex-specific selection remained consistent between the SE and SW populations. Specifically, we addressed the following questions: (1) are there genetic signatures of differential selection between the two study regions? (2) does the genetic divergence between SE and SW populations of D. laureola remain after excluding loci under selection? (3) are there contrasting selective regimes on individuals of different sex? (4) do differences between sexes in the selective patterns remain consistent across regions?

Material and methods

Study Species and Populations

Daphne laureola L. (Thymelaeaceae) is an evergreen shrub with a disjunct Palaearctic distribution, with distribution patches on the main calcareous mountainous ranges of Europe and northern Africa, including the Baetic Ranges in Southern Spain where this study was conducted (**Fig. 1**). Within the Baetic Ranges, *D. laureola* is less frequent in the SW region, where populations are characteristically more isolated and genetically differentiated from those in the SE region, which represents the core area of distribution (Alonso & Herrera, 2011; Castilla et al., unpublished data). The two regions also differ in the composition of pollinator fauna, with SE populations presenting as main pollinator the pollen beetle *Meligethes elongatus Rosenhauer*, which is absent in SW populations (Alonso, 2004; Castilla et al., 2011). Furthermore, overall female frequency is lower in SW populations (Alonso et al., 2007).

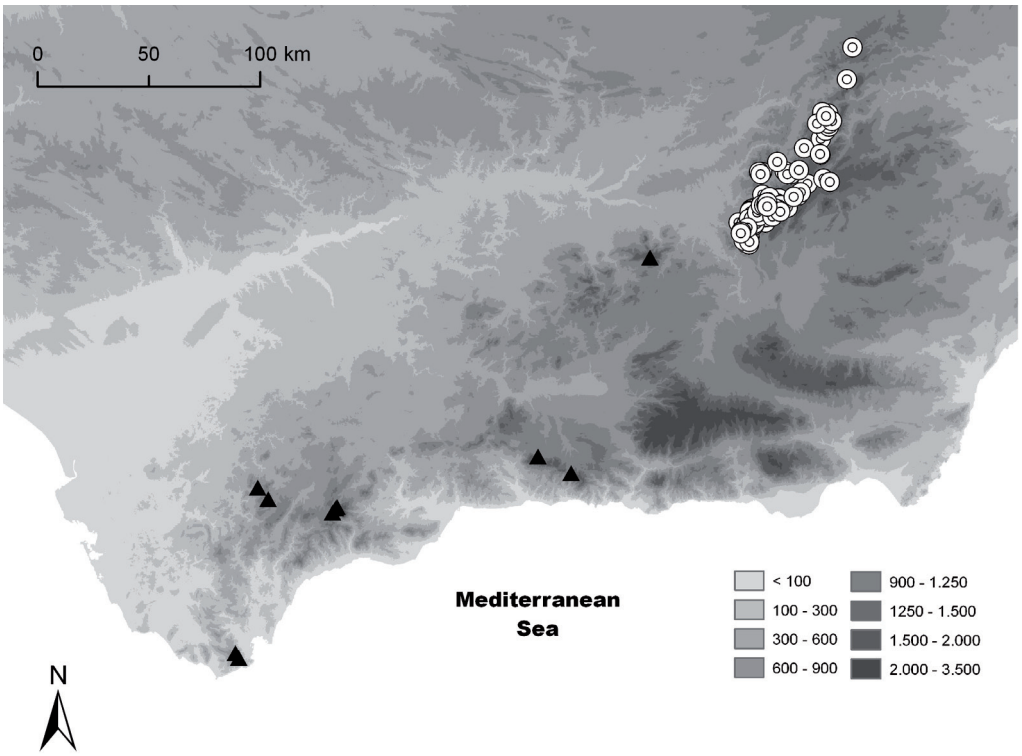

Figure 1. Distribution of Daphne laureola populations in the Baetic Ranges (Southern Spain), and location of the SE and SW populations defined in this study, here denoted by filled circles and black triangles, respectively. The grey scale corresponds to different intervals of altitude (m.a.s.l.).

AFLP analyses

Were sampled 15 populations of *D. laureola*, ten in the most densely populated SE region and five in the marginal SW region (**Fig. 1**). In March 2007, fresh leaf material was collected from 25-40 randomly chosen reproductive individuals per population, placed in small paper envelopes and dried immediately at ambient temperature in sealed containers with abundant silica gel. Dried leaves were homogenized to a fine powder using a Retsch MM 200 mill. Total genomic DNA was extracted from approximately 15 mg of ground leaf material using DNeasy Plant Mini Kit (Qiagen) and following the manufacturer protocol. DNA concentration of extracts was estimated by running electrophoreses of 5 µl aliquots on 0.8 % agarose gels. The AFLP analysis was performed essentially as originally described by Vos et al. (1995), with modifications involving the use of fluo-

rescent dye-labeled selective primers following Applied Biosystems (2005). Restriction-ligation was conducted using EcoRI / MseI and PstI / MseI endonuclease mixtures and doubled-stranded adaptors. A total of 32 EcoRI + 3 MseI + 3 and 32 PstI + 3 MseI + 3 primer pairs were first screened for selective amplification in a pilot study conducted with a random subsample of 8 individuals from four widely scattered populations. We finally selected four EcoRI + 3 / MseI + 3 and four PstI / MseI + 3 primer combinations on the basis of their higher polymorphism and ease of interpretation of band pattern (see **Table 2 in Chapter 1**).

Each plant was fingerprinted using the eight combinations chosen. Fragment separation and detection was made using an ABI PRISM 3100 DNA sequencer. The presence or absence of each marker in each individual plant was scored manually by visualizing electrophoregrams with GeneMapper 3.7 software. All scoring was done by the same person (A.R.C.). Prior to statistical analyses, we excluded markers present in < 1 or > 99 % of individuals. Furthermore, only fragments ≥ 150 base pairs in size were considered, as a way of reducing the potential impact of size homoplasy (Vekemans et al., 2002; Caballero et al., 2008). In addition, five plants that produced noisy sequencer electrophoregrams for some primer combinations after running the analyses several times, were also excluded from the sample. Our final data set consisted of a total of 493 individuals, each scored for presence / absence of 225 polymorphic loci.

Genotyping error rates were determined for each primer combination by running repeated, independent analyses for a total of 32 randomly chosen individual plants, and estimated as the ratio of the total number of loci with discordant scores (all individuals combined) to a product of the number of individuals by the total number of scored loci (Bonin et al., 2004; Pompanon et al., 2005). Error rates varied among primers combinations, being larger in EcoRI combinations than in PstI combinations (see **Table 2 in Chapter 1**). The mean error rate (± SE) for the whole set of eight combinations was 0.9 ± 0.4 %.

Detection of Loci Under Selection

Analyses to identify loci under selection were conducted separately for SE and SW populations of *D. laureola*. In addition, we analysed separately female and hermaphrodite individuals within each population group (57 females and 141 hermaphrodites in SW populations; 102 females and 193 hermaphrodites in SE populations).

The software package BayeScan version 2.01 (Foll & Gagiotti, 2008; Foll et al., 2010) was used to test for the neutrality of the AFLP markers. BayeScan extends Beaumont & Balding's (2004) Bayesian method that implements the multinomial-Dirichlet likelihood, which arises in a wide range of neutral population genetic models. The methodology consists of identifying loci that present population differentiation coefficients (FST) that are distinct from those under neutral expectations. A locus is suspected to be under selection if the locus-specific effect is significantly different from zero. The method developed by Foll & Gagiotti (2008) uses a hierarchical Bayesian approach to estimate the posterior probabilities of two alternative models, one including the effects of the selection and one excluding them. In the context of the multiple testing, we also need to incorporate our scepticism related to statistical error type I about the chance that each locus is under selection. This is done by BayeScan setting the prior odds of the neutral model and using the Posterior Odds instead of Bayes factors to make decisions (Foll et al., 2010). Posterior odds are simply the ratio of posterior probabilities, and indicate how more likely the model with selection is compared to the neutral model. Posterior probabilities directly allow the control of the False Discovery Rate (FDR).

In our analyses, we calculated the posterior probabilities threshold to detect loci under selection leading to a False Discovery Rate ≤ 0.001 and we used a value of prior odds of 10, which is reasonable for the identification of candidate loci within a few hundreds of markers (Foll et al., 2010). We ran BayeScan with 10 pilot runs, burn-in of 50000, total of 550000 iterations, sample size of 10000 and thinning interval of 50.

Genetic Structure of Populations

We removed outlier loci from the analysis of the genetic structure to detect how much of the genetic divergence between SE and SW populations of *D. laureola* across the Baetic Ranges was accounted by differences in the selective regimes. The inclusion of only a few outlier loci among many neutral loci can greatly bias estimates of the effects of population evolutionary history (Luikart et al., 2003; Herrera & Bazaga, 2008). Therefore, to avoid making erroneous conclusions from using outlier loci, population parameters should be estimated without outliers (Luikart et al., 2003; Herrera, 2011). The loci departing significantly from neutral expectation were removed from further analyses of genetic structure and gene flow, altogether 13 loci (Supplementary material). An approach based on statistical inference with Bayesian clustering methods using STRUCTURE 2.2.3. was then used to further elucidate the uppermost level of genetic structure in the study region (Pritchard et al., 2000; Falush et al., 2007). This program probabilistically assigns genotyped individuals into genetic groups in order to minimize departures from Hardy-Weinberg equilibrium and linkage disequilibrium. The number of genetic groups was explored by performing 20 replicates of each simulation from K=1 to K=17, with a burning of 50000 and MCMC of 100000, assuming admixture and correlated allele frequencies as recommended by Pritchard et al. (2000). We applied Evanno et al.'s (2005) modal ΔK parameter as the choice criterion to detect the true number of genetic groups in the set of individuals assayed.

Results

Loci Under Selection and Neutral Genetic Structure of Populations

BayeScan identified six and seven AFLP markers with evidence of purifying selection in the SE and SW populations, respectively (3 % of all markers approximately in both cases; **Fig. 2a**). Note that loci under purifying selection in one group of populations were not under detectable selection in the other group (**Fig. 2b**).

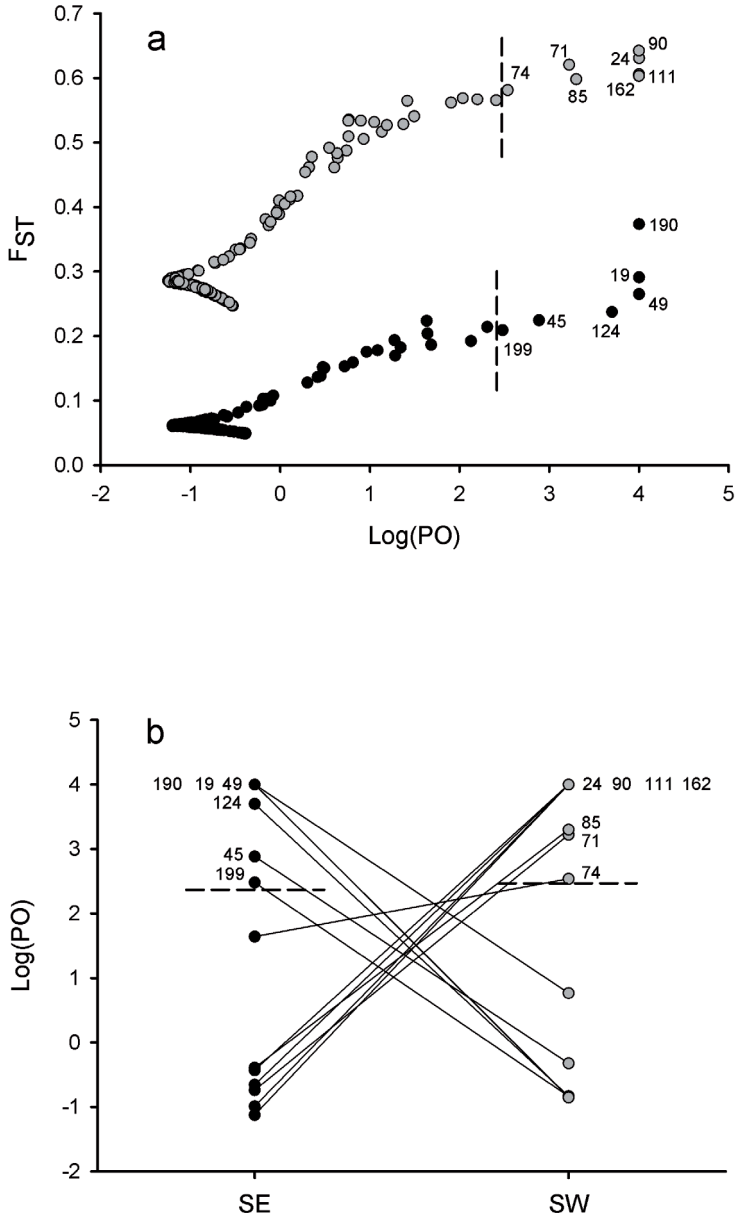

Figure 2. Detection of selection using a Bayesian genomic scan approach in SE and SW populations of *Daphne laureola* (black and grey dots respectively). Each dot corresponds to an AFLP marker (N = 225). Dashed lines indicate the threshold value of the log (PO) beyond which there is evidence for a locus to be subject to purifying selection at a False Discovery Rate of 0.001. Note that a threshold value was calculated independently for SE and SW populations. **(a)** Plot of the estimated FST values against the (log PO) for each locus with labels of the outlier loci. **(b)** Comparison of loci-specific patterns of purifying selection (log PO) in SE and SW populations. Solid lines join values of log (PO) of the same locus in SE and SW populations. Only loci under purifying selection in at least one group of studied populations were represented.

Bayesian analysis of putatively neutral genetic variation (i.e. after removing selected loci from the data), which did not rely on predetermined assignment of samples to regions or populations, showed a distinct modal maximum of ΔK at K = 2 genetic groups. Note that SW populations formed a genetic group differentiated from the rest of populations with nearly all populations being assigned exclusively to the SW genetic group (Fig. 3).

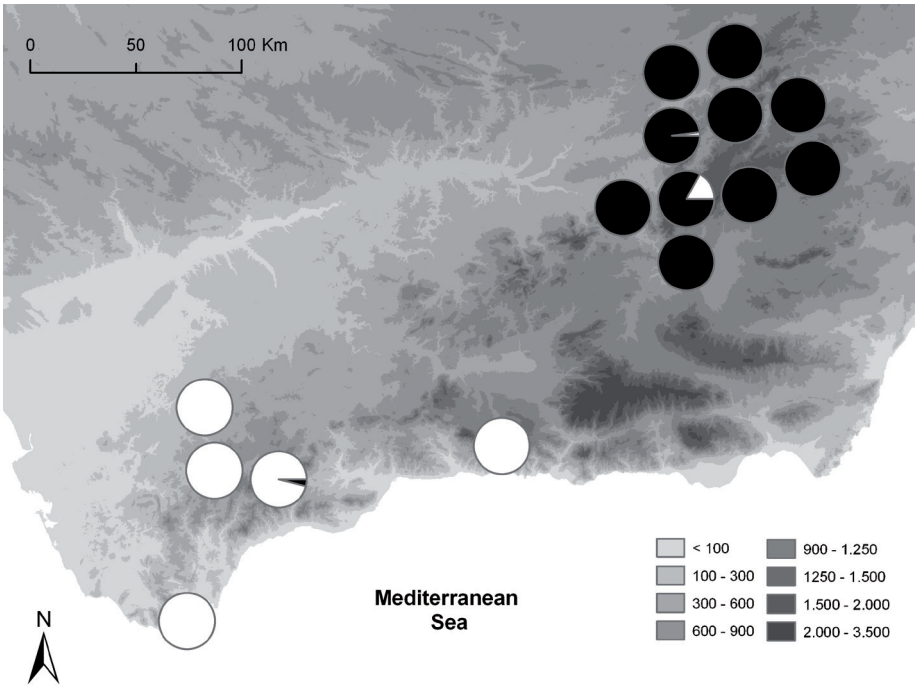

Figure 3. Genetic population structure of *Daphne laureola* in the Baetic Ranges inferred with the model-based Bayesian clustering implemented in STRUCTURE from data on putatively neutral loci alone. The individual pie charts indicate the mean proportion of membership of each local population for the inferred number of K = 2 genetic groups.

Differential Selection on Sexes

Very few loci were subject to purifying selection in female plants. In SE populations, hermaphrodite individuals presented three loci under purifying selection in comparison to only one in females (**Fig. 4 a-c**). The difference between sexes was even sharper in SW populations, where hermaphrodite and female individuals exhibited seven and one only locus under purifying selection, respectively (**Fig. 4d-f**). The separate analyses by sex confirmed that the identity of the loci under selection on each sex was different in SE and SW populations (**Fig. 4**).

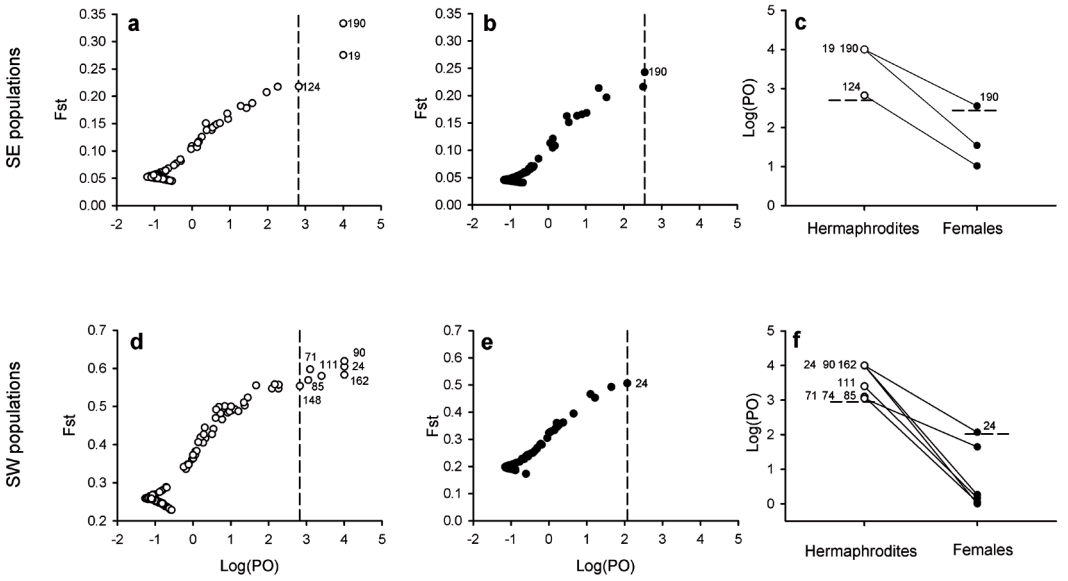

Figure 4. Sex-related selection patterns revealed by Bayesian genomic scan in the SE (upper row) and SW (lower row) populations of the gynodioecious shrub *Daphne laureola*. Within each row, from left to right, the first two graphs plot estimated FST of each locus against its corresponding value of log (PO) with labels of the loci outliers, separately for hermaphrodites **(a, d)** and females **(b, e)**, respectively. The third graph illustrates differences between sexes in the log (PO) values of loci found subject to purifying selection in at least one sex **(c, f)**. Solid lines join the values of log (PO) of the same locus in hermaphrodites and females. Each dot corresponds to an AFLP marker (N = 225; white and black dots, hermaphrodites and females, respectively). On each panel, dashed lines indicate the threshold value of the log (PO) beyond which there is evidence for a locus to be subject to purifying selection at a False Discovery Rate of 0.001. Independent threshold values were calculated for each dataset (SE hermaphrodites, SE females, SW hermaphrodites, SW females).

Discussion

Differential Selection Between Regions and Neutral Genetic Structure

Strong genetic differentiation among disjunct populations is a common feature in plant species of Mediterranean mountainous ranges (Schönswetter et al., 2006; Ronikier et al., 2008; Csergö et al., 2009). This genetic divergence at intraspecific level could be the result of non-selective mechanisms such as random fixation of different alleles in disjunct distribution patches because of founder events during colonization or genetic drift arising from reduction of population sizes during warmer stages in the Holocene (Young et al., 1996; Thompson, 2005). Contrasting selective regimes in disjunct parts of the species distribution range can also lead to strong genetic differentiation among disjunct distribution patches (Kawecki, 2008; Sexton et al., 2009). Divergent selective regimes in disjunct groups of populations because of variation in abiotic and/or biotic factors have

been documented in some plant species (Allan & Pannell, 2009; Hereford & Winn, 2008; Parisod & Joost, 2010). Populations of *D. laureola* in southern Iberian Peninsula present a strong genetic differentiation, exhibiting a clear split between SW and SE populations (Alonso & Herrera, 2011; Castilla et al., unpublished data). Results of the present study suggest that both non-selective and selective mechanisms have most likely contributed to the strong genetic divergence of *D. laureola* populations across the Baetic Ranges. In particular, we found that SE and SW populations of *D. laureola* present a similar number of loci under purifying selection, which may tentatively be interpreted as an indication of equivalent levels of selective pressure. This result contradicts to the expectation of marginal populations representing areas with stronger selective pressures than core populations (Kawecki, 2008; Sexton et al., 2009). However, the non-overlapping identity of the loci under selection in both groups of populations suggests different selective regimes in SE and SW populations. Reduced floral display sizes and increased relative fruit set associated to a change in the pollinator fauna has been reported in SW populations (Castilla et al., 2011). Studies of phenotypic selection by pollinators on floral traits in these two disjunct groups of populations are necessary to understand the role of geographic variation in plant-pollinator interactions on the intraspecific differentiation of *D. laureola* populations.

Our results also supported a role of non-selective mechanisms in contributing to the strong genetic differentiation of the *D. laureola* across the Baetic Ranges. The clear genetic split between SW and SE populations of species previously reported (Alonso & Herrera, 2011; Castilla et al., unpubl. data) remained after removing all the loci under purifying selection. Range shifts associated with climatic oscillations during the Quaternary have led to random fixation of different alleles in disjunct groups of populations contributing to genetic intraspecific differentiation of plant species (Bittkau & Comes, 2005; Schönswetter et al., 2006). In this sense, Baetic Ranges emerge as a mountainous range less affected by the glaciations during the Quaternary than more northern mountain systems (Benett et al., 1991; Médail & Diadema, 2009) and thus the strong genetic divergence of *D. laureola* populations could reflect a long-term history of genetic isolation between the two currently geographically disjunct groups of populations here studied.

Divergent Selection on Sexes

Studies of sex-specific selection in plants under natural conditions are scarce (Charlesworth et al., 2010). However, increasing support to differential selection on individuals of different sex under experimental conditions has been found in several gynodioecious species (Ashman, 1999; Ashman, 2003; Sakai et al., 2007; but see also Culley et al., 2006; Caruso & Yakobowski, 2008). Our results supported differential selection on individuals with different sex in natural gynodioecious populations of *D. laureola*. A larger number of loci were under purifying selection in hermaphrodites than in females in both SE and SW populations. Determining the relationships of those loci with phenotypic traits and the potential selective forces behind these patterns is challenging. Interestingly, the difference between hermaphrodites and females in the selective regimes was greater in populations located on the SW margin of the study area, where female frequency is lower and a change in the pollinator fauna has been documented (Castilla et al., 2011). Thus, selective pressures arising from plant-pollinator interactions emerge as plausible driving forces behind the observed non-neutral genetic divergence among sexes and regions in *D. laureola*. A relevant role of pollinators in determining the extent of sexual dimorphism in floral traits of gynodioecious species has been documented (Ashman, 2000; Case & Ashman, 2009). In our study system, changes in the composition of pollinators could lead to differential selective pressures on hermaphrodite and female individuals through, e.g., variation in selfing levels of hermaphrodites (Ashman, 2006), eventually leading to increased sex-differential reproductive success under strong inbreeding depression (Medrano et al., 2005). Nevertheless, relevance of other selective pressures cannot be ruled out because D. laureola females present larger concentration of specific chemical defences than hermaphrodites (Alonso et al., 2005) suggesting that environment and herbivores could both contribute to sex-specific selective pressures.

Identifying loci under sex-differential selection represents a first step to disentangle how natural selection can contribute to maintenance of sexual polymorphism in plant species. In this sense, the population genomics approach emerges as a powerful tool to detect sex-differential selection in the wild, taking into account all the selective factors that act in natural populations and not

only a few potential selective factors artificially manipulated. In gynodioecious species, increased selection favouring male function in hermaphrodites is expected once females spread within populations. Our results supported more intense selection in hermaphrodite than in female individuals. Moreover, the contrast between sexes in the magnitude of natural selection signatures differed between two regions with different pollinator fauna. This variation in sex-specific patterns of selection across regions differing in the pollinator fauna suggest that contrasting selection patterns by pollinators may cause geographical variation in the extent of dimorphism in floral traits of sexually dimorphic plant species.

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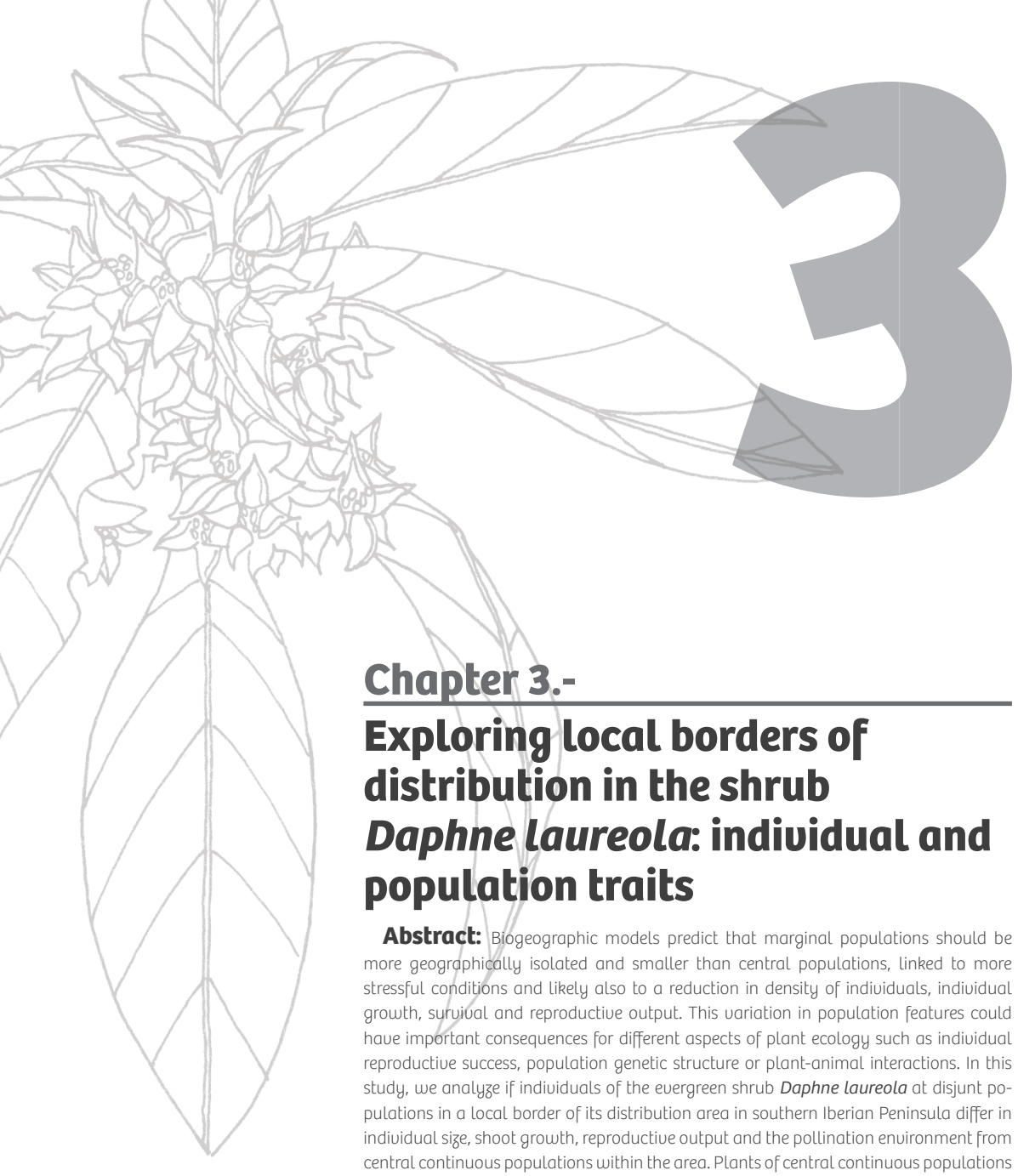

Chapter 3.-

Exploring local borders of distribution in the shrub *Daphne laureola*: individual and population traits

Abstract: Biogeographic models predict that marginal populations should be more geographically isolated and smaller than central populations, linked to more stressful conditions and likely also to a reduction in density of individuals, individual growth, survival and reproductive output. This variation in population features could have important consequences for different aspects of plant ecology such as individual reproductive success, population genetic structure or plant-animal interactions. In this study, we analyze if individuals of the evergreen shrub *Daphne laureola* at disjunt populations in a local border of its distribution area in southern Iberian Peninsula differ in individual size, shoot growth, reproductive output and the pollination environment from central continuous populations within the area. Plants of central continuous populations were larger and produced more flowers and fruits than plants of marginal disjunct populations suggesting more optimal conditions, although they had lower annual shoot growth. In contrast, fruit set was higher in plants at the local border, suggesting a more efficient pollinator service in these populations where the main pollinator in central continuous populations, the pollen beetle *Meligethes elongatus*, was not present. Our results do not support strong differences in the ecological stress between marginal disjunct and central continuous populations of *D. laureola* in the south of the Iberian Peninsula but indicate some changes in plant-pollinator interactions that could be relevant for the sexual polymorphism in this gynodioecious species.

Castilla, A.R., Alonso, C. and Herrera, C.M. 2011. Exploring local borders of distribution in the shrub Daphne laureola. Acta Oecologica 37: 269-276

Resúmen

Los modelos biogeográficos predicen que las poblaciones marginales de las especies vegetales son más pequeñas y están más aisladas geográficamente que las poblaciones centrales debido a que están expuestas a condiciones más estresantes que limitan la densidad de individuos, el crecimiento, la supervivencia y el éxito reproductivo de los individuos. Esta variación en los rasgos poblacionales podrían tener consecuencias importantes en distintos aspectos de la ecología de la especie como son el éxito reproductivo individual, la estructura genética poblacional o las interacciones planta-animal. En este estudio, analizamos si los individuos de poblaciones disjuntas marginales y centrales continuas del arbusto perenne ***Daphne laureola*** en el sur de la Península Ibérica se diferencian en el tamaño y crecimiento de los individuos, su éxito reproductivo y ambiente de polinización. Las plantas de las poblaciones centrales continuas presentaron un mayor tamaño produjeron más flores y frutos que las plantas de las poblaciones marginales disjuntas sugiriendo condiciones ecológicamente más óptimas en el área central, aunque hay que señalar que el crecimiento anual de los individuos fue menor. En cambio, la proporción de flores que dieron frutos ("fruit set") fue mayor en las plantas de las poblaciones marginales, sugiriendo un servicio de polinización más eficiente en estas poblaciones donde ***Meligethes elongatus***, el principal polinizador de la especie en las poblaciones centrales continuas, no está presente. Nuestros resultados no apoyaron por tanto fuertes diferencias de estrés ecológico entre poblaciones centrales continuas y marginales disjuntas de ***D. laureola*** en el sur de la Península Ibérica, pero indican ciertos cambios en las interacciones planta-polinizador que podrían tener una especial relevancia para el polimorfismo sexual de esta especie ginodioica.

Introduction

Distinguishing the factors that determine the limits in the distribution of species has been one of the most important issues both in ecology (e.g. Caughley et al., 1988; Lawton et al., 1994; Brown and Lomolino, 1998) and evolutionary biology (Jablonski, 1987; Sexton et al. 2009). According to niche-based conception of geographical range limits, the spatial variation in the biotic and abiotic factors that define a species' ecological niche determine where the species does or does not occur (Samis and Eckert, 2009). Therefore, along any ecological gradient, a species will be most abundant where the conditions allow maximum survival and reproduction and will be progressively less abundant away from this point until a geographic limit is reached (Hengeveld and Haeck, 1982; Brown, 1984; Lawton, 1993). However, several studies have shown that some species do no present lower fitness in marginal populations (e.g. Jump and Woodward, 2003; Kluth and Bruelheide, 2005; Samis and Eckert, 2007) suggesting that marginal populations may be locally adapted to sub-optimal conditions at the range edge (Caughley et al. 1988; Barton, 2001) and other mechanisms such as dispersal limitation or unstable metapopulation dynamics, rather than departure from ecological optimum, could be also important in limiting geographic distributions of some species (Kawecki and Holt, 2002; Holt, 2003).

Evolutionarily stable range limits are established when marginal populations cannot adapt to conditions beyond the limit because genetic variance is too low to allow adaptation to local conditions on the margin (Kawecki, 2008) or gene flow from central populations recurrently introduces genes adapted to ecological optimum in the center of range and therefore it prevents local adaptation on the distribution borders ("gene swamping"; García-Ramos and Kirkpatrick, 1997; Alleaume-Benharira et al., 2006). Otherwise, genetic drift in combination with responses to local selection pressures could promote genetic divergence, non-reduced fitness, and rapid evolution in marginal populations (Lesica and Allendorf, 1995; Alleaume-Benharira et al., 2006).

Plant-pollinator interactions are an example of selection pressures expected to vary geographically following spatial changes in the abundance and identity of the floral visitors (Moeller, 2005, 2006; Thompson, 2005). In general, small and/or isolated populations attract fewer and less diverse pollinators (Groom, 1998; Sih and Baltus, 1987). Also, marginal populations have been found to have poorer pollinator faunas than central populations (Silva-Montellano and Eguiarte, 2003; Stone and Jenkins, 2008). Pollination regimes differing in composition and abundance of pollinators can in turn lead to differential reproductive success (Herrera et al., 2006; Pérez-Barrales et al., 2007), and in the specific context of the comparison between central and marginal populations, could lead to a higher reproductive success in central populations.

In the present study, we compared central continuous and marginal disjunct populations of *D. laureola* within its distribution range in the south of the Iberian Peninsula. We used a broad array of population features and plant fitness correlates, in addition to consideration of the main pollinator identity and abundance of co-flowering species. Daphne laureola is an evergreen shrub with a wide distribution range within Europe, growing in the understory of mountain forests and presenting distribution patches on the main calcareous mountain ranges of southern Europe and north of Africa (Meusel et al., 1978). In our study area, most populations are gynodioecious with hermaphrodite and female plants coexisting within populations (Alonso et al., 2007). The main pollinator of *D. laureola* in the central area is a small pollen beetle, *Meligethes elongatus Rosenhauer* (Alonso, 2004), but its role as pollinator in marginal populations was uncertain. We analyzed population features such as population size, density of reproductive and non-reproductive individuals within population, identity of main pollinators and alternative floral resources for the pollinators, and individual life-history traits including plant size, shoot growth, flower and fruit production, fruit-set, and mortality. We further studied differences between females and hermaphrodites in size and reproductive traits presuming the existence of more stressful conditions in marginal populations that would allow to test the "sex-differential plasticity hypothesis" (Case and Ashman, 2007; Dorken and Mitchard, 2008). A considerable number of studies support that females are more abundant under stressful conditions because by investing in one only sex function, females

have more available resources for other functions thus increasing their relative fitness compared to hermaphrodites (reviewed in Ashman, 2006). However, according to the sex-differential plasticity hypothesis (Dorken and Mitchard, 2008), if hermaphrodites are plastic in their investment in female reproduction, they will allocate fewer resources to seed production under stressful conditions counteracting the females' advantages. Specifically, we addressed the following questions: (1) do population size and density of individuals differ between central continuous and marginal disjunct populations? (2) do plants in central continuous and marginal disjunct populations differ in size, growth, flower and fruit production? (3) do the identity of main pollinator and the abundance and diversity of other co-flowering species vary in central continuous and marginal disjunct populations? and (4) do hermaphrodite plants present a lower seed output than females in the marginal disjunct populations?

Material and methods

Study Species

D. laureola L. (Thymelaeaceae) is an evergreen shrub with a disjunct Palaearctic distribution associated with the main calcareous mountain ranges of Europe, and north of Africa. In the Iberian Peninsula it shows a disjunct distribution, being abundant in the northern Cantabrian Range and the Pyrenees, and also in the southern Baetic Ranges (Alonso et al., 2007; Fig. 1a). This study was conducted in 2007-2009 in the Baetic Ranges.

Plants of *D. laureola* present a variable number of branches that elongate over several consecutive growing seasons without any secondary branching. Leaves occur only at the distal end of branches, where they form a well-defined rosette, termed "leaf whorl" hereafter. It has a broad winter-flowering period (January-April) and produces a large number of small, tubular, green-yellowish flowers aggregated into several compact axillary inflorescences per stem. *D. laureola* presents gynodioecious populations where female and hermaphrodite individuals coexist, and populations having exclusively hermaphrodite plants, the last ones being rare in our study area (Alonso et al., 2007). Fructification begins in June, presenting one only seed per fruit.

The main pollinator of *D. laureola* in populations of the Sierra de Cazorla, which constitute the center of abundance of the species within the studied southern Iberian region (Fig. 1b), is *M. elongatus Rosenhauer*, a small pollen beetle that moves frequently among flowers of the same individual (Alonso, 2004).

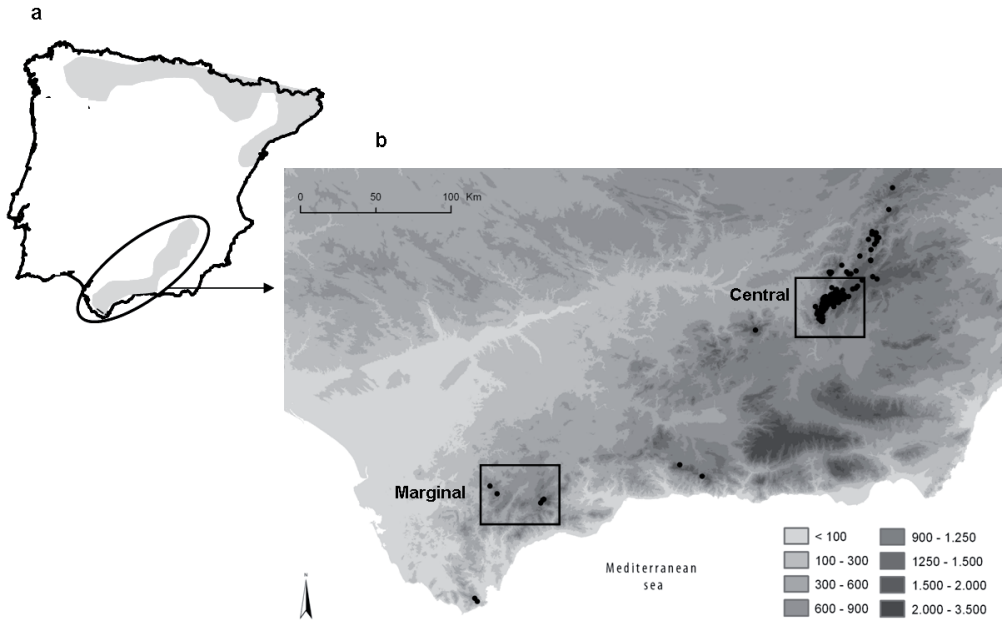

Figure 1. (a) Distribution range of *Daphne laureola* in the Iberian Peninsula. **(b)**More detailed distribution of populations in the study area and location of central and marginal regions defined in this study. Intervals of altitude (m.a.s.l.) in the study area are represented with grey scale (see legend).

Study area

Across the Baetic Ranges, *D. laureola* is especially abundant in the Sierra de Cazorla, with at least 88 populations highly connected among them located along a broad altitudinal range. In fact, considering population as a discrete group of individuals distant from other groups of individuals by at least 1 km, most populations of Cazorla have > 60 neighbouring populations in a radius of 25 km (Castilla et al., 2011). The geographic isolation among population increases towards eastern and western regions of the Baetic Ranges, being isolation more marked in the western populations here analysed (Castilla et al., 2011). In particular, western populations of *D. laureola* have on average < 3 neighbouring populations in a radius of 25 km. Therefore, in this study we

considered the Sierra de Cazorla as the local continuous center ("central region", hereafter) and populations at the western edge as a local disjunct margin ("marginal region", hereafter) of the species' distribution patch in southern Iberian Peninsula (Fig. 1b).

We studied three accessible populations per region. In the central region we used Valdecuevas, Cañada del Espino and Fuente Bermejo, the distance among them being 2-5 km. In the marginal region we used two different mountain ranges. Two populations (Fuente Molina and Cañada de las Animas) were located in the Sierra de las Nieves, with a distance between them < 3 km. The third marginal population was located in the Sierra de Grazalema, distant from the other two populations about 35 km. All study populations were gynodioecious, and the proportion of female and hermaphrodite individuals determined during the flowering period of 2007 by examining 100 randomly chosen individuals was ca. 20 % in all of them (Table 1). At the beginning of the study we haphazardly marked 25 hermaphrodite and 15 female plants in each study population, to avoid potential artefacts due to uncontrolled variation on sample sex ratio.

Population Features: Size, Plant Density and Mortality of Individuals

Populations were classed into one of the following size categories: <500, 500-1000, 1000-1500, 1500-2000, >2000 reproductive individuals. Densities of reproductive and non-reproductive individuals within each population were estimated using 20 randomly located circular areas of 10 m diameter in which the number of reproductive and non-reproductive individuals were counted. In 2009 summer, the mortality of marked individuals was recorded.

Individual Features: Size, Annual Shoot Growth, Floral Display and Seed Production

Four measures related to individual size were obtained for every marked plant: height of the tallest branch ("plant height" hereafter), total number of leaf whorls ("number of leaf whorls" hereafter) and the major and the minor diameter of the plant vertical projection, used to estimate the area of the plant assimilating it to an ellipse ("plant area" hereafter).

Three measures related to floral display were also obtained for every marked plant: proportion of stems bearing flowers; number of inflorescences/stem (based on counts of five stems per plant) and number of flowers/inflorescence (counted in one inflorescence per sampled stem). Total flower production per plant was then estimated as the number of flowering stems x average number of inflorescences/stem x average number of flowers/inflorescence. The annual shoot growth was estimated by measuring the shoot elongation at the end of the growing season in the five stems per plant used for the floral display ("shoot growth" hereafter). In addition, the total number of fruits produced per individual was counted ("fruit production" hereafter) and the proportion of fruits per flower ("fruit set" hereafter) was estimated using the five marked inflorescences.

Main Pollinator and Alternative Floral Resources

Previous studies showed that the pollen beetle *M. elongatus (Nitidulidae)* is the main pollinator of *D. laureola* in Sierra de Cazorla, determining important aspects of species' reproductive biology (Alonso, 2004; Medrano et al., 2005). However, occasional observations on *D. laureola* flowers during the flowering period of 2007 (A.R.C. personal observation) indicated that pollen beetles were apparently absent from marginal populations, where several species of *Hymenoptera*, mainly *Bombus terrestris L, Xylocopa violacea L.* and *Apis mellifera L.*, and another beetle *Tachyporus nitidulus Fabricius (Staphylinidae)* were observed (**Figure 2**). Therefore, in 2009 censuses were conducted to determine the abundance of *M. elongatus* in marginal populations of *D. laureola*. The censuses were done in sunny days. In each census, the observer was close to the plant and counted the number of *M. elongatus* individuals systematically by examining all the flowering stems of the plant during a maximum interval of three minutes. The number of flowering stems, the time spent and whether the plant was in sun or shade during the observation were recorded. Forty individuals per population were censused in all cases. In marginal populations, censuses were conducted in three different dates covering all the flowering period (early, peak and late flowering) to exclude the possibility of a mismatch between the presence of *M. elongatus* and the *D. laureola's* flowering peak. For comparison within the same season, plants in central populations were similarly censused although only in a single date at flowering peak. We censused the same

number of individuals in marginal and central populations, investing 168 minutes in marginal populations during the flowering peak and altogether 539 and 304 minutes in marginal and central populations, respectively.

In addition, the abundance of other flowering species which could potentially share pollinators with *D. laureola* was estimated by counting the number of flowering individuals of each species within five meters around every censused plant.

Figure 2. Pollinator assemblage in marginal populations of *Daphne laureola* in southern Iberian Peninsula. **(a)** Bombus terrestris; **(b)** Xylocopa violacea; **(c)** Apis mellifera.

Data Analyses

All statistical analyses were performed using the SAS statistical package (SAS, Institute 2002). In order to compare marginal and central populations, tests considering the effects of region (central vs. marginal) and sex (female vs. hermaphrodite) as fixed factors and the effect of population as random, were conducted. Density of reproductive and non-reproductive individuals, plant area, plant height, number of leaf whorls, flower production, shoot growth, fruit production and fruit set were the response variables analysed separately. Fruit set was normally distributed and plant height was normalized after being log transformed. Both responses were analyzed by mixed models (Procedure MIXED). We analyzed all the other variables with generalized linear mixed models (Procedure GLIMMIX), using the negative binomial function. Differences among regions in the re-

lationship between flower production and individual size (plant area and number of leaf whorls per plant) were studied with an analysis of covariance (ANCOVA). Flower production was the response variable, plant area, and number of leaf whorls were the explanatory variables and region was the categorical effect. In addition, we explored potential trade-offs between reproduction and shoot growth with an analysis of covariance, distinguishing shoot growth as the response variable, flower and fruit production as the explanatory variables and region as the categorical effect. Finally we used Fisher's exact test to analyze differences in the frequency of dead individuals between central and marginal populations. Means are given ± 1 SE.

Results

Population Features: Size, Plant Density and Mortality of Individuals

All the populations studied had more than 500 reproductive individuals (**Table 1**). The two largest populations occurred in the central region, with > 2000 reproductive plants. The two smallest populations, with 500-1000 reproductive individuals were found one in the central region and the other in the marginal region.

Table 1. Population features of the six *Daphne laureola* populations studied.

Population	Coordinates	Region	Altitude (m.a.s.l.)	Size (No. reproductive individuals)	Female frequency (%)
Valdecuevas	37º 54' N 2º 52' W	Central	1575	>2000	25.5
Cañada del Espino	37º 55' N 2º 53' W	Central	1513	500-1000	20
Fuente Bermejo	37º 55' N 2º 50' W	Central	1380	>2000	21
Grazalema	36º 46' N 5º 25' W	Marginal	1229	1500-2000	10
Fuente Molina	36º 41' N 5º 1' W	Marginal	1380	1000-1500	20
Cañada de las Animas	36º 42' N 5º 1' W	Marginal	1333	500-1000	18.5

There was marginally significant variation among regions for plant density within population ($F_{1,106}$ = 3.86, P = 0.05). Marginal populations were denser than central populations (0.09 ± 0.01 and 0.06 ± 0.01 individuals / 100 m2 respectively). Density of reproductive individuals did not differ significantly between regions ($F_{1,118}$ = 2.48, P > 0.1; **Fig. 2**). However, density of non-reproductive individuals in marginal populations was larger than in central populations ($F_{1,118}$ = 13.84, P < 0.001; **Fig. 2**). The percentage of dead individuals after two years was also larger in marginal than in central populations (17 % and 2 % respectively; Fisher's exact test: P < 0.0001).

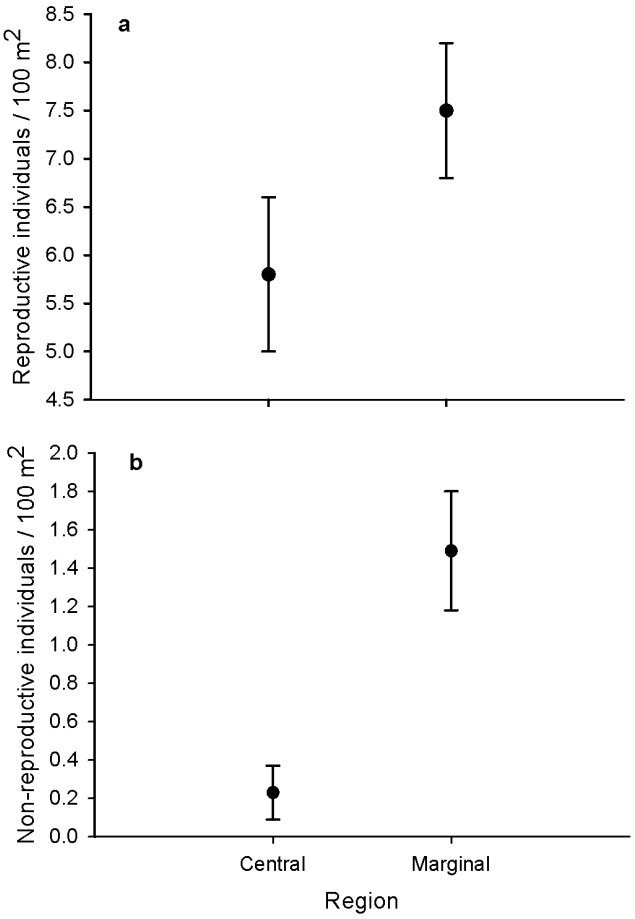

Figure 3. Regional differences in the density of reproductive (a) and non-reproductive individuals of *Daphne laureola* based on data of three populations per region and 20 plots per populations. In each plot, reproductive and non-reproductive individuals were counted in a circular area with a diameter of five meters. Bars represent the least-square means (± s.e.)

Individual Features: Plant Size, Shoot Growth, Floral Display and Fruit Production

Plants of central populations were larger than those growing in marginal populations. The average area of individuals in central populations was more than twice the area of plants in marginal populations ($F_{1,232}$ = 17.65, P < 0.0001; **Fig. 3a**). Also the number of leaf whorls was more than three times larger in central populations ($F_{1,232}$ = 16.92, P < 0.0001; **Fig. 3b**). Plants in marginal populations tended to be taller than those in central populations but their height averages did not differ significantly (84.72 ± 2.44 cm vs. 80.22 ± 2.28 cm; $F_{1,232}$ = 0.71, P = 0.40). Shoot growth was significantly larger in marginal populations (5.9 ± 0.2 cm vs. 3.9 ± 0.1 cm; $F_{1,229}$ = 24.07, P < 0.0001). Also, shoot growth was negatively related to flower and fruit production ($F_{1,235}$ = 36.21, P < 0.0001 and $F_{1,235}$ = 9.01, P < 0.01, respectively). The ANCOVA revealed that negative relationships between growth and flower and fruit production were consistent in the two study regions (P = 0.28 and P = 0.29 for the interaction between region and flower and fruit production, respectively). Furthermore, there were not significant differences between hermaphrodite and female plants in any size-related variable (P > 0.5 in all cases). Absence of differential size between sexes was consistent between the two study regions (P > 0.5 in the interaction region × sex for all size variables).

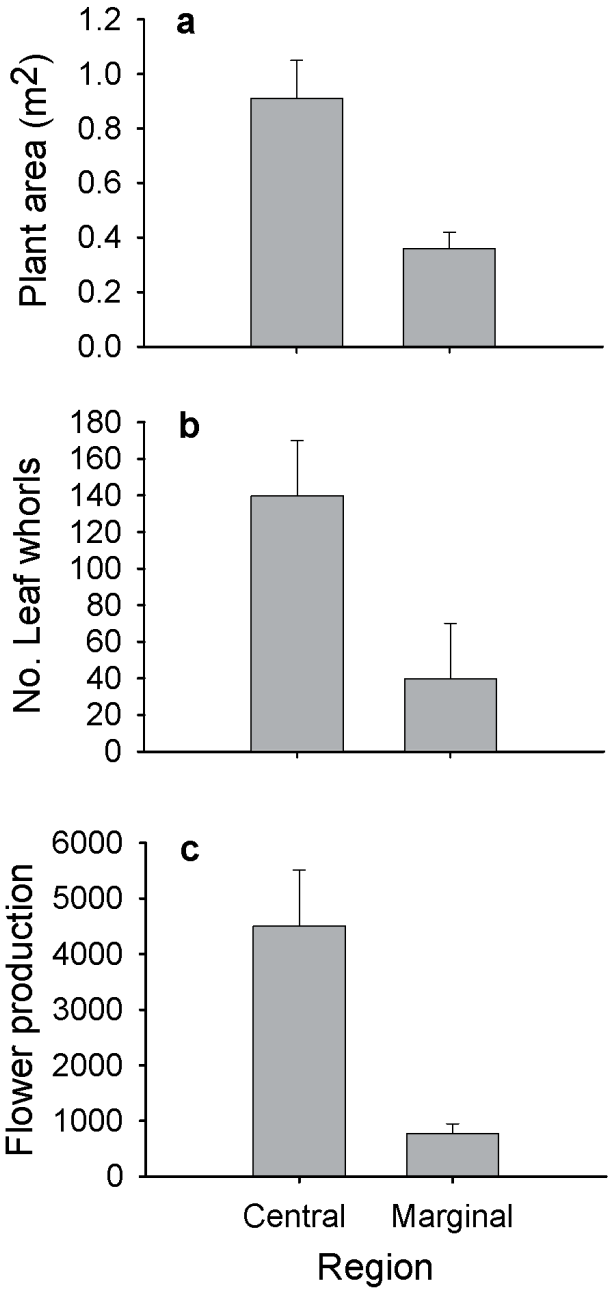

Figure 4. Differences in average plant area **(a)**, number of leaf whorls **(b)** and flower production per plant **(c)** in central and marginal populations of Daphne laureola, based on data of three populations per region and 40 individuals per population. Bars represent the least-square (+ s.e.) after accounting for sex (fixed effect) and population (random effect) variation.

Floral display was larger in plants of central populations (**Table 2**). They produced more reproductive stems/individual, more inflorescences/stem and more flowers/inflorescence, and consequently many more flowers than plants in marginal populations (**Table 2**). No significant differences were found between female and hermaphrodite individuals in any of these reproductive parameters ($P > 0.5$). Absence of differences in any of reproductive parameters between sexes was consistent between the two study regions ($P > 0.5$ in the interaction region × sex for all variables).

Table 2. Reproductive parameters analyzed (mean ± S.E.) in the marginal and central *Daphne laureola* populations studied.

Parameter	Central	Marginal	$F_{1,232}$	P
No. reproductive stems	97 ± 24	30 ± 7.4	11.14	< 0.01
No. inflorescences / stem	5 ± 0.3	4 ± 0.2	19.78	< 0.0001
No. flowers / inflorescence	9 ± 0.5	7 ± 0.4	10.18	< 0.01

Flower production was directly related to plant area in both central and marginal populations (R2 = 0.43, $F_{1,118} = 90.48$, $P < 0.0001$ and R2 = 0.28, $F_{1,118} = 46.87$, $P < 0.0001$, respectively). Nevertheless there was a regional difference in the slope of the relationship between these variables ($F_{1,236} = 6.44$, $P < 0.05$; **Fig. 4a**). Flower production was also positively related to number of leaf whorls in both regions (R2 = 0.56, $F_{1,118} = 142.46$, $P < 0.0001$ and R2 = 0.56, $F_{1,118} = 142.47$, $P < 0.0001$ in central and marginal populations, respectively) and there was a regional difference in the slope of the relationship between flower production and number of leaf whorls ($F_{1,236} = 40.61$, $P < 0.0001$; **Fig. 4b**). In both cases, the slope of the relationship between flower production and individual size variables was steeper in central populations.

Fruit production was greater in central populations ($F_{1,231} = 8.36$, $P < 0.01$; **Fig. 5a**). No effect of sex on fruit production was detected ($P > 0.1$). Absence of differential fruit production between sexes was consistent between the two study regions ($P > 0.5$ region × sex interaction). The proportion of

flowers setting fruit, however, was larger in marginal populations ($F_{1,231}$ = 5.13, P < 0.05; **Fig. 5b**). Fruit set was the only variable for which a significant difference was detected between plants of different sex, with hermaphrodite plants having a larger fruit set than females ($F_{1,231}$ = 7.33, P < 0.01; 0.46 ± 0.04 and 0.52 ± 0.03 fruits/flower in female and hermaphrodite plants respectively). This difference in fruit set between sexes was consistent in central and marginal populations (P > 0.5 region × sex interaction). Note that all sampled populations showed female frequencies ca. 20 % except one, the marginal population of Grazalema with 10 % females (**Table 1**). Therefore, the average fruit set of this population with more hermaphrodites was expected to be even larger, increasing the average fruit set of marginal populations and their difference with respect to central populations.

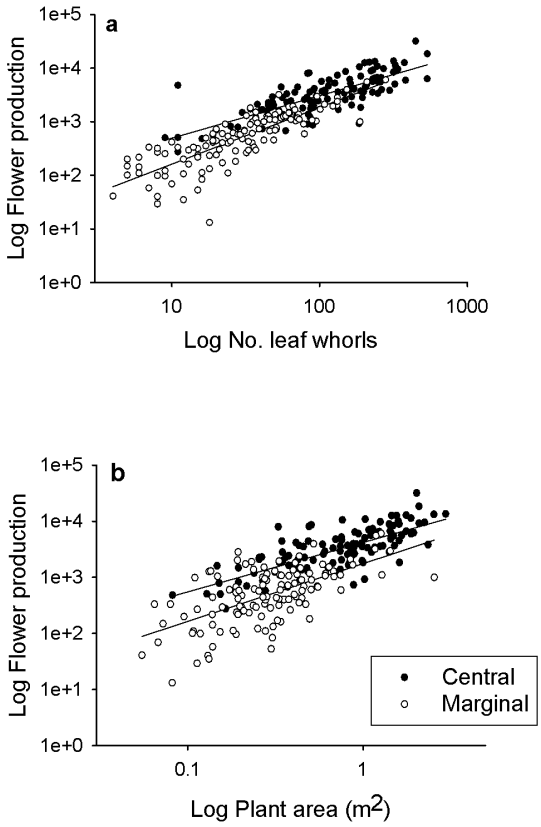

Figure 5. The total number of flowers per individual related to plant area **(a)** and number of leaf whorls **(b)**. Each circle corresponds to an individual. The axes were transformed to a logarithmic scale.

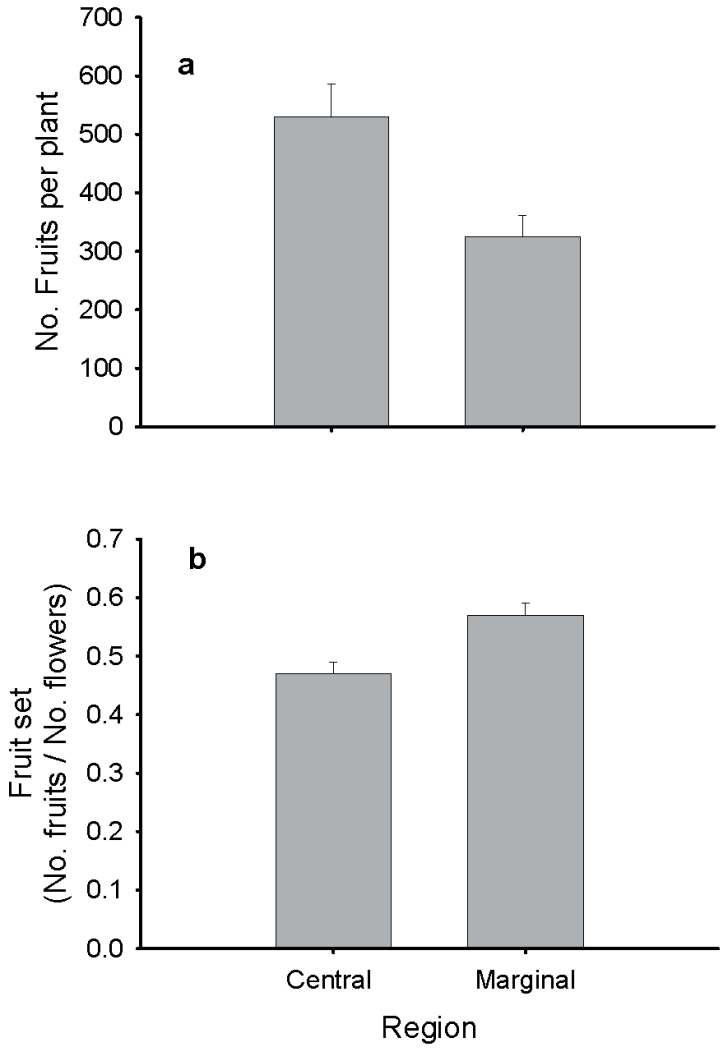

Figure 6. Regional differences in fruit production per plant **(a)** and fruit set **(b)**. Bars represent the least-square means (± s.e.) after accounting for sex (fixed effect) and population (random effect) variation, and are based on three populations per region and 40 individuals per population.

Pollination Environment

In central populations, *M. elongatus* was observed in all censuses and its average abundance at flowering peak in this region was 0.8 beetles / min equivalent to 2 individuals / plant. In contrast, this species was not observed in any of the censuses in marginal populations conducted at early, peak and late flowering (**Table 3**).

Table 3. Abundance of *Meligethes elongatus* in central and marginal populations of *Daphne laureola*. Censuses were conducted in three different dates at early, peak and late flowering in marginal populations and only at flowering peak in the central region.

Population	Meligethes elongatus (individuals/min)	Total time per population (min)
Center		
Cañada del Espino	1.6 ± 0.3	109
Fuente Bermejo	0.8 ± 0.2	95
Valdecuevas	0.1 ± 0.03	100
Marginal		
Fuente Molina	0	125
Cañada de las Animas	0	194
Grazalema	0	220

A marked regional difference was found in the number of local plant species co-flowering with *D. laureola* (Appendix 1). Nine herbaceous species were blooming in the three central populations combined: *Helleborus foetidus, Viola odorata, Lobularia maritima, Primula vulgaris, Narcissus cuatrecasasii, Bellis perennis, Ranunculus ficaria, Scilla reverchonii*. However, one central population (Cañada del Espino) presented a poor flowering community with only *Crocus nevadensis* flowering with *D. laureola* at low density (**Table 4**).

Table 4. Local plant species that bloomed simultaneously with *Daphne laureola* in central and marginal populations, available as alternative floral resources to pollinators.Regional means are presented in Center and Marginal rows.

Alternative floral resources (flowering individuals /100 m2)										
Population	**Helleborus foetidus**	**Viola odorata**	**Lobularia maritima**	**Primula vulgaris**	**Ranunculus ficaria**	**Narcissus cuatrecasasii**	**Scilla reverchonii**	**Bellis perennis**	**Crocus nevadensis**	**Total**
Center										
Cañada del Espino	0	0	0	0	0	0	0	0	0.1	**0.1**
Fuente Bermejo	2.5	1.7	3.4	0.3	0.1	0	0	0.5	0	**8.5**
Valdecuevas	5.8	6.6	0	0.6	0.2	0.1	0.5	0	0	**13.8**
Average	2.8	2.8	1.1	0.9	0.3	0.1	0.5	0.5	0.1	**9.1**
Marginal										
Grazalema	0.4	0	0	0	0	0	0	0	0	**0.4**
Fuente Molina	0.6	0	0	0	0	0	0	0	0	**0.6**
Cañada de las Animas	0.8	0	0	0	0	0	0	0	0	**0.8**
Average	0.6	0	0	0	0	0	0	0	0	**0.6**

In marginal populations, in contrast only *Helleborus foetidus* was flowering together with *D. laureola*, and its abundance was four times lower than in central populations (2.8 and 0.6 individuals / 100 m2 in central and marginal populations respectively). Thus, *D. laureola* was almost the only floral resource available for pollinators in marginal populations, while a broader array of species co-flowered with *D. laureola* in most central populations.

Discussion

Population Features: Size, Plant Density and Mortality of Individuals

Empirical evidence for the hypothesized reduction of population size towards limits of distribution is quite limited (Gaston, 2009). Ongoing studies with *D. laureola*, based on an intensive sampling of 111 populations, revealed a decrease of the frequency of populations and an increase of the geographic isolation of populations towards the geographic limits of the study area (Castilla et al., 2011); both trends were more marked on the western border, examined in this study. However differences in population size between central and marginal populations were not detected (Castilla et al., 2011). In the present study, we found that relatively large populations of *D. laureola* occurred indistinctly in central and marginal locations. In fact, two of the three marginal populations had more than 1000 reproductive individuals, supporting that high quality habitats do exist in both marginal and central locations although likely at different frequency.

A lower density of individuals associated with stressful conditions in marginal populations has been also hypothesized (Brown, 1984; Brussard, 1984; Lawton, 1993). Nevertheless, tests of this hypothesis have provided contradictory results, particularly in plants for which there are a reduced number of studies (Sagarin and Gaines, 2002; Sagarin et al., 2006). A decrease in the density of individuals in marginal populations has been found in only five out of 13 plant species for which this pattern has been analyzed (Carey et al., 1995; Jump and Woodward, 2003; Lönn and Prentice, 2002; Moore, 2009). In other cases, the density of individuals either did not vary between central and marginal populations (Herlihy and Eckert, 2005; Ribeiro and Fernandes, 2000; Samis and Eckert, 2007) or even was higher in marginal populations (Kluth and Bruelheide, 2005; Yakimowski and Eckert, 2007). In the present study, the density of reproductive individuals did not differ significantly between central and marginal populations of *D. laureola* suggesting the absence of strong difference in ecological stress between central and marginal populations. However, we found differences in the density of non-reproductive individuals (**Fig. 2**) and the frequency of dead individuals. Non-reproductive individuals were always smaller than reproductive ones, therefore suggesting they were younger plants. Assuming this, the combination of larger mortality of

adults and higher density of non-reproductive individuals in marginal populations would suggest a higher turnover in these populations. A higher demographic variability in marginal populations of plant species has been found by several studies (Lönn and Prentice, 2002; Moore, 2009; Nantel and Gagnon, 1999). However, more demographic analyses are required to determine if this is a common pattern in plants and to investigate the possible underlying factors.

Individual Features: Plant Size, Annual Growth, Floral Display and Reproductive Output

Adult plants of *D. laureola* vary widely in size and architecture through the Iberian Peninsula (Alonso et al., 2007). The present study found that in its southern distribution area, individuals of central populations were larger than those in marginal populations, which had as a direct consequence a huge variation in flower production. Plants in central populations produced one order of magnitude more flowers per individual than in marginal populations, because they consistently produced more reproductive stems, more inflorescences per stem and more flowers per inflorescence. These results suggested more optimal conditions for the species in the central locations, further supported by the steeper slope of the relationship between flower production and individual size in central populations. We must point out, however, that shoot growth was lower in central populations, likely showing a trade-off between reproduction and growth within the season with different outcomes in central and marginal populations (Obeso, 2002 and references therein; but see also Knops et al., 2007). Shoot growth was negatively related to flower and fruit productions. Plants of central populations produced more flowers and fruits than plants of marginal populations, which could have constrained more the shoot growth in individuals of central populations.

Ecological stress in distribution borders can also result in differential reproduction between central and marginal populations (Hengeveld and Haeck, 1982; Brown, 1984; Lawton, 1993). We found that *D. laureola* individuals of central populations produced more fruits than those on marginal populations, associated with the differences in size and flower production mentioned above. The connection between the number and density of adult individuals in populations and their re-

productive output was not, however, supported because central and marginal populations did not differ in size or density of adult plants (see also Samis and Eckert, 2007). The expectation that population size and density of individuals are mainly determined by individual seed production, however, could be unrealistic in some circumstances. Likely there are other factors constraining seedling establishment, such as abundance and quality of available sites for establishment and further postdispersal factors that affect seed survival, germination and seedling survival (Harper, 1977; Clark et al., 2007) that could be more determinant for population size and density of individuals.

Finally, according to the sex-differential plasticity hypothesis (Dorken and Mitchard, 2008), if the hermaphrodites are plastic in their investment on female reproduction they will allocate fewer resources to the seed production under more stressful conditions, and subsequently their relative female fitness should change. Our results, however, indicated that hermaphrodite individuals did not produce less fruits than female individuals in *D. laureola* central or marginal populations, suggesting either the absence of ecological stress in both study regions or a similar response of the two sexes.

Pollination Environment

Given the synchronic flowering of *D. laureola* individuals (Alonso, 2004), the much higher flower production recorded in central populations may have consequences for the interaction with pollinators, since floral display can influence both the attraction and the behaviour of the pollinators (Eckhart, 1991; Ashman and King, 2005). In addition, poorer pollinator faunas are expected in marginal populations (Moeller, 2006; Silva-Montellano and Eguiarte, 2003; Stone and Jenkins, 2008).

Previous studies conducted in the central region documented both high selfing rates in hermaphrodites and strong inbreeding depression from seedling to reproductive stage in *D. laureola* populations characterized by individuals with large floral displays (Medrano et al., 2005) and the pollen beetle *M. elongatus* as the main pollinator (Alonso, 2004). The reduction of individual size and floral display in marginal populations could therefore have some consequences for hermaphrodite

reproduction through seed production, associated with a decrease in geitonogamy (Klinkhamer and de Jong, 1993). The increased fruit-set recorded in marginal populations suggested that pollinator services might be improved in these sites. A possible explanation could be related to differences in the identity of the main pollinator in central and marginal populations. Pollinator censuses in 2009 confirmed that *M. elongatus* was the main pollinator in the central populations studied, detecting its presence in all populations, with an average of 2 beetles/plant. In contrast, M. elongatus was not found in any census at the marginal populations studied, despite considerably higher and phenologically wider sampling effort than in central populations. Additionally, several species of *Hymenoptera* as *Bombus terrestris, Xylocopa violacea and Apis mellifera* were observed visiting flowers of *D. laureola* at the marginal populations. Thus, the absence of a key pollinator that promotes geitonogamy as *M. elongatus* and the existence of other more dynamic visitors did not support the prediction of poorer pollinator faunas at marginal populations and suggested that the larger fruit set in marginal populations could be related to changes in the pollinator fauna potentially leading to reduced geitonogamy. A more exhaustive sampling effort is required to quantify the abundance of bees and bumblebees as pollinators of marginal populations of *D. laureola* and their consequences for realized mating system. Furthermore, the diversity of alternative floral resources for insects was higher in central populations, with up to six different co-flowering species in the same population. In this context, *D. laureola* flowers appear to be a low-valued resource for most day-flying floral visitors in central populations, where previous studies and anecdotal observations showed that bumblebees and bees only exceptionally visited *D. laureola* flowers and preferred visiting other coexisting flowering species (Alonso, 2004). In contrast, pollinators in marginal populations scarcely have alternative floral resources, mainly *Helleborus foetidus* in low abundance, which could drive them to visit a less attractive species such as *D. laureola*. This shift of pollinators, together with the increased fruit set in marginal populations despite a lower floral display, suggest a more effective pollination environment in marginal populations. Interestingly, one of the central populations presented a very poor flowering community, similar to marginal populations. Further studies in populations with poor winter co-flowering communities could be useful to test if individuals of *D. laureola* are visited more frequently by bees and bumblebees in them.

To summarize, marginal populations of *D. laureola* in the southern Iberian Peninsula did not differ significantly from central ones in size and density of individuals. In addition, the lower reproductive investment of marginal populations and their higher fruit set could be related to observed variation in plant-pollinator interactions, which could be relevant for the sexual polymorphism in this gynodioecious species if levels of geitonogamy were reduced in marginal populations. Differences in reproductive success between central and marginal populations of *D. laureola* seemed therefore more closely related to variation in biotic selective pressures than to changes in the species' abiotic niche. Additional work is currently underway focusing on the effects of changes in the pollinator community on floral traits and the genetic structure of marginal and central populations, which may inform about the importance of other mechanisms potentially involved in the delimitation of distribution ranges (e.g. limited gene flow, unstable metapopulation dynamics). These studies should contribute to a better understanding of the evolutionary potential of marginal disjunct populations.

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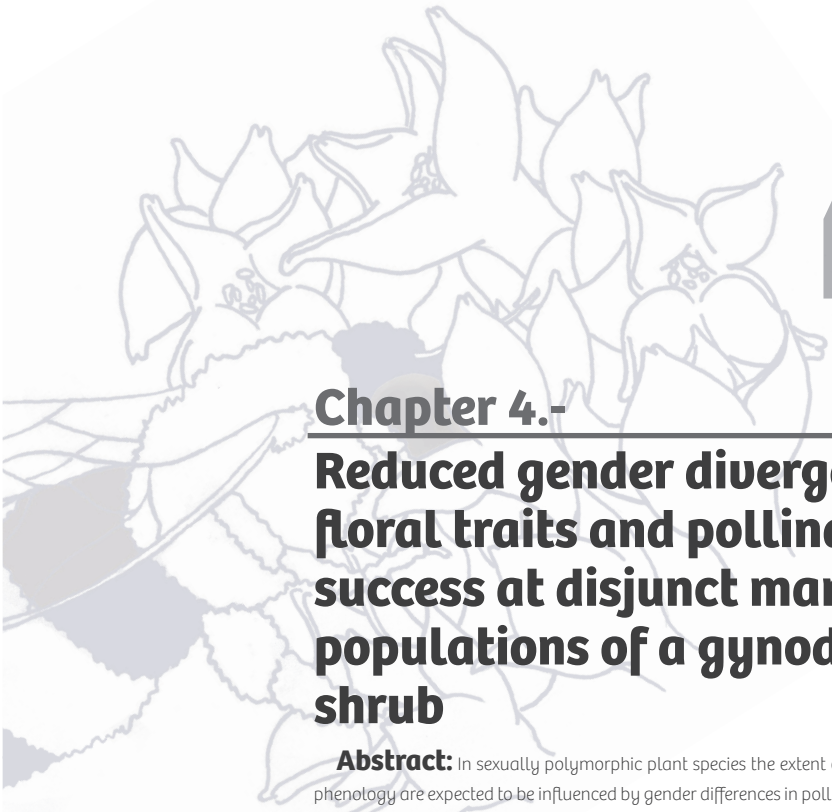

Chapter 4.-

Reduced gender divergence in floral traits and pollination success at disjunct marginal populations of a gynodioecious shrub

Abstract: In sexually polymorphic plant species the extent of gender divergence in floral morphology and phenology are expected to be influenced by gender differences in pollination success and phenotypic selection by pollinators. Margins of distribution constitute natural areas where changes in the pollinator fauna and thus variation in gender divergence of floral traits are expected. Here we indirectly assessed the effects of a change in the identity of main pollinator occurring in a disjunct group of marginal populations of the gynodioecious shrub *Daphne laureola* by analyzing gender relative pollination success and the gender-specific selection patterns of pollinators on floral traits and flowering phenology.

We found that pollinators deposited larger pollen loads on individuals of marginal populations, contrary to the expectation of less efficient pollinator service on distribution margins, and differences between genders in the frequency of visited flowers also vanished. Furthermore, hermaphrodites and females received similar quality pollen in marginal populations contrasting with core populations, where females received higher quality pollen than hermaphrodites. Pollinators selected longer flowers only on hermaphrodite individuals of core populations. In addition, hermaphrodites of core populations with longer flowers presented greater fruit sets and a marginally significant negative association with a locus under purifying selection. Consistent with these selection patterns, the gender divergence in the longitude of flower tube was greater in core populations. On the other hand, pollinators selected also towards late flowering on hermaphrodite individuals of marginal populations, being this phenological trait negatively related to another locus under purifying selection. However, a geographic variation in this phenological trait congruent with this phenotypic selection pattern was not observed.

Our results supported that geographic variation in the composition of the pollinator faunas could lead to changes in both qualitative and quantitative components of pollination success and to differences in the gender-specific selection patterns by pollinators. These changes can have a relevant role in the geographic variation of the gender divergence of floral traits. Therefore, the results of the present study supported that marginal disjunct populations. Reduced gender divergence in floral traits and pollination success at disjunct marginal populations of a gynodioecious shrub constitute natural areas where changes in the evolutionary dynamics of plantpollinator interactions can be expected, especially in sexually polymorphic plant species.

Castilla, A.R., Alonso, C. and Herrera, C.M. In review. Reduced gender divergence in floral traits and pollination success at disjunct marginal populations of a gynodioecious shrub.

Resumen

Las diferencias entre plantas de distinto sexo en el éxito de la polinización y en los patrones de selección fenotípica ejercidos por los polinizadores pueden determinar el grado de divergencia entre sexos en los rasgos florales y en la fenología de la floración en especies vegetales con sistemas sexuales polimórficos. Los márgenes de distribución constituyen áreas naturales donde se esperan cambios en la fauna de polinizadores y por tanto, donde se espera divergencia entre plantas de distinto sexo en los rasgos florales. En este estudio, nosotros hemos evaluado indirectamente el efecto del cambio en la identidad del polinizador principal en poblaciones marginales del arbusto ginodioico ***Daphne laureola***. Para ello, hemos analizado las diferencias entre plantas de distinto sexo en el éxito de polinización y su relación con los rasgos florales y la fenología de floración.

En este trabajo hemos encontrado que los polinizadores en las poblaciones marginales depositaron cargas polínicas mayores en los individuos, contrario a la expectativa de un servicio de polinización menos eficiente en los márgenes de distribución. Los polinizadores visitaron con similar frecuencia las flores de individuos hembras y hermafroditas. Los individuos hermafroditas y hembra recibieron polen de similar calidad en las poblaciones marginales en contraste con las poblaciones centrales, donde los individuos hembras recibieron polen de mejor calidad que los hermafroditas. Por otro lado, los polinizadores seleccionaron positivamente flores más largas sólo en los hermafroditas de las poblaciones centrales. Además, los hermafroditas de las poblaciones centrales con flores más largas también presentaron mayor proporción de flores produciendo frutos. La variación en la longitud del tubo floral estuvo relacionada negativamente, de manera marginal, con la presencia de un locus con signos de estar bajo selección purificadora. Consistente con estos patrones de selección, encontramos que la divergencia entre plantas de distinto sexo con respecto a la longitud del tubo floral fue mayor en las poblaciones centrales. En las poblaciones marginales, los polinizadores seleccionaron positivamente fenologías de floración más tempranas sólo en los individuos hermafroditas. Además la variación en este rasgo fenológico estuvo relacionada negativamente con otro locus con signos de selección purificadora. Sin embargo, la variación geográfica en este rasgo no fue congruente con el patrón de selección fenotípica observado.

Nuestros resultados apoyaron la idea de que los cambios en la composición de la fauna de polinizadores dan lugar a variación en los componentes cuantitativo y cualitativo del éxito de la polinización; y a diferencias en los patrones de selección ejercidos por los polinizadores entre plantas de distinto sexo. Estos cambios podrían tener una especial relevancia sobre la variación geográfica de la divergencia de los rasgos florales entre plantas de distinto sexo. Por tanto, los resultados de este estudio señalan las poblaciones marginales disjuntas de las especies vegetales como áreas naturales importantes para el estudio de los cambios en las dinámicas coevolutivas de las interacciones planta-animal, especialmente en plantas con sistemas polimórficos sexuales.

Introduction

Geographic variation in the composition of the pollinator fauna in plant populations may lead to important changes in the pollination success of individuals through variation in quantity and/or quality of pollen received (Herrera 1987; Herrera 2004; Gómez et al. 2010; Alonso et al. 2011). Variation in the pollination success may have a relevant role in the evolution of sexually polymorphic systems in plant species (Barrett 2002; Ashman 2006). For instance, divergence in pollination success between sex morphs in gynodioecious species can facilitate the spreading of female individuals through increased number and/or better quality of their offspring (Charlesworth & Charlesworth 1978). Many gynodioecious species present self-compatible hermaphrodites (Meagher 2007; Ehlers & Schierup 2008). As a consequence, higher quality seed production by females can result from the inability of that morph to self-fertilize, thereby limiting inbreeding in their offspring (e.g. Thompson & Tarayre 2000; Delph 2004; Chang 2007), that linked to strong inbreeding depression may contribute to female persistence within populations (Charlesworth & Charlesworth 1978; Medrano et al. 2005; Ramsey et al. 2006).

Habitat fragmentation simultaneously affects a variety of abiotic and biotic factors, which directly or indirectly can change the abundance and composition of pollinators (Steffan-Dewenter & Tscharntke 1999; Knight et al. 2005; González-Varo et al. 2009). In this sense, ecological margins in plant distributions constitute areas where populations are geographically more isolated and, thus, where shifts towards poorer pollinator fauna may be expected (Silva-Montellano & Eguiarte 2003; Stone & Jenkins 2008). Different pollinator faunas may exert different patterns of phenotypic selection on floral or phenological traits (Herrera et al. 2006; Harder & Johnson 2009). This differential selection could lead to intraspecific phenotypic differentiation and ultimately to speciation events (Johnson 2006). However, few studies have analyzed specifically the causal role of the spatially variable selection by pollinators on the geographical differentiation in floral traits of plant species (Herrera et al. 2006), despite most of them have found a relevant role of divergent selection by pollinators on intraspecific floral differentiation of plant species (e.g. Totland 2001;

Herrera et al. 2006; Gómez & Perfectti 2010). Clarification of the role of pollinators in the evolution of sexually polymorphic systems awaits studies that document if changes in pollinator fauna composition affect differently to individuals with different sex in their pollination success and their phenotypic traits (Ashman 2006).

Flowers of angiosperms with unisexual flowers frequently exhibit dimorphism in secondary sex characters (Eckhart 1999), including gender differences in flower size, flower shape, nectar production, flower longevity, floral scent or flowering phenology (Eckhart 1999; Asikainen & Mutikainen 2005; Collin & Shykoff 2010). In gynodioecious species, where hermaphrodite and female plants occur in populations, flower size is almost universally larger in hermaphrodites than females (Eckhart 1999; Shykoff et al. 2003; but see Delph & Lively 1992). Gender divergence has been also confirmed in flowering phenology of gynodieocious species. Female individuals frequently flower somewhat earlier than hermaphrodites (Uno 1982; Ashman & Stanton 1991; Asikainen & Mutikainen 2005). Both flower size and flowering phenology may be largely influenced by pollinator's selection patterns (Campbell 1989; Galen 1989; Johnston 1991; Conner 1996). Therefore, the levels of gender divergence in flower size and flowering phenology are expected to vary in populations with different pollinator faunas.

In this study, we analyzed the geographic variation in pollination success and gender divergence in floral morphology and flowering phenology of the long-lived gynodioecious shrub Daphne laureola. We compared core continuous and marginal disjunct populations in southern Iberian Peninsula which differ in the identity of their main pollinator (Alonso 2004; Chapter 3). The persistence and frequency of female individuals in core continuous populations is accounted for a combination of extensive selfing in hermaphrodites and strong inbreeding depression in their offspring (Medrano et al. 2005). In addition, hermaphrodites of *D. laureola* present longer flowers and start their flowering earlier than females in core populations (Alonso 2004; Alonso 2005), but the level of spatial variation of gender divergence has not been analysed in a broader geographic context. In addition, a previous work has shown the existence of different loci under purifying selection

between marginal disjunct and the remaining populations of southern Iberian Peninsula (Chapter 2). In the present study, we addressed specifically the following questions: (1) **is the pollination success in its quantitative and qualitative components lower in marginal disjunct populations of D. laureola in southern Iberian Peninsula?**, (2) **is the level of gender divergence in floral and phenological traits different in core and marginal disjunct populations?**, (3) **is there differential phenotypic selection on floral and phenological traits in continuous core and marginal disjunct populations of *D. laureola?***, (4) **do patterns of phenotypic selection by pollinators match to geographic variation in the level of gender divergence in floral traits?**, and (5) **are floral and phenological traits associated to loci under purifying selection?**

Material and Methods

Study Species

Daphne laureola L. (Thymelaeaceae) is an evergreen shrub with a disjunct Palaearctic distribution associated with the main calcareous mountainous ranges of Europe and northern Africa. In the Iberian Peninsula, it shows a disjunct distribution, being abundant in the northern Cantabrian Range and the Pyrenees and also in the southern Baetic Ranges (**Fig. 1a**; Alonso et al. 2007). This study was conducted in the Baetic Ranges (**Fig. 1b**), where the species flowers from January to April, a period characterized by low temperatures, frequent rains and irregular snowfalls. Each plant produces a large number of small, tubular, green-yellowish flowers aggregated into several compact inflorescences per stem. The two sexes are similar in size, flower and fruit production (Alonso & Herrera 2001). Individual flowers of both genders have a single ovule and remain open continuously during approximately one month. Flowers consist of a corolla tube (4-12 mm) with four lobes and bear eight stamens arranged in two whorls of four stamens each. Hermaphrodite plants are fully self-compatible, but fruit production requires flower visitation by pollinators and an excess of pollen may clog-up the stigma of hermaphrodites, reducing their fruit set (Alonso & Herrera 2001). The small beetle *Meligethes elongatus Rosenhauer* constitute the main pollinator in

continuous core populations (Alonso 2004), but it does not visit *D. laureola* flowers in the marginal disjunct populations studied here, where several bees and bumblebees species constitute the main floral visitors (Chapter 3). Black drupes ripen in June.

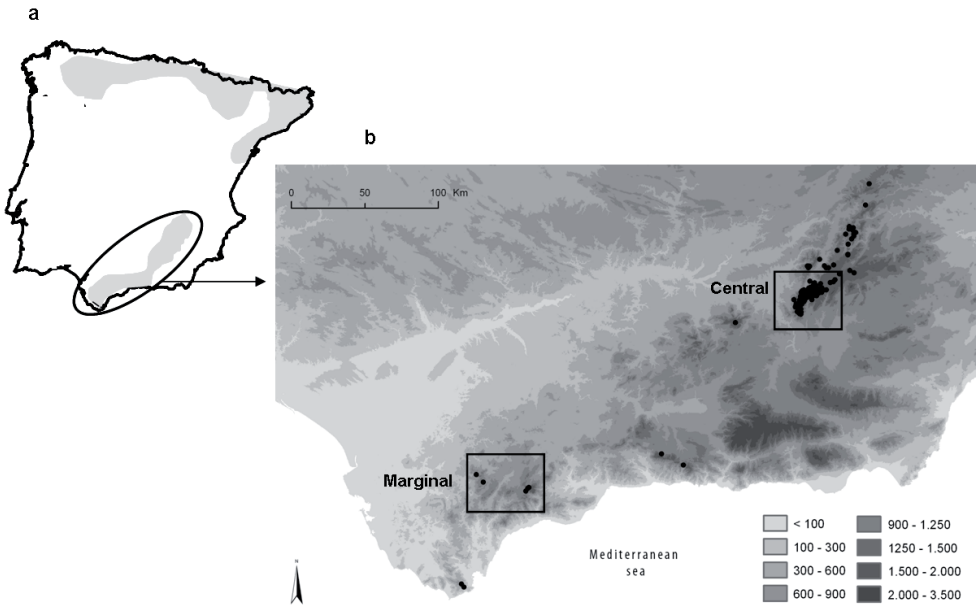

Figure 1. (a) Distribution range of *Daphne laureola* in the Iberian Peninsula. **(b)** More detailed distribution of populations in the study area and location of central and marginal regions defined in this study. Intervals of altitude (m.a.s.l.) in the study area are represented with grey scale (see legend).

Study Area

Across the Baetic Ranges, *D. laureola* is particularly abundant in the Sierra de Cazorla, where populations are largely connected among them and occupy a broad altitudinal range (**Fig. 1; Chapter 1**). The spatial isolation of populations increases towards western and eastern margins of the Baetic Ranges, being the variation in spatial isolation sharper in the western populations here analyzed (**Fig. 1; Chapter 1**). Therefore, in this study we considered the Sierra de Cazorla as the local continuous core ("core region" hereafter) and populations at the western edge as a local disjunct margin ("marginal region" hereafter) of the species' distribution patch in southern Iberian Peninsula. We studied three accessible populations per region (Chapter 3). All study populations

were gynodioecious, and the proportion of females was ca. 20 % in all of them (Chapter 3). At the beginning of the study, we haphazardly marked 25 hermaphrodite and 15 female plants in each study population.

Pollination Success

In 2007, 12 flowers per marked plant were collected in each population in the end of the flowering period (N = 2880 flowers). Flowers were preserved in FAA solution (2.5 % formaldehyde, 2.5 % acetic acid, 95 % ethanol) until their dissection and measurement. Styles of each flower were softened in 1 N KOH at 65 ºC for 20 min, rinsed with distilled water and stained for 20 min at 65 ºC in decolorized aniline blue. Pollen grains on the stigma and pollen tubes at the base of the style were counted under fluorescence microscope. We calculated the individual pollen load averaging the number of pollen grains on the stigma in its 12 collected flowers. Pollen load constitute an estimate of the amount of floral visits (Engel & Irwin 2003). In addition, we quantified the percentage of flowers with at least one pollen grain on their stigmas in each individual, which represents the proportion visited flowers. Differences between sexes and regions in average pollen load and percentage of visited flowers were analyzed by generalized linear mixed models (Procedure GLIMMIX, SAS Institute 2002) with lognormal error distribution for stigmatic pollen load and negative binomial error distribution for the percentage of visited flowers. Sex and region effects were treated as fixed. Population was treated as random. When replicated at this level, the effects due to differences between individual plants within populations were considered as random.

We used the method proposed by Alonso et al. (2011) to analyse the functional relationship of pollen grains to tubes based on piecewise aimed to distinguishing the quantitative and qualitative components of the pollination success (Qt and Ql, respectively). Piecewise regression is a regression technique that allows the inclusion of break points in linear regression when an abrupt change in the slope of the relationship is predicted and allows for the simultaneous evaluation of the location and the standard error of such a break point (Ryan & Porth 2007; Ficetola & Denoël 2009). The relationship between dependent and independent variables can thus be interpreted as

two linear regressions with different slopes (b1 and b2) and the point at which these slopes meet is the 'breakpoint' (c), that is, the point at which differences in the relationship occur. Specifically, the initial slope (b1) provides information about the quality of pollen received: a high b1 (steep rise) reflects receipt of high-quality pollen, and a low b1 (shallow rise) reflects the receipt of low-quality pollen. The value of c reflects the point at which the initial slope (b1) ceases to fit the data and the second regression (b2) becomes more appropriate. The width of the confidence interval around c reflects both the certainty of its location and the range of pollen load sizes for which Ql and Qt are contributing with similar importance to pollination success. The slope b2 reflects the degree to which, even after the quantitative effect is diminished (tends towards 0), pollen quality still plays a role (positive values), or that additional pollen has a detrimental effect on pollen tube recruitment (negative values). Piecewise models were fitted using the NLIN procedure and confidence intervals (BCa, hereafter) for all three parameter estimates (b1, b2 and c) in the piecewise regression were calculated using nonparametric bootstrapping (N = 1000). A chi-square test was used to test for differences in the relative frequencies of data points in the three regions defined by the confidence limits around c (I, $x <$ lower BCa; II, lower BCa $\leq x <$ Upper BCa; III, $x \geq$ Upper BCa). Differences in the relative frequencies of data points in the three regions indicate if the pollination success of a given sample is limited primarily by quantity (I), quality (III) or both (II).

We applied the method to female and hermaphrodite samples separately within each region (core and marginal). Comparisons between sexes with regard to the parameters of piecewise regressions were then conducted within each region. Following Alonso et al. (2011), before running the piecewise analyses the linearity of the relationship between pollen grains and tubes of visited flowers was tested using generalized additive models (GAMs) and a significant spline component indicated a better fit when the model includes a non-linear local component in all data sets (results not shown).

Floral Traits

For each one of the 12 collected flowers per individual were measured the longitude of flower tube and flower tube aperture (longitude of tube and aperture of tube, hereafter) under a dissecting microscope using an ocular micrometer. In addition, we measured the length between the tips of two opposite lobes and between the tips of the other two lobes. Outer corolla diameter was estimated as the mean of both values (outer diameter, hereafter).

Patterns in the phenological synchronization in flowering could be described at among-individual and within-individual levels (Bolmgren 1998; Munguía-Rosas et al. 2011). As adaptative advantages of among-individual synchronization could be confounded by within-individual patterns of synchronization, we need to investigate separately both levels of phenological synchronization (Bolmgren 1998). We counted the number of opened and closed flowers in five inflorescences per marked individual during flowering peak in 2007. Flowering time, a measure of among-individual flowering synchronization, was estimated as the mean percentage of opened flowers per inflorescence of each individual standardized to mean 0 and variance 1. In addition, flowering asynchrony within-individual was quantified through the variation coefficient of the number of opened flowers within each marked individual, standardized to mean 0 and variance 1.

Differences in morphological and phenological traits between sexes and regions were analyzed by generalized linear mixed models (Procedure GLIMMIX) with normal error distribution for morphology traits (longitude of tube, outer diameter and aperture of tube) and negative binomial error distribution for phenology traits (flowering time and flowering asynchrony). Sex and region effects were treated as fixed. Population was treated as random. When replicated at this level, the effects due to differences between individual plants within populations were considered as random.

Phenotypic Selection

Phenotypic selection on floral morphology and flowering phenology via its influence on pollination success and maternal component of fitness was assessed by fitting generalized linear

models for each sex within region. We used the mean number of pollen tubes and the mean fruit set (proportion of fruits per flower) as the response variables related to pollination success and maternal component of individual fitness, respectively. We used relative values of both response variables (estimated by dividing the mean values per individual by the population means; Lande & Arnold 1983). All floral and phenological traits were standardized to mean 0 and variance 1, and were used as independent variables. Among population variation in phenotypic selection on floral morphology was evaluated by testing the homogeneity of slopes of the relations of relative pollination success and maternal fitness to the measures of floral traits among populations, i.e. through the analyses of population x longitude of tube, population x outer diameter, population x aperture of tube, population x flowering time and population x flowering asynchrony interactions (e.g. Strauss et al. 2005; Herrera et al. 2006). We first ran the model with all of the interactions and then excluded the nonsignificant ones (Littell et al. 2006). For simplicity, we focused only on directional selection gradients and do not assess quadratic terms in the phenotypic selection model, as this approach facilitates interpretation of population x traits interactions. Restriction of the analyses to directional selection is also justified in the present context, because gender divergence in sexually polymorphic species is mainly maintained through directional selection.

Adaptative Genetic Basis of the Floral Traits

Amplified fragment length polymorphism (AFLP) markers were used to characterize our 240 study plants genetically. The AFLP analysis was performed essentially as originally described by Vos et al. (1995), with modifications involving the use of fluorescent dye-labelled selective primers following Applied Biosystems (2005). Each plant was fingerprinted using four EcoRI + 3/MseI + 3 and four PstI + 2/MseI + 3 primer combinations. Fragment separation and detection were made using an ABI PRISM 3130xl DNA sequencer, and the presence/absence of each marker in each individual plant was scored manually by visualizing electrophoregrams with GeneMapper 3.7 software. Only fragments ≥ 150 base pairs in size were considered, as a way of reducing the potential impact of size homoplasy (Vekemans et al. 2002). The AFLP data set used here is the same used in the Chapters 1 and 2, where details on primer combinations, number of markers, scoring error rates and levels of

polymorphism can be found. A side study revealed the existence of six loci under purifying selection in core populations (Loci 19, 45, 49, 124, 190 and 199) and seven loci under purifying selection in marginal western populations (Loci 24, 71, 74, 85, 90, 111 and 162) (Chapter 2).

We conducted analyses aimed to verify the hypothesis that floral and/or phenological traits under phenotypic selection may be associated to AFLP loci under purifying selection in *D. laureola* populations. Hermaphrodites and females of each region (core vs. marginal) were separately analyzed; altogether four data sets were analyzed (females_core, hermaphrodites_core, females_marginal, hermaphrodites_marginal). For each set, the analysis was limited to phenotypic traits and loci under selection. A priori, we cannot rule out the existence of variable loci/trait relationship among populations. To test for this possibility, we used generalized linear models including population as a fixed factor. Traits under phenotypic selection, population and their interactions were the independent variables. Loci under selection were the dependent variables, using binomial function as an error distribution. Variable trait/loci relationships among populations were ruled out because there was not any significant interaction trait x population for any loci under purifying selection (results not shown). Therefore, we analyzed the potential relationships between AFLP markers under purifying selection with traits under phenotypic selection using simple logistic regressions for each group (females_marginal, hermaphrodites_marginal, females_core, hermaphrodites_core). Band presence was used as the dependent binary variable and the traits under phenotypic selection as the continuous, independent variables.

Results

Quantity and Quality Components of Pollination Success

Hermaphrodite flowers were quantitatively more successful than females but the magnitude of divergence between the two sexes was highly different in the two regions (**Fig. 2**). Flowers of hermaphrodite individuals received larger mean pollen loads than females and the magnitude of the difference was higher in marginal populations ($F_{1,1865} = 16.14$, $P < 0.0001$ region x sex interaction; Figure 2a). Furthermore the difference between sexes in the mean percentage of visited flowers was widely different among regions ($F_{1,227} = 89.20$, $P < 0.0001$ region x sex interaction), because hermaphrodites had larger mean percentage of visited flowers than females only in core populations (**Fig. 2b**).

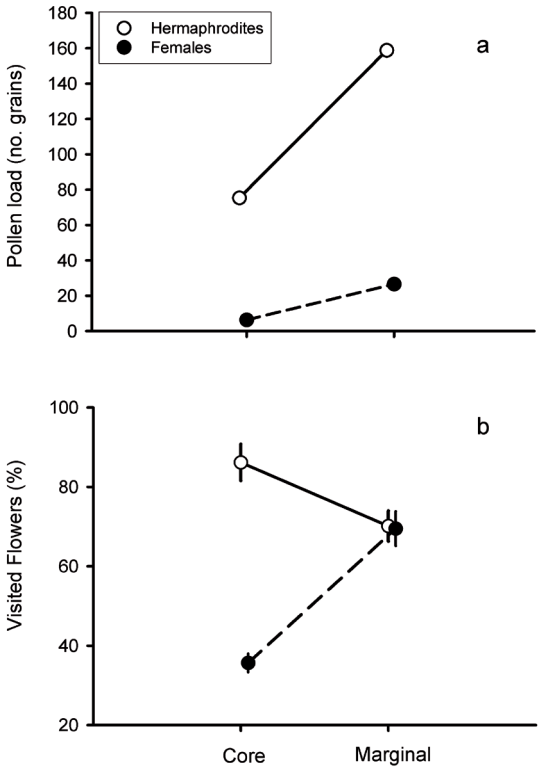

Figure 2. Variation in average pollination success (± SD/SE) between genders (hermaphrodites vs. females) and regions (core vs. marginal) of *Daphne laureola* in southern Iberian Peninsula. **(a)** (a) Pollen load refers to the average number of pollen grains deposited on the stigma. **(b)** Visited flowers refer to the percentage of flowers with at least one pollen grain recorded from a sample of 12 flowers per plant. In both panels N = X females and XX hermaphrodites per region.

Piecewise analyses further indicated that visited female flowers were more frequently limited by quantity than hermaphrodite flowers (22 = 114.39, P < 0.0001 and 22 = 1226.25, P < 0.0001, for core and marginal populations), although with some relevant differences between the two regions. In core populations quantitative limitation was more frequent in females but relatively common in the two sexes, with 64.6 and 36.8 % samples falling below the lower BCa. In marginal populations female flowers were similarly affected by quantity limitation (66.75 %) but the frequency of quantity limitation in hermaphrodite flowers was much lower (6.43 %). As regards limitation by quality, only 11.8 and 11.2 % of visited female flowers fall above the higher BCa in core and marginal populations, respectively. However, 85.4 % of hermaphrodite flowers in marginal populations were limited by quality, in contrast to 50.6 % recorded in core populations.

Furthermore, in core populations the slope of the initial relationship (b1) was nearly twice as high in females as in hermaphrodites whereas in marginal populations it was similar between sexes and substantially lower in the females, thus, indicating a reduction in the quality of pollen received by females of marginal populations (**Table 1 and Figure 3**). Finally, the slope of the second relationship (b2) did not differ from 0 in any case (Table 1 and Figure 3). This last result suggests that the styles were fully saturated with pollen tubes, although the number of tubes corresponding to the breakpoint was 16 in hermaphrodites and 6 in females of core populations, and 8 in hermaphrodites and 13 in females of marginal populations; always higher than the single ovule per flower.

Table 3. Parameters of piecewise regression analysis of the relationship between numbers of pollen grains and pollen tubes for hermaphrodite and female individuals in core and marginal populations of *Daphne laureola*. The breakpoint indicates the number of pollen grains at which the slope of the relationship changes. Bootstrapping Confidence intervals (BCa) were estimated with n = 1000 bootstraps. The slopes of the relationship before and after the breakpoint are represented by b1 and b2, respectively.

Region	Sex	b1	Breakpoint (BCa)	b2
Core	Female	0.55	11 (4.51-19.34)	0.12
	Hermaphrodite	0.34	45.96 (37.06-55.37)	-0.001
Marginal	Female	0.26	50.52 (29.27-71.26)	-0.058
	Hermaphrodite	0.31	24.80 (19.74-43.34)	0.002

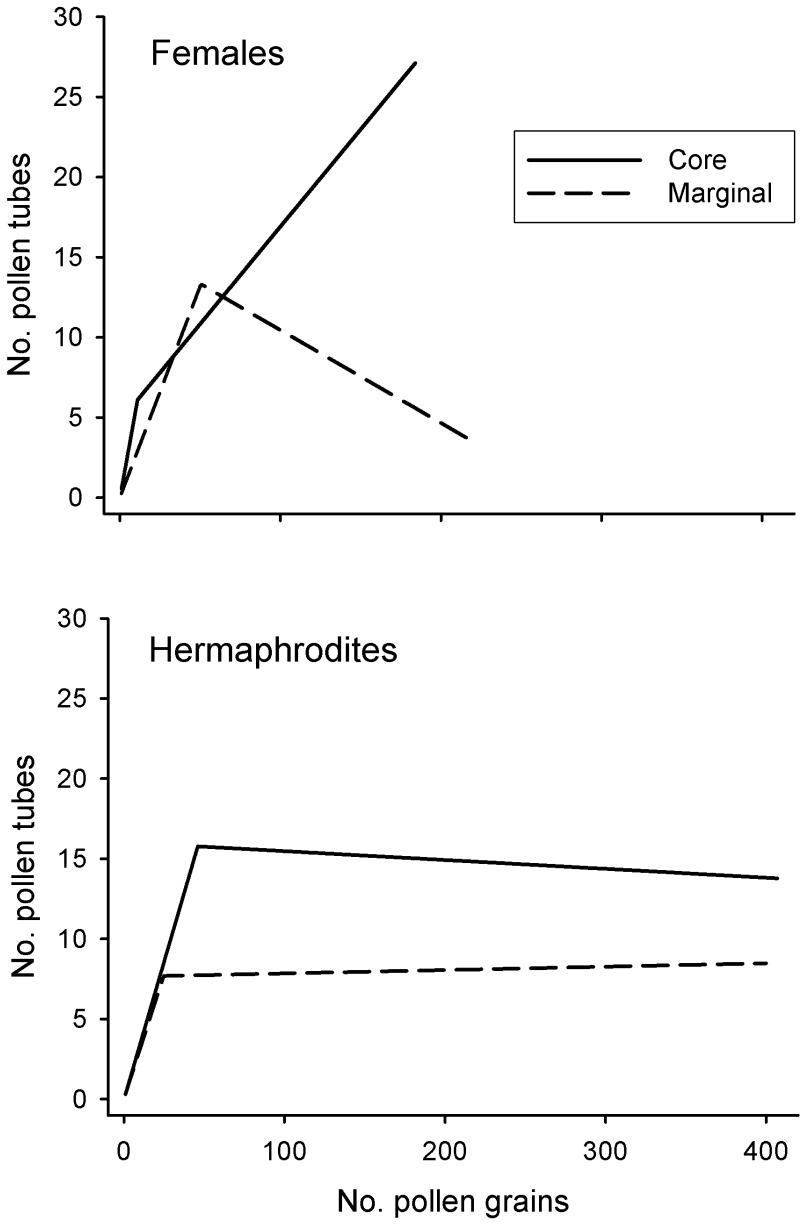

Figure 3. Piecewise regression of the relationship between the number of pollen grains and pollen tubes for females and hermaphrodites of *D. laureola* in core and marginal populations (solid and dashed lines respectively). For comparison, the four piecewise models are presented on a common scale.

Intraspecific Floral Traits Differentiation

Hermaphrodite flowers were longer and wider than female flowers. Floral tubes were longer in hermaphrodites than females, although the magnitude of the gender divergence was lower at marginal populations ($F_{1,2392}$ = 30.63, P < 0.0001; region x sex interaction; **Fig. 4a**). In particular, hermaphrodite flowers of marginal populations had shorter tubes than those of core populations (**Fig. 4a**), whereas female flowers were similar between regions (**Fig. 4a**). Flowers of hermaphrodites had greater mean outer diameter than those of females ($F_{1,2321}$ = 174.08, P < 0.0001; **Fig. 4b**). The magnitude of the difference between both sexes was similar in core and marginal populations (P = 0.8; region x sex interaction; **Fig. 4b**). Flowers of hermaphrodites showed larger mean aperture of tube than those of females in both core and marginal populations ($F_{1,2377}$ = 491.46, P < 0.0001; **Fig. 4c**). The average values of aperture of tube and the magnitude of the difference between both sexes was similar between core and marginal populations ($F_{1,2377}$ = 2.22, P = 0.1; region x sex interaction). In addition, flowers of the two sexes had greater mean aperture of tube in marginal than in core populations ($F_{1,2377}$ = 52.95, P < 0.0001; **Fig. 4c**).

Gender divergence in phenological traits was different in core and marginal populations of *D. laureola*. Sexes differed in the time of flowering in marginal populations, where females were delayed, but not in core populations ($F_{1,232}$ = 25.66, P < 0.0001, interaction region x sex; **Fig. 4d**). Also, individuals in core populations had more open flowers per inflorescence at flowering peak than those of marginal populations. Within-plant asynchrony was higher in core populations and the sign of gender divergence changed between regions ($F_{1,23}2$ = 22.06, P < 0.0001; interaction region x sex). Hermaphrodites presented greater variation in the number of open flowers during the flowering peak in core populations, in contrast to the marginal populations were females were more variable (**Fig. 4e**).

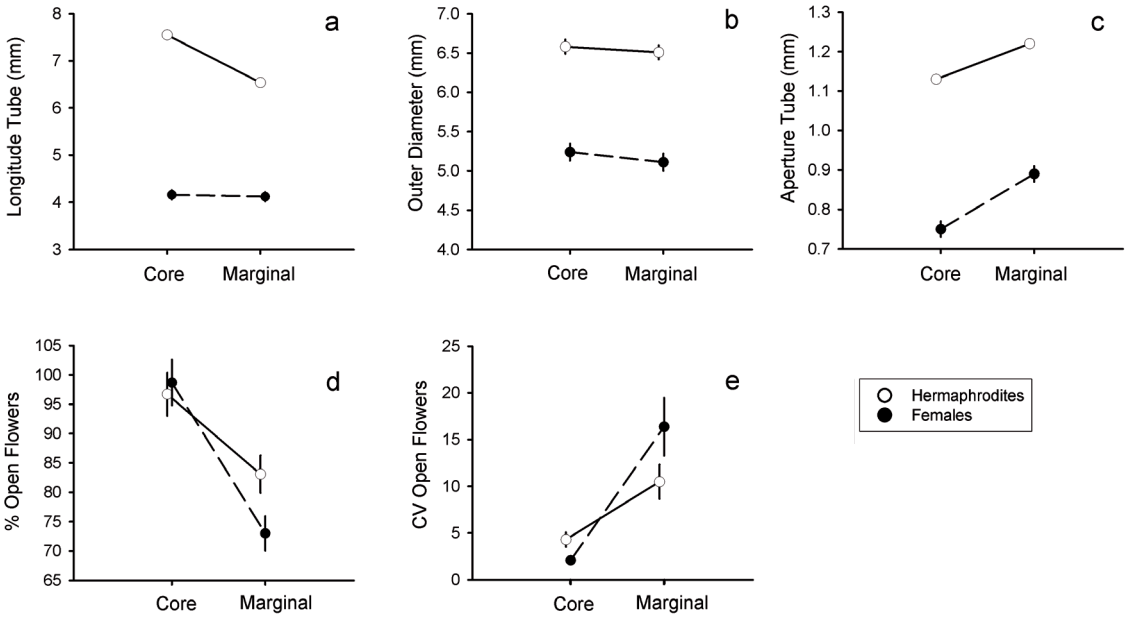

Fig 4. Variation in average of floral phenotypic traits between genders (hermaphrodites vs. females) and regions (core vs. marginal) of *Daphne laureola* in southern Iberian Peninsula. Three morphological traits related to flower size were studied: **(a)** longitude of tube; **(b)** outer corolla diameter; and **(c)** aperture of tube. Additionally, two phenological traits were studied: percentage of open flowers during flowering peak, flowering time; and coefficient of variation of the number of open flowers during flowering peak, flowering asynchrony. Values plotted are the model-adjusted least-squares means (± SE).

Phenotypic Selection Estimated From Pollination Success

Only some floral traits of hermaphrodite individuals were influential for the mean number of pollen tubes recorded per plant, our estimate of individual relative pollination success. In core populations, mean number of pollen tubes was significantly related to longitude of tube and aperture of tube in hermaphrodites, being the level of statistic significance variable among populations (**Table 2**). In particular, the mean number of pollen tubes was positively related to longitude of tube in hermaphrodites of two core populations (CDE and VCV; **Fig. 5**). Furthermore, mean number of pollen tubes showed a marginally significant positive relationship with aperture of tube in hermaphrodites of VCV population (t = 1.85, $P = 0.07$; b = 0.14 ± 0.08). However, there was not any significant relationship in the other two core populations ($P = 0.12$ and $P = 0.43$ for hermaphrodites of CDE and FBJ respectively).

In addition, in marginal populations the pollination success was not related to floral morphology but it was larger in late flowering hermaphrodite individuals (i.e. with lower flowering time; **Table 2**). Neither floral morphological nor phenological traits were related to the mean number of pollen tubes in females of the two study regions (**Table 2**).

Table 2. ANCOVA analyses comparing patterns of selection by pollinators on floral traits in females and hermaphrodites of core and marginal populations of *Daphne laureola*. Dependent variable was the relative mean number of pollen tubes. Independent variables were standardized to mean 0 and variance 1. Significant relationships ($P \leq 0.05$) are in boldface. LT, OD, AT, FT and FA refer to longitude of tube, outer diameter, aperture tube, flowering time and flowering asynchrony respectively. We first ran the model with all of the interactions and then excluded the nonsignificant ones. Only linear selection gradients were considered (see Material and Methods). Results show the standardized selection coefficient (b) and its standard error mean (SE).

		Core Hermaphrodites			**Core Females**			**Marginal Hermaphrodites**			**Marginal Females**		
Effect	d.f.	$F_{(62)}$	*P*	b (SE)	$F_{(33)}$	*P*	b (SE)	$F_{(62)}$	*P*	b (SE)	$F_{(36)}$	*P*	b (SE)
LT	1	6.16	**< 0.05**	0.20 (0.08)	0.41	0.52	0.09 (0.14)	0.00	0.96	0.01 (0.09)	0.12	0.74	-0.04 (0.11)
OD	1	0.98	0.33	0.05 (0.05)	0.07	0.79	-0.04 (0.14)	0.28	0.60	0.05 (0.10)	0.21	0.65	0.05 (0.12)
AT	1	0.50	0.48	0.14 (0.08)	0.03	0.87	0.03 (0.15)	0.44	0.51	0.07 (0.10)	0.47	0.50	0.08 (0.11)
FT	1	0.48	0.49	0.07 (0.11)	0.93	0.34	-0.25 (0.26)	5.30	**< 0.05**	-0.25 (0.11)	1.30	0.26	-0.14 (0.12)
FA	1	0.02	0.90	0.01 (0.10)	1.12	0.30	-0.22 (0.21)	0.05	0.82	-0.03 (0.11)	2.16	0.15	0.16 (0.11)
Pop	2	0.79	0.46		0.41	0.66		0.59	0.56		0.05	0.95	
LT x Pop	2	3.31	**< 0.05**										
AT x Pop	2	3.33	**< 0.05**										

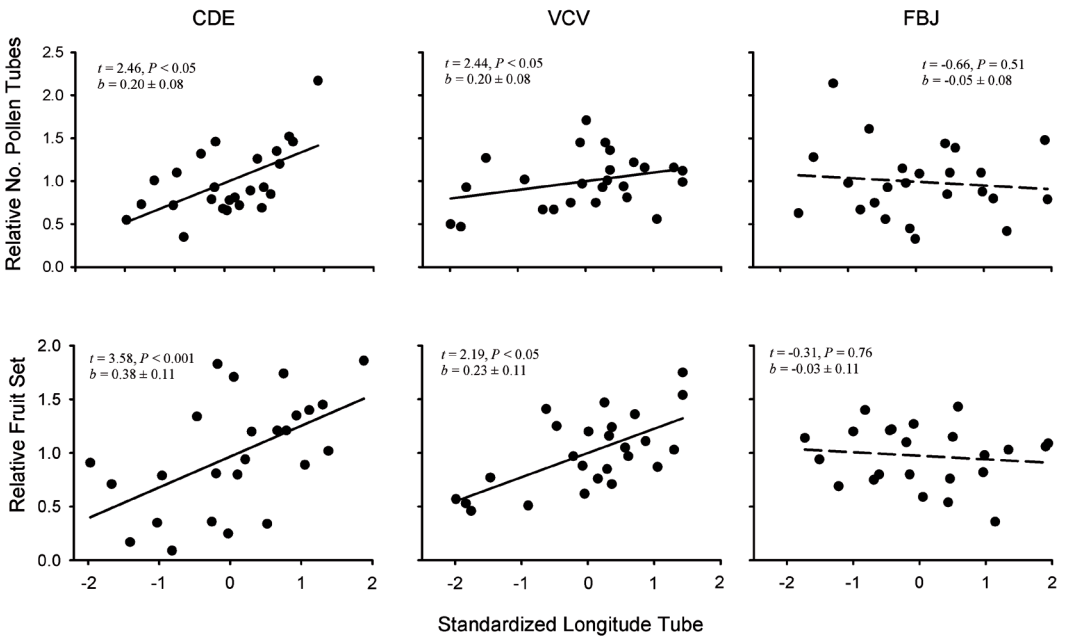

Fig 5. Standardized linear phenotypic selection gradients for longitude of flower tube of *D. laureola*, based on number of pollen tubes per style as an estimate of pollination success (upper panels) and fruit set as an estimate of the maternal component of female fitness (bottom panels). Relative values of dependent variable, estimated by dividing the mean values per individual by the population means, were used. Independent variable was standardized to mean 1 and variance 0. Solid and dashed lines indicate significant and non-significant relationships respectively.

Phenotypic Selection Estimated From Maternal Component of Fitness

Again, phenotypic selection patterns were variable among sexes, populations and regions. In core populations, positive selection on longitude of tube variable among populations was detected in hermaphrodites (**Table 3**). As noted from pollination success (see previous section), hermaphrodites with greater fruit set presented also longer corolla tubes in CDE and VCV populations but not in FBJ (**Fig. 5**). Females of all core populations exhibited positive selection on outer diameter (**Table 3**). Also in females we detected selection on flowering asynchrony that varied among populations (**Table 3**). Higher fruit set was positively related to flowering asynchrony only in CDE (**Fig. 6**).

In marginal populations, we did not detect any selection on phenotypic traits of hermaphrodite plants (**Table 3**). However, phenotypic selection on flowering time and flowering asynchrony

variable among populations in females of marginal populations was detected (**Table 3**). Fruit set was negatively related to flowering time and flowering asynchrony in females of GRZ population (**Fig. 7**). Any phenotypic selection on flowering time was detected neither CDA nor FML (t = 0.19, *P* = 0.85 and t = -0.71, *P* = 0.48). Flowering asynchrony was not related to fruit set neither CDA nor FML (t = -0.23, *P* = 0.82 and t = -0.63, *P* = 0.53).

Table 3. ANCOVA analyses comparing patterns of selection on floral traits in females and hermaphrodites of core and marginal populations of *Daphne laureola*. Dependent variable was relative fruit set, as an estimate of the maternal component of fitness. Independent variables were standardized to mean 0 and variance 1. Significant relationships ($P \leq 0.05$ or better) are in boldface. LT, OD, AT, FT and FA refer to longitude of tube, outer diameter, aperture tube, flowering time and flowering asynchrony respectively. We first ran the model with all of the interactions and then excluded the nonsignificant ones. Only linear selection gradients were considered (see Methods). Results show the standardized selection coefficient (b) and its standard error mean (SE).

		Core Hermaphrodites			**Core Females**			**Marginal Hermaphrodites**			**Marginal Females**		
Effect	d.f.	$F_{(64)}$	*P*	b (SE)	$F_{(34)}$	*P*	b (SE)	$F_{(62)}$	*P*	b (SE)	$F_{(32)}$	*P*	b (SE)
LT	1	**9.59**	**< 0.01**	**0.23 (0.11)**	0.34	0.56	0.04 (0.08)	0.00	0.96	0.002 (0.05)	3.50	0.07	0.15 (0.08)
OD	1	0.13	0.72	-0.02 (0.06)	**8.49**	**< 0.01**	**0.24 (0.08)**	0.11	0.74	-0.02 (0.05)	0.22	0.64	0.04 (0.09)
AT	1	0.05	0.83	-0.01 (0.06)	2.58	0.12	0.17 (0.10)	0.32	0.57	0.03 (0.05)	0.65	0.42	-0.06 (0.08)
FT	1	0.19	0.66	-0.06 (0.14)	3.03	0.09	0.41(0.24)	0.39	0.53	0.04 (0.06)	**5.13**	**< 0.05**	**-0.56 (0.18)**
FA	1	0.03	0.87	-0.02 (0.14)	1.72	0.20	0.09 (0.21)	0.03	0.86	-0.01 (0.06)	**12.84**	**< 0.01**	**-0.91 (0.20)**
Pop	2	1.39	0.26		0.25	0.78		0.34	0.71		2.59	0.09	
LT x Pop	2	**4.02**	**< 0.05**										
FT x Pop	2										**3.34**	**< 0.05**	
FA x Pop	2				**5.15**	**< 0.05**					**7.13**	**< 0.01**	

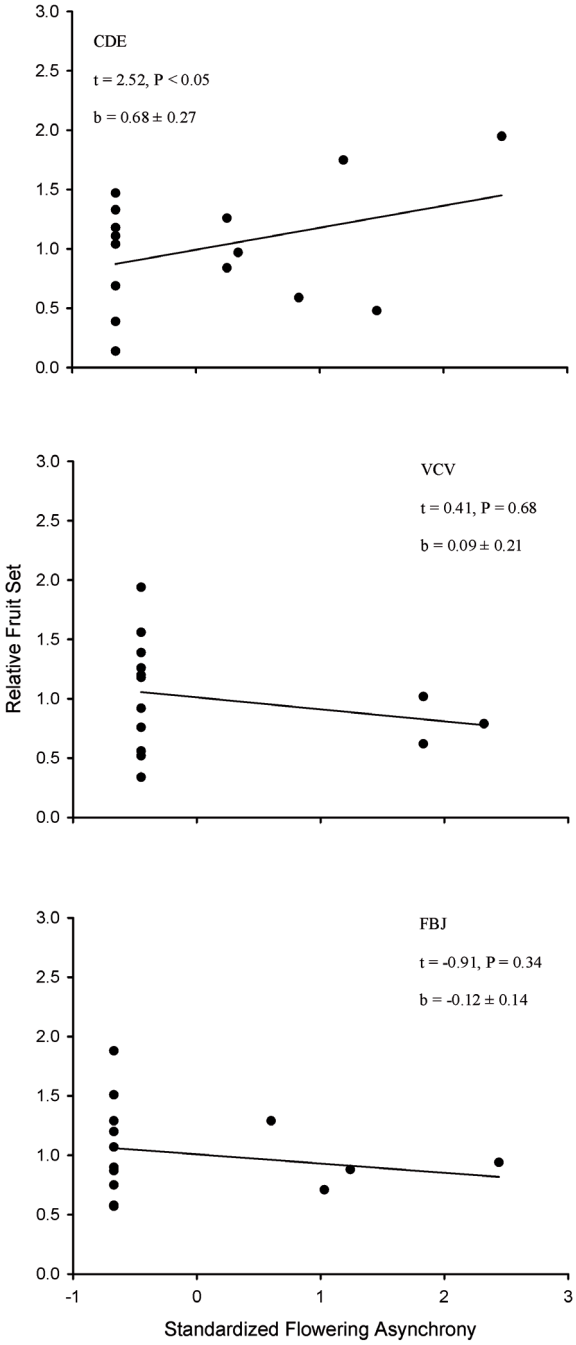

Fig 6. Standardized linear phenotypic selection gradients for flowering asynchrony (FA) in female individuals of core populations of *Daphne laureola*. Flowering asynchrony between inflorescences of the same individual was estimated through the coefficient of variation of the number of open flowers per inflorescence (N = 5 per plant) during the population flowering peak. Dependent variable was the relative fruit set, estimated by dividing the mean values per individual by the population means.

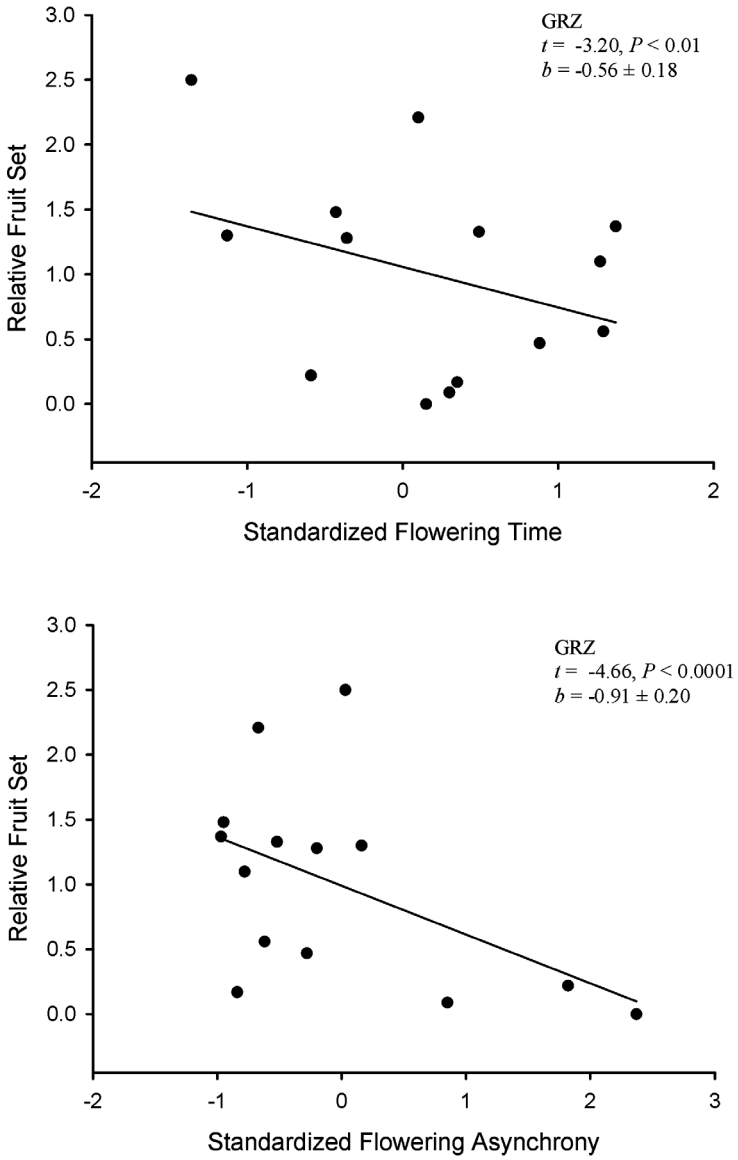

Fig 7. Standardized linear phenotypic selection gradients for flowering time and flowering asynchrony (FT and FA, respectively) in female individuals of the marginal population GRZ. Significant relationships between these phenological variables and relative fruit set were only found in females of this marginal population (see results). Flowering time was estimated as the mean percentage of opened flowers per inflorescence in each individual during the population flowering peak. Flowering asynchrony was estimated through the variation coefficient of the number of opened flowers per inflorescence in each individual during the population flowering peak. Dependent variable was the relative fruit set, estimated by dividing the mean values per individual by the population means.

Adaptative Genetic Basis of Phenotypic Traits

Hermaphrodites of core populations showed a negative association between longitude of tube and the presence of the marker 49, although it was only marginally significant (**Table 4**). Hermaphrodites of core populations presented a negative association between aperture of tube and the presence of 190 and 19 markers (**Table 4**). However, the association with the 19 marker was only marginally significant (**Table 4**).

Females of core populations presented a negative association between outer diameter and the presence of the marker 19 (**Table 4**). There was not any association between flowering asynchrony and the presence or absence of any marker in females of core populations (**Table 4**).

Hermaphrodites of marginal populations showed a negative association between flowering time and the absence of the marker 74 (**Table 5**). In females of marginal populations, neither flowering time nor flowering asynchrony showed any association with the presence or absence of any marker (**Table 5**).

Table 4. Results of separated logistic regressions relating scores for AFLP loci (1-0, presence-absence) to phenotypic traits under phenotypic selection in hermaphrodites and females of core populations of *Daphne laureola*. Only the six loci under purifying selection in core populations were used in the analysis (see Table 2). LT, OD, AT and FA refer to longitude of tube, outer diameter, aperture tube and flowering asynchrony respectively. Regressions model the probability of marker band presence (i.e. score = 1) as a function of the phenotypic trait, hence positive regression parameters denote a positive effect of the dominant allele, and vice versa.

	Hermaphrodites Core				**Females Core**			
	LT		*AT*		*OD*		*FA*	
Locus	Regression Parameter (SE)	*P* value	Regression Parameter (SE)	*P* value	Regression Parameter (SE)	*P* value	Regression Parameter (SE)	*P* value
19	0.54 (0.56)	0.33	0.74 (0.43)	0.08	**-1.60 (0.75)**	**<0.05**	-0.30 (0.67)	0.65
45	-0.02 (0.27)	0.95	-0.28 (0.24)	0.24	-0.33 (0.36)	0.36	0.25 (0.34)	0.46
49	-0.47 (0.27)	0.09	-0.23 (0.23)	0.31	0.24 (0.35)	0.50	0.26 (0.36)	0.47
124	0.18 (0.27)	0.51	-0.24 (0.25)	0.33	0.29 (0.33)	0.38	-0.31 (0.34)	0.36
190	-0.19 (0.27)	0.47	**-0.54 (0.24)**	**< 0.05**	0.08 (0.34)	0.81	0.09 (0.34)	0.79
199	0.27 (0.26)	0.31	0.18 (0.22)	0.43	0.20 (0.32)	0.52	0.09 (0.32)	0.78

Table 5. Results of separated logistic regressions relating scores for AFLP loci (1-0, presence-absence) to phenotypic traits under phenotypic selection in hermaphrodites and females of marginal populations of *Daphne laureola*. Only the seven loci under purifying selection in marginal populations were used in the analysis (see Table 2). FT and FA refer to flowering time and flowering asynchrony respectively. Regressions model the probability of marker band presence (i.e. score = 1) as a function of the phenotypic trait, hence positive regression parameters denote a positive effect of the dominant allele, and vice versa.

	Hermaphrodites Marginal		**Females Marginal**			
	FT		*FT*		*FA*	
Locus	Regression Parameter (SE)	*P* value	Regression Parameter (SE)	*P* value	Regression Parameter (SE)	*P* value
24	0.09 (0.28)	0.73	-0.02 (0.40)	0.97	-0.13 (0.37)	0.73
71	-	-	-	-	-	-
74	**-0.84(0.43)**	**< 0.05**	-0.25 (0.41)	0.55	-0.02 (0.38)	0.97
85	0.09 (0.29)	0.75	0.08 (0.39)	0.84	-0.04 (0.37)	0.91
90	-	-	-	-	-	-
111	-0.06 (0.51)	0.91	-	-	-	-
162	0.23 (0.49)	0.63	-0.75 (0.67)	0.26	0.02 (0.69)	0.98

Discussion

Pollination Success

Geographic variation in pollinator fauna may lead to different pollination success among populations because of changes in the quantity and quality of pollen received (Herrera 2004; Harder & Aizen 2010). The analysis of the relationship between pollen grains and tubes of wilted flowers has been recently proposed as a suitable way to compare the relative relevance of these two components (Alonso et al. 2011). Distinguishing between the two components of pollination success may be particularly important for a gynodioecious species in which geitonogamy and inbreeding depression seems to play a more relevant role than number of seeds produced in the dynamics of the sexual polymorphism (Medrano et al. 2005). Results obtained by this method supported a geographic variation in the quantitative and qualitative components of the pollination success bound to a shift in the identity of the main pollinator in core and marginal populations of *D. laureola* in southern Iberian Peninsula. Several bumblebees and bees species visit *D. laureola* flowers in marginal populations in contrast to core populations where *M. elongatus*, a small pollen-beetle, is the main pollinator of the species (Alonso 2004; Chapter 3) Hermaphrodite flowers were mostly

limited by quality and received similar quality pollen in core and marginal populations. However, female flowers were less visited but received high quality pollen in core populations, contrasting to marginal populations, where visitation rates increased but the quality of pollen received decreased, reducing the gender divergence in the two components. As far as we know, this study is the first empiric evidence for geographic variation in the qualitative component of pollination in female but not in hermaphrodite individuals of a gynodioecious species.

Impoverishment of quantitative pollination success in marginal populations because of lower efficiency of pollinators transporting pollen among individuals has been reported in some studies (e.g. Moeller 2006; Etcheverry et al. 2008). However, quantitatively it does not seem to be the case in marginal populations of D. laureola because female flowers were more frequently visited and received larger pollen loads in marginal than in core populations. Stronger genetic relatedness between pollen donors and maternal plants in marginal than in core populations of D. laureola could account for the decrease of pollination quality of female individuals of these populations (Mazer et al. 2010). Biparental inbreeding was estimated to be low in some core populations (Medrano et al. 2005). An increase of biparental inbreeding in females of marginal populations because of loss of post-pollination mechanisms for relatedness-based pollen selection and/or greater abundance of relatives in their neighbourhood could lead to an impoverishment of the quality of their received pollen (Waser & Price 1989; Teixeira et al. 2009; Mazer et al. 2010). Hermaphrodites of marginal populations did not present lower quality pollen than hermaphrodites of core populations, probably because in both regions, most of pollen deposited on their stigmas is own pollen (see Medrano et al. 2005). In any case, this variation in the quality of pollination in female but not in hermaphrodite individuals could have important consequences for the spreading of females and the evolution of floral gender divergence in a gynodioecious species (see below).

Selection on Floral Traits

Geographic variation in the extent of gender divergence in floral size and morphology has been reported in some sexually polymorphic species (Kohorn 1995; Delph et al. 2002; Delph & Bell

2008). In *D. laureola* we found that only gender divergence of longitude of tube was significantly different between the two study regions. Among all studied traits, was precisely longitude of tube the only one that resulted in congruent phenotypic selection patterns based on pollination success and post-pollination maternal fitness of individuals. Selection on floral traits by pollinators is thought to influence the evolution of separate sexes because of its potential to limit plant reproductive success, constraining or promoting the phenotypic divergence of the sexes (Ashman 2000). However, few studies have investigated the role of pollinators as causal agents of gender differences in floral traits in gynodioecious species (e.g. Ashman & Diefenderder 2001; Case & Ashman 2009). Our results showed that a shift from a pollinator fauna composed almost exclusively by a pollen beetle species in core populations to a another constituted by several bumblebees and bees species in marginal populations lead to differences in the gender-specific phenotypic selection patterns by pollinators on floral traits. Tube longitude was positively and significantly selected for in hermaphrodites of two core populations, and selection could be thus interpreted as a process behind geographic variation in the extent of gender divergence in this trait, that was also higher in core populations. In our study, the absence of relationship between the studied phenotypic traits and the under purifying selection loci did not rule out a genetic basis of the phenotypic traits variation because we only analyzed under selection markers. Alternatively, an association between a phenotypic trait and one or several loci under selection suggested a genetic signature of the selection exerted on this trait. In this sense, we found a negative, although marginally significant, relationship between longitude of tube and the presence of the AFLP marker 49. The association of longitude of tube, a trait under phenotypic selection and whose variation has implications for the fitness of individuals, with a locus under purifying selection suggests a genetic signature of selection linked to longitude of tube. Nevertheless, phenotypic selection by pollinators on longitude of tube was detected in only two of the three studied populations, which did not differ in the average longitude of tube of their individuals (7.5 ± 1.0; 7.7 ± 0.8 and 7.4 ± 1.0 mm in hermaphrodites of FBJ, CDE and VCV populations respectively; the level of gender divergence was 3.3 – 3.4 cm in the three studied populations) indicating that pollinators selection can be indeed variable among populations within a region.

Interestingly, gender divergence in longitude of flower tube was higher in core populations, where different sexes received pollen of different quality. Larger flower tubes in hermaphrodites of core populations could be related to a qualitative improvement of their offspring through an increased gametophytic competition. If longer tubes involve longer styles, pollen tubes may travel greater distances before gaining access to ovules in hermaphrodites of core populations in comparison to those from marginal populations. Therefore, this greater distance provides the opportunity for small differences in pollen tube growth rates resulting in large differences in paternal success which would improve the quality of hermaphrodites' offspring in core populations, where female individuals receive higher quality pollen (Travers & Shea 2001; Mazer et al. 2010). Nevertheless, the short length of D. laureola flowers do a priori little expected this hypothesis. Finally, greater aridity in marginal populations also could reduce the gender divergence in the flower size (Fraze & Marquis 1994; Galen et al. 1999; Herrera 2005). However, core and marginal populations of D. laureola did not differ in mean annual temperature and mean annual precipitation was even larger in marginal populations where hermaphrodite individuals presented shorter corolla tubes (see General Introduction).

Interestingly, post-pollination processes selected positively outer diameter in females of core populations. This result is particularly interesting and intriguing because this floral trait is expected to be positively selected by pollinators but negatively selected by non-pollinator agents (Strauss & Whittall 2006; and references therein). Corolla of flowers of *D. laureola* is marcescent structure that could reduce the attack by fruit predators, as it has been shown for other plant species (Herrera 2010). In addition, the adaptative value of the corolla marcescence is dependent of the ecological context (Herrera 2011), which could explain why outer diameter is only positively selected in females of core populations. Nevertheless, further experimental studies implying the removal of withered corollas help to confirm or reject the previous hypothesis.

Less attention has been paid to gender divergence in phenological aspects (Bolmgren 1998; Munguía-Rosas et al. 2011). We might predict that mating system of hermaphrodites could vary with

within- and among-plant synchronization in flowering. We found consistent across populations phenotypic selection at pollen tubes level only in hermaphrodite individuals of marginal populations, where hermaphrodites with lower percentage of open flowers presented larger number of pollen tubes. In addition, variation on this phenological trait was negatively related to the presence of the under purifying selection marker 74. However, it did not lead to a congruent phenotypic gender differentiation because hermaphrodites of marginal populations had larger average percentage of open flowers during the flowering peak than females in marginal populations. Patterns of phenotypic selection based on fruit-set suggested some significant effects on phenological traits in females of core and marginal populations, but these patterns were variable among populations and in addition, they did not match with the patterns of phenotypic differentiation between sexes in core and marginal populations.

Concluding Remarks

Marginal regions in plant distributions often present large fragmentation and thus they constitute natural areas where shifts in pollinator fauna are feasible. These shifts in the composition of pollinator fauna may have important consequences for both pollination success and selection patterns by pollinators in plant populations, particularly in sexually polymorphic plant species. Results of the present study supported both predictions in *D. laureola* populations in southern Iberian Peninsula. We found that a change in the identity of the main pollinator in core and marginal populations of *D. laureola* lead to changes in both qualitative and quantitative components of pollination success. Interestingly pollinators deposit larger pollen loads on individuals of marginal than core populations, but the quality of received pollen by females was higher in core than in marginal populations. Furthermore, gender-specific selection patterns by pollinators on flower longitude were different in core and marginal populations of *D. laureola* congruent with a geographic variation in the extent of gender divergence in such floral trait. Therefore, the results of the present study support the role of marginal populations as natural areas where changes in the evolutionary dynamics of plant pollinator interactions can be expected. Further work will be necessary to investigate its influence on the evolution of the gynodioecy.

Acknowledgements

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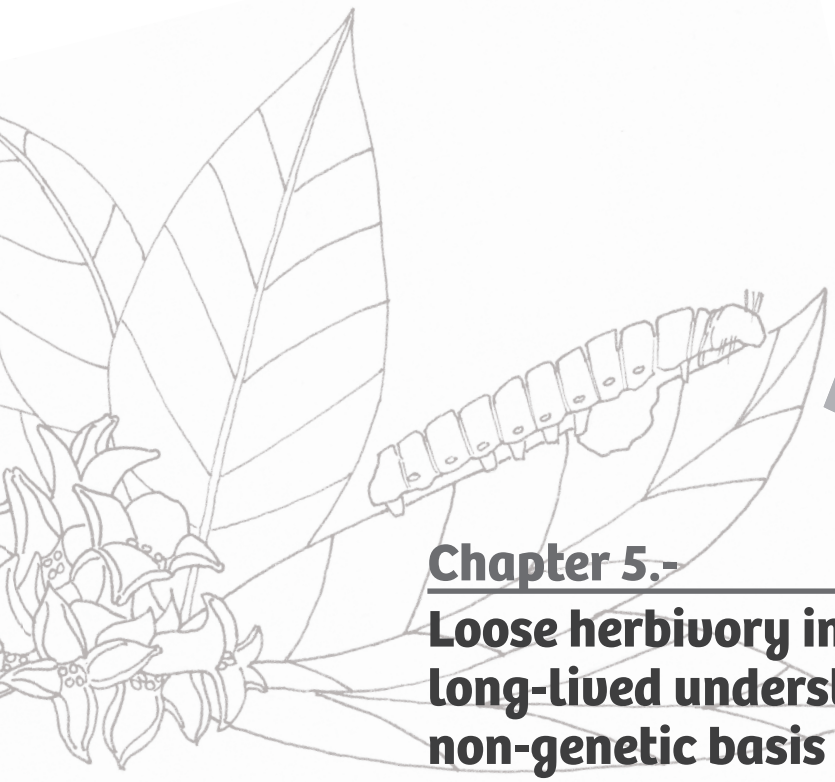

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Chapter 5.-

Loose herbivory interactions in a long-lived understory plant: non-genetic basis for adult defoliation

Abstract: Antagonist interactions are considered an important selective factor on plant species. Geographic variation in the phenotypic selection patterns by herbivores on plant traits could lead to intraspecific differentiation in plant traits relevant for plant-herbivore interaction. In the present study, we analyzed differences in the average herbivory level and its effect on maternal fitness in core continuous and marginal disjunct populations of *Daphne laureola*. Furthermore, we investigated the existence of intraspecific differentiation in vegetative traits and its potential connection to differential selection by herbivores in both groups of populations. Genetic distance among individuals calculated from 225 polymorphic AFLP loci was related to differences in herbivory among individuals to investigate the potential genetic basis of the resistance or tolerance to herbivory. Our results did not support differential herbivory incidence but did a differential effect of herbivory on maternal fitness between core continuous and marginal disjunct populations of *D. laureola*. However, differences in the herbivory level among individuals did not relate to genetic distance among them ruling out a genetic basis of resistance or tolerance to herbivory in *D. laureola*. Changes in the herbivore abundance because of spatial variation of abiotic conditions could explain differences in the level of herbivory among individuals. In addition, herbivores did not exert phenotypic selection consistent with the geographic variation in studied plant traits. Regional differences in the light environment of the understory because of changes in the composition of tree canopy could be related to regional differentiation of vegetative traits of *D. laureola*. Therefore, both among individual differences in the herbivory level within populations and geographic variation in vegetative traits of *D. laureola* seem to be consequence of environmental heterogeneity more than output of geographically differential selection by herbivores.

Castilla, A.R., Alonso, C. and Herrera, C.M. In review. Loose herbivory interactions in a long-lived understory plant: non-genetic basis for adult defoliation.

Resumen

Las interacciones antagonistas están consideradas un agente selectivo importante para las especies vegetales. La variación geográfica en los patrones de selección fenotípica de los herbívoros sobre rasgos fenotípicos de las plantas puede originar diferenciación intraespecífica en tales rasgos. En este estudio comparamos poblaciones centrales continuas y marginales disjuntas de ***Daphne laureola*** en relación a sus niveles medios de herbivoría y al efecto de ésta sobre la proporción de flores que producen frutos. Además investigamos la existencia de diferenciación intraespecífica en rasgos vegetativos y su potencial conexión con selección diferencial llevada a cabo por herbívoros en ambos grupos de poblaciones. Las distancias genéticas entre individuos fueron estimadas mediante el análisis de 225 AFLP loci polimórficos y relacionadas con las diferencias en el nivel de herbivoría de los individuos para investigar la posible base genética de la resistencia o tolerancia a la herbivoría. Nuestros resultados no apoyaron una incidencia diferencial pero sí un efecto diferencial sobre la proporción de flores que producen frutos en poblaciones centrales continuas y marginales disjuntas de ***D. laureola***. Sin embargo, las diferencias en el nivel de herbivoría experimentado por los individuos no se relacionó con la distancia genética entre ellos descartando una base genética de la resistencia o tolerancia a la herbivoría en ***D. laureola***. Cambios en la abundancia de los herbívoros debido a variación espacial de las condiciones abióticas dentro de las poblaciones de la planta podrían explicar las diferencias en los niveles de herbivoría entre individuos de una misma población. Además, los herbívoros no ejercieron selección fenotípica consistente con el patrón de diferenciación geográfica mostrado por los rasgos vegetativos estudiados. Diferencias regionales en el ambiente lumínico del sotobosque debido a cambios en la composición del dosel arbóreo podrían estar relacionadas con la diferenciación regional en los rasgos vegetativos de ***D. laureola***. Por tanto, tanto las diferencias entre individuos dentro de las poblaciones en los niveles de herbivoría como la variación geográfica en los rasgos vegetativos de ***D. laureola*** parecen ser una consecuencia de la heterogeneidad ambiental más que un resultado de selección diferencial llevada a cabo por los herbívoros.

Introduction

Herbivory can reduce growth, fecundity and survival of plants (Crawley 1989; Strauss 1991). Geographic variation in plant-herbivore interactions is also commonly observed and thus both the magnitude and the effects of herbivory on plant populations should be geographically variable (Strauss and Zangerl 2002; Pennings and Silliman 2005; Adams and Zhang 2009). Spatial variation in the intensity of interactions is important because it establishes geographic selection mosaics where intraspecific differentiation processes could occur (Thompson 1994).

Herbivore abundance can vary spatially and temporally because of abiotic (e.g. weather conditions) and biotic (e.g. abundance of their host plant) factors (Schoonhoven et al. 1998). Such variations can be spatially random but we can predict that several factors can lead to changes in plant-herbivore interactions associated with marginal populations (Maron and Chrone 2006; Gaston 2009). Marginal populations of plant species frequently present ecological and genetic peculiarities that could contribute to modify the relationships between plants and their herbivores (e.g. reduced genetic diversity could decrease the adaptative potential of marginal populations to herbivores). First, changes in herbivore fauna have been frequently reported in marginal populations of the host plant (Lewinsohn et al. 2005). Furthermore, the negative impact of herbivory on fitness of individuals could be larger in marginal populations contributing to the maintenance of stable distributional limits (Bruelheide and Scheidel 1999; Maron and Chrone 2006). Therefore, margins of distribution emerge as natural locations where plant-herbivore interactions are expected to vary (Maron and Chrone 2006). However, few empirical studies have examined the influence of natural enemies at range limits (Gaston 2009).

Furthermore, plant traits related to resistance to herbivory should be under differential selective pressure in a landscape where the herbivore-plant interactions vary spatially and temporarily (Zangerl and Berenbaum 2003; Muola et al. 2010a; Vergeer and Kunin 2011). Plant quality from the herbivore's viewpoint depends on a wide variety of phenotypic traits encompassing from nutritio-

nal and secondary chemicals to plant architectural traits. Variation in these traits can reflect not only genetic variation among individuals, but also unevenly distributed resources (Karban 1992). Therefore, the analysis of the geographic variation in phenotypic traits related to herbivory, their connection to differential selection regimes by herbivores and the determination of the genetic or environmental control of such phenotypic variation is a matter of maximal relevance in evolutionary ecology.

In the present study, we compared core continuous and marginal disjunct populations of the shrub Daphne laureola within its distribution range in the South of Iberian Peninsula. In this region, most of populations of the species are gynodioecious with female and hermaphrodite individuals coexisting within populations (Alonso et al. 2007). Four noctuid caterpillars constitute the main herbivores of the species in the study area (Alonso and Herrera 1996). Gender differences in resistance and tolerance to damage by herbivores has been reported in gynodioecious species. Previous study did not find that *D. laureola* herbivores discriminate between leaves of female and hermaphrodite plants (Alonso 2003). However, marginal populations frequently present harsher conditions that could promote sex-differential resistance and tolerance to damage by herbivores (Ashman 2006; Kawecki 2008). Therefore, in the present study we analyze the effect of herbivory on plants with different sex in both core continuous and marginal disjunct populations. Furthermore, intraspecific variation in defoliation levels has been related to plant architecture (Alonso and Herrera 1996) and leaf nutrient composition (Alonso and Herrera 2003). However, the influence of secondary metabolites on this plant-herbivore system is still not fully understood. Specifically we addressed the following questions: (1) **do core continuous and marginal disjunct populations of *D. laureola* differ in their level of herbivory damage?** (2) **does herbivory damage have stronger impact on fitness in individuals of marginal disjunct populations?** (3) **is there differential phenotypic selection by herbivores in core continuous and marginal disjunct populations?** and (4) **do individuals genetically differentiated showed significantly different level of herbivory damage?**

Material and methods

Study Species

Daphne laureola L. (Thymelaeaceae) is a long-lived evergreen shrub with a disjunct Palaearctic distribution associated to the main calcareous mountainous ranges of Europe and north of Africa (Meusel et al. 1978). This study was conducted in 2007-2009 in the Baetic Ranges in southern Iberian Peninsula. The flowering occurs in January-April shortly before new leaves are produced. *D. laureola* plants consist of a variable number of erect stems that rise at ground level from a common trunk. Leaves are found only at the distal end of each branch, forming a single, well-defined leaf whorl. Four polyphagus noctuid species are the main herbivores of the species in the study region (*Trigonophora flammea Esper., Noctua janthe Bkh., Noctua fimbriata Schreber, Pseudenargia ulicis Staud.*). The period of activity of herbivores encompasses from April to mid-June. We only evaluated the importance of caterpillars as consumers of foliage, although they also consume flowers and unripe fruits (Alonso and Herrera 1996).

Study Area

Across the Baetic Ranges, *D. laureola* presents a local continuous center in the Sierra de Cazorla with numerous and largely connected populations (Castilla et al. in press). The spatial isolation increases towards eastern and western margins, although the increase is sharper in western edge (Castilla et al. in press). Therefore, in this study we considered the Sierra de Cazorla as the local continuous center ("core region" hereafter) and populations at the western edge as a local disjunct margin ("marginal region" hereafter) of the species' distribution patch in southern Iberian Peninsula. We studied three accessible populations per region (see Chapter 3). All study populations were gynodioecious, and the proportion of female and hermaphrodite individuals was determined during flowering period of 2007 by examining 100 randomly chosen individuals. The frequency of females was ca. 20 % in all of them (see Chapter 3). At the beginning of the study, we haphazardly marked 25 hermaphrodite and 15 female plants in each study population.

Plant Phenotypic and Genetic Traits

In 2007, we measured the basal diameter, at ground level, of the supporting stem of each marked plant using a Mitutoyo digital calliper (basal diameter, hereafter). A previous study showed basal diameter is under negative phenotypic selection by herbivores in at least some populations of *D. laureola* in the core region (Alonso and Herrera 1996). In addition, we estimated proportion of fruits per flower using randomly marked five inflorescences per individual ("fruit set" hereafter).

In 2009 July, five intact leafs (i.e. without signs of herbivory) were haphazardly removed with scissors of each marked plant, placed into sealed plastic bags and kept in a portable cooler to minimize water loss during their transport to the field station. Afterwards, leaves were individually placed in small paper envelopes and dried immediately at ambient temperature in sealed containers with abundant silica gel. Dried leaves were individually weighted on an analytical balance Mettler Toledo PL 203.S and their area measured using a leaf area meter LI-3000C (LI-COR, Lincoln, USA). Then, dry weight and leaf area were used to calculate the specific leaf area mm2 g-1 for each leaf (SLA, hereafter).

Dry leaves from the same plant (N = 5), were pooled into a single sample for chemical analyses and homogenised to a fine powder using a Retsch MM 301 mill. This fine powder was the starting material for the extraction procedure. 250 mg of fine powder of each individual were extracted with 100 ml of methanol/water (70:30, v/v) by agitation at room temperature for 24 hours. The extraction procedure was repeated twice for each individual. The mixtures were centrifugated at 4000 rpm, filtered through a Whatman filter paper and the filtrate was concentrated by evaporation of the solvent, using a rotatory evaporator (BÜCHI Rotavapor R-215, BÜCHI, Lausanne, Switzerland). The extract was then re-dissolved in 10 ml of methanol/water (80:20, v/v) Total phenolic concentration in plant extracts (Phenolic content, hereafter) were determined spectrophotometrically by the Folin-Ciocalteau assay (Singleton et al. 1999) using gallic acid as a standard. An aliquot of 20 µl diluted with 1580 µl water was mixed with 100 µl of Folin-Ciocalteau phenol reagent

and allowed to react for 5 min. Afterwards, we added 300 µl of saturated Na2CO3 solution and allowed to stand for 120 min at room temperature before the absorbance of the reaction mixture was measured in triplicate at 765 nm. Phenolic content of the plant extracts was expressed as mg gallic acid equivalents per g of plant material. Moreover, we quantified the concentration in leaves of the three most abundant coumarins in *D. laureola* using a subsample of 15 individuals (Alonso et al. 2005; Alonso et al. 2009). Phenolic content showed a strong positive correlation with concentration of the three coumarin compounds analyzed (**Fig. 1**).

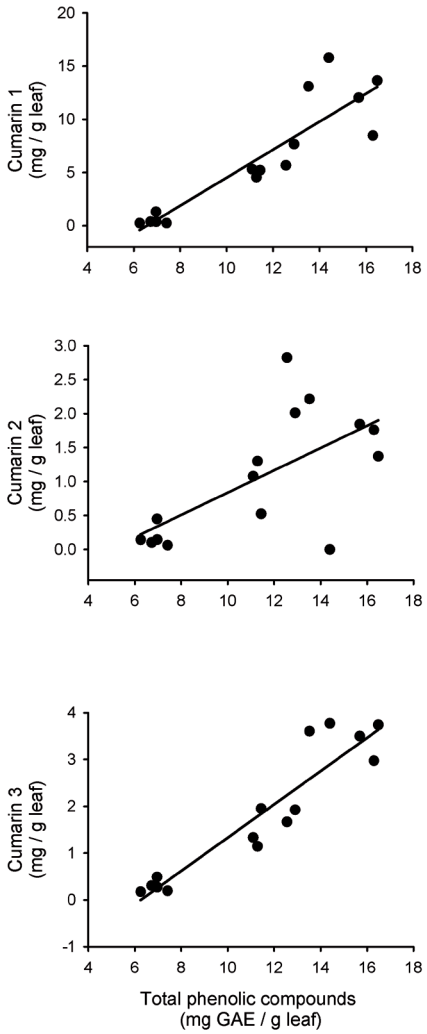

Figure 1. Relationship between total phenolic content and the three most abundant coumarins in leaves of *D. laureola* (N = 15). Pearson correlation was used.

Amplified fragment length polymorphism (AFLP) markers were used to characterize 240 study plants genetically. The AFLP analysis was performed essentially as originally described by Vos et al. (1995), with modifications involving the use of fluorescent dye-labelled selective primers following Applied Biosystems (2005). Each plant was fingerprinted using four EcoRI + 3/MseI + 3 and four PstI + 2/MseI + 3 primer combinations. Fragment separation and detection were made using an ABI PRISM 3130xl DNA sequencer, and the presence/absence of each marker in each individual plant was scored manually by visualizing electrophoregrams with GeneMapper 3.7 software. Only fragments ≥ 150 base pairs in size were considered, as a way of reducing the potential impact of size homoplasy (Vekemans et al. 2002)., Details on primer combinations, number of markers, scoring error rates and levels of polymorphism can be found in Castilla et al. in press.

Herbivory Incidence

The proportion of leaf area removed by herbivores was quantified in the leaf whorls of 20 % of total stems per plant, except for individuals with ≤ 12 stems, in which the proportion of leaf area removed was quantified in all stems (Herbivory level, hereafter). Herbivory level of individuals was quantified in two different years (2007 and 2009). In each leaf whorl, each leave was classified into one of 6 herbivory classes according to percentage of leaf area removed: 0, no signs of herbivory; 1, 1-5% area removed; 2, 6-25%; 3, 26-50%; 4, 51-75%; and 5, >75%. An overall estimate of herbivory level for each leaf whorl was obtained following the method proposed by Alonso and Herrera (1996). Finally we calculated the mean value of herbivoy level per individual averaging the herbivory values of all its leaf whorls.

Data Analysis

All statistical analyses were performed using SAS statistical package (SAS Institute 2002). Differences in the level of herbivory between regions and sexes were analyzed by generalized linear mixed models using the negative binomial function (Procedure GLIMMIX). Effects of region

(core vs marginal), population and sex (hermaphrodite vs female) were treated as fixed factors. Differences in the level of herbivory in 2007 and 2009 were analyzed with separated analyses. In addition, temporal variability in the level of herbivory of individuals was explored with spearman rank correlation.

Differences between sexes and regions in basal diameter, phenolic content and SLA of individuals were analyzed by generalized linear mixed models (Procedure GLIMMIX) with normal error distribution for phenolic content and negative binomial distribution for both basal diameter and SLA. Sex, population and region effects were treated as fixed.

We studied the relationship between herbivory and fruit set in 2007, analyzing separately hermaphrodite and female individuals within each region, because it is expected that the effect of herbivory on fitness varies between hermaphrodite and female individuals in gynodioecious species (Ashman 2006, and references therein). For each sex, we explored potential among population differences in the relationship between herbivory level and fruit set using an analysis of covariance (Procedure MIXED). Fruit set was the response variable, showing a normal distribution. Herbivory level was the continuous explanatory variable and population was the categorical effect.

Phenotypic selection at each region was examined by Lande-Arnold selection gradient models (Lande and Arnold 1983). Within each region, we previously analyzed phenotypic selection patterns by herbivores on individuals of different sex separately. However, the similarity between sexes in phenotypic selection analyses together with the absence of differences in the herbivory level and phenotypic traits lead us to analyzed hermaphrodite and female individuals of each region jointly to get larger statistical power in the analyses. Therefore, we conducted separated phenotypic selection analyses for individuals of core and marginal populations without tacking into account sex of individuals. We quantified phenotypic selection only in 2009 because we only measured foliar traits (SLA and phenolic content) in that year and foliar traits frequently present among-year variation. We used relative values of the response variable, herbivory level (estima-

ted by dividing the mean values per individual by the population means). All phenotypic traits were standardized to mean 0 and variance 1, and were used as independent variables. Among population variation in phenotypic selection on floral morphology was evaluated by testing the homogeneity of slopes of the relations of relative level of herbivory to the measures of phenotypic traits among populations with Population x basal diameter, Population x phenolic content and Population x SLA (e.g. Strauss et al. 2005, Rey et al. 2006). We first ran the model with all of the interactions and then excluded the nonsignificant ones (Littell et al. 2006). For simplicity, we focused only on directional selection gradients and do not assess quadratic terms in the phenotypic selection model, as this approach facilitates interpretation of population x traits interactions. Restriction of the analyses to directional selection is also justified in the present context, because directional selection seems to play a central role in phenotypic diversification at the species level and above (Rieseberg et al. 2002).

A pairwise genetic distance matrix among individuals was built for each population using GenAlEx 6.3. In addition, a pairwise distance of herbivory matrix among individuals also was built using the Procedure Distance implemented in SAS 9.2. To test if differences in the level of herbivory and genetic differentiation among individuals were related, we conducted separated mantel tests per population using the Vegan library of R 2.13 statistical package (R Development Core Team 2009).

Results

Spatial Variation in Herbivory Level

The level of herbivory on the individuals in 2007 and 2009 were positively correlated (r_S = 0.39, P < 0.0001, N = 240; **Fig. 2**). On average, individuals of core region presented larger herbivory than those of marginal region, but the difference was only statistically significant in 2009 ($F_{1,231}$ = 2.60, P = 0.11 and $F_{1,214}$ = 4.24, P < 0.05 in 2007 and 2009 respectively; **Fig. 3**). Population was the only factor with a significant effect on the herbivory level of individuals in both years ($F_{4,231}$ = 12.57, P < 0.0001 and $F_{4,214}$ = 39.40, P < 0.0001 in 2007 and 2009 respectively; **Fig. 3**). This among population

variation in average herbivory level was sharper in marginal than core region. GRZ population presented the maximum values of herbivory in both years, whereas CDA and FML showed the minimum values (**Fig. 3**).

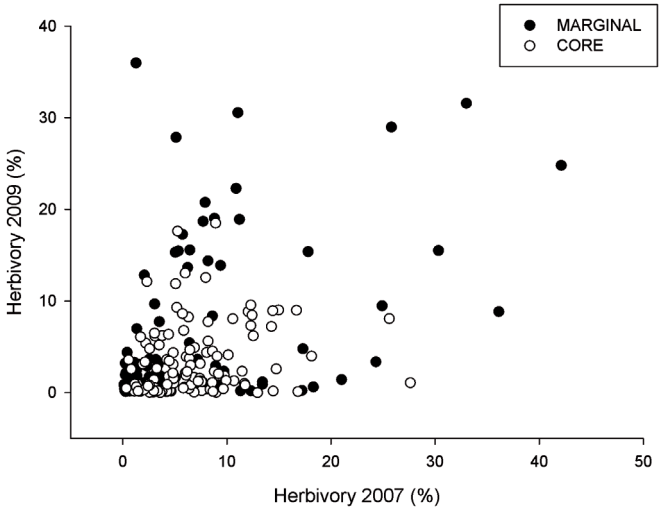

Figure 2. Relationship between the herbivory levels on individuals of core and marginal populations of *D. laureola* in different years (2007 and 2009). Spearman correlation was used to study the relationship between both years. The herbivory level is expressed as percentage of leaf removed per individual (see material and methods for a more detailed description). White and black dots represent herbivory level scores per individual in core and marginal populations respectively.

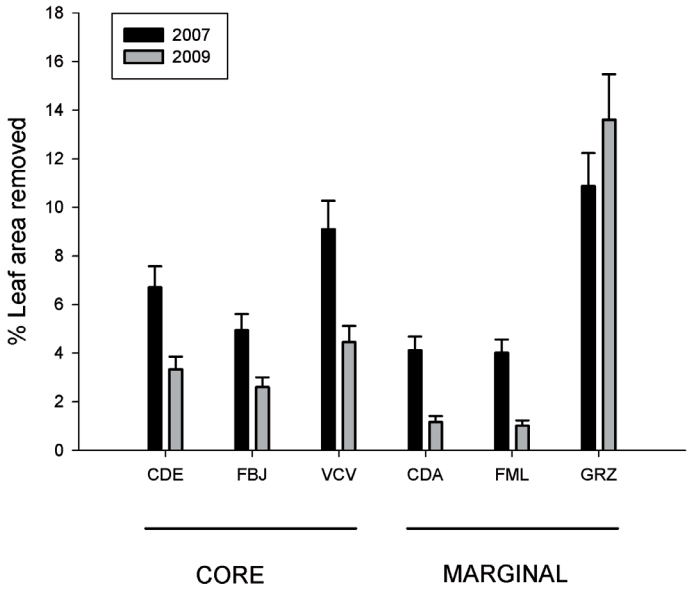

Figure 3. Among population differences in the average herbivory level in two different years (2007 and 2009). Herbivory level is expressed as average percentage of leaf removed. Bars represent the least-square means (± s.e.) after accounting for region and sex (fixed factors).

Regional Differentiation in Phenotypic Traits

The three studied phenotypic traits showed differences between core and marginal populations (**Fig. 4**). On average, individuals of marginal populations presented larger SLA than those of core populations ($F_{1,209}$ = 30.07, P < 0.0001; **Fig. 4a**). Nevertheless, populations also varied widely in the average SLA of their individuals ($F_{4,209}$ = 5.28, P < 0.0001). In addition, sexes did not differ significantly in their SLA ($F_{6,209}$ = 1.93, P = 0.1). Basal diameter was larger in individuals of the marginal region than in those of the core region ($F_{1,215}$ = 23.88, P < 0.0001), although there was a strong among population variation ($F_{4,215}$ = 6.61, P < 0.0001). However, sexes did not differ in their average basal diameter ($F_{6,215}$ = 0.69, P = 0.7). The average phenolic content in marginal populations was lower than in core populations ($F_{1,207}$ = 269.65, P < 0.0001). In addition, populations differed widely in their average phenolic contents ($F_{4,207}$ = 9.52, P < 0.0001). Again, sexes did not differ in their phenolic content ($F_{6,207}$ = 0.32, P = 0.9).

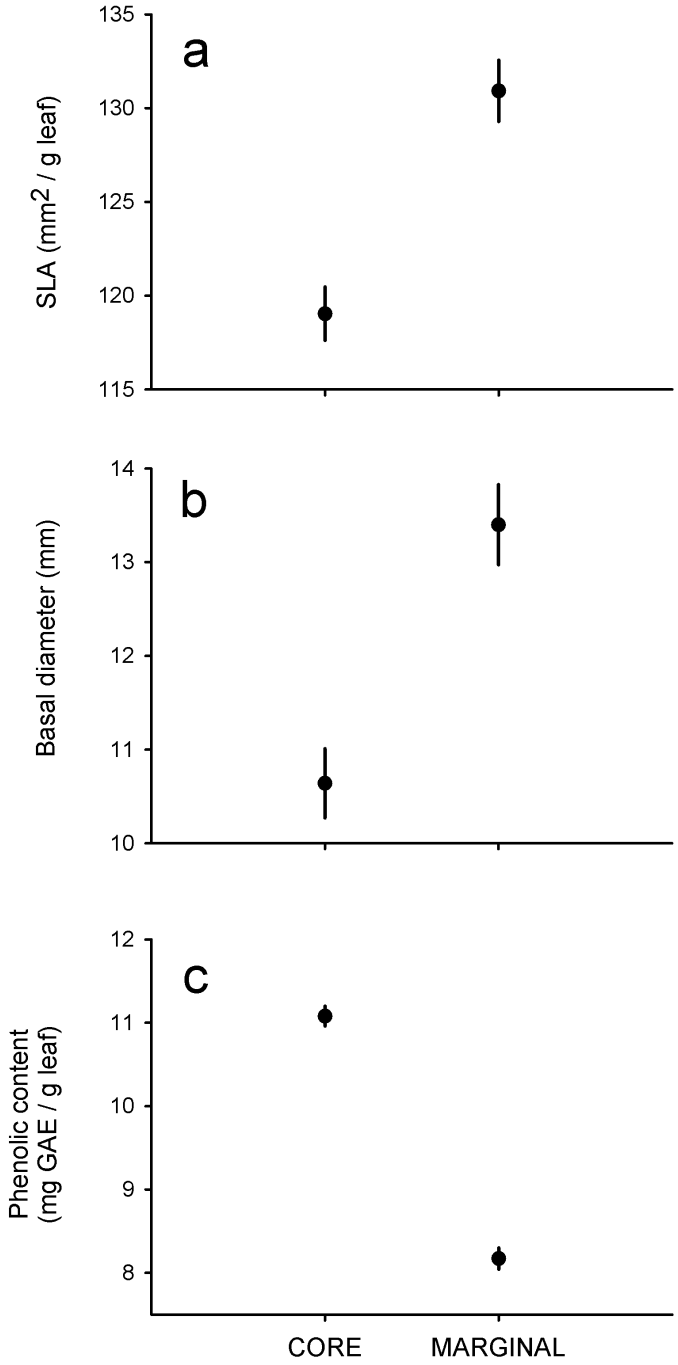

Figure 4. Regional differences in vegetative traits of *D. laureola*. Three vegetative traits were studied: specific leaf area (SLA), basal diameter, at ground level, of the supporting stem of each plant (BD) and total phenolic content (PC). Dots represent regional least-square means (± s.e.) for each vegetative trait.

Did Herbivores Discriminate Among Genetically Distinct Individuals?

A significant and temporarily consistent relationship between differences in herbivory and genetic differentiation among individuals was not found in any of the studied populations (**Table 1**). In the core region, a positive relationship between differences in herbivory and genetic differentiation of individuals was observed in two populations (significant in FBJ and marginally significant in CDE) in only one year (**Table 1**). In the marginal region, relationships between differences in herbivory and genetic differentiation among individuals were not found in any population (**Table 1**).

Table 1. Relationship between differences in the herbivory level and genetic differentiation among individuals. Mantel tests per population and year were conducted. Mantel statistic (r_M) and significance level are presented for each population in both years (2007 and 2009). Populations coded as in Table 1. Significant relationships ($P < 0.05$ or better) are in boldface. Marginally significant relationships ($0.05 \leq P < 0.09$) are in italics.

	2007		**2009**	
Population	r_M	*P*	r_M	*P*
Core				
CDE	-0.04	0.59	*0.12*	*0.08*
FBJ	**0.18**	**< 0.05**	-0.03	0.64
VCV	-0.02	0.62	-0.001	0.52
Marginal				
CDA	-0.09	0.89	-0.07	0.84
FML	-0.03	0.61	0.008	0.43
GRZ	-0.10	0.93	-0.14	0.99

Effects of Herbivory on Maternal Fitness

The level of herbivory affected differentially to fruit set in core and marginal populations. In core populations, herbivory did not show any significant effect on individual fruit set in any of two sexes ($F_{1,69} = 0.08$, $P = 0.77$ and $F_{1,37} = 1{,}31$, $P = 0.26$ respectively). However, in marginal populations herbivory level negatively affected fruit set in both hermaphrodite and female individuals ($F_{1,67} = 59.04$, $P < 0.0001$ and $F_{1,41} = 19.63$, $P < 0.0001$ respectively; **Fig. 5**).

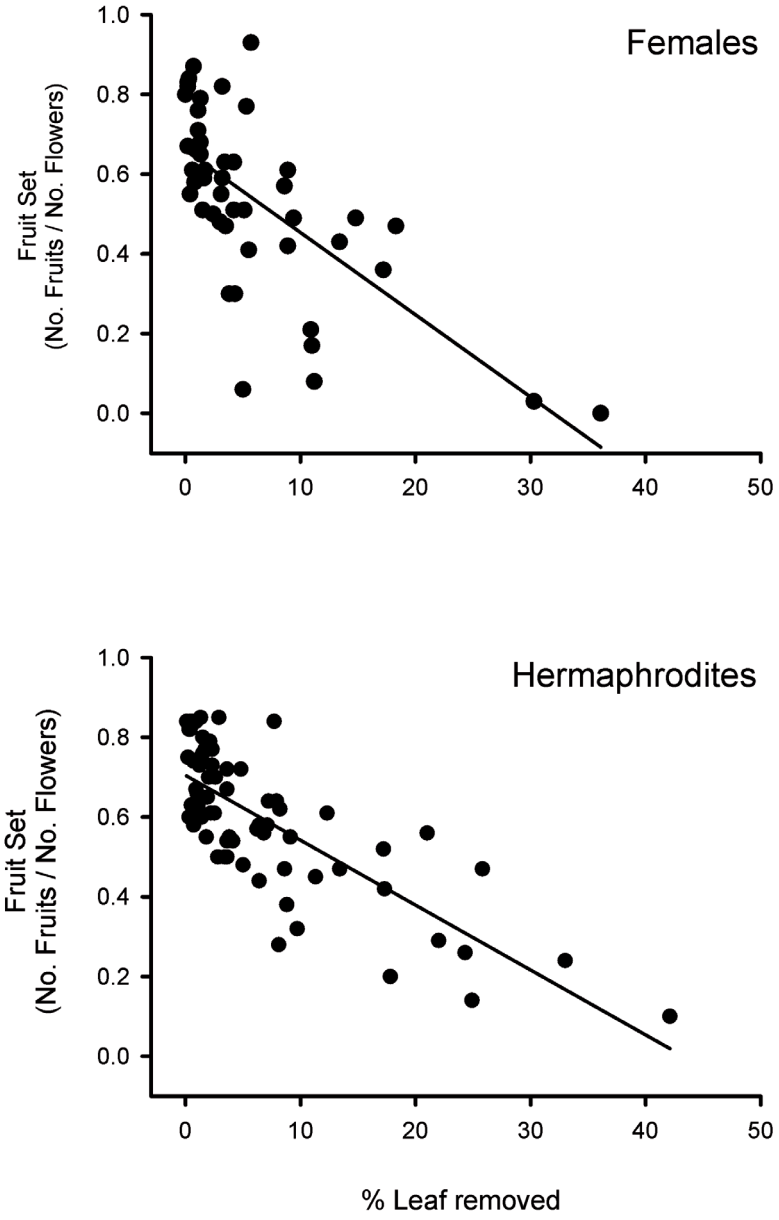

Figure 5. Fruit set related to herbivory level in female and hermaphrodite individuals of marginal populations of *D. laureola*. Fruit set was quantified as the proportion of flowers developing fruits per individual. The herbivory level is expressed as percentage of leaf removed per individual. Each circle corresponds to an individual.

Selection by Herbivores on Plant Traits

According to the previous results, we could expect stronger phenotypic selection on individuals of marginal populations. In core populations plants with lower phenolic content presented marginally larger herbivory level, in contrast to marginal populations where the phenolic content had not any effect (**Table 2**).

In marginal populations, herbivores also exerted phenotypic selection on basal diameter but this effect was variable among populations (**Table 2**). Individuals with larger basal diameter presented also larger herbivory level in FML population, but there were not any effect in the other two populations (**Fig. 6**). In core populations, there was not any relationship between herbivory level and basal diameter (**Table 2**). Finally, herbivores did not exert any phenotypic selection on SLA neither core nor marginal populations (**Table 2**).

Table 2. ANCOVA analyses comparing patterns of selection by herbivores on vegetative and foliar traits in core and marginal populations of ***D. laureola***. Dependent variable was the level of herbivory. BD, PC and SLA represent basal diameter, phenolic content and specific leaf area respectively. Independent variables were standardized to mean 0 and variance 1. Significant relationships ($P < 0.05$ or better) are in boldface. Marginally significant relationships ($0.05 \leq P < 0.09$) are in italics. Only linear selection gradients were considered (see Material and Methods). Results show the standardized selection coefficient (b) and its standard error mean (SE).

	Core				**Marginal**		
Effect	d.f.	$F_{(108)}$	*P*	b (SE)	$F_{(101)}$	*P*	b (SE)
BD	1	2.64	0.11	-0.18 (0.11)	0.71	0.40	-0.19 (0.12)
PC	1	3.90	*0.05*	*-0.21 (0.11)*	2.71	0.10	-0.13 (0.08)
SLA	1	0.11	0.74	-0.04 (0.11)	1.26	0.27	-0.09 (0.08)
Pop	2	0.03	0.97		0.11	0.89	
BD x Pop	2				**7.31**	**< 0.01**	

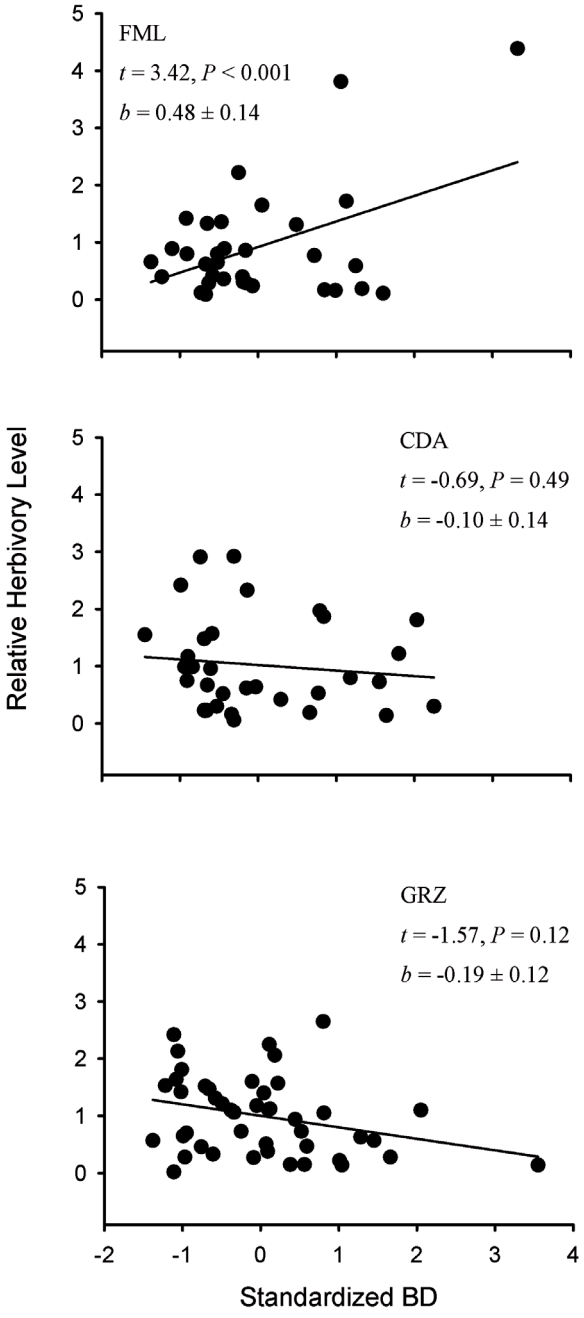

Figure 6. Standardized linear phenotypic selection gradients for the basal diameter (BD) in individuals of marginal populations of *D. laureola* (FML, CDA, GRZ). Dependent variable was the relative fruit set, estimated by dividing the mean values per individual by the population means.

Discussion

Herbivory and Its Effect on Fitness in Core and Marginal Populations

Antagonistic interactions are considered important agents limiting geographic distribution of species (Gomulkiewicz et al. 2000; Nuismer et al. 2000; Paterson et al. 2010). For instance, escape from predators or pathogens has been reported as a causal agent of geographic expansion in numerous invasive species (Maron and Vilá 2001; Torchin et al. 2003). In addition, a growing body of experimental literature demonstrating a key role of herbivory in the spatial structure of plant populations is emerging (Bruelheide and Scheidel 1999; Fine et al. 2004; Gómez et al. 2005; Miller et al. 2009). However, herbivory has not been traditionally considered an important factor limiting geographic distribution of plant species (Gaston 2009).

Core populations of *D. laureola* presented larger herbivory level in 2009, whereas core and marginal populations did not differ in the average herbivory level in 2007. Decrease of herbivory damage in marginal populations has been reported in other studies (Moore 2009; Vergeer and Kunin 2011). However, herbivory affected negatively to fruit set of individuals of both sexes in marginal populations, but did not in core populations. Interestingly, individuals of marginal populations had greater fruit set than those of core populations (Castilla et al. 2011a). Therefore, results of the present study give still more support to our hypothesis of a more efficient pollination environment in marginal populations of *D. laureola* (Castilla et al. 2011a). Furthermore, we found low average levels of herbivory in populations of *D. laureola*, but the differences among individuals were temporarily consistent in line with previous investigations of the study species at other core populations (Alonso and Herrera 1996). Nevertheless, this result does not rule out a relevant role of *D. laureola* herbivores as selective agents because chronic levels of relatively insubstantial herbivory (that is, damage that results in relatively little tissue lost) can have significant impacts on long-lived plants over their lifetimes (Doak 1992; Miller et al. 2009).

Selection by Herbivores in Core and Marginal Populations of *D. laureola*

Herbivores frequently exert phenotypic selection on vegetative traits of plant species, although the intensity of such phenotypic selection frequently varies geographically (Gómez et al. 2005; Pennings et al. 2009; Muola et al. 2010a; Vergeer and Kunin 2011). This geographic variation in phenotypic selection could lead to intraspecific differentiation among plant populations in phenotypic traits important for such interactions (e.g. Herrera et al. 2006 for selection by pollinators). A rigorous demonstration of a relevant role of *D. laureola's* herbivores as selective agents requires demonstrating that observed differences in the herbivory level among individuals have a genetic basis. Resistance and tolerance to herbivory are commonly genetically determined traits that show heritable genetic variation within species (e.g. Muola et al. 2010b; Kuzina et al. 2011; Evans et al. 2011). In model organisms and crop species, great strides have been made in understanding the evolution of plant defence, as well as expression changes in response to herbivory contrasting to non-model organisms where few studies have examined this issue (Anderson and Mitchell-Olds 2011). Our results did not support a predictable, and temporarily constant relationship between differences in the herbivory level among individuals and their genetic distance in wild populations of *D. laureola*. Furthermore, although all studied vegetative traits did differ between core and marginal populations of *D. laureola*, herbivores did not promote such phenotypic differentiation through phenotypic selection. Herbivores exerted negative selection on the phenolic content of leaves in individuals of core populations, whereas herbivores did not exert any selection on this trait at marginal populations. This result seems to match with the geographic variation in the chemical composition of *D. laureola* populations, with an increase of the level of phenolic compounds of defence in populations where herbivores exert negative phenotypic selection on this trait. However, we must be cautious here because selection in core populations was only marginally significant. On the other hand, neither SLA nor basal diameter geographic variations matched with the phenotypic selection patterns by herbivores in core and marginal populations.

Therefore both differences in the level of herbivory among individuals within populations and regional differences in vegetative traits seem to be related to abiotic variation. On the one hand,

abundance of terrestrial insect herbivores can be limited by abiotic conditions, particularly temperature and moisture (Kingsolver 1989; Hunter and Prince 1998; Ritchie 2000). Therefore, our results suggest that environmental heterogeneity among and within *D. laureola* populations lead to spatial variation in the abundance of herbivores, promoting differences in the herbivory level among individuals and populations. On the other hand, individuals of core populations presented lower average basal diameter and leafs with lower SLA but larger phenolic content than those individuals of marginal populations. Differences in SLA and phenolic content could be related to contrasting light environments in core and marginal populations of *D. laureola*. Plants growing in shaded environments frequently present leaves with larger SLA which are more efficient in capturing a limiting light resource (Chen et al. 1996; Mezíane and Shipley 1999; Barber and Marquis 2011). On the other hand, light exposure can alter leaf quality, especially in plants with carbon-based defences, resulting in plants with lower phenolic content in leaves in shaded environments (Nichols-Orions 1991; Dudt and Shure 1994; Barber and Marquis 2011). In core region, D. laureola grows in the understory of mixed forests characterized by shading heterogeneity (Alonso and Herrera 2008). In marginal areas, individuals of *D. laureola* are established in the shrub stratum of closed fir forests, *Abies pinsapo Boiss*, characterized by more homogeneous and closed canopy (Arista 1995). Therefore, the larger SLA and lower phenolic content in leaves of individuals of marginal populations could be a consequence of living in more shaded environments.

Therefore, results of the present study suggested that herbivores did not have a relevant role promoting intraspecific differentiation in vegetative traits of adult plants among *D. laureola* populations. However, we only have studied the effects of foliar herbivory on adult plants and some studies point out the relevance of herbivory differs among different life stages in plant species. For instance, slugs also seem to have a relevant role in the survival of *D. laureola* seedlings (Alonso personal comm.), but we did not analyze their effect in the present study. In this sense, Bruelheide and Scheidel (1999) found that slug herbivory was an important factor limiting the geographical distribution of *Arnica montana*. On the other hand, foliar herbivores of *D. laureola* also consume flowers and fruits but their effects are still waiting to be evaluated.

Concluding Remarks

Results of the present study showed important differences in the herbivory level among nearby populations more than clear regional divergence. Among population and among individual differences seem to be related to environmental heterogeneity determining spatial variation in the abundance of herbivores more than differences in the resistance of individual plants with a genetic basis. In addition, our results ruled out a regional differentiation in vegetative traits conducted by divergent phenotypic selection by herbivores in core and marginal populations of *D. laureola*. Rather contrasting light environments because of differences in the species composition of the tree canopy in core and marginal populations of *D. laureola* could be related to regional differences in foliar traits. In addition, sexes did differ neither vegetative traits nor phenotypic selection by herbivores supporting the widely accepted issue of scarce gender differentiation of vegetative traits in contrast to reproductive traits in gynodioecious species.

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Síntesis y discusión general

El estudio de la variación geográfica intraespecífica en rasgos relevantes para las interacciones planta-animal y su posible relación con una selección divergente efectuada por animales, es crucial para entender el vínculo entre micro y macroevolución en estos caracteres y el papel de las interacciones planta-animal en la evolución de los sistemas sexuales en plantas (Barrett et al. 2001; Zangerl & Berenbaum 2003; Herrera et al. 2006). La teoría del mosaico geográfico de coevolución propuesta por Thompson (1994, 2005) y los modelos genéticos asociados a ella (Gomulkiewicz et al. 2000; Alleaume-Benharira et al. 2006) proporcionan un modelo teórico que permite el acercamiento al estudio del papel causal de los animales en la aparición y mantenimiento de diferencias intraespecíficas en los rasgos fenotípicos importantes para las interacciones planta-animal (Vermeer et al. 2011). Sin embargo, este marco teórico no predice las áreas concretas donde el cambio de las dinámicas coevolutivas en las interacciones planta-animal sea esperable a priori. El objetivo general de esta Tesis Doctoral ha sido tratar de mostrar cómo los márgenes de distribución de las especies vegetales, debido a sus peculiaridades ecológicas y genéticas, constituyen áreas donde cabe esperar cambios en las interacciones planta-animal. La integración de información obtenida mediante muestreos de campo (cuantificación del éxito de polinización y reproducción, censos de polinizadores, estimas de herbivoría), técnicas moleculares (diversidad y diferenciación genética, detección de loci bajo selección natural) y medición de rasgos fenotípicos en laboratorio (morfología floral y foliar, fenología, análisis de la concentración de fenoles en hojas) han permitido inferir la importancia de polinizadores y herbívoros como agentes del cambio en los rasgos fenotípicos de *D. laureola* en márgenes de su distribución.

La Estructuración Geográfica de las Poblaciones de *D. laureola*

El análisis espacialmente explícito llevado a cabo con la mayor parte de las poblaciones de *D. laureola* existentes en las Cordilleras Béticas reveló que la estructuración geográfica de las po-

blaciones varió hacia los márgenes de la distribución. Los resultados de este estudio mostraron un incremento del aislamiento espacial de las poblaciones hacia los márgenes de distribución de la especie en el área de estudio (Capítulo 1). Sin embargo, no hubo evidencias que apoyasen una reducción del tamaño poblacional hacia los márgenes de distribución (Capítulo 1). De hecho, las poblaciones marginales de D. laureola en la región de estudio se caracterizaron por presentar en muchos casos más de 100 individuos reproductivos. Estos resultados sugirieron la existencia de localidades ecológicamente óptimas para la especie en todo el área de estudio, aunque la abundancia de las mismas disminuyó hacia los márgenes de distribución (Eckert et al. 2008; Yakimowski and Eckert 2008; Sexton et al. 2009). Por otro lado, aunque el aislamiento espacial de las poblaciones aumentó tanto hacia el margen oriental como hacia el margen occidental, la magnitud del aislamiento no fue equivalente. Las poblaciones del margen occidental presentaron una nivel de aislamiento espacial mucho mayor que las poblaciones del margen oriental. El análisis de la estructura genética de las poblaciones demostró que estas diferencias en el aislamiento espacial de las poblaciones tienen importantes consecuencias sobre el patrón de diferenciación genética de las poblaciones (Capítulo 1). En esta Tesis Doctoral demostramos que la estructuración genética de las poblaciones puede ser diferente en distintos márgenes de distribución (Hampe and Petit 2005; Sexton et al. 2009). Las poblaciones béticas del margen oriental fueron genéticamente muy similares a las poblaciones centrales y además, constituyeron un grupo genéticamente homogéneo, constituyendo un límite en expansión (*"expanding edge"*). En cambio, las poblaciones disjuntas del margen occidental estuvieron genéticamente muy diferenciadas del resto de las poblaciones béticas y presentaron además una mayor heterogeneidad genética entre ellas, características típicas de un margen de distribución de retaguardia (*"rear or trailing edge"*). Esta distinción genética entre diferentes bordes de distribución de una especie vegetal ha sido propuesta a escala de la distribución geográfica global de las especies, donde la distancia geográfica entre bordes ocurre a escala continental (Hampe and Petit 2005). Sin embargo, nosotros hemos demostrado que la estructuración genética diferencial en distintos bordes de distribución puede producirse igualmente a escalas geográficas más reducidas, como son cada uno de los parches de distribución de las especies vegetales con distribuciones disjuntas. Este tipo de situaciones pueden ser especialmente

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frecuentes en sistemas montañosos que no se vieron afectados drásticamente por los períodos glaciares, como es el caso de las Cordilleras Béticas (Benett et al. 1991; Carrión 2002; Carrión et al. 2003; Médail & Diadema 2009). Este patrón diferencial de estructuración genética puede influir de manera diferente en las dinámicas planta-animal a través de la remezcla de caracteres vía flujo génico, deriva genética y dinámicas de extinción/colonización (Thompson 1994, 2005).

La existencia de una intensa selección natural diferencial podría explicar la fuerte diferenciación genética entre las poblaciones disjuntas del margen occidental frente al resto de poblaciones. Por ello, analizamos los perfiles genéticos de los individuos siguiendo un enfoque genómico poblacional destinado a detectar la presencia de marcadores sujetos a selección entre los marcadores polimórficos usados (Capítulo 2). Nuestros resultados confirmaron la existencia de selección natural diferencial entre ambas regiones. Los loci que mostraron signos de selección natural en las poblaciones disjuntas del margen occidental no mostraron tales signos en el resto de poblaciones, y viceversa. Una vez detectados los loci outliers, se retiraron del análisis de la estructura genética de las poblaciones para discernir cuánto de la diferenciación genética entre los grupos disjuntos de poblaciones se podía atribuir a selección diferencial y cuánto a variación neutral afectada por efectos históricos de deriva y flujo génicos. La fuerte diferenciación genética entre las poblaciones disjuntas del margen occidental y el resto de poblaciones persistió después de retirar los loci outliers del análisis. Por tanto, nuestros resultados confirmaron que tanto la selección natural diferencial como el fuerte aislamiento genético parecen estar contribuyendo a la marcada diferenciación genética entre las poblaciones disjuntas del margen occidental y el resto de poblaciones béticas de *D. laureola*.

El papel de la selección diferencial sobre plantas de distinto sexo en el mantenimiento del polimorfismo sexual en plantas ha sido demostrado en otras especies ginodioicas (Ashman 1999, 2003; Sakai et al. 2007). Nuestros resultados confirmaron un patrón diferencial entre individuos de distinto sexo en ambas regiones (Capítulo 2). Los individuos hermafroditas siempre presentaron un mayor número de loci con frecuencias alejadas del patrón neutral que los individuos hembra.

Estos resultados obtenidos en las poblaciones de *D. laureola* son interesantes por dos motivos. Por un lado, si suponemos que un mayor número de loci bajo selección natural implica una mayor presión selectiva sobre el individuo, entonces los individuos hermafroditas estarían expuestos a una mayor exigencia en términos de selección natural que los individuos hembra. Nuestros resultados sugieren que para los individuos hermafroditas, mantener ambas funciones sexuales durante el período reproductivo, supone un coste que se traduce en un ambiente selectivo más exigente. Por otro lado, la diferencia entre sexos fue más marcada en las poblaciones disjuntas del margen occidental, donde no estaba presente el principal polinizador de la especie en las poblaciones centrales del área de estudio (Capítulo 3). Este resultado confirma por tanto la importancia de estudiar posibles cambios en la interacción planta-polinizador en plantas de distinto sexo en poblaciones disjuntas del margen occidental de *D. laureola*.

Las peculiaridades genéticas y ecológicas de las poblaciones marginales occidentales de D. laureola hacen de ellas buenas candidatas para investigar posibles cambios en las interacciones planta-animal que puedan dar lugar a diferenciación intraespecífica en las poblaciones de esta especie. En primer lugar, debido a su distribución disjunta es esperable que en ellas tengan lugar cambios en las comunidades de animales interactuantes (Lewinsohn et al. 2005; Moeller 2006; Tepedino et al. 2007). En segundo lugar, las poblaciones disjuntas del margen occidental presentan una diversidad genética total similar a las poblaciones centrales por lo que, al menos, la probabilidad de que aparezcan variantes genéticas adaptadas a las condiciones locales debería ser similar a la de las poblaciones centrales continuas (Lesica & Allendorf 1995; Alleaume-Benharira et al. 2006). En tercer lugar, diversos estudios han documentado que el flujo génico desde las poblaciones centrales puede reducir la adaptación local de las poblaciones marginales (García-Ramos & Kirkpatrick 1997; Alleaume-Benharira et al. 2006). Sin embargo, nuestro análisis de la estructura genética de las poblaciones sugiere que el flujo génico entre poblaciones occidentales y centrales debe ser reducido, a lo sumo, a eventos infrecuentes de dispersión a larga distancia. Esta situación contrasta con la de las poblaciones del margen oriental, donde encontramos evidencias de flujo génico extensivo con las poblaciones centrales y por tanto, donde es previsible la entrada de alelos

mal adaptados a las condiciones locales del margen oriental. Por tanto, las poblaciones disjuntas del margen occidental de *D. laureola* constituyen un lugar idóneo para verificar si los cambios en las dinámicas coevolutivas de las interacciones planta-animal son comunes en los márgenes de distribución.

Variación en las Interacciones Planta-Animal en las Poblaciones Marginales Disjuntas

Conforme aumenta la distancia al centro ecológico de la especie (región "core") se espera que las poblaciones estén expuestas a unas condiciones más alejadas del óptimo ecológico de la especie (Brussard 1984; Hoffmann and Blows 1994; Sexton et al. 2009). Sin embargo, el análisis comparativo de los rasgos poblacionales e individuales en poblaciones centrales y marginales disjuntas de *D. laureola* reveló un patrón de mayor complejidad, más que apoyar simplemente un mayor estrés ecológico hacia los márgenes de distribución (Brussard 1984; Hoffmann and Blows 1994; Sexton et al. 2009). Este resultado está en la misma línea que los obtenidos para otras especies vegetales (Sagarin & Gaines 2002; Herlihy and Eckert 2005; Kluth & Bruelheide 2007; Moore 2009). Las poblaciones marginales disjuntas de *D. laureola* estaban compuestas por un mayor número de individuos y tenían una densidad similar de individuos reproductivos que las poblaciones centrales continuas. Únicamente el reemplazamiento de individuos en las poblaciones (*"population turnover"*) fue mayor en poblaciones marginales disjuntas, lo que frecuentemente se ha interpretado como una evidencia de mayor estrés ecológico en los márgenes de distribución (Lammi et al. 1999). Sin embargo, este mayor reemplazamiento de individuos no se tradujo en menor densidad, menores tamaños poblacionales, ni menor diversidad genética media en dichas poblaciones. Por lo tanto, no hay evidencias de que este mayor reemplazamiento de los individuos en las poblaciones marginales estudiadas esté ligado a un mayor estrés ecológico en estas poblaciones.

Cuando se consideraron rasgos individuales de las plantas, nuestros resultados revelaron un mayor tamaño e inversión reproductiva en los individuos de las poblaciones centrales continuas. Este resultado ha sido tradicionalmente interpretado como una evidencia de mayor estrés ecológico en los márgenes de distribución (Hengeveld & Haeck 1982; Brown 1984; Gaston 2009). Sin

embargo otros resultados de este estudio, junto con los de investigaciones anteriores sobre la especie, aconsejan una interpretación más cautelosa. Previos estudios llevados a cabo en la región central demostraron que el principal polinizador de la especie es *Meligethes elongatus* un pequeño coleóptero que se alimenta de polen (Alonso 2004). Este coleóptero se desplaza principalmente entre flores del mismo individuo promoviendo la geitonogamia (transferencia de polen propio entre flores distintas de un mismo individuo). El elevado nivel de endogamia en la descendencia de los individuos hermafroditas, unido a una fuerte depresión por endogamia, sugieren que los individuos hembra se mantienen en las poblaciones gracias a su capacidad para evitar la endogamia vía fecundación cruzada obligatoria (Medrano et al. 2005).

En el capítulo 3 encontramos un cambio en la identidad del principal polinizador de *D. laureola* y una reducción del despliegue floral de los individuos en las poblaciones disjuntas del margen occidental. En lugar de *M. elongatus*, diversas especies de Himenópteros (e.g. *Bombus terrestris, Xylocopa violacea, Apis mellifera*) aparecen como los principales polinizadores de la especie en las poblaciones marginales disjuntas. Estos cambios en el ambiente de polinización podrían alterar el nivel de endogamia en la descendencia de los individuos hermafroditas y, por tanto, tener importantes consecuencias para el mantenimiento del polimorfismo sexual. En esta línea encontramos que la eficiencia de la polinización fue mayor en las poblaciones marginales disjuntas, donde una mayor proporción de flores produciendo frutos fue encontrada en sus individuos (Capítulo 3). Por tanto, el cambio en la fauna de polinizadores hacia una comunidad más diversa, junto con una posible disminución de la geitonogamia vía reducción del despliegue floral de los individuos, podrían estar mejorando la eficiencia de la polinización en las poblaciones marginales disjuntas de *D. laureola* (Klinkhamer & de Jong 1993).

El cambio en la identidad del polinizador principal en poblaciones marginales disjuntas estuvo relacionado con variaciones en el éxito de la polinización de los individuos tanto cuantitativamente como cualitativamente (Capítulo 4). Los polinizadores de las poblaciones centrales visitaron con mayor frecuencia y depositaron mayores cargas polínicas en las flores de los individuos her-

Discusión

mafroditas. En las poblaciones marginales disjuntas, los polinizadores no mostraron preferencia o discriminación por las flores de distinto sexo. Además depositaron cargas de polen mayores en individuos de ambos sexos en comparación con los de las poblaciones centrales. Por tanto, desde el punto de vista cuantitativo, los polinizadores de las poblaciones marginales disjuntas de *D. laureola* fueron más eficaces que *M. elongatus* en las poblaciones centrales. Este resultado es contrario a la tendencia esperada, y encontrada en diversos estudios, de una menor deposición de polen en poblaciones marginales debido a un peor servicio de polinización (Moeller 2006; Etcheverry et al. 2008). Por otro lado, la diferencia de calidad en el polen recibido por individuos de distinto sexo no fue igual en ambas regiones. En las poblaciones centrales los individuos hembra recibieron polen de mayor calidad que los individuos hermafroditas. Sin embargo, en las poblaciones marginales disjuntas individuos hembra y hermafrodita recibieron polen de calidad similar. Estudios anteriores han mostrado que la permanencia de individuos hembra en poblaciones de *D. laureola* parece relacionarse con ventajas de tipo cualitativo (p.ej. mayor vigor en su descendencia debido a que es producida por fecundación cruzada) que de tipo cuantitativo (p.ej. mayor producción de semillas en plantas hembra) (Alonso & Herrera 2001; Medrano et al. 2005). Por tanto, la ausencia de diferencias en la calidad del polen recibido por hembras y hermafroditas en las poblaciones marginales disjuntas podría estar contribuyendo al mantenimiento del polimorfismo sexual de la especie en dicha región. En esta dirección, es interesante señalar que en el margen occidental se ha detectado una reducción en la frecuencia poblacional de hembras, llegando incluso a existir poblaciones compuestas exclusivamente por individuos hermafroditas (Alonso et al. 2007). Estas diferencias en la divergencia de calidad del polen recibido por individuos de distinto sexo en poblaciones centrales y marginales no se tradujeron en una menor proporción de flores produciendo frutos, ni en menores tamaños poblacionales, ni tampoco en densidades de individuos dentro de las poblaciones. En este sentido, nuestros resultados parecen sugerir una posible reducción de la depresión por endogamia en poblaciones marginales disjuntas de *D. laureola*, como ha sido mostrado recientemente para otras especies vegetales (Pujol et al. 2009; Levin 2010). Sin embargo, una demostración rigurosa de esta hipótesis requiere una comparación de las tasas de fecundación cruzada en individuos adultos y en su descendencia.

Los cambios en la composición de la comunidad local de polinizadores pueden tener consecuencias sobre la variación intraespecífica de los rasgos florales (Herrera et al. 2006; Harder & Johnson 2009; Gómez & Perfectti 2010) y los resultados de esta Tesis Doctoral apoyan esta predicción para *D. laureola* (Capítulo 4). El análisis de selección fenotípica señaló a los polinizadores de las poblaciones centrales como agentes que contribuyen al dimorfismo sexual en la longitud del tubo del periantio. Detectamos una variación geográfica en el dimorfismo sexual de este rasgo floral conectada a una selección diferencial por polinizadores en poblaciones centrales y marginales disjuntas de *D. laureola*. Los polinizadores seleccionaron flores con tubos más largos sólo en los individuos hermafroditas de las poblaciones centrales, donde por otro lado, el dimorfismo sexual de este rasgo floral fue mayor. Por tanto, nuestros resultados apoyaron la predicción general planteada al inicio de esta Tesis Doctoral de que los márgenes de distribución locales de las especies son áreas proclives a la aparición de cambios en las dinámicas evolutivas de las interacciones planta-polinizador.

Los rasgos vegetativos de las especies ginodioicas, a diferencia de los rasgos florales, no suelen exhibir dimorfismo sexual y cuando lo presentan suele ser menos marcado (Ashman 2005). En esta línea, no encontramos que ninguno de los rasgos vegetativos estudiados presentase dimorfismo sexual en *D. laureola* (Capítulo 5). Tampoco hallamos evidencias de un papel relevante de los herbívoros promoviendo el dimorfismo sexual en tales rasgos o la diferenciación de los mismos entre las poblaciones centrales y marginales disjuntas de esta especie (Capítulo 5). El patrón de herbivoría en los individuos fue consistente en el tiempo, pero sólo afectó a la fitness materna de los individuos de las poblaciones disjuntas del margen occidental, independientemente de su sexo. De acuerdo con esto, esperábamos una selección fenotípica por herbívoros de mayor intensidad en las poblaciones marginales. Además apoyando esta idea, encontramos una importante diferenciación regional en los rasgos vegetativos estudiados. Sin embargo, nuestros resultados no apoyaron que las diferencias en el nivel de herbivoría experimentado por los individuos guardasen relación con su nivel de similaridad genética. Además, los patrones de selección fenotípica por herbívoros sobre los rasgos vegetativos, cuando existieron, no fueron congruentes con la diferenciación regional en dichos rasgos. Por tanto, nuestros resultados sugieren que tanto las diferencias de herbivoría entre

individuos como la diferenciación en los rasgos vegetativos podrían ser resultado de variación en las condiciones abióticas tanto dentro como entre las poblaciones de *D. laureola*. La diferenciación regional en los rasgos vegetativos podría ser consecuencia de un cambio en la especie dominante del dosel arbóreo. Las poblaciones disjuntas del margen occidental estudiadas se localizan en el sotobosque de pinsapares. En comparación con los pinares del margen oriental, los pinsapares del margen occidental forman una cobertura muy cerrada que reduciría mucho la entrada de luz al sotobosque (Dudt and Shure 1994; Meziane & Shipley 1999; Barber & Marquis 2011). Sin descartar otras posibilidades, este cambio en el régimen lumínico podría estar detrás de la variación en rasgos vegetativos tales como la concentración de fenoles de las hojas o el área específica foliar.

Futuras Líneas de Trabajo

Esta Tesis Doctoral representa un intento de localizar áreas en la distribución natural de una especie donde se esperan cambios en las dinámicas coevolutivas de las interacciones planta-animal, así como de comprender los mecanismos responsables de tales cambios. Sin embargo, somos conscientes de las limitaciones de nuestro trabajo. Unas veces porque no pudieron ser tenidos en cuenta otros factores selectivos potenciales por limitaciones de tipo práctico, y otras veces por la aparición de nuevos interrogantes a medida que profundizamos en el conocimiento del sistema de estudio, quedan áreas inexploradas que merecen consideración en un futuro.

Una de estas líneas de estudio podría ir encaminada a analizar si las diferencias en la calidad del polen que reciben individuos de distinto sexo en poblaciones centrales y marginales de *D. laureola* son debidas a una mayor estructuración genética a escala intrapoblacional en las poblaciones marginales. Un análisis del parentesco genético de los individuos basado en marcadores moleculares podría verificar la predicción anteriormente mencionada. En este mismo sentido, un estudio de los mecanismos de selección post-polinización en el estilo basados en parentesco (p.ej. duración del período receptivo de los estigmas, competencia espermática en el estilo) podría aclarar si las flores de individuos hembra y hermafroditas presentan distintas capacidades selectivas.

Por otro lado, los resultados de esta tesis parecen sugerir una reducción del nivel de depresión por endogamia en las poblaciones marginales disjuntas de *D. laureola*. Esta hipótesis podría verificarse con relativa facilidad mediante el estudio comparado de las tasas de cruzamiento de individuos adultos parentales y su descendencia en estas poblaciones (Ritland 1990).

Finalmente, el papel de otras especies animales interactuantes debería ser evaluado en estudios adicionales. Por ejemplo, el efecto de los vertebrados depredadores y dispersantes de semillas sobre el reclutamiento y los patrones de estructuración genética en poblaciones centrales y marginales de *D. laureola*. Finalmente, así como el impacto de virus y hongos patógenos sobre las poblaciones también, merecerían ser estudiados a lo largo del mismo marco geográfico considerado en esta Tesis Doctoral. En este trabajo encontramos que la mortalidad de individuos fue mayor en poblaciones marginales, aunque esto parece estar contrarrestado por una mayor cantidad de reclutantes. La causa de la muerte parece guardar relación con un agente patógeno. De hecho los síntomas previos a la muerte coincidieron con los descritos para otras especies del género *Daphne* atacadas por el virus del mosaico de Daphne (Fránová et al. 2006). Ya que el genoma de este virus está secuenciado (Petrzik & Fránová 2006), un primer paso sería intentar identificar a través de técnicas moleculares si el agente patógeno de *D. laureola* es efectivamente el virus del mosaico del Daphne y evaluar el impacto real de este patógeno en poblaciones de la especie. El estudio de las interacciones planta-patógeno ha sido mostrado como un modelo experimental particularmente adecuado para el análisis de la teoría del mosaico geográfico de coevolución, por lo que la propuesta anterior podría abrir una prometedora línea de investigación en un futuro.

Conclusiones

A continuación se enumeran las principales conclusiones obtenidas durante el desarrollo de la presente Tesis Doctoral:

1. Las poblaciones de *D. laureola* del sur de la Península Ibérica muestran una reducción en la abundancia de localidades óptimas para la especie hacia los márgenes de distribución, más que un alejamiento progresivo del óptimo ecológico. Esta menor abundancia de localidades óptimas se traduce en un mayor aislamiento geográfico de las poblaciones marginales, que es más pronunciado en las poblaciones del margen disjunto occidental.

2. Las diferencias en el grado de aislamiento geográfico en los márgenes de distribución oriental y occidental dan lugar a dos bordes completamente distintos desde el punto de vista genético. Un margen oriental genéticamente homogéneo y similar a las poblaciones centrales y un margen occidental muy diferenciado genéticamente del resto de poblaciones y con mayor heterogeneidad genética interna.

3. El estudio basado en marcadores moleculares reveló que las poblaciones del margen disjunto occidental muestran signos de selección diferencial con respecto al resto de poblaciones.

4. Los individuos hermafroditas presentaron un mayor número de loci bajo selección purificadora que los individuos hembra, siendo esta diferencia más acusada en las poblaciones del margen disjunto occidental.

5. Las diferencias entre individuos en niveles de herbivoría no tienen una base genética, al menos en relación con los marcadores moleculares empleados, y parecen estar más relacionadas con variación ambiental dentro de las poblaciones. Del mismo modo, la diferenciación regional en los rasgos ve-

getativos parece estar relacionada con variación en el ambiente lumínico del sotobosque debido a cambios en la composición del dosel arbóreo.

6. El principal polinizador de la especie en la región de estudio está ausente en las poblaciones del margen disjunto occidental. Sin embargo, el ambiente de polinización en estas poblaciones parece ser más eficiente ya que sus individuos presentan una mayor proporción de flores produciendo frutos y menores despliegues florales que podrían reducir la geitonogamia en dichas poblaciones.

7. A pesar de contar con un servicio de polinización más eficiente, los individuos hembra de las poblaciones del margen disjunto occidental reciben polen de calidad similar al recibido por los individuos hermafroditas. Esta situación contrasta con la de poblaciones centrales donde las hembras reciben polen de calidad superior. Estas diferencias entre sexos en la calidad del polen recibido podrían tener consecuencias importantes para el mantenimiento del polimorfismo sexual de la especie.

8. Los polinizadores en poblaciones centrales podrían contribuir a mantener las diferencias regionales en la magnitud del dimorfismo sexual floral, seleccionando positivamente flores con tubos más largos solo en hermafroditas de poblaciones centrales.

9. Aunque hay tres loci bajo selección purificadora relacionados con los rasgos fenotípicos estudiados, para el resto de loci bajo selección purificadora no hemos encontrado relación alguna. Por tanto, otros factores selectivos no considerados podrían estar contribuyendo a la diferenciación genética entre las poblaciones del margen disjunto occidental y el resto de poblaciones del sur de la Península Ibérica.

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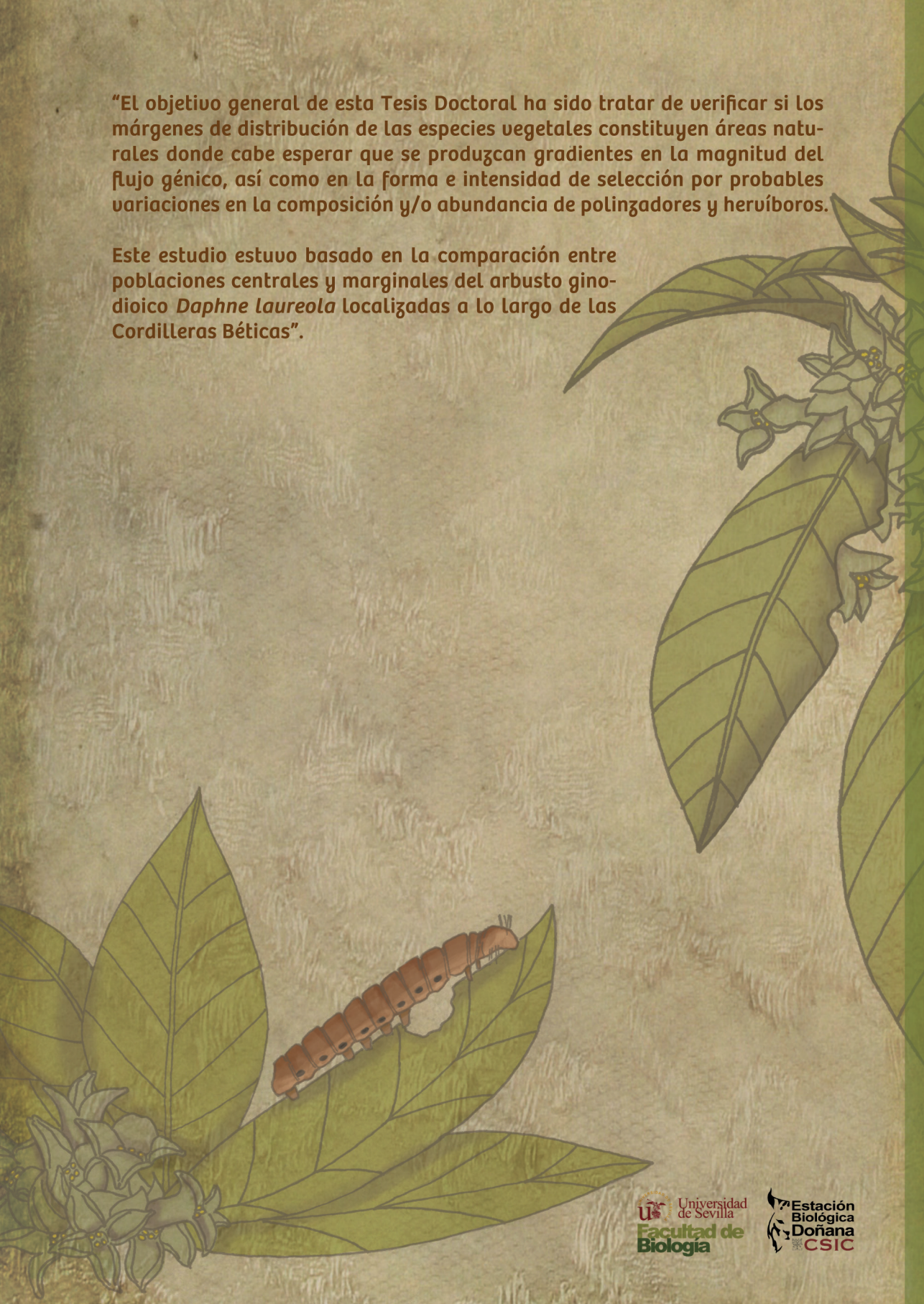
"El objetivo general de esta Tesis Doctoral ha sido tratar de verificar si los márgenes de distribución de las especies vegetales constituyen áreas naturales donde cabe esperar que se produzcan gradientes en la magnitud del flujo génico, así como en la forma e intensidad de selección por probables variaciones en la composición y/o abundancia de polinzadores y herviboros.

Este estudio estuvo basado en la comparación entre poblaciones centrales y marginales del arbusto ginodioico *Daphne laureola* localizadas a lo largo de las Cordilleras Béticas".



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