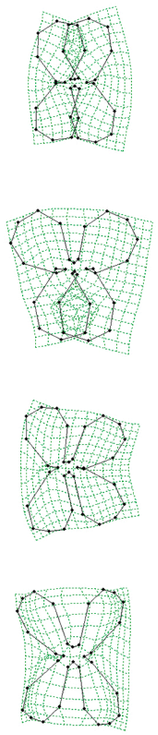

Department of Genetics
Department of Ecology

ugr Universidad de Granada

How species are evolutionarily maintained?
Pollinator-mediated divergence and hybridization in *Erysimum mediohispanicum* and *E. nevadense*

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HOW SPECIES ARE EVOLUTIONARILY MAINTAINED? POLLINATOR-MEDIATED DIVERGENCE AND HYBRIDIZATION IN *ERYSIMUM MEDIOHISPANICUM* AND *E. NEVADENSE*

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Mohamed Abdelaziz Mohamed

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HOW SPECIES ARE EVOLUTIONARILY MAINTAINED? POLLINATOR-MEDIATED DIVERGENCE AND HYBRIDIZATION IN *ERYSIMUM MEDIOHISPANICUM* AND *E. NEVADENSE*

Memoria que el Licenciado Mohamed Abdelaziz Mohamed presenta para aspirar al Grado de Doctor por la Universidad de Granada

Esta memoria ha sido realizada bajo la dirección de:
Dr. José María Gómez Reyes y Dr. Francisco Perfectti Álvarez

Ldo. Mohamed Abdelaziz Mohamed

Aspirante al Grado de Doctor

Granada, enero de 2013

Dr. José María Gómez Reyes, Catedrático de Ecología de la Universidad de Granada **y Dr. Francisco Perfectti Álvarez**, Profesor Titular de Genética de la Universidad de Granada

CERTIFICAN

Que los trabajos de investigación desarrollados en la Memoria de Tesis Doctoral: "*How species are evolutionarily maintained? Pollinator-mediated divergence and hybridization in Erysimum mediohispanicum and E. nevadense***", son aptos para ser presentados por el Ldo. Mohamed Abdelaziz Mohamed ante el Tribunal que en su día se designe, para aspirar al Grado de Doctor por la Universidad de Granada.**

Y para que así conste, en cumplimiento de las disposiciones vigentes, extendemos el presente certificado a 16 de enero de 2013

Dr. José María Gómez Reyes **Dr. Francisco Perfectti Álvarez**

Durante el tiempo de realización de esta Tesis Doctoral he disfrutado de una Beca del Programa Nacional de Formación de Personal Investigador del Ministerio de Educación y Ciencia Ref. (BES–2007–14206).

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Felicidad.

(Del lat. *felicĭtas, -ātis*)

Estado del ánimo que se complace en la posesión de un bien.

Real Academia Española
Diccionario 22ª edición

...y que luego digan que el conocimiento no tiene ideología...

"Los animales se emplean en un esfuerzo por la existencia; por los recursos, por evitar ser comidos y reproducirse. Los factores ambientales influencian a los organismos a desarrollar nuevas características que aseguren su supervivencia, transformándolos así en nuevas especies. Los animales que sobreviven hasta reproducirse pueden pasar sus características exitosas a su descendencia."

Libro de los Animales
Abu Uthman Amr ibn Bahr al-Kinani al-Fuqaimi al-Basri, Al-Jahiz
(781 - h. 869)

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Resumen

En la presente tesis doctoral se aborda el estudio de ciertos procesos evolutivos que están involucrados en la generación y mantenimiento de las especies. Hemos explorado el estímulo evolutivo resultante de la interacción antagonista entre procesos que promueven la homogenización y otros que favorecen la divergencia entre linajes evolutivos próximos. En especial hemos atendido al papel de los polinizadores en promover la divergencia o la hibridación entre plantas. A la vez exploramos el uso de métodos cuya combinación podría ayudar a la identificación de especies crípticas en grupos en los que la evolución ha generado patrones filogenéticos de complejo encuadre taxonómico. Para ello hemos usado especies del género *Erysimum* L. (Brassicaceae) presentes en España y el norte de África. Las especies norteafricanas se usaron para poner a prueba técnicas útiles para la detección de especies crípticas. Sin embargo, el resto de la presente memoria de tesis se centra en el estudio de una zona de contacto entre *E. mediohispanicum* Polatschek y *E. nevadense* Reut. en Sierra Nevada (SE España), mediante la combinación de estudios observacionales y experimentales. Dicha zona de coexistencia se ha estudiado mediante una aproximación multidisciplinar que integra análisis ecológicos, genéticos y filogenéticos, con el fin de comprender cuáles son los mecanismos que promueven la hibridación entre ambas especies y cuáles la previenen. Así pues, en el ***capítulo 1*** confirmamos la existencia de especies crípticas en el género *Erysimum*. Mediante la combinación de técnicas moleculares, métodos cuantitativos basados en el análisis de caracteres complejos y métodos morfológicos de la taxonomía estándar, conseguimos mejorar la capacidad de delimitar especies con marcadas similitudes morfológicas. Esto nos permitió, no solo delimitar especies crípticas, sino también describir una nueva especie endémica de las montañas del Rif, *Erysimum riphaeanum* sp.nov. En el ***capítulo 2*** abordamos el análisis de las relaciones filogenéticas de todas las especies que habitan los sistemas Béticos. Dicho análisis sugierenque *E. mediohispanicum* y *E. nevadense* no son especies hermanas, lo cual convierte las zonas en las que coinciden en contactos secundarios. El ***capítulo 3*** se dedica a la cuantificación en condiciones controladas de las tasas de limitación de polén autógamo, depresión por endogámia y depresión por exogámia que

E. mediohispanicum y *E. nevadense* presentan. Esto nos ayudó a entender mejor los sistemas reproductivos de ambas especies, así como a inferir el efecto sobre estos sistemas reproductivos de posibles fenómenos de adaptación local. Por otro lado, se demuestra que ambas especies son inter-fértiles, aunque presentaron ciertas tasas asimétricas de inviabilidad híbrida. En el ***capítulo 4*** se analizan las correlaciones existentes entre caracteres fenotípicos y las tasas de depresión por endogamia individuales que presentan las plantas de *E. mediohispanicum*. Se encontró que el diámetro de la corola, uno de los caracteres que definen el tamaño de la flor, está asociado a mayor tolerancia a endogamia. Dicho carácter a su vez se correlacionó con los niveles de heterocigosidad en un grupo independiente de plantas que se analizaron en el campo. Ésto indica que dicho carácter podría estar relacionado con eventos de reproducción clasificada, incrementando la tolerancia de determinados individuos a la reproducción endógama. En el ***capítulo 5*** se utilizan diez marcadores microsatélites para analizar las características genéticas de las poblaciones de estudio. Se confirmó la existencia de una zona híbrida entre ambas especies. Además exploramos los patrones de estructuración y diferenciación genética, las tasas de flujo génico, y los tamaños efectivos de las poblaciones que conforman el contacto secundario. En el ***capítulo 6*** se abordó el estudio de los polinizadores como posibles barreras reproductivas pre-zigóticas y post-zigóticas. Para ello, analizamos experimentalmente las preferencias de los diferentes polinizadores por plantas parentales e híbrida, tanto en habitats parentales como en la zona híbrida. Así confirmamos que los polinizadores no actúan como barreras impermeables a la reproducción inter-específica. En el ***capítulo 7*** se presenta el cálculo de las heredabilidades para diversos caracteres relacionados con el tamaño de la planta, el tamaño de la flor y la forma floral en *E. mediohispanicum*. La heredabilidad fue significativa para la mayoría de los caracteres estudiados, confirmándose la capacidad de respuesta a presiones selectivas. Además en ese capítulo se aborda el estudio de las correlaciones genéticas entre dichos caracteres. Finalmente, en el ***capítulo 8*** estudiamos durante dos años consecutivos la selección fenotípica en las poblaciones parentales que conforman el contacto secundario, así como en la zona híbrida.

Por otro lado, se cuantificó la seleción divergente entre *E. mediohispanicum* y *E. nevadense*. El carácter que presentó un gradiente de selección más intenso en la zona híbrida fue un carácter asociado al vigor híbrido, el número de flores. Existieron caracteres sometidos a presiones selectivas divergentes entre *E. mediohispanicum* y *E. nevadense*, tanto a nivel poblacional como específico. En conclusión, todos estos procesos y mecanismos que ocurren en una zona de contacto entre dos especies como *E. mediohispanicum* y *E. nevadense*, no sólo explicarían los patrones de flujo génico que impedirían la divergencia entre especies, sino por qué ambas especies siguen siendo aun hoy diferentes.

Introducción General

El proceso de especiación, sus causas y consecuencias, ha recibido una gran atención por un amplio grupo de biólogos evolutivos, llegando a generarse debates muy productivos en torno a los mecanismos involucrados en dicho proceso (véase, Otte & Endler, 1989; Coyne & Orr, 2004, y las referencias en ellos incluidas, entre otros). Especial mención merecen las investigaciones llevadas a cabo sobre especiación ecológica, entendiendo la misma como el proceso por el que evolucionan barreras al flujo génico entre poblaciones como resultado de la adaptación a diferentes ambientes (Schluter, 2001; Rundle & Nosil, 2005; Funk *et al*., 2006). En este sentido han destacado, por un lado, los estudios sobre el éxito biológico (en adelante fitness) diferencial de diferentes fenotipos a lo largo de ambientes divergentes, y, por otro, los trabajos que exploran el aislamiento reproductivo que sufren dos especies que están divergiendo evolutivamente (Schluter, 1993; Hendry *et al*., 2007; Bomblies, 2010; The Marie Curie SPECIATION Network, 2012, entre otros). De este modo, tanto el cuerpo teórico generado como las evidencias empíricas obtenidas resaltan la importancia de las presiones selectivas divergentes y de los fenómenos de adaptación local como generadores de los primeros pasos de la diversificación (Gavrilet, 2004; Via, 2009), como el papel crucial del aislamiento reproductivo para la consolidación y mantenimiento de los grupos recién divergidos (Ramsey *et al*., 2003; Nosil *et al*., 2005; Lowry *et al*., 2008).

A este respecto, han sido bastantes los trabajos que, centrándose a nivel poblacional, han abordado el estudio de la divergencia evolutiva en presencia de flujo génico (e. g., Smith *et al*., 2001; Michalak *et al*., 2001; Pressoir & Berthaud, 2004; Rice *et al*., 2011, entre otros). Sin embargo, a pesar de la importancia del nivel poblacional, cada vez son más los autores que abordan el estudio de la especiación ecológica y del aislamiento reproductivo desde un punto de vista inter-específico (Greenberg *et al*., 2003; McKinnon *et al*., 2004; Rosemblum, 2006; Savolainen *et al*., 2006; Papadopoulos *et al*., 2011; Rice *et al*., 2011). Así pues, son las intensidades de las presiones selectivas de carácter divergentes y de las tasas de flujo génico las que modelan un continuo con dos extremos. En un extremo tendríamos una elevada tasa de flujo génico

(por ejemplo, migración o reproducción cruzada) que impediría la adaptación local promovida por la selección divergente y homogenizaría las poblaciones, colapsando la divergencia entre grupos (Slatkin, 1987; Kirkpatrick & Barton, 1997; Lenormand, 2002; Rosenblum, 2006). En el extremo opuesto habría tasas de selección divergentes que anularían el efecto homogenizador del flujo génico, permitiendo la divergencia evolutiva de los grupos (Turrelli *et al*., 2001; Doebeli & Dieckmann, 2003; Rosenblum, 2006). De ahí que explorar las interacciones entre poblaciones de especies cercanas, nos permitirá cuantificar y valorar los mecanismos que favorecen y/o anulan la divergencia evolutiva y la especiación.

En un contexto de especiación ecológica, bajas tasas de flujo génico incrementan la probabilidad de desarrollo de barreras reproductivas eficientes (Coyne & Orr, 2004), que disminuyen la capacidad de fecundación cruzada efectiva, favoreciendo el efecto divergente de la selección pudiendo desembocar en eventos de especiación. Sin embargo, las poblaciones divergentes o las especies incipientes no tienen por qué mantenerse aisladas reproductivamente durante todo el proceso de especiación, pudiendo entrar en contacto o experimentar episodios de flujo génico de forma más o menos continuada durante dicho período. Cuando esto es así, son varios los escenarios que podemos llegar a encontrar en condiciones naturales (Fig. i.1).

El primer escenario vendría caracterizado por unas tasas de flujo génico tan altas que no permitirían el desarrollo de barreras de aislamiento reproductivo eficientes (Fig. i.1). Generalmente, en este escenario se da una homogenización continua de las poblaciones que intercambian genotipos, aunque éstas estén sometidas a selección divergente (Slatkin, 1987; Coyne & Orr, 2004). En el caso en que el efecto de las presiones selectivas divergentes fuera mayor que el efecto homogenizador del flujo génico, aun existiría la posibilidad de que se diera especiación. Este proceso se denomina especiación en presencia de flujo génico (Turrelli *et al*., 2001; Coyne & Orr, 2004), llegándose recientemente a asociar a casos muy paradigmáticos de especiación simpátrica (Savolainen *et al*, 2006; Papadopoulos *et al*., 2011). Aunque la situación más

clara en el que podría darse este escenario sería en simpatría, hay que tener en cuenta que la divergencia en presencia de flujo génico puede darse también entre poblaciones o taxones que estén en alopatría (con eventos temporales o accidentales de flujo) o más frecuentemente en parapatría (Coyn 2004).

Otro escenario posible sería el que tendría lugar entre dos poblaciones que desarrollaron ciertos niveles de aislamiento reproductivo (por ejemplo, tras haberse anulado temporalmente el flujo génico en alopatría), pero que aún preservan la capacidad de reproducirse. En este caso, cuando los taxones se encuentren puede ocurrir que una de las especies incipientes esté mucho más favorecida por la selección que la otra especie o sus híbridos en la zona de contacto, en cuyo caso se podría dar fenómenos de introgresión que llegaran incluso a extinguir genéticamente a la otra especie.(Rhymer & Simberloff, 1996; Levin *et al*., 1996; Allendorf *et al*., 2001).

Por otro lado podrían darse fenómenos de especiación híbrida, que consistirían en que los híbridos se aíslan ecológica y/o genéticamente de las especies parentales generando un linaje independiente (Soltis & Soltis, 2009). Por último, puede aparecer una zona híbrida. Si los mecanismos de aislamiento reproductivo desarrollados no son muy eficientes, las altas tasas de reproducción inter-específica generarían una zona híbrida unimodal (Fig. i.1, B). Ésta se caracteriza por una alta frecuencia de individuos cuyos genomas están formados por una mezcla de los genomas de los dos grupos taxonómicos que hibridan (Jiggins & Mallet, 2000). Esta zona híbrida podría mantenerse porque las formas parentales estén favorecidas en los extremos de la distribución del transecto entre ambas especies, generado un gradiente en los principales caracteres responsables de la divergencia (Endler, 1977). Sin embargo, puede ocurrir que dicho gradiente no exista y que los híbridos estén desfavorecidos incluso en la zona híbrida, a pesar de lo cual dicha zona se mantendría gracias al aporte continuo de individuos desde las poblaciones parentales, en cuyo caso a la zona híbrida se la conoce como zona de tensión (Barton & Hewitt, 1985).

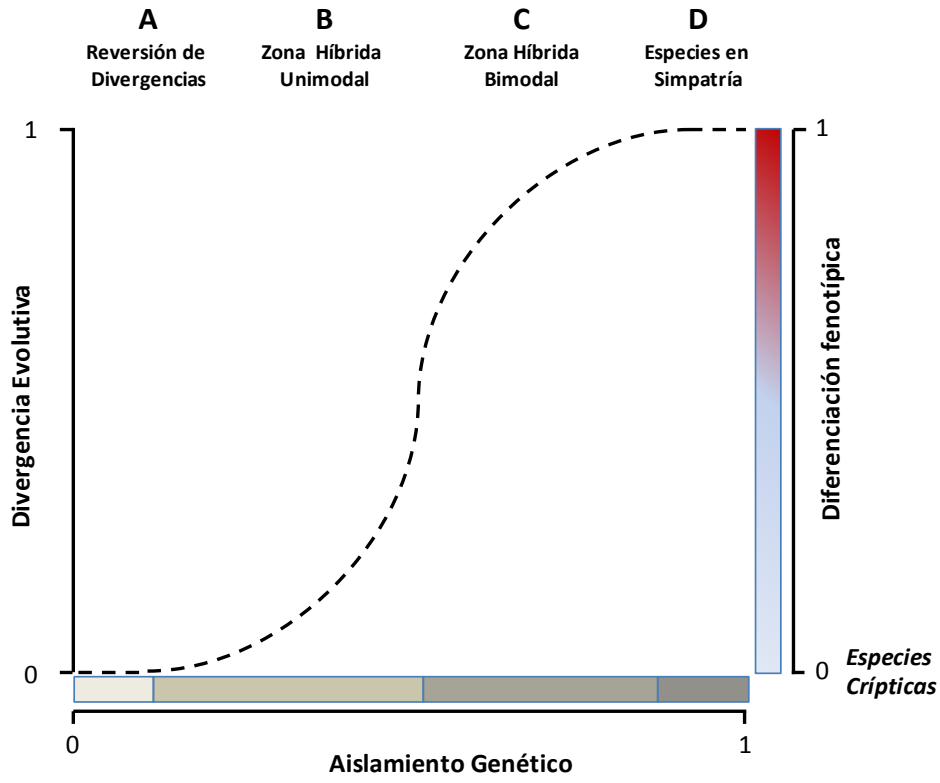

Figura i.1. Posibles escenarios que resultan del contacto entre poblaciones durante el proceso de divergencia evolutiva. La intensidad de flujo génico entre las poblaciones afectará a las tasas de divergencia y la diferenciación fenotípica. Menor flujo génico (mayor aislamiento genético) permite mayores tasas de divergencia evolutiva, pudiendo resultar en especiación . La aparición de especies crípticas dependerá del grado de diferenciación fenotípica que las especies generadas hayan acumulado durante dicho proceso. Cero y uno representan los respectivos mínimos y máximos teóricos de los procesos representados.

En caso de que la divergencia evolutiva haya sido suficiente para permitir el desarrollo de barreras de aislamiento reproductivo más eficientes que en el caso anterior, pero no totalmente impermeables, lo que se generará en un contacto entre dos especies incipientes será una zona híbrida bimodal (Fig. i.1, C) (Harrison & Bogdanowicz, 1997; Jiggins and Mallet, 2000). Este tipo de zonas híbridas se caracterizan porque la mayor parte de los individuos que la componen presentan genotipos adscribibles a una de las especies

parentales, apareciendo híbridos en muy baja frecuencia. La separación entre zonas híbridas unimodales y bimodales no es discreta, pudiendo encontrarse diferentes estados que representan un tránsito entre ambas (Jiggins & Mallet, 2000).

Por último, cuando dos especies han completado el proceso de especiación, desarrollando barreras de aislamiento reproductivo impermeables, podrán aún interactuar ecológicamente pero no genéticamente incluso cuando están en simpatría (Fig. i.1, D).

En todo ese tránsito, desde poblaciones pertenecientes a la misma especie hasta especies en la que la divergencia evolutiva se ha completado, las unidades divergentes pueden ir reflejando dicha separación en su fenotipo. De este modo, y dependiendo de la intensidad de las presiones selectivas y del número de caracteres sometidos a las mismas (responsables de dicha divergencia), la especiación generará dos unidades evolutivas independientes que se habrán diferenciado en mayor o menor grado en su fenotipo (Fig. i.1). Sin embargo, podemos encontrarnos en la situación en la que dos (o más) especies que han completado su separación sean consideradas, erróneamente, la misma especie. Esto puede deberse a varias causas, entre las que destacan que los caracteres que han divergido no sean considerados de interés taxonómico, que se hayan dado procesos de evolución convergente o que las especies estén sometidas a presiones selectivas que promuevan la estasis morfológica (Bickford *et al.*, 2007). Éstas especies indiferenciadas fenotípicamente se denominan especies crípticas (Grant, 1981), pudiendo presentar un reciente origen común o no (Bickford *et al.*, 2007). Además estas especies crípticas pueden involucrar a más de dos especies dando lugar a lo que se conoce como complejo (o enjambre) de especies (Grant, 1981).

La identificación y delimitación de especies crípticas es esencial a la hora de abordar estudios sobre las mismas, ya que su falsa identificación complica y confunde cualquier esfuerzo en explorar cuestiones biológicas que las ataña. Por otra parte, la adecuada identificación de especies crípticas es de gran importancia para el desarrollo de adecuadas estrategias de conservación

(Schönrogge *et al*., 2002). En este sentido, las técnicas moleculares aplicadas a los análisis filogenéticos han demostrado sin duda ser muy útiles a la hora de identificar especies cuando éstas no son fáciles de delimitar morfológicamente (Knowles & Bryan, 2007; Judd *et al*., 2008). Esto añade una información indispensable para abordar el estudio de los fenómenos (presiones selectivas, flujo génico, etc.) que se pueden observar en las zonas en las que se da la divergencia o el contacto de especies y nos ayuda a comprender cuáles y cómo han sido los mecanismos de aislamiento que se erigieron entre dichas especies. Sin embargo, estas técnicas de filogenias moleculares no son la panacea, ya que pierden eficiencia cuando hay involucrados clados hermanos de evolución reciente o linajes con separación incompleta (Maddison & Knowles, 2006; Rubinoff *et al*., 2006; Bickford *et al*., 2007). En este sentido, el uso de técnicas que nos permitan el manejo de caracteres fenotípico complejos nos ayudará no solo a la delimitación, sino al estudio de los procesos evolutivos a los que las especies están siendo sometidas (Roy & Foote, 1997).

El estudio de las barreras reproductivas entre especies sirve para determinar los mecanismos involucrados en la prevención del flujo génico (Arnold, 2006). Los híbridos pueden presentar alguna desventaja biológica intrínseca, como la inviabilidad o esterilidad, o extrinseca, como disminución en fitness (Schluter, 1996; Arnold, 2006), o por el contrario pueden presentar alguna ventaja biológica, como el vigor híbrido (Rieseberg *et al*., 1999). Las barreras reproductivas generadas pueden ser simétricas, cuando el éxito reproductivo híbrido es independiente de si una especie actúa como macho o hembra (Tiffin *et al*., 2000; Ramsey *et al*., 2007), o asimétricas, cuando existen diferencias reproductivas dependientes de si unas de las especies esté actuando como macho o hembra. Estas barreras reproductivas asimétricas pueden llegar a afectar a los fenómenos de introgresión génica, favoreciéndola en uno de los sentidos (Anderson & Hubricht, 1938; Arnold, 2006).

En Angiospermas el principal mecanismo de aislamiento reproductivo son los polinizadores (Grant, 1981). Éstos, mediante sus diferentes preferencias florales, actúan como barreras pre-zigóticas a la reproducción inter-específica,

generando lo que se conoce como aislamiento etológico (Grant, 1981; Campbell & Aldridge, 2006). Pero dichos polinizadores no tienen porqué limitarse a afectar a los mecanismos de aislamiento prezigótico, ya que pueden actuar también como barrera de aislamiento post-zigótico, en caso de que reduzcan la fertilidad de los individuos híbridos debido a que los caracteres fenotípicos que desarrollen éstos no sean atractivos para dichos polinizadores (Campbell & Aldridge, 2006).

Los polinizadores son además importantes agentes selectivos para las Angiospermas (Darwin, 1876; Grant, 1949; Levin, 1978). La variación espacial en polinizadores con diferentes patrones de preferencia, comportamiento de pecoreo y eficiencia puede resultar en variación espacial en las presiones selectivas que sufren las plantas (Ashman & Morgan, 2004; Harder & Barrett, 2006; Gómez *et al*., 2008a,b; 2009a). Ésto puede generar mosaicos de escenarios selectivos (Gómez and Zamora, 2000; Rudgers and Strauss, 2004; Thompson, 2005; Rey *et al*., 2006; Gómez *et al*., 2009a,b), que puede resultar en patrones geográficos de adaptación local a los polinizadores. Incluso dicha selección podría derivar en divergencias evolutivas entre poblaciones, si dichas presiones selectivas fueran divergentes y con cierto grado de constancia temporal (Turrelli *et al*., 2001; Doebeli and Dieckmann, 2003; Greenberg *et al*., 2003; McKinnon *et al*. 2004). Si estas presiones selectivas pueden tener importancia para la divergencia de taxones, sin duda la tendrán para la consolidación de dicha divergencia. Es decir, las presiones selectivas jugarán un papel fundamental en prevenir la reversión de la divergencia contrarrestando los efectos de la hibridación entre linajes divergentes (Becker *et al*., 2006; Leimu & Fischer, 2008; McKinnon *et al*. 2004).

El hecho de que los polinizadores sean frecuentes agentes selectivos y actúen como barreras de aislamiento reproductivo entre plantas da la razón a los autores que llegaron a aventurar el papel esencial que los polinizadores ejercen en la especiación y diversificación de las plantas (Darwin, 1876; Grant, 1949; Levin, 1978). Sin embargo, a pesar de que las especies de plantas con sistemas de polinización generalistas son frecuentes en la naturaleza (Waser

et al., 1996; Herrera, 1996; Gómez & Zamora, 2006), la especiación mediada por polinizadores ha sido estudiado más intensamente en plantas con sistemas de polinización especialistas (Faegri & van der Pijl, 1979; Ramsey *et al*., 2003; Martin *et al*., 2008; Kay & Sargent, 2009; Schiestl & Schlüter, 2009; Natalis & Wesseligh, 2012). En estos sistemas generalistas, la variación en composición, abundancia y diversidad de los polinizadores son los que definen el solapamiento de los nichos interactivos de poblaciones o especies de las plantas con las que interactúan (Price *et al*., 2005). Este solapamiento puede afectar a los patrones de adaptación local y divergencia (Gómez *et al*., 2009a, b), y modificar el acervo genético de las poblaciones (Liu *et al*., 1998; 1999).

Sin duda las plantas son un sistema de estudio ideal para abordar no solo las cuestiones hasta ahora planteadas, sino otras relacionadas con los procesos que dirigen hacia la especiación (Rieseberg & Willis, 2007). El género *Erysimum* L. puede servir para explorar los mecanismos que subyacen a dichos procesos. Esto es debido a que la historia evolutiva del género es compleja, asociada a continuos eventos de radiación, hibridación inter-específica y poliploidización (Clot, 1992; Ancev, 2006; Marhold & Lihová, 2006). Esto hace que la evolución reticulada y la separación incompleta de linajes sean frecuentes, enriqueciendo al género en complejos de especies y especies crípticas, que debido a sus similitudes morfológicas complican su taxonomía (Faverger, 1978; Nieto Feliner, 1991; Ancev, 2006; Turner, 2006). Esto ha hecho que el género *Erysimum*, con más de 200 especies, sea uno de los más numerosos dentro de la familia Brassicaceae, habiéndo sido separado recientemente en una única tribu llamada Erysimeae (Couvreur *et al*., 2010, Al-Shebaz, 2012).

El género *Erysimum* presenta una distribución amplía en el hemisferio norte (Polatschek, 1986), presentando especies en norte y centro América y en África, aunque la mayoría de especies aparecen en el continente Euroasiático (Al-Shehbaz *et al*., 2006; Warwick *et al*., 2006; Koch & Al-Shehbaz, 2008). La región mediterránea representa uno de los centros de diversificación

más importantes del género, albergando más de un centenar de especies (Greuter *et al.*, 1986). Sin embargo, es el norte de la cuenca mediterránea el que acumula mayor diversidad, como refleja la diferencia en el número de especies encontradas a ambos lados del estrecho de Gibraltar. Son 22 las especies descritas en la Península Ibérica (Nieto Feliner, 1993), mientras que solo cuatro las reconocidas en todo el norte de la África continental (Ball, 1877; Jahandiez & Maire, 1932; Maire, 1967; Valdés *et al.*, 2002). Dentro de la península Ibérica, los sistemas Béticos constituyen una de las zonas con mayor biodiversidad de la cuenca mediterránea (Sainz-Ollero & Hernández Bermejo, 1985; Domínguez *et al.*, 1996; Blanca *et al.*, 1998; Médail & Quézel, 1999; Quézel & Médail, 1995). Esta alta biodiversidad parecen haber sido producida, al menos en parte, porque estas sierras Béticas, junto a las montañas del Rif, parecen haber funcionado como los refugios glaciares más importantes a ambos lados del estrecho de Gibraltar (Medail & Diadema, 2009). Ésta diversidad biológica ha podido estar influenciadas por las altas tasas de migración entre ambas regiones (Lavergne *et al.*, 2012). Estos dos macizos montañosos, asimismo, presentan un origen geológico común, y diferente al de las regiones continentales a las que están unidas, conformando lo que se conoce como Arco Bético-Rifeño (Lonergan & White, 1997).

De ambas zonas son las especies de *Erysimum* que se han incluido en el presente trabajo de tesis. Así se usaron poblaciones de E. nervosum s.l. de las montañas del Atlas y del Rif para abordar estudios de delimitación de especies crípticas, mientras que el resto de la tesis se centró en poblaciones de las especies de *Erysimum* de las Sierras Béticas, más particularmente, poblaciones de Sierra Nevada de *E. mediohispanicum* y *E. nevadense*. Así, E. nervosum s.l. está descrita como una especie herbácea perenne y monocárpica endémica de las montañas del Rif, y del Atlas, en las que aparece ampliamente distribuidas desde los 1500 a los 2500 m. de altitud (Pomel, 1875; Valdés *et al.*, 2002). A este lado del estrecho, *E. mediohispanicum* también es una especie montañosa que aparece distribuida a lo largo de un rango altitudinal que va de entre los 600 a los 2300 m. y con una distribución geográfica que comprende parte

de las submesetas Norte y Sur de la Península Ibérica (Fig. i.2), donde puede llegar a coexistir con otros *Erysimum* endémicos de las Sierras Béticas. Uno de estos endemismos es *E. nevadense* (Fig. i.2), especie exclusiva de Sierra Nevada donde alcanza altitudes de hasta 2800 metros (Gómez *et al.*, 2012). Ambas especies son herbáceas perennes que crecen durante dos o tres años como rosetas antes de desarrollar escapos florales, habitualmente uno en *E. mediohispanicum* y multiples en *E. nevadense* (Fig. i.2), sobre los que se pueden hayar de decenas a miles de flores (Gómez, 2003).

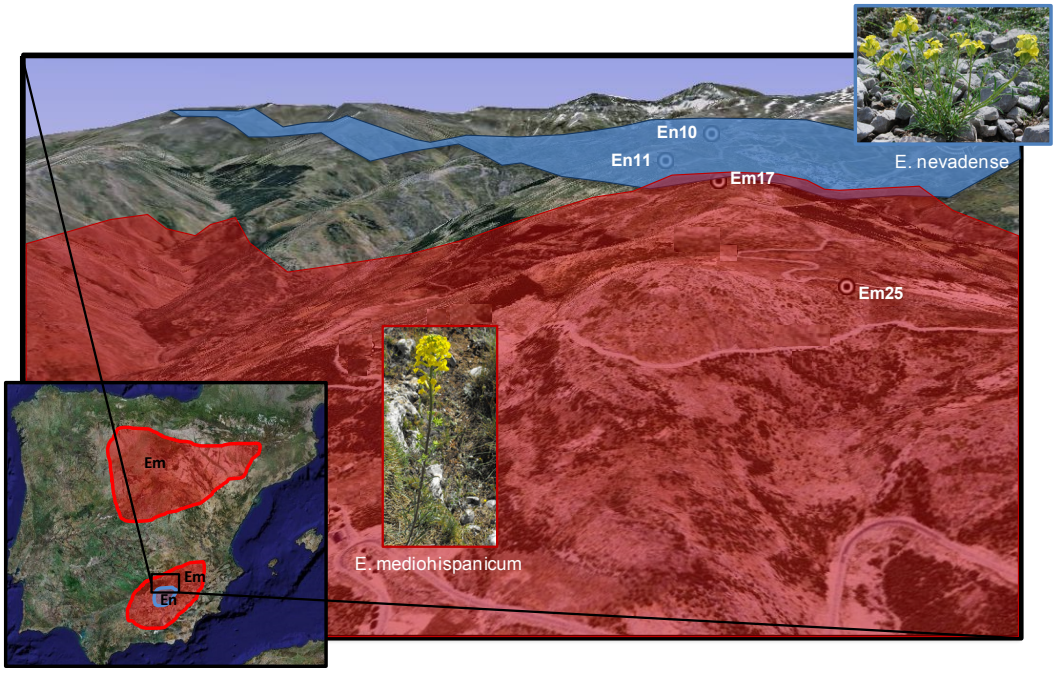

Figura i.2. Distribución de las poblaciones de *E. mediohispanicum* y *E. nevadense* en la cara norte de las montañas de Sierra Nevada (España). Mientras que las poblaciones de *E. nevadense* se restringen a las cumbres silíceas de Sierra Nevada, *E. mediohispanicum* está asociada a suelos calizos en dicha sierra, presentando además una distribución más amplia en la Península Ibérica. Ambas especies pueden presentar zonas de contacto en sus límites de distribución.

Estas dos especies son generalistas presentando gran diversidad de vistantes florales(Gómez *et al.*, 2007; Ortigosa & Gómez, 2010). Aunque ambas especies son autocompatibles, su producción de semillas se incrementa con el concurso

de los polinizadores. Mientras que *E. mediohispaniucm* crece asociada a suelos calizos de la media y alta montaña en Sierra Nevada, *E. nevadense* lo hace sobre los suelos silíceos de sus cumbres (Blanca *et al.*, 2009). Sin embargo ambas especies presentan al menos una zona en las que contactan a lo largo de una estrecha franja alrededor de los 2200 metros de altitud, y en la cual se pueden encontrar poblaciones de ambas especies separadas por menos de 100 metros.

OBJETIVOS

El objetivo general de la presente tesis es ***explorar los mecanismos genéticos y ecológicos que determinan, ya sea diluyéndolas o magnificándolas, las barreras de aislamiento que se erigen entre dos especies con sistemas de polinización generalista y que comparten vectores polínicos***. Este objetivo general puedo a su vez ser diferenciado en los siguientes objetivos particulares:

Objetivo 1: *Explorar la posibilidad de mejorar la delimitación e identificación de especies mediante la combinación de metodologías dispares.*

Para este objetivo combinaremos métodos morfológicos estándares, usados tradicionalmente en análisis taxonómico, junto con métodos moleculares y métodos que permiten analizar caracteres complejos (como la forma y el color de la flor) multidimensionalmente. Todos estos métodos conjuntamente mejoran la delimitación e identificación de especies en taxones de evolución compleja, en los que el uso de uno solo de estos métodos no fuera suficiente (***Capítulo 1***).

Objetivo 2: *Analizar la relación filogenética de las especies objeto de estudio de la presente tesis.*

Para este objetivo se abordará el estudio de las relaciones filogenéticas de las especies de *Erysimum* que habitanlas Sierras Béticas, usando marcadores

genéticos cloroplastidiales y nucleares, con el fin de discernir, por un lado, si estas especies conforman unidades evolutivas independientes, y por otro, identificar la naturaleza de las posibles zonas de contacto que dichas especies presenta (***Capítulos 1*** y ***2***).

Objetivo 3: *Describir los gremios de polinizadores que interactúan con las especies estudiadas.*

Aquí nos centraremos en el estudio de la zona de contacto entre *E. mediohispanicum* y *E. nevadense*, explorando para ello los gremios de polinizadores que ambas especies presentan en condiciones naturales, con el fin de analizar el grado de solapamiento de sus nichos interactivos (***Capítulos 6*** y ***8***).

Objetivo 4: *Describir los sistemas reproductivos y explorar su efecto en la estructuración poblacional de las especies focales.*

Para este objetivo calcularemos las tasas de depresión por endogamia y sus posibles efectos sobre el sistema reproductivo de cada una de las especies. Además, analizaremos el posible efecto de caracteres fenotípicos sobre dicha depresión por endogamia, y por tanto sobre la tolerancia individual de la endogamia. Por otro lado, analizamos las tasas de depresión por exogamia de cada una de las especies, completando la información sobre el sistema reproductivo de ambas especies estudiadas (***Capítulos 3*** y ***4***).

Objetivo 5: *Explorar la posible existencia de barreras de aislamiento intrínsecas entre las especies de estudio.*

Se analizaran dichas barreras de aislamiento realizando cruzamientos dirigidos en condiciones controladas con el fin de evaluar el éxito de dichos cruzamientos y la viabilidad y fertilidad de los híbridos resultantes (***Capítulos 3*** y ***6***).

Objetivo 6: *Estudiar los mecanismos de aislamiento reproductivos extrínsecos entre las especies de estudio.*

Aquí abordaremos el estudio de los mecanismos de aislamiento reproductivo que se dan en dicha zona de contacto. Con este fin se atenderá principalmente a los polinizadores como barreras extrínsecas de aislamiento pre-zigótico y post-zigótico, analizando las preferencias de los polinizadores en cada uno de los hábitats de las especies parentales, así como dicha preferencia sobre los posibles individuos híbrido en la zona de contacto (***Capítulo 6***).

Objetivo 7: *Analizar la selección existente en la zona de contacto entre E. mediohispanicum, E. nevadense.*

Cuantificaremos la selección e identificaremos los caracteres sobre la que ésta actúa en cada una de las poblaciones estudiadas, prestando especial atención a la que pueda favorecer el mantenimiento a largo plazo de una zona híbrida. Asimismo, se explorarán la posible existencia de selección divergente a nivel inter-poblacional e inter-especifico, y respecto a la zona híbrida (***Capítulos 6*** y ***8***).

Objetivo 8: *Cuantificar la heredabilidad y correlaciones genéticas de los caracteres sobre los que la selección puede estar actuando en E. mediohispanicum.*

Para poder inferir la capacidad que tiene la selección mediada por polinizadores de producir evolución fenotípica, analizaremos la heredabilidad y correlación genética de caracteres relacionados con la atractividad y el comportamiento de los polinizadores (tamaño de la planta, tamaño de la flor y forma floral) (***Capítulo 7***).

Objetivo 9: *Explorar las características genéticas de las poblaciones que conforman el contacto entre E. mediohispanicum y E. nevadense, asi como los patrones de estructuración, divergencia y flujo génico entre ellas.*

Analizaremos marcadores microsatelites para inferir la estructuración y diferenciación genética entre las poblaciones con-específicas y las inter-especificas. Así mismo, estimaremos las tasas de flujo génico que se dan entre las poblaciones y los tamaños efectivos de las mismas (***Capítulo 5***).

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CHAPTER 1

USING COMPLEMENTARY TECHNIQUES TO DISTINGUISH CRYPTIC SPECIES: A NEW *ERYSIMUM* (BRASSICACEAE) SPECIES FROM NORTH AFRICA

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ABSTRACT

Cryptic species are superficially morphologically indistinguishable and therefore erroneously classified under one single name. The identification and delimitation of these species is usually a difficult task. The main aim of this study is to provide an inclusive methodology that combines standard and new tools to allow accurate identification of cryptic species. We used *Erysimum nervosum s.l.* as a model system. Four populations belonging to *E. nervosum s.l.* were sampled at the two distribution ranges where they appear in Morocco (the Atlas Mountains and the Rif Mountains). Fifteen individuals per population were collected to assess standard taxonomic traits. Additionally, corolla color and shape were quantified in 30 individuals per population using spectrophotometry and geometric morphometrics, respectively. Finally, we collected tissue samples from each population per species in order to study the phylogenetic relationships among them. Using the standard taxonomic traits, the four populations were indistinguishable. Nonetheless, there were differences in corolla color and shape between plants from two different mountain ranges. The population differentiation based on quantitative morphological differences were confirmed and supported by the phylogenetic relationships obtained for these populations and the rest of the Moroccan *Erysimum* species. The joint use of the results obtained from standard taxonomic traits, quantitative analyses of plant phenotype, and molecular data suggests the occurrence of two species within *E. nervosum s.l.* in Morocco, one located in the Atlas Mountains (*E. nervosum s. str.*) and the other one in the Rif Mountains (*E. riphaeanum sp. nov.*). Consequently, we suggest that combining quantitative and molecular approaches with standard taxonomy greatly benefits the identification of cryptic species.

Key words

Atlas Mountains; corolla color; corolla shape; cryptic species; *Erysimum nervosum*; *Erysimum riphaeanum sp. nov.*; geometric morphometrics; Rif Mountains; taxonomy.

INTRODUCTION

Plant taxonomy has traditionally relied on morphological trait analysis (Sivarajan, 1991). This analysis, based on the use of diagnostic traits, has been complemented in the last decades with phenetic analysis tools (Rohlf and Marcus, 1993). These morphological approaches have been very useful to describe new species, construct keys, or to differentiate between species in the field. Nevertheless, in some plant groups with low morphological differences between taxa, distinguishing species using only these morphological traits is a difficult task. Since the seminal work of Grant (1981), it is widely acknowledged that these assemblages of species, called species complexes, represent a very intriguing evolutionary problem because they probably represent lineages where speciation is recent or yet incomplete (Nosil *et al.*, 2009; Schulter and Conte, 2009). In such situations, ascribing a new described population to a new species will depend on the species concept used by the plant taxonomists. Under the evolutionary and phylogenetic concepts of species (Wiley, 1978; Cracraft, 1989; de Queiroz and Donoghue, 1990), this new population should be an independent monophyletic lineage to be considered as new species. In this context, DNA sequencing analysis could be crucial to diagnose the polyphyletic status in a species complex, and to recognize individual species.

Molecular techniques have helped to solve taxonomic problems when species are difficult to separate morphologically (Knowles and Bryan, 2007; Judd *et al.*, 2008). However, those analyses can time- and resource-consuming, making them unfeasible in many regions with poor resources where paradoxically there is much unclassified biodiversity (Hillis, 1987). In this context, the development of quantitative techniques for assessing important taxonomic traits may be very useful. Since it is very difficult to measure the whole plant phenotype, these techniques should focus on characters known to be of ecological and evolutionary significance (Roy and Foote, 1999). In this sense, traits such as corolla shape and color, widely used to discriminate or arrange taxa (e.g., Heywood *et al.*, 2007), are particularly relevant. Consequently, a rising number of ecological studies have used either geometric morphometric

analysis of the corolla shape (e.g., Gerie *et al.*, 1997; Medel *et al.*, 2003; Gómez *et al.*, 2008b, 2009) or spectrophotometry quantification of the corolla color (e.g., Galsterer *et al.*, 1999; Whitney *et al.*, 2009). The combination of these two not commonly used techniques may be useful to distinguish cryptic species.

Accurately detecting cryptic species may be also important for developing adequate conservation agendas. Schönrogge *et al.* (2002) showed the dual problem of cryptic species complexes for conservation programs: 1) the species considered for conservation would be composed of more than one species, each of them more threatened than the group as a whole; 2) thus, these different species, composing a cryptic complex, would require a more specific conservation strategy. For this reason, any technique helpful to detect cryptic species may be useful for improving our conservation strategies.

Erysimum L. (*Brassicaceae)* is composed of over 200 species mainly distributed in the Northern Hemisphere (Polatschek, 1986), having in the western Mediterranean region an important diversification center (Greuter *et al.*, 1986). According to Koch and Al-Shehbaz (2008), this genus is centered primarily in Eurasia, with eight species inhabiting Northern Africa and the Macaronesia, and 15 more species distributed in North America. The genus has been traditionally placed in the broadly circumscribed *Camelineae* De Candolle (sensu Al-Shehbaz *et al.*, 2006). However, recent molecular studies have suggested that *Erysimum* could be a sister genus of the tribe *Descurainieae* Al-Shehbaz, Beilstein & E. A. Kellogg (Beilstein *et al.*, 2008) or could even be a unigeneric tribe, *Erysimeae* Dumortier (Bailey *et al.*, 2006; Koch and Al-Shehbaz, 2008). Taxonomic problems also arise intragenerically (e.g., Faverger, 1978; Nieto Feliner, 1991), as manifested by the recognized number of *Erysimum* species, which varies between 180 to 223 species depending on the author (Al-Shehbaz *et al.*, 2006; Warwick *et al.*, 2006; Koch and Al-Shehbaz, 2008). These taxonomic difficulties arise as a consequence of the morphological similarities among most *Erysimum* species, probably reflecting rapid speciation processes occurring within the genus. These rapid speciation events generate sibling or cryptic species that, although being almost identical morphologically, are

ecologically and/or geographically isolated from each other.

The main goal of this study is to test whether the joint use of standard taxonomic tools, quantitative techniques, and phylogenetic tools can improve our ability to identify cryptic species. We have made use of the standard taxonomic analysis of diagnostic traits together with geometric morphometric analysis of corolla shape, spectrophotometric determination of corolla color, and phylogenetic analysis of molecular data to identify species within *Erysimum nervosum sensu lato* (*s.l.*).

MATERIAL AND METHODS

STUDY SYSTEM

Two main mountain ranges occur in North Africa, the Atlas and the Rif (Fig. 1.1). The Atlas extends ca. 2400 Km through Morocco, Algeria and Tunisia. In Morocco, the Atlas is subdivided into three ranges (from north to south): Middle Atlas, High Atlas and Anti-Atlas. In the northern Morocco, and parallel to the Mediterranean coast, the Rif Mountains cover ca. 50,000 km^2. This latter mountain range, having a geological origin common to southern Spain Baetic ranges, with which it forms the Baetic-Rifean Arc, is geologically distinct from the Atlas range (Lonegran and White, 1997).

According to the floristic studies carried out in the northern Africa (Ball, 1877; Jahandiez and Maire, 1932; Maire, 1967; Valdés *et al.*, 2002), four autochthonous *Erysimum* taxa inhabit the area: (i) *E. incanum* Kunze, widely distributed in the region; (ii) *E. semperflorens* Schousb., found in the west coast of Morocco and in the north coast, between Morocco and Algeria; (iii) *E. wilczekianum* Braun.-Blanq. & Maire, inhabiting the Middle Atlas; and (iv) *E. nervosum* Pomel, which inhabits the two Moroccan mountain ranges, the Atlas and the Rif mountains. Within this latter taxon, some authors have recognized several varieties, subspecies and even species (Ball, 1877; Maire, 1967; Faverger and Galland, 1982). However, in recently published reviews,

all the infra-specific categories haven been included in the *E. nervosum* species complex (Valdés *et al.*, 2002; Koch and Al-Shehbaz, 2008).

E nervosum s.l. is a monocarpic perennial herb endemic to the Atlas Mountains, where the species was firstly described (Pomel, 1875), and the Rif Mountains (Valdés *et al.*, 2002). In the Atlas Mountains it grows on oligotrophic soils (schists) in alpine and subalpine grasslands and scrublands from 1500 to 2500 m. In contrast, the species in the Rif Mountains inhabits forest and shrubland canopies between 1200 and 1800 m, always on basic

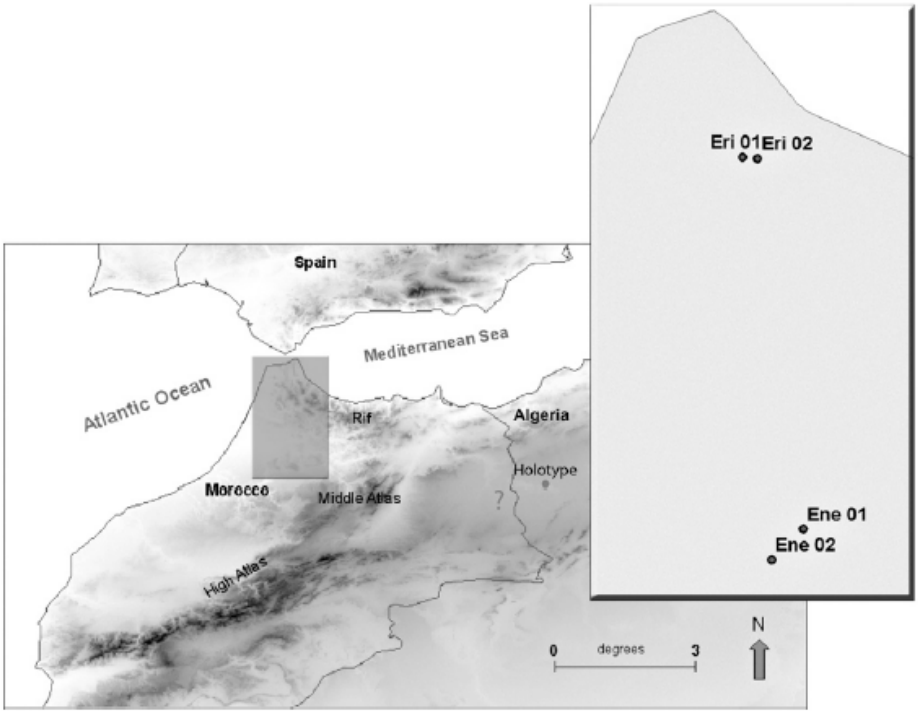

Figure 1.1. Location of the studied populations of the *Erysimum nervosum* complex in Morocco. ?=Locations unsuccessfully prospected; Holotype=Location of the holotype (Pomel, 1879).

soils (limestones). In both regions, this species is biennial, growing two years as a vegetative rosette and then dying after producing stalks with between a few and hundreds of yellow bisexual flowers. The flowers are self-compatible and are pollinated by a diverse assemblage of pollinators (Abdelaziz *et al.*, unpublished data).

Between 2006-2009 we studied *E. nervosum s.l.* in both of the ranges where this species occurs in Morocco (i.e. Atlas and Rif Mountains) (Fig. 1.1). In each range, we selected two populations, which are hereafter referred to as "Ene" for the Atlas populations and "Eri" for the Rif populations (Fig. 1.1 and Table 1.1).

STANDARD TAXONOMIC STUDY

For the taxonomic study, we collected 15 plants per population, totaling 60 samples. Plants were dried, pressed and mounted on herbarium sheets, and registered at the herbarium of the University of Granada (GDA). Afterwards, we measured 30 quantitative and qualitative variables that have been widely used in several floras to differentiate species in this genus (see Appendix 1.1). The traits were measured with digital calipers with ± 0.1 mm resolution, except plant height, which was measured with measuring tape with ± 0.5 cm resolution.

Pops	Mountain Range	Lat.	Long.	Altitude (m)	Habitat type	Flower app.
Ene 01	Atlas	33º 26.308'	-4º 56.188'	1711	Perennial grassland	
Ene 02	Atlas	33º 17.661'	-5º 5.159'	1802	Perennial grassland	
Eri 01	Rif	35º 11.14'	-5º 13.32'	1650	Open forest	
Eri 02	Rif	35º 10.742'	-5º 9.106'	1398	Shrubland	

Table 1.1. Location, habitat type and flower appearance of the studied populations of the *Erysimum nervosum* complex. Flower photos were chosen to show the average shape in each population.

These traits were compared by nested ANOVAs, including range (Atlas vs. Rif) as the main factor and population as a random factor nested within range. All statistical analyses were performed with the software JMP® 7.0 (SAS Institute Inc., 2007).

GEOMETRIC MORPHOMETRIC ANALYSIS OF COROLLA SHAPE

Corolla shape was quantified in 30 randomly selected plants per population by means of landmark-based geometric morphometric tools (Bookstein, 1991; Rohlf, 2003; Zelditch *et al.*, 2004). We took a digital photograph of one flower per plant using a standardized procedure (front view and planar position). Flowers were photographed at anthesis to avoid ontogenetic effects (Gómez *et al.*, 2006), and always in the same position to ensure the conservation of petal homology across flowers. We defined 32 coplanar landmarks (Fig. 1.2 and Appendix 1.2) located along the outline of the flowers and the aperture of the corolla tube; the landmarks were chosen to provide comprehensive coverage of the flower shape (Roth, 1993; Zelditch *et al.*, 2004). Landmarks were defined by reference to the midrib (landmarks 1, 9, 17, and 25), primary veins (landmarks 2, 8, 10, 16, 18, 24, 26, and 32) and secondary veins (landmarks 3, 4, 6, 7, 11, 12, 14, 15, 19, 20, 22, 23, 27, 28, 30, and 31) of each petal as well as the connection between petals (landmarks 5, 13, 21, and 29) (Fig. 1.2).We captured the landmarks using the software tpsDig version 1.4 (available at the Stony Brook Morphometrics website: http://life.bio.sunysb.edu/morph/morphmet.html).Afterwards, the two-dimensional coordinates of these landmarks were determined for each plant, and the generalized orthogonal least-squares Procrustes average configuration of landmarks was computed using the Generalized Procrustes Analysis (GPA) superimposition method (Rohlf and Slice, 1990; Slice, 2001).

Differences in corolla shape between Atlas and Rif populations were quantified by means of a Canonical Variate Analysis (CVA) (Zelditch *et al.*, 2004; Klingenberg and Monteiro, 2005). CVA is a specific multivariate analysis

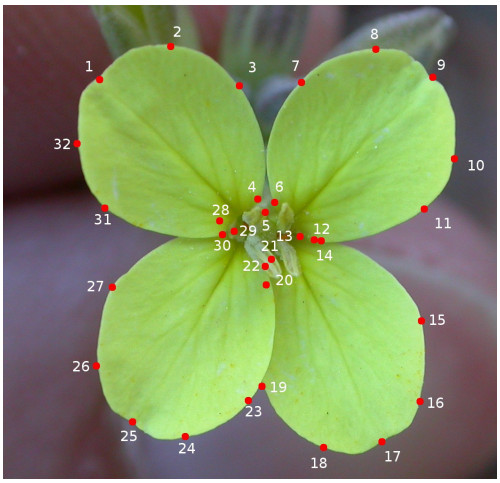

Figure 1.2. A planar view of the *Erysimum nervosum s.l.* corolla, showing the location of the 32 landmarks used in the geometric morphometric analysis.

optimizing the between-group differences relative to within-group variation. It generates several CV axes and computes the Procrustes distances among groups in the CV space. We additionally performed a Procrustes discriminant analysis, which examines the separation between two groups of observations. These two types of analyses are complementary, since discriminant analysis is more useful for comparisons of specific groups, whereas CVA may be more useful for general analysis of group structure in a dataset. The statistical significance of the between-groups Procustes distances was determined by randomization tests using 10,000 permutations with the software MorphoJ (Klingenberg, 2008).

COROLLA COLOR ANALYSIS

The corolla color was quantitatively measured in situ in each plant used in the geometric morphometric study by means of spectrophotometry, using an USB4000 miniature fibre optic spectrometer with a USB-DT Deuterium Tungsten Halogen Source (Ocean Optics, Dunedin, Florida, USA). This method has several advantages over the traditional visual evaluation. Namely, it gives accurate and objective measurements of reflectance (i.e. spectral reflectance curve) over the entire color spectrum including ultraviolet (300-700 nm), and the data can be stored automatically in computer spread-

sheets (Chittka and Kevan, 2005). Following Vorobyev and Osorio (1998) and Montgomerie (2006), we used a Hue-Saturation-Brightness (HSB) color assessment model (Andersson and Prager, 2006; Sharma, 2004) to characterize the corolla color of the studied populations by calculating brightness, chroma, and hue. Brightness, an achromatic measure that shows the maximum reflectance, was measured as the cumulative reflectance values of the entire spectrum (Andersson and Prager, 2006; Montgomerie, 2006). Chroma, which is an estimate of a color purity and perceived intensity, was calculated as the difference between the maximum and minimum reflectance values divided by the average reflectance (Andersson and Prager, 2006; Montgomerie, 2006). Hue is the degree to which a stimulus can be described as similar to, or different from, stimuli that are described as red, green, blue, or yellow. Hue was estimated as the wavelength with maximum reflectance (Andersson and Prager, 2006; Montgomerie, 2006). Between-population differences in color parameters were quantified by one-way ANOVAs with Tukey-Kramer HSD post-hoc comparison.

ANALYSIS OF PHYLOGENETIC RELATIONSHIPS

We collected fresh leaf tissue material from each population (Table 1.1). In addition we also collected fresh tissue from the other Moroccan *Erysimum* species (*E. incanum*, *E. semperflorens* and *E. wilczekianum*). This material was dried and conserved in silica gel until DNA extraction. We extracted DNA by using *GenElute™ Plant Genomic DNA Miniprep Kit* (Sigma-Aldrich) with at least 60 mg of plant material crushed in liquid nitrogen.

We amplified four different DNA regions: two plastid (*ndhF*, ~2000 bp and *trnT-L*, ~1300 bp) and two nuclear (ITS1, ~350 bp and ITS2, ~350 bp). We used the primers ndhF5 and ndhF2100 (Olmstead and Sweere, 1994) to amplify *ndhF*; tabA and tabD (Taberlet *et al.*, 1991) for *trnT-L*; ITS1 and ITS2 primers for the ITS1 region (White *et al.*, 1990); ITS3 and ITS4 primers for the ITS2 region (White *et al.*, 1990). PCR reactions were performed in

a total volume of 50 µl, with the following composition: 5 µl 10X Buffer containing $MgCl_2$ at 1.5 mM (New England BioLabs), 0.1 mM each dNTP, 0.2 µM each primers and 0.02 U Taq DNA polymerase (New England Biolabs). PCRs were performed in a Gradient Master Cycler Pro S (Eppendorf) using a initial denaturing step of 3 min. at 94°C and a final extension step of 3 min at 72°C in all the reactions. Reactions for ndhF included 35 cycles of 94 °C for 15 s, 47 °C for 30 s, and 72 °C for 90 s. Reactions for trnT-3'trnL included 35 cycles (94 °C 15 s, 53 °C 30 s and 72 °C 90 s). Reactions for ITS1 also included 35 cycles (94 °C 15 s, 64 °C 30 s and 72 °C 45 s). For ITS2, reactions included 35 cycles of 94 °C 15 s, 53 °C 30 s and 72 °C 45 s).

PCR products were mixed with 0.15 vol. 3 M sodium acetate, pH 4.6 and 3 vol. 95% (v/v) ethanol and subsequently purified by centrifuging at 4ºC. Amplicons were then sent to Macrogen Inc. (http://dna.macrogen.com/eng/) to be sequenced using the respective PCR primers and additional internal primers for *ndhF* (ndhF-599, ndhF-989-R, and ndhF-1354) and *trnT-L* regions (tabB and tabC).

Chromatograms were reviewed using Finch TV v1.4.0 (Geospiza Inc.) and the sequences edited using BioEdit v7.0.5.3 (Hall, 1999; Larkin *et al.*, 2007). As outgroup we used *Arabidopsis thaliana* sequences from GenBank. This species was used because it is a close relative of *Erysimum* (Al-Shehbaz *et al.*, 2006). We tested for incongruence between the nuclear and plastid genes using a ILD-test (Farris *et al.*, 1995) as implemented in ILD-bionj v1.0 (Zelwer and Daubin, 2004), obtaining that phylogenetic data showed by the two sequence types are not significantly incongruent (P=0.528). Sequences of different markers were concatenated on a individual basis and then aligned using *ClustalW* (Thomson *et al.*, 1994) tool in BioEdit (Hall, 1999; Larkin *et al.*, 2007). The sequences reported in the present study have been deposited in GenBank (Appendix 1.3).

Alignments were manually reviewed, and a region of indels and a string of adenines in the *trnT-L* (positions 2880–3300 of the concatenated alignment) were deleted using the GBlocks Server (http://molevol.cmima.

csic.es/castresana/Gblocks_server.html; Castresana, 2000) with the less stringent selection.

We built phylogenetic trees using both maximum likelihood (Felsenstein, 1973) with PhyML v2.4.4 (Guindon and Gascuel, 2003) and Bayesian MCMC inference (Yang and Rannala, 1997) using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). PhyML analysis was performed with default options and assuming a GTR model. This was the best fitting evolutionary model for the four concatenated regions as estimated by ModelTest v3.7 using the Akaike Information Criterion (Akaike, 1974; Posada and Crandall, 1998). Base frequencies, the proportion of invariable sites, substitution rates, and the alpha parameter of the gamma distribution were estimated by PhyML. Branch support was calculated both with the approximate likelihood ratio test (SH-like supports option) and the bootstrap (Felsenstein, 1985; 1,000 replicates). For Bayesian analysis, we used MrBayes allocated in Bioportal, University of Oslo (http://www.bioportal.uio.no/), partitioning the data into four regions, one for each locus cited above (ITS regions treated as a single locus), and we estimated the best fitting evolutionary model for each region using MrModelTest v2.3 (Nylander, 2004). Analysis lasted for four million MCMC generations, with a sample frequency of every 100 generations and we removed the first 25% of trees as burn-in, after checking trace files with Tracer v1.4 (Rambaut and Drummond, 2007) to determine the convergence of the two independent Bayesian MCMC runs. The consensus trees were visualized, edited and exported using MEGA v4.0.2 (Tamura *et al.*, 2007).

RESULTS

STANDARD TAXONOMIC STUDY

According to the Nested ANOVAs, only one quantitative trait (petal width) significantly differed between the Atlas and Rif populations (Table 1.2 and Appendix 1.4). Similarly, no differences were found for the qualitative traits, with the exception of a marked dark-rib in the fruit, which is less conspicuous

in Rif populations (Table 1.3).

GEOMETRIC MORPHOMETRIC ANALYSIS OF COROLLA SHAPE

The two main Canonical Variate axes accounted for 90% of the variance in corolla shape (Fig. 13). As figure 1.3 shows, the two Atlas populations did

Trait	Atlas (n=30)	Rif (n=30)	F-ratio	P
Number of stems	9.23±1.03	4.81±0.97	7.56	n.s.
Plant height (cm)	20.20±0.74	18.21±1.10	0.27	n.s.
Leaf length (mm)	14.54±1.24	21.82±1.45	9.48	n.s.
Leaf width (mm)	0.85±0.047	1.22±0.09	6.53	n.s.
Number of flower (mm)	43.92±5.65	34.44±6.53	0.47	n.s.
Sepal length (mm)	7.69±0.14	8.53±0.25	1.46	n.s.
Petal length (mm)	13.87±0.31	15.04±0.31	4.86	n.s.
Petal width (mm)	2.96±0.11	3.72±0.17	63.89	<0.0001
Filament length (mm)	8.99±0.17	9.39±0.15	2.71	n.s.
Number of fruits	23.92±3.65	13.19±2.72	2.66	n.s.
Length of fruit pedicel (mm)	2.79±0.10	3.53±0.19	12.58	n.s.
Fruit length (mm)	14.50±0.97	18.05±1.79	0.62	n.s.
Fruit width (mm)	0.55±0.30	0.65±0.04	0.61	n.s.

Table 1.2. Results of the comparison between *E. nervosum s.l.* populations from Rif and Atlas region (mean ± SE) for quantitative morphological traits. F-values refer to Nested ANOVA, using population as a random effect (results not shown), d.f. = 3; n.s. = not significant (P>0.05).

not differ according to their Procrustes distance in the Canonical Variate space, but they did differ from the Rif populations. The two Rif populations, however, differed in the Canonical Variate space, although the variation was mainly through the second axis. Discriminant Analyses outcomes were similar to that of Canonical Variate Analyses, and are not shown.

COROLLA COLOR ANALYSIS

We obtained similar spectral profiles for populations belonging to the same mountain range, but considerable differences between mountain ranges.

The spectral profile of the flowers was very different between Rif and Atlas populations (Fig. 1.4). Moreover, a reflectance peak obtained around 450-475 nm in the Rif populations was completely absent in the Atlas populations. Brightness and chroma also statistically differed among the four studied populations, although they were more similar between populations belonging to the same range (Fig. 1.4). In contrast, hue was only significantly different for one of the populations (Fig. 1.4).

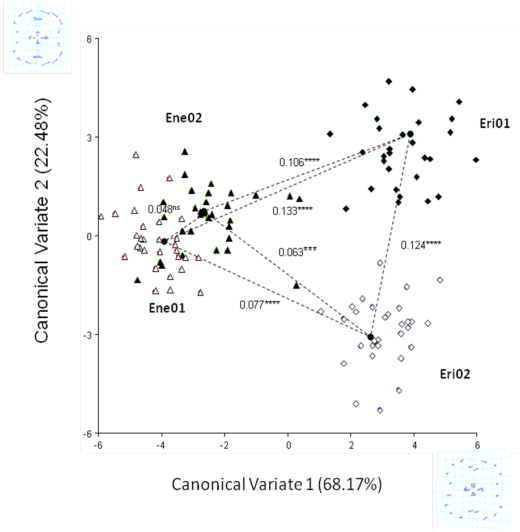

Figure 1.3. Results of the Canonical Variate Analysis. The figure represents the position of the plants belonging to each of the four studied populations in the two-axis CV space. The variance in shape explained by each CV axis, the change in shape produced by each axis, and the Procrustes distances between each population (^{ns} non significant, $^{***}P < 0.001$, $^{****}P < 0.0001$) are shown.

PHYLOGENETIC ANALYSIS

The topologies of the phylogenetic trees using maximum likelihood and Bayesian inference approaches are similar (Fig. 1.5). The only difference between them is the position of one *E. incanum* population (Ei03, Fig. 1.5).

The Rif populations of *E. nervosum* (Eri01 and Eri02) are clearly separated from the Atlas populations of *E. nervosum* (Ene01 and Ene02). In fact, the Atlas populations were always associated with *E. semperflorens* forming a clade, and never appeared sister to Rif populations. These relationships are strongly supported by bootstrap, approximate likelihood ratio test values, and

posterior probabilities (Fig. 1.5). Therefore, Rif and Atlas populations appear to represent two different evolutionary lineages.

Trait	Atlas (n=30)	Rif (n=30)
PLife cycle	Monocarpic perennial	Monocarpic perennial
Stem shapes	Erect to ascending	Erect to ascending
Plant surface	Hairy	Hairy
Hair shape	All medifixed	All medifixed
Lower leaves arrangement	Rosette-forming	Rosette-forming
Lower leaves	Simple and entire	Simple and entire
Cauline leaves	Simple and entire	Simple and entire
Base of cauline leaves	Sessile	Sessile
Inflorescence type	Simple	Simple
Inflorescence position	Terminal	Terminal
Stigma shape	Bi-lobed	Bi-lobed
Indument of fruit pedicel	Hariry	Hairy
Fruit rib	dark-marked	slightly marked
Fruit patent	Erect	Erect
Persistence of fruits	Deciduous	Deciduous
Valve surface	Hairy	Hairy

Table 1.3. Results of the comparison between *E. nervosum s.l.* populations from Rif and Atlas Mountains (mean ± SE) for qualitative morphological traits.

DISCUSION

Our study identifies three main points that may be useful when trying to identify cryptic species. First, it seems that standard taxonomic traits could be uninformative in some study systems. Here this standard phenotypic analysis revealed weak differences in the morphology of *E. nervosum* plants inhabiting the two distribution areas, since they differed in only one quantitative trait and one qualitative trait. This outcome reflects the difficulty of discriminating between Atlas and Rif populations, and open the possibility that they are potential cryptic species.

Second, we have seen that using quantitative complex traits, which may be important during the evolutionary divergence process of species pairs, could be also useful to distinguish some groups of populations. Under these

circumstances, additional approaches can allow a better identification and determination of species within syngameons and cryptic species complexes (Bickford *et al.*, 2007). In this sense, corolla shape and color could be used as important taxonomic key characters, not only when the differences are evident (e.g., zygomorphic flower vs. actinomorphic flower, red corolla vs. white corolla) but also when they are subtle and quantitative.

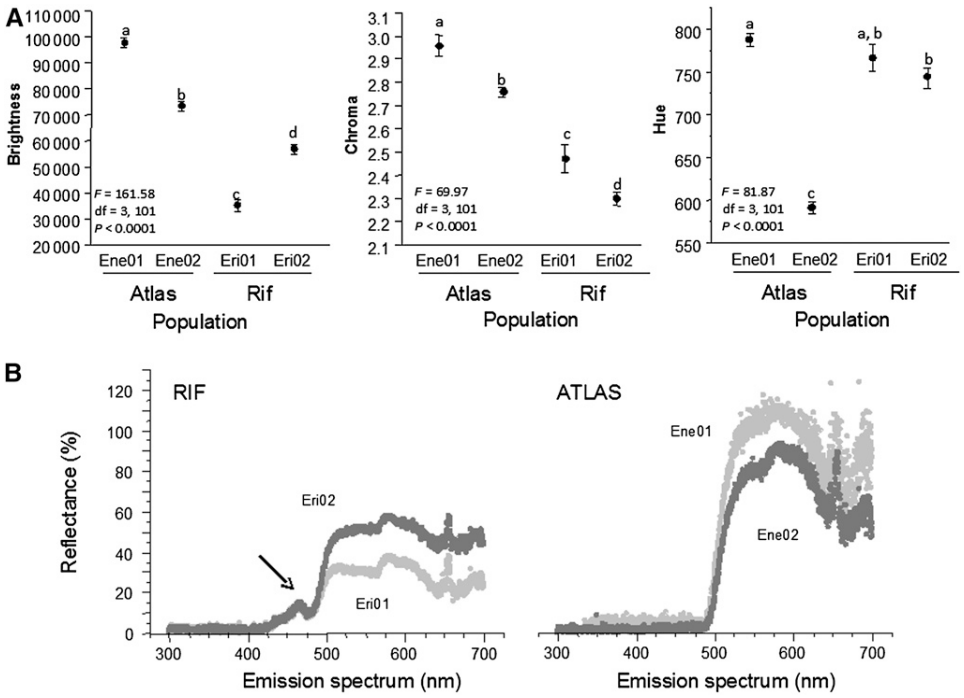

Figure 1.4. A) Mean values for brightness, chroma and hue per each population in the two studied regions. F ratios refer to one-way ANOVA. Letters indicate the groups where the differences are significant, according to a Tukey HSD comparison. B) Comparative spectral profile for the percentage of reflectance for each population in the two studied regions. The arrow shows a local maximum obtained in the populations from the Rif Mountains and not present in the populations from Atlas Mountains.

Most *Erysimum* species have a yellow corolla with similar shapes which, in principle, makes these traits somewhat subjective and difficult to use for differentiating closely-related species. However, the approach used in the current study, in which both corolla shape and color were quantitatively

measured by geometric morphometrics and field spectrophotometry, respectively, seems to be useful for discerning groups in those cases where the standard taxonomic tools are insufficient. These two traits, furthermore, are especially useful in *Erysimum* because they are associated with pollination and reproductive success in many species, and they do not change whether measured in the field or in the greenhouse.

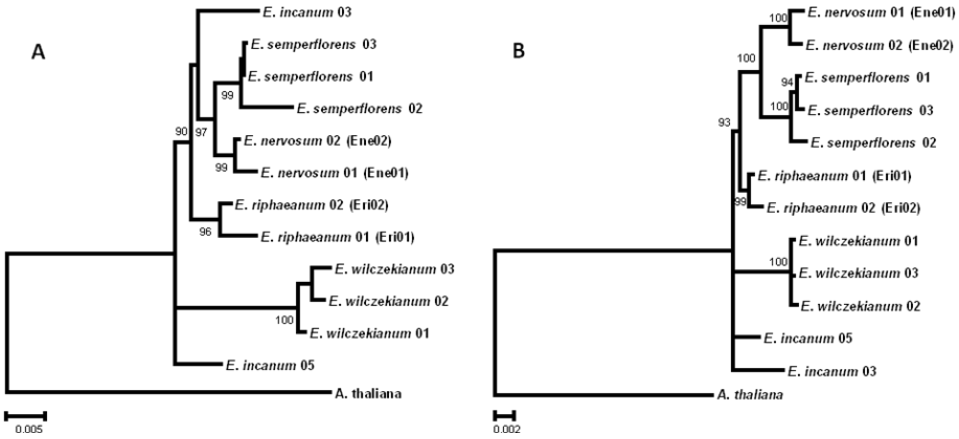

Figure 1.5. Phylogenetic position of *E. nervosum* and *E. riphaenum* populations within the North African species of the genus *Erysimum*. A) ML tree obtained with PhyML; branch reliability supports, calculated by approximate likelihood ratio test, appear below branch lines, and bootstrap values above branch lines. B) Tree obtained using Bayesian MCMC inference; branch supports values are posterior probabilities. Only branch supports higher than 75% are shown.

The use of corolla shape and color has further interest since they are known to have important evolutionary and ecological implications in many plant species (Dyer, 2004; Chittka *et al.*, 1999; Schemske and Bradshaw, 1999). We have previously shown that corolla shape is under pollinator-mediated selection, playing an important role in the adaptation to their pollinators of some *Erysimum* species from the Iberian Peninsula (Gómez *et al.*, 2006, 2008a, 2008b; Gómez *et al.*, 2009). Because the selective pressures exerted by pollinators seem to be similar across many *Erysimum* species (Gómez

et al., 2006, 2008a, 2008b; Gómez *et al*., 2009; Gómez and Perfectti, 2010; Ortigosa and Gómez, 2010), these complex traits will presumably be also similar among different species. Consequently, the study of these traits may help to disentangle the evolutionary divergence resulting in morphological differences between closely-related species.

The analysis of quantitative traits in common garden or greenhouse conditions may be crucial in some occasions, since it allows distinguishing between genetically controlled and environmentally controlled variance. However, the traits quantified in the current study, corolla shape and color, are identical between greenhouse and natural populations of a large amount of *Erysimum* species that we are currently growing, including one species analyzed in this study, *Erysimum semperflorens* (authors' personal observation). For this reason, we think *E. nervosum* and *E. riphaeanum* behave as the other congenerics and will display similar corolla shape and color in field and greenhouse conditions.

Finally, we have shown that molecular data may be used to corroborate what it is found with previous data. Molecular techniques (mainly molecular phylogenies) are very useful for the identification and description of new species, but they are not a panacea for species delimitation in some complex situations involving cryptic species (Bickford *et al*., 2007). However, such techniques have proven very useful for identifying cryptic species with polyphyletic origins, as shown in our present study. This kind of non-sister cryptic species can be produced by phenotypic convergence, in which the different species have similarly responded to the same selective pressures (Futuyma, 1997; Keller and Lloyd, 1992), or by phenotypic stasis, which is common for traits undergoing selective pressures from generalist interactions (Williamson, 1987). However, molecular phylogenetic analyses based on only a few sequences could fail identifying recently derived sibling species, since these species usually show insufficient sequence variation (Rubinoff *et al*., 2006) or incomplete lineage sorting (Maddison and Knowles, 2006).

There are several advantages of using complementary techniques to

identify cryptic species in plants. First, it could unravel hidden biodiversity not previously detected (Blaxter, 2004). We presume the existence of many undescribed and undetected cryptic species in plants, since cryptic plant species have received less attention than cryptic animals species (e.g., Schönrogge *et al.* 2002; Bickford *et al.*, 2007; Pfenninger and Schwenk, 2007). Second, the use of complementary techniques can help to identify the mechanisms responsible for the formation of cryptic species. Combining the study of phylogenetic relationships with the analysis of the adaptive role of traits contributing to the speciation may be useful to discern among evolutionary convergence, phenotypic stasis or cryptic speciation. In our case, since *E. riphaeanum* and *E. nervosum* are not sister species in the molecular phylogeny, they have not been produced by cryptic speciation. Third, from an ecological point of view, the identification of species in cryptic complexes is fundamental to accurately establish the degree of generalization/specialization in the ecological interactions of those species (Molbo *et al.*, 2003). A nominal species previously categorized as a generalist could actually be a group of specialist cryptic species. In our case, the pollination system of the two *Erysimum* cryptic species appears much more generalist if we erroneously consider them as one single species (Abdelaziz *et al.*, unpublished data).

The dual problem of cryptic species complexes for conservation programs showed by Schönrogge *et al.* (2002) suggests that an accurate determination of the taxonomy of a given group is a first step for the establishment of efficient conservation policies (Leadlay and Jury, 2006; Bickford *et al.*, 2007). Our results illustrate this matter, showing that the widely distributed species *E. nervosum s.l.* is actually two species, one of which, *E. riphaeanum*, is narrowly distributed and therefore more prone to extinction (Bickford *et al.*, 2007). Considering the scarce knowledge about the population features of the new species, *E. riphaeanum* must be considered Data Deficient (DD), according to IUCN (2001). Nevertheless, due to its restricted distribution area (less than 2,000 km^2) and its severely fragmented populations, the most plausible category for this species is Vulnerable (Vu)

(IUCN 2003).

The Rif Mountains are one of the most important Mediterranean glacial refugia in North Africa (Battandier, 1894; Haffer, 1982) and, consequently, they represent a biodiversity hotspot structured by dramatic climatic cycles (Médail and Diadema, 2009). In spite of this high biodiversity, the endangered flora of the Rif Mountains is poorly known. To date, there is only a preliminary, still uncompleted, Red List of endangered, rare and endemic plants of Morocco (Fennane and Tattou, 1998). In the last decade some taxonomic and ecological studies have been conducted in the area (e.g., Valdés *et al*., 2002), and it is urgent to update and extend this list by incorporating these results as a basis to prioritize conservation measures. Hence, *E. riphaeanum* forms part of the rich biodiversity of these mountains, and could benefit from conservation programs.

In addition to biodiversity conservation, ecological interactions also have important conservation interest (Kearns *et al*., 1998; Bronstein *et al*., 2004). *E. riphaeanum* acts as an important node in the interaction network between plants and their pollinators, because this species shows a generalized and highly diverse pollinator assemblage (Abdelaziz *et al*., unpublished data). Consequently, from a conservation point of view, this species has special interest given its direct and indirect effects on the biodiversity of these mountains.

In summary, this study shows that the combination of different morphological and molecular analyses can facilitate the identification of cryptic species, help in the design of conservation policies, and be useful for studying the evolutionary processes taking place in these recently diverged taxa.

Description of a new species— The results gathered from morphological traits, quantitative corolla color, corolla shape and phylogenetic data, led us to conclude that populations from western Rif Mountains constitute a new species, clearly separately from *E. nervosum* Pomel. Moreover a dichotomous key of Moroccan species of *Erysimum* is included in Table 1.4.

Erysimum riphaeanum *J. Lorite, M. Abdelaziz, A. J. Muñoz-Pajares, F. Perfectti & J. M. Gómez,* **sp.nov.**

Diagnosis— Hemicriptophytum caespitosum 15-25 cm altum. Caules floriferi (2)3-5(6), erecti vel adscendentes, plerumque simplices, sparse foliosi, pilis navicularibus raris praediti. Flores in racemo c. 20-40-floro, terminali atque simplici compositi, actinomorfi, tetrameri; sepalis 8-9 mm longis, viridi-flavis; petalis 13-16 mm ítem longis, longe ungulatis flavisque; staminibus 6, tetradynamis, filamentis 9-10 mm longis. Fructus quidem siliquosi, plus minusve nervosi, pilis navicularibus induti, erecti, 16-19 mm longi, pedicello autem 3-4 mm longo atque stigmate bilobo aut capitato. A simili specie nostra, *E. nervosum* Pomel nerviis prominentibus atque valde obscuris praecipue aperteque differt. Floret a mense Aprili usque ad mensem Iunium, fructificat autem a Iunio usque ad Iulium.

Holotype— MOROCCO: Western Rif, Chefchaouen, jbel Talassemtane. Coord: 35º 10.742' N, 5º 9.106' W, 1398 m. 17.VI.2009, Leg: M. Abdelaziz & A. J. Muñoz-Pajares, Collection number: Eri010801 (GDA 55655).

Description— Hemicriptophyte caespitose of 15-25 cm. height, with erect to ascendent (2)3-5(6) flowered stems, usually not ramified, sparsely leafy, with dispersed medifixed hairs. Leafs of 15-20 x 1-2 mm, linear, entire, sessile with medifixed hairs. Flowers arranged in simple and terminal racemes with c. 20-40 flowers, actinomorphic, hermaphrodites and tetrameric. Sepals of 8-9 mm length, green-yellowish; petals of 13-16 mm length, long clawed, light-yellow. 6 tetradynamous stamens with long filaments of 9-10 mm. Fruits in siliques of 16-19 mm length, erect, pubescent, with pedicels of 3-4 mm length and stigma bi-lobed to capitate. This new species has a less conspicuous dark-

marked rib in the fruit than *E. nervosum*.

***Flowering time*—** April-June. *Fruiting time*. June-July.

***Habitat description and distribution*—** Inhabits gaps of holm-oak (*Quercus ilex* subsp. *ballota*) and fir (*Abies pinsapo* subsp. *maroccana*) in forests and shrublands from 1200-2800 m on limestone. The species is distributed along the western Rif Mountains, being more abundant in the Talasemtane massif.

Etymology. The name *E. riphaeanum* refers to the Rif Mountains (Northern Morocco), where the species is endemic.

Other observed specimens*— *Erysimum nervosum; ALGERIA: O. Ghar-Rouban, 10-06-1879, A.N. Pomel, MPU005845 Holotype. ALGERIA: O. Aïn-Ghoraba près Terni, 06-1875, A.N. Pomel, MPU005844 Syntype?. MOROCCO: Medio Atlas, P. N. de Taza, paredones sobre calizas, 6-VII-1986, G. Blanca, M. Cueto, J. Garrido, C. Morales, J. F. Mota & A. Ortega, GDAC29192. MOROCCO: Ouarzazate à proximité du Tizi n' Melloul (Jab. Siroua), lat. 30.78, long. -7.6, 05-06-1980, A. Charpin, J. Fdez. Casas, F. Jacquemoud & D. Jeanmonod, MA395675-1. MOROCCO: High Atlas, S. From Marrakech, 2 km below ski resort of Oukaïmeden on road to Vallée de l'Ourika. 2500 m., 31.22 -7.85 1997-07-26 S. L. Jury & al., MA617106-1. MOROCCO: Ksar-es-Souk. Akeimeden, versant N au dessus de la MF de Midkane prés de la MF, 2100 m., lat. 32.52, long. -4.97, 09-06-1980, A. Charpin, F. Jacquemoud & D. Jeanmonod, MA395601-1. MOROCCO: Middle Atlas, Azrou; 1 km along road to Ain Leuh from junction with main Azrou - Midelt road above Azrou, 1650 m, 1997-07-14, S.L. Jury, A. Abaouz, M. Ait Lafkih & A.J.K. Griffiths, MA614866-1. MOROCCO: Medio Atlas, Carretera Ifrane a Boulemane, praderas de alta montaña sobre caliza, coord 33º 26.308' N, 4º 56.188' W, 1711 m., 16-05-2009, Abdelaziz & A. J. Muñoz, GDA55658 (16 samples). MOROCCO: Medio Atlas, Carretera Azrou a Timahdite, coord. 33º 17.661' N, 5º 5.159' W, 1802 m., 16-05-2009, Abdelaziz & A. J. Muñoz, GDA55657 (14 samples).

1.	Biennial or perennial	2
1´.	Annual	4
2.	White or white- yellowish flowers. Stems markedly woody	*E. semperflorens* Schousb.
2´.	Yellow flowers. Stems slightly woody at the base	3
3.	Silique with a dark-marked rib. Deep-yellow flowers. Atlas and Middle Atlas mountains	*E. nervosum* Pomel
3´.	Silique with a not dark-marked rib. Light-yellow flowers. Rif mountain	***E. riphaeanum sp. nov.* Lorite** ***et al.***
4.	Leaves pinnately lobed	*E. wilczekianum* Braun-Blanq. & Maire
4´.	Leaves dentate	*E. incanum* Kunze

Table 1.4. Key to the genus *Erysimum* in Morocco.

E. riphaeanum sp. nov.; MOROCCO: Rif, In collibus inter montes Kalaa et Tisuka, 1500 m., 15-VI- 1928-1932 (*Iter Maroccanum*, year not specified), Font Quer, (GDA28249) (*sub E. grandiflorum*). MOROCCO: Chefchaouen, Djbel Talmssemtane, 1699 m., M.C. García, M.A. Mateos, F.J. Pina & I. Sánchez, 25-07-1996, SEV155811-1 (*sub E. nervosum*). Ibidem SEV155811-1 (*sub E. nervosum*). MOROCCO: Chefchaouen, Djbel Bouhalla, 1230 m., M.A. Mateos, A. Ortega & F.J. Pina, 25-07-1995, SEV218136-1 (*sub E. nervosum*). MOROCCO: Rif occidental, jbel Talassemtane, claros de matorral sobre calizas, coord. 35º 10.742' N, 5º 9.106' W, 1398 m., 15-05-2009, M. Abdelaziz & A. J. Muñoz, GDA55669. Ibidem, GDA55699 (two samples). Ibidem GDA55671. Ibidem GDA55670. MOROCCO: Rif occidental, jbel Lakraa, claros de matorral sobre calizas,coord. 35º 11.14' N, 5º 13.32' W, 1650 m., 17-05-2009, M. Abdelaziz & A. J. Muñoz, GDA55656 (10 samples).

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APPENDICES

Appendix 1.1. Summary of the morphological traits measured in the four selected populations of the *E. nervosum s.l.* Note: variables cover the morphological traits observed in the genus *Erysimum* from the western Mediterranean region.

Variable	Values
1. Life cycle	1=Annual; 2=Biennial; 3=Monocarpic perennial
2. Number of stems	Number of stems
3. Stem shapes	1=Erect; 2=Erect to ascending; 3=Ascending; 4=Otherwise.
4. Plant height	Maximum height in cm
5. Plant surface	1=Glabrous; 2=Sparsely hairy; 3=Hairy
6. Hair shape	1=All medifixed; 2=Mostly medifixed; 3=Medifixed with stellate mixed; 4=Mostly stellate; 5=All stellate
Leaf characters	
7. Lower leaves arrangement	1=Rosette-forming; 2=No rosette-forming
8. Lower leaves	1=Simple and entire; 2=Variously serrate; 3=Lobed or pinnatisect
9. Cauline leaves	1=Simple and entire; 2=Variously serrate; 3=Lobed or pinnatisect
10. Cauline leaves base	1=Sessile; 2=Petiolate; 3. Otherwise (Amplexicaul, Decurrent, Ligulate or Perfoliate)
Inflorescence characters	
11. Inflorescence type	1=Simple; 2=Ramified
12. Inflorescence position	1=Terminal; 2=Terminal and axillary; 3=Axillary
Flower characters	
13. Number of flowers	Number of flowers
14. Sepal length	Mean length in mm
15. Petal colour	1=White; 2=Light yellow; 3=Yellow, 4=Purple
16. Petal length	Mean length in mm
17. Petal width	Mean width in mm
18. Filament length	Mean length in mm
19. Stigma shape	1=Capitate; 2=Capitate to bi-lobed; 3=Bi-lobed
Fruit characters	
20. Fruit pedicel length	Mean length in mm
21. Fruit pedicel indument	1=Glabrous; 2=Glabrous to hairy; 3=Hairy
22. Fruit length	Mean length in mm
23. Fruit width	Mean width in mm
24. Number of seeds in fruit	Mean number of seeds per fruit
25. Fruit patent	1=Erect; 2=Erect to spreading; 3=Spreading; 4=Adpressed to the stem
26. Fruit persistence	1=Deciduous; 2=Persistent
27. Valve surface	1=Glabrous; 2=Glabrous to slightly hairy; 3=Hairy
Seed characters	
28. Seed length	Mean length in mm
29. Seed width	Mean width in mm

Appendix 1.2. Description of Landmarks definition in genus Erysimum. Dividing the flower in four quadrants and following the trigonometric name for each one, here we define the landmark used for the study of corolla shape for each petal (Fig. 1.2):

Landmark name	Location
1 (quadrant 2), 9 (q. 1), 17 (q. 4), 25 (q. 3)	Intersection of midrib (if necessary, its continuation) and petal margin.
2 (q. 2),10 (q. 1), 18 (q. 4), 26 (q. 3)	Intersection between first primary veins on the right side of the midrib (if necessary, its continuation) with the petal margin.
32(q. 2), 8 (q. 1), 16 (q. 4), 24 (q. 3)	Intersection between first primary veins on the left side of the midrib (if necessary, its continuation) with the petal margin.
3(q. 2), 11(q. 1), 19(q. 4), 27(q. 3)	Intersection of secondary veins on right side of the midrib (if necessary, its continuation) and the petal margin.
31(q. 2), 7(q. 1), 15(q. 4), 23(q. 3)	Intersection of secondary veins on left side of the midrib (if necessary, its continuation) and the petal margin.
4(q. 2), 12(q. 1), 20(q. 4), 28(q. 3)	Point where petal inflect to corolla on right side of midrib.
30(q. 2), 6(q. 1), 14(q. 4), 22(q. 3)	Point where petal inflect to corolla on left side of midrib.
5, 13, 21, 29	Point where the both petal contact with the sepals.

Appendix 1.3. Origin of the material used in the phylogenetic analyses and GenBank accession numbers: ***Taxon***; Code; ITS1 GenBank accession; ITS2 GenBank accession; *ndh*F GenBank accession; *tabAD* GenBank accession; *Voucher specimen*, Collection locale; Herbarium.

Arabidopsis thaliana *(L.) Heynh.; X52322; X52322; AP000423; AP000423.* ***Erysimum incanum*** *Kunze; Ei03; HM235723; HM235735; HM235747; HM235759; GDA56843; Morocco, Ifrane; GDA.* ***E. incanum;*** *Ei05; HM235724; HM235736; HM235748; HM235760; Morocco, Chefchaouen; GDA.* ***E. nervosum*** *Pomel; Ene01; HM235725; HM235737; HM235749; HM235761; GDA55657; Morocco, Ifrane; GDA. E. nervosum; Ene02; HM235726; HM235738; HM235750; HM235762; GDA55658; Morocco, Ifrane; GDA.* ***E. riphaeanum*** *sp. nov.; Eri01; HM235727; HM235739; HM235751; HM235763; GDA55655; Morocco, Chefchaouen; GDA.* ***E. riphaeanum*** *sp. nov.; HM235728; HM235740; HM235752; HM235764; GDA55672; GDA.* ***E. semperflorens*** *Wettst.; Esem01; HM235729; HM235741; HM235753; HM235765; GDA56846; Morocco, Essaouira; GDA.* ***E. semperflorens*** *Wettst.; Esem02; HM235730; HM235742; HM235754; HM235766; GDA56847; Morocco, Essaouira; GDA. E. semperflorens Wettst.; Esem03; HM235731; HM235743; HM235755; HM235767; Morocco, Essaouira.* ***E. wilczekianum*** *Braun.-Blanq. & Marie; Ewi01; HM235732; HM235744; HM235756; HM235768; GDA56844; Morocco, Ifrane; GDA.* ***E. wilczekianum;*** *Ewi02; HM235733; HM235745; HM235757; HM235769; GDA56845; Morocco, Ifrane; GDA. E. wilczekianum; Ewi03; HM235734; HM235746; HM235758; HM235770; Morocco, Ifrane.*

Appendix 1.4. Among-population means variation in the quantitative morphological traits (mean±SE).

Trait	Atlas populations		Rif populations	
	Ene01 (n=15)	Ene02 (n=15)	Eri01 (n=15)	Eri02 (n=15)
Number of stems	8.43±1.66	10.17±1.11	6.11±1.40	3.14±1.10
Plant height (cm)	20.66±0.86	19.67±1.29	15.44±0.51	21.77±1.65
Leaf length (mm)	16.16±1.56	12.66±1.91	20.56±2.22	23.45±1.69
Leaf width (mm)	0.97±0.05	0.71±0.06	1.19±0.13	1.25±0.12
Number of flower (mm)	32.07±6.51	57.75±8.18	42.22±9.70	24.43±7.30
Sepal length (mm)	8.11±0.16	7.21±0.16	7.96±0.26	9.27±0.28
Petal length (mm)	14.11±0.41	13.58±0.49	14.58±0.38	15.63±0.45
Petal width (mm)	2.96±0.12	2.96±0.20	3.88±0.20	3.52±0.28
Filament length (mm)	9.14±0.26	8.82±0.22	9.25±0.22	9.58±0.20
Number of fruits	28.93±6.04	18.08±3.05	14.89±4.11	11±3.44
Length of fruit pedicel (mm)	2.92±0.10	2.63±0.18	3.42±0.28	3.67±0.25
Fruit length (mm)	17.37±1.37	11.14±0.38	14.09±0.95	23.13±3.03
Fruit width (mm)	0.65±0.03	0.45±0.03	0.56±0.03	0.77±0.04

CHAPTER 2

PHYLOGENETIC RELATIONSHIPS OF *ERYSIMUM* L. (BRASSICACEAE) FROM THE BAETIC RANGES (SE IBERIAN PENINSULA)

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ABSTRACT

The Baetic Ranges, located in the southern Iberian Peninsula, are a major hotspot of biodiversity in the Mediterranean Basin, constituting one of the most important glacial refuges for vascular plants in Europe. Despite their relatively limited extension, the Baetic Ranges contain almost 50% of the total endemic *Erysimum* species in the Iberian Peninsula. The broadly distributed *Erysimum* genus has diversified profusely in the Mediterranean region, with more than a hundred species described in the area, out of a total of some 200 species included in the genus. We used two cpDNA regions (ndhF and trnT-L) and one nDNA region (ITS1-5.8S-ITS2), with a 3,556 bp total length, to carry out phylogenetic analysis by Bayesian inference, maximum likelihood and maximum parsimony, in order to explore the evolutionary relationship between the *Erysimum* species inhabiting these Ranges. Two main clades were identified in our analysis. The different populations included per species appeared congruent, since they belonged to the same monophyletic group. However, our phylogenetic analysis exposed incongruence with previous taxonomic groups defined in the genus (E. nevadense group). Moreover, it also revealed the recurrent origin of the flower color in the *Erysimum* species inhabiting the Baetic Ranges.

Key words

cpDNA, flower color, nDNA, *Erysimum* nevadense group, secondary contact, sister species.

INTRODUCTION

Erysimum L. is one of the largest genera of the *Brassicaceae*, comprising more than 200 species, recently grouped in the unigeneric tribe *Erysimeae* (Couvreur *et al.*, 2010; Al-Shebaz, 2012). The evolutionary history of this genus is very complex, with events of inter-specific hybridization and polyploidization (Clot, 1992; Ancev, 2006; Marhold and Lihová, 2006). In fact, *Erysimum* is one of the few crucifer polybasic genera (i.e., characterized by multiple base chromosome numbers; Warwick *et al.*, 2006). Incomplete sorting and reticulate evolution have been frequent in the evolution of *Erysimum*. This speciation pattern has resulted in many species complexes and cryptic species with high morphological similarities (Ancev, 2006; Turner, 2006; ***Chapter 1***), and it has also caused many taxonomic conflicts (Faverger, 1978; Nieto-Feliner, 1991). As a consequence of these disputes, the number of species comprising the genus ranges from 180 to 223, depending on the author (Al-Shehbaz *et al.*, 2006; Warwick *et al.*, 2006; Koch and Al-Shehbaz, 2008).

The genus *Erysimum* is primarily distributed in Eurasia, with some species in North America and North Africa (Al-Shehbaz *et al.*, 2006; Warwick *et al.*, 2006; Koch and Al-Shehbaz, 2008). Apart from its broad distribution (Polatschek, 1986), the genus has diversified profusely in the Mediterranean region, with more than one hundred *Erysimum* species described in the area (Greuter *et al.*, 1986). Twenty-two *Erysimum* species have been described in the Iberian Peninsula (Nieto-Feliner, 1993). Molecular evidence suggests that many of these species are inter-fertile and have hybridized in the past (Muñoz-Pajares, 2013).

In fact, the Baetic Ranges, located in the southern Iberian Peninsula (Fig. 2.1), are a major hotspot of biodiversity in the Mediterranean Basin (Sainz-Ollero and Hernández Bermejo, 1985; Domínguez *et al.*, 1996; Blanca *et al.*, 1998; Médail and Quézel, 1999; Quézel and Médail, 1995). The geological history and origin of the Baetic Ranges sets them apart from the rest of Iberian Peninsula. They share a common geological origin with the

Rif Mountains in northern Morocco, and together the two ranges form the Baetic-Rifean Arc (Lonergan and White, 1997). More than 3,000 vascular plant species live in this 45,000 km^2 area, 40% of them endemic to the Iberian Peninsula (Medina-Cazorla *et al.*, 2010). This high plant diversity has several non-exclusive causes. This area has acted as one of the most important glacial refuges for vascular plants in the Mediterranean region (Medail and Diadema, 2009), while local diversification after allopatric and peripatric speciation has been favoured by the complex topography (Lavergne *et al.*, 2012). Furthermore, its biogeographical connection with the North African Rif has led to migration from the Maghreb, enhancing the plant diversity still further (Lavegne *et al.*, 2012). Ten *Erysimum* species inhabit the Baetic System, seven of them endemic to the area (Blanca *et al.*, 2009). In this study we explore the phylogenetic relationship between the *Erysimum* species inhabiting the Baetic Ranges.

Figure 2.1. Geographic distribution of the Baetic System in South-East Spain and the location of the sampled populations included in the present study.

MATERIAL AND METHODS

STUDY SYSTEM

According to *Flora Iberica* (Nieto-Feliner, 1993), nine outcrossing species of *Erysimum* are native to the Baetic Ranges: *Erysimum cazorlense* (Heywood) Holub, *Erysimum fitzii* Polatschek, *Erysimum popovii* Rothm, *Erysimum baeticum* Polatschek, *Erysimum myriophyllum* Lange, *Erysimum gomezcampoi* Polatschek, *Erysimum mediohispanicum* Polatschek, *Erysimum nevadense* Reut., *Erysimum rondae* Polatschek. All these species belong to the section *Erysimum* L, along with an autogamous annual species *Erysimum incanum* Kunze (see Table 2.1 and Fig. 2.1).

Erysimum cazorlense (Heywood) Holub, is a monocarpic biennial species endemic to the Sierra de Cazorla, Segura in Jaén province and Alcaraz in Albacete province.

Erysimum fitzii Polatschek, is a perennial polycarpic species endemic to the Sierra de la Pandera (Jaén).

Erysimum popovii Rothm. is a perennial and polycarpic species distributed on most mountains in Jaén, Granada and Córdoba provinces.

Erysimum baeticum Polatschek, a biennial to perennial species, mostly monocarpic. We will consider two subspecies, E. b. baeticum from the Sierra Nevada of Almería, and E.b. bastetanum from the Sierra de Baza in Granada province and the Sierra de María in Almería province (Blanca *et al*., 2009).

Erysimum myriophyllum Lange is a biennial to perennial monocarpic species inhabiting dolomitic soils in mountains in Granada, Málaga and Jaen provinces.

Erysimum gomezcampoi Polatschek is a polycarpic herb living along the Eastern Iberian peninsula, from Alicante in the South to Tarragona in the North, at altitudes of 600 to 1,400 m a.s.l..

Erysimum mediohispanicum Polatschek is a monocarpic biennial herb endemic to the Iberian Peninsula that lives at altitudes of 600 to 2,300 m. a.s.l., distributed in two isolated regions in the north-east and south-east of the Iberian Peninsula.

Species	Author	Sub-sp.	Short name	Nevadense group	Distribution	Baetic Range Endemism
E. incanum	Kunze	incanum	Ei	0	S of Spain and N of África	0
E. incanum	Kunze	mairei	Eim	0	Spain and N of Africa	0
E. gomezcampoi	Polatschek		Ego	1	Eastern mountain ranges of Spain	0
E. mediohispanicum	Polatschek		Em	1	Center and south of Spain and Baetic Range	0
E. rondae	Polatschek		Er	1	Western Baetic Range (Serranía de Ronda s.l.)	1
E. nevadense	Reut.		Ene	1	Sierra Nevada	1
E. myriophyllum	Lange		Emy	0	Baetic Ranges (Tejeda-Almijara, calcareous Sierra Nevada and Cazorla)	1
E. baeticum	(Heywood) Polatschek	baeticum	Eba	0	Sierra Nevada, Filabres and neighboring sierras	1
E. baeticum	(Heywood) Polatschek	bastetanum	Ebab	0	Sierra de Baza and Maria	1
E. popovii	Rothm.		Epo	0	Center of Baetic Range	1
E. cazorlense	(Heywood) Holub		Eca	0	Sierra of Cazorla s.l.	1
E. fitzii	Polatschek		Ef	0	Sierra de la Pandera	1

Table 2.1. Authors, short names, distribution and altitude range, main growth substrate, life history, plant height and flower color of the *Erysimum* species and sub-species inhabiting the Baetic System, as well as their status as a Baetic endemism (1=endemism, 0=non-endemism) and their inclusion in the *nevadense* group (1=included, 0= excluded).(*Continued on next page*).

Species	substrate	Altitude range	Life history	Plant height (cm)	Flower color
E. incanum	Indiferent	900-1500	annual	2-25	yellow
E. incanum	Calcareous	1000-1600	annual	10-40	yellow
E. gomezcampoi	Calcareous	600-1400	perennial policarpic	15-30(50)	yellow
E. mediohispanicum	Calcareous	700-2100	perennial monocarpic	25-50(70)	yellow
E. rondae	Calcareous	700-1700	perennial monocarpic	10-40	yellow
E. nevadense	Siliceous	1700-2800	perennial monocarpic	5-25	yellow
E. myriophyllum	Calcareous	700-1900	perennial monocarpic	14-40	purple
E. baeticum	Indiferent	1600-2600	perennial policarpic	25-60(70)	purple
E. baeticum	Calcareous	1000-2000	perennial policarpic	25-60(70)	purple
E. popovii	Calcareous	700-2000	perennial monocarpic	20-40(50)	purple
E. cazorlense	Calcareous	1200-1900	perennial monocarpic	(15)25-80	yellow
E. fitzii	Calcareous	1200-1800	perennial monocarpic	15-35	yellow

Table 2.1. (*Continued from previous page***).**

Erysimum nevadense Reut. is a perennial polycarpic herb endemic to the high mountains of the Sierra Nevada in Granada province.

Erysimum rondae Polatschek is a monocarpic biennial or perennial herb endemic to the Sierra de Ronda in Málaga province and the Sierra de Grazalema in Cádiz province, in the westernmost part of the Baetic Ranges. The last four species (*E. gomezcampoi*, *E. mediohispanicum*, *E. nevadense* and *E. rondae*) are considered microspecies that form a natural group called *nevadense* (Nieto-Feliner, 1993).

There is one additional *Erysimum* especies inhabiting the Baetic Ranges, *Erysimum incanum* Kunze. This species, in contrast with all the above, is an annual autogamous species (Nieto-Feliner, 1991) inhabiting ruderal, arid and semi-arid lowland habitats in the Iberian Peninsula, SW France and North Africa, at 200-2,100 m a.s.l. (Nieto-Feliner, 1993).

ANALYSIS OF PHYLOGENETIC RELATIONSHIPS

Fresh leaf tissue material was collected from at least two populations from each Baetic *Erysimum* species (except for *E. gomezcampoi*, which inhabits only one locality on the Baetic Ranges) for inclusion in the phylogenetic reconstruction (Table 2.2). In total, 23 populations were sampled. This material was dried and preserved in silica gel until DNA extraction. We extracted DNA by using GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, Missouri, USA) with at least 60 mg of plant material crushed in liquid nitrogen.

We amplified three different DNA regions: two plastid (*ndhF*, ~2000 bp and *trnT-L*, ~1300 bp) and one nuclear (ITS1-5.8S-ITS2, ~710 bp). We used the primers ndhF5 and ndhF2100 (Olmstead and Sweere, 1994) to amplify *ndhF*; tabA and tabD (Taberlet *et al.*, 1991) for *trnT-L*; ITS1 and ITS4 primers for the ITS1-5.8S-ITS2 region (White *et al.*, 1990). PCR reactions were performed in a total volume of 50 μL, with the following composition: 5 μL 10× buffer containing $MgCl_2$ at 1.5 mmol/L (New England BioLabs), 0.1 mmol/L each dNTP, 0.2 μmol/L each primer and 0.02 U *Taq* DNA polymerase (New England Biolabs). PCRs were performed in a Gradient

Master Cycler Pro S (Eppendorf Ibérica, Spain) using an initial denaturing step of 3 min at 94°C and a final extension step of 3 min at 72°C in all the reactions. Reactions for ndhF included 35 cycles of 94°C for 15 s, 47°C for 30 s, and 72°C for 90 s. Reactions for trnT-3´ trnL included 35 cycles (94°C 15 s, 53°C 30 s, and 72°C 90 s). Reactions for ITS1 also included 35 cycles (94°C 15 s, 64°C 30 s, and 72°C 45 s). For ITS2, reactions included 35 cycles of 94°C 15 s, 53°C 30 s, and 72°C 45 s).

PCR products were mixed with 0.15 volume of 3 mol/L sodium acetate, pH 4.6 and 3 volumes 95% (v/v) ethanol and subsequently purified by centrifuging at 4°C. Amplicons were then sent to Macrogen (Maryland Rockville, USA) to be sequenced, using the respective PCR primers and additional internal primers for *ndhF* (ndhF-599, ndhF-989-R, and ndhF-1354) and *trnT-L* regions (tabB and tabC).

Chromatograms were reviewed using the program Finch TV v1.4.0 (Geospiza, Seattle, WA, USA) and the sequences were edited using the program BioEdit v7.0.5.3 (Hall, 1999; Larkin *et al.*, 2007). *Moricandia moricandioides* and sequences from GenBank for *Arabidopsis thaliana* (ITS1: X52322; ITS2: X52322; ndhF: AP000423; tabAD: AP000423) were used as outgroups, as well as the Iranian species *Erysimum passgalense* Boiss., which was used as an internal outgroup. We tested for incongruence between the nuclear and plastid genes using Congruence Among Distance Matrices tests (CADM, Legendre and Lapointe, 2004), as implemented in APE (Paradis, 2004; R Development Core Team. 2011); the phylogenetic information resulting from the three analyzed sequences were significantly congruent (W= 0.708, χ^2=688.6, P = 0.001).

Sequences of different markers were concatenated on an individual basis and then aligned using the ClustalW (Thompson *et al.*, 1994) tool in BioEdit (Hall, 1999; Larkin *et al.*, 2007). The sequences reported in the present study will be deposited in GenBank (Table 2.2).

Taxon	Pop.	Sample origin
A. thaliana Heynh.	-	GeneBank
E. baeticum Polatschek	01	Spain: Granada, Sierra de Baza
	04	Spain: Granada, Sierra de Baza
	06	Spain: Almería, Sierra de María
	08	Spain: Almería, Sierra Nevada
E. cazorlense (Heywood) Holub	01	Spain: Jaén, Sierra de Cazorla, Segura y las Villas
	02	Spain: Albacete, Sierra de Alcázar
E. fitzii Polatschek	02	Spain: Jaén, Sierra de la Pandera
	04	Spain: Jaén, Sierra de la Pandera
E. incanum Kunze	03	Morocco: Granada, Cúllar-Baza
	04	Spain: Cuenca, Altobuey
E. mediohispanicum Polatschek	18	Spain: Almeria, Sierra de Gador
	21	Spain: Granada, Sierra Nevada
	25	Spain: Granada, Sierra Nevada
	27	Spain: Granada, Sierra de Lújar
E. gomezcampoi	-	Spain: Alicante, Font Roja
E. myriophyllum Lange	01	Spain: Granada, Sierra de Huétor
	02	Spain: Granada, Sierra de Baza
E. nevadense A. Heller	05	Spain: Almeria, Sierra Nevada
	10	Spain: Granada, Sierra Nevada
E. passgalense Boiss.	01	Iran: Karaj-Chalus road
E. popovii Rothm.	04	Spain: Granada, La Peza
	13	Spain: Granada, Sierra de Huetor
E. rondae Polatschek	03	Spain: Cádiz, Sierra de Grazalema
	04	Spain: Cádiz, Sierra de Grazalema
M. moricandioides (Boiss.) Heywood	-	Spain: Almería,

Table 2.2. Population origin for each species used in the phylogenetic analyses and GenBank accession numbers for the nuclear and plastidial markers used in the present study.

The alignments were manually reviewed, and a region of indels and a string of adenines in the *trnT-L* (positions 2880–3300 of the concatenated alignment) were deleted using the GBlocks Server (http://molevol.cmima.csic.es/castresana/Gblocks.html; Castresana, 2000) with the less stringent selection. We built phylogenetic trees for nuclear markers, for plastidial markers and for the total concatenated marker, using maximum parsimony with PAUP v4.0 (Swofford, 2002), maximum likelihood (Felsenstein, 1973) with the program PhyML v2.4.4 (Guindon and Gascuel, 2003) and Bayesian Markov chain Monte Carlo (MCMC) inference (Yang and Rannala, 1997) using the program MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). The PhyML analysis was performed with default options, assuming a general time reversible (GTR) model. This was the best-fitting evolutionary model implemented in PhyML for the three concatenated regions as estimated by the program ModelTest

v3.7, using the Akaike information criterion (AIC) (Akaike, 1974; Posada and Crandall, 1998). Base frequencies, the proportion of invariable sites, substitution rates and the alpha parameter of the gamma distribution were estimated by PhyML. Branch support was calculated with both the approximate likelihood ratio test (SH-like supports option) and the bootstrap (Felsenstein, 1985; 1,000 replicates). For Bayesian analysis, we used MrBayes on the online Bioportal of the University of Oslo (http://www.bioportal.uio.no/), partitioning the data into three regions, one for each locus cited, and we estimated the best-fitting evolutionary model for each region using MrModelTest v2.3 (Nylander, 2004). The best-fitting evolutionary model obtained for nDNA region was GTR+Γ, and for *ndhF* and *trnT-L* it was GTR+I and GTR+Γ, respectively. The analysis lasted for 4 million MCMC generations, with a sample frequency of every 100 generations, and we removed the first 25% of trees as burn-in, after checking trace files with the program Tracer v1.4 (Rambaut and Drummond, 2007) to determine the convergence of the two independent Bayesian MCMC runs. The consensus trees were visualized, edited, and exported using the program MEGA v4.0.2 (Tamura *et al.*, 2007).

RESULTS

The phylogenetic analysis performed using either nDNA or cpDNA and the both set of sequences together identified the external outgroup and established it with the con-generic outgroup as basal species for the rest of the *Erysimum* species included in the present work (Fig. 2.2, Appendices 2.1 and 2.2). In the inclusive analyses of nDNA and cpDNA, *E. gomezcampoi* proved to be separate from and older than the rest of the *Erysimum* species on Baetic Ranges, which were distributed into two main clades. The first of these clades did not have enough significant support to be separated from *E. gomezcampoi* and contained: *E. fitzii*, *E. cazorlense*, *E. myriophyllum* and *E. rondae*. The second main group presented a significant support and included: *E. baeticum* (both sub-species), *E. popovii*, *E. mediohispanicum* and *E. nevadense* (Fig. 2.2). We did

not find any incongruence between the populations belonging to the same species, which shared clades, i. e. they were monophyletic.

Despite their significant congruence, the phylogenetic relationships shown by the nuclear region and the plastidial markers were at variance. In this respect, the two major clades remained the same when the analysis was performed with nDNA alone; the only difference being that *E. gomezcampoi* appeared as the basal node of the newest clade, comprised of: *E. baeticum*, *E. popovii*, *E. mediohispanicum* and *E. nevadense* (Appendix 2.1). However, this species distribution disappeared with the phylogenetic relationship obtained by cpDNA. Here one population of *E. popovii* appeared as the oldest OTU for the rest of species, except for internal and external outgroups. After that, there was a polytomy that included the other populations of *E. popovii*, *E. myriophyllum*, *E. fitzii*, *E. cazorlense* and *E. baeticum*, as well as a well-supported clade of three populations of *E. mediohispanicum*. The remaining species were included in the newest polytomy, with the clade including *E. baeticum*, *E. nevadense* and a population of *E. fitzii*, being this clade the only one that was significantly supported (Appendix 2.2).

DISCUSSION

Our analysis suggests that the Baetic *Erysimum* forms a well-supported monophyletic clade. More species from outside the Baetic Ranges would need to be included to verify whether this clade comprises only Baetic species or whether it encompasses other species from surrounding Mediterranean areas. We included two populations of *E. incanum* from outside the Baetic Ranges, arranged in basal position close to the Iranian *Erysimum*, *E. passgalense*. Our analysis also suggests that there are two main *Erysimum* clades in the Baetic Ranges. One clade is composed of *E. baeticum*, *E. popovii*, *E. nevadense* and *E. mediohispanicum*, whereas the other comprises *E. fitzii*, *E. cazorlense*, *E. myriophyllum* and *E. rondae*. Nevertheless, only the first clade is well supported in our phylogenetic analysis, and so this is the only one we are confident about.

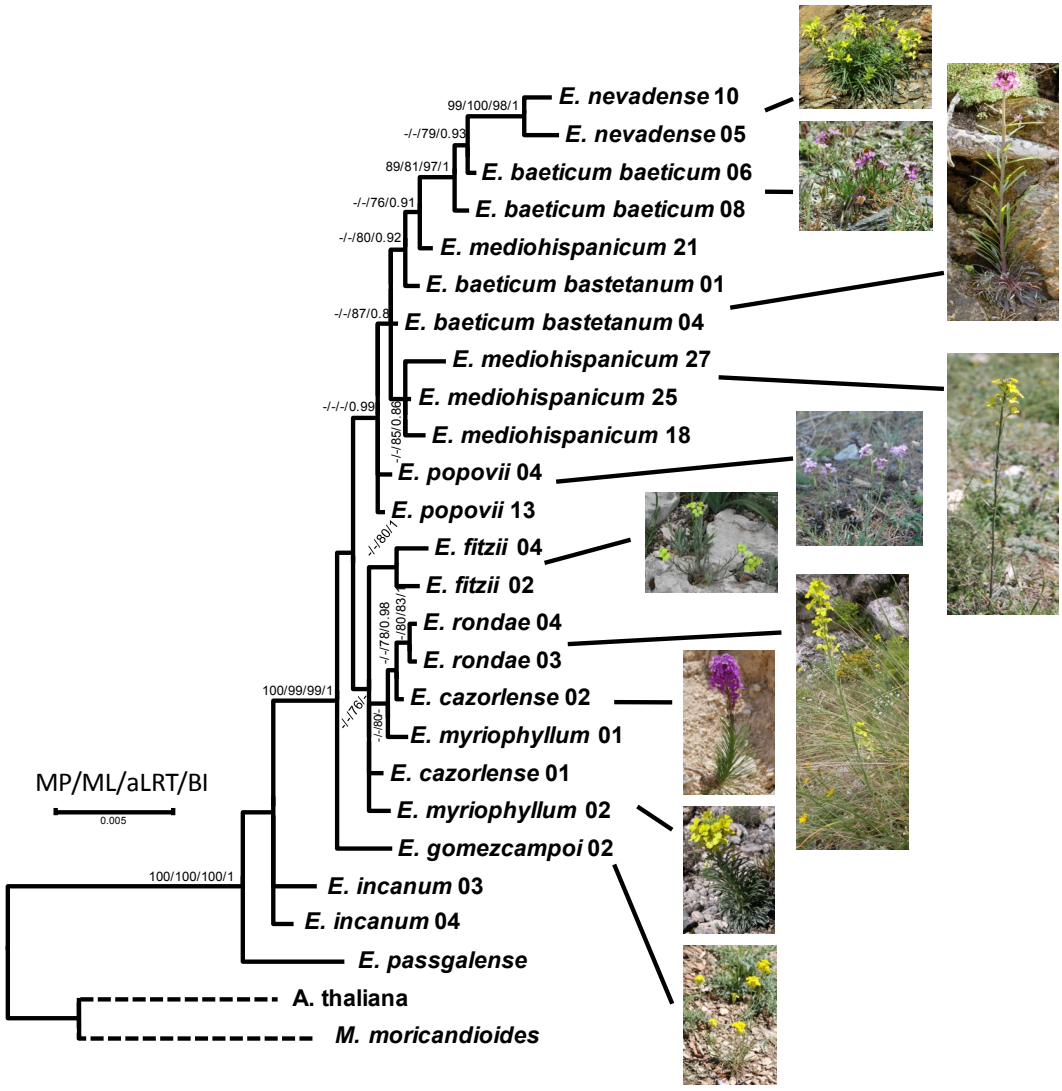

Figure 2.2. Phylogenetic relationships between *Erysimum* species inhabiting the Baetic ranges using the combined information from nuclear (ITS1-5.8S-ITS2) and plastidial (ndhF and trnT-L) DNA. MP: Maximum Parsimony tree obtained using PAUP, branch support was calculating by bootstrapping/ aLRT: Maximum likelihood tree obtained with PhyML, calculating branch reliability support by approximate likelihood ratio test/ ML: Maximum likelihood tree obtained with PhyML, calculating branch reliability support by bootstrapping/ BI: Tree obtained using Bayesian Markov chain Monte Carlo inference; branch supports values are posterior probabilities. Only branch supports higher than 75% are shown.

The species from the other clade could be located in a basal position together with *E. gomezcampoi*, and we are not able to conclude whether they form a true clade or not.

According to our outcomes, it seems that the E. *nevadense* group, as considered by some authors (e.g., Nieto-Feliner, 1993; Blanca *et al.*, 2009), appears to be only a morphological arrangement of species with no phylogenetic support. In fact, four of the six species in the *nevadense* group inhabiting the Iberian Peninsula did not form a monophyletic group in our results. Thus, two *nevadense* species, *E. nevadense* and *E. mediohispanicum*, are located in the first clade, whereas another, *E. rondae*, is located in the other lineage, and the fourth *nevadense* species, *E. gomezcampoi*, seems to be basal to all the Baetic *Erysimum*. Nieto-Feliner (1993) indicates that *E. rondae* sometimes displays an intermediate phenotype between the *nevadense* group and *E. myriophyllum*. In keeping with this notion, our analysis suggests a deeper evolutionary relationship between *E. rondae* and the latter species. However, Blanca *et al.* (2009) consider *E. rondae* a subspecies of *E. mediohispanicum*, a taxonomical consideration that is not supported by our phylogenetic analysis.

Furthermore, the two subspecies of *E. baeticum*: *E. baeticum* subsp. *baeticum* and *E. baeticum* subsp. *bastetanum*, do not seem to form a monophyletic clade. It is notable that the *E. baeticum bastetanum* from the Sierra de María is grouped with the *E. baeticum baeticum* population from the Sierra Nevada, even though the Sierra de Baza (where the other two *E. baeticum bastetanum* populations were collected) is located between these two mountain ranges. Nieto-Feliner (1992a) has suggested that *E. baeticum baeticum* could have evolved as a consequence of hybridization between *E. nevadense* and the widespread subspecies *E. baeticum bastetanum*. This author invoked several circumstantial findings to support his hypothesis. First, *E. nevadense* and *E. baeticum baeticum* are phenotypically identical, apart from differing in petal color (yellow and purple, respectively). Both taxa are short and polycarpic with multiple flowering stems arising directly from the rootstock rather than from the axillary leaflet fascicles (Nieto-Feliner, 1992b). Moreover, *E. nevadense* is diploid (2n =14), *E. baeticum*

bastetanum is tetraploid (2n = 38), but *E. baeticum baeticum* can be tetraploid or octoploid (2n = 56 [n = 28]) (Blanca *et al.*, 1992). Effectively, our phylogenetic outcome suggests a close relationship between *E. baeticum baeticum* and *E. nevadense*. We cannot rule out the hypothesis of *E. baeticum baeticum* evolving by hybridization, although further studies with more populations and markers are needed to throw more light these questions.

Most *Erysimum* species bear flowers with yellow petals. In fact, only seven Irano-Turanian species, five-six Macaronesian species (Polatschek, 1975), one Himalayan species and *Erysimum pallasii* (Pursh) Fernald from the North American and Siberian Arctic (Kevan, 1972) have purple flowers. Despite the small geographical area of the Iberian Peninsula, it contains six purpled-flowered taxa, four of them (*E. cazorlense*, *E. baeticum baeticum*, *E. baeticum bastetanum* and *E. popovii*) inhabiting the Baetic Ranges (Nieto-Feliner, 1993). They have usually been considered a monophyletic group (Ball, 1990, Nieto-Feliner, 1992c) or even a single species (Heywood, 1954). However, our phylogenetic analysis shows that these species are not monophyletic. In contrast, it seems that purple flowers appeared at least three times (Fig. 2.2). Evolution of petal color is not difficult, since it would entail few mutations in the genes evolved in the pigment biosynthetic pathways (Davies *et al.* 1998; Ono *et al.*, 2006; Dick *et al.*, 2011). Nevertheless, it is intriguing that in the Mediterranean region purpled-flowered *Erysimum* appear only in the Iberian Peninsula, even though this genus is frequent in other Mediterranean areas, such as Morocco (5 *Erysimum* species, ***Chapter 1***), Italy (17 species and one subspecies, Polatschek, 1982), Greece (26 species, Polatschek, 1986) or Turkey (40 species and 6 subspecies, Cansaran *et al.*, 2007). It would be interesting to find out which ecological factors are determining the recurrent evolution of purple flowers in the Iberian Peninsula

Most *Erysimum* species inhabiting the Baetic Ranges are allopatric, since their distribution does not overlap (Blanca *et al.*, 2009). However, several species co-occurr in the same mountains and have some sympatric populations. Therefore, *E. baeticum bastetanum* and *E. myriophyllum* are sympatric in some

localities on the Sierra de Baza, whereas *E. mediohispanicum* is sympatric with *E. popovii* in one locality on the Sierra Nevada and in another locality on the Sierra de Huétor, with *E. myriophyllum* in one locality of the Sierra de Cazorla and with *E. nevadense* in a narrow part of the Sierra Nevada. According to our phylogenetic analysis, it seems that all these are secondary contacts. We can conclude that the sympatric *Erysimum* species in the Baetic Ranges evolved allopatrically and probably made secondary contact as a result of the expansion of the geographic range of one or both members of the sympatric pair. The consequences of these secondary contacts are completely unknown so far, although we can envision that they have influenced the evolutionary history of the genus in the region.

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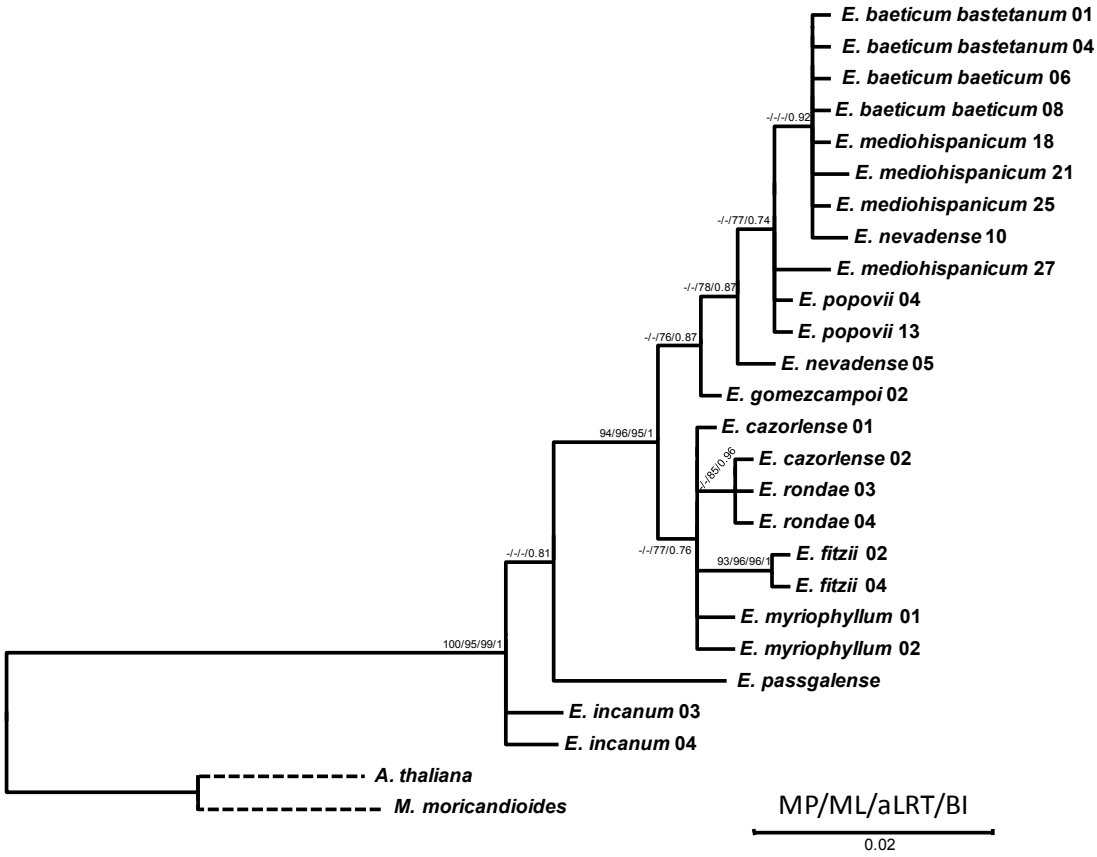
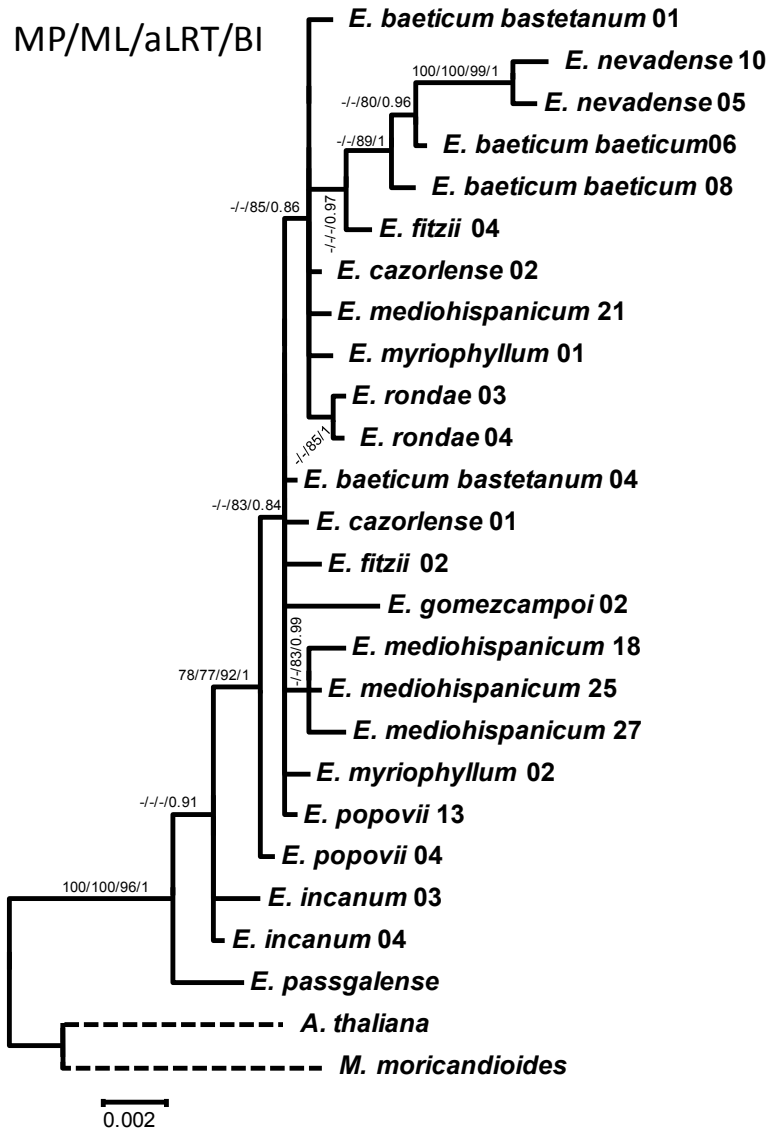
APPENDICES



Appendix 2.1. Phylogenetic relationship obtained using only the nuclear region *ITS1-5.8S-ITS2* for the *Erysimum* species inhabiting the Baetic Ranges. MP: Maximum Parsimony tree obtained using PAUP, branch support was calculating by bootstrapping/ aLRT: Maximum likelihood tree obtained with PhyML, calculating branch reliability support by approximate likelihood ratio test/ ML: Maximum likelihood tree obtained with PhyML, calculating branch reliability support by bootstrapping/ BI: Tree obtained using Bayesian Markov chain Monte Carlo inference; branch supports values are posterior probabilities. Only branch supports higher than 75% are shown.

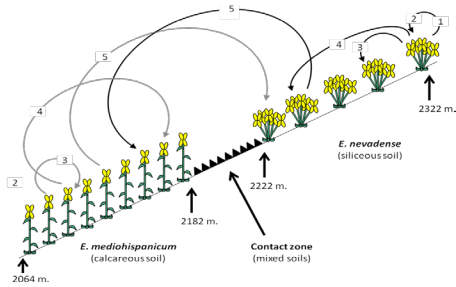


Appendix 2.2. Phylogenetic relationship obtained using only the plastidial DNA (ndhF and trnT-L) for the *Erysimum* species inhabiting the Baetic Ranges. MP: Maximum Parsimony tree obtained using PAUP, branch support was calculating by bootstrapping/ aLRT: Maximum likelihood tree obtained with PhyML, calculating branch reliability support by approximate likelihood ratio test/ ML: Maximum likelihood tree obtained with PhyML, calculating branch reliability support by bootstrapping/ BI: Tree obtained using Bayesian Markov chain Monte Carlo inference; branch supports values are posterior probabilities. Only branch supports higher than 75% are shown.

CHAPTER 3

INBREEDING DEPRESSION, OUTBREEDING DEPRESSION AND ASYMMETRIC HYBRID INVIABILITY IN TWO CO-OCCURRING *ERYSIMUM* SPECIES (BRASSICACEAE)

Mohamed Abdelaziz, A. Jesús Muñoz-Pajares, Modesto Berbel, Francisco Perfectti, José M. Gómez



ABSTRACT

Plant reproduction is lowered due to mating with close relatives (inbreeding depression), mating with distant relatives (outbreeding depression), and mating with other species (hybrid inviability). Although these three processes may act simultaneously in the same plant populations, they are usually studied separately. Here we explore inbreeding depression (ID), outbreeding depression (OD) and hybrid inviability (HI) in two *Erysimum* species (*E. mediohispanicum* and *E. nevadense*) by means of controlled crosses. We also calculated the selfing rate of each species using molecular markers. The studied species differed in ID, with *E. mediohispanicum* showing higher levels than *E. nevadense*. The latter species was more selfing than the former, and was self-pollen limited. These differences could be partially due to their contrasting pollinator fauna: whereas *E. mediohispanicum* flowers were visited by bees, *E. nevadense* flowers were visited mostly by ants. *Erysimum mediohispanicum* and *E. nevadense* are inter-fertile but they differ as regards hybrid inviability. Decrease in hybrid performance was only significant when *E. mediohispanicum* was the pollen donor and *E. nevadense* the pollen recipient. We believe that this difference may be a consequence of the abovementioned differences in selfing rate. Our findings suggest that the isolation barrier between these two *Erysimum* species is asymmetrical.

Key words

Erysimum mediohispanicum; *Erysimum nevadense*; hybrid inviability; introgression; mating system; microsatellites markers; outcrossing; pollen limitation; selfing; Sierra Nevada.

INTRODUCTION

The origin of pollen grains may have significant effects on plant reproductive success. Reproductive output may be reduced when pollen comes from close relatives, a phenomenon leading to inbreeding depression (Darwin, 1876; Charlesworth and Willis, 2009). Furthermore, a reduction in fitness may also occur after mating with distant relatives – a phenomenon called outbreeding depression (Edmands, 2002; 2007). Inbreeding and outbreeding depression are two non-exclusive processes that can occur simultaneously in the same plant populations (Lynch, 1991; Quilichini *et al.*, 2001; Escobar *et al.*, 2008; Forrest *et al.*, 2011; Hufford *et al.*, 2012).

Inbreeding depression has received much more attention than outbreeding depression (Edmands, 2007), and it has usually been studied to investigate the range of mating systems and their evolution at intra-specific levels (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Uyenoyama and Waller, 1991; Winn *et al.*, 2011; Goodwillie *et al.*, 2005). When the levels of inbreeding depression vary geographically from one population to another, we can expect to find populations with different mating systems. Populations where inbreeding is frequent would evolve towards a reduction in inbreeding depression (Lloyd, 1979; Husband and Schemske 1996; Armbruster and Reed, 2005). Under these circumstances, the genetic purge could produce a rapid transition to selfing, due to the decrease in inbreeding depression resulting from a loss of deleterious recessive alleles or a reduction in their harmful effects (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987). For example, inbreeding depression seems to be lower in peripheral than in central plant populations (Barringer *et al.*, 2012). The low availability of self-pollen will limit the reproduction in selfing populations more often than in outcrossing populations, where deleterious recessive alleles have not been purged; accordingly, an increase in the amount of self pollen deposited on stigma will not entail an increase in reproductive output.

Outbreeding depression has usually been explored with reference to processes of local adaptation and reinforcement during speciation (Coyne

and Orr, 2004; Servedio, 2000; 2004; Gavrilets, 2004). Elevated levels of gene flow reduce inter-population genetic structure, preventing local adaptation and population divergence (Slatkin, 1985; 1987; Lenormand, 2002). While populations (or species) with high level of endogamy would purge their genetic load for deleterious recessive alleles affecting inbreeding depression (Charlesworth and Charlesworth, 1987), this scenario may result in locally adapted populations that exhibit high outbreeding depression when receiving foreign, locally maladapted alleles (Bierne *et al.*, 2002; Epinat and Lenormand, 2009; Edelaar and Bolnick 2012). Locally adapted populations , whether selfer or outcrosser, will undergo outbreeding depression as a consequence of gene flow breaking up co-adapted gene complexes (Lenormand, 2002).

Inter-specific hybridization can be considered the extreme exponent of outcrossing, and the corresponding hybrid inviability could therefore be a severe expression of outbreeding depression (Lynch and Walsh, 1998; Coyne and Orr, 2004; Waser *et al.*, 2000). In this case, inter-specific hybridization may also lower the reproductive output of the parental species due to post-zygotic barriers such as hybrid inviability and sterility (Arnold, 2006). These post-zygotic isolation barriers could be symmetric, with both species suffering a similar magnitude of reproduction loss after hybridization (Tiffin *et al.*, 2000; Ramsey *et al.*, 2007), or asymmetric, with one of the species more likely to receive and/or accept pollen from the other. In the latter case, introgressive hybridization may occur as a result of a net transfer of alleles from one species to another via repeated backcrossing of hybrids to the parental species (Anderson and Hubricht, 1938; Arnold, 2006).

The effects of inbreeding depression, outbreeding depression and inter-specific hybridization can manifest themselves at different stages of a plant's life cycle (Husband and Schemske, 1996; Lázaro and Traveset, 2006; Escobar *et al.*, 2008). It seems, however, that the harmful effects of inbreeding and inter-specific hybridization appear more intensely during pre-dispersal phases, when the recessive deleterious alleles have strong effects (Husband and Schemske, 1996; Harder *et al.*, 2011). Nevertheless, it is still not clear

which life cycle component most intensely affects outbreeding depression, partly because of the paucity of studies on this phenomenon (Edmands, 2007). In this respect, the measurement of fitness components across the whole life cycle could help us to disentangle and understand the mechanisms responsible for the establishment and maintenance of reproductive barriers between closely related species.

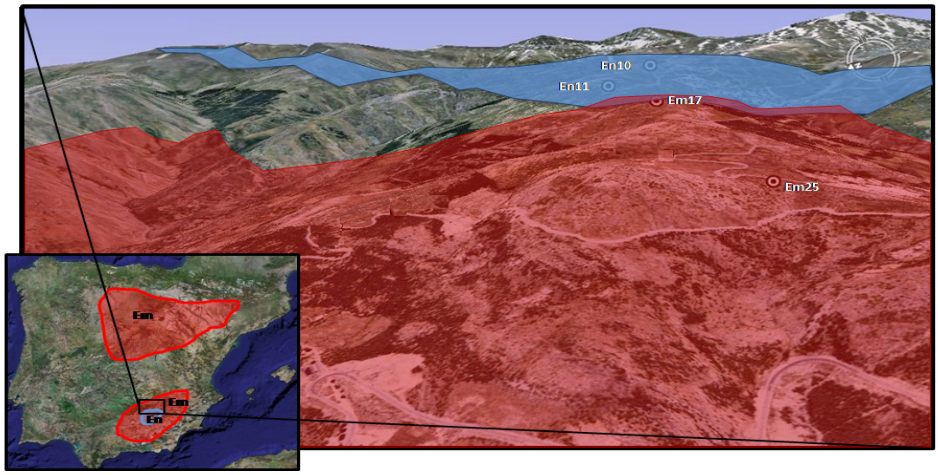


Figure 3.1. Distribution of *E. mediohispanicum* and *E. nevadense* populations on the North face of the Sierra Nevada Mountains (Spain). While *E. nevadense* is restricted to the top of the Sierra Nevada, *E. mediohispanicum* presents a wider distribution in the Iberian Peninsula. The two species come into contact in a narrow area in which they produce viable hybrids.

In this work we explore the inbreeding depression, outbreeding depression and intespecific hybrid inviability of two related species belonging to the genus *Erysimum* L. (Brassicaceae), *E. mediohispanicum* Polatschek and *E. nevadense* Reut. The genus *Erysimum* L. (Brassicaceae) comprises 180-223 species distributed primarily in Eurasia, with some species in North America and North Africa (Al-Shehbaz *et al.*, 2006; Warwick *et al.*, 2006; Koch and Al-Shehbaz, 2008). Apart from its broad distribution (Polatschek, 1986), the genus has diversified profusely in the Mediterranean region, with more

than a hundred *Erysimum* species described in this area (Greuter *et al.*, 1986). Molecular evidence suggests that many of these species are inter-fertile and have hybridized in the past (Muñoz-Pajares, 2013). In fact, the two focal *Erysimum* species, although living in slightly different habitats, coexist in a narrow zone in the Sierra Nevada Mountains of SE Spain (Fig. 3.1). In this study, we experimentally quantify 1) the spatial variation in inbreeding-outbreeding depression, 2) the magnitude of self-pollen limitation, inbreeding coefficient, and selfing rate for both species, and 3) the potential capacity for inter-specific hybridization. Our main goal is to investigate the mechanisms underlying the evolution of mating systems and divergence in these two closely related species.

MATERIALS AND METHODS

STUDY SYSTEM

Erysimum mediohispanicum (Fig. 3.2A) is endemic to the Iberian Peninsula, where it is distributed in two extended and isolated regions in the Northeast and Southeast of the peninsula, respectively. This species lives at an altitude of 600 to 2,300 m. (Fig. 3.1). *E. mediohispanicum* is often monocarpic, although its life history varies between individuals and populations. As it is a facultative biennial, plants spend 2 to 3 years growing like a vegetative rosette on calcareous soils. After that period they display flowers on 1-3 stalks (Gómez, 2003), ranging in number from only a few to several hundred. These flowers are visited by a highly diverse assemblage of insects (Gómez *et al.*, 2007), which may significantly influence the selection of many floral traits (Gómez *et al.*, 2008a; 2008b). These pollinators discriminate between different combinations of heritable phenotypic traits (Gómez *et al.*, 2009a; 2009b; 2009c).

Erysimum nevadense (Fig. 3.2,B) is mostly polycarpic and is endemic to the peaks of the Sierra Nevada Mountains (Fig. 3.1). This species presents populations in siliceous soils at 2,300 to 2,700 m on the north face of the range and at 2,130 to 2,800 m on the south face. They grow like a rosette for

2 to 3 years, before displaying anything from a few to several hundred flowers on various floral stalks. It is also a pollination-generalist plant, but it does not present a pollinator assemblage as diverse as that of *E. mediohispanicum*, due to the harsh conditions of its habitat (Gómez *et al.*, 2007; Ortigosa and Gómez, 2010).

CROSSING DESIGN

In September 2009, we collected 120 individual plants belonging to two populations each of *E. mediohispanicum* (Em25: 37º 7.230' N, 3º 26.082' W, 2,064 m. altitude; Em17: 37º 6.698' N, 3º 25.45' W, 2,182 m altitude) and *E. nevadense* (En11: 37º 6.750' N, 3º 25.048' W, 2,222 m altitude; En10: 37º 6.658' N, 3º 24.301'W, 2,322 m altitude) (Fig. 3.1). These individuals were transplanted to individual pots (11 x 11 x 15 cm), using the same soil in which they were growing, and moved to a common garden in the University of Granada (approx. 700 m altitude).

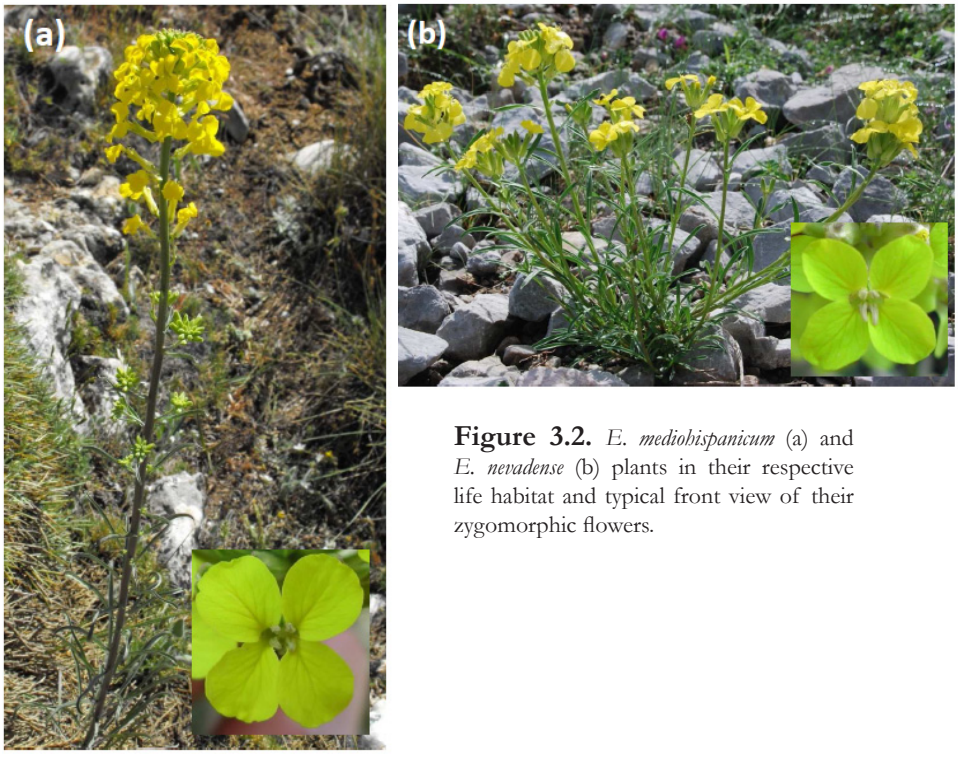


Figure 3.2. *E. mediohispanicum* (a) and *E. nevadense* (b) plants in their respective life habitat and typical front view of their zygomorphic flowers.

By May 2010, 44 individuals had survived (30 *E. mediohispanicum* plants and 14 *E. nevadense* plants).These were moved to a greenhouse before they started blooming to exclude them from pollinators. Each of these plants was subjected to five treatments: (a) Autonomous Selfing (AS), in which some flowers were not manipulated and left for spontaneous self-pollination; (b) Facilitated Selfing (FS), in which some flowers were emasculated and hand-pollinated with their own pollen; (c) Intra-Populational Outcrossing (CO), in which some flowers were emasculated before opening and pollinated with pollen from a different conspecific individual from the same population; (d) Inter-Populational Outcrossing (DO), in which some flowers were emasculated before opening and pollinated with pollen from a different conspecific individual from a different population; (e) Inter-Specific Hybridization (HO), in which some flowers were emasculated before opening and pollinated with pollen from an individual from the other species. In total, 1,275 flowers were used in the experiment, with a mean of 29 ± 17 flowers per plant (9.6 ± 7.3 for AS, 10.4 ± 6.1 for FS, 2.5 ± 1.9 for CO, 2.2 ± 2.0 for DO, and 4.3 ± 3.3 for HO).

Once the blooming period was over, we recorded the number of flowers per plant and treatment setting ripe fruits or aborting without producing any fruits. The total number of ovules, unfertilized ovules, aborted seeds and ripe seeds produced per ripe fruit were recorded in the lab using magnifying glasses. A total of 4,661 seeds were harvested at the end of the experiment. Subsequently, when possible, fifteen seeds per plant and treatment were taken at random and sown haphazardly in a greenhouse. Their germination was recorded twice a week for the first month and their survival was recorded every month during the next 10 months.

ESTIMATION OF FITNESS COMPONENTS

The following pre- and post-dispersal components of plant reproductive output were quantified for each treatment and plant: (a) Fruit set, the

proportion of labeled flowers setting fruit; (b) Ovule fertilization, the number of ovules within ripe fruits that were effectively fertilized; (c) Seed abortion, the number of fertilized ovules aborting before seed ripening. Aborted seeds were distinguished from unfertilized ovules because, as in other crucifers (Gómez *et al.*, 2010), they are dark brown, with shriveled cotyledons and embryo. In contrast, unfertilized ovules are creamy white and smaller; (d) Seed production, number of seeds produced per ovule in a given fruit; (e) Seedling emergence, the proportion of sown seeds germinating and emerging as seedlings; and (f) Seedling survival, the proportion of seedlings surviving until the end of the experiment. Afterwards, we calculated the cumulative pre-dispersal fitness (W_{PRE}), as fruit set x seed production, and the cumulative total fitness (W_{TOT}), as fruit set x seed production x seedling emergence x seedling survival.

ESTIMATION OF INBREEDING AND OUTBREEDING DEPRESSION

For each studied plant we calculated 1) Inbreeding Depression (ID) by comparing fitness between any of the two selfing treatments and the intra-populational outcrossing treatment; 2) Outcrossing Depression (OD), by comparing fitness between intra-populational and inter-populational outcrossing treatments; 3) Self-Pollen Limitation (SPL), by comparing fitness between autonomous and facilitated selfing; and 4) Hybrid Inviability (HI), by comparing fitness between the intra-populational outcrossing and inter-specific hybridization treatments. In all cases, we used both pre-dispersal and total fitness. These four variables were computed using the Agren and Schemske (1993) approach, as:

$$1-w_s/w_o;\ w_s<w_o$$

$$w_s/w_o-1;\ w_s>w_o$$

where w_o is the fitness of the intra-populational outcrossing treatment and w_s is the fitness of the other treatments, except for SPL, where w_o is the fitness of facilitated selfing treatment and w_s is the fitness of the autonomous selfing

treatment. In all cases, the values of the four variables ranged between -1 and +1. Positive values indicate that selfing crosses have lower fitness than outcrosses (occurrence of inbreeding depression), inter-populational outcrosses have lower fitness than intra-populational outcrosses (occurrence of outbreeding depression), inter-specific hybrids have lower fitness than intra-specific crosses (occurrence of hybrid inviability), and autonomous selfing have lower fitness than facilitated selfing (occurrence of self-pollen limitation). The significant values of these variables were calculated by computing the 95% confidence intervals by means of bootstrapping with 1,000 permutations, using package boot in R (Canty and Ripley, 2009).

ESTIMATION OF INBREEDING COEFFICIENT (F_{IS}) AND SELFING RATES

In 2007, we genotyped 30 additional plants for each of the four parental populations used in our experiment (120 plants in total). The genotyping was carried out with 10 microsatellite markers, previously described by Muñoz-Pajares *et al.* (2011) for *E. mediohispanicum* and other *Erysimum* species. For this purpose, we collected fresh tissue that was stored in silica gel for subsequent DNA isolation, using the GenElute Plant Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, Missouri, USA). We performed PCR in 15 µL of reaction mixture containing 0.17 ng/µL of template genomic DNA, 1x buffer (ref. M0273S, New England BioLabs), 0.16 mM each dNTP (Sigma-Aldrich), 0.33 µM each forward (fluorescently tagged) and reverse primer, and 0.02 U/µL *Taq* polymerase (ref. M0273S, New England Biolabs). PCR was conducted in a Gradient Master Cycler Pro S (Eppendorf, Hamburg, Germany) with an initial 30s of denaturation at 94 °C, 35 cycles at 94 °C for 15s, annealing temperatures per single microsatellite marker described by Muñoz-Pajares *et al.* (2011) for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 3 min. PCR products were diluted 1:15 and analyzed by MACROGEN analyzers (Geumchun-gu, Seoul, South Korea; http://www.macrogen.com), using 400HD ROX as standard. Alleles were called using Peak Scanner

Software version 1.0 (Applied Biosystems).

We estimated the inbreeding coefficient (F_{IS}) by Bayesian inference using BayesAss v3.0 (Wilson and Rannala, 2003) for each of the studied populations, and overall for the two studied species. Analysis lasted for 10 million MCMC iterations, with a sample frequency of every 1,000 generations, optimizing the mixing parameter for allele frequencies and for inbreeding coefficients. After that, we removed the first 10% of total iterations and we checked trace files with the program Tracer v1.4 (Rambaut and Drummond, 2007) to determine the convergence of the independent Bayesian MCMC runs. The Selfing Rate (SR) was calculated following Hartl and Clark (2007) as:

$$SR = 2\ F_{IS}/(1 + F_{IS})\ x\ 100$$

DATA ANALYSIS

The effect of treatments on each reproductive component and the two cumulative fitness estimates was tested using generalized linear mixed models (GLMM), including treatment and species as fixed factors and individual plant as random factors. GLMMs were performed using Normal distribution and identity link function for the cumulative fitness variables, and the remaining variables were compared using Binomial distribution and logit link function. The significance of each source of variation included in these models was tested by comparing the values of their Akaike Information Criterion (AIC) and Schwarz's Bayesian criterion (BIC), as well as the difference in deviance, using likelihood ratio tests (LRT) (Zuur *et al.*, 2009). We decided to use LRTs in addition to AICs because the ratio between our sample size and the levels of the fixed factors was always low (Bolker *et al.*, 2009). We determined the significance of fixed factors by comparing the AICs of a model with only the intercept against the models built, including each fixed factor one at a time and in all appropriate combinations (Bates, 2011). The individual plant was included in all models as random to control for over-dispersion. A fixed factor significantly predicted the dependent variable when the LRT of the model

with vs. without that factor was significant, and when the AIC of the model including that factor was lower than the AIC of the model including only the intercept. Afterwards, between-population and species comparisons of the reproductive output were performed using Kruskall-Wallis tests. All analyses were performed using stats and lme4 packages in R (R Development Core Team, 2011).

RESULTS

There were significant effects of treatment on every fitness component, except for seedling survival (Table 3.1, Fig. 3.3 and 3.4). The GLMMs indicate that the effect of the treatment on fruit set, seedling emergence, W_{PRE} and W_{TOT} were similar between species, since the best models for those variables only included treatment but not species as a factor (Table 3.1). In all cases, the two selfing treatments had significantly lower reproductive output that the two outcrossing treatments and the inter-specific hybridization (Fig. 3.3, 3.4 and 3.5). Nevertheless, the differences between selfing and outcrossing treatments in *E. nevadense* were significant on fruit set but were not clearly different on abortion, W_{PRE} and W_{TOT}; with no significant differences in ovule fertilization, seedling emergence and seedling survival (Fig. 3.3, 3.4 and 3.5). In contrast, the effect of treatment on ovule fertilization and seed set varied between species, as indicated by the significant Treatment * Species interaction (Table 3.1). There were no clear between-treatment differences in ovule fertilization (Fig. 3.3). In contrast, seed set varied between selfing and non-selfing treatments in both species, although in *E. nevadense* the intra-population outcrossing produced the highest amount of seeds (Fig. 3.3). No model fitted significantly for seedling survival (Table 3.1C).

Table 3.1. (*Next page*) Outcome of the GLMMs testing the effect of treatment and species on plant reproductive outputs. The significance of each factor was found by comparing the AICs of a model with only the intercept vs. models built by including each fixed factor one at a time (maintaining the random component of the complete model). Those factors that were significant are shown in bold (the model with them had a smaller AIC than alternative models without them).Id was significant according to doplot analysis across all the dependent variables.

		Fruit Set						Ovule Fertilization					
Model	df	AIC	BIC	LogLik	LRT	df	*P*	AIC	BIC	LogLik	LRT	df	*P*
Intercept	2	576.16	582.71	-286.08				623.98	630.08	-309.99			
Sp+Id[Sp]	5	577.10	593.49	-283.55	5.058	3	0.168	619.78	635.03	-304.89	10.20	3	**0.017**
Treat+Id	6	316.71	336.38	-152.36	262.38	1	**0.000**	554.76	573.06	-271.38	67.02	1	**0.000**
Sp+Treat+Id[Sp]	9	319.97	349.48	-150.99	2.74	3	0.433	552.27	579.72	-267.14	8.48	3	**0.037**
Sp+Treat+Sp*Trat+Id[Sp]	13	321.23	363.84	-147.61	6.75	4	0.150	549.38	589.03	-261.69	10.89	4	**0.028**

		Seed Abortion						Seed Production					
Model	df	AIC	BIC	LogLik	LRT	df	*P*	AIC	BIC	LogLik	LRT	df	*P*
Intercept	2	819.60	825.70	-407.80				821.34	827.44	-408.67			
Sp+Id[Sp]	5	822.07	837.32	-406.04	3.532	3	0.317	820.60	835.85	-405.30	6.736	3	0.081
Treat+Id	6	649.54	667.84	-318.77	174.53	1	**0.000**	558.83	577.13	-273.42	263.77	1	**0.000**
Sp+Treat+Id[Sp]	9	651.54	678.99	-316.77	3.995	3	0.262	559.01	586.45	-270.50	5.828	3	0.120
Sp+Treat+Sp*Trat+Id[Sp]	13	637.69	677.34	-305.85	21.852	4	**0.000**	543.77	583.42	-258.89	23.233	4	**0.000**

		Seedling Emergence						Seedling Survival					
Model	df	AIC	BIC	LogLik	LRT	df	*P*	AIC	BIC	LogLik	χ^2	df	*P*
Intercept	2	187.01	192.31	-91.50				118.47	123.578	-57.235			
Sp+Id[Sp]	5	190.09	203.36	-90.05	2.91	3	0.405	122.18	134.949	-56.09	2.29	3	0.514
Treat+Id	6	183.41	199.34	-85.71	8.68	1	**0.003**	121.995	137.318	-54.997	2.18	1	0.139
Sp+Treat+Id[Sp]	9	185.78	209.66	-83.89	3.64	3	0.303	125.74	148.725	-53.87	2.25	3	0.521
Sp+Treat+Sp*Trat+Id[Sp]	13	191.99	226.49	-82.99	1.79	4	0.774	130.452	163.652	-52.226	3.29	4	0.511

		W_{PRE} Cumulative Fitness						W_{TOT} Cumulative Fitness					
Model	df	AIC	BIC	LogLik	χ^2	df	*P*	AIC	BIC	LogLik	χ^2	df	*P*
Intercept	3	-10.17	-0.46	8.09				-91.62	-82.82	48.81			
Sp+Id[Sp]	6	-10.35	9.07	11.17	6.18	3	0.103	-88.82	-71.21	50.41	3.20	3	0.362
Treat+Id	7	-123.76	-101.10	68.88	115.41	1	**0.000**	-145.70	-125.15	79.85	58.87	1	**0.000**
Sp+Treat+Id[Sp]	10	-124.10	-91.74	72.05	6.35	3	0.096	-144.54	-115.20	82.27	4.85	3	0.183
Sp+Treat+Sp*Trat+Id[Sp]	14	-125.00	-79.69	76.50	8.90	4	0.064	-143.25	-102.17	85.63	6.71	4	0.152

The two *Erysimum* species differed in their level of inbreeding depression when considering both W_{PRE} and W_{TOT}. Inbreeding depression quantified from facilitated selfing during W_{PRE} was 0.84 for *E. mediohispanicum* and 0.59 for *E. nevadense* (Tables 3.2 and 3.3). The difference was even greater when ID was quantified for W_{TOT}, since it was significant 0.89 for *E. mediohispanicum* but not significant 0.32 for *E. nevadense* (Tables 3.2 and 3.3). However, *E. mediohispanicum* and *E. nevadense* presented similarly high significant values of autonomous inbreeding depression at W_{PRE} (Table 3.2). These results are consistent with the values of self-pollen limitation, which were high and significant in *E. nevadense* and low and non-significant in *E. mediohispanicum*, for both W_{PRE} and W_{TOT} (Table 3.2).

No significant outbreeding depression was found for either species, apart from a low value found for W_{TOT} in *E. mediohispanicum* (Table 3.3). However, we found significant hybrid inviability for the crosses performed on *E. nevadense*, but not for the hybrid crosses performed on *E. mediohispanicum* (Table 3.3).

The inbreeding coefficients (F_{IS}) showed significant positive values in all the populations, being slightly higher in *E. nevadense* (Tables 3.2 and 3.3). A higher level of inbreeding coefficient was found for *E. nevadense* (Table 3.3). Both results show an excess of homozygotes at population and species level. The highest selfing rate (SR) was found in the *E. nevadense* population at a higher altitude, while the lowest value was estimated in one of the *E. mediohispanicum* population (Table 3.2). When the two species were compared, *E. nevadense* presented a higher selfing rate (38.71% to 33.75%, Table 3.3).

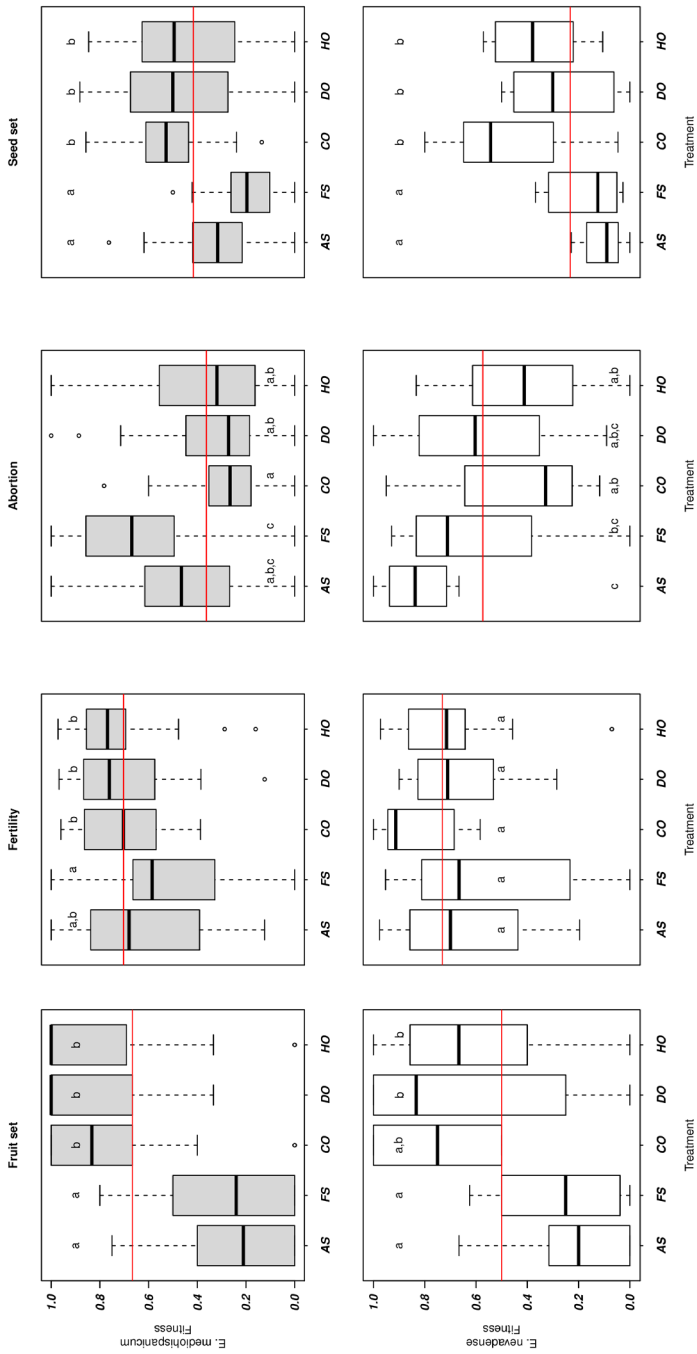


Figure 3.3. Absolute fitness for pre-dispersal life-cycle stages represented by box plots for the five treatments carried out in our control-crossing experiment. Top and bottom boxes indicate upper and lower quartiles of the distribution, while vertical lines show 1.5x the interquartile range; open circles indicate points falling beyond that range. The heavy horizontal bar indicates the median for each treatment, while the median fitness value for each life-cycle stage and species is represented by a red horizontal bar. Distributions with no statistically significant differences by Kruskall-Wallis pairways comparison show the same letter. AS: Autonomous Selfing; FS: Facilitated Selfing; CO: Intra-Populational Outcrossing; DO: Inter-Populational Outcrossing; HO: Inter-Specific Hybridization.

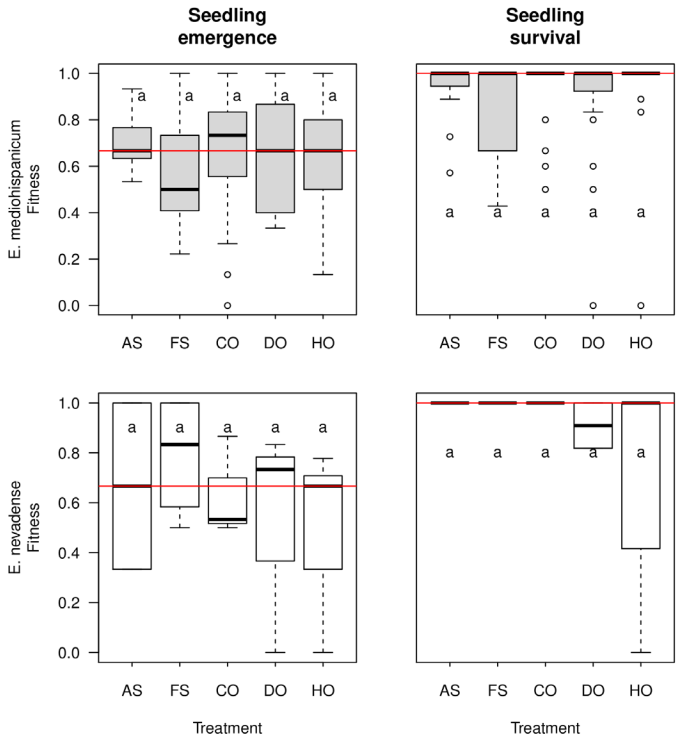


Figure 3.4. Absolute fitness for the components of post-dispersal life-cycle stages represented by box plots for the five treatments carried out in our control-crossing experiment. The top and bottom boxes indicate upper and lower quartiles of the distribution, while vertical lines show 1.5x the interquartile range; open circles indicate points falling beyond that range. The heavy horizontal bar indicates the median for each treatment; while the median fitness value for each life-cycle stage and species is represented by a red horizontal bar. Distributions with no statistically significant differences by Kruskall-Wallis pairways comparison show the same letter. AS: Autonomous Selfing; FS: Facilitated Selfing; CO: Intra-Populational Outcrossing; DO: Inter-Populational Outcrossing; HO: Inter-Specific Hybridization.

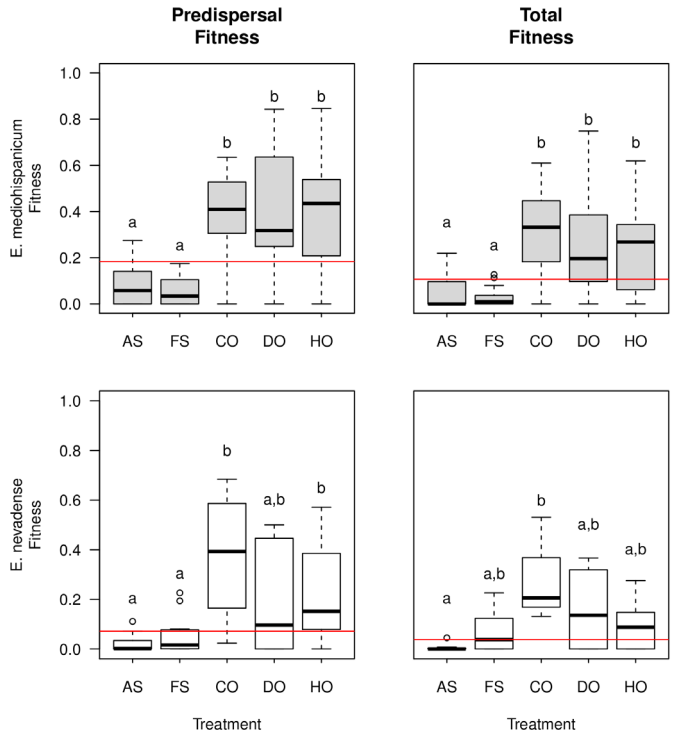


Figure 3.5. Multiplicative pre-dispersal and total fitness represented by box plots for the five treatments carried out in our control-crossing experiment for *E. mediohispanicum* and *E. nevadense*. The top and bottom boxes indicate upper and lower quartiles of the distribution, while vertical lines show 1.5x the interquartile range; open circles indicate points falling beyond that range. The heavy horizontal bar indicates the median for each treatment, while the median fitness value for the species is represented by a red horizontal bar. Distributions not statistically significantly different by Kruskall-Wallis pairways comparison show the same letter. AS: Autonomous Selfing; FS: Facilitated Selfing; CO: Intra-Populational Outcrossing; DO: Inter-Populational Outcrossing; HO: Inter-Specific Hybridization.

DISCUSSION

The decrease in fitness due to selfing was much more intense during the pre-dispersal phase of the life cycle than during the post-dispersal phase, both in *E. mediohispanicum* and *E. nevadense* (Fig. 3.3 and 3.4). These strong differences between treatments in our studied species highlight the pre-dispersal components of their life cycle as the stages in which inbreeding depression manifests itself most effectively, as demonstrated by previous empirical and theoretical works (e. g., Husband and Schemske, 1996; Harder *et al.*, 2011). This explains the high inbreeding depression values found at W_{PRE} and maintained, but not increased, at W_{TOT} in all cases, except for facilitated inbreeding depression in *E. nevadense* (Table 3.3). This finding may affect the efficiency of genetic purging, as the genetic load expressed early in the life cycle may be purged more easily than the load expressed later on (Husband and Schemske, 1996; Stift *et al.*, 2012). Furthermore, we can presume that inbreeding depression is even stronger in the natural conditions of both plant species, as our experiment was performed in greenhouse conditions, where inbreeding depression is usually weak (Dudash, 1990; Willis, 1993; Montalvo, 1994; Koelewijn, 1998).

Inbreeding depression was lower, and the selfing rate higher, in *E. nevadense* than in *E. mediohispanicum*. These results suggest that *E. nevadense* has historically undergone a stronger purge of its genetic load than *E. mediohispanicum* because of its greater history of selfing. Moreover, self-pollen limitation varied between species, being strong and significant in *E. nevadense* but very weak and non-significant in *E. mediohispanicum* (Table 3.3). This means that although both species have the mechanical capacity for self-pollen autodeposition, the reproductive output of *E. nevadense* seems to be partially limited by the amount of self-pollen landing onto its stigmas. The difference in the habitat occupied by each species may partly explain these differences. In fact, *E. nevadense* occurs in the high mountains of the Sierra Nevada, whereas *E. mediohispanicum* is distributed along its low- and medium-altitude mountain areas (Blanca *et al.*, 2009).

Species	Pops	N_{ID}	Facilitated Inbreeding Depression		N_{SR}	F_{IS}	SR	Pollinator assemblage		
			ID_{PRE}	ID_{TOT}				Main pollinators	Abundance	S_{obs}
E. mediohispanicum										
	Em 25	7	0.93	0.91	30	0.363±0.248	53.32	Large and small bees (41%)	0.65	20
	Em 17	23	0.86	0.81	30	0.213±0.045	35.13	Large bees and beetles (42%)	0.58	41
E. nevadense										
	En 11	10	0.55	0.32	30	0.298±0.068	45.95	Ants (42%)	0.18	20
	En 10	4	0.74	-	30	0.360±0.249	52.97	Ants (48%)	0.27	23

Table 3.2. Number of plants used in the control-crossing experiment (N_{ID}) and the estimation of the allele Fixation Index (F_{IS}) and Selfing Rate (SR) using 10 microsatellite markers (N_{SR}). Selfing Rate was calculated as: $2\ F_{IS}/(1 + F_{IS})$ x 100. Facilitated Inbreeding Depression (comparing Facilitated Selfing and Intra-Populational Outcrossing) at W_{PRE} (ID_{PRE}) and W_{TOT} (ID_{TOT}) calculated for the *E. mediohispanicum* and *E. nevadense* populations included in the present study. *Main pollinators* refer to the functional groups accumulating higher numbers of interaction with *Erysimum* plants in the studied population. *Abundance* is expressed as insect visits flower^{-1} h^{-1}. S_{obs} is the observed number of pollinator species censused per population. Pollinator assemblage refers to sampling carried out in 2007 (***Chapter 8***).

Alpine environments are characterized by the scarcity and temporal unpredictability of pollinators (Ashman et al., 2004; Burd et al., 2009), which cause many alpine plants to have their reproduction limited by pollen availability (Garcia-Camacho and Totland, 2009; Arroyo et al., 1982). The Sierra Nevada, in particular, presents a harsher climate than most alpine environments due to severe summer droughts. Consequently, the most frequent pollinators in the high-mountain of the Sierra Nevada are low-efficiency insects such as ants and small flies (Gómez and Zamora, 1992; Gómez et al., 1996). In fact, previous observation has shown that these two types of insects dominate the pollinator assemblage of *E. nevadense* (Ortigosa and Gómez, 2010; ***Chapter 6*** and ***8***). In contrast, the pollinator assemblage of *E. mediohispanicum* is more diverse and is dominated by bees, bee-flies and other high-efficiency insects (Gómez et al., 2007). These findings concur with our observations in the present study. The pollinators visiting *E. nevadense* were scarcer and less diverse (Flower visitor abundance = 0.23 visits flower^{-1} h^{-1} and Flower visitor species richness = 21.5 spp.) than the pollinators visiting *E. mediohispanicum* (Flower visitor abundance = 0.62 visits flower^{-1} h^{-1} and Flower visitor species richness = 30.5 spp.) (***Chapter 8***). In these harsh conditions, selfing would probably have ensured plant reproduction (Lloyd, 1979), increasing the frequency of genetic load purging events (Uyenoyama and Waller, 1991; Crnokrak and Barret, 2002). The purging events associated with reproductive assurance would have caused *E. nevadense* plants to be more tolerant to selfing, or at least to those deleterious effects manifested in their early development (Husband and Schemske 1996).

It seems that outbreeding depression was higher in *E. mediohispanicum* than in *E. nevadense*. This finding agrees with previous reports showing pollinator-mediated local adaptation (Gómez *et al.*, 2008a; 2008b; 2009a; 2009c) and possible intra-population genetic structure (***Chapter 4***) in *E. mediohispanicum*. Nevertheless, the magnitude of outbreeding depression is low in both species, suggesting that local adaptation is not important for either of them. Our experiment thus suggests that the genetic load due to inbreeding is higher

	F_{IS}	SR	Self-Pollen Limitation	Autonomous Inbreeding Depression	Facilitated Inbreeding Depression	Outbreeding Depression	Hybrid Inviability
E. mediohispanicum	0.20±0.03	33.75%					
W_{PRE}			-0.11 [-0.57, 0.04]	**0.86 [0.77, 0.89]**	**0.84 [0.82, 0.92]**	0.17 [-0.21, 0.21]	0.21 [-0.18, 0.23]
W_{TOT}			0.02 [-0.90, -0.01]	**0.81 [0.78, 0.94]**	**0.89 [0.84, 0.95]**	**0.09 [0.02, 0.46]**	0.23 [-0.01, 0.46]
E. nevadense	0.24±0.04	38.71%					
W_{PRE}			**0.58 [0.39, 1.16]**	**0.95 [0.88, 1.00]**	**0.59 [0.57, 0.93]**	0.31 [-0.04, 1.02]	**0.08 [0.07, 0.73]**
W_{TOT}			**0.16 [0.06, 1.74]**	**0.97 [0.92, 1.01]**	0.32 [-0.13, 0.60]	-0.08 [-0.28, 1.31]	**0.34 [0.37, 0.99]**

Table 3.3. Selfing rates (SR = 2 F_{IS}/(1 + F_{IS}) x 100) calculated using the Inbreeding Coefficient (F_{IS}) for *E. mediohispanicum* (N=60) and *E. nevadense* (N=60). Predispersal (W_{PRE}) and total (W_{TOT}) multiplicative fitness were used to calculate Self-Pollen Limitation, Autonomous and Facilitated Inbreeding Depression (relative performance of Autonomous and Facilitated Selfing to Intra-Populational Outcrossing, respectively), Outbreeding Depression (relative Inter-Populational Outcrossing and Intra-Populational Outcrossing performance) and Hybrid Inviability (comparing Inter-Specific Hybridization and Intra-Populational outcrossing).

than the genetic load due to outbreeding.

Our study has shown that *E. mediohispanicum* and *E. nevadense* are inter-fertile. And this capacity for inter-specific hybridization occurs in both directions, i.e., irrespective of which species is pollen donor or recipient. Hybridization is frequent in crucifers, where it is an important evolutionary force (Marhold and Lihová, 2006; Lysak and Koch, 2011). *Erysimum* is a highly diversified genus (> 200 spp.) of recent origin (Couvreur et al., 2010). The evolution of the genera is complex, with recurrent inter-specific hybridization, polyploidization, incomplete sorting, and reticulate evolution (Clot, 1992; Ancev, 2006; Marhold and Lihová, 2006), frequently resulting in species complexes and cryptic species (Ancev, 2006; Turner, 2006; ***Chapter 1***). In the present study, a phylogenetic analysis performed using one nuclear (ITS1-ITS2) and two plastidial (trnT-L, ndhF) molecular markers indicates that the two species included here are not sister species (Appendix 3.1). Furthermore, despite their environmental divergence, they come into a secondary contact along a narrow area at an altitude of approximately 2,300 m. Populations from each species may be located a mere 100 m apart. For this reason, we believe that the inter-specific hybridization observed in experimental conditions may also occur in natural populations.

Our experiment also found hybrid inviability, as the performance of hybrid crosses was poorer than that of intraspecific crosses. However, the magnitude of this inviability was very low. In fact, the performance of the hybrids was, on average, only 23 to 34% below that of non-hybrid progeny. Our experiments were performed in a greenhouse, where the optimal conditions would have probably reduced the differences between hybrid and non-hybrid crosses (Chèvre et al., 2000). A lower rate of hybridization could thus be expected in the wild. It is also worth noting that the performance of hybrids was superior to that of the progeny resulting from autogamy, whether autonomous or facilitated. This outcome suggests that hybridization between *E. mediohispanicum* and *E. nevadense* in the wild will be more intense in years with a depauperated pollinator assemblage, when pollen limitation is stronger

and there is a greater probability of self-pollination (Ramsey and Vaughton, 1996; Gómez et al., 2010). In this situation, hybridization will be selected over self-pollination.

It seems that the two studied species differ slightly in hybrid inviability (Table 3.3). *E. mediohispanicum* did not present any significant differences in fitness between hybrid and intra-specific outcrossing in any component of the life cycle. In contrast, *E. nevadense* presented a significant and consistent hybrid inviability when considering both W_{PRE} and W_{TOT}. This finding suggests the presence of an asymmetric reproductive barrier (Tiffin *et al.*, 2000). Asymmetrical hybridization has frequently been documented in plants (Rieseberg and Carney, 1998; Tiffin et al., 2000) and it has several possible causes (Arnold et al. 2010). In our case, however, as asymmetry had already been observed for W_{PRE}, we believe that the factors causing this asymmetry acted between pollination and seed production. For example, both species may differ in pollen success, as occur in *Ipomopsis* (Aldridge and Campbell, 2006), *Senecio* (Chapman et al., 2005), *Iris* (Emms et al., 1996) and *Silene* (Montgomery et al., 2010). In fact, we found a slight reduction in the ovule fertilization of *E. nevadense* pollinated by *E. mediohispanicum* pollen, but not the opposite (Fig. 3.3). Differences in the mating system may also produce asymmetrical hybridization (Tiffin et al., 2000). The present study suggests that *Erysimum nevadense* is more selfing and less negatively affected by self-pollen than *E. mediohispanicum*. Selfing seems to be important for the development and maintenance of coadapted gene complexes (Fenster et al., 1997, Volis *et al.*, 2011). This would make heterospecific pollen flow a disadvantage when *E. nevadense*, the more selfing species, receives pollen from *E. mediohispanicum*, the outcrossing species, but not *vice versa* (Price and Waser, 1979). An increase in selfing, an asset in the harsh environment inhabited by *E. nevadense*, seems to have also produced a decrease in its ability to hybridize. It would be necessary to extend this study to other *Erysimum* species with a different mating system to test the prevalence of this association between selfing and hybrid inviability.

In summary, our study shows that the two *Erysimum* species, although ecologically very similar, present different mating systems. Whereas *E. mediohispanicum* experiences more intense inbreeding and outbreeding depression but lower hybrid inviability, *E. nevadense* does not present any outbreeding depression, has lower inbreeding depression but shows higher levels of hybrid inviability. Further ecological and genetic studies are necessary to find out the putative mechanisms causing this difference in the mating systems.

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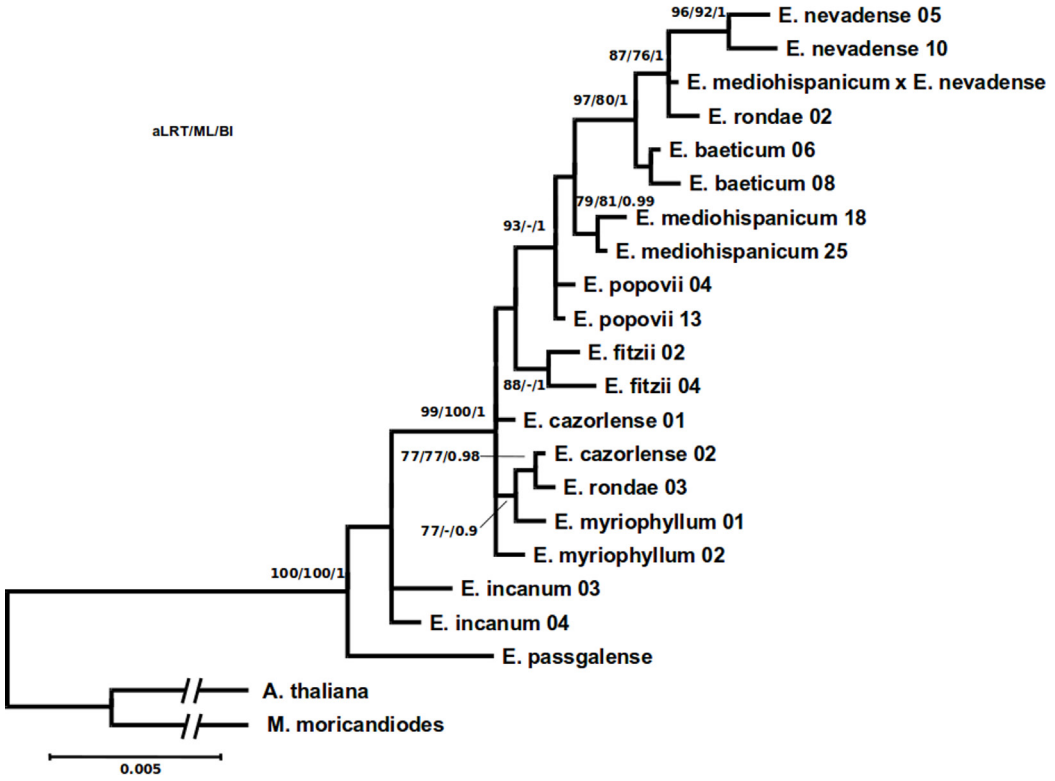
APPENDICES



Appendix 3.1. Phylogenetic position of *E. mediohispanicum* and *E. nevadense* populations within the phylogeny of *Erysimum* species inhabiting the Baetic ranges. aLRT: Maximum likelihood tree obtained with PhyML, calculating branch reliability support by approximate likelihood ratio test/ ML: Maximum likelihood tree obtained with PhyML, calculating branch reliability support by bootstrapping/ BI: Tree obtained using Bayesian Markov chain Monte Carlo inference; branch supportsvalues are posterior probabilities. Only branch supports higher than 75% are shown. For additional information about phylogenetic analyses, see ***Chapter 3*** of the present thesis.

CHAPTER 4

ASSOCIATION BETWEEN INBREEDING DEPRESSION AND FLORAL TRAITS IN *ERYSIMUM MEDIOHISPANICUM*

Mohamed Abdelaziz, A. Jesús Muñoz-Pajares, Modesto Berbel, Francisco Perfectti, José M. Gómez

ABSTRACT

Individual variation in the magnitude of inbreeding depression (ID) in plants and its association with phenotypic traits may have important consequences for mating system evolution. This association has been investigated only scarcely, and always considering traits mechanically related to autogamy. Here we explore the association between individual variation in ID and floral traits associated to pollinator attractiveness (corolla size, corolla shape, flowering stalk height, number of flowers) in two populations of *Erysimum mediohispanicum* (Brassicaceae). ID was calculated along the entire life cycle of the plant. In addition, we also explore the relationship between phenotypic traits and heterozygosity. We found a significant association between ID and corolla diameter, with plants having larger corollas undergoing a lower intensity of inbreeding depression. Furthermore, we found a negative relationship between corolla diameter and heterozygosity, suggesting that plants with large flowers have purged their genetic load. All these finding suggest that plants with large flowers have secularly suffered frequent inbreeding in the study populations. Because corolla diameter is a trait frequently selected by pollinators in the study plant, we believe that the observed relationship between this trait and ID could be mediated by pollinators, probably throughout an increasing in biparental inbreeding.

Key words

Biparental inbreeding, corolla diameter, heterozygosity, microsatellite markers, outcrossing, purging, self-pollination.

INTRODUCTION

The evolution of self-fertilization in hermaphroditic organisms has been the focus of a long-standing debate (Darwin, 1876; Fisher, 1941; Baker, 1955; Kimura, 1959; Nagylaki, 1976; Lloyd, 1979; Takebayashi and Morell, 2001). Several ecological and genetic advantages of self-fertilization have been claimed to explain the evolution of selfing in plants. Selfing may evolve because it has an automatic advantage over outcrossing (automatic selection) with respect to transmission. While outcrossers pass on only one set of genes to their offspring, selfers transmit their entire set of genes (Fisher, 1941; Nagylaki, 1976; Busch and Delph, 2012). Selfing may also evolve because it allows plants to maintain their reproductive output when mates or pollinators are scarce – a phenomenon called reproductive assurance (Darwin, 1876; Baker, 1955; Lloyd, 1979). Finally, self-pollination may evolve because it is more efficient energetically (Darwin, 1877; Waller, 1979; Aarsen, 2008). Despite these advantages, Goodwillie *et al.* (2005) and Barrett and Eckert (1990) found that only 14% and 25% out of 345 and 129 studied plants, respectively, were predominantly selfers (outcrossing rates lower than 20%). Inbreeding depression (ID), the reduced survival and fertility of the offspring of related individuals, was soon recognized as a major obstacle to the evolution of self-fertilization (Darwin, 1876; Charlesworth and Charlesworth, 1987). Inbreeding depression occurs as a result of an increase in homozygosity in detrimental partially recessive alleles (dominance hypothesis), or in alleles at loci with a heterozygous advantage (overdominance hypothesis) (Charlesworth and Charlesworth, 1987; Charleswoth and Willis, 2009).

When selfing is frequent and inbreeding depression is intense, plant populations can be expected to purge their genetic load and thereby counteract ID (Byers and Waller, 1999). Decreasing levels of ID via efficient purging of the genetic load would favor the transition from outcrossing to selfing in a given population within a relatively low number of generations (Charlesworth and Charlesworth, 1987; Charlesworth *et al.*, 1990); and more easily in organisms in which genetic load express in early life history stages

than in laters (Husband and Schemske, 1996). Recent evidence suggests that genetic purging may occur over a short time period in many plants (Crnokrak and Barrett, 2002; Roels and Kelly, 2011). Under these circumstances, natural selection may cause an association between the selfing rate and those traits correlating with selfing probability. In fact, selfing has been found to be associated with some floral traits in self-compatible plants that facilitate autogamy, such as decreased spatial and temporal separation between anthers and stigmas (herkogamy and dichogamy, respectively) (Brunet and Eckert, 1998; Takebayashi and Delph, 2000; Elle and Hare, 2002; Takebayasi *et al.*, 2006; Fishman and Willis, 2008; Kalisz *et al.*, 2012) and small flower size (Elle and Carney, 2003). Attractive plant traits may also influence the selfing rate in those plants in which selfing is mediated by pollinators (facilitated self-pollination; Lloyd and Schoen, 1992). Floral display size or plant size, for example, may increase the selfing rate through geitonogamy (Karron *et al.*, 2004; Williams, 2007; Duminil *et al.*, 2009). Attractive plant traits may also correlate with selfing when it occurs as a consequence of biparental inbreeding (Ritland, 1984; Goodwillie *et al.*, 2010). Flowering phenology, for example, may increase the probability of reproduction between relatives by increasing population structuring and assortative mating. (Weis, 2005; Elsinga *et al.*, 2007). Furthermore, species with limited dispersion and spatially structured populations may have reduced genetic neighborhoods, implying an increase in the breeding between relatives (e.g., Carillo-Angeles *et al.*, 2011).

Uyenoyama and Waller (1991a; 1991b; 1991c) have explored the association between individual ID and phenotypic traits as a consequence of selfing evolution. They found that when ID is caused by deleterious recessive mutations (dominance hypothesis), individuals with traits promoting selfing should exhibit lower levels of ID than individuals with traits that promote outcrossing (Uyenoyama and Waller, 1991a). This is because individuals with selfing-associated traits belong to lineages with a longer history of selfing and have exposed their deleterious recessive alleles to genetic purging by selection more frequently than heterozygotic individuals. Consequently, individuals

bearing selfing-associated traits would have a lower frequency of deleterious recessive alleles than other individuals in the population, reinforcing the association between trait value and selfing tolerance (Uyenoyama and Waller, 1991a; 1991c). In keeping with the theoretical expectations of Uyenoyama and Waller (1991a), some studies have found that the traits influencing the selfing rate, such as herkogamy, may be associated with a decrease in the inbreeding depression (Takebayashi and Delph, 2000; Stone and Motten, 2002).

Plant-pollinator systems range from specialist to generalist. Specialized pollination systems have long been recognized as the result of important co-evolutionary processes that promote the effective transfer of outcross pollen (Stebbins, 1970; Fenster *et al.*, 2004). In contrast, the flowers of generalist plants are visited by a wide range of flower visitors that differ dramatically in their pollinator effectiveness, foraging behavior and preference patterns (Gómez and Zamora, 1999; Wilcok and Neiland, 2002; Perfectti *et al.*, 2009). Consequently, the floral traits in generalist plants undergo contrasting selective pressures both inter- and even intra-populationally (Gomez *et al.*, 2009a; 2011). In this scenario, plants from the same population but with a different phenotype may be visited by different floral visitors, with varying preference patterns and pollination efficiencies (Gómez *et al.*, 2011). This intra-population variation in pollination effectiveness may produce concomitant intra-population and inter-individual variations in the selfing rate. Generalist systems are, therefore, appropriate scenarios for exploring any putative association between ID and floral traits that favor both autogamy and pollinator-mediated selfing (Uyenoyama and Waller, 1991a). Here we investigate the association between pollinator-attracting floral traits and ID in a generalist plant, *Erysimum mediohispanicum* (Brassicaceae). Its flowers are visited by over 160 insect species belonging to six orders (Gomez *et al.*, 2007), with varying efficiency as pollinators as regards the floral traits they prefer and the magnitude and direction of the selection they exert (Gómez *et al.*, 2006; 2008a; 2008b; 2009a). Specifically, in this study we quantify the association between pollinator-attracting floral traits and the individual levels of both

heterozygosity and inbreeding depression.

MATERIALS AND METHODS

STUDY SYSTEM

Erysimum mediohispanicum is a monocarpic hermaphroditic herb endemic to the Iberian Peninsula. As a facultative biennial, the plants spend 2 to 3 years as a rosette in a vegetative stage. After that period they display anything from a few to several hundreds of flowers located on 1-3 stalks (Gómez, 2003). Its flowers are visited by a highly diverse assemblage of insects (Gómez *et al.*, 2007) with different pollination effectiveness and preference patterns (Gómez *et al.*, 2008a; 2009a; 2009b). These pollinators exert significant selection on many floral traits (Gómez *et al.*, 2008a; 008b).

EXPERIMENTAL DESIGN

In September 2009, 30 juvenile plants of *E. mediohispanicum* from two populations in the Sierra Nevada (Spain), located at 2,064 and 2,182 m. a. s. l., respectively, were transplanted to individual pots (11x11x15 cm) and moved to a common garden in the University of Granada (700m. a. s. l. approx.). At the beginning of May 2010, 30 plants had survived and before they started blooming they were moved to a greenhouse in order to isolate them from pollinators. We carried out three treatments on each of these plants: 1) Outcrossing (OC), in which flowers were emasculated and hand-pollinated with pollen from different individuals from the same population; 2) Facilitated Selfing (FS), in which flowers were emasculated before opening and hand-pollinated with their own pollen; and 3) Autonomous Selfing (AS), in which flowers were not manipulated and left to self-pollinate spontaneously. This latter treatment was a procedural control for our manipulations. In total, 717 flowers were used in the experiment, with a mean of 22±14 experimental flowers per plant (10 ± 8 AS flowers, 10± 6 FS flowers and 3 ± 2 OC flowers).

Once the blooming period was over, we recorded the number of flowers per plant and treatment setting ripe fruits or aborted without producing fruits. The total number of ovules, unfertilized ovules, aborted seeds and ripe seeds produced per ripe fruit were recorded in the lab by using magnifying glasses. Fifteen seeds per plant and treatment were taken at random and sown haphazardly in a greenhouse. Their germination was recorded twice a week during the first month and seedling survival was recorded every month during the next 10 months.

QUANTIFICATION OF PHENOTYPIC TRAITS

The following phenotypic traits were quantified for each experimental plant: (1) stalk height: the height of the tallest stalk from the ground to the top of the stalk at the end of flowering period; (2) corolla diameter: the distance between the edge of two opposite petals (±0.1 mm error); (3) corolla tube width: the diameter of the corolla tube aperture as the distance between the bases of two opposite petals; (4) corolla tube length: the distance between the corolla tube aperture and the base of the sepals; (5) corolla shape: determined by means of geometric morphometric tools, using a landmark-based methodology that eliminates the effect of variations in the location, orientation and scale of the specimens (Zelditch *et al.*, 2004). We took a digital photograph of one flower per plant using a standardized procedure (front view and planar position). Flowers were photographed at anthesis to avoid ontogenetic effects. We defined 32 coplanar landmarks located along the outline of the flowers and the aperture of the corolla tube, the number of landmarks being chosen to provide comprehensive coverage of the flower shape (see Gómez *et al.*, 2006 and ***Chapter 1*** for a detailed description of the landmark locations and software used). The two-dimensional coordinates of these landmarks were determined for each plant, and the generalized orthogonal least-squares Procrustes average configuration of landmarks was computed using the Generalized Procrustes Analysis (GPA) superimposition method (Zelditch *et al.*, 2004). After GPA, the relative warps (RWs, principal components of

the covariance matrix of the partial warp scores) were computed (Zelditch *et al.*, 2004). This procedure generates 2p-4 orthogonal RWs (p= number of landmarks). Each RW explains a given variation in shape between specimens. Thus, RWs summarize shape differences between specimens, and their scores can be used as a data matrix to perform standard statistical analyses (Zelditch *et al.*, 2004). In all subsequent analyses we used the first four RWs, because each explained more than 5% of variation in shape.

ESTIMATION OF FITNESS COMPONENTS

The following pre- and post-dispersal components of the plant reproductive output were quantified for each treatment and plant: (a) fruit set, the proportion of labeled flowers setting fruit; (b) seed production, number of seeds produced per ovule in a given fruit; (c) seedling emergence, calculated as the proportion of sown seeds germinating and emerging as seedlings; and (d) seedling survival, calculated as the proportion of seedlings surviving until the end of the experiment. Afterwards, we calculated the cumulative pre-dispersal absolute fitness (W_{pre}) as fruit set x seed production, and the cumulative total absolute fitness (W_{tot}), as fruit set x seed production x seedling emergence x seedling survival.

ESTIMATION OF INBREEDING DEPRESSION

We calculated inbreeding depression (**δ**) per plants $1-w_S/w_X$, where w_S and w_X are the fitness associated with self crosses and outcrosses, respectively, for each fitness component (Lande and Schemske, 1985). The statistical significance of inbreeding depression was calculated by bootstrapping, using the package stats and boot in R (R Development Core Team, 2011).

ESTIMATION OF HETEROZYGOSITY AND INBREEDING COEFFICIENTS (F_{IS})

The association between phenotype and heterozygosity was determined during 2007 in 60 additional individuals growing in the same populations in which our experimental plants were collected. For estimating heterozygosity, we collected fresh tissue that was stored in silica gel for their subsequent genotyping, using 10 microsatellite markers described for *E. mediohispanicum* (Muñoz-Pajares *et al.* 2011). DNA was isolated using the GenElute Plant Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, Missouri, USA). PCR was performed in 15 µL of reaction mixture containing 0.17 ng/µL of template genomic DNA, 1x buffer (ref. M0273S, New England BioLabs), 0.16 mM each dNTP (Sigma-Aldrich), 0.33 µM each forward (fluorescently tagged; Applied Biosystems, Foster City, California, USA) and reverse primer, and 0.02 U/µL *Taq* polymerase (ref. M0273S, New England Biolabs). PCR was conducted in a Gradient Master Cycler Pro S (Eppendorf, Hamburg, Germany) with an initial 30s of denaturation at 94 °C, 35 cycles at 94 °C for 15s, annealing temperatures per single microsatellite marker described by Muñoz-Pajares *et al.* (2011) for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 3 min. PCR products were diluted 1:15 and analyzed by MACROGEN analyzers (Geumchun-gu, Seoul, Korea; http://www.macrogen.com) using 400HD ROX (Applied Biosystems) as standard. Alleles were called using Peak Scanner Software version 1.0 (Applied Biosystems), with which we counted the number of heterozygotic and homozygotic loci in order to calculate the proportion of heterozygotic loci for each individual.

The inbreeding coefficient (F_{IS}) was estimated using FSTAT software v2.9.3 (Goudet, 1995), for studied populations, and after obtaining similar positive values we estimated the overall F_{IS}. We also calculated the expected and observed heterozygosity for the studied individuals. The selfing rate (s) was estimated using F_{IS} from the classical way $F_{IS}=s/(2-s)$ (Hartl and Clark, 1989).

DATA ANALYSIS

Between-treatment differences in reproductive output during each life-cycle stage and for the cumulative fitness estimates were tested with the non-parametric Kruskal-Wallis test, using the software JMP 7.0. The potential relationship between inbreeding depression and the phenotypic traits was first explored by means of a GLM that also included populations and their interaction with plant phenotype as random factors. As no single interaction was significant (data not shown), we explored the relationship between inbreeding depression and the phenotypic traits using Sperman's rank correlation. We decided to use non-parametric correlations due to our small sample size, and in order to avoid any assumption about linearity in the relationship between variables. Sperman's correlation was also used to explore the correlations between phenotypic traits, as well as the association between plant phenotype and heterozygosity. These analyses were performed using the package stats in R (R Development Core Team, 2011).

RESULTS

Reproductive output was significantly higher in outcrossed flowers than in selfed flowers for all fitness components except seedling survival, where no differences were found (Table 4.1). However, no difference in reproductive output was found between autonomous and facilitated selfing for any of the life-cycle stages analyzed in this study. This indicates that our experimental manipulations did not produce significant side effects. Inbreeding depression was significant for every life-cycle stage except seedling survival (Table 4.1). The highest intensity of inbreeding depression was found for seed production (0.69), W_{re} (0.86) and W_{ot} (0.81). There was marked variation in inbreeding depression between maternal plants (Fig. 4.1).

There was significant positive correlation between corolla diameter and corolla tube length and stalk height, whereas there was significant negative correlation between corolla tube length and corolla tube width (Table 4.2). No other traits

Life-cycle stage	Autonomous Selfing	Facilitated Selfing	Outcrossing	χ^2 (AS-FS)	*P*	χ^2 (FS-OC)	*P*	**δ**	95% Confidence interval
Fruit set	0.24±0.04	0.28±0.04	0.80±0.05	0.45	0.498	42.04	0.000	**0.32**	[0.25 to 0.44]
Seed production	0.17±0.03	0.15±0.03	0.51±0.04	0.07	0.790	36.02	0.000	**0.67**	[0.61 to 0.81]
Seedling emergence	0.28±0.07	0.32±0.07	0.63±0.06	0.08	0.772	13.38	0.001	**0.46**	[0.28 to 0.72]
Seedling survival	0.89±0.06	0.88±0.07	0.85±0.06	0.09	0.762	0.10	0.950	0.06	[-0.25 to 0.19]
W_{pre}	0.07±0.01	0.06±0.01	0.42±0.03	0.09	0.766	44.93	0.000	**0.84**	[0.82 to 0.92]
W_{tot}	0.04±0.01	0.03±0.01	0.29±0.04	0.01	0.940	35.22	0.000	**0.89**	[0.84 to 0.95]

Table 4.1. Performance of plants under each of the three treatments carried out in the experiment, and mean inbreeding depression (δ) values calculated using outcrossing and facilitated selfing treatment [± 95% confidence interval found by boostrapping].

were correlated in the experimental plants (Table 4.2). The first four shape components (RWs) were associated to similar changes in corolla shape in both experimental and natural plants (Fig. 4.2), explaining altogether more than 78% of its variance.We found significant correlation between the intensity of individual inbreeding depression and some phenotypic traits. Plants with larger corollas suffered lower inbreeding depression during most of their progeny's life-cycle stages (Table 4.3). Plants with taller flowering stalks also expressed lower inbreeding depression (Table 4.3).

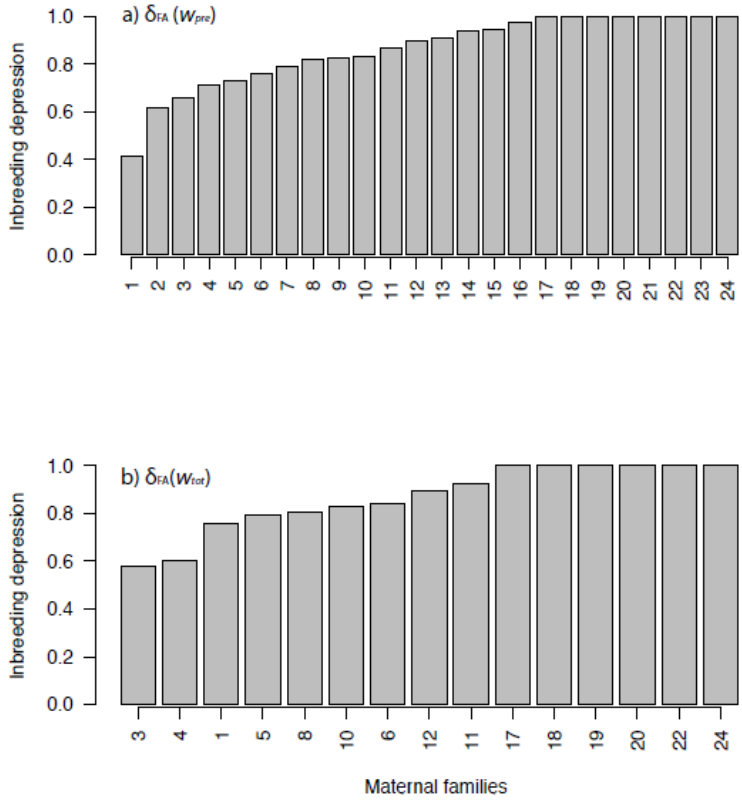


Figure 4.1. Differences among *Erysimum mediohispanicum* families in inbreeding depression, calculated as: a) pre-dispersal absolute fitness (W_{pre}), and b) cumulative total absolute fitness (W_{tot}). Families are arranged in order of decreasing inbreeding depression.

Experimental plants	Mean±1 SE	Stalk height	Corolla diameter	Corolla tube length	Corolla tube width	RW1	RW2	RW3
Stalk height	29.43±2.06							
Corolla diameter	11.43±0.31	0.5232**						
Corolla tube length	10.31±0.25	0.2018	0.5005**					
Corolla tube width	1.21±0.11	-0.1346	-0.0127	-0.3867*				
RW1	0.01±0.02	0.2240	0.3558	0.1819	-0.1417			
RW2	0.00±0.01	0.0174	-0.2904	-0.2200	0.2115	-0.0637		
RW3	0.00±0.01	0.0684	-0.2325	-0.1416	-0.2607	0.0609	0.0433	
RW4	0.00±0.01	0.1901	-0.1583	-0.0293	-0.0845	-0.0751	0.0151	-0.0140
Natural plants	Mean±1 SE	Stalk height	Corolla diameter	Corolla tube length	Corolla tube width	RW1	RW2	RW3
Stalk height	27.00±1.48							
Corolla diameter	12.32±0.25	0.1024						
Corolla tube length	10.96±0.24	0.0984	0.7468****					
Corolla tube width	1.36±0.10	-0.2365	0.1894	0.1249				
RW1	-0.01±0.01	0.2313	-0.2235	-0.1444	-0.0037			
RW2	-0.01±0.01	-0.2520	0.0375	-0.0508	0.2069	0.1043		
RW3	0.01±0.01	-0.2350	-0.1422	0.0498	-0.1053	-0.0917	0.1443	
RW4	0.01±0.01	0.0841	-0.2415	-0.2139	-0.0471	0.1126	-0.2644	-0.1092

Table 4.2. Mean values of phenotypic traits included in the study (N=30) in *Erysimum mediohispanicum* and Sperman's correlations values between them. *Experimental plants* refers to plant flowering at greenhouse conditions; *natural plants* refers to plants flowering in the field. *P<0.05, **P<0.01, ****P<0.0001

Individual heterozygosity was significantly and negatively correlated with corolla diameter and corolla tube length (r_s= -0.5362, P<0.0001 and r_s= -0.5319, P<0.0001, respectively). In contrast, no other phenotypic traits present a significant correlation with the individual level of heterozygosity. Corolla diameter and corolla tube length were the only two phenotypic traits presenting a significant positive correlation (r_s=0.7468, P<0.0001; Table 4.2).

The observed heterozygosity was lower than expected (H_O=0.586 and H_E=0.683, respectively) and the inbreeding coefficients showed positive values (F_{IS}=0.091 and 0.193), showing a significant overall inbreeding coefficient value by bootstrapping, F_{IS}=0.156 (95% confidence interval=0.016-0.251). Both results revealed an excess of homozygotes with respect to the Hardy-Weinberg equilibrium condition. Positive values of selfing rates were found, based on the aforementioned F_{IS} values, s= 0.17 and 0.32. The overall selfing value was 0.27.

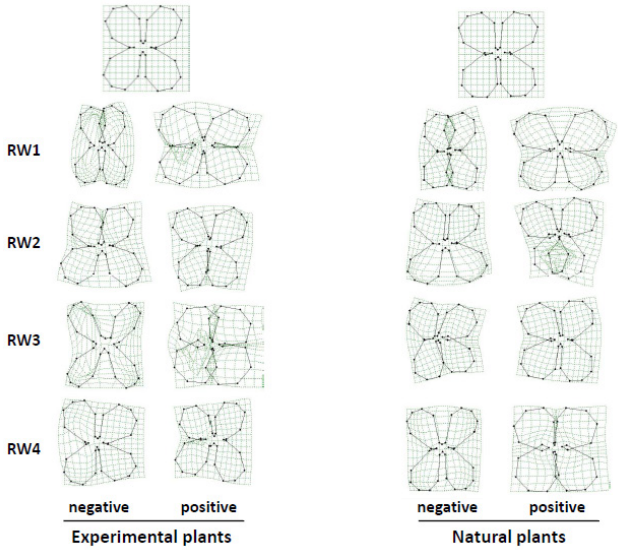


Figure 4.2. Summary of the geometric morphometric for the experimental (N=30) and natural plants (N=60) used in the present study. Consensus morphology (uppermost panels) and relative warps explaining more than 5% of the overall variation in shape for both groups of plants are shown.

	δ_{FS}					
Traits	Fruit set	Seed production	W_{pre}	Seedling emergence	Seedling Survival	W_{tot}
Stalk height	-0.526**	-0.410	-0.501*	-0.447	0.600	-0.425
Corolla diameter	-0.441*	-0.636**	-0.601**	-0.613*	0.676	-0.757**
Corolla tube width	-0.114	-0.078	-0.179	-0.391	0.338	-0.280
Corolla tube length	-0.199	-0.108	-0.069	-0.014	0.676	-0.121
RW1	-0.165	-0.115	-0.094	-0.162	0.676	-0.130
RW2	0.171	0.233	0.145	0.266	-0.034	0.306
RW3	-0.064	-0.098	-0.079	0.307	-0.338	0.061
RW4	0.223	0.184	0.177	0.156	-0.034	0.248

Table 4.3. Spearman's rank correlation between phenotypic traits and inbreeding depression calculated at each life cycle stage and their cumulative: pre-dispersal absolute fitness (W_{pre}) and cumulative total absolute fitness (W_{tot}). *P<0.05, **P<0.01

DISCUSSION

The magnitude of inbreeding depression in *E. mediohispanicum* (up to 0.8) was very high, much higher than the values found in most outcrossing and mixed-mating plants (Barrett and Eckert, 1990; Husband and Schemske, 1996; Goodwillie *et al.*, 2005; Winn *et al.*, 2011). And this high ID was found even though we performed the experiment in controlled conditions, where the strength of ID used to be significantly smaller than in the field (Dudash, 1990; Willis, 1993; Montalvo, 1994; Koelewijn, 1998). In addition, studies evaluating the impact of inbreeding depression tend to focus on a single life-history stage, or only a few. However, the studies accounting for lifetime inbreeding depression have shown that this tends to accumulate across life-history stages (Szulkin *et al.*, 2007; Grindeland, 2008; Grueber *et al.*, 2010). In our case, we quantified inbreeding depression across most of the life cycle of the progeny – a further explanation of why inbreeding depression was so strong. All of these findings suggest that *E. mediohispanicum*, although self-compatible, greatly benefits from outcross pollen and behaves functionally as outcrossing or mixed-mating (Goodwillie, 2000).

The main finding of our study is the significant correlation between the individual level of inbreeding depression and the values of several phenotypic traits. Takebayashi and Delph (2000) and Stone and Motten (2002) have previously reported association between phenotype and inbreeding depression. The phenotypic trait associated with ID in those studies was the level of herkogamy, a trait directly affecting the rate of spontaneous autogamy. For this reason, this association between phenotype and ID is explained by invoking the occurrence of genetic purging, mostly in those genotypes exhibiting a lower magnitude of herkogamy and consequently a higher rate of spontaneous autogamy. This option is not possible under the overdominance hypothesis, when individuals with selfing-associated traits would show lower levels of fitness, due to their low level of heterozygosity as a consequence of promoting self-fertilization (Uyenoyama and Waller, 1991b). In our study, the trait associated with ID does not affect spontaneous autogamy. In contrast,

the trait associated with *E. mediohispanicum* ID was corolla diameter, a trait affecting the interaction of plants with pollinators. Specifically, we found that plants with larger corollas underwent a lower intensity of inbreeding depression after self-pollination. This suggests that pollinators should mediate any association between corolla diameter and ID. In fact, corolla diameter influences pollinator preference pattern, as well as being under pollinator-mediated selection, being heritable and associated with floral reward (Gómez *et al.*, 2006; 2008a; 2008b; 2009b; 2009a). *Erysimum mediohispanicum* flowers are visited in any population by many diverse insects with varying foraging behavior and effectiveness (Gómez *et al.*, 2007; 2009a). And different insects are attracted by different plant traits (Gomez *et al.*, 2008a). This results in reproductively structured populations, where groups of phenotypically similar individuals tend to mate with each other more frequently than with the rest of the population because they are visited by a similar subset of the whole pollinator assemblage (Gómez *et al.*, 2011). This reproductive structure probably means that genetically related individuals frequently mate with each other, causing pollinator-mediated biparental inbreeding. Since the probability of biparental inbreeding depends on the value of various phenotypic traits that influence the identity of the insects visiting the flowers, any genetic purging will also be associated with the phenotypic traits mediating biparental inbreeding.

The negative correlation found between corolla diameter and heterozygosity level supports our argument. Thus, we found that plant families with corollas that promote self-fertilization were also more homozygous. This finding may indicate that these families have evolved after a long period of self-fertilization (Takebayashi and Delph, 2000). This reduction in heterozygosity would entail the purging of deleterious recessive alleles, producing reproductive lines that endure higher rates of selfing and thus present less ID. This may explain why, despite the high level of ID found in *E. mediohispanicum*, its selfing rate was fairly high (s=0.27, F_{IS}=0.156), closer to a mixed-mating species than to a outcrossing species. If we consider that pollinators were the main agent producing this pattern, we can assert that a

high intra-population structure exists, due to the assortative mating promoted by the different species of pollinator interacting with *E. mediohispanicum*.

The presence of plants with different levels of ID and, consequently, different degrees of tolerance to selfing in the same population could help to explain the existence of mixed mating systems (Chang and Rausher, 1999; Goodwillie *et al*., 2005; Winn *et al*., 2011). Under low or null ID, theoretical models predict the replacement of outcrossing by selfing as the primary mating system (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Charlesworth *et al*., 1990). In contrast, when ID is high, outcrossing should be the most probable mating system. In this scenario, a mixed-mating population is considered an evolutionary transition between outcrossing and selfing (Winn *et al*., 2011), but its high frequency (Barret and Eckert 1990; Goodwillie *et al*., 2005) requires alternative theories to explain the stable levels of mixed mating systems. Recently, Winn *et al.* (2011), after exploring the relationship between ID and mating system in 68 taxa, suggested that mixed-mating systems could be maintained by selective interference and called for more empirical studies focused on understanding mixed systems in plants. In the present work we describe an association between a singular flower trait (corolla diameter) and the degree of self-tolerance in *E. mediohispanicum*. In this species, hot and cold selection spots has been described on a geographic selective mosaic by contrasting significant selective pressures acting on them (Gómez *et al*., 2009a; 2009c), due to the differences in pollination effectiveness, foraging behavior and preference patterns exhibited by its huge flower visitor assemblage (Gómez *et al*., 2006; 2007; 2008a; 2008b; 2009a). We suggest that these differences could play an important role in the differential selfing degrees and subsequent genetic purge experienced by the different families at intra-population level. In this respect, plants exhibiting phenotypic traits preferred by pollinators that increase selfing or biparental inbreeding would increase their fitness by genetic purge, thereby reducing their dependence on more effective, abundant or outcross-promoting pollinators. This could eventually produce structured populations composed of plants reproducing mainly by outcrossing coexisting with plants reproducing mostly by selfing. The excess

of homozygotes (positive F_{IS} values) found in the studied populations despite their high ID support this idea (Agrawall, 2010).

In summary, in this study we have shown a significant correlation between individual ID and the value of some floral traits related to pollination in *E. mediohispanicum*. We think that this outcome may be the result of different plant families experiencing contrasting selfing histories, thereby contributing to the long-term stability of mixed-mating systems by mating structure. Nevertheless, further evidence is needed to demonstrate this hypothesis.

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CHAPTER 5

GENE FLOW DYNAMICS IN A SECONDARY CONTACT ZONE BETWEEN TWO *ERYSIMUM* SPECIES

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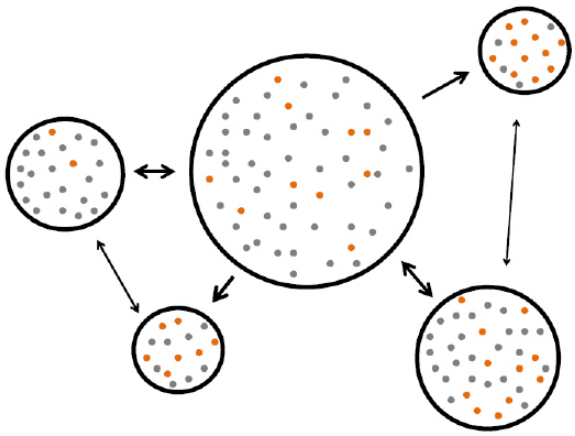


ABSTRACT

Hybrid zones have the potential to shed light on evolutionary processes driving adaptation and speciation. Secondary contact hybrid zones are particularly powerful natural systems where study the interaction between divergent genomes to understand the mode and rate at which reproductive isolation accumulates during speciation. We have studied 210 plants belonging to five populations from two *Erysimum* (Brassicaceae) species presenting a secondary contact zone in Sierra Nevada Mountains (SE Spain). We used 10 previously developed microsatellites markers to study the population genetics of this contact zone. We have estimated the genetic molecular variances, the genetic differentiation, and the genetic structure by means of F-statistics and Bayesian inference. We have also deduced effective population sizes and the amount of recent gene flow between populations. The high levels of genetic diversity and heterozygosity were congruent with a high level of outcrossing. However, the inbreeding coefficients showed by both species suggest that some level of inbreeding or assortative mating is also taking place. We found a narrow unimodal hybrid zone where the hybrid genotypes were more frequent than the parental genotypes. This hybrid zone presented a high effective population size and high level of gene flow from the parental populations. These facts flows would help to explain the long-term maintenance of the hybrid population.

Key words

Effective population size, *Erysimum mediohispanicum*, *E. nevadense*, genetic differentiation, genetic structure, hybrid zone.

INTRODUCTION

Hybrid zones represent scenarios of great interest to explore the evolutionary interactions between divergent but related taxa (Barton and Hewitt, 1985; Harrison, 1993). Even since the first conceptual models to explain hybrid zones were proposed (Dobzhansky, 1940; Mayr, 1942), gene transfer between neighbor species have been recognized as an important evolutionary process (Anderson, 1949). Two main classifications of hybrid zones have been proposed, the first attending to the distribution of hybrid and parental phenotypes/genotypes, and the second depending on the species genealogical origin (Harrison and Bogdanowicz, 1997; Jiggins and Mallet, 2000). The first classification considers three main types of hybrid zones: bimodal, unimodal and flat. In a bimodal hybrid zone, individuals belonging to the parental species co-occur with a very low frequency of their hybrids (Cruzan and Arnold, 1993; McMillan *et al.* 1997; Jiggins and Mallet, 2000; Vedenina and Helversen, 2003). This kind of hybrid zone appears when strong reproductive isolation barriers between the species previously exist (Harrison and Bogdanowicz, 1997; Jiggins and Mallet, 2000; Coyne and Orr, 2004). However, when reproductive isolation between taxa is only partial, a stable hybrid zone would arise depending, in one hand, on fitness differences between parentals and hybrids, and in the other hand, on the migration rates from the parental zones (Key, 1968; Barton and Hewitt, 1985; Jiggins and Mallet, 2000). A second kind is the *unimodal hybrid zone*, where the more frequent individuals are those representing intermediate genotypes between both parental species (Jiggins and Mallet, 2000). A special case of unimodal hybrid zones appears when the hybrid zone form a single panmictic population exhibiting individuals with variable degrees of genetic similarity to each parental forms, forming which was termed as *hybrid swarms* (Harrison and Bogdanowicz, 1997; Jiggins and Mallet, 2000). Hybrid swarms could present individuals coming from first or second hybrid generations, or different levels of back-crossing. The third kind is the flat hybrid zone, representing an intermediate situation between the unimodal and bimodal hybrid zones where hybrid and parental genotypes

appear with similar frequencies (Harrison and Bogdanowicz, 1997).

The second classification of hybrid zones relies on the origin of the hybridizing species (Mayr, 1942; Endler, 1977; Harrison and Bogdanowicz, 1997; Coyne and Orr, 2004). *Primary hybrid zones* are found when two taxa are in the initial phases of the speciation process and show diverging phenotypes at the ends of their continuous distribution (Endler, 1977). Among them no complete isolating barriers exist, implying that hybridization and introgression could commonly occur. The diffusion of diverged alleles among the incipient taxa would depend on the selective pressures acting on hybrids (Endler, 1977; Barton and Gale, 1993). In contrast, a *secondary contact hybrid zone* result from the contact of two already diverged genomes (Mayr, 1942), usually after allopatric speciation. In both cases, hybridization would reduce the genetic differences between species when complete isolating mechanisms are not yet appeared, but if hybrids are unfit, reinforcement could arise and maintain the genetic differentiation between the species (Noor, 1999; Taylor *et al.*, 2006).

Morphological, behavioral and genetic characters in a contact zone can present a clinal distribution, with traits changing gradually from one of the parental species to the other, and the hybrid zone being a transition area between them (Endler, 1977; Barton and Hewitt, 1985). In contrast, Harrison and Rand (1989) suggested the possibility of more complex geographic distribution shaped by exogenous factors, especially in secondary contacts, and termed them *mosaic zones*.

Molecular markers could be used to make inferences on the genetic dynamics of hybrid zones (Barton and Hewitt, 1989; Keller *et al.*, 2001; Avise, 2004). In this sense, the study of the genetic differentiation and structure between populations (or related species) are very useful to infer the extent of admixture between them, as well as to establish the origin of particular alleles across a set of populations (Rousset, 1997; Falush *et al.*, 2003). However, the indirect estimates about long-term gene flow using genetic divergence are based on models with strong assumptions (Rousset, 1997; Falush *et al.*, 2003; Wilson and Rannala, 2003), such as constant population

size, symmetrical and constant migration rates, and population persistence for periods allowing genetic equilibrium (Wright, 1931; 1969). New methods making use of the transient disequilibrium observed at multilocus genotypes and making, in comparison, few assumptions, manage to extract information about recent migration events (Wilson and Rannala, 2003). However, both approaches are complementary, providing information about migration on different timescales (Wilson and Rannala, 2003). Finally, the estimation of the effective population size (*Ne*), defined as the number of breeding individuals in a panmictic population with a binomial distribution of the number of successful offspring per parent (Fisher, 1930; Wrigth, 1931), will complete our understood on the structure and dynamics in secondary contact zones, because it summarizes many demographic parameters of a given population (Caballero, 1994; Wang, 2005).

Hybridization is much more frequent and evolutionary relevant in plants than in animals, but surprisingly it has been more explored in these latter (Ellstrand *et al*., 1996; Dowling and Secor, 1997). However, interesting examples of contact zones and hybrid populations have been described in plants, such as those described in *Phlox* (Levin, 1967), *Irises* (Cruzan and Arnold, 1993; Young, 1996; Emms and Arnold, 1997), *Helianthus* (Rieseberg *et al*., 1998), and recently those described in *Ipomopsis* (Campbell and Aldridge 2006) and *Silene* (Minder *et al*., 2007).

In the present study, we explore a hybrid zone between two species of the genus *Erysimum* L, *Erysimum mediohispanicum* and *E. nevadense*. This genus presents a complex evolutionary history due to events of inter-specific hybridization and polyploidization, producing a highly diversified genus (with more than 200 species) enriched in species complexes and cryptic species (Clot, 1992; Ancev, 2006; Turner, 2006; Marhold & Lihová, 2006; Couvreur *et al*., 2010, Al-Shebaz, 2012; ***Chapter 1***). *Erysimum mediohispanicum* and *E. nevadense* form a secondary contact zone (***Chapter 2***) in Sierra Nevada Mountain (SE Spain), and are able to interbreed in controlled conditions (***Chapter 3***). The main goals of this study are to determine the existence

and nature of hybrid population between *E. mediohispanicum* and *E. nevadense* and to explore the genetic differentiation and structure at intra- and inter-specific levels, as well as to quantify the gene flow between those species and their consequences on effective population sizes.

MATERIAL AND METHODS

STUDY SYSTEM

Erysimum mediohispanicum is an endemism of the Iberian Peninsula, where it is distributed in two extended and disconnected regions in the Northeast and Southeast of the peninsula, respectively. Its life cycle varies among individuals and populations, being usually monocarpic. *E. mediohispanicum* is facultative biennial, spending two to three years growing like a vegetative rosette on calcareous soils up to 2,300 m. altitude. After that period, plants display from only a few to several hundred flowers on one to three stalks (Gómez, 2003). These flowers are visited by a highly diverse assemblage of insects (Gómez *et al.*, 2007).

Erysimum nevadense is mostly polycarpic and endemic to the peaks of the Sierra Nevada Mountains, where it grows on siliceous soils at 2,300 to 2,700 m. They spend two to three years like a rosette before to display anything from a few to several hundred flowers on various floral stalks. It is also a pollination-generalist plant, but it does not present a pollinator assemblage as diverse as that of *E. mediohispanicum*, probably due to the harsh conditions of its habitat (Gómez *et al.*, 2007; Ortigosa and Gómez, 2010; ***Chapter 6*** and ***8***).

Both species come into a secondary contact (***Chapter 2***) along a narrow area at an altitude of approximately 2,200 m. In this contact zone, populations from each species may be located at a mere 100 m apart. These two species were reported as inter-fertile in glasshouse conditions, presenting relatively low values of hybrid inviability (***Chapter 3***).

Population characteristics				Sampling effort	
Population	Latitude	Longitude	Altitude	Sampled plants	Genotyped plants
Em25	37º 7.230' N	3º 26.082' W	2064	30	30
Em17	37º 6.698' N	3º 25.450' W	2182	30	30
H01	37° 6.908' N	3° 25.250' W	2200	90	80
En11	37º 6.750' N	3º 25.048' W	2222	30	27
En10	37º 6.658' N	3º 24.301' W	2322	30	30

Table 5.1. Geographic information and sampling effort in the five populations included in the transect (ordered by altitude).

In 2007, we established a transect between these species in the north face of Sierra Nevada encompassing five population (Table 5.1), two *E. mediohispanicum* populations (Em25 and Em17), two *E. nevadense* populations (En11 and En10), and a putative hybrid population (H01), where both species contact geographically.

DNA ISOLATION AND GENOTYPING

We sampled 30 plants at each of the four parental populations included in the transect and 90 from the hybrid population (in total 210 plants). We collected plant fresh tissues and stored them in silica gel for subsequent DNA isolation, using the GenElute Plant Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, Missouri, USA). The isolated DNA was used for individual genotyping, which was carried out with ten microsatellite markers, previously described by Muñoz-Pajares *et al.* (2011). We performed PCR in 15 μL reaction mixture containing 0.17 ng/μL of template genomic DNA, 1X buffer (ref. M0273S, New England BioLabs), 0.16 mM each dNTP (Sigma-Aldrich), 0.33 μM each forward (fluorescently tagged) and reverse primer, and 0.02 U/μL Taq polymerase (ref. M0273S, New England Biolabs). PCR was conducted in a Gradient Master Cycler Pro S (Eppendorf, Hamburg, Germany) with an initial step of 30s of denaturation at 94 °C followed with 35 cycles at 94 °C

for 15s, annealing for 30 s with a temperature for each microsatellite marker described in Muñoz-Pajares *et al.* (2011), extension at 72 °C for 30 s, and a final extension step at 72 °C for 3 min. PCR products were diluted 1:15 and sent toMACROGEN (Geumchun-gu, Seoul, South Korea; http://www.macrogen.com) to obtain the electropherograms, using 400HD ROX as standard. Alleles were called using Peak Scanner Software version 1.0 (Applied Biosystems).

GENETIC DATA ANALYSIS

After established multilocus genotipes, we excluded for the genetic analyses the individuals presenting levels of missing data higher to 30%, including three individuals form En11 population and 10 individuals form H01 (Table 5.2). To characterize genetically each studied population, we estimated the following parameters: a) number of non-redundant multilocus genotypes (N_G), as the number of genotypes showing at least one different allele, excluding missing data; b) mean number of alleles per locus (n_a); c) observed heterozygosity (*Ho*), as the actual frequency of heterozygous individuals in the sample. We estimated the mean of the individual heterozygosities per population using the ratio between the number of heterozygote loci and the number of successfully genotyped loci; d) gene diversity (*Hs*), as the expected proportion of heterozygous individuals assuming Hardy-Weinberg equilibrium. Gene diversity was calculated using Nei (1987) estimator; e) mean allelic richness per locus (*Rs*), estimated as the probability of sampling the allele i at least once among the 2n genes of a sample, being independent of sample size; f) the mean private allelic richness (R_p), estimated by hand as the mean number of singular alleles per locus presented at each population. All the previous parameter were calculated using the package hierfstat v. 0.04-6 (Goudet, 2005) or using scripts developed by ourselves, both in R (R Development Core Team, 2011). g) inbreeding coefficient (F_{IS}), which provide information about Hardy-Weinberg equilibrium departures due to either excess or defect of heterozygotes. We estimated F_{IS} by Bayesian inference using BayesAss v3.0 (Wilson and Rannala, 2003) for each population, and overall for the two

studied species. Analysis lasted for 10 million MCMC iterations, sampling each 1,000 generations, optimizing the mixing parameter for allele frequencies and for inbreeding coefficients. After that, we removed the first 10% of total iterations and we checked trace files with the program Tracer v1.4 (Rambaut and Drummond, 2007) to determine the convergence of the independent Bayesian MCMC runs. h) selfing rate (S_R), which represent the occurrence level of inbreeding crosses in a given population, was calculated following Hartl and Clark (2007) as:

$$SR = 2\,F_{IS}/(1 + F_{IS}) \times 100;$$

And, finally, i) effective population size (N_e), which represent the number of parental individuals from the previous generation contributing to the gene pool of the sampled population. N_e was computed with NeEstimator v.1.3 (Ovenden *et al.* 2007) by means of a point estimation method using linkage-gametic disequilibrium (Hill, 1981).

Microsatellite based genetic differentiation among groups of populations belonging to the same species, among populations within groups, and among individuals within population were estimated using a hierarchical analysis of molecular variance (AMOVA) as implemented in Arlequin (Excoffier and Lischer, 2010), using 1,000 permutations to test significance. We performed this analysis twice: the first one excluding the hybrid population and the second one including it as a different group. In addition, pairwise comparison for genetic differentiation between populations were computed using package hierfstat in R (R Development Core Team, 2011) by the computation of F_{ST} statistics (Weir and Cockerham, 1984) and D_{ST} (Jost, 2008). F_{ST} significance was calculated from 1,000 permutations, while D_{ST} was estimated as the harmonic mean across loci.

The genetic relationship among multilocus genotypes was inferred by a Bayesian procedure using the model-based clustering algorithm as implemented in Structure v.2.2 (Pritchard *et al.,* 2000; Falush *et al.,* 2003). The number of multilocus genotype clusters (K) was analysed using diploid

setting, and using *admixture* and *prior information* as ancestry models and *correlation* as allele frequency model using prior information. We performed simulations with ten replicates for each K value, ranging from K=1 to K=6. Each run consisted on 50,000 MCMC (Markov Chain Monte Carlo) steps after 20,000 burn-in steps. To detect the optimum value of K, we used the Structure Harvester website (Earl and von Holdt, 2012) that implements Evanno method (Evanno *et al.*, 2005).

Finally, we estimated gene flow rates among the studied populations and their significances by means of Bayesian inference using BayesAss v3.0 (Wilson and Rannala, 2003). Analysis lasted for 10 million MCMC iterations, with a sample frequency of every 1,000 generations, optimizing the mixing parameter for allele frequencies and for inbreeding coefficients. After that, we removed the first 10% of total iterations and we checked trace files with the program Tracer v1.4 (Rambaut and Drummond, 2007) to determine the convergence of the independent Bayesian MCMC runs.

RESULTS

GENETIC DIVERSITY AND DIFFERENTIATION

A total of 197 plants were successfully genotyped from the five sampled populations (Table 5.2). The 197 genotyped plants showed 196 multilocus genotypes, finding no identical multilocus genotypes among populations, but only one repeated multilocus genotype at population En11 (Table 5.2). Mean number of alleles per locus (n_a) ranged between 6.0 and 9.9, corresponding these values to En11 and H01, respectively, and presenting *E. mediohispanicum* slightly higher values than *E. nevadense* (Table 5.2). The observed heterozygosity (H_O) presented values from 0.50 to 0.63, corresponding these extremes to En11 and Em25, respectively, and being again *E. mediohispanicum* values slightly higher. Moreover, the mean gene diversity (H_S) presented the same pattern showed by H_O, but being the values of H_S higher than H_O in all the considered populations and species (Table 5.2).

Population	N	N_G	n_a	H_O	H_S	R_S	R_P	F_{IS}	S_R	N_e
Em25	30	30 (30)	7.8±1.31	0.63±0.04	0.70±0.06	7.39±1.30	0.50±0.22	0.363±0.248	53.32	18.8 (15.7-22.9)
Em17	30	30 (30)	7±1.12	0.54±0.05	0.67±0.07	6.58±0.97	0.10±0.10	0.213±0.045	35.13	24.9 (20.0-32.0)
H01	80	80 (80)	9.9±1.57	0.61±0.05	0.70±0.06	7.54±1.12	1.20±0.36	0.139±0.019	24.47	100 (80.1-130.5)
En11	27	26 (26)	6±1.16	0.50±0.07	0.64±0.06	5.87±1.09	0.20±0.13	0.298±0.068	45.95	9.3 (7.9-11.1)
En10	30	30 (30)	7.8±1.57	0.57±0.07	0.68±0.06	7.06±1.34	0.70±0.33	0.360±0.249	52.97	40.4 (30.2-58.9)
Total Em	60	60 (60)	7.4±0.85	0.59±0.03	0.68±0.04	6.98±0.79	0.30±0.13	-	-	-
Total En	57	56 (56)	6.9±0.97	0.53±0.05	0.66±0.04	6.47±0.85	0.45±0.18	-	-	-

Table 5.2. Genetic diversity parameters, breeding characteristic and effective population sizes for *E. mediohispanicum* (Em), *E. nevadense* (En), and the hybrid populations (Total N = 197). For each population, parameters include: number of genotyped plants (N) with 10 nuclear microsatellite loci, number of multilocus genotypes (N_G), mean number of alleles per locus (n_a), mean observed heterozygosity (H_O), mean gene diversity (H_S), mean allelic richness (R_S) and mean private allelic richness (R_P), inbreeding coefficient (F_{IS}), selfing rate (S_R), effective population size (N_e). Standard deviation (±SD) values are indicated. Mean (±SD) values for each population and species are also given.

Mean allelic richness (R_S) ranged from 5.87 at En11 to 7.54 at H01, presenting higher values *E. mediohispanicum* plants (Table 5.2). The pattern exhibited by mean private allelic richness (R_p) was different, being higher for *E. nevadense* and presenting their minimum value at Em17 (0.10) and the maximum value at H01 (1.20).

The inbreeding coefficient (F_{IS}) exhibited its minimum value at the hybrid population (0.139) and the maximum values at both extremes of the transect (Em25=0.363 and En10=0.360), although the standard errors associated to these values were high (Table 5.2). Finally, the selfing rate showed the same pattern observed for F_{IS} (Table 5.2), because the calculation dependence of the former on the latter.

Hierarchical analysis of molecular variance (AMOVA) indicated that population groups (species) were not genetically differentiated for these markers (Table 5.3). And this also happened when H01 was included as a proper group. In contrast, the among-populations level (at both, including and excluding H01 in the analysis) were significantly differentiated although accounting for low amounts of genetic variance (Table 5.3). The genetic variance at among-populations level decreased when the hybrid population was included (Table 5.3). In both cases, the among-individuals within population levels accounted for almost all genetic variance (Table 5.3).

Pairwise F_{ST} comparisons between populations showed values ranging from the maximum differentiation of 0.0368 between Em17 and En10, to the minimum differentiation value of 0.0107 exhibited between Em25 and H01 (Table 5.4). Despite to the low pairwise F_{ST} values found in the transect, all of pairwise F_{ST} were significant after Bonferroni correction (Table 5.4). In addition, pairwise D_{ST} comparisons between populations indicated a similar pattern of genetic differentiation, with the highest D_{ST} value obtained Em25 and Em17 and between Em17 and En10, and the lower one between Em25 and H01 (Table 5.4).

	A: [Em25-Em17][En11-En10]				B: [Em25-Em17]-H01-[En11-En10]			
Source of variation	d.f.	Variation (%)	Fixation index	P-value	d.f.	Variation (%)	Fixation index	P-value
Among groups	1	0.000	0.000	0.69306	2	0.000	0.000	0.93939
Among populations within groups	2	2.31	0.023	0.00489	2	1.48	0.015	0.00782
Within populations	230	97.69	0.034	0.00391	389	98.52	0.033	0.00293
Total	233				393			

Table 5.3. Hierarchical analysis of molecular variance (AMOVA). A) Two groups (*E. mediohispanicum; E. nevadense*) were established, excluding the H01 population. B) three groups were established (*E. mediohispanicum populations*; *E. nevadense* populations; hybrid population). Degrees of freedom (*d.f.*), percentage of variation in each hierarchical level, and their respective fixation indexes with their *P*-values are shown.

	Em25	Em17	H01	En11	En10
Em25		0.0321**	0.0107*	0.0250*	0.0325**
Em17	0.055		0.0228**	0.0265**	0.0368**
H01	0.013	0.038		0.0179**	0.0128**
En11	0.043	0.042	0.032		0.0267**
En10	0.032	0.054	0.014	0.037	

Table 5.4. Pairwise F_{ST} (above diagonal) and D_{ST} (bellow diagonal) values along the transect, estimated by means of Weir and Cockerham methods (1984) and as the harmonic mean across loci, respectively. The significance for F_{ST} values were calculated from 1,000 permutation and the P-values resulted after Bonferroni correction are indicated as *P<0.05, **P<0.01

GENETIC STRUCTURE, GENE FLOW AND EFFECTIVE POPULATION SIZE

The Bayesian inference of genetic structure assigned the studied populations to two genetic clusters (K=2), being the second more probable model that one considering K=4 (Appedix 5.1). Considering K=2, the plants belonging to the populations Em25, En10 and H01 exhibited very high membership proportions to a given ancestral genetic cluster, while the plants belonging to Em17 and En11 showed higher or medium values of membership proportions to the second cluster (Fig 5.1). However, when we consider the model assuming K=4, the multilocus genotypes from *E. mediohispanicum* and *E. nevadense* populations showed medium to high membership proportions to four ancestral genetic clusters (Fig 5.1), while the individuals from the hybrid population exhibited medium values for their assignment probabilities to the most frequent genetic clusters in Em25 and En10, respectively (Fig. 5.1).

The Bayesian inference indicated that the highest gene flows were taking place at intrapopulational level, representing self-recruitment per population (Table 5.5). The gene flow among populations were no significant, except to the gene flow from Em25, En11 and En10 to the hybrid population (Table 5.5), being higher the gene flow from Em25 (29%) and En10 (28%) to H01, than that from En11 (14%) (Table 5.5). These values were consistent to the estimations of effective population sizes (N_e) for each population, in

which the only two populations presenting higher N_e than the number of individuals sampled were H01 and En10 (100 and 40.4, respectively) (Table 5.2). Ne were not significantly different for *E. mediohispanicum* populations and lower than the number of sampled plants (Table 5.2), while *E. nevadense* populations exhibited significantly different N_e values (Table 5.2).

		Population *j*				
		Em25	Em17	H01	En11	En10
Pop *i*	Em25	**0.677±0.019**	0.010±0.019	**0.287±0.042**	0.018±0.028	0.008±0.016
	Em17	0.011±0.022	**0.879±0.076**	0.055±0.065	0.045±0.053	0.010±0.018
	H01	0.004±0.009	0.017±0.021	**0.947±0.046**	0.027±0.041	0.005±0.009
	En11	0.011±0.023	0.019±0.034	**0.141±0.099**	**0.818±0.098**	0.011±0.020
	En10	0.008±0.017	0.017±0.026	**0.279±0.047**	0.017±0.031	**0.677±0.021**

Table 5.5. Recent migration rates (m_{ij}) from *i* populations to *j* populations in the transect between *E. mediohispanicum* and *E. nevadense*, inferred from variation at ten microsatellite DNA loci using BayesAss. Significant gene flow rates are shown in bold. Values in the diagonal represent rates of self-recruitment per population.

DISCUSSION

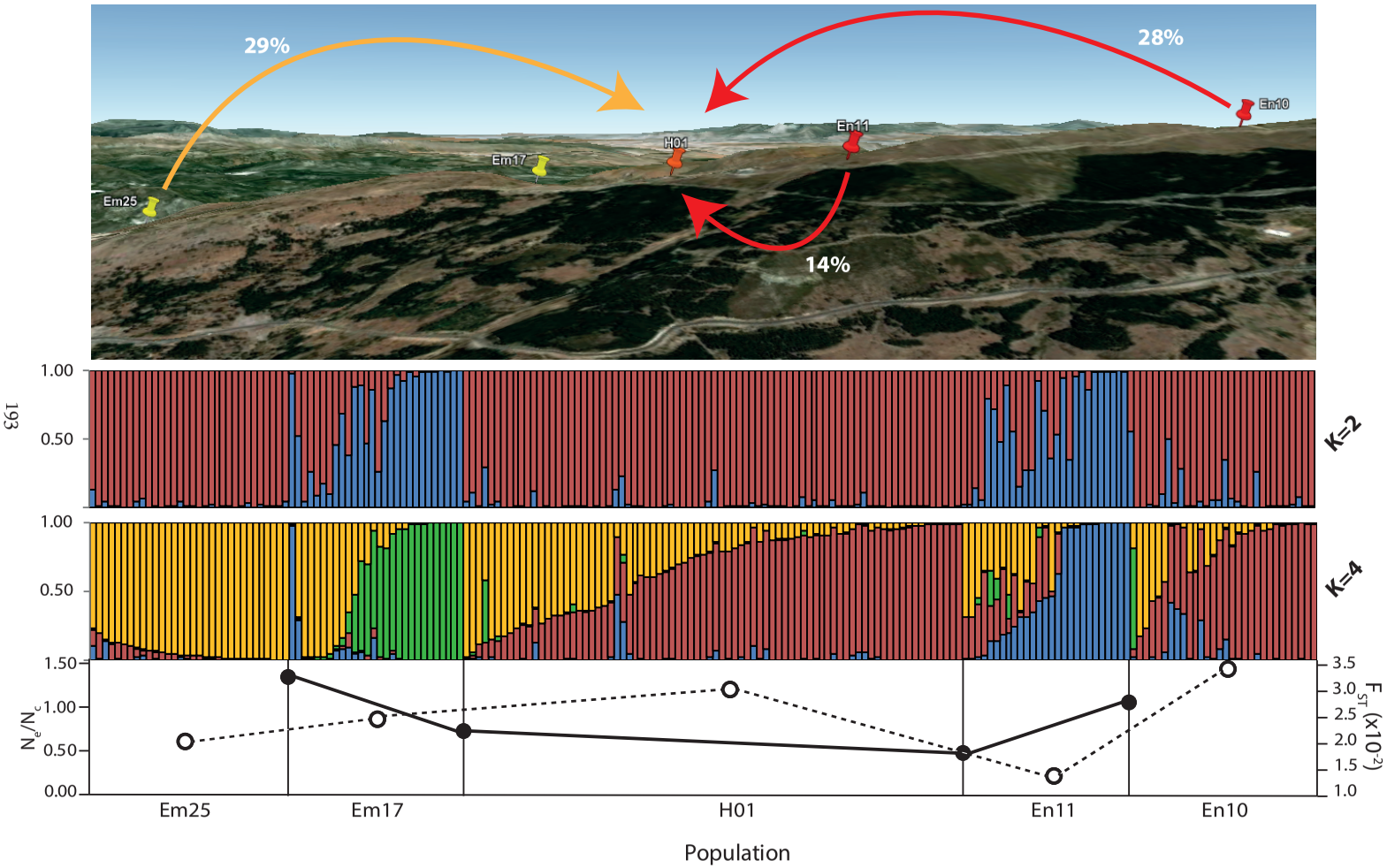
The genus *Erysimum* presents a complex evolutionary history, with recent speciation events and probable hybridizations (Clot, 1992; Ancev 2006; Marhold and Lihová, 2006). *Erysimum mediohispanicum* and *E. nevadense* are two non sister species that contact in a reduced area that should be considered a secondary contact zone (***Chapter 2***). These species also exhibit a capacity to inter-breed producing viable descent, at least in glasshouse conditions (***Chapter 3***). Moreover, the microsatellites markers developed for *E. mediohispanicum* (Muñoz-Pajares *et al.*, 2011) have been successfully amplified in *E. nevadense*, implying that genetic divergence is reduced.

The number of multilocus genotypes in all the populations was high (Table 5.2), comparable to the number of inviduals sampled, pointing to high levels of outcrossing within all the studied population. High outcrossing rates help to mix migrant alleles and novel mutation appearing in the populations (Gomaa *et al.,* 2011). In the same way, the genetic parameters analyzed (n_a, R_S, H_O and H_S, Table 5.2) have revealed high levels of genetic diversity, which would indicate that both species

were mainly outcrossing species, such as nucleotide diversity at the phosphoglucose isomerase locus indicate in *Leavenworthia* (Liu *et al.*, 1998; Liu *et al.,* 1999). In this sense, *E. mediohispaniucm* plants presented slightly higher values for the mentioned parameters, suggesting that this species could present higher outcrossing. These results highly agree with previous studies carried out on independent groups of plants in this same transect (***Chapter 3*** and ***Chapter 4***). These works found levels of inbreeding depression pointing to an auto-incompatible reproductive system for *E. mediohispanicum*. However, lower values of inbreeding depression were found for *E. nevadense* (***Chapter 3***), suggesting that populations from this species had being the scenario of historical inbreeding events (***Chapter 3***). This idea is supported by the increased levels of mean private allelic richness found in *E. nevadense* (Table 5.2), a feature associated to selfing species (Stenoien *et al.,* 2005; Bomblies *et al.,* 2010).

H_O were lower that H_S in all studied populations, a result in accordance with the positive values found for F_{IS} in every population (Table 5.2). These results could be explained by two non-excluding processes: the first one invokes the existence of inbreeding events within populations, increasing the levels of homozygosity (Uyenoyama and Waller, 1991a; 1991b); and the second one lies on intra-population subdivision implying assortative matting between relatives (bi-parental inbreeding; Uyenoyama, 1986). In the case of *E. mediohispanicum*, and considering the high levels of inbreeding depression showed by this species (***Chapter 3***), we suggest that the main factor generating these positive F_{IS} values, and consequently the lower than expected H_O, is the presence of bi-parental inbreeding. However, in the case of *E. nevadense* populations, this output could be the consequence not only of intra-population sub-structure, but a consequence of historically more frequent inbreeding events (***Chapter 3***). This fact would be associated to the harsher condition of the alpine environments where these plants growth (Ashman *et al.*, 2004; Burd *et al.*, 2009).

Figure 5.1. (*Next page*) **Top:** Sinificant values of recent gene flow among the population composing the transect from *E. mediohispanicum* populations to *E. nevadense* populations. Populations are shown on a surface map using data coming from Table 1. **Center:** Diagram with individual barplots from STRUCTURE (K=2 and K=4) based on the variation of 10 unlinked microsatellite loci. Diferent colours identify the cluster assigned by the software. **Bottom:** Pairwise FST values (solid lines) and relative value of effective population size to sample size per each population (broken lines).


29%
28%
14%
Em25
Em17
H01
En11
En10
1.00
0.50
K=2
1.00
0.50
K=4
1.50
1.00
0.50
0.00
N_e/N_c
3.5
3.0
2.5
2.0
1.5
1.0
F_ST (x10^-2)
Em25
Em17
H01
En11
En10
Population

It is remarkable that the lowest levels of F_{IS} were found in the hybrid population, where the outcrossing and/or non-assortative mating rates should be the highest in the present transect (Table 5.2).

The Bayesian analysis of the genetic structure identified two or four clusters as the more plausible scenarios taking place in the present transect, with a small difference in likelihood between them (ΔK_2=2.37 and ΔK_4=1.96; Fig 5.1, Appendix 5.1). In the first case (K=2), one of the clusters included the hybrid population with the two more distant parental populations, Em25 and En10 (Fig. 5.1, Appendix 5.1). The second scenario (K=4) identified each of the four parental population as different genetic clusters and the hybrid population appeared as presenting individuals with intermediate probability to be included in the clusters dominated by Em25 and En10 (Fig 5.1). In both cases (K=2 and K=4), the analysis associated the hybrid population with Em25 and En10. Taking into account that Em25 and En10 belong to different non-sister species (***Chapter 2***), we suggest the existence of four clusters as the more probable scenario. On top of that, the population H01 appeared as a unimodal hybrid population because intermediate hybrid genotypes are predominant, probably because frequent hybridization events are taking place in the population between both parental species (Jiggins and Mallet, 2000).

The hierarchical analysis of the genetic variance agreed with the Bayesian genetic structure analysis, because no amount of genetic variance was found at between-group level, including or not the hybrid population into the analysis (Table 5.3). However, low but significant values of genetic variance were found at population level, being the majority of that variance at the within population level (Table 5.3). The same pattern was found when we calculated the differentiation by pairwise F_{ST}, exhibiting low, but significant, F_{ST} values every population pairwise comparison. D_{ST} better reflects the genetic differentiation when markers show more than two alleles per locus (Jost, 2008; Gerlach *et al.*, 2010). D_{ST} comparisons were congruent with the pairwise F_{ST} values (Table 5.4). Indeed, both genetic differentiation values exhibited their minimum when comparing respectively Em25 and En10 with the hybrid population (Table 5.4). Both, the genetic structure and genetic differentiation analyses suggest that this transect represents a mosaic hybrid zone (Harrison

and Rand, 1989; Paige *et al.,* 1991; Harrison, 1993), with a no clear geographic pattern between *E. mediohispanicum* and *E. nevadense*.

The inferred migration rates between the studied populations (Table 5.5) support the pattern described previously. We detected high (30%) unidirectional gene flows from Em25 and En10 to the hybrid population, and a lower and more variable migration rate from En11 to the hybrid population (Table 5.5 and Fig. 5.1). These results would explain not only the low differentiation between hybrid population and Em25 and En10, but also the occurrence in hybrid population of a high percentage of individuals with intermediate genotypes (Fig. 5.1). Finally, these levels of gene flow incoming to the hybrid zone could be the driving force behind the high effective population size values estimated for this population.

The effective population size is almost always lower than total population sizes (Ellegren, 2009). Assuming that our sampling effort have been adequate, we can conclude that the hybrid population is receiving immigrants (via seeds or pollen) contributing new alleles. The other population exhibiting higher effective than census population size was En10, and because the other sampled populations appear did not significantly contribute migrants to En10, it is possible that this population was receiving migrants of gene flow from other non sampled populations. Another possibility is that En10 present an important seed bank, which could contribute with new multilocus genotypes along the time. This effect of seed banks has been reported previously in A. thaliana (Lundemo *et al.,* 2009; Bomblies *et al.,* 2010), an annual plant with a mostly selfing mating system. The gene flow patterns together with the effective population sizes founded at the studied populations suggest that the hybrid population could represent a tension zone where the hybrid plants would present stable fitness or decreased fitness, suggesting the occurrence of a tension zone (Key, 1968; Barton and Hewitt, 1985; 1989; Harrison, 1993).

The highly coherent results obtained by the analysis of genetic variances, the genetic structure, the pairwise genetic differentiation, and

the gene flow analysis allow us to conclude that in the secondary contact between *E. mediohispanicum* and *E. nevadense* studied in the present work exist a narrow unimodal hybrid zone between both species. The hybrid population appears to be maintained by high levels of gene flow from two of the parental populations, explaining the high effective population size of the hybrid population. Finally, the hybrid population would represent a tension zone where the hybrids are confined. This population would be maintained due to the continuous contribution of individuals from the parental populations.

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APPENDICES

K	Reps	Mean LnP(K)	Stdev LnP(K)	ΔK
1	10	-5838.21	0.5343	NA
2	10	-5781.12	16.3434	2.367319
3	10	-5762.72	43.0608	0.704121
4	10	-5714	46.3427	1.962121
5	10	-5756.21	92.1796	0.796054
6	10	-5725.04	85.3463	NA

Appendix 5.1. Mean of the Log posterior probabilities (Ln P(K)), their standard deviation (Stdev LnP(K)) (Pritchard *et al.,* 2000), and ΔK values (Evanno *et al.,* 2005) for alternative numbers of ancestral genetic clusters (K) as estimated from STRUCTURE.

CHAPTER 6

POLLINATORS AS POTENTIAL PREZYGOTIC AND POSTZIGOTIC ISOLATION BARRIERS BETWEEN *ERYSIMUM MEDIOHISPANICUM* AND *E. NEVADENSE*

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ABSTRACT

Hybridization is frequent in flowering plants. In animal-pollinated plants, heterospecific gene flow is mainly driven by the preference pattern and foraging behaviour of the pollen vectors. Using experimental arrays of 40 plants, we explored the role of pollinators in the prezygotic and postzygotic reproductive isolation between *Erysimum mediohispanicum* and *E. nevadense* and its respective hybrids. Pollinators belonging to 106 species made 3,448 floral visits to the experimental plants on three different sites: hybrid population and two parental sites. Pollinator preference on plant phenotype and treatments were analyzed, as well as the reproductive output from each type of cross observed in the experimental array. We found no evidence of ethological isolation between the two studied parental species, or between them and their F1 hybrids. In *mediohispanicum* site, however, pollinators showed preferences in both the *E. mediohispanicum* types of plants, pure and hybrid, while on the *nevadense* site these were generally avoided. F1 hybrids presenting any level of heterosis were more visited on the *hybrid* site. These patterns of attractiveness suggest not only the higher hability of hybrids attracting pollinator, but also a reduction in hybrid viability and consequently the occurrence of recurrent hybridization between pure plants for the long-term maintenance of the narrow hybrid population.

Key words

Ethological isolation, prezygotic and postzygotic isolating barriers, hybridization, hybrid fitness, pollinator discrimination.

INTRODUCTION

Hybridization is very common in flowering plants (Arnold, 2006; Soltis and Soltis, 2009). Some recent estimates suggest that it may occur in as many as 25% of plant species (Ellstrand *et al.*, 1996; Mallet, 2005). Hybridization has various evolutionary consequences. It may cause the production of new hybrid species via a process known as hybrid speciation (Soltis and Soltis, 2009), which can occur after polyploidization (allopolyploid speciation) or without any duplication of genetic material (homoploid hybrid speciation). Hybridization may also entail the transfer of genetic material from one species to another, a process called introgression (Anderson, 1949). In extreme situations introgression may cause the complete assimilation of the genome of one species by that of another, thereby triggering its extinction (Rhymer and Simberloff, 1996; Levin *et al.*, 1996; Allendorf *et al.*, 2001). In some cases this process may also cause the transfer of adaptation from one species to another (Kim and Rieseberg, 1999). Finally, contact between two hybridizing species very frequently gives rise to a hybrid zone in which pure individuals from both parental species coexist with a wide range of F_1, F_2 and backcrossed hybrids (Barton and Hewitt, 1985; Harrison, 1993; Arnold, 2006). Hybrid zones may be primary, as a consequence of an ongoing process of sympatric or parapatric speciation, or secondary, as a consequence of the contact between two related species that have evolved allopatrically (Campbell and Aldridge, 2006). Two main models have been developed to describe hybrid zones according to their selective processes. The first covers to the hybrid zone produced by a geographical selection gradient determined by geographical variations in the relative fitness of pure and hybrid individuals, in accordance with concomitant variations in the ecological setting (Endler, 1977). A second model, called tension zone, considers that hybrid fitness is always weaker and that the hybrid zone is maintained by a balance between dispersal of pure individuals into the hybrid zone and selection against hybrid individuals (Barton and Hewitt, 1985). Whereas the tension zone model predicts a narrow but geographically fixed hybrid zone (Kingston *et al.*, 2012),

the geographical-selection-gradient model assumes that ecological variables determine the width and position of the hybrid zone (Moore and Price, 1993).

When hybridization is selected against, hetero-specific gene flow between potentially hybridizing species is impeded by several multi-stage mechanisms when they come into contact in hybrid zones (Arnold, 2006). In animal-pollinated plants, the preference pattern and foraging behavior of specific pollinators may help maintain reproductive isolation between two congeneric plants when they occur in sympatry (Grant, 1981). This prezygotic reproductive isolating barrier is called ethological isolation (Grant, 1981, Campbell and Aldridge, 2006). Pollinators showing strong fidelity to their host plant may act as strong prezygotic pre-pollination barriers (Ramsey *et al*., 2003; Martin *et al*., 2008; Ellis and Johnson, 2012; Marques *et al*., 2007). Pollinator-mediated pre-pollination isolation is strong in plants (Lowry *et al*., 2008), particularly when they have specialized pollination systems or belong to different pollination syndromes (Ramsey *et al*., 2003; Martin *et al*., 2008; Kay and Sargent, 2009; Schiestl and Schlüter, 2009; Natalis and Wesselingh, 2012). However, the role of pollinators in preventing hetero-specific pollen flow in pollination-generalist plants is still unknown.

Pollinators may still prevent hybridization once the hybrid has been formed if they discriminate against hybrids (Campbell and Aldridge, 2006). In this case, the pollinator preference pattern results in a decrease in a hybrid's fertility, a postzygotic reproductive isolation barrier related to hybrid sterility. Pollinators may exhibit less preference for hybrids if their floral traits do not attract pollinators visiting parental species (Lee and Snow, 1998; Emms and Arnold, 2000; Ippolito *et al*., 2004; Aldridge and Campbell, 2007). Once again, pollinator-mediated postzygotic isolation would be stronger when parental plants differ drastically in floral traits and pollinator fauna. Under these circumstances, the hybrids formed between two species with different pollination syndromes will surely have intermediate phenotypes, and they will be unattractive to all the pollinator types visiting any of the parental plants; this is the intermediate hypothesis postulated by Grant (1949). Decreased

hybrid performance should be one of the main factors responsible for the scarcity of hybrid taxa in pollinator-specialist plants (Schemske and Bradshaw, 1999; Emms and Arnold, 2000). Lee and Snow (1998) even found a similar pattern when exploring the preference patterns of insects visiting the flowers of pure and hybrid individuals of the pollination generalist *Raphanus raphanistrum* (Brassicaceae). In contrast with this empirical evidence, some studies have failed to find any reduction in hybrid fitness as a result of pollinator discrimination (Campbell *et al*., 2003; Tierney and Wardle, 2008). Consequently, the magnitude and strength of pollinator-mediated postzygotic isolation barriers remain an open question.

In this study we explored the role of pollinators in the prezygotic and postzygotic reproductive isolation between *Erysimum mediohispanicum* and *E. nevadense*. These two species come into secondary contact in the Sierra Nevada Mountains (SE Spain), where they form a narrow hybrid zone. Our specific goals were: 1) to test whether these two species present any ethological isolation between each other (pre-pollination prezygotic barrier); 2) to quantify the relative attractiveness of F_1 hybrids for pollinators (postzygotic isolation barrier); 3) to check the role of phenotype in this attraction; and 4) to quantify hybrid sterility (postzygotic isolation barrier). These results would help us understand the maintenance of the hybrid zone between these two species.

MATERIAL AND METHODS

STUDY SYSTEM

The two species used in this study, *Erysimum mediohispanicum* and *Erysimum nevadense*, are not sister species, even though they belong to the same lineage within the genus *Erysimum* (***Chapter 2***). Both species inhabit the Sierra Nevada (SE Iberian Peninsula). Whereas *E. nevadense* is endemic to the high mountains above the treeline (from 2,200 m a.s.l. to 2,700 m a.s.l.), *E. mediohispanicum* extends from 900 m a.s.l. to 2,200 m a.s.l, inhabiting both pine and oak woodlands and high-mountain shrublands. They come into

contact along a narrow zone, and although this contact is secondary, they may hybridize occasionally, producing a narrow hybrid zone less than 500 m wide (***Chapter 5***).

PLANT MATERIAL

For this study, we used *E. mediohispanicum*, *E. nevadense* and their reciprocal F_1 hybrids, encompassing four treatments: 1) *E. mediohispanicum* parental plants; 2) *E. nevadense* parental plants; 3) *E. mediohispanicum* hybrid plants, where *E. mediohispanicum* were pollen recipients and *E. nevadense* were pollen donors; and 4) *E. nevadense* hybrid plants, where *E. nevadense* were pollen recipients and *E. mediohispanicum* were pollen donors. Both hybrid types were obtained by reciprocally hand-pollinating flowers from each species in 2009 with pollen from the other species (***Chapter 3***). Parental plants were also obtained in 2009 by hand-pollinating plants from the same population. The plants grew for two years in a greenhouse and were then moved, one month before flowering, to a common garden located at 1,980 m a.s.l., within the plants' natural distribution area.

EXPERIMENTAL SET UP

Pollinator preference and activity were observed in two experimental arrays in June 2012 for a total of 30 hr of observation. Each array consisted of 40 potted plants (10 per treatment), separated from each other by 0.5 m and randomly arranged in a square. The experiment was performed on three sites, corresponding to the habitat of *E. mediohispanicum* (population Em25 in ***Chapters 3***, ***5*** and ***8***), the habitat of *E. nevadense* (population En10 in ***Chapters 3***, ***5*** and ***8***) and a hybrid zone between these two species (population H01 in ***Chapters 3***, ***5*** and ***8***), respectively. The same experimental plants were tested on all the sites every day. To do this, we randomly moved each array from one site to the other on every study day. The array was set up on each site for one hr per day, and over this period we observed the number of

visits made to the flowers by natural pollinators. All the observations were made by three observers between 10:30 am and 17:30 pm, local time.

POLLINATOR MOVEMENTS

We also recorded the pattern of insect movement between flowers in each observation period. To do this, two of the observers followed the flight behavior of each single insect seen in the plant arrays, and the third noted the sequence of plants visited and the number of flowers visited on each plant. Insects were observed for the entirety of their foraging bouts, whenever possible. When more than one insect was visiting our experimental plants, we randomly ruled out one of them , although we still noted the plants it visited to know its preference. In total, we observed 2,275 between-plant flights made by 34 species (Appendix 6.1).

PLANT PHENOTYPIC TRAITS

Prior to the experiment, we quantified the following phenotypic traits for each labeled plant: (1) Number of stalks: number of flowering stalks borne by each plant. (2) Stalk diameter: diameter in mm of the tallest stalk at its base. (3) Stalk height: the height in cm of the tallest stalk, from the ground to the top of the highest open flower. (4) Flower number: the entire production of flowers in each plant. (5) Corolla diameter: the distance between the edges of two opposite petals, measured with a digital caliper (± 0.1 mm error). (6) Corolla tube length: the distance between the corolla tube aperture and the base of the sepals. (7) Corolla tube width: the diameter of the corolla tube aperture as the distance between the bases of two opposite petals. (8) Corolla shape: determined in each plant by means of geometric morphometric tools, using a landmark-based methodology (Zelditch *et al.*, 2004) (see Gómez and Perfectti, 2010; and ***Chapter 1*** for a detailed description of landmark locations). To describe corolla shape, we used the first four relative warps (RW) (Zelditch *et al.*, 2004). Each RW explains a given variation in shape between specimens,

and their scores can be used as a data matrix to perform standard statistical analyses (Zelditch *et al.*, 2004).

REPRODUCTIVE SUCCESS OF F_1, F_2 AND BACKCROSSING HYBRIDS

We experimentally tested the reproductive success of hybrid and pure crossing. We performed the following crosses in the greenhouse: 1) Intra-specific crossing, by hand-pollinating flowers from each species with pollen from a different individual from the same species; 2) F_1 crossing, by hand-pollinating pure plants from each species with pollen from the other species. This crossing produces two type of hybrids, depending on the species acting as pollen donor or recipient; 3) F_2 crossing, by hand-pollinating F_1 plants with pollen from another F_1 plant; 4) Intra-specific backcrossinging, by crossing F_1 plants with pure plants from the same species. We considered a hybrid to belong to the same species of a pure plant when they shared the same cytoplasm, i.e., when both acted as pollen recipient in the previous generation. This crossing produces another two types of plants, depending on whether the F_1 hybrid acted as pollen donor or recipient. 5) Inter-specific backcrossing, by crossing F_1 plants with pure plants from the other species. We considered a hybrid to belong to a different species of a pure plant when did not share the same cytoplasm, i.e., when one acted as pollen recipient in the previous generation and the other as pollen donor. This crossing also produced two types of plants, depending on whether the F_1 hybrid acted as pollen donor or recipient. 6) Open pollination, a control treatment to determine the natural fruit set of both hybrid and pure plants. After pollination, we evaluated the number of fruits and aborted flowers per crossing. Reproductive success was quantified as the proportion of flowers setting fruits (fruit set).

REPRODUCTIVE ISOLATION

We calculated an index of prezygotic reproductive isolating barrier due to pollinators, $RI_{pollinator}$, as 1-[(observed/expected) hetero-specific flights/

(observed/expected) con-specific flights] (Lowry *et al.*, 2008), while the postzygotic isolating barrier due to pollinator attractiveness, $RI_{attractiveness}$, was calculated as 1- (hybrid attractiveness/mean parental attractiveness) (Lowry *et al.*, 2008). Finally, the postzygotic isolating barrier due to hybrid sterility, $RI_{hybrid\ sterility}$, was calculated as 1-(hybrid reproductive success/mean parental reproductive success) (Lowry *et al.*, 2008).

STATISTICAL ANALYSES

We first tested the effect of the experiment on the visitation rate of both, the overall flower visitor community and large bees, small bees and beetles (the three main insect groups visiting the experimental flowers), by means of Generalized Linear Mixed Models (GLMMs). We included as fixed factors: Pollen recipient species, with two levels (*E. mediohispanicum* and *E. nevadense*), Type of crossing, with two levels (Pure and Hybrid), and Site, with three levels (*mediohispanicum* habitat, *nevadense* habitat, and hybrid zone). We included as random factors the array and their interactions with fixed factors. Furthermore we included an individual random factor to control for over-dispersion. The dependent variables were fitted to a Poisson, with logarithm link function. As the two-way and three-way interactions were significant, we repeated the GLMMs separately for each habitat. Between-treatment comparison in phenotype was tested by a Generalized Linear Model, including as factors the Pollen recipient species and the Type of crossing. The relationship between pollinator visitation rate and the phenotype of the plants was tested by mean of Multiple Poisson Regression. Between-treatment comparison of the pollinator assemblage composition was performed by permutational multivariate analyses of variance using distance matrices (ADONIS)**,** which test whether similarity in pollinator assemblage was significantly higher within treatments than between them. ADONIS partitions dissimilarities for the sources of variation, and uses permutation tests to inspect the significance of those partitions. Dissimilarity was calculated as a Bray-Curtis distance. The ADONIS function was performed with package vegan in R (Oksanen, 2008).

We tested whether the transition probability was consistent across sites by a nominal logistic model, including as independent variables the origin of the flight and the site, and as dependent the destination of the flight. Because the interaction between independent variables was significant, we performed independent analyses on each site.

RESULTS

DIFFERENCES IN PHENOTYPE BETWEEN HYBRID AND PURE PLANTS

Only three phenotypic traits differed between parental and F_1 hybrids: number of stalks, stalk height and number of flowers (Table 6.1). Stalk height differed between the two parental species, *E. mediohispanicum* plants being taller than *E. nevadense* plants (Fig. 6.2). Nevertheless, the Species x Hybridization interaction term was significant for stalk height. As observed in Figure 6.2, whereas *E. mediohispanicum* hybrids were significantly shorter than their parental plants, *E. nevadense* hybrids were taller than their parentals.

The patterns of variation in the number of stalks and number of flowers were similar. Pure *E. mediohispanicum* plants bore the lowest number of stalks and flowers, both pure and hybrid *E. nevadense* had an intermediate number of stalks and flowers, and *E. mediohispanicum* hybrids had the highest number of stalks and flowers (Fig. 6.2).

BETWEEN-TREATMENT DIFFERENCES IN FLORAL VISITOR ASSEMBLAGE

Our experimental plants received 3,448 floral visits from 106 species (Appendix 6.1), all of them pollinators of natural populations. Most of the visits (73%) were made by high-efficiency large bees (*Anthophora* spp., *Lasioglossum xanthophus*, etc.), while small bees (*Andrena* spp., *Lasioglossum* spp., *Ceratina* spp., etc.) made 13.2% of the visits and low-efficiency beetles made

	df	No of stalks	Stalk diameter	Stalk height	No of flowers	Corolla diameter	Corolla tube length	Corolla tube width	RW1	RW2	RW3	RW4
Sp	1	0.001	0.193	10.366**	0.785	0.247	2.065	1.223	0.189	0.231	0.101	0.123
Hybrid	1	3.800*	2.033	1.034	17.231****	0.054	3.375	0.046	0.504	0.617	0.005	0.114
Sp x Hybrid	1	6.887**	0.848	6.372*	64.210****	1.060	2.670	0.027	2.073	0.657	0.668	0.000

Table 6.1. Summary of the Generalized Linear Models comparing the phenotypic traits between plants belonging to different treatments. All phenotypic traits were fitted to a normal distribution, except the number of stalks and number of flowers, which were fitted to a Poisson distribution. We show the Likelihood Ratio Chi Squares. *P<0.05, **P<0.01, ****P<0.0001.

Figure 6.1. Corolla of the two parental species (*E. mediohispanicum* and *E. nevadense*) and their F_1 hybrids.than their parental plants, *E. nevadense* hybrids were taller than their parentals.

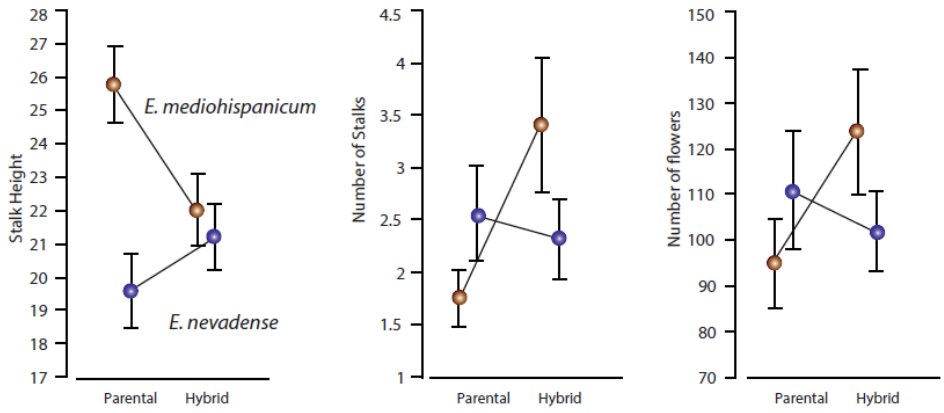

Figure 6.2. Value of the three phenotypic traits differing between treatments.

4.78%. The remaining visits were made by beeflies, hoverflies, ants and wasps (Appendix 6.1). There was no effect on the type of crossing in the pollinator assemblage composition on any of the three sites (Table 6.2). In the *mediohispanicum* habitat, the most abundant insects were large bees (81.5% of visits), small bees (9.1%), beetles (4.4.%), beeflies (1.0) and ants (0.7%). In the *nevadense* habitat the visitors were large bees (68.1%), small bees (16.1%), ants (6.1%), beetles (2.0%) and beeflies (1.8%). In the hybrid zone, the pollinator assemblage was composed of large bees (68.3%), small bees (14.6%), beetles (5.4%), beeflies (5.7%) and ants (1.4%).

	df	F. Model	P-values	F. Model	P-values	F. Model	P-values
Species	1	0.928	0.495	0.917	0.366	0.239	0.960
Type of crossing	1	0.607	0.584	0.780	0.545	0.388	0.861
Species x Type of crossing	1	0.417	0.812	1.586	0.148	1.923	0.069

Table 6.2. Outcome of the ADONIS analyses testing for between-treatment differences in pollinator assemblage composition using Bray dissimilarity distances.

EFFECT OF PLANT PHENOTYPE ON INSECT VISITATION RATE

The abundance of insects visiting the flowers was influenced by the phenotype of the plants (Table 6.3). Furthermore, the relationship between plant phenotype and insect visits varied between the sites and between the pollinator functional groups (Table 6.3). The only trait affecting the visitation rate of the whole community of flower visitors was the number of flowers per plant (Table 6.3). Plants with more flowers received more visits on all three sites (Fig. 6.3). In the *mediohispanicum* habitat plants with low values of RW1 and high corolla tube width also received more visits.

Large bees also visited plants with more flowers more often on all the sites, although this preference was significant only on the two parental sites (Table 6.3). On these two sites, large bees also preferred plants with narrow stalks, wide corolla tubes and negative values of RW1 (Table 6.3).

The effect of plant phenotype on small-bee and beetle visits was weaker (Table 6.3). After Bonferroni, we found that only small bees were attracted to plants with thicker stalks on the *mediohispanicum* site (Table 6.3).

DIFFERENCES BETWEEN HYBRID AND PURE PLANTS IN FLOWER VISITOR ATTRACTIVENESS

The pollinator visitation rate differed significantly between sites (Appendix 6.2), being significantly lower in the hybrid zone (11.1±0.5 insects/plant) than in the habitats of the two parental species (*E. mediohispanicum* habitat:15.7±1.0; *E. nevadense* habitat: 16.8±0.8; Fig. 6.4). Moreover, the interactions between pollen recipient species and site, as well as between type of crossing and site, were significant (Appendix 6.2), suggesting that the effect of the experiment was different on each site (Fig. 6.4). A similar pattern was found when we separately analyzed the visitation rate of the main pollinator functional groups (Appendices 6.3-6.13).

	Em25		H01		En10	
Total						
Number of stalks	0.03±0.02		0.04±0.02		0.05±0.02	*
Stalk diameter	-0.23±0.07	***	0.04±0.08		-0.10±0.06	
Stalk height	0.01±0.01		-0.01±0.01		0.01±0.01	
Corolla diameter	0.06±0.03	*	0.04±0.03		0.02±0.02	
Corolla tube	0.01±0.04		0.03±0.04		-0.06±0.03	
Corolla width	**0.26±0.05**	****	0.10±0.06	*	0.15±0.05	**
Number of flowers	**0.10±0.00**	****	0.08±0.04	*	**0.10±0.01**	****
RW1	**-1.14±0.35**	***	-1.01±0.42	*	0.01±0.34	
RW2	-0.86±0.50		0.45±0.58		-0.43±0.48	
RW3	0.75±0.56		0.46±0.67		0.25±0.55	
RW4	0.83±0.63		-0.86±0.77		-0.39±0.61	
Large bees						
Number of stalks	0.04±0.02		0.02±0.03		0.07±0.02	**
Stalk diameter	**-0.34±0.08**	****	0.22±0.09	*	**-0.32±0.08**	****
Stalk height	0.01±0.01		-0.01±0.01		0.02±0.01	*
Corolla diameter	0.04±0.03		0±0.04		0.05±0.03	
Corolla tube	0±0.04		0.07±0.05		-0.10±0.04	*
Corolla width	**0.32±0.06**	****	0.05±0.07		**0.21±0.06**	***
Number of flowers	**0.01±0.00**	****	0.01±0.01		**0.10±0.01**	***
RW1	**-1.31±0.38**	***	-0.12±0.5		-0.20±0.41	
RW2	-0.89±0.57		1.01±0.7		-0.61±0.59	
RW3	0.67±0.62		-0.68±0.81		0.26±0.66	
RW4	1.34±0.69	*	-1.63±0.96		0.93±0.73	
Small bees						
Number of stalks	0.02±0.09		0.08±0.06		0.03±0.05	
Stalk diameter	**0.89±0.24**	***	-0.39±0.22		0.34±0.15	*
Stalk height	0.02±0.02		0.03±0.02		-0.02±0.02	
Corolla diameter	0.22±0.08	**	0.14±0.08		-0.02±0.06	
Corolla tube	-0.06±0.12		-0.08±0.11		0.01±0.09	
Corolla width	0.04±0.16		0.32±0.16	*	0.09±0.12	
Number of flowers	0.01±0.01		0.01±0.01		0.01±0.01	
RW1	1.86±1.18		-1.81±1.12		1.67±0.83	*
RW2	0.81±1.56		-0.11±1.58		-0.45±1.17	
RW3	1.81±1.76		3.48±1.70	*	-0.12±1.44	
RW4	-0.79±2.14		2.59±1.91		-4.29±1.60	**
Beetles						
Number of stalks	-0.19±0.1		0.29±0.15	*	-0.19±0.20	
Stalk diameter	-0.85±0.34	**	0.32±0.43		0.21±0.48	
Stalk height	0.03±0.04		0.02±0.05		-0.05±0.05	
Corolla diameter	0.13±0.12		0.24±0.15		0.08±0.17	
Corolla tube	0.07±0.17		-0.38±0.25		0.35±0.24	
Corolla width	-0.14±0.25		0.09±0.30		0.04±0.29	
Number of flowers	0.01±0.01	*	-0.01±0.01		0.01±0.01	
RW1	**-4.89±1.8**	**	-0.08±2.35		-1.89±2.39	
RW2	-4.38±2.3	*	0.45±3.13		6.95±3.38	*
RW3	1.57±2.67		-3.15±3.52		-1.21±3.34	
RW4	-3.64±3.03		-5.63±4.50		2.08±4.76	

Table 6.3. Summary of the Multiple Poisson Regression comparing the effect of the plant phenotypic traits on the visitation rate of flower visitors. We show the Likelihood Ratio Chi Squares. *P<0.05, **P<0.01, ****P<0.0001. In bold, those relationships significant after Bonferroni correction.

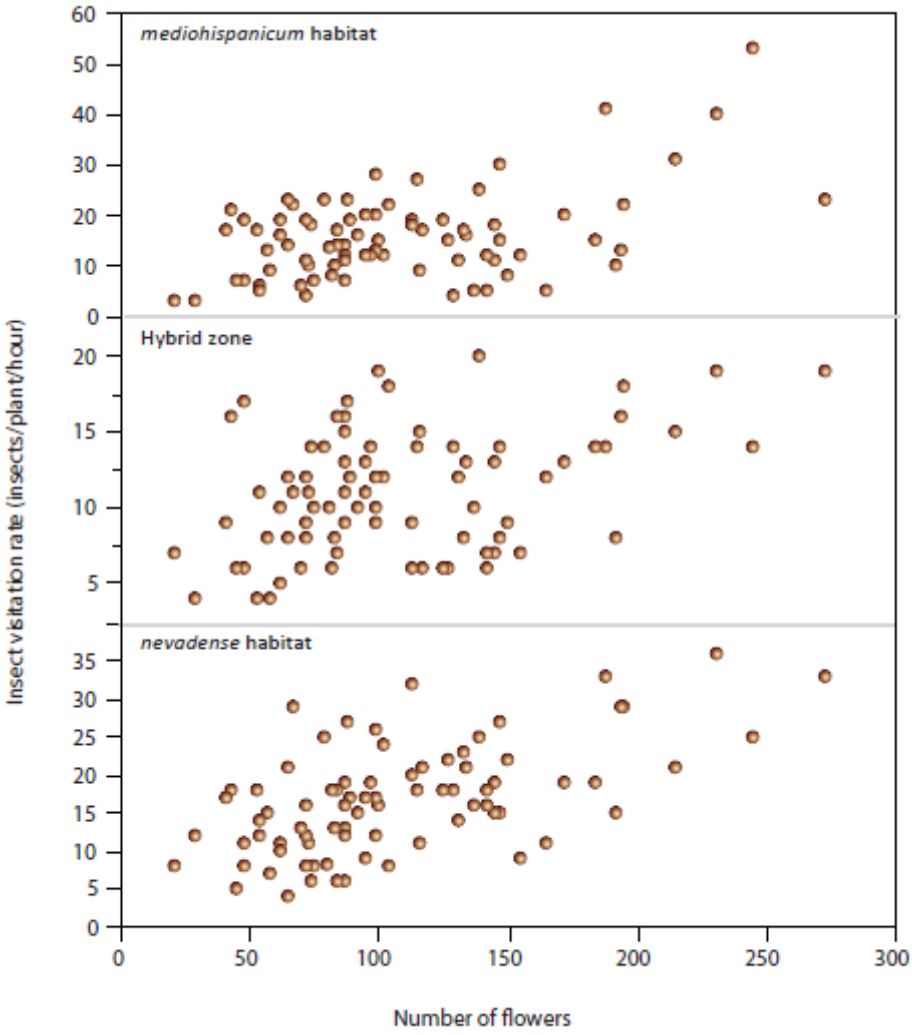

Figure 6.3. Relationship between number of flowers per plant and number of visits in each of the three sites.

When comparing each site separately, we found interesting patterns. In the *E. mediohispanicum* habitat, pollinators made more frequent visits to *E. mediohispanicum* plants, both pure and hybrids, than to *E. nevadense* plants (Table 6.4, Fig. 6.4). However, in the *E. nevadense* habitat, the hybrids from both species received more visits than the pure plants, although this preference was

more intense for *E. mediohispanicum* hybrids (Table 6.4, Fig. 6.4). Finally, in the hybrid zone, the *E. mediohispanicum* hybrids were significantly preferred over the other plants by the overall community of flower visitors (Table 6.4, Fig. 6.4). Consequently, if we pool the results from the three sites, the most visited plants were *E. mediohispanicum* hybrids (P<0.001, Poisson GLM).

The preference pattern of large bees was almost identical to the above-described pattern for the whole community of flower visitors, except that in the hybrid zone the between-treatment differences were not significant (Table 6.4, Fig. 6.4). Thus, it seems that *E. mediohispanicum* hybrids were more visited by large bees than the other plants.

		Absolute abundance			Residual abundance vs plant phenotype		
	df	Em25	H01	En10	Em25	H01	En10
TOTAL							
Species	1	5.390*	1.771	0.325	0.146	0.583	2.876
Type of crossing	1	0.786	1.413	5.333*	8.036**	0.859	0.469
Species x Type of crossing	1	0.458	5.993*	4.610*	3.932*	2.142*	0.021
Large Bees							
Species	1	7.436****	1.299	0.099	0.134	0.545	2.502
Type of crossing	1	0.017	0.395	4.014*	3.046	2.288	0.045
Species x Type of crossing	1	1.988	1.496	2.073	2.172	1.839	0.546
Small Bees							
Species	1	0.600	0.211	1.332	0.226	0.350	0.624
Type of crossing	1	6.256**	0.560	0.388	5.537*	0.022	0.475
Species x Type of crossing	1	9.831***	2.524	12.151****	7.567**	0.807	8.057***
Beetles							
Species	1	0.162	1.059	2.492	1.027	2.349	2.901ms
Type of crossing	1	0.409	0.005	0.859	1.356	0.944	2.488ms
Species x Type of crossing	1	0.799	0.005	4.472*	0.061	0.421	1.173

Table 6.4. Summary of the Generalized Linear Models comparing the visitation rate of flower visitors between plants belonging to different treatments, separated by population, with the data from the two trials pooled All flower visitors were fitted to a Poisson distribution. We show the Likelihood Ratio Chi Squares. *P<0.05, **P<0.01, ****P<0.0001.

In contrast, the small-bee preference pattern varied between sites. They preferred pure *E. mediohispanicum* in the *mediohispanicum* habitat, pure *E. nevadense* and *E. mediohispanicum* hybrids in the *nevadense* habitat, and did not show any significant preference pattern in the hybrid zone (Table 6.4, Fig. 6.4). Similarly, the beetle preference pattern also varied between sites (Table 6.3), as they visited *E. nevadense* hybrids more frequently in the *nevadense* habitat but had no preferences on the other two sites (Fig. 6.4).

The preference pattern displayed by flower visitors can be partly explained by the between-plant differences in phenotype (Table 6.4). So, when controlling for the phenotype of the plants, the plants most visited by the whole pollinator assemblage in the *E. mediohispanicum* site were the two parentals, particularly the pure *E. mediohispanicum*, rather than the *E. mediohispanicum* hybrids. Something similar happened in the *nevadense* habitat, where the preference for *E. mediohispanicum* hybrids also disappeared after controlling for the plant phenotype. The pattern of the large-bee visitation rate changed in a similar way after controlling for plant phenotype. In contrast, the visitation rate pattern of both small bees and beetles was constant before and after controlling for plant phenotype (Table 6.4).

FLIGHT MOVEMENTS BETWEEN PURE AND HYBRID PLANTS

We observed flight movements between all the possible combinations of plant types (Fig. 6.5). However, the distribution of pair-wise flight movements departed significantly from the distribution expected from random movements ($\chi^2 = 38.49$, df = 9, $P < 0.0001$, N = 2,275 flights, Nominal Logistic Model). Furthermore, the pattern of movement differed between sites, according to the significant interaction term ($\chi^2 = 35.86$, df = 18, $P = 0.007$). The percentages of hetero-specific flights between pure plants were 24.4% and 24.9% in the *mediohispanicum* and *nevadense* habitats, respectively, and 26.9% in the hybrid zone (Fig. 6.5). The percentage of backcrossing flights was 43.7% in the hybrid zone, and 50.5% and 53.7% in the parental habitats (Fig. 6.5).

The percentage of flights between F_1 hybrids was 24.6% and 21.8% in the two pure habitats, and 29.3% in the hybrid zone (Fig. 6.5).

REPRODUCTIVE SUCCESS OF DIFFERENT TYPES OF CROSSING

There were significant differences in fruit set between different types of crossing (χ^2 = 157.09, P<0.0001, Binomial GLM), but not between pollen recipient species (χ^2 = 2.31, P=0.129) or their interaction (χ^2 =8.59, P=0.283, binomial GLM). The fruit set of F1 crossing was almost identical to that of intra-specific crossing (Fig. 6.6). In contrast, the fitness of F_2 crossing was much lower (Fig. 6.6). Backcrossing fitness depended on the pollen recipient plant. Thus, when the pollen recipient plant was a hybrid, the fitness was very low, irrespective of the pollen donor species, whereas when the pollen recipient plant was pure, the fitness was always higher (Fig. 6.6). As expected, open pollination showed a medium value in fruit set (Fig. 6.6).

REPRODUCTIVE ISOLATION

$RI_{pollinator}$ between *E. nevadense* and *E. mediohispanicum* was very low: 0.054 in the *mediohispanicum* habitat, 0.085 in the hybrid zone and -0.290 in the *nevadense* habitat. On the latter site, insects flew more often between species and within species. As can be seen in figure 6.5, $RI_{pollinator}$ was symmetrical, since it had similar values for *E. nevadense* and *E. mediohispanicum*.

$RI_{attractiveness}$ was 0.045 in the *mediohispanicum* habitat, -0.088 in the hybrid zone and -0.137 in the *nevadense* habitat. In other words, on the latter two sites, insects preferred hybrid plants to pure plants.

$RI_{hybrid\ sterility}$ was 0.622 when calculated using only F_2 crossing, and 0.563 when including all crossings and backcs.

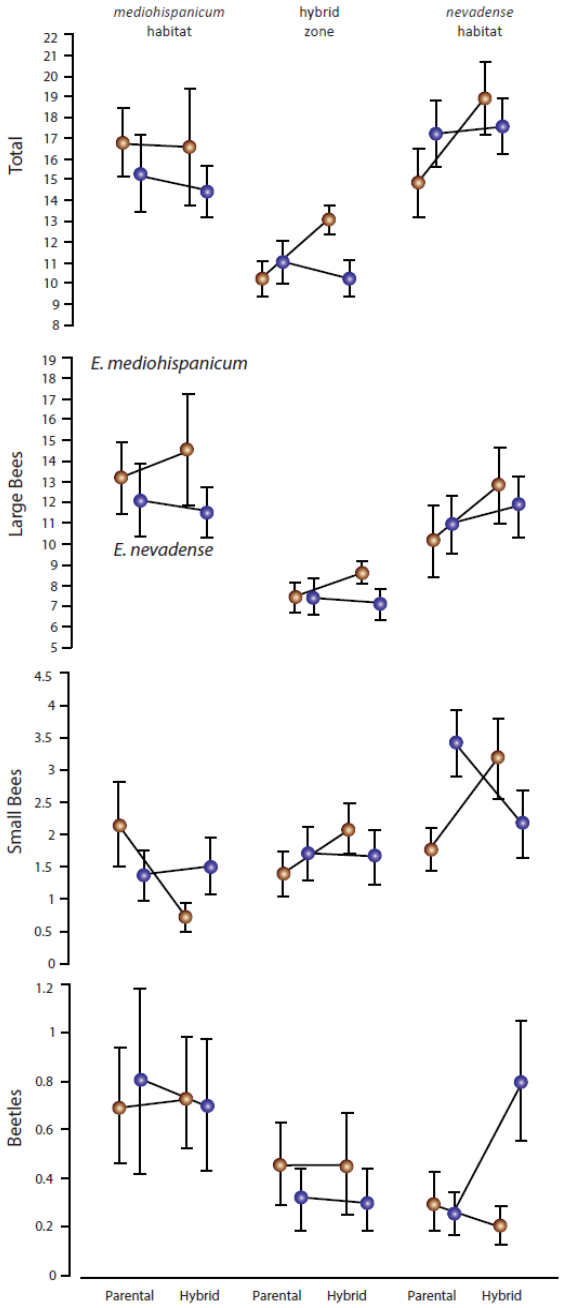

Figure 6.4. Between-treatment differences in visitation rate of the overall community of flower visitors, as well as of large bees, small bees and beetles.

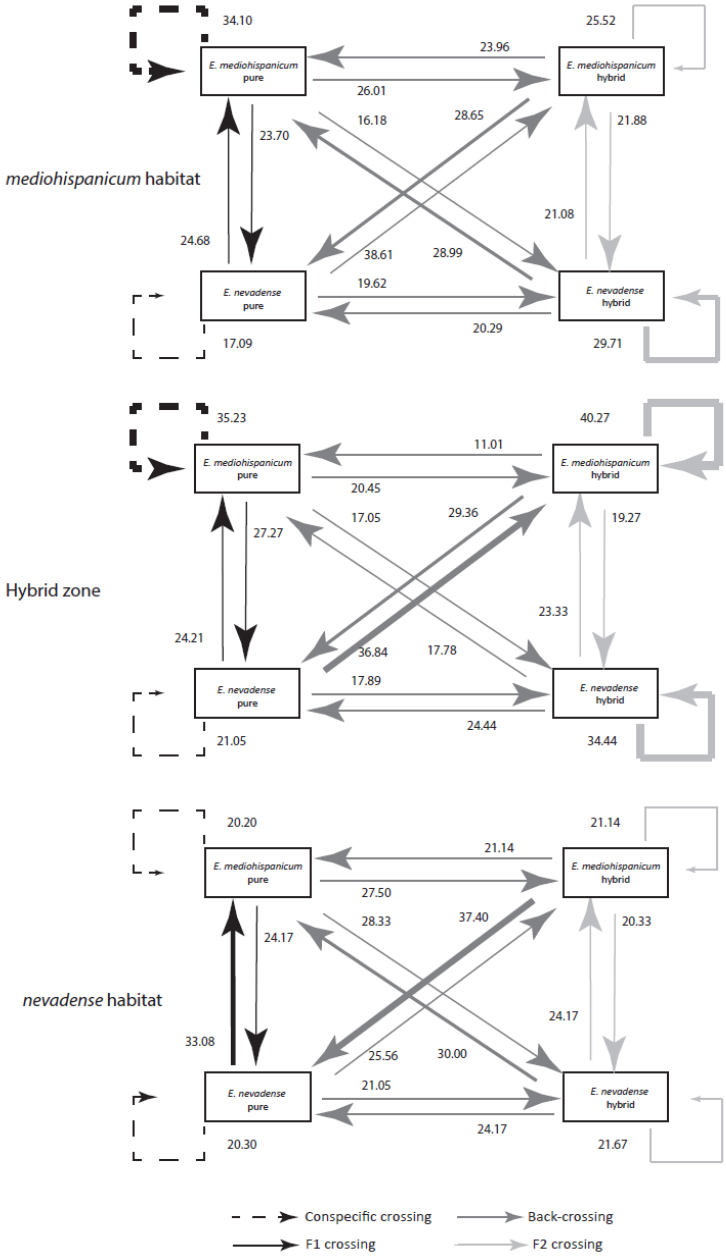

Figure 6.5. Transactions between different types of plants (species and hybrids), showing the observed deviation in the probability of flight movements between plant types with regard to the expected probabilities based on random flights. In black: positive deviation (preferred flights); in gray: negative deviations (avoided flights). The numbers indicate the magnitude of the deviations, $*P < 0.05$, $**P < 0.01$, $*** P< 0.001$.

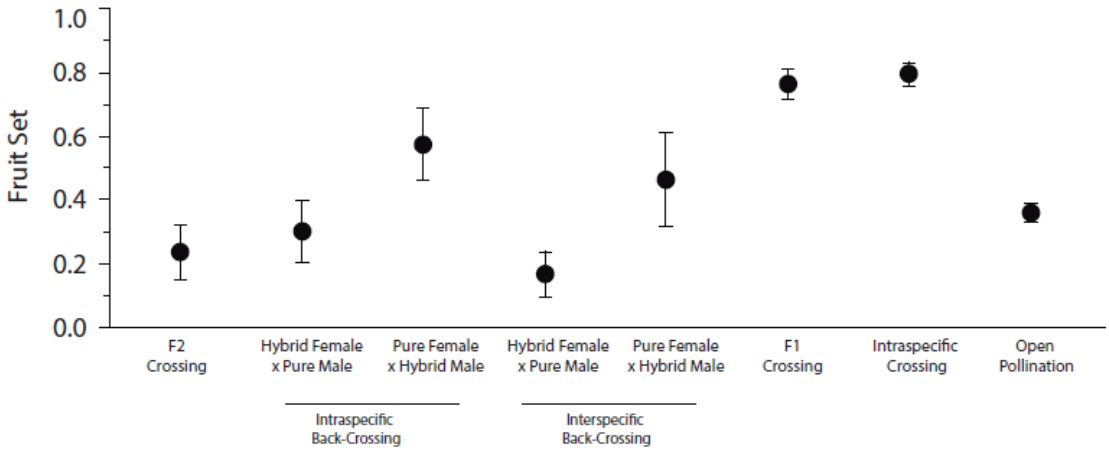

Figure 6.6. Reproductive output, assessed as proportion of flowers setting fruits (fruit set) of parental plants after con-specific and hetero-specific crossing and of hybrid plants after F_2 crossing and backcrossinging.

DISCUSSION

ETHOLOGICAL ISOLATION BETWEEN E. MEDIOHISPANICUM AND E. NEVADENSE

Erysimum mediohispanicum and *E. nevadense*, despite being pollination generalists, are visited in the Sierra Nevada by different assemblages of insects (Gómez *et al*., 2007; Ortigosa and Gómez, 2010; ***Chapters 3*** and ***8***). Whereas *E. mediohispanicum* flowers are preferentially visited by large bees, beeflies, small bees and beetles, the flowers of *E. nevadense* are mostly visited by ants, flies and beetles. However, our experiment shows that, when the pollinator assemblages of *Erysimum mediohispanicum* and *E. nevadense* occur in sympatry they are indistinguishable. So, all the plants were visited by the same insects, irrespective of the treatment, with the large bees belonging to the genus *Anthophora* the most frequent floral visitors on all the sites. This correlation may be partly due to tdifference in the ability of these two groups of insects to detect flowering plants. We believe that, whereas large bees can easily find new plants in the population, other insects like ants, flies and small beetles

would need more time to identify and develop a prospective image of these new plants. This could explain why the pollinator assemblages on all three experimental sites were dominated by bees, insects typical of *mediohispanicum* sites. Nevertheless, it is true that plants were visited on each site by insects abundant in natural plants on those sites. For example, ants, major pollinators of *E. nevadense* (***Chapter 8***), were one of the most frequent flower visitors on the *nevadense* site. This suggests that the differences found in natural populations in term of pollinator identity were also related to between-plant differences in altitude and habitat. Plants are visited by the main insects inhabiting those habitats:large bees and beeflies in low and medium-high mountain areas, and ants and flies in high mountain areas. The sharing of local pollinator fauna has been detected in other co-generic plants when they occur in sympatry (Smith *et al.*, 2008). This similarity in pollinator assemblage in sympatric conditions may have important consequences for the ethological isolation between the two *Erysimum* species (Campbell and Aldridge, 2006; Marques *et al.*, 2007).

Ethological isolation is a strong prezygotic pre-pollination barrier when hybridizing plants have specialized pollination systems or belong to different pollination syndromes (Ramsey *et al.*, 2003; Martin *et al.*, 2008; Kay and Sargent, 2009; Schiestl and Schlüter, 2009; Natalis and Wesselingh, 2012). In pollination-generalist plants, in contrast, congeneric plant species are used to sharing some or many pollinators. In this type of system, pre-pollination isolation is weak (Pascarella, 2007; Ellis and Johnson, 2012). In fact, our study shows that the pre-pollination barrier between *E. nevadense* and *E. mediohispanicum* is very permeable. Almost 25% of the times a pollinator left a flower of one pure species it visited a flower of the other pure species, thereby contributing to inter-specific crossing. This high frequency of inter-specific flights is to be expected, taking into account the phenotypic similarity of the two species. As stated above, *E. mediohispanicum* and *E. nevadense* only differ in some attraction traits, such as stalk height, number of stalks and number of flowers. Furthermore, this heterospecific phenotypic similarity leads us to believe that mechanical isolation is low in these species, and therefore most

of the pollen transported by insects between species will reach the stigmas. As a consequence, ethological isolation, quantified as $RI_{pollination}$, is extremely low between these two species (close to zero).

FLORAL TRAITS AND HYBRID ATTRACTIVENESS

Our experiment also shows that F_1 hybrid plants were visited by the same pollinators as the two parental species. This similarity in pollinator fauna may be another consequence of the strong phenotypic resemblance between plant groups. Only three traits – number of flowers, number of stalks and stalk height - were different from one species to another and, from the parentals to the hybrids, in our experimental plants. Hybrid plants showed an intermediate phenotype for stalk height, the expected outcome after hybridization (Arnold, 2006). However, the other two traits showed asymmetric hybrid vigor. Only the hybrids obtained after pollinating *E. mediohispanicum* flowers with *E. nevadense* pollen differed from their parentals, producing more stalks and, above all, more flowers than both *E. mediohispanicum* and *E. nevadense* (Fig. 6.2). These two traits influence fitness and are positively selected in both the pure species (Gómez *et al.*, 2003; 2009a; ***Chapter 8***). Furthermore, as this increase in phenotypic values appears in first-generation hybrids it may indicate the occurrence of heterosis (Rieseberg *et al.*, 1999). Hybrid vigor in flower numbers has been reported in many plant species, such as the Californian wild radish (*Raphanus sativus* x *raphanistrum*) (Campbell *et al.*, 2006). This trait is strongly associated with plant performance and colonization ability in many plants, and it seems to enhance the ability of hybrids to colonize new areas (Hovic *et al.*, 2012).

We found striking differences across the sites in the attractiveness of the different treatments. So, in *mediohipanicum* sites insects were more abundant in both the *E. mediohispanicum* types of plants, pure and hybrid. As more than 80% of the insects visiting flowers on these sites were large bees, we believe that this preference was partly influenced by the significant preference shown by these insects for high-flowered plants (Appendix 6.8). In fact, these two

types of plants were the ones that produced the most flowers (Fig. 6.2). This could also explain why *E. mediohispanicum* hybrids were the most visited plants in the hybrid zone. On the *nevadense* site, insects avoided the flowers of pure *E. mediohispanicum* plants. This may because these plants are taller than the rest. In natural populations of *E. nevadense* pollinators selectively prefer short plants (***Chapter 8***), partly because ants are the most abundant pollinators. It is widely known that ants visit short plants more often than tall plants on account of their foraging behavior (Hickman, 1974; Rico-Gray and Oliveira, 2007). The hybrid *E. mediohispanicum* plants also appeared to be visited very often, again probably due to their abundant flower production. To sum up, these plants are both short and high-flowered, making them very attractive on the *nevadense* site.

CONSEQUENCES FOR THE STABILITY OF THE HYBRID ZONE

The pattern found in this study may help us understand the existence of a hybrid zone between *E. mediohispanicum* and *E. nevadense*. We have found that the reproductive isolation between these two species due to pollinators is very low, almost zero, when they coexist on the same site. This absence of a pre-pollination barrier may promote inter-specific pollen flow. Hersch and Roy (2007) also reported that pollinator constancy to each of three species of *Castilleja* was weakened in the hybrid zone, thereby increasing the pollen flow between species. Similarly, Aldridge and Campbell (2007) found that pollinator preference and constancy were weaker, and hetero-specific pollen flow was stronger, in an *Ipomopsis aggregata/I. tenuituba* hybrid zone in which hybrids were abundant. It seems that, in general, hetero-specific pollen flow is fueled by the presence of hybrids in the population. Any contribution by the hetero-specific flights observed in our experiment to hybridization would depend on both the intensity of pollen carryover and the efficiency of post-pollination isolation barriers. In this respect, we also found that F_1 hybrid inviability is very low between these two *Erysimum* species. In fact, the reduction in the viability of artificially produced *E mediohispanicum* x *E. nevadense* hybrids with respect to

con-specific outcrossing (=hybrid inviability) was only 0.23 to 0.34 (***Chapter 3***). Consequently, we can imagine that hybrids would arise frequently on sites where these two species co-occur. This study shows that F_1 hybrid phenotype is similar to that of the pure plants. We have also found heterosis for some traits involved in pollinator attraction, such as the number of stalks and the number of flowers. We therefore also found that hybrid attractiveness is very high, higher than that of pure plants in the hybrid zone. In fact, we found that postzygotic reproductive isolation due to pollinator attractiveness was very low. When plant reproduction is limited by pollinator availability (i.e. pollen limitation), as seems to be the case in these plants (Gómez *et al.*, 2010, Abdelaziz *et al.* unpublished data), this difference in visitation rate may produce a difference in seed production. This effect is magnified even more by the fact that the least visited plants, pure *E. mediohispanicum* plants, are also unable to reproduce by selfing (***Chapter 3***). If we also take into account that *E. mediohispanicum* hybrid plants are the ones that produce the most flowers (Fig. 6.2), we can presume that, all things being equal, they will produce more seeds than the other plant types. Nevertheless, there would still be selection against hybrids when their fertility is lower than that of pure plants. Our study suggests that the fertility of hybrids, quantified as fruit set, is much lower than the fertility of pure plants (Fig. 6.6), and consequently we found high levels of postzygotic isolation barrier due to hybrid sterility. This would indicate that the hybrid zone cannot maintain itself in the long term without recurrent hybridization between pure plants. And this would therefore cause a high level of historic gene flow from the pure population into the hybrid zone, a phenomenon already reported in this system (***Chapter 5***). However, the hybrid zone may still be maintained with high levels of backcrossing. Our study shows that any kind of back-crossing, when the pollen recipient plant is pure and the pollen donor plant is hybrid, results in higher levels of fertility. In other words, hybridizing individuals may be produced if there is a sufficient number of pure plants colonizing the hybrid zone. So, the gene flow into the hybrid zone needs to be effected by seeds (to introduce pure plants) rather than by pollen (because the backcrossing fertility of the hybrid female and

pure male is very low). All of these reasons may explain the presence of a hybrid zone between these two *Erysimum* species in the geographical area in which they come into contact (***Chapter 5***).

Why do hybrid plants fail to colonize parental habitats and extend the geographical range of the hybrid zone? We think that there are several non-exclusive explanations; two in particular are very convincing. First, it is true that the high fertility of backcrossing between pure females and hybrid males suggests the possibility of hybrid individuals being produced in any of the parental habitats if there is enough pollen flow (=pollinator movements) from the hybrid zone to the parental sites. However, the fertility of the plants produced by this backcrossing is lower that of plants produced by pure intra-specific crossing in any of the parental habitats. Moreover, our experiment shows that pollinators prefer pure plants over hybrids in both the parental habitats. So, we presume that hybrids would be selected against, owing to the high frequency of intra-specific crosses in pure populations. Second, we have found that selection in the hybrid zone diverges from that of the two parental habitats (***Chapter 8***). Specifically, we found that there was a consistent selection for a high number of flowers per plant in the hybrid zone. This selection was weaker in the parental habitats. If we take into account that the hybrid plants showed hybrid vigor for this trait, we believe that they will be selected mainly in the hybrid zone, rather than in the parental habitats.

On all the sites we have also detected a high frequency of flights between hybrids, and between them and pure individuals. This is an indication of the potential for backcrossing and F1 hybrid crossing in the hybrid zone, where there is a natural occurrence of hybrids (***Chapter 5***). It is worth noting that most of the crosses in the hybrid zone involved some hybrids (Fig. 6.5). As hybrids, most particularly those from a *E. mediohispanicum* mother and *E. nevadense* father, were significantly preferred by pollinators (Fig. 6.2), the frequency of backcrossing and hybrid crossing would be even higher. The low intensity of hybrid inviability found in these species probable ensures the long-term stability of the hybrid zone. It is interesting that although flights

between hybrid and pure plants were also detected on the parental sites, where the attractiveness of hybrids was not much lower, the frequency of hybrids on these sites was negligible (***Chapter 5***). We think that other factors, possibly related to ecological conditions, may act to prevent the expansion of hybrids toward adjacent pure parental areas, thus maintaining the hybrid zone as a narrow strip.

In conclusion, three important outcomes emerge from our study on the pollinator preference in *E. mediohispanicum*, *E. nevadense* and their hybrids. (1) Despite the differences in pollinator assemblage in the studied zones, these pollinators do not prevent gene flow between parental species or between these and their hybrids, and they therefore generate virtually no prezygotic isolation barriers. (2) The pollinators present a significant preference for the hybrid plants in the hybrid zone, because of the heterosis patterns exhibited by hybrid individuals for the number of flowers, which could explain the short-term maintenance of the hybrid zone. (3) However, *E. mediohispanicum* and *E. nevadense* presented a postzygotic isolation barrier because of the low fertility of hybrids, suggesting that a constant migration of plants from the parental population would be necessary for the long-term maintenance of the hybrid zone.

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APPENDICES

Appendix 6.1. List of pollinator species visiting our plant arrays during the development of our experiment in the field. Species in bold were those in which we could measure flights between plants.

Pollinator species	Visits	Pollinator species	Visits
Abeja mediana	1	Escarabajo trompa larga	1
Halictus mediano	3	*Eupeodes corollae*	11
Allotarsus	1	*Eurydema ornata*	1
Andrena flavipes	5	*Exechia dorsalis*	1
***Andrena* grande**	1	*Fannius*	5
***Andrena* mediana**	34	*Forficula auricularia*	1
***Andrena* mediana dorada**	9	**Hesperido marron**	6
Andrena nigroaenea	13	*Hoplitis*	2
***Andrena* pequeña**	178	Hormiga pequeña roja	2
Andrena pequeña verde	1	***Ilaeus***	11
***Andrena* Roja**	6	*Isturgia famula*	17
Andrena sardoa	2	*Lachnaea*	5
Anthaxia	6	***Lasioglossum* diminuta**	5
Anthophora acervorum	163	***Lasioglossum* mediano**	46
Anthophora aestivalis	21	***Lasioglossum* pequeño**	147
Andrena agilisima	5	***Lasioglossum xantopus***	140
Anthophora atroalba	2084	*Lasius*	4
***Anthophora* marron**	56	**Licenido**	1
Anthophoraretusa	9	*Ligaeus*	1
Aplocnemus	14	***Malachius***	42
Attagenus	1	*Meligethes*	1
Bibionido	1	*Mordellistena*	1
Bombilido	39	Mosca dros	3
Bombilido gris	10	Mosquita	1
Bombilido negro	12	Mosquito diminuto	3
Bombilido rubio	21	*Muscidae* marron	2
Bombylius major	2	*Muscidae* pequeño	3
Brachionido	25	Muscido	5
Brachionido grande	1	Ortoptero ninfa	1
Brachionido pequeño	2	*Osmia brevicornis*	1
Brachonido negro	1	***Osmia* dorada**	6
Bruchido	2	***Osmia* parietina**	1
Calcidido	2	**Osmia claviventris**	1
Califora vomitoria	1	*Hoplitisrufohirta*	1
Ceratina	1	*Platycheirus manicatus*	1
Chisura	1	*Poliomnatus telina*	1
Chloropido	1	*Proformica longiseta*	96
Coccinella septempunctata	3	*Ceutorhynchus chlorophanus*	1
Coleoptero Rojo alargado	1	*Psilotrix*	1
Colias crocea	12	***Pyrgus***	1
Coreido	2	*Ramphomyia*	3
Crisido	8	***Scaeva albomaculata***	2
Crisomelido rojo	3	**Scatophagidae**	26
Curculionido	2	*Sphecodes*	4
Dasites subaeneus	15	Sirphido	2
Dasites torminalis	5	Tabanido	10
Dasitido pequeño	1	Tentredinido	2
Elaterido	1	Tifidido	4
Empidido pequeño	2	Tifidido negro	3
Empis negro	3	***Vanessa cardui***	9
Eristalis tenax	7	*Villa*	2
Escarabajo redondo	1		

Appendix 6.2. Outcome of experiment on TOTAL floral visitor abundance on flowers.

Fixed Effects	Estimate	Std. Error	z value	Pr(>|z|)	
SpEn	-0.07855	0.14786	-0.531	0.5953	
TratH	-0.03467	0.11141	-0.311	0.7557	
POPEn10	-0.11853	0.16899	-0.701	0.483	
POPH01	**-0.44598**	**0.17335**	**-2.573**	**0.0101**	*
SpEn:TratH	-0.02521	0.15957	-0.158	0.8745	
SpEn:POPEn10	**0.23704**	**0.11262**	**2.105**	**0.0353**	*
SpEn:POPH01	0.14476	0.12617	1.147	0.2512	
TratH:POPEn10	**0.26173**	**0.11021**	**2.375**	**0.0176**	*
TratH:POPH01	**0.27513**	**0.12209**	**2.253**	**0.0242**	*
SpEn:TratH:POPEn10	-0.1808	0.15735	-1.149	0.2505	
SpEn:TratH:POPH01	-0.28216	0.17685	-1.596	0.1106	

Appendix 6.3. Outcome of experiment on LARGE BEE abundance on flowers.

Fixed Effects	Estimate	Std. Error	z value	Pr(>|z|)
SpEn	0.004682	0.173954	0.027	0.979
TratH	0.097726	0.08515	1.148	0.251
POPEn10	-0.272225	0.421707	-0.646	0.519
POPH01	-0.441773	0.423764	-1.042	0.297
SpEn:TratH	-0.183122	0.125471	-1.459	0.144
SpEn:POPEn10	0.165372	0.131939	1.253	0.210
SpEn:POPH01	-0.040741	0.149775	-0.272	0.786
TratH:POPEn10	0.132223	0.126511	1.045	0.296
TratH:POPH01	0.054465	0.141359	0.385	0.700
SpEn:TratH:POPEn10	-0.010842	0.182291	-0.059	0.953
SpEn:TratH:POPH01	0.006098	0.206117	0.03	0.976

Appendix 6.4. Outcome of experiment on SMALL BEE abundance on flowers.

Fixed Effects	Estimate	Std. Error	z value	Pr(>|z|)	
SpEn	-0.4201	0.28438	-1.477	0.139606	
TratH	**-1.08642**	**0.337**	**-3.224**	**0.001265**	**
POPEn10	0.03343	0.66122	0.051	0.959681	
POPH01	-0.25523	0.67176	-0.38	0.703986	
SpEn:TratH	**1.16087**	**0.45472**	**2.553**	**0.010683**	*
SpEn:POPEn10	**1.1109**	**0.32159**	**3.454**	**0.000552**	***
SpEn:POPH01	0.63591	0.37348	1.703	0.088636	.
TratH:POPEn10	**1.70219**	**0.37251**	**4.569**	**4.89E-06**	***
TratH:POPH01	**1.55014**	**0.40886**	**3.791**	**0.00015**	***
SpEn:TratH:POPEn10	**-2.26006**	**0.49731**	**-4.545**	**5.50E-06**	***
SpEn:TratH:POPH01	**-1.69267**	**0.55815**	**-3.033**	**0.002424**	**

Appendix 6.5. Outcome of experiment on BEETLE abundance on flowers.

Fixed Effects	Estimate	Std. Error	z value	Pr(>|z|)	
SpEn	-0.38567	5.05E-01	-7.64E-01	0.4451	
TratH	0.11622	4.19E-01	2.78E-01	0.7813	
POPEn10	**-1.02589**	**5.56E-01**	**-1.85E+00**	**0.0648**	.
POPH01	-0.43797	4.61E-01	-9.50E-01	0.3423	
SpEn:TratH	0.30657	6.50E-01	4.71E-01	0.6374	
SpEn:POPEn10	-0.32438	7.97E-01	-4.07E-01	0.684	
SpEn:POPH01	-0.52699	6.68E-01	-7.89E-01	0.4299	
TratH:POPEn10	-0.57523	8.46E-01	-6.80E-01	0.4964	
TratH:POPH01	-0.06722	6.18E-01	-1.09E-01	0.9134	
SpEn:TratH:POPEn10	**2.01777**	**1.10E+00**	**1.84E+00**	**0.0659**	.
SpEn:TratH:POPH01	0.19603	9.39E-01	2.09E-01	0.8347	

Appendix 6.6. Outcome of experiment on BEEFLY abundance on flowers.

Fixed Effects	Estimate	Std. Error	z value	Pr(>|z|)
SpEn	0.287509	0.764252	0.376	0.7068
TratH	-0.070992	0.833173	-0.085	0.9321
POPEn10	0.009607	2.287033	0.004	0.9966
POPH01	1.640209	2.179281	0.753	0.4517
SpEn:TratH	-0.709886	1.201871	-0.591	0.5548
SpEn:POPEn10	0.271818	0.987981	0.275	0.7832
SpEn:POPH01	0.272247	0.881855	0.309	0.7575
TratH:POPEn10	-0.271388	1.143061	-0.237	0.8123
TratH:POPH01	0.866761	0.926855	0.935	0.3497
SpEn:TratH:POPEn10	1.509614	1.516376	0.996	0.3195
SpEn:TratH:POPH01	-0.50155	1.333533	-0.376	0.7068

Appendix 6.7. Outcome of experiment on ANT abundance on flowers.

Fixed Effects	Estimate	Std. Error	z value	Pr(>|z|)	
SpEn	-16.3225	2,112.3251	-0.008	0.99383	
TratH	-0.1112	1.0281	-0.108	0.91385	
POPEn10	**2.1866**	**0.8003**	**2.732**	**0.00629**	****
POPH01	-16.0964	2241.62	-0.007	0.99427	
SpEn:TratH	17.1807	2,112.3252	0.008	0.99351	
SpEn:POPEn10	15.9788	2,112.3251	0.008	0.99396	
SpEn:POPH01	32.319	3,080.0612	0.01	0.99163	
TratH:POPEn10	0.3829	1.0688	0.358	0.72015	
TratH:POPH01	16.1298	2,241.6202	0.007	0.99426	
SpEn:TratH:POPEn10	-17.2528	2,112.3253	-0.008	0.99348	
SpEn:TratH:POPH01	-32.0036	3,080.0614	-0.01	0.99171	

GLMs per population including covariables

Appendix 6.8.

Total Insects		Em25		H01		En10	
Source	**DF**	**L-R c2**	**P value**	**L-R c2**	**P value**	**L-R c2**	**P value**
Sp	1	0.146	0.7022	0.582295	0.4454	2.8756568	0.0899
Hybrid	1	8.036	0.0046	0.8594535	0.3539	0.4686214	0.4936
Sp*Hybrid	1	3.932	0.0474	2.1419082	0.1433	0.0207362	0.8855
N Stalk	1	2.587	0.1078	1.8652013	0.1720	6.9356641	0.0084
Stalk	1	14.512	0.0001	0.5856141	0.4441	2.2215698	0.1361
Height	1	0.551	0.4578	0.6235574	0.4297	2.9925791	0.0836
Cross	1	7.882	0.0050	1.575292	0.2094	0.4461915	0.5041
Corolla tube	1	0.138	0.7105	0.4162867	0.5188	2.366464	0.1240
Corolla width	1	31.727	<.0001	2.2848435	0.1306	10.177451	0.0014
N Flower	1	35.815	<.0001	2.0242992	0.1548	13.142014	0.0003
RW1	1	12.322	0.0004	4.8413161	0.0278	0.0056255	0.9402
RW2	1	4.944	0.0262	1.0850928	0.2976	0.7095144	0.3996
RW3	1	3.044	0.0810	0.4458572	0.5043	0.089742	0.7645
RW4	1	2.689	0.1010	1.6411221	0.2002	0.3021938	0.5825

Appendix 6.9.

LB		Em25		H01		En10	
Source	**DF**	**L-R c2**	**P value**	**L-R c2**	**P value**	**L-R c2**	**P value**
Sp	1	0.1338871	0.7144	0.5455685	0.4601	2.5022704	0.1137
Hybrid	1	3.046199	0.0809	2.2876392	0.1304	0.0450388	0.8319
Sp*Hybrid	1	2.1722699	0.1405	1.8391044	0.1751	0.5460064	0.4600
N Stalk	1	3.357779	0.0669	0.1688756	0.6811	11.925049	0.0006
Stalk	1	22.390877	<.0001	6.9544922	0.0084	16.734108	<.0001
Height	1	0.4937185	0.4823	0.7458043	0.3878	6.7431178	0.0094
Cross	1	3.5099999	0.0610	0.0123018	0.9117	2.0734231	0.1499
Corolla tube	1	0.0071448	0.9326	2.233409	0.1351	4.587672	0.0322
Corolla width	1	35.317618	<.0001	0.2074366	0.6488	14.714792	0.0001
N Flower	1	34.179667	<.0001	0.1395959	0.7087	11.591407	0.0007
RW1	1	12.85369	0.0003	0.0040859	0.9490	0.5045936	0.4775
RW2	1	3.6515253	0.0560	3.1612197	0.0754	1.1796399	0.2774
RW3	1	1.9125834	0.1667	0.6528896	0.4191	0.1226139	0.7262
RW4	1	4.4465238	0.0350	3.2720713	0.0705	2.1225697	0.1451

Appendix 6.10.

SB		Em25		H01		En10	
Source	**DF**	**L-R c2**	**P value**	**L-R c2**	**P value**	**L-R c2**	**P value**
Sp	1	0.2262698	0.6343	0.3497923	0.5542	0.6234911	0.4298
Hybrid	1	5.5375171	0.0186	0.0222139	0.8815	0.4753097	0.4906
Sp*Hybrid	1	7.5673236	0.0059	0.8068787	0.3690	8.0569284	0.0045
N Stalk	1	0.0047025	0.9453	1.882645	0.1700	0.5729036	0.4491
Stalk	1	10.561911	0.0012	3.0320387	0.0816	6.0353485	0.0140
Height	1	0.0050311	0.9435	2.0893468	0.1483	0.0205035	0.8861
Cross	1	10.718774	0.0011	3.136466	0.0766	0.2777307	0.5982
Corolla tube	1	1.0327416	0.3095	0.4571791	0.4989	0.031488	0.8592
Corolla width	1	0.4352212	0.5094	3.7367545	0.0532	0.33991	0.5599
N Flower	1	0.0209609	0.8849	0.9048298	0.3415	0.6534809	0.4189
RW1	1	1.7591919	0.1847	2.3431936	0.1258	4.1715896	0.0411
RW2	1	0.008966	0.9246	0.0005773	0.9808	0.0170185	0.8962
RW3	1	0.8086788	0.3685	3.8169407	0.0507	0.010138	0.9198
RW4	1	0.0000131	0.9971	1.8488081	0.1739	7.6621117	0.0056

Appendix 6.11.

B		Em25		H01		En10	
Source	**DF**	**L-R c2**	**P value**	**L-R c2**	**P value**	**L-R c2**	**P value**
Sp	1	1.0267247	0.3109	2.3489043	0.1254	2.9010871	0.0885
Hybrid	1	1.3564603	0.2442	0.9440872	0.3312	2.4876213	0.1147
Sp*Hybrid	1	0.0614359	0.8042	0.4214515	0.5162	2.173628	0.1404
N Stalk	1	3.650041	0.0561	2.4858444	0.1149	0.9732502	0.3239
Stalk	1	7.1929083	0.0073	0.4550834	0.4999	0.7400464	0.3896
Height	1	0.1997623	0.6549	0.067268	0.7954	1.4484645	0.2288
Cross	1	1.8587032	0.1728	4.0538475	0.0441	0.1419761	0.7063
Corolla tube	1	0.0128678	0.9097	3.8472946	0.0498	4.7421151	0.0294
Corolla width	1	0.1899565	0.6630	0.2403942	0.6239	0.0058893	0.9388
N Flower	1	5.3465541	0.0208	1.595419	0.2066	0.0331037	0.8556
RW1	1	7.3216902	0.0068	0.0009739	0.9751	1.2665109	0.2604
RW2	1	4.398273	0.0360	0.0015928	0.9682	4.7039197	0.0301
RW3	1	0.6405332	0.4235	0.4172134	0.5183	1.2339296	0.2666
RW4	1	1.1135054	0.2913	1.9325731	0.1645	0.2006239	0.6542

Appendix 6.12.

BF		Em25		H01		En10	
Source	**DF**	**L-R c2**	**P value**	**L-R c2**	**P value**	**L-R c2**	**P value**
Sp	1	1.2884257	0.2563	0.029513	0.8636	0.3525038	0.5527
Hybrid	1	1.2967902	0.2548	0.0034172	0.9534	0.251722	0.6159
Sp*Hybrid	1	0.0088922	0.9249	0.4441189	0.5051	0.4424868	0.5059
N Stalk	1	0.172823	0.6776	6.5183334	0.0107	1.6185631	0.2033
Stalk	1	7.0365138	0.0080	0.5557383	0.4560	11.528718	0.0007
Height	1	0.4147827	0.5196	0.3324103	0.5642	0.4406226	0.5068
Cross	1	2.9436653	0.0862	0.0150844	0.9023	0.6890323	0.4065
Corolla tube	1	0.0074824	0.9311	0.0092155	0.9235	0.2976931	0.5853
Corolla width	1	1.5893562	0.2074	0.0130285	0.9091	0.3088099	0.5784
N Flower	1	6.0501535	0.0139	0.1210867	0.7279	0.9924527	0.3191
RW1	1	0.0619763	0.8034	0.5453181	0.4602	9.7360382	0.0018
RW2	1	0.1171052	0.7322	0.789633	0.3742	0.3660479	0.5452
RW3	1	0.1875418	0.6650	0.6098099	0.4349	0.0745132	0.7849
RW4	1	0.4609744	0.4972	0.0243814	0.8759	0.0054208	0.9413

Appendix 6.13.

A		Em25		H01		En10	
Source	**DF**	**L-R c2**	**P value**	**L-R c2**	**P value**	**L-R c2**	**P value**
Sp	1	1.2141985	0.2705	4.6407162	0.0312	0.1398073	0.7085
Hybrid	1	5.5096381	0.0189	3.0462752	0.0809	1.3986108	0.2370
Sp*Hybrid	1	2.5021102	0.1137	0.1525154	0.6961	1.5699357	0.2102
N Stalk	1	0.8769178	0.3490	0.0249314	0.8745	11.952188	0.0005
Stalk	1	1.2231442	0.2687	1.4192715	0.2335	1.87442	0.1710
Height	1	3.3117131	0.0688	0.4061706	0.5239	0.0043992	0.9471
Cross	1	0.0236093	0.8779	0.4758924	0.4903	4.7013347	0.0301
Corolla tube	1	0.3837966	0.5356	0.3292555	0.5661	2.4057203	0.1209
Corolla width	1	0.035363	0.8508	0.3302316	0.5655	1.4266422	0.2323
N Flower	1	0.0411914	0.8392	2.2383677	0.1346	6.8433527	0.0089
RW1	1	2.0624915	0.1510	6.2688946	0.0123	2.5295449	0.1117
RW2	1	2.0836451	0.1489	0.0144399	0.9044	0.1860921	0.6662
RW3	1	0.6116044	0.4342	0.076684	0.7818	0.6519121	0.4194
RW4	1	0.7327069	0.3920	0.1360006	0.7123	1.1535434	0.2828

CHAPTER 7

HERITABILITY AND GENETIC CORRELATION OF COROLLA SHAPE AND SIZE IN *ERYSIMUM MEDIOHISPANICUM*

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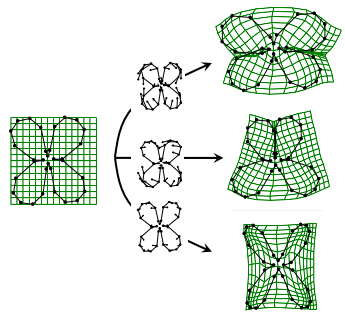

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ABSTRACT

Flower shape has evolved in most plants as a consequence of pollinator-mediated selection. Unfortunately, no study has explored the genetic variation of flower shape, despite that this information is crucial to understand its adaptive evolution. Our main goal here is to determine heritability of corolla shape in *Erysimum mediohispanicum* (Brassicaceae). Also, we explore heritability of other pollinator-selected traits in this plant species, such as plant size, flower display, and corolla size. In addition, we investigate genetic correlations between all these traits. We found significant heritability for one plant-size trait (stalk height), for number of flowers, for all corolla-size traits (corolla diameter, corolla tube length and corolla tube width), and for corolla shape. Consequently, this species retains a high ability to respond to the selection exerted by its pollinators. Genetic correlation was strong between all functionally related traits and between flower number and plant size, weak between corolla size and plant size and no correlation between corolla shape and any other trait. Thus, selection affecting some *E. mediohispanicum* traits would also indirectly affect other functionally-related and unrelated traits. More importantly, the observed genetic correlation seems to be at least partially adaptive, since positive correlational selection currently acts on the covariance between some of these traits.

Key words

Brassicaceae, corolla shape heritability, *Erysimum mediohispanicum*, genetic correlation, geometric morphometrics, phenotypic integration.

INTRODUCTION

Adaptive floral evolution requires the occurrence of two important factors, phenotypic selection on floral traits caused by pollinator activity and genetic variation for those selected traits (Lynch and Walsh, 1998). Pollinator-mediated phenotypic selection has been widely documented for floral traits in many plant species (for recent reviews, see Ashman and Morgan, 2004; and Harder and Barrett. 2006). In contrast, genetic variation and heritability has been much less frequently studied for these types of plant traits (Geber and Griffen, 2003; Ashman and Majetic, 2006). Furthermore, most quantitative genetic studies on floral traits have focused on variables related to size, such as corolla size, corolla tube length, number of stamens, number of flowers, etc. (Conner and Via, 1993; Mitchell and Shaw, 1993; Ashman, 1999; Kaczorowski *et al*., 2008). By contrast, studies on genetic architecture of shape-related floral traits are scarce, and the few examples studying the heritability of floral shape divide shape into several simple linear variables (Venable and Búrquez, 1989; Galen and Cuba, 2001). However, floral shape is a complex multidimensional trait that can only partially be described by its linear components. Exploring the quantitative genetics of floral shape, thereby, requires a multivariate approach (Monteiro, 1999; Klingenberg and Leamy, 2001; Monteiro *et al*., 2002; 2003; Klingenberg, 2003; Klingenberg and Monteiro, 2005). This is probably the main reason explaining the paucity of studies affronting the investigation of the genetic basis of corolla shape. This happens despite that most theoretical and empirical studies on floral evolution postulate that corolla shape has evolved as a response to strong selection exerted by pollinators (Coen *et al*., 1995; Endress, 2001; Schemske and Bradshaw, 1999; Sargent, 2004).

Selection on floral traits has both a direct and an indirect component. The relative importance of these two components depends on the strength of the genetic correlation between the target traits and other phenotypic traits. Under these circumstances, response to selection is mediated not only by trait heritability but also by the genetic correlations among traits. We are still far from knowing how strongly the genetic correlations constrain the responses of plant

traits to selection (Conner, 2002; Ashman and Majetic, 2006; Kaczorowski *et al.*, 2008). In addition, genetic correlations also contribute to the phenotypic integration of complex structures. In scenarios where integration is beneficial, selection should increase the genetic correlation among functionally related traits, leading to the evolution of complex integrated structures (Venable and Búrquez, 1990). Genetic correlation is expected to be stronger between functionally and developmentally related traits than between unrelated ones (Berg, 1959; 1960). For example, genetic correlation between vegetative and floral traits is expected to be lower than among floral traits or vegetative traits themselves (Armbruster *et al.*, 1999). Correlational selection would cause higher genetic correlation between traits belonging to the same complex structure than between traits from different structures (Sinervo and Svensson, 2002; McGlothlin *et al.*, 2005). This enhanced genetic correlation can result through the build up of linkage disequilibrium, by favoring pleiotropic mutations, or through linkage between genes affecting traits under correlational selection (Lynch and Walsh, 1998).

Erysimum mediohispanicum (Brassicaceae) is a pollination-generalist plant that shows high phenotypic variation for fitness-related traits (Gómez *et al.*, 2006). Despite its being a generalist, we have found that pollinators exert strong phenotypic selection on many traits, associated mostly with plant size and corolla size and shape (Gómez *et al.*, 2006; 2008a; 2009). Our main objective in this paper is to estimate the heritability and genetic correlation for the major pollinator-selected phenotypic traits in this plant species, as a way to predict their response to selection. Estimating the quantitative genetics of size-related traits would require the use of standard methodology. However, it is not adequate to use such methodology to estimate the quantitative genetics of complex multidimensional traits such as corolla shape. For this reason, following the approach proposed by some evolutionary biologists (Monteiro, 1999; Monteiro *et al.*, 2002; 2003; Klingenberg, 2003; Kingenberg and Monteiro, 2005), we have estimated corolla shape heritability and genetic correlation using a multivariate approach that does not break it down into

linear components. Very few attempts have been made so far to use this approach to explore the heritability of complex shapes (see Myers *et al.,* 2006; Santos *et al.*, 2005).

MATERIALS AND METHODS

STUDY SYSTEM

E. mediohispanicum Polatschek (Brassicaceae) is a biennial to perennial monocarpic herb abundant in the N and SE of the Iberian Peninsula. Plants usually grow for 2-3 years as vegetative rosettes, and then die after producing one to eight reproductive stalks which can display between a few and several hundred hermaphroditic, slightly protandrous bright yellow flowers (Gómez, 2003). Although this crucifer is self-compatible, it requires pollen vectors to produce a full seed set (Gómez, 2005a). Flowers are visited by many different species of insects, from large bees and butterflies to tiny beetles and ants (Gómez *et al.*, 2007; 2008a). Selective exclusion experiments have demonstrated that even minute, unspecialized flower visitors are important pollinators of *E. mediohispanicum* (Gómez, 2005a) and can exert strong selective pressure (Gómez *et al.*, 2006; 2008a). Mean seed dispersal distance is extremely short in this species, less than 20 cm (Gómez, 2007).

The field study was conducted between 2005 and 2007 in eight *E. mediohispanicum* populations of the Sierra Nevada high mountains (Granada province, SE Spain; Table 7.1). Genetic differentiation among populations is high, based on both nuclear markers (Bayesian G_{st} = 0.27± 0.02 based on 160 RAPDs) and plastidial haplotypes (F_{st} = 0.35 based on trnL-trnF cpDNA) (Gómez *et al.*, 2009).

Pollination ecology and phenotypic selection of floral traits were previously measured at these same sites (Gómez *et al.*, 2008a; 2008b; 2009). In these populations, flowers are visited by more than 150 insect species, ranging from beetles to beeflies and bees (Gómez *et al.*, 2007).

EXPERIMENTAL DESIGN

Ninety plants were marked in each of the eight populations (720 plants in total), at the onset of the 2005 flowering period (April) using aluminum tags attached to the base of the flowering stalks. Plants were monitored throughout the entire reproductive season. At the end of the season, when seeds are mature but prior to dispersal (September), we collected 30-40 seeds per plant from each of the surviving individuals (N=335 plants; Table 7.1). Losses are very frequent under natural conditions due to summer drought and ungulate damage (Gómez, 2005b).

We planted 10 seeds per surviving maternal plant on October 2005 in a University of Granada (UGR) glasshouse. Seeds were located in individuals pots 15 cm apart to avoid competition. To avoid environmental covariance, pots were distributed according to a completely randomized design. Seedlings were transferred to an UGR outdoors common garden when they had produced the cotyledons but before true leaf development. Plants were watered once weekly during winter (October-January), twice weekly during spring (February-May) and daily during summer (June-September). The watering regime was identical for all plants. Plants flowered when they were two years olds, on April-May 2007. In total, 1675 plants belonging to 332 families reached adulthood (Table 7.1).

Code	Latitude	Longitude	Habitat	Altitude	No. maternal plants	No. planted seeds	No. flowering offspring
Em01	37º 8.00' N	3º 25.69' W	Forest	1750	65	650	334
Em02	37º 7.33' N	3º 25.86' W	Shrubland	2099	14	140	33
Em08	37º 8.00' N	3º 25.91' W	Shrubland	1690	65	650	370
Em21	37º 8.07' N	3º 25.71' W	Forest	1723	41	410	237
Em22	37º 7.86' N	3º 25.70' W	Forest	1802	57	570	370
Em23	37º 7.74' N	3º 25.58' W	Shrubland	1874	32	320	97
Em24	37º 7.51' N	3º 26.14' W	Forest	1943	34	340	147
Em25	37º 7.27' N	3º 26.05' W	Shrubland	2064	26	260	64

Table 7.1. Location and characteristics of the eight plant populations studied, and sample size of the individuals used in this study.

QUANTIFICATION OF FLORAL TRAITS

The following phenotypic traits were determined for both the maternal (2005) and the offspring (2007) plants:

(1) Plant size, estimated by (i) Stalk height, quantified as the height of the tallest stalk, measured to the nearest 0.5 cm as the distance from the ground to the top of the highest open flower; (ii) Number of stalks; and (iii) Stalk diameter, quantified as the basal diameter in mm of the tallest stalk. These traits were measured with a digital caliper with ± 0.1 mm resolution. All plant-size-related traits were measured when plants were in full bloom.

(2) Flower number, counting the entire production of flowers of each plant.

(3) Corolla size, estimated in one flower per plant by (i) Corolla diameter, estimated as the distance in mm between the edges of two opposite petals; (ii) Corolla tube length, the distance in mm between the corolla tube aperture and the base of the sepals; (iii) Corolla tube width, the diameter of the corolla tube aperture, estimated as the distance between the bases of two opposite petals. These traits were also measured with a digital caliper.

(4) Corolla shape, determined in each of the plants by means of geometric morphometric tools, using a landmark-based methodology (Bookstein, 1991; Rohlf, 2003; Zelditch *et al.*, 2004). We took a digital photograph of the same flower as above using a standardized procedure (front view and planar position). Flowers were photographed at anthesis to avoid ontogenetic effects. We defined 32 coplanar landmarks located along the outline of the flowers and the aperture of the corolla tube, the number of landmarks being chosen to provide comprehensive coverage of the flower shape (Roth, 1993; Zelditch *et al.*, 2004). Landmarks were defined by reference to the midrib (landmarks 1, 9, 17, 25), primary veins (landmarks 2, 8, 10, 16, 18, 24, 26 and 32), and secondary veins (landmarks 3, 4, 6, 7, 11, 12, 14, 15, 19, 20, 22, 23, 27, 28, 30 and 31) of each petal as well as the connection between petals (landmarks 5, 13, 21,and 29; see Fig. 7.1). We captured the landmarks using the software tpsDig vs. 1.4 (available in the Stony Brook Morphometrics website at http://life.bio.sunysb.

edu /morph/morphmet.html). Afterwards, the two-dimensional coordinates of these landmarks were determined for each plant, and the generalized orthogonal least-squares Procrustes average configuration of landmarks was computed using the Generalized Procrustes Analysis (GPA) superimposition method (Rohlf and Slice, 1990; Slice, 2001). We used this method because of its low bias (Rohlf, 2003). This procedure was performed using the software tpsRelw vs. 1.11 (available in the Stony Brook Morphometrics website at http://life.bio.sunysb.edu /morph/morphmet.html). In these analyses, we considered the flower as a non-articulated structure because the relative position of the petals does not change during their functional life. After GPA, the relative warps (RWs, which are principal components of the covariance matrix of the partial warp scores) were computed (Walker, 2000; Adams *et al.*, 2004). Unit centroid size was used as the alignment-scaling method and the orthogonal projection as the alignment-projection method. This procedure generates a consensus configuration, the central trend of an observed sample of landmarks, which is similar to a multidimensional average. In addition, this procedure generates 2p-4 orthogonal RWs (p= number of landmarks). Each RW is characterized by its singular value, and explains a given variation in shape among specimens. Thus, RWs summarize shape differences among specimens (Adams *et al.*, 2004), and their scores can be saved to be used as a data matrix to perform standard statistical analyses (Zelditch *et al.*, 2004).

ESTIMATION OF HERITABILITY AND GENETIC CORRELATIONS

Heritability of plant and corolla size.

Heritability was quantified using a mother-offspring regression (Falconer and Mackay, 1996) as

$$h^2_{\phi} = 2B$$

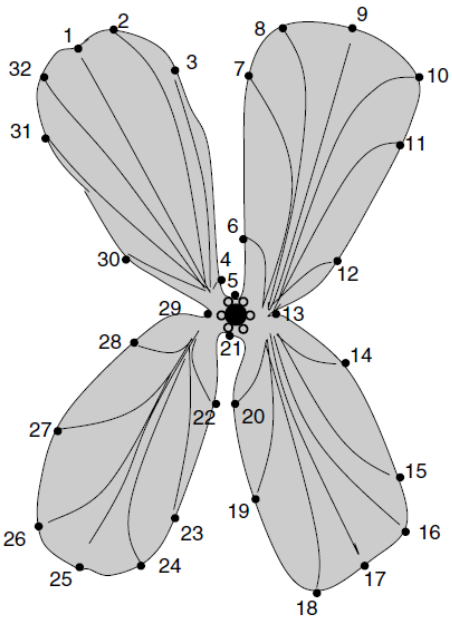

Figure 7.1. A schematic planar view of the *Erysimum mediohispanicum* corolla, showing the location of the 32 landmarks used in the geometric morphometric analysis.

where B is the slope of the regression of offspring trait values on the mother trait values. However, h^2_{OP} is dependent not only on the additive genetic variation in the parent generation, but also on the additive genetic variation in the offspring generation grown in the greenhouse (see appendix by R. Lande in Coyne and Beecham, 1987). Under these circumstances, estimates of h^2_{OP} may not be accurate estimates of the heritabilities in the natural population. Therefore, we also used a method developed by Riska *et al.* (1989) to calculate the heritability in the natural population from the offspring–parent regression, which corrects for the additive genetic variation of the offspring generation in the greenhouse,

$$h^2_{RISKA} = 4B^2\left(\frac{\sigma^2_P}{\sigma^2_O}\right)$$

where s^2_{PP} is the phenotypic variance in the natural population, estimated from

the parental plants, and s^2_{GO} is the additive genetic variance of the offspring in the greenhouse (Kleunen and Ritland, 2004). The squared coefficient of parent-offspring regression is multiplied by 4 following the suggestions by Riska *et al.* (1989) when information exists only for mothers. We estimated s^2_{GO} from the analysis of variance on the offspring plants under the conservative assumption that offspring of the same seed family are half sibs. Consequently, the Riska estimator is a minimum estimate of the actual heritability and therefore it yields low values of heritability. Standard errors and significance levels of the Riska estimator of heritability were calculated by bootstrapping, producing 1,000 bootstrap replicates for each phenotypic trait in the "boot" package of R (R Development Core Team. 2007).

Since we had unequal family sizes, we used weighted least-squares regression to find h^2_{OP} and h^2_{RISKA} (Lynch and Walsh, 1998). Weight was the inverse of the residual sampling variance of family means about the mother-offspring regression (Lynch and Walsh, 1998). Because of the hierarchical nature of the design, all the regressions were performed on residuals from an analysis of variance that included the population as the random factor (Campbell, 1996). Considering population as random avoids problems associated to heritability overestimation when fixed factors are included in the models (Wilson, 2008). Nevertheless, to check whether this hierarchical design could affect our conclusions on heritability, we determined heritability separately for the only three populations for which we obtained information from more than 50 families: Em01, Em08 and Em22.

Heritability of corolla shape

Since shape is an inherently multivariate concept, estimating heritability of corolla shape is not possible by using the above-described standard univariate methods (Monteiro *et al.*, 2002; 2003; Klingenberg, 2003). Monteiro *et al.* (2002) recommended the calculation of shape heritability as the ratio of the total variances of the **G** and **P** matrices from the relative warps. We first used

this method to calculate the overall heritability for corolla shape.

However, this approach does not consider the directionality of variation in **G** and **P** (Klingenberg and Monteiro, 2005). Some authors have proposed the use of a multivariate approach, such as Generalized Multivariate Regression (GMR),to overcome this problem and to accurately estimate heritability of shape (Monteiro, 2000; Klingenberg and Leamy, 2001; Klingenberg, 2003; Klingenberg and Monteiro, 2005). Here, we followed this suggestion and estimated heritability on flower shape by means of a GMR, including as dependent variables the 60 RWs of the offspring, averaged per family, and as independent variables the 60 RWs of the mother plants. The GMR measures only the magnitude of shape differences, but ignores their direction, and consequently no coefficient is associated to this analysis (Klingerberg, 2003). The significance of the whole shape heritability was performed by a Wilks' Lambda (Zelditch *et al.*, 2004). In addition, we calculated a multivariate regression coefficient for each of the first four RWs, since each of these explained more than 5% of the variance in corolla shape (over 70% of variability all together, see below).

These multivariate coefficients are multivariate analogues to the standard univariate regression coefficients; they represent the association between each parent and offspring pair of RWs, while controlling for the remaining RWs in the matrix. After this, following the procedure explained above, we found the h^2_{RISKA} for the shape components.

Genetic correlation

Genetic correlations r_A between traits x_i and x_j were calculated as

$$r_A = \frac{Cov(x_i, x_j)}{\sqrt{Var(x_i) \times Var(x_j)}}$$

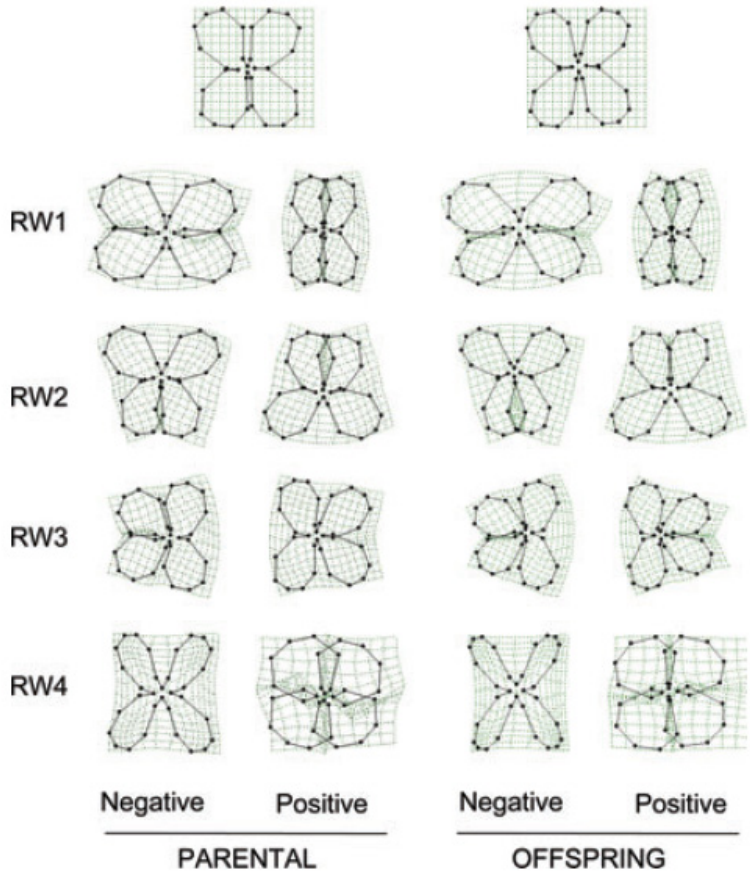

Figure 7.2. Summary of the geometric morphometric analysis (N= 720 plants in the parental group and 1635 in the offspring group) showing the consensus morphology (uppermost panels) and the variation in flower morphology produced by the Relative Warps explaining more than 5% of the overall variation in shape (see Appendix 7.2). The distribution of each RW statistically fitted a normal distribution with mean=0 (χ^2<0.993, p>0.34 in all cases, Shapiro-Wilks' W test).

where *Cov* (x_i, x_j) are additive covariances and *Var* (x_i) are additive variances (Falconer and Mackay, 1996; Lynch and Walsh, 1998). However, since we studied the parent population in the wild and the offspring population in the greenhouse, we also estimated genetic correlations between traits x_i and x_j as

$$r_A^* = \frac{0.5\left[Cov(x_{i,O}x_{j,P}) + Cov(x_{j,O}x_{i,P)}\right]}{\sqrt{Cov(x_{i,O}x_{i,P}) \times Cov(x_{j,O}x_{j,P})}}$$

where O and P refer to offspring and parent values, respectively. This method allows the estimation of genetic correlation without any previous knowledge concerning the relatedness between measured individuals in the natural population (Lynch, 1999; Kleunen and Ritland, 2004). Because r_A* is not a product-moment correlation, it can sometimes be estimated out of the ±1 boundary (Lynch and Walsh, 1998).

The standard error for the mean of the genetic correlation was calculated as

$$\mathrm{SE} = \left[\frac{1 - r_A^*}{\sqrt{2}}\right]\sqrt{\frac{\sigma_{h_x^2}\sigma_{h_y^2}}{h_x^2 h_y^2}}$$

Falconer and Mackay (1996).

RESULTS

HERITABILITY OF TRAITS

Phenotypic values for floral traits were very similar in parental and offspring plants, except for flower number that was almost twice as high in the offspring plants (Table 7.2). The number of flowers, stalk diameter, and number of stalks had non-significant heritability values (Table 7.3). The heritability of these traits was also low in each of the three populations analyzed separately (Appendix 7.1). By contrast, stalk height presented a significant heritability, which remained high even when estimated using the Riska method (Table 7.3).

	Parental Generation (N=332)		Offspring Generation (N=1665)		
	Mean±1 SE	V_p	Mean±1 SE	V_p	Vg
Stalk diameter (mm)	1.85±0.03	0.32	2.40±0.09	13.70	1.15
Number of stalks	1.35±0.98	0.95	1.86±0.84	2.29	1.23
Stalk height (cm)	36.97±0.85	239.73	38.06±0.35	207.84	148.05
Number of flowers	40.90±0.85	926.17	78.84±1.96	6435.13	6428.12
Corolla diameter (mm)	10.89±0.09	2.64	12.41±0.04	2.77	1.05
Corolla tube length (mm)	10.52±0.08	1.94	10.65±0.03	1.98	1.06
Corolla tube width (mm)	0.52±0.05	0.95	1.47±0.02	0.64	0.18
Corolla shape (x 10^2)		2.82		2.64	1.11
RW1*	0.90±0.68	1.54	0.17±0.29	1.39	0.64
RW2*	0.45±0.47	0.74	0.02±0.19	0.63	0.25
RW3*	0.58±0.34	0.38	0.31±0.15	0.39	0.13
RW4*	0.10±0.31	0.31	0.15±0.11	0.21	0.09

*RW's refer to the first four relative warps obtained from the geometric morphometric analysis of corolla shape.

Table 7.2. Descriptive statistics of floral traits in *Erysimum mediohispanicum* (N=332 families). Vp= Phenotypic variance (calculated as the among-individual variance, N=332 parental plants and 1665 offspring plants), Vg= Genetic variance (calculated as the among-family variance, N=332 families).

Heritability was significant for the traits related to flower size, such as corolla diameter, corolla-tube length and corolla-tube width (Table 7.3). Corolla diameter and tube length displayed higher R^2 value than did corolla tube width (Table 7.3). Corolla diameter heritability was significant in two of the populations while corolla tube length and width heritability was significant in one of the populations analyzed separately (Appendix 7.1).

The main components describing the variation in corolla shape were similar for parental as well as offspring generations (Fig. 7.2). Thus, the first four RWs explained more than 70% of the variance in corolla shape in these two groups of plants (Appendix 7.2), and were associated with the same patterns of shape variation (Fig. 7.2). Thus, RW1 was associated with changes in petal parallelism, RW2 was associated with changes in corolla zygomorphy, RW3 was associated with lateral symmetry, and RW4 was associated with corolla roundness.

Table 7.3. Heritability of floral traits in *Erysimum mediohispanicum* (N=332 families).

Significant heritabilities are shown in bold.

Plant traits	h^2_{OP} €					h^2_{Riska} §
	Values± 1 SE	R^2^	Wilk's l	F	P	Value± 1 SE_b ∞
Stalk diameter¶	0.180±0.110	0.020		0.86	0.389	0.009±0.028
Number of stalks¶	0.002±0.063	0.001		0.16	0.876	0.004±0.014
Stalk height¶	**0.190±0.043**	0.015		2.21	0.027	**0.361±0.162**
Number of flowers¶	0.001±0.149	0.001		0.14	0.887	0.050±0.199
Corolla diameter¶	**0.270±0.033**	0.046		3.97	0.0001	**0.239±0.199**
Corolla tube length¶	**0.392±0.039**	0.071		5.00	0.0001	**0.267±0.152**
Corolla tube width¶	**0.094±0.024**	0.011		1.94	0.050	**0.019±0.094**
Corolla shape¥	**0.423**		0.642		0.0001	
RW1*	**0.184±0.056**	0.277	0.917		0.002	0.045±0.059
RW2*	**0.038±0.055**	0.363	0.933		0.018	0.001±0.047
RW3*	0.004±0.055	0.312	0.967		0.407	0.012±0.034
RW4*	**0.500±0.061**	0.415	0.917		0.003	0.001±0.024

¶ Models including population and population*phenotypic traits as random factors were solved by REML and were weighted by the inverse of the variance of the residuals.

¥ Overall heritability for corolla shape was calculated as the ratio of the total variances of the **G** and **P** matrices (Monteiro *et al.* 2002), and its significance was estimated by a generalized multivariate regression (GMR) between the 60 parental RWs and the 60 offspring RWs, including population as the random factor.

* Heritability values of each shape component were the multivariate regression coefficients resulting from the GMR.

^ R^2 was multivariate for corolla shape components and univariate for the remaining traits.

€ h^2_{OP}=2B, where B is the slope of the regression of offspring-trait values on the mother trait values.

§ $$h^2_{RISKA} = \gamma^2 h^2 = 4B^2\left(\frac{\sigma_P^2}{\sigma_O^2}\right).$$

∞ The standard error and the significance level of the Riska estimator of heritability were found by bootstrappin

The multivariate regression indicates that heritability was also significant for corolla shape (Table 7.3). Specifically, three shape components had significant heritability, namely RW1, RW4 and to a lesser extent RW2 (Table 7.3). Thus, non-parallel petals and narrow petals were highly heritable. In these cases, the multivariate R^2 were very high, consistently above 25% of the variance explained (Table 7.3). When the three populations were studied separately, we found significant heritability for corolla shape in two populations, due mostly to RW4 and RW1components (Appendix 7.1).

GENOTYPIC CORRELATIONS AMONG TRAITS

There was a significant and high positive genetic correlation amongst all plant-size-related phenotypic traits (number of stalks, stalk diameter, and stalk height), irrespective of the method used to quantify it (Table 7.4). Many of the genetic correlations, when quantified using the standard method, remained significant when the three populations were analyzed separately (Appendix 7.1). Furthermore, number of flowers was positively correlated with all these plant-size traits, both when all populations were analyzed together (Table 7.4) as well as when the three populations were studied separately (Appendix 7.1).

Flower-size related traits (corolla diameter, corolla tube length, and corolla tube width) were also positively correlated amongst them according to the standard r_A. However, according to r_A* the correlations between corolla tube width and the other two flower-size related traits vanished (Table 7.4). When studied separately, there was again significant genetic correlation, when estimated as r_A, between corolla diameter and the other two floral-size-related traits (Appendix 7.1). Flower-size traits were significantly correlated with plant-size traits when estimated as r_A*(Table 7.4). However, most of this correlation disappeared when populations where studied separately (Appendix 7.1).

There was no significant genetic correlation, either r_A or r_A*, amongst flower-shape components. Also remarkable was the low correlation observed between corolla shape and other plant traits (Table 7.5). Only number of flowers significantly correlated with flower shape (Table 7.5). In addition, number of stalks was negatively correlated with floral roundness (RW1), stalk height was positively correlated with floral zygomorphy (RW2), and number of stalks and flower size were correlated with floral asymmetry (RW3; Table 7.5). However, when we calculated the genetic correlations separately for the populations Em01, Em08 and Em22, we found some striking outcomes.

A	Number of stalk	Diameter of stalk	Stalk height	Number of flower	Corolla diameter	Corolla tube length	Corolla tube width	RW1	RW2	RW3	RW4
Number of stalk		0.990****	0.990****	0.980****	-0.049	-0.399***	0.980****	-0.010	0.004	-0.005	0.002
Diameter of stalk	0.109*		0.999****	0.980****	0.698****	-0.138	0.599****	0.001	-0.005	-0.002	0.000
Stalk height	0.145**	0.255****		0.551****	0.323***	0.351***	-0.034	0.067	-0.067	-0.003	-0.033
Number of flower	0.703****	0.311****	0.458****		0.143	0.144	-0.109	-0.528****	0.194*	-0.515****	-0.034
Corolla diameter	-0.004	0.078	0.122*	0.115*		0.579****	-0.092	-0.001	0.000	-0.005	0.000
Corolla tube length	-0.094	0.037	-0.021	0.023	0.542****		0.023	0.000	0.003	-0.002	0.002
Corolla tube width	-0.095	-0.029	-0.060	-0.016	0.237****	0.486****		0.002	-0.000	-0.001	0.001
RW1	-0.110*	0.017	0.068	-0.082	-0.008	0.005	0.052		-0.000	-0.000	0.000
RW2	0.078	-0.094	-0.109*	0.048	0.010	0.067	-0.018	-0.012		-0.000	-0.000
RW3	-0.126*	-0.047	-0.007	-0.180***	-0.123*	-0.048	-0.053	-0.099	-0.002		0.001
RW4	0.045	0.003	-0.089	-0.014	0.015	0.057	0.072	0.092	-0.047	-0.014	

B	Number of stalk	Diameter of stalk	Stalk height	Number of flower	Corolla diameter	Corolla tube length	Corolla tube width	RW1	RW2	RW3	RW4
Number of stalk		0.061	0.005	0.264	0.396	0.477	0.011	0.602	1.294	4.025	0.376
Diameter of stalk	5.401		0.004	1.938	0.838	2.849	1.608	4.374	9.597	29.491	2.772
Stalk height	0.439	2.813		3.687	0.159	0.138	0.351	0.346	0.864	2.502	0.243
Number of flower	3.916	66.755	4.451		5.172	4.661	9.673	14.552	16.739	96.978	6.235
Corolla diameter	0.379	2.558	0.206	5.341		0.066	0.273	0.273	0.595	1.842	0.173
Corolla tube length	0.373	2.411	0.217	5.320	0.071		0.220	0.246	0.535	1.657	0.155
Corolla tube width	0.598	4.127	0.360	8.862	0.191	0.116		0.393	0.860	2.652	0.249
RW1	0.661	4.304	0.346	10.304	0.275	0.245	0.374		0.939	2.893	0.272
RW2	1.198	10.447	0.898	19.771	0.589	0.501	0.875	0.950		6.309	0.594
RW3	4.510	30.815	2.512	75.534	2.059	1.733	2.790	3.179	6.322		1.830
RW4	0.360	2.764	0.256	6.114	0.170	0.147	0.232	0.247	0.622	1.857	

Table 7.4. A) Genetic correlations among *E. mediohispanicum* phenotypic traits (N= 332 families) calculated by the standard r_A (below diagonal) and alternative r_A* method (above diagonal). Functionally related traits appear in gray. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. B) Standard errors of the genetic correlations.

Thus, different shape components correlated significantly with different traits in each population (Appendix 7.1). RW1 correlated positively with some plant-size traits in Em01, but negatively in the other two populations, whereas RW3 correlated with plant-size traits only in Em08, and the other two shape components did not correlate with any trait (Appendix 7.1).

DISCUSSION

HERITABILITY OF SIZE AND SHAPE

We found that traits associated with pollinator attraction and plant fitness (Gómez *et al*., 2006; 2008a; 2009), such as stalk height, corolla diameter, corolla tube length and corolla tube width, showed high levels of heritability. By contrast, traits not associated with fitness, like number of flowering stalks and diameter of the flowering stalks, showed low heritability. This outcome partly agrees with a recent review on heritability comprising more than 60 systems (Ashman and Majestic, 2006). However, contrasting with the above-mentioned review, we found no heritability for number of flowers per plant. As stated above, *E. mediohispanicum* is self-compatible (Gómez, 2005), and according to Ashman and Majectic´s review, heritability tends to be lower in self-compatible than in self-incompatible species. In any case, our finding suggests that variation in *E. mediohispanicum* flower number is partially caused by environmental factors. Many studies have indeed found that additive genetic variation is more common for floral traits than for flower number (Campbell, 1997; Elle, 1998; Worley and Barrett, 2000). Remarkably, genetic variance for flower number was much higher than the phenotypic variance of the parental generation, suggesting that flower number is very similar among different genotypes in field conditions as a consequence of some environmental factors constraining its variation.

We also found significant heritability for corolla shape, both for the overall trait and for three of the four main shape components. To our knowledge, this is the first study that has demonstrated heritability corolla shape considering it as

a complex and single multidimensional trait rather than decomposing it in its linear components.. Indeed, as far as we know, Galen and Cuba (2001)'s study on *Polemonium viscosum* is the only study exploring corolla shape heritability to date. However, these authors estimated corolla shape by two linear surrogates, corolla flare and length. Our findings suggest that not only size-related floral traits but also shape-related traits have enough genetic variation to respond to selection exerted by pollinators.

Our results suggest that *E. mediohispanicum* traits under strong pollinator-mediated selection had high heritability (Gómez *et al*., 2008a; 2009). In fact, the only highly selected trait showing low heritability was number of flowers. This is counterintuitive, since a wide amount of information suggests that those traits more tightly related with fitness have low heritability (Merilä and Sheldon, 2000). In our case, several non-exclusive factors can promote enough genetic variation even in pollinator-selected traits. First, herbivore-pollinator conflicting selection occurs on many *E. mediohispanicum* traits, weakening the strength of the net selection exerted by the pollinators (Gómez, 2003; 2005a; 2008). Another possible explanation for this phenomenon is that the selection acting on these traits is relatively recent and it has not yet exhausted all deleterious variation. Finally, the high values of heritability could be related to our experimental design. We estimated the phenotypic traits of the progeny in a common garden rather than in its natural environment where the traits of the parents were studied. This approach may overestimate heritability, since the environmental-variance component is much higher in the field than in the greenhouse (Schoen *et al*., 1994; Conner *et al*., 2003). We tried to circumvent this pitfall by estimating heritability following the Riska method (Riska *et al*., 1989; Kleunen and Ritland, 2004). In fact, heritability estimates were smaller when using this method than when using the standard method (Table 7.2), suggesting that heritability can actually be overestimated when quantified in the greenhouse. Nevertheless, Young *et al.* (1994) found for *Raphanus sativus* (Brassicaceae) that heritability on several floral traits is similar both in the greenhouse and the field. Another important caveat

of our experimental design is related to the fact that it does not consider maternal effect explicitly. Maternal effects may overestimate heritability estimates since it inflates the parent-offspring regression coefficients. Since we did not control the sire in our experiment, it is not possible to compare maternal covariance to paternal covariance in order to estimate the maternal effects (Roff, 1998). Nevertheless, we tried to minimize the maternal effects by randomly distributing the seeds in the greenhouse in order to lower the within-family environmental correlation. In addition, most studies have shown that maternal effects have a stronger influence on juvenile traits than on adult traits (Shaw and Byers, 1998). Since all phenotypic traits considered in this study are displayed during flowering, we presume that maternal effects are not very important. Finally, including in the same analysis families from eight populations could also affect our heritability estimates. Nevertheless, we believe that our estimates are good proxy of real heritability estimates, since we include in the analysis population as a random effect, and furthermore we repeated the analysis in three populations independently and the outcomes were similar.

GENETIC CORRELATIONS AMONG TRAITS

There was a strong genetic correlation between functionally related traits (except between corolla-shape components, since a non-zero correlation between them is possible only when phenotypic covariance matrices do not conform to the model of common principal components), both for all plant populations analyzed together and for the three populations studied separately. This outcome suggests the occurrence of phenotypic integration for plant size and for flower size in *E. mediohispanicum*.

Number of flowers was significantly and positively correlated with plant-size traits, also whether analyzing all populations together or separately. This relationship, reported for a wide number of species, is shown as a typical example of environmental covariation among traits. First, being modular

organisms, bigger plants produce more modules, which means more flowers. Second, plants located in high-quality microsites have more resources to produce both more vegetative and reproductive tissue, resulting in a spurious correlation between them. Our outcomes suggest, nonetheless, that the relationship between plant size and flower number may be also genetic, at least in *E. mediohispanicum*.

There was also positive genetic correlation between flower-size traits and plant-size traits. In fact, five out of the nine potential correlations between these groups of traits were positive and significant. Nevertheless, most of these correlations vanished for the populations studied separately, suggesting that floral-size traits are not actually coupled with vegetative traits in *E. mediohispanicum*. This finding agrees with most studies, which have shown that genetic and phenotypic correlations between floral and vegetative character suites are low and mostly statistically non-significant (Conner and Via, 1993; Conner and Sterling, 1996; Waitt and Levin, 1998; Armbruster *et al*., 1999; Worley and Barrett, 2000; Juenger *et al*., 2005; Ashman and Majetic, 2006).

Theoretical models predict a trade-off between flower number and size (Sakai, 1995; Schoen and Ashman, 1995; Harder and Barrett, 1996; de Jong and Klinkhamer, 2005; Sargent *et al*., 2007). However, many empirical studies have failed to find such a negative genetic correlation between these plant traits (Mazer, 1989; Meagher, 1992; Andersson, 1996; Elle, 1998; Worley and Barrett, 2000; 2001; Ashman and Majestic, 2006; Caruso, 2006; Lehtilä and Holmén Bränn, 2007; but see Caruso, 2004). Worley and Barrett (2000; 2001) even showed for *Eichhornia paniculata* that genetic correlations for flower-size number can range from negative to positive in different localities. In our case, we have found no correlation, whether positive or negative, between flower number and flower size, both for all populations analyzed together and for the three populations studied separately (except between flower number and corolla tube length in Em08, where correlation was significantly negative). Worley and Barrett (2000) suggest that a potential cause of the absence of a genetic correlation between plant size, flower number and flower size may be

the genetic variation in module size and resource status. That is, the genetic correlation between module size and flower size and between module size and flower number disrupted any potential for flower size and number genetic trade-off. We do not have enough information to test this hypothesis, although we believe that it could also apply to *E. mediohispanicum*, since we found a high positive correlation between plant size and flower number, and between plant size and flower size. Nevertheless, we also think that our analyses had low power to detect negative correlations between flower number and flower size (power <0.5 in all analyses). In fact, although not significant, all estimates of correlations between these traits were negative in Em01, Em08 and Em22.

Remarkably, corolla shape was only slightly correlated with flower number, flower size, and plant-size traits. This absence of strong correlation suggests that corolla shape is genetically decoupled from other floral traits and from vegetative traits in *E. mediohispanicum*. The decoupling found in this study suggests that this complex trait can respond to pollinator-mediated selection without any constraint by indirect selection through other plant traits. Specialized, zygomorphic flowers tend to have higher phenotypic integration and more decoupling between different trait suites than generalist and actinomorphic flowers (Ashman and Majetic, 2006). Our findings show that *E. mediohispanicum* corolla shape but not size is decoupled from other traits, behaving more as zygomorphic than as actinomorphic species. Actually, zygomorphy and specialized flower shapes are currently selected by pollinators in several *E. mediohispanicum* populations (Gómez, 2008; Gómez *et al*., 2006).

CONCLUDING REMARKS

This study has shown that most *E. mediohispanicum* floral traits, even complex traits such as corolla shape, can have significant heritability. Consequently, this species retains a high ability to respond to the selection exerted by its pollinators. Furthermore, this study has also shown genetic correlation between flower number, flower size, and plant size. Under these circumstances, selection

affecting a given *E. mediohispanicum* trait would also indirectly affect other traits, functionally related and unrelated traits. This finding agrees with the frequent indirect selection detected in this plant (Gómez, 2003; 2008; Gómez *et al*., 2006; 2008a; 2009). More importantly, genetic correlation, if beneficial, can be maintained and even promoted by correlational selection (Sinervo and Svensson, 2002; McGlothlin *et al*., 2005). In fact, we have previously reported positive correlational selection acting on the covariance between flower number and plant size (Gómez, 2003) and between flower number and flower size (Gómez *et al*., 2006) of *E. mediohispanicum*. This suggests that the genetic correlation detected between *E. mediohispanicum* traits could be at least partially adaptive.

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Appendix 7.1. Heritability and genetic correlations (calculated by the standard rA below diagonal and alternative rA* method above diagonal) for the three populations with enough number of families studied (>50 families). Functionally related traits are shown in grey. In bold, those values significant at $\alpha<0.05$.

Traits	Heritability		Genetic correlations										
A	h^2_{op} ±SE	h^2_{riska} ±SE_b	Number of stalk	Stalk diameter	Stalk height	N flowers	Corolla diameter	Corolla tube length	Corolla tube width	RW1	RW2	RW3	RW4
Em01 (65 families)			Values										
Number of stalks	-0.033±0.109	0.006±0.097		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Stalk diameter	0.092±0.241	0.009±0.030	0.088		0.816	0.003	0.952	0.456	3.168	-0.412	-2.233	1.334	0.894
Stalk height	0.344±0.248	0.119±0.251	-0.015	**0.480**		-0.018	0.424	0.110	1.697	-0.758	-0.491	2.325	-0.184
Number of flowers	**0.522±0.247**	**0.265±0.221**	**0.335**	**0.737**	**0.456**		0.308	-0.171	1.717	-0.081	-1.483	2.988	0.383
Corolla diameter	**0.405±0.149**	**0.422±0.393**	0.007	**0.300**	-0.048	0.098		0.409	-0.050	-1.006	-0.091	1.797	0.192
Corolla tube length	**0.484±0.173**	**0.444±0.335**	0.170	**0.263**	-0.222	-0.010	**0.528**		0.256	-1.266	0.628	-0.223	0.623
Corolla tube width	0.089±0.128	0.031±0.197	-0.032	**0.326**	0.071	0.124	**0.494**	0.240		1.301	0.451	4.778	-0.976
Corolla shape													
RW1	0.092±0.175	0.017±0.063	0.056	**0.356**	**0.249**	**0.301**	0.082	0.070	0.186		-0.921	-3.385	0.960
RW2	0.168±0.122	0.117±0.187	-0.225	0.151	0.165	-0.118	-0.086	0.007	0.167	0.071		-3.062	0.983
RW3	0.003±0.125	0.000±0.073	0.002	-0.040	0.162	-0.031	0.132	-0.188	0.214	0.051	-0.016		-1.721
RW4	0.249±0.183	0.114±0.203	0.110	-0.205	**-0.395**	-0.218	0.174	0.201	-0.040	-0.045	-0.113	-0.109	
			Standard Errors										
Number of stalks				0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Stalk diameter			0.000		0.178	0.784	0.033	0.371	2.965	2.227	3.148	2.406	0.104
Stalk height			0.000	0.505		0.420	0.209	0.319	0.501	1.456	0.762	5.013	0.610
Number of flowers			0.000	0.207	0.225		0.204	0.340	0.417	0.725	1.028	6.091	0.257
Corolla diameter			0.000	0.485	0.381	0.266		0.151	0.538	1.185	0.398	2.152	0.297
Corolla tube length			0.000	0.503	0.438	0.293	0.121		0.376	1.320	0.134	3.254	0.136
Corolla tube width			0.000	0.922	0.667	0.510	0.259	0.384		0.352	0.395	20.145	1.433
RW1			0.000	1.016	0.622	0.469	0.542	0.542	0.950		1.597	26.975	0.033
RW2			0.000	0.826	0.427	0.463	0.396	0.357	0.600	0.772		15.423	0.009
RW3			0.000	7.495	3.171	3.160	2.343	3.160	4.190	5.839	3.857		10.401
RW4			0.000	1.181	0.718	0.508	0.304	0.289	0.755	0.875	0.575	4.239	

Traits	Heritability		Genetic correlations									
B	h^2_{op} ±SE	h^2_{riska} ±SE$_b$	Number of stalk	Stalk diameter	Stalk height	N flowers	Corolla diameter	Corolla tube length	Corolla tube width	RW1	RW2	RW3	RW4
Em08 (65 families)			Values										
Number of stalks	1.173±0.848	0.118±0.135		0.784	1.041	1.098	2.042	0.136	0.000	-0.789	0.000	-1.417	-1.351
Stalk diameter	0.313±0.194	0.158±0.230	**0.547**		1.513	0.947	0.134	-0.559	0.000	0.657	0.000	0.060	0.141
Stalk height	0.093±0.130	0.032±0.102	**0.265**	**0.526**		1.264	0.988	-0.644	0.000	0.421	0.000	0.918	-0.218
Number of flowers	0.283±0.573	0.015±0.147	**0.866**	**0.762**	**0.404**		1.760	-0.716	0.000	0.207	0.000	-1.901	-1.362
Corolla diameter	0.066±0.157	0.011±0.232	-0.146	0.046	-0.023	-0.100		-0.516	0.000	-0.651	0.000	0.283	-0.742
Corolla tube length	0.259±0.173	0.138±0.169	**-0.313**	-0.129	-0.081	**-0.297**	**0.564**		0.000	-0.071	0.000	0.216	0.786
Corolla tube width	-0.023±0.084	0.005±0.217	-0.137	0.099	-0.208	-0.066	**0.636**	0.240		0.000	-0.195	0.000	0.000
Corolla shape													
RW1	**0.180±0.126**	0.114±0.167	**-0.455**	-0.177	-0.187	**-0.406**	0.039	-0.029	0.238		0.000	-0.073	-0.094
RW2	0.006±0.120	0.004±0.148	0.172	-0.171	-0.233	0.073	-0.080	0.035	-0.229	0.054		0.000	0.000
RW3	0.139±0.101	0.067±0.197	**0.284**	0.235	0.193	**0.252**	-0.069	-0.070	**-0.245**	-0.223	0.051		0.134
RW4	**0.371±0.116**	**0.567±0.387**	-0.111	-0.051	0.033	-0.157	-0.216	0.130	-0.194	0.207	0.101	0.038	
			Standard Errors										
Number of stalks				0.102	0.056	0.162	1.875	0.822	0.000	1.787	0.000	2.769	1.528
Stalk diameter			0.215		0.175	0.022	0.385	0.366	0.000	0.085	0.000	0.266	0.138
Stalk height			1.016	0.162		0.315	0.016	1.127	0.000	0.416	0.000	0.067	0.571
Number of flowers			0.223	0.097	0.712		1.182	1.411	0.000	0.685	0.000	2.871	1.327
Corolla diameter			2.062	0.424	1.326	1.710		1.353	0.000	1.547	0.000	0.771	1.063
Corolla tube length			1.249	0.265	0.741	1.066	0.389		0.000	0.530	0.000	0.445	0.069
Corolla tube width			0.000	0.000	0.000	0.000	0.000	0.000		0.000	3.182	0.000	0.000
RW1			1.454	0.290	0.854	1.214	0.901	0.510	0.000		0.000	0.640	0.370
RW2			0.000	0.000	0.000	0.000	0.000	0.000	3.273	0.000		0.000	0.000
RW3			0.820	0.216	0.666	0.741	1.149	0.608	0.000	0.730	0.000		0.336
RW4			0.722	0.169	0.453	0.650	0.742	0.280	0.000	0.268	0.000	0.374	

Traits	Heritability		Genetic correlations									
C	h^2_{op} ±SE	h^2_{riska} ±SE_b	Number of stalk	Stalk diameter	Stalk height	N flowers	Corolla diameter	Corolla tube length	Corolla tube width	RW1	RW2	RW3	RW4
Em22 (57 families)			Values										
Number of stalks	0.170±0.208	0.048±0.315		0.000	0.000	0.000	0.268	0.590	-0.115	-0.248	0.000	1.211	0.375
Stalk diameter	-0.004±0.211	0.000±0.120	**0.502**		-15.248	-7.132	0.000	0.000	0.000	0.000	-0.193	0.000	0.000
Stalk height	-0.019±0.183	0.001±0.136	0.209	**0.413**		-5.007	0.000	0.000	0.000	0.000	0.657	0.000	0.000
Number of flowers	-0.219±0.380	0.024±0.101	**0.759**	**0.815**	**0.327**		0.000	0.000	0.000	0.000	0.257	0.000	0.000
Corolla diameter	**0.364±0.146**	**0.405±0.324**	0.022	0.024	**0.270**	-0.150		0.796	0.367	-0.041	0.000	0.450	-0.140
Corolla tube length	0.219±0.151	0.148±0.207	-0.159	-0.079	0.027	-0.197	**0.485**		0.304	0.096	0.000	-0.232	-0.401
Corolla tube width	**0.175±0.091**	**0.253±0.257**	-0.194	0.029	0.095	-0.232	**0.327**	0.164		0.138	0.000	1.574	-0.384
Corolla shape													
RW1	**0.209±0.161**	0.120±0.220	-0.238	-0.098	**-0.277**	-0.154	-0.059	**0.421**	-0.035		0.000	-0.758	-0.320
RW2	-0.166±0.118	0.140±0.168	0.012	-0.080	-0.189	-0.165	**0.319**	0.000	0.007	-0.015		0.000	0.000
RW3	0.060±0.121	0.018±0.076	-0.185	-0.180	-0.222	-0.207	-0.055	0.086	0.015	0.159	-0.139		0.647
RW4	**0.431±0.128**	**0.680±0.351**	0.155	-0.023	0.087	0.187	0.023	-0.184	-0.166	-0.046	-0.201	-0.070	
			Standard Errors										
Number of stalks				0.000	0.000	0.000	0.365	0.268	0.632	0.861	0.000	0.237	0.268
Stalk diameter			0.000		258.073	54.742	0.000	0.000	0.000	0.000	5.122	0.000	0.000
Stalk height			0.000	9.319		17.425	0.000	0.000	0.000	0.000	0.634	0.000	0.000
Number of flowers			0.000	1.246	1.952		0.000	0.000	0.000	0.000	0.583	0.000	0.000
Corolla diameter			0.488	0.000	0.000	0.000		0.076	0.204	0.409	0.000	0.352	0.278
Corolla tube length			0.757	0.000	0.000	0.000	0.192		0.294	0.465	0.000	1.031	0.448
Corolla tube width			0.677	0.000	0.000	0.000	0.218	0.353		0.385	0.000	0.417	0.384
RW1			0.854	0.000	0.000	0.000	0.416	0.298	0.462		0.000	1.555	0.446
RW2			0.000	4.640	2.201	0.914	0.000	0.000	0.000	0.000		0.000	0.000
RW3			1.331	0.000	0.000	0.000	0.675	0.765	0.716	0.744	0.000		0.194
RW4			0.362	0.000	0.000	0.000	0.238	0.378	0.324	0.353	0.000	0.588	

Appendix 7.2. Outcome of the geometric morphometric analysis describing corolla shape in the parental (720 plants) and offspring generations (1635 plants). SV= Singular values of each RW; Var = Percentage of variance in overall flower shape explained by each individual RW; CumVar = Cumulative variance in overall flower shape explained by the RWs.

RW	Parental			Offspring		
	SV	Var.	CumVar	SV	%	%Cum
1	3.30124	35.17	35.17	4.82391	39.98	39.98
2	2.44050	19.22	54.38	3.25134	18.16	58.14
3	1.73122	9.67	64.06	2.55805	11.24	69.39
4	1.38967	6.23	70.29	1.93682	6.45	75.83
5	1.14129	4.20	74.49	1.31434	2.97	78.80
6	0.96975	3.03	77.52	1.14371	2.25	81.05
7	0.86632	2.42	79.95	1.04370	1.87	82.92
8	0.77857	1.96	81.90	1.01955	1.79	84.70
9	0.76281	1.88	83.78	0.94349	1.53	86.23
10	0.71661	1.66	85.44	0.94019	1.52	87.75
11	0.65065	1.37	86.80	0.85222	1.25	89.00
12	0.61038	1.20	88.01	0.72646	0.91	89.91
13	0.56999	1.05	89.05	0.64219	0.71	90.61
14	0.51252	0.85	89.90	0.62948	0.68	91.30
15	0.48462	0.76	90.66	0.61826	0.66	91.95
16	0.46293	0.69	91.35	0.58996	0.60	92.55
17	0.43663	0.62	91.97	0.56001	0.54	93.09
18	0.42373	0.58	92.55	0.51141	0.45	93.54
19	0.40920	0.54	93.09	0.46827	0.38	93.92
20	0.37736	0.46	93.55	0.44860	0.35	94.26
21	0.36143	0.42	93.97	0.43901	0.33	94.59
22	0.35874	0.42	94.38	0.42927	0.32	94.91
23	0.33290	0.36	94.74	0.41259	0.29	95.20
24	0.32799	0.35	95.09	0.40035	0.28	95.48
25	0.31871	0.33	95.41	0.39693	0.27	95.75
26	0.31292	0.32	95.73	0.38255	0.25	96.00
27	0.29725	0.29	96.02	0.37315	0.24	96.24
28	0.29068	0.27	96.29	0.37014	0.24	96.47
29	0.28358	0.26	96.55	0.35863	0.22	96.69
30	0.27550	0.24	96.79	0.34607	0.21	96.90
31	0.25931	0.22	97.01	0.33825	0.20	97.10
32	0.25125	0.20	97.21	0.33371	0.19	97.29
33	0.23390	0.18	97.39	0.32834	0.19	97.47
34	0.23143	0.17	97.56	0.31700	0.17	97.65
35	0.22482	0.16	97.73	0.31169	0.17	97.81
36	0.21961	0.16	97.88	0.30052	0.16	97.97
37	0.21594	0.15	98.03	0.29490	0.15	98.12
38	0.20786	0.14	98.17	0.28920	0.14	98.26
39	0.20498	0.14	98.31	0.27977	0.13	98.40
40	0.19987	0.13	98.44	0.27726	0.13	98.53
41	0.19747	0.13	98.56	0.26505	0.12	98.65
42	0.19319	0.12	98.68	0.25872	0.11	98.76
43	0.19082	0.12	98.80	0.25077	0.11	98.87
44	0.18818	0.11	98.91	0.24166	0.10	98.97
45	0.17949	0.10	99.02	0.23730	0.10	99.07
46	0.17362	0.10	99.11	0.22966	0.09	99.16
47	0.16471	0.09	99.20	0.22149	0.08	99.24
48	0.16107	0.08	99.29	0.22107	0.08	99.33
49	0.15495	0.08	99.36	0.21567	0.08	99.41
50	0.15246	0.08	99.44	0.20812	0.07	99.48
51	0.15172	0.07	99.51	0.20452	0.07	99.55
52	0.14541	0.07	99.58	0.20302	0.07	99.62
53	0.14414	0.07	99.65	0.19449	0.06	99.69
54	0.14227	0.07	99.71	0.19112	0.06	99.75
55	0.13670	0.06	99.77	0.18089	0.06	99.81
56	0.13587	0.06	99.83	0.17445	0.05	99.86
57	0.12314	0.05	99.88	0.15351	0.04	99.90
58	0.11733	0.04	99.93	0.14515	0.04	99.94
59	0.11015	0.04	99.97	0.14041	0.03	99.97
60	0.10300	0.03	100.00	0.13022	0.03	100.00

CHAPTER 8

DIVERGENT SELECTION MEDIATED BY POLLINATORS CONSTRAINS HYBRID ZONE IN A SECONDARY CONTACT WITH GENE FLOW

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ABSTRACT

When two recently diverged species contact, they could hybridize and interchange genes in their overlapping distribution areas. The dynamics of such contact areas will depend of several genetic and ecological factors, including local selective pressures producing local adaptation. However, the strength and direction of such selective pressures have been scarcely explored in secondary hybrid zones. In plants, pollinators have been characterized as an important factor promoting hybridization (or divergence) between co-occurring species and also act as an important selective factor. In the present work we explore the morphological and ecological distances in a secondary contact between *Erysimum mediohispanicum* and *E. nevadense* in Sierra Nevada (SE Spain). Besides, we analyze the selection occurring in this secondary contact, particularly the divergent selection acting on phenotypic characters among the hybrid and the parental populations. Corolla shape was the main phenotypic trait presenting divergent selective gradients between *E. mediohispanicum* and *E. nevadense*. However, both species presented divergent selection on number of flowers when we compare them to the hybrid population. The number of flowers (a heterotic character in hybrids) was strongly selected at the hybrid population. This last trait, together with a rich and diverse pollinator assemblage, could be favoring the formation of hybrids in the contact zone. However, the divergent selection between hybrids and the parental species appears as a constraining force limiting the hybrids to a narrow strip in the contact zone and preventing inter-specific gene flow between *E. mediohispanicum* and *E. nevadense*.

Key words

E. mediohispanicum, *E. nevadense*, evolutionary divergence, introgression, hybrids, local adaptation, natural selection.

INTRODUCTION

In an allopatric situation, gene pools suffer a progressive differentiation mediated by genetic drift and, local selective pressures. Divergent selection may drive the population differentiation despite the ocurrence of some levels of gene flow (Doebeli and Dieckmann, 2003; Rosenblum, 2006). However, gene flow may overwhelm the local selective pressures, preventing local adaptation by homogenizing the diverging gene pools (Slatkin, 1987; Lenormand, 2002). Depending on the evolutionary divergence driven by natural selection and gene flow, the diverging populations would exhibit variable levels of reproductive isolation.

Secondary contacts arise when taxa with variable differentiation rates through allopatric speciation come together (Endler, 1977; 1982; Otte and Endler, 1989; Coyne and Orr, 2004). When species with partial reproductive isolation contact, they would produce a bimodal hybrid zone with most individuals belonging to each of both parental species (Jiggins and Mallet, 2000). If the secondary contact happens before complete reproductive isolation, inter-specific gene flow it is frequent (Anderson, 1949). The consequences of these inter-fertility range between the extinction of one species, due to the complete assimilation of the genome of the other (Rhymer and Simberloff, 1996; Levin *et al.*, 1996; Allendorf *et al.*, 2001) to the production of a unimodal hybrid zone, in which hybrid individuals coexist with pure individuals (Barton and Hewitt, 1985; Harrison, 1993; Arnold, 2006). In the latter case, other biological mechanisms are required to maintain the pre-existing species. In this sense, divergent local selective pressures driving the local adaptation and, ultimately, the evolutionary divergence between the contacting species would play an important role counterbalancing the homogenizing effect of gene flow (Coyne, 1992; Dieckmann and Doebeli, 1999).

While ecologically based divergent selection have recently emphasized as an important factor for local adaptation (Kawecki and Ebert, 2004; Hall and Willis, 2007) and the evolution of reproductive isolation (Schluter,

2001; Rundle and Nosil, 2005), much less is known about its effect on the maintenance of the boundaries between closely related species hybridizing upon a secondary contact. The exploration of the divergent selection occurring in secondary contacts will help to discern the mechanisms maintaining the evolutionary independence of the species. In this sense, it has been pointed out the importance of temporal replicates for the study of strength selection in natural populations (Schemske and Horvitz, 1989; Kingsolver *et al.*, 2001).

In flowering plants, pollinators are main selective pressures (Faegri and van der Pijl, 1979; Wilson and Thomson, 1996; Totland, 2001; Alexanderson and Johnson, 2002; Gómez, 2003; Gómez *et al.*, 2006; 2009). They play an important role for plant local adaptation and differentiation by their effect on floral phenotype (Wilson and Thompson, 1996; Aigner, 2005; 2006; Gómez *et al.*, 2009c). Pollinator-mediated selection may contribute to maintain reproductive isolation between two diverging plant species when occurring in sympatry (Grant, 1981), because their fidelity to one plant species (Ramsey *et al.*, 2003; Martin *et al.*, 2008; Ellis and Johnson, 2012; Marques *et al.*, 2007; 2012). Pollinator-mediated reprodctive isolation has been mostly explored in specialized systems systems (Ramsey *et al.*, 2003; Martin *et al.*, 2008; Kay and Sargent, 2009; Schiestl and Schlüter, 2009; Natalis and Wesselingh, 2012). In contrast, despite of been very frequent in nature, the role of pollinators as reproductive barriers in generalist systems has been seldom studied.

In this work we explore the strength and direction of phenotypic selection in a secondary contact zone of two related species, in order to detect the occurrence of divergent selection. We have used two species from the genus *Erysimum* L. (Brassicaceae), *E. mediohispanicum* Polatschek and *E. nevadense* Reut. Both species were previously reported to successful interbreed producing hybrids with low inviability in greenhouse conditions (***Chapter 3***). We have found a hybrid zone in a secondary contact in Sierra Nevada (SE Spain) between these two species (***Chapter 5***), which was included in the present study. The main goals of the present study are: 1) to study the phenotypic and ecological differences between both species; 2) to analyze the selection taking

place both in the parental populations conforming the secondary contact and in the hybrid population; 3) to identify and quantify the occurrence of divergent selection in this system.

MATERIAL AND METHODS

STUDY SYSTEM

The genus *Erysimum* is as complex genus, with recurrent inter-specific hybridization, polyploidization and reticulate evolution (Clot, 1992; Ancev, 2006; Marhold and Lihová, 2006), frequently resulting in species complexes and cryptic species (Ancev, 2006; Turner, 2006; ***Chapter 1***).

Erysimum mediohispanicum is a mostly monocarpic hermaphroditic herb endemic to the Iberian Peninsula distributed in two extended and isolated regions in the north-west and south-west of the peninsula, respectively, where it inhabits between 600 to 2300 m. altitude. As a facultative biennial, the plants spend 2 to 3 years growing like a vegetative rosette on calcareous soils. After that period they display from few to several hundred of flowers located on 1-3 stalks (Gómez, 2003). Their flowers are visited by a highly diverse assemblage of insects(Gómez *et al*., 2007), which may exert significant selection on many floral traits (Gómez *et al*., 2008a; 2008b). These pollinators show the ability to discriminate between different combinations of heritable phenotypic traits(Gómez *et al*., 2009a; 2009b; 2009c).

Erysimum nevadense is a mostly polycarpic hermaphroditic herb endemic to the top of Sierra Nevada Mountains. They grow like a rosette for 2 to 3 years, displaying from few to hundreds of flowers on several floral stalks (personal observation). This species presents populations on siliceous soils up to 2300 m in north face of the range, and from 2130 in the south face. It also displays a generalist pollination system, but it does not present a pollinator assemblage as diverse as *E. mediohispanicum*, probably due to the harsh conditions of its habitat (Ortigosa and Gómez, 2010; ***Chapter 3*** and ***6***)

These two species are interfertile, presenting relatively lower values of hybrid inviability in greenhouse conditions (***Chapter 3***). In addition, they contact in the Sierra Nevada along a narrow area at an altitude of approximately 2,200 m, where an hybrid zone is established Populations from each species may be located a mere 100 m apart from the hybrid zone. Previous analysis of gene flow reported the highest levels of gene flow occurring from the parental areas to the hybrid population (***Chapter 5***).

To study divergent selection, we marked permanently 90 plants in the hybrid zone (Table 8.1). In addition, we marked 90 plants in the adjacent populations of both *E. mediohispanicum* and *E. nevadense* (Table 8.1). Finally, we marked another 90 plants in a *E. mediohispanicum* and *E. nevadense* populations located about 1 km away from the hybrid zone. We performed this study during two years (2007 and 2008), comprising a total of 5 populations and 450 plants per year.

PLANT PHENOTYPIC TRAITS

The following phenotypic traits were quantified for each experimental plant: (1) stalk height: the height of the tallest stalk from the ground to the top of the stalk at the end of flowering period; (2) number of flower: the total number of flower produced by each plant; (3) corolla diameter: the distance between the edge of two opposite petals (± 0.1 mm error); (4) corolla tube width: the diameter of the corolla tube aperture as the distance between the bases of two opposite petals; (5) corolla tube length: the distance between the corolla tube aperture and the base of the sepals; (6) corolla shape: determined by means of geometric morphometric tools, using a landmark-based methodology that eliminates the effect of variations in the location, orientation and scale of the specimens (Zelditch *et al.*, 2004). We took a digital photograph of one flower per plant using a standardized procedure (front view and planar position). Flowers were photographed at anthesis to avoid ontogenetic effects. We defined 32 coplanar landmarks located along the outline of the flowers and

Population characteristics				Sampling effort							
				2007				2008			
Population	Latitude	Longitude	Altitude	Plants	Minutes	Flowers	Pollinators	Plants	Minutes	Flowers	Pollinators
Em25	37º 7.230' N	3º 26.082' W	2064	90	120	9805	142	90	150	15222	211
Em17	37º 6.698' N	3º 25.450' W	2182	90	180	10456	204	90	150	11297	206
H01	37º 6.908' N	3º 25.250' W	2200	90	165	19516	335	90	90	16761	126
En11	37º 6.750' N	3º 25.048' W	2222	90	250	11266	94	90	240	9313	91
En10	37º 6.658' N	3º 24.301' W	2322	90	345	7470	131	90	330	4848	134

Table 8.1. Location and sampling effort for the populations of *Erysimum* species included in the present study in Sierra Nevada (SE Spain).

the aperture of the corolla tube, the number of landmarks being chosen to provide comprehensive coverage of the flower shape (see Gómez *et al.* 2006 and ***Chapter 1*** for a detailed description of the landmark locations and software used). The two-dimensional coordinates of these landmarks were determined for each plant, and the generalized orthogonal least-squares Procrustes average configuration of landmarks was computed using the Generalized Procrustes Analysis (GPA) superimposition method (Zelditch *et al.*, 2004). After GPA, the relative warps (RWs, principal components of the covariance matrix of the partial warp scores) were computed (Zelditch *et al.*, 2004). This procedure generates 2p-4 orthogonal RWs (p= number of landmarks). Each RW explains a given variation in shape between specimens. Thus, RWs summarize shape differences between specimens, and their scores can be used as a data matrix to perform standard statistical analyses (Zelditch *et al.*, 2004). In all subsequent analyses we used the first four RWs, because each explained more than 5% of variation in shape.

PLANT FITNESS

Lifetime female fitness was estimated for each labeled plant as the number of seeds produced per plant at the end of the reproductive season. For this, we counted the number of mature fruits for each plant, and collecting five of these fruits in order to count the number of seed produced per fuit. The total number of seeds produced by plant was obtained by multiplying the number of fruits per plant by number of seeds determined per fruit (Gómez *et al.*, 2006).

POLLINATOR ASSEMBLAGE

For each of the studied population we conducted pollinator censuses under sunny conditions and no wind. The number of censuses per population depended to the local abundance of insects, fitting them to an adequate number of interactions to characterize the pollinator assemblage (Gómez *et al.*, 2007).

We identified as many pollinators possible in the field, but some specimens were captured to be identified in the laboratory or sent to specialists. The observed pollinators interacting with our labeled plants were grouped into functional groups (Fenster *et al.*, 2004; Wilson *et al*, 2004) depending on the manner they interact with the plant following the description done by Gómez *et al.* (2009a). We identify nine functional groups: (1) ants, including species collecting nectar; (2) beeflies, long-tongued nectar-collecting Bombyliidae; (3) beetles, including species collecting nectar and/or pollen; (4) butterflies, all nectar collectors; (5) hoverflies, nectar- and pollen-collecting Syrphidae and short-tongued Bombyliidae; (6) large bees, mostly pollen- and nectar-collecting, females bigger than 10 mm in body length; (7) small bees, mostly pollen- and nectar collecting, females smaller than 10 mm in body length; (8) wasps, aculeate wasps, large parasitic wasps, and cleptoparasitic bees collecting only nectar; (9) others, including various species of small flies, small parasitic wasps, bugs, and grasshoppers.

For each population and year we estimate following characteristic defining pollinator assemblage: main functional group, abundance and diversity (Magurran, 2004). We identified the functional group with highest relative abundance in the community of pollinators interacting with the marked plants. The estimation of pollinator abundance was calculated standardizing the number of visits per open flower and time unit (expressed as visits flowers–1 h–1). While the pollinator diversity was defined by: (1) richness (S_{obs}), calculated as the number of pollinator species found visiting flowers in each population; diversity, as the Shannon–Wiener index and Hulbert's PIE index (Colwell, 2005). Hulbert's PIE is the probability that two randomly sampled individuals from the community pertain to two different species. It is an evenness index that combines the two mechanistic factors affecting diversity: dominance and species abundance; and dominance, calculated as the relative abundance of the most abundant pollinator species. We used pollinator rank-abundance plots as a way to visualize the structure of the pollinator communities (Magurran, 2004). Finally, niches overlap was estimated at species and functional group

level among each of studied plant species and their hybrids using Pianka index (Pianka, 1973). All these indexes were generated by a randomization process using EcoSim (Gotelli and Entsminger, 2005) (http://www.homepages.together.net/~gentsmin/ecosim.htm).

SELECTION GRADIENTS

Direction and magnitude of selection for each quantitative trait was estimated by means of selection gradients. This is a multivariate technique that measures the selection acting on each studied trait independently of any other traits (Lande and Arnold, 1983). In our case we explored three types of selection gradients: (1) linear selection gradient, β, computed from the standardized partial-regression coefficients of a linear regression of relative fecundity on all the traits; (2) direct nonlinear selection gradient for each character i, γii, which was computed from the second-order standardized coefficient in a quadratic regression of relative fecundity on the character i; and (3) correlational selection gradients for character i and j, γii, computed from the standardized coefficient in a quadratic regression of relative fecundity on the product of i and j character value, describing the selection acting on the correlation between both i and j character (Lande and Arnold, 1983). The last estimates of selection introduce multiple peaks in the model to improve the method for estimating selection surfaces (Mitchell-Olds and Shaw, 1987; Schluter, 1988). The multivariate models of selection gradients were built introducing as independent variables the standardized data of the original phenotypic traits and as a dependent variable the estimate of the lifetime relative fecundity of the plants.

We also examined stabilizing/disruptive and correlational selection on the multivariate phenotype through canonical analysis of the matrices of nonlinear selection (Reynolds *et al.*, 2010). This analysis has been strongly recommended to get a straightforward interpretation of non-linear selection (Phillips and Arnold, 1989). This method finds the major axes of nonlinear

selection by means of a diagonalized matrix M. We found as many axis as number of traits in the analyses, but because the off-diagonal terms of M are zero, the power to detect nonlinear selection is higher than standard selection gradients (Phillips and Arnold, 1989). The strength of selection along each axis is indicated by their eigenvalues whereas the loading of each original trait on each axis provided by each eigenvector (Blows and Brooks, 2003). The significance of the eigenvalues was tested using permutation tests following Reynolds *et al.* (2010). The sign of the eigenvalues determined the type of fitted quadratic surface (Phillips and Arnold, 1989). Positive eigenvalues indicate concave (stabilizing) nonlinear selection whereas negative eigenvalues indicate convex (disruptive) nonlinear selection. When eigenvalues there is a mixture of positive and negative eigenvalues the fitness surface will be a multivariate saddle (Chenoweth *et al.*, 2012).

To determine the effect of each phenotypic trait on fecundity while removing the confounding effects of the other traits, we used the partial regression leverage plots of each trait on fecundity residuals (Rawling *et al.*, 1998). Leverage plots were made by regressing each variable residual against the residuals from the regression of the dependent variable on all the remaining independent variables. The slope of the linear regression is the partial regression coefficient for that independent variable in the full model (Rawling *et al.*, 1998). This plot also calculates a confidence function with respect to each variable, from which it can give the sign and percentage of the variability in fecundity explained by each variable.

DIVERGENT SELECTION

We explored how selection diverged both between species and between populations within species in 2007 and 2008. For the first, we analyzed divergent selection among the studied species and between them and the hybrid population. At this level, we computed the divergent selection from the linear, nonlinear and correlational selection gradients including the species

and its interaction with each of the studied character affecting the fitness in the analyses.

At population level, we analyzed the number of traits under divergent selection between each of the studied population in our transect of *E. mediohispanicum* and *E. nevadense*. Here we include the studied population and its interaction in the multivariate analyses of the gradients of selection. We compute these analyses by pairwise comparison, obtaining which character and number of them are under different type of divergent selection across the contact zone. All statistical analyses of selection gradients and their divergence were performed with the software JMP, version 7.0 (SAS Institute, Cary, North Carolina, USA).

RESULTS

POLLINATOR ASSEMBLAGES

The populations included in this work received in 2007 and 2008 a total of 906 and 768 floral visits, respectively. These floral visits belonged to 106 and 104 different species in six orders, being the majority Hymenoptera and Coleoptera for both *E. mediohispanicum* (16 and 20 species of Hymenoptera and 13 and 21 species for Coleoptera, in 2007 and 2008, respectively) and *E. nevadense* (10 and 14 Hymenoptera species and 11 and nine Coleoptera species in 2007 and 2008 respectively). Whereas, the hybrid population present the majority of species belonging to Coleoptera and Lepidoptera both studied years (12 and 10 Coleoptera species and 10 and five Lepidoptera species, for 2007 and 2008, respectively). Despite this diversity only between three and eight species made up more than 5% of the total visits per population and year (Table 8.2).

The observed species richness (Sobs) per population ranged from 19 to 42 in *E. mediohispanicum* populations, from 20 to 36 in *E. nevadense* populations and being 26 and 54 in the hybrid population (Table 8.2). This

fact presented the studied species as generalized pollination systems at both specific and local level (Table 8.2). Shannon-Weiner H´ and Hulbert´s PIE indices established similar relationships comparing the population diversities (Table 8.2). Moreover, Hulbert´s PIE indices were high ranging between 0.85 and 0.95 (Table 8.2) reflecting high probabilities of random selected insects belong to different species. Despite these high levels of diversity, we found similar structure in the pollinator assemblage with few abundant species and high number of scarce species.

Population	Main polinator Funtional Group	Abundance	S_{obs}	Dominance	Shannon-Wiener H´	Hulbert PIE
2007						
Em25	LB and SB (41%)	0.65	19^{3}	21^{3}	2.41^{3}	0.88^{3}
Em17	LB and B (42%)	0.58	41^{1}	9^{5}	3.23^{1}	0.95^{1}
H01	A and BT (50%)	0.56	54^{1}	13^{4}	3.35^{1}	0.95^{1}
En11	A (42%)	0.18	20^{2}	19^{2}	2.53^{2}	0.90^{2}
En10	A (48%)	0.27	23^{2}	34^{1}	2.36^{3}	0.85^{4}
2008						
Em25	LB (49%)	0.50	42^{1}	21^{3}	2.93^{2}	0.90^{2}
Em17	LB and B (48%)	0.66	33^{2}	26^{2}	2.79^{3}	0.90^{2}
H01	LB and SB (66%)	0.45	26^{2}	32^{1}	2.46^{4}	0.85^{3}
En11	A (34%)	0.22	22^{2}	$20^{2,3}$	$2.63^{2,3}$	0.91^{2}
En10	A and O (51%)	0.45	36^{1}	13^{4}	3.18^{1}	0.95^{1}

Table 8.2. Among-population differences in the main pollinator funtional group, abundance and diversity. Sobs Observed number of pollinator species censused per population, Dominance percentage of the most abundant species within a population. Significant differences in the indexes were indicated with different letter or number following the value.

However, there were among-species differences in abundances, presenting in both years higher abundances levels the *E. mediohispanicum* (Table 8.2). These abundance and diversity levels were distributed differentially between the defined functional group depending on *Erysimum* species. Thus, while the majority of pollinators interacting with *E. mediohispanicum* plants were large bees, small bees and beetles (from 41% to 49%, depending on the population and year), in *E. nevadense* populations the main pollinator visits were done by ants (from 34% to 51%, depending on population and year)

(Table 8.2). The Hybrid population presented the assemblage of pollinator more similar to *E. nevadense* in 2007 and similar to *E. mediohispanicum* in 2008 (Table 8.2). Because this, the pollination niche overlap was higher between the species and the hybrid population than among-species (Table 8.3).

	Niche Overlap	
	Pollinator Functional Group	Pollinator Species
2007		
Em-H01	0.76	0.46
En-H01	0.70	0.30
Em-En	0.66	0.37
2008		
Em-H01	0.69	0.26
En-H01	0.54	0.16
Em-En	0.60	0.19

Table 8.3. Interactive niche overlaps among *E. mediohispanicum* (Em), *E. nevadense* (En) and the hybrid population (H01). Niche overlaps were estimated at species and functional group level using Pianka index.

VARIATION IN PHENOTYPIC TRAITS AND CORRELATIONS

The population presenting the higher total number of flower was the hybrid population at both studied years (Table 8.1). In 2007 the closer populations to hybrid zone presented medium number of flower, while in 2008 were the *E. mediohispanicum* populations which presented higher number of flower after hybrid population (Tabla 1). The geometric morphometric analysis showed four RWs explaining more that 5% of the variance in flower shape in 2007 and 2008 (Appendix 8.1). These four RWs were consistent between both years and were conserved with respect the RWs described in previous works for *E. mediohispanicum* and *E. nevadense*. Using one-way ANOVAs we found among-population significant differences for all phenotypic trait measured for plant size and flower size, but for corolla tube width in 2007 and RWs, which only RW3 in 2007 and RW4 in 2008 exhibited significant differences between populations (Table 8.4). The *E. mediohispanicum* populations

presented the lower number of stalks at both studied years, but for the rest of phenotypic traits defining plant size (stalk diameter and stalk height) were *E. mediohispanicum* population which present higher values. For number of flower the hybrid population presented highest values and the *E. nevadense* populations the lowest for 2007 and 2008 (Table 8.4). However, the among-population differences in phenotypic traits for flower size varied between years. Corolla diameter and corolla tube length presented higher values around hybrid zone in 2007 (Table 8.4), while in 2008 were the *E. mediohispanicum* populations which presented larger flowers, because their higher values for corolla diameter, corolla tube length and corolla tube width (Table 8.4). RW3 and RW4 were the only two components of flower shape presented significant among-population differences in 2007 and 2008, respectively (Table 8.4). In 2007, Em25, H01 and En10 presented negative mean values for RW3, and En11 and Em17 presented positive mean values, while in 2008 were Em25 and En11 which presented mean negative values for RW4, and Em17, H01 and En10 presented positive values for RW4 (Table 8.4). RW2 presents marginal significant differences between populations at both studied years (Table 8.4).

We found in almost all populations and years significant correlation between the phenotypic traits defining the plant size (stalk height and the number of flower), and between phenotypic traits defining the flower size (corolla diameter, corolla tube length and corolla tube width) (Appendix 8.2). No significant negative correlation was found in any of the studied population between plant size traits and flowers size trait, which means that larger plants do not present smaller flowers (Appendix 8.2). However, with the only exception of En11, the larger plants present larger flower showed by the significant correlations found between plant size traits and flower size traits in our transect (Appendix 8.2). At the same time, significant correlation were found between the flower size traits and flower shape vectors (Appendix 8.2), suggesting that some flower shape were associated to flower size.

Character	*E. mediohispanicum* Em25	*E. mediohispanicum* Em17	Hybrid zone H01	*E. nevadense* En11	*E. nevadense* En10	*F*	*P*
2007							
No. stalk	2.11 ± .21 [c]	3.09 ± .26 [b,c]	4.57 ± .40 [a]	3.11 ± .33 [b,c]	3.73 ± .33 [a,b]	8.30	<.0001
Stalk diam (mm)	2.85 ± .10 [a]	2.69 ± .11 [a,b]	2.46 ± .10 [b,c]	2.19 ± .08 [c,d]	1.87 ± .06 [d]	17.87	<.0001
Stalk height (cm)	27.36 ± 1.08 [a]	20.43 ± .98 [b]	19.51 ± .75 [b]	21.33 ± .75 [b]	15.72 ± .60 [c]	24.43	<.0001
No. flowers	108.94 ± 7.87 [b]	116.18 ± 8.24 [b]	216.84 ± 21.51 [a]	125.18 ±13.94 [b]	83.00 ± 6.51 [b]	15.69	<.0001
Cor. Diam (mm)	12.24 ± .19 [c]	13.13 ± .17 [a,b]	12.79 ± .16 [b,c]	13.77 ± .19 [a]	12.99 ± .16 [b]	10.07	<.0001
Cor. Tube (mm)	11.01 ± .19 [c]	11.73 ± .13 [a,b]	11.50 ± .14 [a,b,c]	11.92 ± .15 [a]	11.21 ± .13 [b,c]	6.19	<.0001
Cor. Width (mm)	1.22 ± .07	1.49 ± .08	1.44 ± .10	1.38 ± .07	1.39 ± .10	1.48	.208
RW1	-.010 ± 0.01	.00 ± 0.01	-.010 ± .010	.010 ± .010	.010 ± .010	.58	.678
RW2	.013 ± 0.008	.010 ± 0.008	-.009± .008	-.003± .008	-.011± .008	2.06	.085
RW3	-.003 ± .005 [a,b]	.011 ± .005 [a]	-.001 ± .005 [a,b]	.006 ± .005 [a,b]	-.013 ± .005 [b]	2.82	.025
RW4	.009 ± .005	-.004 ± .005	.000 ± .005	-.002 ± .005	-.003 ± .005	1.05	.383
2008							
No. stalk	1.66 ± .18 [c]	2.38 ± .29 [b,c]	3.13 ± .31 [a,b]	3.72 ± .41 [a]	2.67 ± .26 [a,b,c]	6.71	<.0001
Stalk diam (mm)	2.94 ± .10 [a]	2.61 ± .10 [a]	2.80 ± .11 [a]	2.06 ± .07 [b]	2.09 ± .08 [b]	18.99	<.0001
Stalk height (cm)	31.97 ± 1.00 [a]	26.36 ± 1.01 [b]	23.94 ± .97 [b,c]	23.73 ± .99 [b,c]	21.52 ± .68 [c]	18.11	<.0001
No. flowers	169.13 ± 20.21 [a,b]	125.52 ± 19.93 [a,b]	186.23 ± 24.97 [a]	103.48 ± 8.90 [b,c]	53.87 ± 4.07 [c]	9.17	<.0001
Cor. Diam (mm)	13.15 ± .17 [a]	12.76 ± .15 [a,b]	12.36 ± .16 [b]	12.26 ± .16 [b]	12.36 ± .15 [b]	5.57	.0002
Cor. Tube (mm)	11.42 ± .13 [a]	10.95 ± .13 [a,b]	10.85 ± .13 [b]	10.52 ± .12 [b]	10.74 ± .10 [b]	7.19	<.0001
Cor. Width (mm)	1.41 ± .08 [a]	1.46 ± .07 [a]	1.31 ± .08 [a,b]	1.11 ± .08 [b]	1.37 ± .07 [a,b]	3.14	.0145
RW1	.010±.011	.003±.011	-.014±.011	-.010±.011	-.007±.011	.82	.513
RW2	.000±.007	-.007±.007	.014±.007	-.015±.007	-.001±.007	2.02	.090
RW3	-.002±.006	-.007±.006	.003±.006	-.001±.006	-.002±.006	.37	.827
RW4	-.011±.005 [b,c]	.008±.005 [a,b]	.002±.005 [a,b,c]	-.014±.005 [c]	.011±.005 [a]	4.43	.0016

Table 8.4. Values of the phenotypic traits for each studied population per year. *F*-ratios refer to one-way ANOVAs. Letters indicate the groups where the differences are significant, according to a Tukey HSD comparison.

VARIATION IN SELECTION ON PLANT TRAITS

In 2007, we found only a significant positive correlational selection gradient for the interaction between corolla tube length and RW4 in Em25, but no other linear, nonlinear or correlational significant selection gradient was found on the studied phenotypic traits at *E. mediohispanicum* population (Appendix 8.3). However, in 2008, the population Em25 presented a significant positive linear selection on number of flower and RW1; a significant positive nonlinear selection on number of flowers; positive significant correlational selection for the interaction between number of flowers and corolla tube length, corolla diameter and corolla tube width, corolla width and RW1, corolla tube length and RW2, RW1 and RW4, and RW2 and RW4 (Appendix 8.3). While at Em17, we found a significant positive linear selection on corolla tube length and RW1, and singnificant negative selection on number of flower (Appendix 8.3).

At the hybrid population, there was an intense and significant positive linear selection on number of flower both studied years (Appendix 8.3), showing that selection is favoring in this population those plants with many flowers. Besides, significant positive correlational selection was found for the interaction of stalk height and corolla diameter, and for corolla tube width and RW1, in 2007, and for the interaction of corolla diameter and corolla tube width, stalk height and RW1, number of flowers and RW1, corolla width and RW2, and corolla diameter and RW4, in 2008. While, there were significant negative correlational selection for the interaction of stalk height and corolla tube length and stalk height and corolla tube width, in 2007; and for the interaction of stalk height and corolla tube length, number of flowers and corolla tube length, and number of flower and RW3, in 2008 (Appendix 8.3). There was no significant nonlinear selection in hybrid population in the studied years (Appendix 8.3).

E. nevadense presented more among-population differences in the selection regimens on its populations. The population En11 presented a significant negative linear selection on stalk height, maintained between years,

and on RW4 only in 2008. There was a significant positive linear selection on corolla diameter in 2007 and on number of flowers in 2008. Significant positive correlational selection were found in this population only for the interaction of corolla width and RW1 in 2007 and for the interaction of number of flower and RW3 in 2008, while for the interaction of number of flowers and RW1 the correlational selection was negative (Appendix 8.3). At the population En10, significant linear selection was found only in 2008, being positive for number of flower and negative for RW4 (Appendix 8.3). Finally, there were significant positive correlational selection in 2008 for the interaction of corolla width with number of flowers and corolla tube length (Appendix 8.3), while these correlational selections were significatively negative for the interaction of corolla tube length and RW3 and RW1 and RW4, in 2007; and for the interaction of number of flowers and RW4 in 2008. There was no significant nonlinear selection on any phenotypic trait for *E. nevadense* populations (Appendix 8.3). However, using canonical analysis, we found statistical significant nonlinear selection only in 2008, and in one *E. mediohispanicum* (Em25) and one *E. nevadense* (En11) population. In these cases we found three significant latent axes (Appendix 8.4). Because they differed in the sign of their eigenvalues, the selection surfaces were a saddle in both cases. In addition, the curvatures of these nonlinear selection surfaces were very steep according to the magnitudes of the eigenvalues (Appendix 8.4).

DIVERGENT SELECTION

Significant divergent selection gradients were found between *E. mediohispanicum* and *E. nevadense*, and between each species and the hybrid population (Tables 8.5A, B, C and Fig. 8.1). In 2007, significant divergence was found for linear selection between *E. mediohispanicum* and the hybrid population on number of flowers; for nonlinear selection on number of flowers and RW2; and for correlative selection for the interaction of stalk height and RW2 (Table 8.5A, Fig. 8.1). Nevertheless, no significant divergent selection was found between *E. mediohispanicum* and hybrid population in 2008 (Table 8.5A).

Between hybrid population and *E. nevadense* we found significant divergence at linear selection on number of flowers and RW4; for nonlinear selection on number of flowers; and correlational selection for the interaction of number of flowers and corolla tube length, in 2007 (Table 8.5B, Fig. 8.1). While, in 2008, the only divengent selection found between hybrids and *E. nevadense* was the correlational selection on the number of flower and RW1 interaction (Table 8.5B).

Finally, more phenotypic traits under divergent selection gradient were found when comparing *E. mediohispanicum* and *E. nevadense*. In 2007, there was significant divergence for the linear selection on RW4; for the nonlinear selection on the RW2; and for the correlational selection acting on the interaction of stalk height and RW2 and corolla tube length and RW3 (Table 8.5C, Fig. 8.1). While, in 2008, the significant divergence was found on linear selection on RW1 and on correlational selection of six phenotypic interactions: stalk height and number of flower, number of flowers and corolla diameter, corolla tube length and corolla tube width, number of flowers and RW1, RW1 and RW4, and RW3 and RW4 (Table 8.5C).

In addition, divergent selection gradients were found at population level when we compare the *E. mediohispanicum* population and *E. nevadense* population, attending to their closeness to the hybrid population. In this sense, we found an increasing number of phenotypic traits under divergent selection when comparing the two populations closer to the hybrid population (three phenotypic trait and an interaction under divergence selection comparing Em17 and En11), than when comparing the two more distant populations from hybrid population (four phenotypic traits and six interactions under divergent selection when comparing Em25 and En10) (Table 8.6). A total of six and nine divergent selection gradients were found when we compare Em 17 to En10 and Em25 to En11, respectively, supporting the increasing level divergence from the centre to the extreme of the distribution of our studied populations (Table 8.6).

Table 8.5. Analysis of linear, quadratic, and correlational divergent selection on phenotypic traits between *E. mediohispanicum* and *E. nevadense* (A), E. mediohispaniucm and the hybrid population (B), and *E. nevadense* and the hybrid population (C), through lifetime female fecundity, both studied years. Bold values represent significant divergent selective gradients. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001

A)

Em-H01			Character j							
			No. Flowers	Corolla diameter	Corolla tube length	Corolla width	RW1	RW2	RW3	RW4
Character i	β	γ_{ii}	γ_{ij}	γ_{ij}	γ_{ij}	γ_{ij}	γ_{ij}	γ_{ij}	γ_{ij}	γ_{ij}
2007										
Stalk height	.164	.026	.101	1.161	.233	.498	.414	**3.934***	.050	1.309
No. Flowers	**32.345****	**21.858****		.003	3.276	.564	.855	.005	.818	1.316
Corolla diameter	.501	.004			.382	.149	.018	.270	.496	.145
Corolla tube length	.014	.015				1.592	.334	.596	.085	.246
Corolla width	.036	.742					.611	.216	1.888	1.511
RW1	.404	.015						.023	.671	.946
RW2	.653	**5.281***							.002	.528
RW3	1.540	.006								.144
RW4	.001	.083								
2008										
Stalk height	1.056	.064	.076	.113	.103	.006	.950	1.910	.036	.000
No. Flowers	1.609	1.125		.231	1.181	.001	.258	.000	.940	.000
Corolla diameter	.493	.101			.551	.329	.000	.422	.214	1.097
Corolla tube length	1.986	.278				.074	1.596	1.102	1.128	.758
Corolla width	.059	.232					.071	3.439	.738	.161
RW1	1.473	1.201						.350	.254	.600
RW2	1.268	.031							.084	.052
RW3	.703	.100								.006
RW4	.564	.008								

B)

En-H01			Character j							
			No. Flowers	Corolla diameter	Corolla tube length	Corolla width	RW1	RW2	RW3	RW4
Character i	β	γ_{ii}	γ_{ij}	γ_{ij}	γ_{ij}	γ_{ij}	γ_{ij}	γ_{ij}	γ_{ij}	γ_{ij}
2007										
Stalk height	1.696	.020	.259	.000	.017	.828	.105	.080	.982	.334
No. Flowers	**28.733******	**18.107******		.206	**6.664***	.010	.644	1.326	1.027	.056
Corolla diameter	2.005	.324			.073	.171	.001	.148	.121	.026
Corolla tube length	.027	.091				2.844	.934	.064	2.692	.000
Corolla width	.409	.647					.967	.280	2.027	2.298
RW1	1.360	.570						.795	3.095	.666
RW2	.022	.266							.094	.116
RW3	.231	1.034								.868
RW4	**4.796***	.433								
2008										
Stalk height	.162	.871	1.474	.258	.422	.014	.016	1.195	.845	.042
No. Flowers	2.582	1.888		.755	.973	.000	**4.341***	.010	1.445	.313
Corolla diameter	1.334	1.084			.000	.160	.328	.221	.025	.040
Corolla tube length	.999	.495				.981	.803	.533	1.157	.391
Corolla width	.269	.153					.667	2.045	.867	.617
RW1	.351	.000						.079	.073	.158
RW2	.674	.419							.391	.806
RW3	.025	.002								.641
RW4	.787	.294								

C)

Em-En			Character j							
Character i	β	γ_{ii}	No. Flowers γ_{ij}	Corolla diameter γ_{ij}	Corolla tube length γ_{ij}	Corolla width γ_{ij}	RW1 γ_{ij}	RW2 γ_{ij}	RW3 γ_{ij}	RW4 γ_{ij}
2007										
Stalk height	3.844	.111	.000	2.279	1.157	.054	.003	**5.483***	1.036	2.871
No. Flowers	.327	.158		.287	1.317	.872	.528	.002	.474	1.343
Corolla diameter	.750	.183			.055	.337	.000	2.688	.249	.049
Corolla tube length	.003	.198				.253	.001	.006	**4.774***	2.036
Corolla width	1.155	.199					.562	.534	.026	.107
RW1	.327	.057						.950	.173	.486
RW2	.586	**7.276****							.218	2.528
RW3	3.740	.001								1.900
RW4	**5.353***	1.007								
2008										
Stalk height	.833	2.318	**4.453***	.570	.724	1.347	.688	1.438	2.115	.199
No. Flowers	.332	.889		**4.807***	.000	.093	**11.282*****	.370	1.051	.015
Corolla diameter	.274	1.592			.171	1.200	2.318	.030	.570	2.102
Corolla tube length	.306	.377				**4.217***	1.965	.823	.061	.199
Corolla width	.114	.005					1.436	.008	3.095	1.265
RW1	**4.720***	2.092						.001	1.358	**8.989****
RW2	.124	.642							.004	1.838
RW3	1.897	.002								**6.008***
RW4	.030	.494								

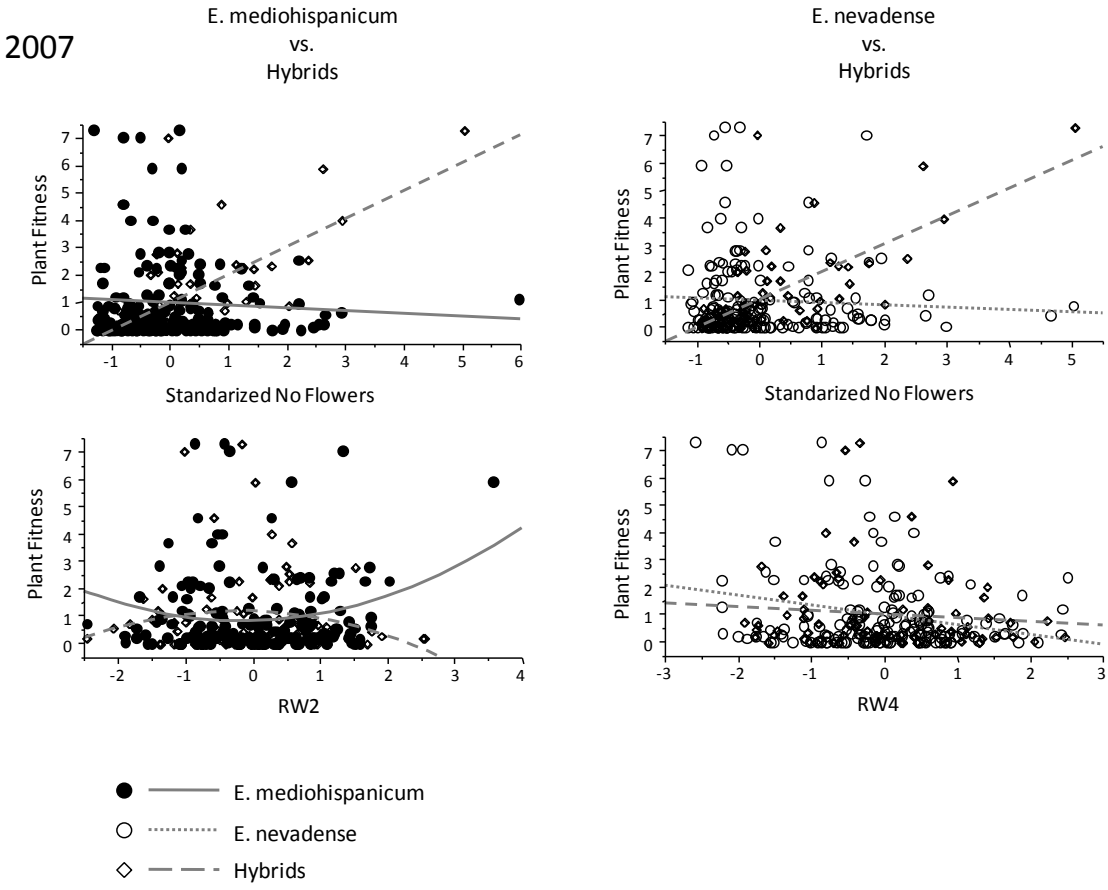

Figure 8.1. Significant divergent selection gradients on phenotypic traits among *E. mediohispanicum* and *E. nevadense*, and between them and the hybrid population at both studied years.

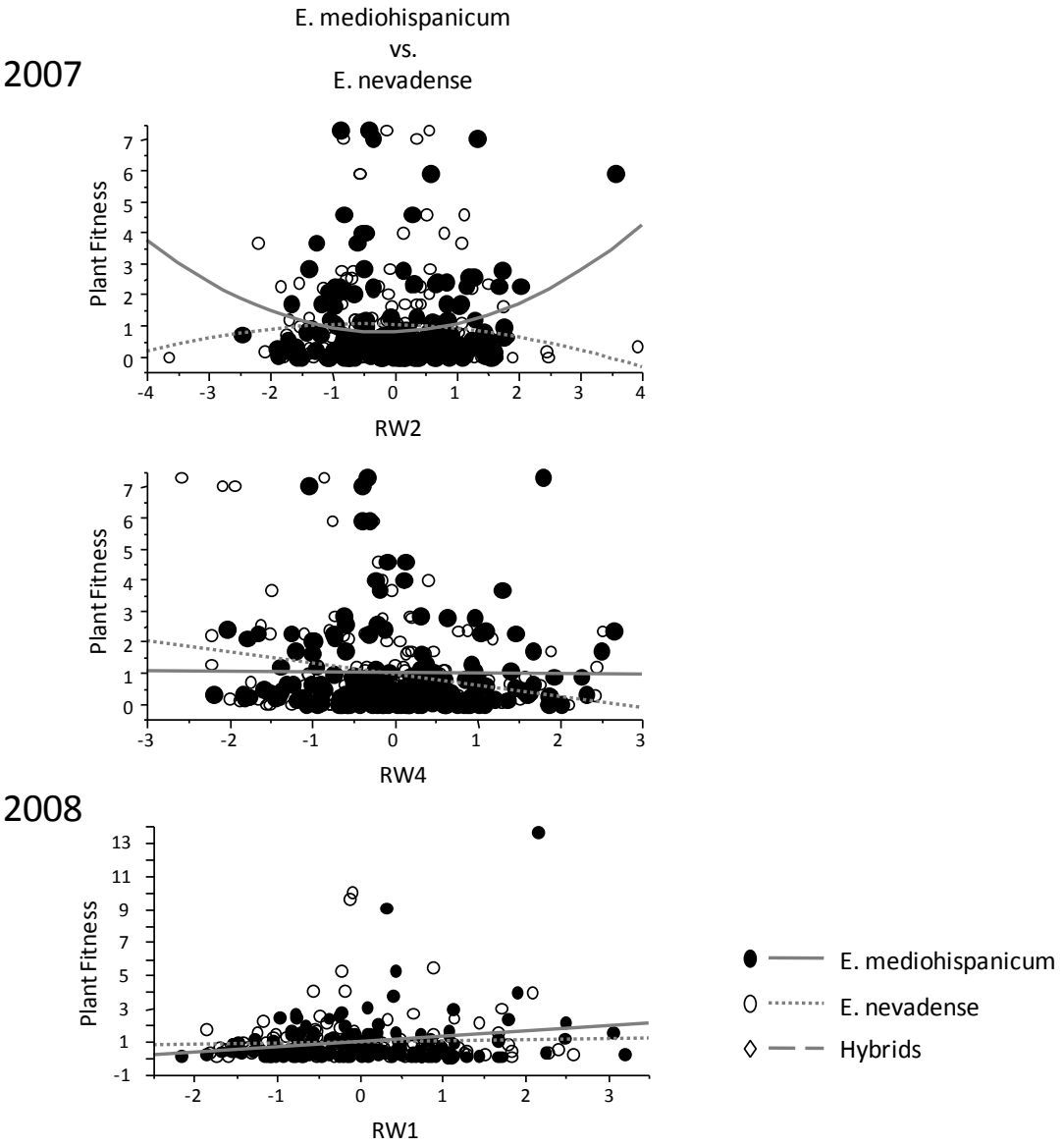

Figure 8.1. (Continuation from previous page)

Population		Divergent selection on traits		
Em	En	β	γ_{ii}	γ_{ij}
Close	Close	*RW4**, RW3*	RW4*	*C. width x RW1***
Close	Far	RW1*, C. tube length*		*RW2 x RW4**, RW1 x RW4*, C. tube length x RW4*
Far	Close	*Stalk height**, Stalk height*	*RW2*, RW4**	*Stalk height x RW3***, No. Flowers x C. width*, No. Flowers x RW3*, C. diameter x RW4*, C. width x RW1****
Far	Far	*RW2**, No. Flowers*, RW4*	*RW2**	*C. tube length x RW4**, No. Flowers x RW3*, C. diameter x C. width**, C. tube length x C. width*, C. width x RW1*, RW2 x RW4*

Table 8.6. Phenotypic character under divergent selection comparing populations attending to their relative distance. Linear (β), quadratic (γ_{ii}), and correlational (γ_{ij}) divergent selection were analysed. "Close" and "Far" were respect to the hybrid zone. Italic letter denote phenotypic trait under divergent selection in 2007. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001

DISCUSSION

INTERACTIVE NICHE OVERLAP IN GENERALIST SYSTEMS

The two studied species, *E. mediohispanicum* and *E. nevadense*, and their hybrids were visited by extremely generalized pollinator assemblages in both years, with more than one hundred species visiting their flowers. Despite these degree of generalization, the studied populations varied significantly in pollinator fauna, mostly in abundance and identity of the main pollinator functional group. In this respect, *E. mediohispanicum* and the hybrid population presented higher pollinator abundances both years, and the main pollinator functional groups (large and small bees) were highly efficient; while *E. nevadense* plants presented lower abundances and were mainly visited by inefficient functional groups, such as ants (Armstrong, 1981; Puterbaugh, 1998). These differences would be explained by differences in altitude and habitat. *E. nevadense* occurs in the high mountains of the Sierra Nevada, above the treeline, whereas *E. mediohispanicum* distributes along its low- and medium-altitude mountain areas (Blanca *et al.*, 2009). Alpine environments are characterized by the scarcity and temporal unpredictability of pollinators (Ashman *et al.*, 2004; Burd *et al.*, 2009), which cause many alpine plants to have their reproduction limited by pollen availability (Garcia-Camacho and Totland, 2009; Arroyo *et al.*, 1982). The Sierra Nevada, in particular, presents a harsher climate than most alpine environments due to severe summer droughts. Consequently, the most frequent pollinators in the high-mountains of the Sierra Nevada are low-efficiency insects such as ants and small flies (Gómez and Zamora, 1992; Gómez *et al.*, 1996). In this sense, previous studies have shown these same differences in the *E. nevadense* and *E. mediohispanicum* pollinator assemblages (Gómez et. al., 2007; Ortigosa and Gómez, 2010). Therefore, despite of the extremely generalized pollinator assemblage exhibited by both species, they presented low values of pollination niche overlap. The relative frequency of effective pollinator species and functional groups will influence the phenotypic variation of host species in the subsequent generations (Faegri and van der Pijl, 1979; Grant, 1993). The pollinator assemblage of the hybrid population resembled any of

the two parental species depending on the year. One year was more similar to the *E. nevadense* assemblage whereas the other was more similar to the *E. mediohispanicum* one. If this temporal fluctuation in the type of floral visitors is maintained through time, the later generations should exhibit intermediate characters shaped by combined selective pressures (Grant, 1993). In fact, we found that plants from the hybrid zone had an intermediate phenotype between *E. mediohispanicum* and *E. nevadense* for most studied traits (see also ***Chapter 6***).

SELECTION IN A SECONDARY CONTACT ZONE

In 2007, our study revealed significant selection only in one *E. nevadense* population, En11, and in the hybrid zone. However, in 2008, there were more phenotypic traits under selection. However, we did not find temporal variation in selection on any trait whitin population (data not shown). The occurrence of temporal constancy in selective patterns makes more relevant the spatial pattern that we found, giving more importance to any geographic variation in selective pressures (Kelly, 1992; Kingsolver *et al*., 2001; Caruso *et al*., 2003).

The number of flowers was the trait exhibiting the most consistent and strongest selection. It was positively selected in all but one populations in 2008 and in the hybrid zone in 2007. Flower number increases the fitness of many plant species through two pathways. First, flower number enhances fitness indirectly, through its effects on pollinator visitation rate and attraction (e.g., Conner and Rush, 1996; Ohashi and Yahara, 1998; Rademaker and de Jong, 1998; Vaughton and Ramsey, 1998; Thompson, 2001; Benítez-Vieyra *et al*., 2006; Makino and Sakai, 2007). In addition, flower number also increases fitness directly, because having more flowers means producing more ovules per plant, a major component of the potential reproductive output (e.g., Herrera, 1993; Conner and Rush, 1996; Gross *et al*., 1998; Gómez, 2000; Gómez and Zamora, 2000). In our system, we presume that the former pathway is more important, mostly in *E. mediohispanicum* because its high level of inbreeding

depression (***Chapter 3***). However, the lower inbreeding depression and the higher self-pollen limitation exhibited by *E. nevadense* (***Chapter 3***) suggest that both direct and indirect pathways could affect this species.

Attending to traits related to plant size, stalk height exhibited consistently negative directional selection in *E. nevadense* populations. This outcome could be explained because the main *E. nevadense* pollinators were ants, a non-flying insect which could prefer short plants because they can reach flowers more easily. Pollination by ants has been previously described in other plants from the alpine of Sierra Nevada (Gómez and Zamora, 1992; Gómez *et al*., 1996). Nevertheless, ants are considered poor pollinators favoring autogamy (Armstrong, 1981; Herrera et al., 1984; Gómez and Zamora, 1992). However, *E. nevadense* would presumably cope the effects of autogamy (***Chapter 3***). Consequently, ants could act as important pollinators for this plant species.

Whereas no trait related to flower size underwent selection, two different flower shape components presented significant directional selection: RW1 in *E. mediohispanicum* and RW4 in *E. nevadense*. *E. mediohispanicum* plants with positive values of RW1 (i.e., corollas with non parallel petals) and *E. nevadense* plants with negative values of RW4 (i.e., corollas with narrow petals) produced more seeds. Selection in corolla shape in *E. mediohispanicum* has been demostrated previously (Gómez *et al*., 2006; 2008a; 2009a). In this plant species, corolla shape is selected as a consequence of its association with reward in pollen and nectar that affect pollinator preferences and behavior (Gómez *et al*., 2008a; 2008b).

SELECTION IN THE HYBRID ZONE

The hybrid zone studied here is unimodal and it is maintained by high levels of gene flow from populations Em25 and En10 (***Chapter 5***). The plants from the hybrid zone were those bearing the highest number of flowers during both studied years. This finding may indicate the occurrence of heterosis

(Rieseberg *et al.* 1999). Indeed, the number of flowers is associated to hybrid vigor in many plant species (Campbell *et al.*, 2006). Our study showed that the phenotypic trait exhibiting the strongest directional positive selection in the hybrid zone was the number of flowers (R2=0.49, P<.0001, in 2007; and R2=0.35, P<.0001, in 2008). Number of flowers affect positively the plant fitness in other *E. mediohispanicum* populations (Gómez *et al.*, 2003, 2009a), and also affect positively the pollinator visitation rate in our study system (***Chapter 6***).

Hybrid plants exhibited a reduction in fitness due to their sterility (***Chapter 6***). These outcomes show the antagonistic interaction between intrinsic and extrinsic postzygotic compatibilities. Thus, while the hybrids between *E. mediohispanicum* and *E. nevadense* exhibited intrinsic postzygotic incompatibilities that lead on the hybrid sterility (***Chapter 6***), they exhibited a fitness advantage due to their hybrid vigor, generating an extrinsic postzygotic benefit. This postzygotic advantage would be an by-product of local adaptation to pollinators through their preference for plant with high number of flowers (Gómez *et al.*, 2003, 2009a, ***Chapter 6***) and their null effect as an ethological isolation barrier (***Chapter 6***). In this sense, the absence of pre-pollination isolation is common in generalist plants (Pascarella, 2007; Ellis and Johnson, 2012). Therefore, the frequency of hybrid plants in the contact zone will depend on the equilibrium between intrinsic postzygotic barriers and extrinsic postzygotic advantages (positive selection on heterotic characters). In our case, we suggest that the selection favoring high number of flowers in the hybrid zone is the driving force behind the actual frequency of hybrids (***Chapter 5***).

DIVERGENT SELECTION

We have found divergent selection among *E. mediohispanicum* and *E. nevadense* acting on three components of corolla shape (RW2 and RW4 in 2007, and RW1 in 2008). These selection could cause differential adaptation of each

species to their own pollinators. We also found divergent selection for RW2 and RW4 when comparing the hybrid zone with the parental population. The strongest divergent selection was found on flower number when comparing the hybrid zone with both parental species. The number of flowers exhibited a strong directional positive selection in the hybrid zone. The existence of hybrid individuals with large number of flowers (***Chapter 6***) could increase the intensity of selection occurring in the hybrid zone. So, the adaptive divergence is not only taking place between *E. mediohispanicum* and *E. nevadense*, but among both species and the hybrid zone where they contact secondarily. The ecological local adaptation and the intrinsic postzygotic disadvantage exhibited by hybrids would determine the width of the hybrid zone and consequently the boundary of the parental species. These outcomes agree with the widely accepted idea that pollinators are important selective agents, which can drive plant phenotypic evolution and even speciation (Grant and Grant, 1965; Johnson, 1996; Kay and Sargent, 2009). If speciation occurs as an indirect consequence of adaptive divergence (Jiggins *et al*, 2001), we can imagine that these same selection could prevent the genetic swamping of two species when they co-occur in a secondary contact.

There was an increasing number of divergent selection gradients between more distant populations. The studied populations exhibited significant level of genetic structure and differentiation using microsatellites markers (***Chapter 5***). Similar high genetic structure values were found when con-specific populations of *E. mediohispanicum* were studied using different genetic markers (Gómez *et al*., 2009a, 2009c). Short-lived and self-compatible species are expected to show stronger local adaptation because their tendency to be more strongly differentiated at smaller scales (Linhart and Grant, 1996). So, the increasing number of phenotypic traits under divergent selection with increasing distance would be reflecting increasing degrees of local adaptation because of reduced gene flow among population with the distance. Increasing patterns of local adaptation related to geographic distance were reported in other plant species (Galloway and Fenster, 2000; Becker *et al*., 2006) In our

case, we think that the reduction in the probability of sharing pollinators not only will affect the degree of gene flow between species, but will also influence the local adaptation patterns and finally the phenotypic traits related to pollination (Grant, 1993).

Four important outcomes emerge from the study of natural selection in a secondary contact of *E. mediohispanicum* and *E. nevadense*: 1) Despite to their interaction with generalist pollinator assemblages, both species present a low pollination niche overlap; 2) There are local selective pressures taking place at each studied population, that could be behind the significant phenotypic differentiation found among populations; 3) In the hybrid population, there was strong selection favouring a heterotic phenotypic trait, flower number; and 4) The between-population and between-species divergent selection found in the study area could act preventing inter-specific gene flow and circumscribing the hybrid zone to a narrow strip.

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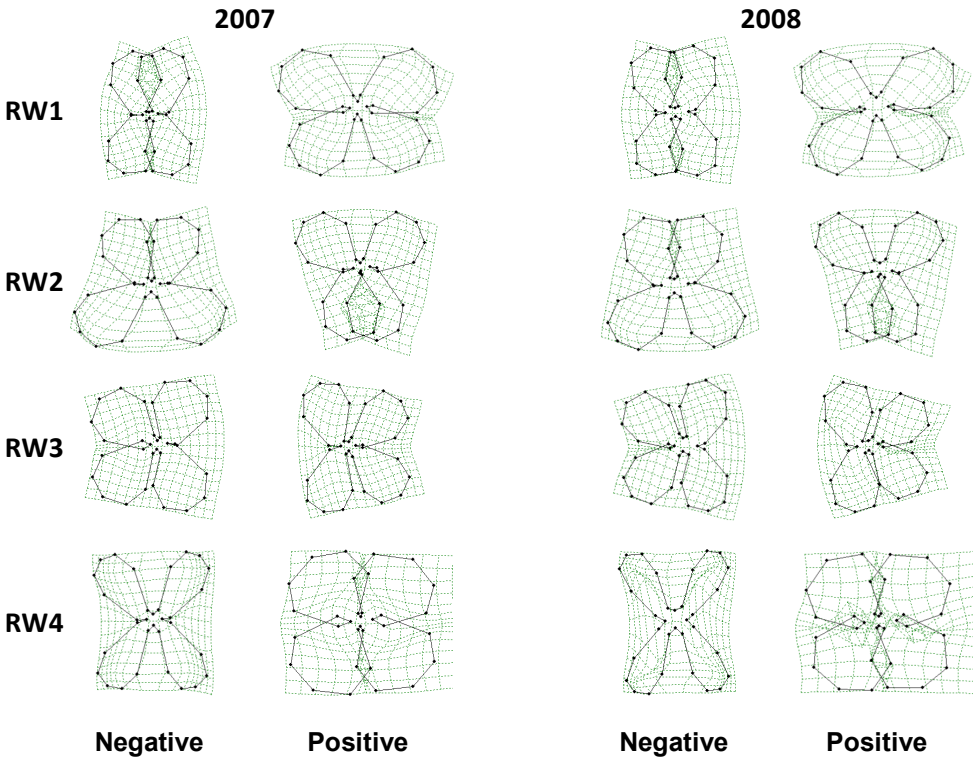
APPENDICES



Appendix 8.1. Summary of the geometric morphometric analysis (*N* = 540 plants per year) showing the consensus morphology (uppermost panels) and the variation in flower morphology produced by the Relative Warps explaining more than 5% of the overall variation in shape.

Appendix 8.2. (*Next pages*) Phenotypic correlations between plant traits. For each studied population 90 plants were measured per year. Above diagonal are product-moment correlations, below diagonal are covariances, and in diagonal is the variance. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001

Em25	Stalk height	No. flowers	Corolla diameter	Corolla tube length	Corolla width	RW1	RW2	RW3	RW4
2007									
Stalk height	105.612	.410 ****	.354 ***	.210 *	.053	-.207	.129	-.062	.007
No. flowers	314.509	5577.986	.043	-.075	-.073	-.173	.004	-.113	-.065
Corolla diameter	6.468	5.670	3.167	.559 ****	.182	-.037	-.145	-.208 *	-.236 *
Corolla tube length	3.843	-9.978	1.770	3.160	.012	-.074	-.007	-.137	-.136
Corolla width	.362	-3.620	.214	.014	.436	.124	-.215 *	-.304 **	-.050
RW1	-.133	-.565	-.001	-.035	.005	.011	-.001	.000	.000
RW2	-.022	.191	-.012	-.007	-.001	.000	.005	.000	.000
RW3	.008	.622	-.008	-.013	.004	-.000	.001	.002	-.000
RW4	-.059	-.064	-.008	-.009	-.000	.000	.001	.000	.002
2008									
Stalk height	90.058	.122	.163	.130	-.053	-.191	.055	-.136	.091
No. flowers	221.144	36745.465	.022	.234 *	-.034	-.002	-.137	.085	.142
Corolla diameter	2.444	6.532	2.490	.552 ****	.420 ****	-.198	-.050	.168	-.186
Corolla tube length	1.530	55.738	1.081	1.540	.145	-.250 *	.060	.093	.025
Corolla width	-.399	-5.192	.520	.142	.616	-.047	-.273 **	.208 *	-.360 ****
RW1	.181	-.101	.032	.032	.003	.010	.000	.000	.000
RW2	-.002	-1.733	-.005	.003	-.013	-.001	.005	.000	.000
RW3	-.055	1.808	.006	.005	.002	-.000	-.000	.003	.000
RW4	.075	.263	-.019	-.001	-.017	.000	.000	-.000	.002

Em17	Stalk height	No. flowers	Corolla diameter	Corolla tube length	Corolla width	RW1	RW2	RW3	RW4
2007									
Stalk height	87.366	.314 **	.355 ***	.250 *	.005	-.094	-.321 **	.055	-.192
No. flowers	229.731	6105.990	.198	.349 ***	.254 *	-.063	-.198	-.051	-.230 *
Corolla diameter	5.401	25.125	2.649	.655 ****	.157	-.095	.046	-.077	-.084
Corolla tube length	2.861	33.410	1.307	1.503	.113	-.087	.082	-.003	-.222 *
Corolla width	.038	14.944	.192	.105	.568	-.014	-.012	-.032	.091
RW1	-.012	.060	-.008	.005	.008	.010	.000	.000	.000
RW2	.067	1.050	-.009	.001	.000	-.000	.006	.000	.000
RW3	-.049	-.274	.011	-.004	.000	-.000	-.001	.003	.000
RW4	-.081	-.690	-.020	-.006	-.001	-.000	-.000	-.000	.002
2008									
Stalk height	91.983	.228 *	.324 **	.299 **	.242 *	-.238 *	-.059	-.099	.079
No. flowers	413.528	35756.455	.119	-.019	.156	-.133	.191	-.077	-.025
Corolla diameter	4.534	32.970	2.127	.648 ****	.479 ****	-.014	-.034	-.359 ***	-.222 *
Corolla tube length	3.566	-4.443	1.175	1.547	.257 *	-.068	-.131	-.170	-.135
Corolla width	1.651	20.965	.497	.228	.507	.114	-.029	-.203	-.174
RW1	.230	3.100	-.001	.005	-.009	-.059	.000	.000	.000
RW2	-.086	1.620	-.003	-.012	.001	-.002	.005	.000	.000
RW3	-.076	-.031	-.010	-.004	-.001	-.000	.000	.003	.000
RW4	-.027	-1.037	-.033	-.015	-.010	-.001	.000	.000	.003

H01	Stalk height	No. flowers	Corolla diameter	Corolla tube length	Corolla width	RW1	RW2	RW3	RW4
2007									
Stalk height	50.509	.328 **	.246 *	.303 **	-.083	-.063	.171	-.012	-.135
No. flowers	475.995	41655.549	.133	.067	-.030	-.170	-.020	.143	-.118
Corolla diameter	2.624	40.656	2.245	.608 ****	.422 ****	-.090	.081	.096	-.049
Corolla tube length	2.824	18.006	1.194	1.720	-.014	-.037	.107	-.048	-.099
Corolla width	-.545	-5.683	.584	-.017	.852	.229 *	-.171	-.017	.088
RW1	-.053	1.064	-.006	-.000	-.004	.008	.000	.000	.000
RW2	-.014	-.913	.002	-.004	-.001	.000	.004	.000	.000
RW3	-.059	-.783	.015	.015	.014	.000	-.000	.003	-.000
RW4	.030	-.601	.008	.005	-.003	-.000	.000	-.000	.003
2008									
Stalk height	85.291	.374 ***	.340 **	.205	.266 *	-.124	-.270 *	-.108	.077
No. flowers	817.514	56135.844	.361 ***	.153	.197	-.136	-.106	-.060	-.093
Corolla diameter	4.783	130.376	2.318	.525 ****	.347 ***	-.199	.078	-.062	-.120
Corolla tube length	2.356	45.263	.995	1.550	.239 *	-.075	.117	-.120	-.002
Corolla width	1.815	34.504	.390	.220	.544	-.062	-.115	-.121	-.231 *
RW1	.129	3.214	.029	.009	.004	.010	.000	.000	.000
RW2	-.194	-1.893	.008	.009	-.007	.000	.006	.000	.000
RW3	.017	.145	.005	.011	.003	-.000	.000	.004	.000
RW4	.023	-1.465	-.013	-.003	-.010	-.001	-.000	-.000	.003

En11	Stalk height	No. flowers	Corolla diameter	Corolla tube length	Corolla width	RW1	RW2	RW3	RW4
2007									
Stalk height	50.253	**.445 ******	.181	.199	-.063	-.018	.049	-.151	.054
No. flowers	416.985	17480.013	.095	.019	.073	-.141	.063	.082	.096
Corolla diameter	2.343	22.948	3.315	**.558 ******	**.344 *****	-.162	.099	**-.306 ****	.118
Corolla tube length	2.036	3.728	1.466	2.081	.003	-.192	-.007	-.197	-.152
Corolla width	-.300	6.516	.421	.003	.453	-.062	.068	-.158	-.022
RW1	-.018	-1.968	-.033	-.029	-.004	.011	.000	.000	.000
RW2	.020	.664	.010	-.001	.003	-.000	.006	.000	.000
RW3	-.008	.603	-.004	-.015	-.003	.000	.000	.002	.000
RW4	-.057	.438	-.027	-.007	-.005	-.001	-.000	.000	.003
2008									
Stalk height	88.989	.080	.149	.111	.027	-.118	-.080	.058	-.100
No. flowers	63.797	7125.646	.041	-.031	.129	-.125	-.101	-.122	-.085
Corolla diameter	2.141	5.267	2.306	**.640 ******	.153	**-.324 ****	.155	-.142	**-.298 ****
Corolla tube length	1.220	-3.060	1.130	1.354	-.143	-.135	-.003	-.127	-.199
Corolla width	.190	8.252	.175	-.125	.570	.018	-.096	-.031	**-.256 ***
RW1	.140	1.296	.055	.018	-.001	.014	.000	.000	.000
RW2	-.045	-.448	.020	-.000	-.006	.001	.005	.000	.000
RW3	.042	-.372	.006	.000	.002	.000	.000	.003	.000
RW4	-.011	-.615	-.028	-.015	-.007	-.000	-.000	.000	.003

En10	Stalk height	No. flowers	Corolla diameter	Corolla tube length	Corolla width	RW1	RW2	RW3	RW4
2007									
Stalk height	32.944	.084	.200	**.228 ***	-.099	-.114	**.210 ***	-.167	**-.221 ***
No. flowers	29.635	3.812	.128	.194	**.225 ***	-.162	**-.260 ***	-.046	-.022
Corolla diameter	1.777	12.244	2.404	**.501 ******	**.254 ***	-.092	.024	-.182	**.256 ***
Corolla tube length	1.618	14.823	.962	1.533	.104	-.066	.078	.035	.047
Corolla width	-.544	13.382	.380	.124	.929	-.188	-.123	.000	.053
RW1	-.037	.166	.006	-.001	.011	.010	.000	.000	.000
RW2	-.046	.095	-.022	-.003	.010	-.000	.006	.000	.000
RW3	-.007	-.219	.015	.001	.003	-.001	-.000	.003	.000
RW4	-.011	-.010	.007	.007	.000	.001	-.001	-.000	.002
2008									
Stalk height	41.629	**.377 *****	**.261 ***	**.311 ****	.199	.000	.042	-.149	-.261 *
No. flowers	93.801	1490.409	.174	.121	**.247 ***	.076	-.155	-.131	-.078
Corolla diameter	2.355	9.392	1.958	**.641 ******	**.336 ****	-.249 *	.012	-.021	-.164
Corolla tube length	1.959	4.572	.875	.952	.183	-.315 **	.030	.082	-.230 *
Corolla width	.825	6.114	.301	.114	.412	.038	-.068	-.019	-.163
RW1	.006	-.271	.034	.030	-.002	.009	.000	.000	.000
RW2	.014	-.444	.001	.002	-.003	-.000	.005	.000	.000
RW3	-.061	-.268	-.002	.005	-.001	.000	-.000	.003	.000
RW4	-.078	-.126	-.009	-.009	-.005	.000	-.000	-.000	.002

Appendix 8.3. (*Next pages*) Outcome of the linear, quadratic, and correlational phenotypic selection analysis through lifetime female fecundity on characters related to plant size, flower size and shape in the studied population in 2007 and 2008. Bold values represent significant selection gradients. * P< .05, ** P< .01, *** P< .001, **** P< .0001

Em25			Character j							
			No. flowers	Corolla diameter	Corolla tube length	Corolla width	RW1	RW2	RW3	RW4
Character i	$\beta \pm$ SE	$\gamma_{ii} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE
2007										
Stalk height	.150 ± .199	-.100 ± .324	.232 ± .581	-.475 ± .865	-.554 ± .859	-1.142 ± 0.96	-.479 ± .697	.684 ± .498	-1.063 ± .615	-.529 ± .645
No. flowers	-.190 ± .182	-.071 ± .132		.246 ± .931	.538 ± .962	1.448 ± 1.005	-.509 ± .884	-.358 ± .616	.062 ± .687	.675 ± .693
Corolla diameter	.004 ± .218	-.161 ± .235			-.046 ± .611	.638 ± .882	-.148 ± .873	-.687 ± .555	.516 ± .682	-.400 ± .831
Corolla tube length	-.279 ± .197	-.202 ± .230				.942 ± .892	.345 ± 1.074	.604 ± .621	-.136 ± .781	1.882 ± .927*
Corolla width	-.202 ± .177	-.029 ± .199					1.065 ± .631	-.030 ± .423	-.456 ± .323	.486 ± .519
RW1	-.111 ± .177	.487 ± .331						.877 ± .594	.211 ± .617	-.572 ± .749
RW2	.176 ± .168	.420 ± .230							-.691 ± .548	-.820 ± .573
RW3	.176 ± .171	.088 ± .235								1.152 ± .576
RW4	-.194 ± .168	-.155 ± .313								
2008										
Stalk height	.099 ± .078	-.093±0.129	.224 ± .183	-.175 ± .119	-.193 ± .116	.301 ± .165	-.117 ± .129	-.114 ± .119	-.011 ± .119	-.051 ± .102
No. flowers	**.905 ± .074******	**.134±0.044****		.111 ± .175	.448 ± .186*	-.360 ± .314	.203 ± .228	-.427 ± .235	.310 ± .167	-.331 ± .189
Corolla diameter	.015 ± .096	.045±0.107			-.026 ± .113	.347 ± .127*	.012 ± .137	.091 ± .160	.007 ± .107	.253 ± .132
Corolla tube length	.066 ± .092	-.043±0.102				-.140 ± .136	-.260 ± .121*	.273 ± .125*	-.031 ± .109	.062 ± .125
Corolla width	.028 ± .093	.192±0.12					**.504 ± .143*****	-.051 ± .124	.038 ± .105	-.003 ± .132
RW1	.176 ± .075*	-.035±0.112						.131 ± .108	-.168 ± .100	.241 ± .114*
RW2	-.057 ± .075	.061±0.116							-.187 ± .105	.300 ± .115*
RW3	.068 ± .076	-.123±0.126								-.116 ± .109
RW4	.069 ± .080	-.163±0.131								

Em17			Character j							
			No. flowers	Corolla diameter	Corolla tube length	Corolla width	RW1	RW2	RW3	RW4
Character j	$\beta \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE
2007										
Stalk height	-.023±0.203	-.32±0.318	.700 ± .930	-1.313 ± .966	.263 ± .886	.417 ± .846	-.588 ± .920	-.694 ± 1.068	.695 ± .952	-.082 ± .716
No. flowers	-.132±0.195	-.016±0.314		-.001 ± 1.284	.211 ± 1.086	.303 ± .680	.395 ± .628	1.175 ± .936	.229 ± .880	-.446 ± .754
Corolla diameter	.024±0.235	-.187±0.250			.094 ± .604	-1.024 ± 1.044	1.006 ± .986	.392 ± .946	.132 ± .778	-.398 ± .870
Corolla tube length	.193±0.236	-.005±0.296				.418 ± .754	-.702 ± .932	-.376 ± .828	-.218 ± .950	-.26 ± .856
Corolla width	-.146±0.174	.018±0.284					-.752 ± .630	-.147 ± .674	-.41 ± .604	1.51 ± .810
RW1	.052±0.174	-.257±0.322						-.214 ± .711	.224 ± .590	-.728 ±.814
RW2	-.152±0.196	.604±0.506							.574 ± .694	-.826 ±.772
RW3	.187±0.169	-.125±0.246								-.048 ± .542
RW4	.111±0.186	.238±0.282								
2008										
Stalk height	-.009 ± .283	.054 ± .447	.198 ± 2.915	-.04 ± 1.601	-1.286 ± 1.384	-.456 ± 1.214	.653 ± .779	.141 ± .997	-.255 ± 1.056	.035 ± .940
No. flowers	.486 ± .344	-.856 ± .401*		-1.853 ± 4.352	3.991 ± 4.088	1.131 ± 2.527	.383 ± 2.515	-1.146 ± 5.135	.565 ± 3.841	.516 ± 2.457
Corolla diameter	-.484 ± .393	-.275 ± .557			-1.800 ± 1.522	-1.594 ± 2.023	-2.681 ± 1.865	.373 ± 1.614	-2.341 ± 2.623	.927 ± 1.473
Corolla tube length	.732 ± .331*	.229 ± .368				.016 ± 1.818	3.379 ± 2.092	-1.124 ± 1.273	1.262 ± 2.335	-2.718 ± 1.555
Corolla width	.150 ± .269	-.296 ± .318					-.444 ± 1.219	-.310 ± 1.169	.763 ± 1.105	.581 ± 1.212
RW1	.566 ± .264*	.325 ± .402						.123 ± 1.120	-.811 ± 1.137	-.692 ± 1.109
RW2	.342 ± .279	.031 ± .454							.479 ± .772	-1.306 ± 1.705
RW3	-.333 ± .284	.015 ± .512								-1.027 ± 1.493
RW4	-.366 ± .251	.728 ± .360								

H01			Character j							
			No. flowers	Corolla diameter	Corolla tube length	Corolla width	RW1	RW2	RW3	RW4
Character i	$\beta \pm SE$	$\gamma_{ij} \pm SE$	$\gamma_{ij} \pm SE$	$\gamma_{ij} \pm SE$	$\gamma_{ij} \pm SE$	$\gamma_{ij} \pm SE$	$\gamma_{ij} \pm SE$	$\gamma_{ij} \pm SE$	$\gamma_{ij} \pm SE$	$\gamma_{ij} \pm SE$
2007										
Stalk height	-.035 ± .138	-.420 ± .230	-.651 ± .374	1.075 ± .509*	-1.279 ± .478*	**-1.655 ± .555****	.045 ± .491	-.485 ± .393	-.074 ± .323	.110 ± .286
No. flowers	**1.017 ± .123******	.107 ± .128		-1.204 ± .696	.382 ± .453	-.183 ± .392	.212 ± .455	-.232 ± .565	.445 ± .465	-.097 ± .386
Corolla diameter	-.153 ± .187	-.079 ± .233			.538 ± .337	-.241 ± .259	.148 ± .41	-.339 ± .449	-.110 ± .461	-.055 ± .366
Corolla tube length	-.113 ± .163	-.094 ± .184				.33 ± .447	.728 ± .462	-.119 ± .502	-.497 ± .378	.144 ± .333
Corolla width	-.121 ± .150	.045 ± .087					1.134 ± .431*	.823 ± .462	.412 ± .404	-.857 ± .428
RW1	-.156 ± .126	.193 ± .210						.206 ± .332	-.591 ± .332	.710 ± .371
RW2	-.070 ± .119	-.012 ± .203							.199 ± .358	-.112 ± .223
RW3	-.032 ± .117	-.039 ± .166								.245 ± .324
RW4	-.042 ± .115	-.096 ± .227								
2008										
Stalk height	-.234 ± .182	-.222 ± .184	.230 ± .919	1.499 ± .743	-2.465 ± .865*	-.281 ± 1.023	1.359 ± .548*	.200 ± .460	-.211 ± .816	-1.608 ± .716
No. flowers	**1.224 ± .262******	-.117 ± .604		-.563 ± 1.339	-2.99 ± .956*	1.885 ± .945	**3.119 ± .578*****	-2.087 ± 1.007	**-5.760 ± 1.258****	.844 ± 1.268
Corolla diameter	.126 ± .204	.421 ± .457			.081 ± .914	**1.945 ± .522****	-.950 ±.562	.626 ± .483	.497 ± .438	1.248 ± .529*
Corolla tube length	.013 ± .186	-.065 ± .341				.719 ± .625	-1.323 ± .614	.171 ± .415	.525 ± .468	-.341 ± .402
Corolla width	-.004 ± .158	.042 ± .160					-.453 ± .706	1.645 ± .584*	-.158 ± 1.046	1.943 ± .863
RW1	.151 ± .141	-.193 ± .226						-.055 ± .378	-.552 ± .349	-.793 ± .532
RW2	.229 ± .148	-.321 ± .241							-.561 ± .495	1.226 ± .439*
RW3	.122 ± .175	-.002 ± .321								.479 ± .568
RW4	.048 ± .150	.070 ± .262								

En11			Character j							
			No. flowers	Corolla diameter	Corolla tube length	Corolla width	RW1	RW2	RW3	RW4
Character i	$\beta \pm$ SE	$\gamma_{ii} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE
2007										
Stalk height	**-.470 ± .175****	-.182 ± .261	-.050 ± .642	-.491 ± .887	1.109 ± .826	.643 ± .902	.103 ± .500	.477 ± .572	1.543 ± .803	-.185 ± .737
No. flowers	.103 ± .170	.016 ± .166		.473 ± .871	.152 ± .793	-1.253 ± 1.004	-.275 ± .871	-.438 ± 1.055	-1.158 ± .750	.160 ± .829
Corolla diameter	.485 ± .204*	-.029 ± .259			-.382 ± .538	-.016 ± .540	-.298 ± .632	.917 ± .597	-.204 ± .537	-.994 ± .610
Corolla tube length	-.099 ± .197	-.137 ± .192				.002 ± .778	-.181 ± .556	-1.225 ± .693	-1.185 ± .695	-.386 ± .629
Corolla width	-.062 ± .179	.013 ± .261					**1.92 ± .609****	-.053 ± .394	-.103 ± .487	.086 ± .552
RW1	-.038 ± .155	-.128 ± .253						-.871 ± .545	1.041 ± .598	-.215 ± .542
RW2	.148 ± .146	-.216 ± .147							.329 ± .731	-.920 ± .657
RW3	-.141 ± .158	-.002 ± .247								.205 ± .553
RW4	**-.522 ± .164****	.381 ± .253								
2008										
Stalk height	-.574 ± .270*	.293 ± .625	-1.427± 1.089	-1.851 ± 1.907	-.069 ± 1.737	-.246 ± .915	.435 ± 1.075	.416 ± 1.068	-1.122 ± 1.009	.859 ± 1.240
No. flowers	**.660 ± .238****	-.368 ± .320		-.667 ± 1.356	-.014 ± 1.342	2.467 ± 1.394	-2.141 ± .946*	.388 ± 1.202	4.631 ± 1.878*	-.414 ± 1.180
Corolla diameter	-.348 ± .344	-.316 ± .357			.458 ± .985	3.085 ± 2.027	1.992 ± 1.586	3.079 ± 1.936	-.544 ± 1.608	-3.164 ± 2.059
Corolla tube length	.482 ± .337	.078 ± .497				-.457 ± 1.361	-.594 ± 1.370	-1.959 ± 1.975	-.267 ± 1.425	1.852 ± 1.348
Corolla width	.177 ± .247	.596 ± .372					-2.324 ± 1.171	-.867 ± .924	-.540 ± 1.094	1.386 ± 1.062
RW1	-.090 ± .264	-.322 ± .456						1.285 ± 1.073	1.223 ± 1.045	.794 ± 1.071
RW2	.031 ± .244	.029 ± .492							.184 ± .664	.938 ± .954
RW3	.480 ± .241	.002 ± .293								-1.863 ± 1.335
RW4	-.074 ± .275	-.666 ± .511								

En10			Character j							
			No. flowers	Corolla diameter	Corolla tube length	Corolla width	RW1	RW2	RW3	RW4
Character i	$\beta \pm$ SE	$\gamma_{ii} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE	$\gamma_{ij} \pm$ SE
2007										
Stalk height	-.048 ± .186	-.165 ± .300	.402 ± .557	.255 ± .620	-.357 ± .666	.109 ± .452	-.736 ± .576	-.198 ± .468	-.087 ± .534	-.532 ± .660
No. flowers	-.103 ± .178	-.036 ± .351		-.084 ± .683	.460 ± .683	.213 ± .600	.873 ± .559	.427 ± .465	.732 ± .681	-.477 ± .568
Corolla diameter	-.084 ± .207	-.217 ± .274			.127 ± .468	.305 ± .631	-.105 ± .585	.211 ± .599	-.053 ± .685	-.677 ± .505
Corolla tube length	-.064 ± .191	.120 ± .349				-.797 ± .649	.642 ± .617	-.463 ± .501	-1.304 ± .525*	.000 ± .558
Corolla width	.072 ± .173	-.087 ± .134					.281 ± .637	.691 ± .620	-.035 ± .708	.272 ± .638
RW1	.196 ± .166	.177 ± .274						.445 ± .507	-.216 ± .604	**-1.843 ± .527****
RW2	-.342 ± .178	-.240 ± .288							.385 ± .546	.519 ± .672
RW3	-.086 ± .169	.077 ± .275								.446 ± .562
RW4	-.243 ± .176	.272 ± .250								
2008										
Stalk height	.101 ± .091	.016 ± .162	-.112 ± .282	.364 ± .267	-.416 ± .314	-.348 ± .262	.028 ± .247	-.096 ± .219	-.080 ± .289	.012 ± .364
No. flowers	**.667 ± .086******	.184 ± .113		-.600 ± .328	.752 ± .424	.643 ± .290*	.129 ± .226	.264 ± .197	-.520 ± .261	-.741 ± .276*
Corolla diameter	-.044 ± .101	-.168 ± .14			.165 ± .169	-.417 ± .260	-.193 ± .237	.123 ± .224	.224 ± .278	.147 ± .228
Corolla tube length	.089 ± .104	.092 ± .117				.547 ± .243*	.273 ± .210	-.203 ± .280	.164 ± .298	-.068 ± .339
Corolla width	.071 ± .08	.058 ± .106					.226 ± .217	.045 ± .208	-.289 ± .203	.048 ± .151
RW1	.003 ± .079	.037 ± .138						-.260 ± .233	.187 ± .246	.187 ± .258
RW2	.012 ± .084	.004 ± .166							-.170 ± .246	.005 ± .208
RW3	-.041 ± .082	-.155 ± .123								-.094 ± .194
RW4	**-.209 ± .078****	.024 ± .119								

Appendix 8.4. Eigenvalues (λi) and eigenvectors (Mi) of the nine latent axes with statistically significant nonlinear selection. *P*-values are from a permutation test (see Reynolds *et al.* 2010).

Population	Mi	Stalk height	Number of flowers	Corolla diameter	Corolla tube length	Corolla tube width	RW1	RW2	RW3	RW4	λi	P-value
2007												
Em17	None											
Em25	None											
En11	None											
En10	None											
2008												
Em17	None											
Em25	M2	-0.420	-0.350	-0.212	0.311	-0.458	-0.511	0.287	0.031	0.075	0.864	0.043
Em25	M6	0.406	0.175	0.128	-0.143	-0.501	0.110	0.268	0.230	0.617	-0.308	0.031
Em25	M8	-0.189	-0.054	-0.076	0.217	0.060	-0.026	-0.735	-0.056	0.600	-0.745	0.026
En11	M3	-0.009	-0.388	0.451	0.135	0.553	0.062	0.097	-0.527	0.175	2.902	0.048
En11	M4	-0.292	0.267	0.273	0.416	0.397	-0.061	-0.387	0.357	-0.396	2.194	0.055
En11	M8	-0.185	-0.307	0.095	0.148	0.012	-0.621	0.341	0.467	0.347	-4.572	0.038
En10	None											

Síntesis General

A lo largo de la presente memoria de tesis hemos analizado ciertos mecanismos involucrados en el mantenimiento de las especies como unidades evolutivas independientes. A la vez hemos explorado posibles herramientas metodológicas que permiten identificar a especies crípticas en linajes con evolución compleja, es decir implicando procesos como la hibridación, linajes con separación incompleta, introgresión, etc. Para ello hemos utilizado especies pertenecientes a un género tan diverso como evolutivamente complejo, como es el género *Erysimum* (Clot, 1992; Ancev, 2006; Marhold & Lihová, 2006). Más especificamente, nos hemos centrado en el estudio de una zona de contacto entre *E. mediohispanicum* y *E. nevadense* en Sierra Nevada (SE España).

Para comprobar la utilidad de diferentes métodos para delimitar especies críptica hemos usado poblaciones de E. nervosum s.l. localizadas en el macizo del Rif y en el Medio Atlas (***Capítulo 1***). Mientras que las técnicas de morfología estándar no permitían la suficiente resolución como para diferenciar nítidamente entre las poblaciones rifeñas y las del Atlas, el uso de caracteres morfológicos complejos, como son la forma floral y el color de la flor, nos permitió hacer un agrupamiento más acertado de estas poblaciones. Para ello, tuvieron que analizarse ambos caracteres con métodos cuantitativos multidimensionales, como las técnicas de morfometría geométrica y las técnicas de espectrofotometría para la forma y el color floral, respectivamente (***Capítulo 1***). Finalmente, el uso de técnicas moleculares fue conclusivo, ya que identificaron claramente el origen polifilético de E. nervosum s.l.. Así, esta combinación de técnicas permitió separar a las poblaciones rifeñas de E. nervosum s.l. y describirlas como una nueva especie, E. riphaeanum Lorite *et al.* (***Capítulo 1***).

Erysimum mediohispanicum y *E. nevadense* ya habían sido descritas previamente como especies diferentes (Polatschek, 1979), a pesar de ser dos especies muy similares morfológicamente y de existir zonas en las que ambas especies pudieran coexistir (Blanca *et al.* 2009).

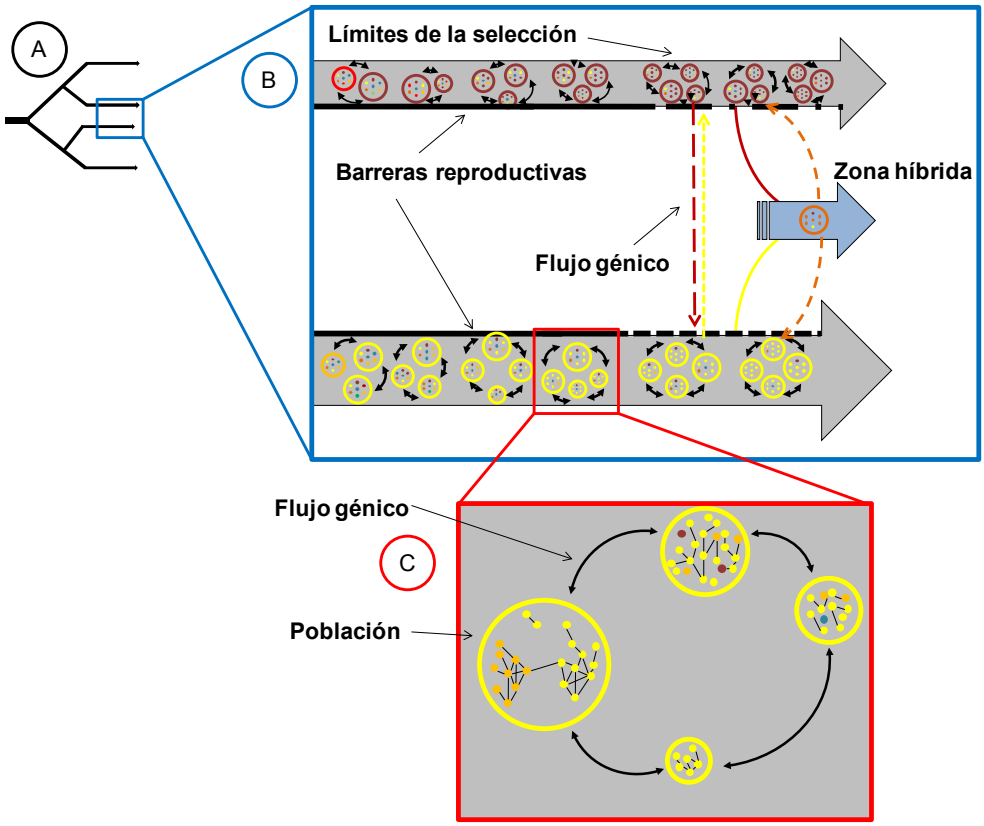


Figura s.1. Representación esquemática de los principales resultados obtenidos en el marco conceptual de la divergencia e hibridación de especies. A: Relación filogenética de especies que conforman linajes no hermanos. B: Sucesión de poblaciones de cada especie a lo largo del tiempo. Están representadas las poblaciones, el flujo génico entre ellas y los límites evolutivos impuestos por la selección. Se pueden observar las barreras de reproducción asimétricas aparecidas y el flujo génico no significativo (líneas discontinuas) entre ambas especies. Aparición de una zona híbrida entre ambas especies gracias al flujo génico efectivo hacía dicha zona desde poblaciones parentales (líneas continuas). La zona híbrida también presentará constricciones selectivas. C: Poblaciones de una de las especies entre las que puede existir flujo génico. La reproducción clasificada dentro de población puede llegar a producir subestructuras dentro de las mismas.

Nuestro estudio filogenético ha demostrado que pertenecen a linajes diferentes dentro del género, y por lo tanto son especies distintas (***Capítulo 2***). Esto nos permite adherirnos al tratamiento taxonómico realizado previamente por Polatschek (1979) en el que identifica a *E. mediohispanicum* como taxón independiente de *E. nevadense* (Polatschek, 1979), pero esta vez atendiendo al concepto evolutivo y filogenético de especie. Pero quizás el aspecto más

destacable es que nuestro estudio filogenético muestra que las zonas de contacto entre ambas especies en Sierra Nevada son de carácter secundario. En la figura s.1 se intenta integrar esquemáticamente los principales resultados que se han obtenido de la tesis dentro del marco conceptual de la divergencia e hibridación entre especies. Esta figura intenta representar la divergencia evolutiva que ha acontecido entre *E. mediohispanicum* y *E. nevadense*. Así, el panel A de la figura s.1 representa el hecho de que ambas especies no son linajes hermanos.

A pesar de no ser especies hermanas, *E. mediohispanicum* y *E. nevadense* mantienen la capacidad de reproducirse inter-específicamente generando híbridos (***Capítulo 3***). Observamos que los cruzamientos inter-específicos no reducen el fitness de forma importante respecto a los cruzamientos intra-específicos. Sin embargo, como representamos en la figura s.1, panel B, las consecuencias del cruzamiento inter-específico son asimétricas. Los cruzamientos en los que las madres fueron *mediohispanicum* y los padres *nevadense* tuvieron mayor probabilidad de éxito que los cruzamientos recíprocos (***Capítulo 3***). Dichos híbridos presentaron, a su vez, heterosis para algunos caracteres como el número de flores o el número de escapos florales (***Capítulo 6***). Sin embargo, la reproducción de los individuos híbridos (ya sea cuando se reproducen entre sí o con un parental), tuvieron una baja probabilidad de éxito medido como producción de frutos (***Capítulo 6***). Esto nos indica la posible existencia de incompatibilidades postzigóticas intrínsecas a los híbridos que estarían limitando las posibilidades de introgresión entre ambas especies. Estas disminución en éxito reproductivo de los cruzamientos inter-específicos podrían ser mayores en condiciones naturales, ya que las condiciones favorables de invernadero habitualmente mitigan dichas diferencias (Chèvre *et al.*, 2000).

Los fenómenos de hibridación son frecuentes en plantas (Emms *et al.*, 1996; Chapman *et al.*, 2005; Aldridge & Campbell, 2006; Montgomery *et al.*, 2010, entre otros), pudiendo deberse a diversas causas (Arnold *et al.*, 2010) y ser asimétricos los patrones que generan (Rieseberg & Carney, 1998; Tiffin *et al.*, 2000), como es el caso que nos ocupa (***Capítulo 3***). Para explicar estos patrones asimétricos en el éxito de los híbridos, en nuestro caso, nos

ayudó el estudio de las tasas de limitación de polen autógamo, depresión por endogamia y depresión por exogamia (***Capítulo 3***). Dichas tasas informan de las estrategias reproductivas (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987; Uyenoyama & Waller, 1991; Goodwillie *et al*., 2005; Winn *et al*., 2011) y los posibles procesos de adaptación local (Coyne & Orr, 2004; Servedio, 2000; 2004; Gavrilets, 2004) a los que estén sometidos ambas especies. *Erysimum mediohispanicum* mostró valores superiores de depresión por endogamia que *E. nevadense*, sugiriendo que esta última especie ha podido estar sujeta a eventos históricos continuados de endogamia. Alta frecuencia de reproducción endógama mantenida en el tiempo permiten la purga de álelos deletereos recesivos y disminuyen el lastre genético, haciendo a las poblaciones más resistentes a la endogamia (Charlesworth & Charlesworth, 1987). Además, los valores significativos de limitación de polen autógamo mostrados por *E. nevadense* indican su mayor resistencia a los efectos perjudiciales de la endogamia (***Capítulo 3***). Sin embargo, cuando atendemos a las tasas de depresión por exogamia, las diferencias entre ambas especies se reducen, aunque es *E. mediohispanicum* la que presentó valores ligeramente más altos (***Capítulo 3***). Este resultado coincide con trabajos previos que demuestran adaptación local en *E. mediohispanicum* como consecuencia del ajuste a la fauna local de polinizadores (Gómez *et al*., 2008a, 2008b; 2009a, 2009b).

Los análisis genéticos de las poblaciones que conforman el contacto secundario entre *E. mediohispanicum* y *E. nevadense* mostraron tasas significativas de estructuración y diferenciación entre las mismas (***Capítulo 5***). Las plantas autocompatibles de vida corta, como las especies objeto de estudio, suelen mostrar tasas elevadas de diferenciación y estructuración genética (Linhart and Grant, 1996), así como mayor capacidad de desarrollar patrones de adaptación local (Leimu and Fischer, 2008). Así mismo, el análisis genético nos confirmó la existencia de una zona híbrida entre ambas especies mantenidas por tasas significativas de flujo génico desde ciertas poblaciones parentales (Fig. s.1, B; ***Capítulo 5***). Precisamente, las altas tasas de flujo génico que existen desde poblaciones parentales hacia zonas híbridas son características de las zonas de tensión y las que explican el mantenimiento a largo plazo de este tipo de zona

híbrida (Barton & Hewitt, 1985; Harrison, 1993, ***Capítulos 5*** y ***6***). Estos patrones están representados en la figura s.1, B, en la que los únicos flujos génicos efectivos son los que se dan hacia la zona híbrida.

La hibridación parece estar favorecida por los patrones de preferencia exhibidos por los polinizadores cuando éstos encuentran individuos de *E. mediohispanicum* y *E. nevadense* en simpatría (***Capítulo 6***). De hecho, el aislamiento reproductivo pre-zigótico generado por los diferentes gremios de polinizadores, tanto en las zonas parentales como en la población híbrida, es casi nulo, existiendo tasas de vuelo inter-específico comparables a las tasas de vuelo intra-específico (***Capítulo 6***). Los polinizadores no solo no discriminan entre parentales e híbridos, sino que también favorecen la generación de toda case de híbridos de segunda generación (***Capítulo 6***). Si esta patrones de preferencia por parte de los polinizadores parecen circunscribirse a la zona de contacto entre ambas especies, contribuyendo a la persistencia temporal de la zona híbrida (Fig. s.1, B). Estos patrones ineficientes de aislamiento reproductivo pre-zigótico son propios de plantas con polinización generalista (Pascarella, 2007; Ellis & Johnson, 2012) como *E. mediohispanicum* y *E. nevadense* (***Capítulos 6*** y ***8***).

Por último, el análisis de la selección natural, sobre caracteres fenotípicos heredables (***Capítulo 7***), realizado durante dos años consecutivos, nos desveló la existencia de gradientes de selección locales, que podrían estar favoreciendo patrones de divergentes de adaptación local (***Capítulo 8***). Los gradientes de selección divergentes, encontrados entre las especies parentales y, entre éstas y la zona híbrida, pensamos que son fundamentales para el mantenimiento de dichas especies como unidades evolutivas independientes (***Capítulo 8***). Estas presiones selectivas divergentes se mantuvieron en los dos años en los que se llevó a cabo el estudio de selección natural (***Capítulo 8***), y parecen estar causadas, al menos en parte, por las preferencias diferenciales exhibidas por la gran número de especies polinizadoras (Gómez *et al*., 2008a, 2008b; 2009a, 2009b; Ortigosa & Gómez, 2010; ***Capítulos 6*** y ***8***). Así pues, mientras que las plantas de *E. mediohispanicum* están principalmente visitadas por abejas grandes y abejas pequeñas, *E. nevadense* presenta un gremio de

polinizadores dominado por hormigas y escarabajo (***Capítulos 6*** y ***8***). Estas diferencias estarían causando las presiones selectivas divergentes y generando un patrón geográfico de presiones selectivas caracterizado por el incremento de las selección divergente en los extremos del transecto (***Capítulo 8***). Así, el número de caracteres fenotípicos sometidos a gradientes de selección divergente son mayores entre las poblaciones de los extremos de la distribución y menores entre las dos poblaciones colindantes con la población híbrida (***Capítulo 8***). Las diferencias en gremios de polinizadores han sido identificadas con patrones de adaptación local (Gómez *et al*., 2009a, 2009b), así como con patrones de divergencia evolutiva y especiación (Grant, 1993; Schemske and Bradshaw, 1999). Este tipo de selección divergente prevendrá el flujo génico efectivo inter-específico (Fig s.1, B). Sin embargo, los intensos gradientes de selección positiva asociados a un carácter heterótico, como es el número de flores (***Capítulo 8***), permitirá la persistencia y éxito de los híbridos. En la zona híbrida, los individuos parentales no solo hibridan, sino que su descendencia (híbridos de primera generación) estaría favorecida debido a la preferencia de los polinizadores con los que interactua (***Capítulos 5***, ***6*** y ***8***). Sin embargo, la reproducción híbrida manifiesta una reducción en fitness que complica la persistencia temporal autónoma de la zona híbrida. Esta situación implica el aporte continuo de genotipos parentales hacia la zona híbrida para asegurar el mantenimiento temporal de la misma, y caracteriza a la zona híbrida entre *E. mediohispanicum* y *E. nevadense* como un ejemplo de zona de tensión.

En síntesis podemos decir que el mantenimiento de la integridad evolutiva de especies que aún conservan la facultad de hibridar, implica el concurso de diversos procesos y mecanismos, tanto genéticos como ecológicos. En el caso de *E. mediohispanicum* y *E. nevadense*, el mantenimiento de su independencia evolutiva implica la presencia de barreras de aislamiento tanto intrínsecas como extrínsecas, donde la interacción con los vectores polínicos juega un papel fundamental. Los resultados y métodos aquí presentados pueden ser útiles para estudiar otras posibles zonas de contacto secundario entre las especies de este complejo género. Asímismo, también

pueden ayudarnos a explorar la evolución de otros linajes con taxonomía compleja, en los que sean frecuentes los fenómenos de hibridación interespecífica, poliploidización, separación incompleta y evolución reticulada, como parece haber sido frecuente en la historia evolutiva del género *Erysimum*.

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Conclusiones

Ad astra per aspera
Lucius Annæus Seneca
(4 a. C. - 65 d. C.)

1. La combinación del análisis de caracteres morfológicos, tanto cualitativos como cuantitativos, y marcadores moleculares permite la identificación de especies crípticas, el estudio de los procesos evolutivos involucrados en la aparición de dichas especies. La combinación de estas herramientas nos permitió la identificación y descripción de una nueva especie en la cordillera del Rif, *Erysimum riphaeanum* Lorite *et al.*, cuyas poblaciones se consideraban anteriormente pertenecientes a la especie *E. nervosum*.

2. *E. mediohispanicum* y *E. nevadense* son dos especies que no forman un clado monofilético. Por tanto, la zona de contacto que presentan en Sierra Nevada es una zona de contacto secundario. Este mismo hecho se repite para la mayoría de las parejas de especies que presentan zonas de contacto en las Sierras Béticas. Así mismo, nuestro análisis filogenético nos permite concluir que el color violeta de las flores en *Erysimum* parece tener un origen polifilético, apareciendo reiteradamente a lo largo de la evolución del género.

3. *E. mediohispanicum* y *E. nevadense* no han perdido la capacidad de hibridar. Sin embargo, presentan cierto grado de inviabilidad híbrida que se da de forma asimétrica entre ambas especies. *E. nevadense* presenta mayor grado de inviabilidad híbrida que *E. mediohispanicum*.

4. *Erysimum mediohispanicum* presenta mayor depresión por endogamia que *E. nevadense*, con tasas cercanas a la autoincompatibilidad. Esto nos lleva a concluir que ambas especies pueden estar sometidas a diferentes tasas históricas de reproducción endogámica, ya sea por autofecundación o por reproducción con parientes (depresión biparental).

5. A pesar de la alta tasa de depresión por endogamia en *E. mediohispanicum*, nuestros resultados indican que existe variación individual dentro de población. Plantas con diámetro de corola mayor toleraron mejor la endogamia. Por otra parte, también encontramos una relación significativa

entre el diámetro de la corola y la heterozigosidad individual, de tal forma que plantas con corolas grandes presentaron niveles más bajos de heterozigosis. Dicho rasgo floral posiblemente determine los patrones de reproducción clasificada dentro de población mediante su efecto sobre el comportamiento de los polinizadores.

6. Existe una zona híbrida en la región de contacto entre *E. mediohispanicum* y *E. nevadense*, en la que aparecen individuos cuyos genotipos son intermedios a los de las especies parentales. Debido que la distribución de los genotipos multilocus parentales en la población no es uniforme, pensamos que la frecuencia de cruces entre híbridos y de retrocruzamientos es alta en la zona híbrida.

7. Los individuos híbridos presentan heterosis para el número de flores y el número de escapos florales, mientras que presentaron fenotipos intermedios entre ambos parentales para la altura del escapo floral. Esto patrón fue observado tanto en los condiciones controladas como en la zona híbrida natural.

8. El flujo génico entre todas las poblaciones parentales incluidas en el presente trabajo es muy bajo. A pesar de éste, encontramos una gran asimetría en la dirección del flujo génico, ocurriendo éste principalmente desde las poblaciones parentales del extremo de la distribución hacia la población híbrida. Por el contrario, apenas detectamos flujo génico desde la población híbrida hacia cualquiera de las otras poblaciones parentales.

9. *E. mediohispanicum* presenta un gremio de polinizadores dominado por abejas, mientras que *E. nevadense* presenta un gremio de polinizadores más propio de una especie de alta montaña, dominado por escarabajos y hormigas.

10. Aunque los visitantes florales son diferentes en los hábitats de *E. mediohispanicum*, *E. nevadense* y en la zona híbrida, dichos polinizadores no discriminan ni entre las especies parentales ni entre éstas y sus híbridos cuando aparecen en simpatría. No hay por lo tanto aislamiento prezigótico mediado por polinizadores.

11. En la zona híbrida, los polinizadores prefieren visitar los individuos híbridos. Esta preferencia contribuye posiblemente al mantenimiento a corto plazo de dicha zona híbrida.

12. Sin embargo, tanto los experimentos de cruzamientos controlados como los experimentos de campo sugieren que existen barreras postzigóticas entre ambas especies de *Erysimum*, ya que tanto los híbridos de primera generación como los de segunda generación presentan una reducción significativa en su fitness. Es necesaria la entrada de individuos parentales para el mantenimiento a largo plazo de la zona híbrida.

13. Encontramos presiones selectivas divergentes entre las especies parentales. La existencia de estas presiones selectivas divergentes ayudaría a prevenir la hibridación entre ambas especies.

14. Hay selección divergente entre cada una de las especies parentales y la zona híbrida. Además, el carácter sobre el que se manifiesta esta divergencia selectiva es el número de flores, que es seleccionado intensamente y de forma positiva en la zona híbrida. Como este carácter es el único que presenta vigor híbrido, la selección positiva contribuiría al mantenimiento de la zona híbrida a corto plazo.

15. La cantidad de rasgos sobre los que actúa la selección divergente va aumentando a medida que las poblaciones están más distantes geográficamente. Este patrón no concuerda con la idea de reforzamiento mediado por polinizadores.

16. Los rasgos florales seleccionados por los polinizadores presentan heredabilidad significativa, al menos en el caso de *E. mediohispanicum*, lo que sugiere que son capaces de responder evolutivamente a las presiones que dichos mutualistas ejerzan.

17. La existencia de presiones selectivas divergentes entre *E. mediohispanicum* y *E. nevadense*, su coexistencia en una zona de contacto, la no existencia de barreras prezigóticas y la capacidad de producir híbridos interespecíficos, unido a las altas tasas observadas de flujo génico desde las poblaciones parentales hacia la zona híbrida y a una mayor eficacia de los híbridos F_1 pero una menor eficacia de los híbridos de segunda generación en la zona de contacto, sugieren que la zona híbrida descrita en este trabajo es consistente con un modelo de zona de tensión híbrida estable temporalmente.

18. Como conclusión final, pensamos que *E. mediohispanicum* y *E. nevadense*, a pesar de tener conjuntos de polinizadores extremadamente generalistas y ser interfértiles, se mantendrán como unidades evolutivas independientes.

ÍNDICE DE APÉNDICES

88 **Appendix 1.1** Summary of the morphological traits measured in the four selected populations of the *E. nervosum s.l.* Note: variables cover the morphological traits observed in the genus *Erysimum* from the western Mediterranean region.

89 **Appendix 1.2** Description of Landmarks definition in genus Erysimum. Dividing the flower in four quadrants and following the trigonometric name for each one, here we define the landmark used for the study of corolla shape for each petal (Figure 1.2).

90 **Appendix 1.3** Origin of the material used in the phylogenetic analyses and GenBank accession numbers: ***Taxon***; Code; ITS1 GenBank accession; ITS2 GenBank accession; *ndhF* GenBank accession; *tabAD* GenBank accession; *Voucher specimen*, Collection locale; Herbarium.

91 **Appendix 1.4** Among-population means variation in the quantitative morphological traits (mean±SE).

115 **Appendix 2.1** Phylogenetic relationship obtained using only the nuclear region *ITS1-5.8S-ITS2* for the *Erysimum* species inhabiting the Baetic Ranges. MP: Maximum Parsimony tree obtained using PAUP, branch support was calculating by bootstrapping/ aLRT: Maximum likelihood tree obtained with PhyML, calculating branch reliability support by approximate likelihood ratio test/ ML: Maximum likelihood tree obtained with PhyML, calculating branch reliability support by bootstrapping/ BI: Tree obtained using Bayesian Markov chain Monte Carlo inference; branch supports values are posterior probabilities. Only branch supports higher than 75% are shown.

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