Generalización Estructurada:
Dinámica Evolutiva a Escala Espacial Fina en un Sistema Generalista

Universidad de Granada
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Generalización Estructurada:
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Memoria de Tesis Doctoral presentada por el licenciado Francisco Javier Valverde Morillas para optar al grado de doctor por la Universidad de Granada.

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Dedicado a mi familia,
a mi Angelita
y a mi gente.
Por los que están
y los que nos dejaron.

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Lista de publicaciones

- Valverde J., Gómez J.M., García C., Sharbel T.F., Jiménez M.N. y Perfectti F. (2016) Inter-annual maintenance of the fine-scale genetic structure in a biennial plant. Scientific reports, 6:1–11.

  (Factor de impacto: 5,228)

- Valverde J., Gómez J.M. y Perfectti F. (2016) The temporal dimension in individual-based plant pollination networks. Oikos, 125:468–479.

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- Valverde J., Calatayud J., Gómez J.M. y Perfectti F. (2014) Variación intraestacional en los visitantes florales de *Erysimum mediohispanicum* en Sierra Nevada. Ecosistemas, 23:83–92.

Resumen

La evolución de los sistemas de polinización constituye uno de los debates clave en la biología de la polinización. En el gradiente de especialización-generalización, las plantas generalistas existen en una frecuencia inesperadamente alta para lo predicho por algunas hipótesis. En este sistema de polinización, existe una gran variación espacio-temporal en la composición y frecuencia sus polinizadores, la cual se manifiesta a diferentes escalas, desde la poblacional a la individual. A escala individual, la variación en el grado de generalización se ha considerado como un patrón aleatorio exento de funcionalidad, a pesar de las evidencias que apuntan lo contrario.

Nosotros pensamos que la evolución mediada por polinizadores podría promover la evolución hacia la generalización en lo que hemos denominado la hipótesis de la generalización estructurada. En esta, la generalización individual estaría asociada a rasgos fenotípicos y genotípicos de la planta como resultado de diferentes preferencias de los visitantes florales. El grado de generalización individual afectaría al éxito reproductivo de cada planta y resultaría en unos patrones de fecundación clasificada. Consecuentemente, la estructura genética poblacional se vería modificada, y podrían surgir divergencias fenotípicas y genéticas si la estructura se mantiene a largo plazo.

En la presente tesis abordamos las bases de la generalización estructurada desde los procesos causales hasta los efectos sobre la estructura genética de la población. Para ello, usamos la especie generalista *Erysimum mediohispanicum* (Brassicaceae) como modelo de estudio. En una población natural y en dos parcelas experimentales monitorizamos una serie de individuos durante la floración, caracterizando su fenotipo y microambiente. Realizamos un seguimiento a lo largo de la floración de las interacciones con los diferentes visitantes florales. Además, genotipamos cada individuo de la población natural y una muestra de su progenie. Finalmente, determinamos la eficacia de polinización de los diferentes grupos funcionales de insectos que visitan las flores de *E. mediohispanicum*.

En el capítulo 1 evidenciamos que los genotipos en la población natural se distribuyen de forma no aleatoria en el espacio, siguiendo una estructura que se mantuvo entre los dos años de estudio. Además del patrón típico de plantas dispersadas a corta distancia, los genotipos estudiados se ajustaron a un gradiente de disponibilidad de luz. Este resultado indica un movimiento sesgado de genes que bien podría ser vía polen, sugiriendo un patrón de visita de polinizadores no aleatorio. En el capítulo 2 demostramos que ese patrón no aleatorio existe en la población estudiada, existiendo diferentes grados de generalización individual que en gran parte se asociaron a dos factores: la disponibilidad de luz y el tiempo de floración de la planta.

El tiempo de floración juega un papel muy importante en la estructuración de las interacciones. En el estudio llevado a cabo en la parcela experimental (capítulo 3), observamos como en ausencia de heterogeneidad ambiental y de autocorrelación espacial de fenotipos, existe un recambio temporal en las interacciones con los polinizadores. Este estudio indica que plantas que florecen durante más tiempo interactúan con un mayor número de especies polinizadoras. El mismo patrón lo observamos en la población natural (capitulo 4). Aquí, encontramos que la acción conjunta de la fenología de floración y de forrajeo modula la estructura de las redes planta-planta de compartición de polinizadores. De este modo, estas redes muestran una variación temporal a lo largo de la floración en cuanto a su estructura, demostrando que son contexto-dependientes. Además, la integración de todas las interacciones ocurridas durante el periodo de floración resulta en estructuras emergentes de la red. Estos resultados, evidencian que algunas características del fenotipo de la planta como la fenología de floración pueden jugar un papel importante en la generalización individual.

Para que la generalización individual tenga efecto sobre el éxito reproductivo, las especies polinizadoras deben mostrar diferentes habilidades en transferir polen de forma efectiva. Encontramos una gran variación en la eficacia de polinización entre los diferentes polinizadores de *E. mediohispanicum* (capitulo 5). Estas diferencias se deben en gran parte a la frecuencia de visita, apuntando a que el paisaje de eficacia es dependiente de contexto. Además demostramos que el polen depositado puede variar entre grupos funcionales en diversidad.

Finalmente en el capítulo 6 estudiamos los patrones de flujo de polen en la población natural. Encontramos que la dispersión de polen en esta especie ocurre fundamentalmente a corta distancia y se encuentra constreñida por la fenología de floración. Además, los patrones de flujo de polen en la población también se encuentran influenciados por el ambiente lumínico. De este modo, la red de flujo de polen resultante presentó una estructura no aleatoria bien definida. La distribución de grado siguió una ley potencial truncada indicando un crecimiento de la red constreñida a los nodos muy conectados. Además esta red fue modular, indicando que los entrecruzamientos ocurren más frecuentemente entre grupos de plantas. Finalmente, mostramos cómo el estudio del movimiento de forrajeo de los polinizadores es una buena aproximación para el estudio de los patrones de flujo de polen.

Abstract

The evolution of plant pollination systems is one of the main subjects of debate in the fore of pollination biology. Along the gradient of specialization-generalization, generalist plants exist in higher frequencies than those expected by some theoretical expectancies. Plants with this pollination system exhibit great spatio-temporal variations in the pollinator assemblage that manifests at different scales, from the populational to the individual level. At the individual level this variation has been considered to be random and extent of any functionality. However, there exist evidences against this thought.

We think that generalization might result from a pollinator-mediated evolution in what we have labeled structured generalization. In this hypothesis the individual level of generalization is associated to plant phenotype and genotype as a result of the different preferences of pollinators. The individual generalization will affect to the plant's reproductive output and result in an assortative mating. Consequently, the populational genetic structure may be affected and phenotypic and genetic divergences may arise if this structure is maintain over time.

In this study we explore the basis of the structured generalization from its causes to its effects on the population genetic structure. To do so, we use the generalist plant species *Erysimum mediohispanicum* (Brassicaceae) as a species model. In a natural population and in two experimental plots we monitorized a series of marked individuals during their flowering time. For each individual plant, we characterized its phenotype and microenvironment and surveyed all plant interactions with its pollinators along the flowering season. Additionally, we genotyped each marked individual as well as part of their progeny. Finally, we characterized the effectiveness of pollination of the different functional groups visiting the flowers of *E. mediohispanicum*.

In the chapter 1 we show that genotypes in the natural population presented a non-random spatial distribution and that the spatial structure was maintained among consecutive flowering cohorts. Genotypes showed the typical spatial distribution of plants dispersed at short distances, but also were distributed following a environmental light gradient. This result indicates a constrained gene flow that could be due pollen dispersal, suggesting a non-random pattern of pollinator visits. In the chapter 2 we showed that this non-random pattern of pollinator visit exists in the natural population and that results in differing degrees of individual generalization. We also demonstrate that the individual generalization was associated to two main factors: light availability and flowering duration.

Flowering time plays an important role in the structuration of interactions. In the experimental plots (chapter 3) we observed that in the absence of

spatial autocorrelation of environmental variables and phenotypes, there exists a temporal turnover in the interactions with the pollinators. This study indicates that plants flowering for longer periods interact with more species of pollinators. This same pattern was observed in the natural population (chapter 4). We showed that the joint action of the flowering and pollinator foraging phenologies modulates the structure of the plant-plant networks built on the pollinator sharing. These networks show a temporal variation in their topology along the flowering season demonstrating that they are context-dependent. Moreover, the integration of all the interactions occurring along the flowering season result in emerging network properties: network cohesion, transitivity y centrality. These results evidence that some phenotypic characteristics such as flowering time can be important in the level of individual generalization.

For the individual generalization to affect the reproductive output of pants the pollinating species should differ in the abilities in transferring pollen efficiently. We have found a great variation in the pollinator effectiveness among the different functional groups of pollinators of *E. mediohispanicum* (chapter 5). These differences result in great part from the visitation frequency indicating that the effectiveness landscape in context-dependent. Moreover we have demonstrated that the diversity of transferred pollen also varies among functional groups.

Finally, in the chapter 6 we study the patterns of pollen effective flow occurring in the natural population. We found that pollen dispersal mainly occurs at short distances and that it is constrained by the flowering time. Moreover, the patterns of pollen flow are also influenced by the light availability. As a result, the mating network presents a non-random topology. The network showed a truncated power-law structure indicating a growing mechanism constrained to highly conected nodes. Additionally the network was modular, indicating that mating occurred more frequently among well defined groups of plants. Finally, we demonstrate that foraging movement of pollinators represents a good proxy of the mating networks.

Parte I

Prólogo | Prologue

Introducción general

Generalización estructurada.

En las plantas polinizadas por animales los sistemas de polinización varían a lo largo de un gradiente de especialización-generalización (Waser *et al.*, 1996), donde las plantas especialistas son aquellas cuyas flores son visitadas por una o unas pocas especies de animales similares, mientras que las generalistas son visitadas por un conjunto diverso de animales. En el estudio de la evolución de los sistemas de polinización, la corriente principal de pensamiento aboga por la hipótesis del polinizador más efectivo (Stebbins, 1970). Esta hipótesis predice una tendencia general a la especialización derivada de la ventaja de desarrollar rasgos florales que aumenten la tasa de visita de los polinizadores más eficaces. Sin embargo, y contrario a las predicciones derivadas de esta hipótesis, la generalización ocurre en la naturaleza con una frecuencia inesperadamente alta (Herrera, 1996; Waser *et al.*, 1996).

Entre los mecanismos propuestos para explicar la persistencia de la generalización se encuentra la variación espacio-temporal de los agentes selectivos. Así, algunos estudios han demostrado que la generalización no es una propiedad invariante de las especies (Herrera, 2005). Varios autores han observado una gran variación en el grado de generalización entre poblaciones (Moeller, 2005; Price *et al.*, 2005; Petanidou *et al.*, 2008; Dupont *et al.*, 2009; Gómez *et al.*, 2009b, 2014a), al igual que una gran variación en el gremio de polinizadores a diferentes escalas temporales (Herrera, 1988; Ashman y Stanton, 1991; Cane *et al.*, 2005; Price *et al.*, 2005; Dupont *et al.*, 2009). En la dimensión espacial los diferentes regímenes de selección que actúan sobre los rasgos vegetales como consecuencia de la variación en composición y abundancia de polinizadores estarían diluidos por la existencia de eventos de flujo genético. En la dimensión temporal, la propia fluctuación en el gremio de polinizadores conllevaría una fluctuación en las presiones selectivas ejercidas por estos. Estas variaciones anularían la acción de la selección natural direccional, dando lugar a una generalización no adaptativa. Hay que apuntar, sin embargo, que la generalización también puede ser adaptativa. Este tipo de generalización surgiría a partir de la convergencia de varios agentes selectivos en su eficacia de polinización y preferencia sobre un mismo rasgo floral (Armbruster *et al.*, 2000; Gómez y Zamora, 2006), dando lugar a la adaptación a un conjunto diverso de polinizadores.

Las variaciones espacio-temporales en el grado de generalización se han observado también entre individuos de una misma población (Herrera, 2005; Gómez *et al.*, 2011; Dupont *et al.*, 2014). La variación a esta escala se asume que es el resultado de un proceso aleatorio y que carece de funcionalidad

al no conferir ningún efecto en el éxito reproductivo. Sin embargo, existen evidencias suficientes para pensar lo contrario. En primer lugar, diversos estudios han demostrado la preferencia de algunos polinizadores por diferentes rasgos fenotípicos y microambientales (Herrera, 1995a,b; Lee *et al.*, 2001; Benitez-Vieyra *et al.*, 2006; Gómez *et al.*, 2006b; Norgate *et al.*, 2010; Malerba y Nattero, 2012), sugiriendo que la variación interindividual no es aleatoria. En segundo lugar, se conoce que el grado de generalización individual afecta al éxito reproductivo medido como producción de semillas y frutos (Kremen *et al.*, 2002; Klein *et al.*, 2003), evidenciando la funcionalidad de dicha variación. Finalmente, existen evidencias de cruzamientos no aleatorios a nivel intrapoblacional que resultan del pecoreo no aleatorio de los polinizadores (Kamm *et al.*, 2010; Dyer *et al.*, 2012; Ison *et al.*, 2014), confiriendo a la variación interindividual una clara importancia en los procesos evolutivos a una escala espacial fina. El estudio de la variación individual en el grado de generalización es, por tanto, fundamental para comprender los procesos evolutivos que promueven la generalización o especialización en los sistemas de polinización.

Nosotros pensamos que en las especies generalistas la evolución mediada por polinizadores no conllevaría necesariamente una evolución hacia la especialización, tal como sostiene la hipótesis del polinizador más efectivo. Por el contrario, pensamos que los sistemas de polinización generalistas puede evolucionar tanto macro- como micro-evolutivamente como consecuencia de cambios en los nichos de polinización generalistas. Esta hipótesis, que hemos denominado hipótesis de la generalización estructurada, se sustenta en las siguientes premisas:

1. A nivel micro-evolutivo, los individuos de una población varían en los nichos de polinización, es decir, interactúan con subconjuntos diferentes de polinizadores.

2. La variación en nichos de polinización está asociada funcionalmente con diferencias en rasgos fenotípicos y genotípicos, ya sean caracteres florales o fenológicos, como consecuencia de diferencias en los patrones de preferencia de los polinizadores.

3. Como consecuencia hay un éxito reproductivo diferencial y una reproducción clasificada, de tal forma que los individuos que comparten nichos de polinización tienen una mayor probabilidad de entrecruzarse entre sí que con individuos que presentan diferentes nichos de polinización.

Si se dan estas tres condiciones, esperaríamos:

1. Aparición de una estructura genética a nivel intra-poblacional debido a al cruzamiento no aleatorio.

2. Divergencia fenotípica y genética a medio y largo plazo como fruto de la interacción continuada con diferentes conjuntos de polinizadores, siempre que se mantenga en el tiempo la estructura en las interacciones.

No obstante, esta divergencia podría verse diluida por cualquier factor extrínseco que produzca covarianza ambiental (van Benthem *et al.*, 2017), o por las propias fluctuaciones del sistema. Por tanto, todo estudio serio sobre este fenómeno debería tener en cuenta estos factores ambientales.

La estructura genética espacial como producto de la generalización estructurada.

La disparidad entre plantas en el conjunto de polinizadores puede verse reflejado en una estructura genética espacial (*SGS*, 'spatial genetic structure'). Esta se refiere a la distribución no aleatoria de los genotipos en el espacio y resulta del equilibrio entre la deriva genética, la selección local y el flujo de genes (Wright, 1943; Epperson, 1993; Vekemans y Hardy, 2004). En relación a lo último, tanto la dispersión de semillas como la de polen pueden estar sesgadas y provocar un movimiento de alelos no aleatorio. La gran mayoría de trabajos que estudian la *SGS* achacan estos patrones a la dispersión de semillas, sobretodo a las restricciones espaciales para su dispersión, las cuales provocan una disminución del grado de parentesco con la distancia conocido como aislamiento por distancia (*IBD*, 'isolation by distance'; Wright, 1943). Sin embargo, el *IBD* también puede producirse cuando el flujo de polen ocurre principalmente entre plantas cercanas en el espacio (Zhou y Chen, 2010). La optimización de los patrones de forrajeo supone la prevalencia de vuelos a corta distancia (Pyke *et al.*, 1977; Zimmerman, 1982), promoviendo los cruzamientos cercanos en el espacio y contribuyendo a generar una SGS.

El *IBD* normalmente se ha computado de forma isotrópica (Vekemans y Hardy, 2004), es decir, obviando posibles patrones de variación en intensidad a diferentes direcciones espaciales. Varios estudios han demostrado, sin embargo, la existencia de estructuras genéticas anisotrópicas, donde la intensidad del *IBD* realmente varía con la dirección (Born *et al.*, 2011; Rhodes *et al.*, 2014; DiLeo *et al.*, 2014; Wang *et al.*, 2016). La heterogeneidad espacial en factores ambientales puede favorecer o restringir el reclutamiento (Howe y Smallwood, 1982) modificando el flujo genético en una dirección dada. Pero, por otro lado, existen suficientes evidencias de que el flujo de polen puede también producir *SGS* anisotrópicas. En concreto se ha visto que los patrones de cruzamiento pueden verse favorecidos entre plantas que comparten ciertas características. Entre estas, se han citado algunas de carácter ambiental, como la disponibilidad de luz bajo el dosel (Kamm *et al.*, 2010; Dyer *et al.*, 2012), pero también fenotípicas como el tiempo de floración (Weis *et al.*, 2014) o caracteres florales

(Kulkarni, 1999). No obstante, el papel del flujo polínico en la configuración espacial de genotipos ha permanecido en un segundo plano.

Así, nosotros pensamos que la *SGS* puede ser un síntoma que evidencie la estructuración de los patrones de interacción entre plantas y polinizadores. Puesto que ante dicha estructuración las plantas que compartan más polinizadores tendrán una mayor probabilidad de cruzamiento, se espera que el fujo de polen no sea aleatorio. Por ende, la exploración de la existencia de una *SGS* y su asociación con factores ambientales o fenológicos ha de ser uno de los pilares en los que se base el estudio de la generalización estructurada.

Generalización individual

Como se ha comentado anteriormente, la generalización estructurada comprendería la estructuración de las interacciones entre plantas y polinizadores a nivel individual. La compartimentación y segregación de nicho han sido ampliamente estudiada en animales (Bolnick *et al.*, 2003), aunque también ha sido observada en plantas (Dupont *et al.*, 2014). Concretamente, en nuestra especie de estudio, Gómez *et al.* (2011) observaron una amplia variación entre plantas en composición y frecuencia de polinizadores. Estos autores además observaron que esa disparidad afectaba al éxito reproductivo individual (Gómez y Perfectti, 2012), sugiriendo que las diferencias entre individuos en el gremio de polinizadores puede suponer un flujo genético vía polen no aleatorio.

La variación de nicho entre individuos de una misma especie es importante para cualquier proceso evolutivo (Hallgrímsson y Hall, 2005). Esto ha hecho que actualmente exista un creciente interés en determinar dicha variación (Bolnick *et al.*, 2003; Araújo *et al.*, 2011; Pires *et al.*, 2011; Tur *et al.*, 2014). Esta determinación ha sido abordada usando diversas estrategias e índices. Entre otros, se ha estudiado en función de la amplitud nicho (Araújo *et al.*, 2008), composición (Johnson, 2010; Pauw, 2013) y solapamiento con el total de la población (Bolnick *et al.*, 2002). La estructuración de nicho puede deberse a varios factores. Por ejemplo, Tur *et al.* (2015) estudiaron la interacción entre individuos de polinizadores y diferentes especies de plantas. Estos autores encontraron que las diferencias en fenología de floración estructuraban las interacciones en grupos de plantas y polinizadores bien definidos en el tiempo. Por otro lado, Herrera (1995a) demostró en *Lavandula latifolia* cómo la variación interindividual en características microambientales influye en la composición de polinizadores. Finalmente, en nuestra especie de estudio, existen evidencias de atracción diferencial de los polinizadores por algunas características fenotípicas (Gómez *et al.*, 2006b, 2008b; Gómez y Perfectti, 2010).

En este sentido, la validación de la existencia de una estructuración en las interacciones y el estudio de los factores asociados a cada descriptor de dicha

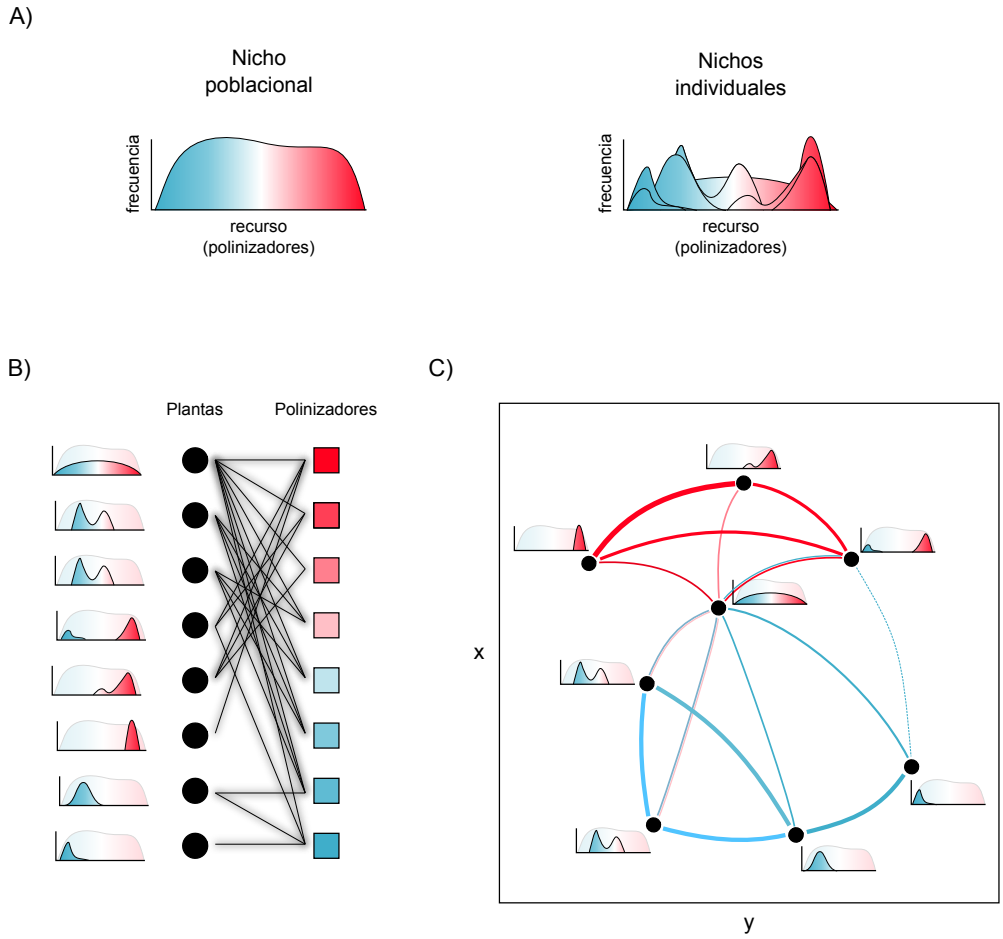


Figura 1: *Hipotética generalización individual y similitud en nicho de polinización. A) Ideograma del nicho de una población (izquierda) y su estructuración en nichos individuales (derecha). B) Red bipartita de esta interacción. C) Localización espacial de estas mismas plantas en el espacio y red unipartita de compartición de polinizadores. Las conexiones y su grosor son función de la compartición de nicho.*

variación asentaría posteriores líneas de trabajo que exploren los mecanismos que subyacen en la generalización estructurada.

Factores que afectan a la generalización individual

Entre las razones más citadas como promotoras de la evolución hacia los sistemas generalistas se encuentra la fluctuación espacio-temporal en la abundancia y composición de los polinizadores. En lo referente a la dimensión espacial, en *E. mediohispanicum* se ha demostrado que la variación en la forma de la corola existente entre diferentes poblaciones de Sierra Nevada

(Granada) está asociada a una variación espacial en la composición del gremio de polinizadores. Junto con los estudios de selección realizados en esta misma especie, estos resultados sugieren que diferentes abundancias de polinizadores modelan el fenotipo floral, y por tanto, demostrarían un mosaico selectivo a nivel de mesoescala (Gómez *et al.*, 2006b, 2008b, 2009a,b; Gómez y Perfectti, 2010). Sin embargo, a nivel intrapoblacional esta asociación aún no ha sido observada, a pesar de los estudios demostrando la variación interindividual en polinizadores (Gómez *et al.*, 2011; Gómez y Perfectti, 2012).

Otro rasgo fenotípico candidato a estructurar las interacciones es la fenología de floración. Las poblaciones de plantas están formadas por individuos que varían en su tiempo de floración (Primack, 1980; Augspurger, 1983; Marquis, 1988; Elzinga *et al.*, 2007). Igualmente, los insectos polinizadores pecorean en diferentes momentos del año (Herrera, 1988; Cane *et al.*, 2005). Esto supone que la probabilidad de interacción de una planta individual con una especie de polinizador va a depender del grado de solapamiento entre sus fenologías. De tal modo que individuos que florezcan en diferentes momentos van a interaccionar con diferentes subconjuntos de polinizadores (Ison y Wagenius, 2014). Como resultado cabe esperar que la acción conjunta de ambas fenologías estructure las interacciones entre plantas y polinizadores (Tur *et al.*, 2015) y, además, determine la verosimilitud de cruzamiento entre ellas (Weis *et al.*, 2005; Ison *et al.*, 2014).

Los efectos de la fenología en las interacciones entre especies de plantas y de polinizadores se han estudiado mediante redes bipartitas (Olesen *et al.*, 2008, 2011b). Una red bipartita esta formada por dos conjuntos de participantes o nodos (en este caso, plantas y polinizadores), en el que cada nodo sólo se conecta con nodos del otro conjunto (Jordano, 1987; Jordano *et al.*, 2003) (Figura 1b). En el caso que nos concierne, las conexiones son eventos de polinización, o al menos de contacto legítimo de un polinizador con las flores de una planta. Los estudios que han utilizado redes bipartitas para estudiar la dinámica temporal de estas interacciones han demostrado que el recambio temporal de plantas y polinizadores supone también un recambio en las interacciones (Olesen *et al.*, 2008, 2011b). A nivel de individuo, sin embargo, el efecto de la fenología en las redes de interacción están aún por explorar.

El uso de las redes adquiere mayor relevancia cuando estas expresan una función. A nivel intrapoblacional y desde el punto de vista fitocéntrico, una función de gran relevancia es la probabilidad de cruzamiento. La transformación de una red bipartita a una red unipartita donde los nodos representan las plantas y los enlaces el grado de similitud en el gremio de polinizadores (Figura 1c) se ha utilizado como símil de una red de probabilidad de cruzamiento (Gómez *et al.*, 2011; Gómez y Perfectti, 2012). Nosotros pensamos que el uso de estas redes planta-planta podría ayudar a estudiar desde una perspectiva más funcional los efectos de la fenología en la estructuración y dinámica de las

interacciones planta-polinizador.

Efectividad de polinización

La variación espacio-temporal determina gran parte de los efectos que los diferentes polinizadores tienen sobre el éxito reproductivo de las plantas. La frecuencia de visita afecta a la capacidad de cada especie animal en transferir polen de forma eficiente, constituyendo la efectividad[1] de polinización (Ne'eman *et al.*, 2010; Freitas, 2013; Rodríguez-Rodríguez *et al.*, 2013). En plantas generalistas, donde el componente espacio-temporal tiene un peso importante en la frecuencia y composición del conjunto de polinizadores (Herrera, 1989; Gómez y Zamora, 1999; Sahli y Conner, 2007; Robertson y Leavitt, 2011; Watts *et al.*, 2012), es esperable que la efectividad de un polinizador ('effectiveness') varíe ante diferentes regímenes de polinización (Waser *et al.*, 1996).

Las diferencias entre taxones en la efectividad de polinización ponen en evidencia que el grado de generalización observada (generalización estructural) puede verse reducida al considerar la capacidad de transferencia de polen (generalización funcional). Así, la caracterización de la eficacia de polinización en plantas generalistas debería ser un pilar fundamental del estudio de la evolución de estos sistemas de polinización, puesto que nos permite estimar la importancia potencial de cada polinizador como agente selectivo (Van Der Niet *et al.*, 2014). Además, a nivel de planta individual esta caracterización ayudaría a responder a preguntas relacionadas con la dinámica poblacional. Por ejemplo, frente a un escenario de generalización estructurada, ayudaría a cuantificar la contribución de cada taxón polinizador al éxito reproductivo asociado a diferentes grados de generalización. Por otro lado, y siguiendo los trabajos de Gómez *et al.* (2011) y de Gómez y Perfectti (2012), la determinación de esta eficacia ayudaría a perfilar las redes de similitud antes descritas como símil de probabilidades de cruzamiento.

[1] En inglés estas definiciones han sido motivo de controversia (Inouye, 1994; Freitas, 2013). En castellano decidimos usar eficacia como sinónimo de la capacidad de producir semillas tras las visita a una flor virgen. Usamos efectividad (equilibrio entre eficacia y eficiencia) para hablar de la contribución total al éxito reproductivo de la planta, es decir, la eficacia pesada por la frecuencia de visita.

Figura 2: *En la figura de la página contigua mostramos el que pensamos que sería el esquema funcional de la generalización estructurada. Para ello, usamos una población hipotética y nos centramos en ocho individuos. Los paneles superiores presentan la plantilla espacial de partida, con la distribución de los individuos, de su fenotipo y genotipo, y además muestra la heterogeneidad espacial ambiental. Los paneles del medio muestran los efectos del ambiente y del fenotipo sobre la compartición de polinizadores y los hipotéticos efectos sobre la probabilidad de flujo de polen. Los paneles inferiores muestran flujo de polen realizado y la estructura espacial que genera.*

A) La distribución espacial de todas las plantas en esta población, destacando las ocho plantas elegidas.

B) El valor de cualquier caracter fenotípico discreto y la distribución espacial de dos ambientes distintos.

C) Estructura genética espacial definida por las ocho plantas focales. La distribución de genotipos en el espacio se representan con una variable continua ficticia.

D) Efectos ambientales sobre la compartición de polinizadores. Las plantas están conectadas en función de las preferencias dispares de dos polinizadores (1 y 2) hacia los dos ambientes distintos. El subpanel inferior muestra la preferencia de cada polinizador sobre el ambiente A. El papel de las constricciones ambientales en la generalización estructurada es dudoso debido a que en un principio no esta asociado al genotipo.

E) Efectos del fenotipo. Constricciones de la fenología de floración sobre la compartición de polinizadores. Las plantas están conectadas si sus fenologías solapan. El subpanel inferior muestra el tiempo de floración de cada planta.

F) Efectos del fenotipo. Igual que en el panel D, las plantas están conectadas en función de la preferencia de dos polinizadores (3 y 4) sobre un fenotipo concreto. En este caso solamente mostramos las conexiones definidas por el polinizador 3 como resultado de su preferencia sobre el fenotipo A.

G) Distribución de la generalización individual y patrón de flujo de polen realizado. Al lado de cada planta se muestra su nicho de polinización (ver Figura 1). La flechas representan flujo polínico entre plantas. El grosor de estas indica la intensidad de este flujo, definida por las diferentes constricciones al flujo polínico destacadas en los paneles D, E y F.

H) Proceso dispersivo a corta distancia de la progenie resultante. El subpanel inferior muestra un kernel de dispersión típico de estos procesos constreñidos en el espacio.

I) Estructura genética espacial de la progenie resultante de todos los procesos antes descritos.

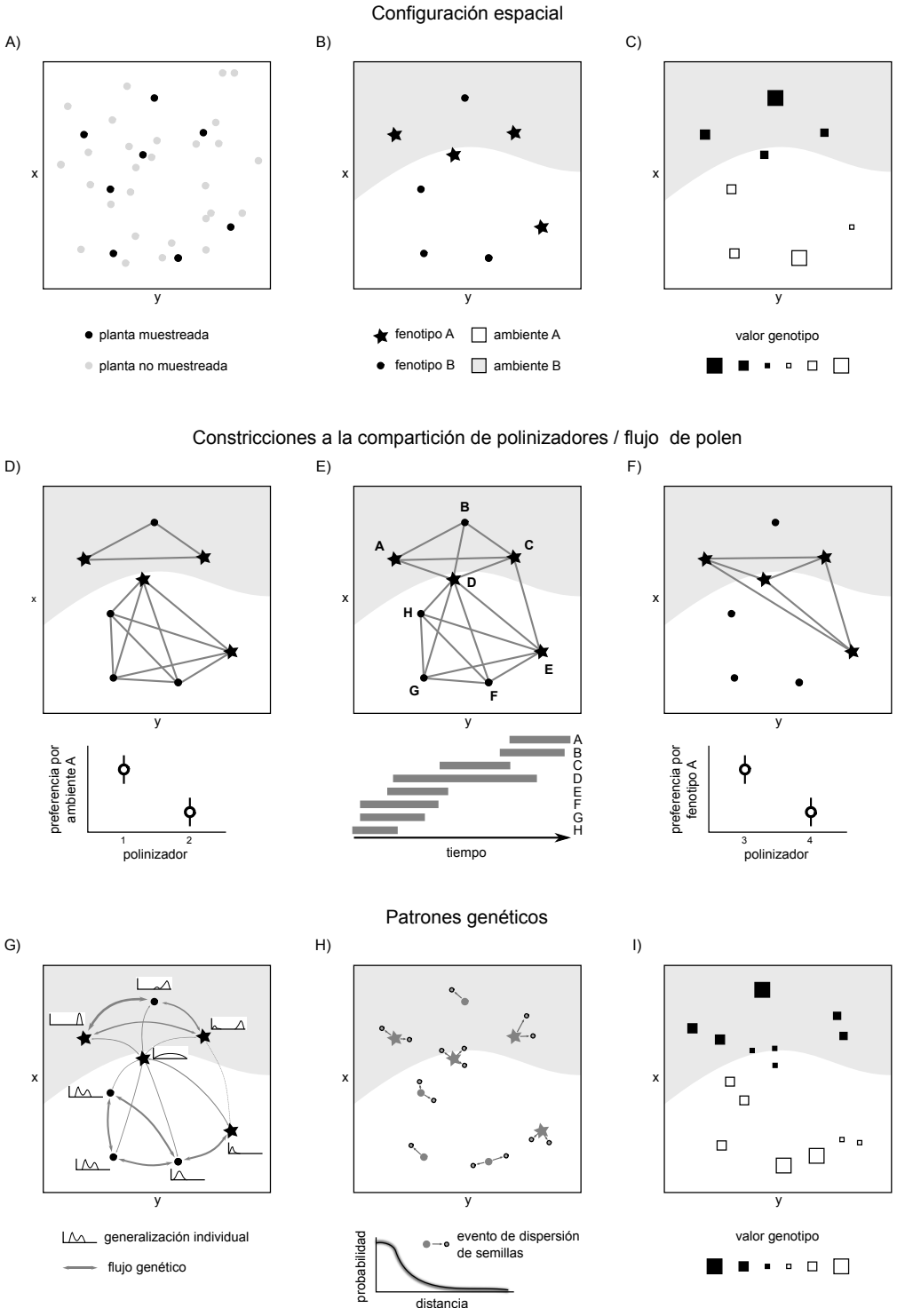


Figura 2: *Esquema funcional de la generalización estructurada.*

Redes de flujo de polen

El movimiento de polinizadores determina el flujo de polen entre plantas de una misma especie. Este flujo normalmente presenta ciertos patrones no aleatorios que resultan de constricciones al vuelo de los polinizadores. El patrón más frecuente es aquel en el que la probabilidad de cruzamiento disminuye con la distancia entre pares de plantas (Buehler *et al.*, 2012; Fortuna *et al.*, 2008). Este tipo de flujo polínico, junto con la dispersión de semillas a corta distancia, es responsable de la estructura genética espacial más común en las poblaciones de plantas, aquella en la que el grado de parentesco disminuye con la distancia. Este patrón puede ser modificado por otra series de constricciones como, por ejemplo, por las impuestas por la fenología de floración. Como hemos visto, diferentes fenologías de floración en conjunción con un recambio en el conjunto de polinizadores condiciona las interacciones entre plantas y polinizadores. Además restringe las probabilidades de cruzamiento entre pares de plantas de tal modo que puede modular el efecto de la distancia espacial en los patrones de cruzamiento (Ison *et al.*, 2014; Ison y Wagenius, 2014).

Por otro lado, existen otros factores que influyen en los patrones de flujo de polen, como pueden ser la distribución espacial de plantas (Dyer y Sork, 2001; Meagher y Vassiliadis, 2003), la heterogeneidad de hábitat (Lander *et al.*, 2013), la disponibilidad de luz (Kamm *et al.*, 2010; Dyer *et al.*, 2012), o diversos caracteres fenotípicos (Jones y Reithel, 2001; Kobayashi *et al.*, 2010; Van Der Niet *et al.*, 2014). Como resultado, estos factores pueden también generar una red de flujo polínico no aleatoria y con una estructura definida (Fortuna *et al.*, 2008).

Nosotros pensamos que bajo la hipótesis de la generalización estructurada, la estructuración de las interacciones planta-polinizador conllevaría unos patrones de reproducción clasificada. Los mismos factores determinantes de la segregación de nicho entre polinizadores actuarían concomitantemente facilitando el flujo de polen entre plantas con similares características. Finalmente, esta reproducción clasificada afectaría a la estructura genética espacial de la población.

Objetivos de la tesis

En la presente tesis exploramos la hipótesis de la **generalización estructurada**, respondiendo a los siguientes objetivos generales: Cuantificar la **generalización individual** (capítulos 2 y 4), identificar los **factores que influyen a la generalización individual** (capítulos 2, 3 y 4), conocer las **consecuencias funcionales de la generalización estructurada** (capítulos 1 y 6), y cuantificar la **eficacia de los polinizadores** de *Erysimum mediohispanicum* (capítulo 5).

A continuación resumimos los objetivos específicos de cada capítulo:

Capítulo 1

1. Caracterizar la estructura genética espacial de una población natural de *E. mediohispanicum*.
2. Testar la concordancia temporal entre cohortes de floración de esta estructura.

Capítulo 2

4. Determinar la variación en el grado de generalización individual en esta población en dos años consecutivos.
5. Explorar la asociación de esta variación con caracteres fenotípicos, así como con características ambientales.

Capítulo 3

6. Analizar la dinámica temporal en los patrones de interacción en una población experimental ausente de heterogeneidad ambiental y estructuración espacial en caracteres fenotípicos.

Capítulo 4

7. Analizar la dinámica temporal en los patrones de interacción en la población natural en dos años consecutivos.
8. Explorar los efectos de esta dinámica sobre las redes planta-planta de compartición de polinizadores.

Capítulo 5

9. Caracterizar la eficacia de polinización del gremio de polinizadores de *E. mediohispanicum* mediante la integración del componente de cantidad (frecuencia de vista) y de calidad (efecto sobre el éxito reproductivo por visita a una flor).

10. Comparar la capacidad de transferencia de polen diverso de los polinizadores más efectivos.

Capítulo 6

11. Estudiar los factores que influyen en los eventos de polinización efectiva. En concreto, los efectos de la distancia espacial y la fenología de floración, además de varios rasgos fenotípicos y factores ambientales.

12. Explorar la estructura de la red de flujo de polen, su distribución de grado y modularidad.

13. Analizar la relación de la red de polinización con tres redes planta-planta: red de compartición de polinizadores, red de compartición de polinizadores pesada por la eficacia de polinización, y red de movimiento de polinizadores.

Material y métodos generales

Los datos de campo de la presente tesis doctoral se colectaron durante los años 2010 al 2014 en el parque natural de Sierra Nevada, Granada. Los diferentes experimentos que comprenden la tesis se llevaron a cabo en dos zonas: una población natural de *E. mediohispanicum* (Em21) y dos parcelas experimentales (HP). En ambas zonas, se monitorizaron una serie de individuos durante la época de floración y se realizó una caracterización minuciosa de su fenotipo y microambiente, así como un seguimiento pormenorizado en el tiempo de las interacciones de estas con los diferentes visitantes florales. Además, genotipamos los individuos de la población natural Em21 y una muestra de su progenie. Adicionalmente en los años 2011-2014, llevamos a cabo un experimento con el que caracterizamos la eficacia de polinización de los diferentes grupos funcionales de insectos que visitan las flores de *E. mediohispanicum*.

Especie de estudio

Erysimum mediohispanicum (Brassicaceae) es una especie endémica de la Península Ibérica (Nieto-Feliner, 2003), en la que se distinguen dos zonas de distribución (Figura 1a). Se trata de una planta bienal y semélpara. Los individuos crecen vegetativamente como una roseta a ras del suelo durante dos o tres años, tras los cuales entran en fase reproductiva y mueren. En esta fase, producen de uno a ocho escapos florales con una inflorescencia corimbiforme. A medida que el escapo crece, las flores se desarrollan a lo largo de este desde la parte basal a la apical, siendo los primordios florales inferiores de desarrollo más temprano que los superiores. Las flores son actinomorfas, hermafroditas, con cuatro pétalos amarillos y cuatro sépalos libres que confieren una forma un tanto tubular a la corola. Poseen un androceo tetradínamo y un gineceo súpero con dos carpelos soldados ($\oplus$ ☿ K_{2+2} C_4 A_{4+2} $\underline{G}_{(2)}$; Figura 1b y c) (Subrahmanyam, 2009). Las flores son ligeramente protándicas, y el gineceo, al madurar, crece llegando incluso a sobrepasar las anteras.

Erysimum mediohispanicum es parcialmente autocompatible (Abdelaziz, 2013). No obstante, requiere del concurso de polinizadores para maximizar la producción de semillas (Gómez, 2005), las cuales se dispersan por barocoria a cortas distancias. Su sistema de polinización se caracteriza por ser ampliamente generalista, siendo varios cientos de especies las que visitan sus flores (Gómez *et al.*, 2009b, 2014a) (Figura 1d). En el Apéndice 1 se ofrece una descripción detallada de los grupos funcionales y se muestran algunas de las especies polinizadoras.

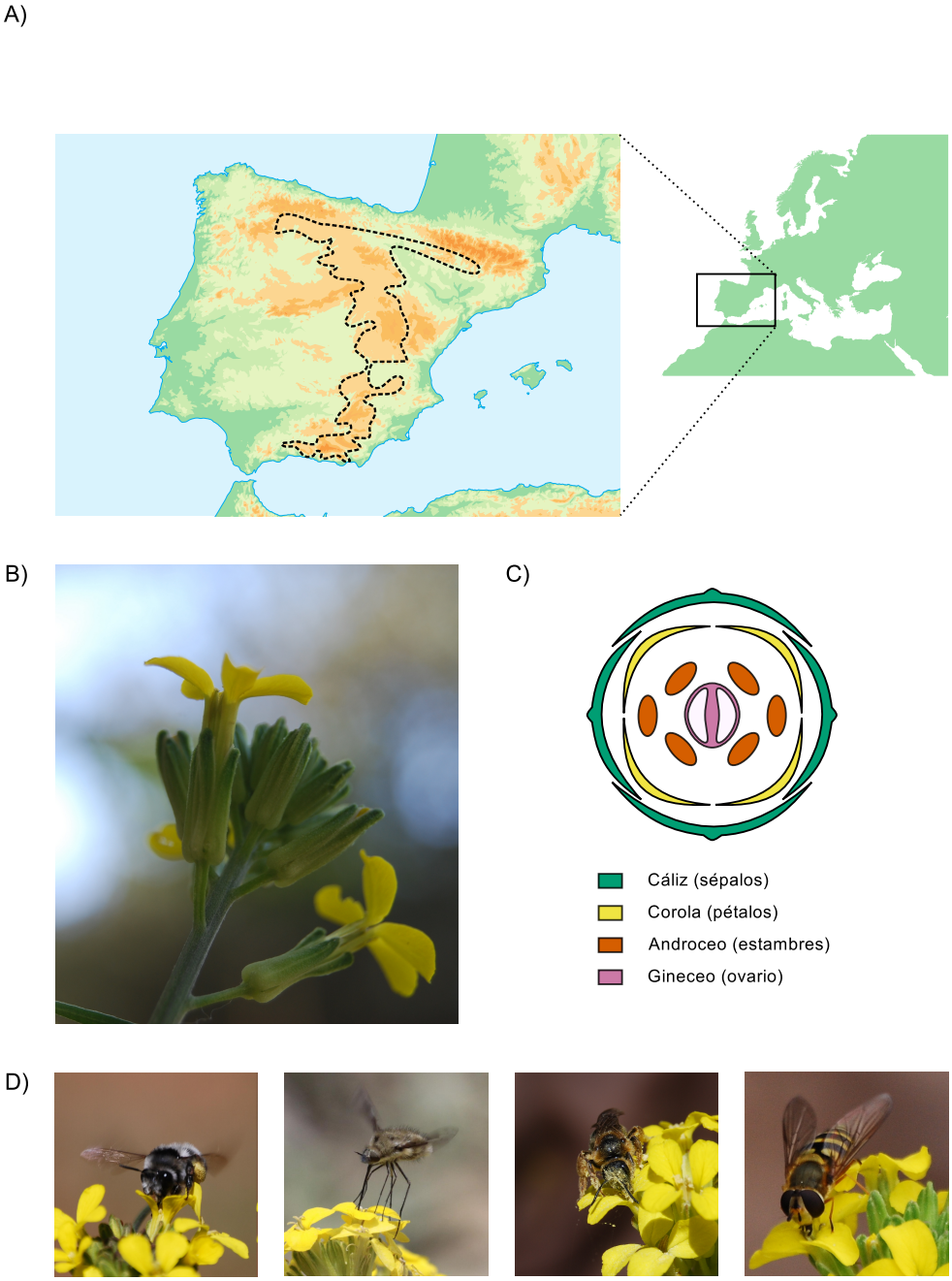


Figura 1: Erysimum mediohispanicum. *A) Area de distribución. B) Vista lateral de la flor. C) Diagrama floral. D) Algunos de los polinizadores más comunes, de izquierda a derecha:* Anthophora leucophaea *(Apidae, Hymonptera),*Bombylius major *(Bombyliidae, Diptera),*Lasioglossum xanthopus *(Halictidae, Hymenoptera) y* Syrphus sp. *(Syrphidae, Diptera)*

.

Parcelas de estudio

Población Em21

Durante los años 2010 y 2011 instalamos un cercado delimitando una parcela de 20×20 metros incluyendo una población natural de *E. mediohispanicum* situada a 1723 m.s.n.m (37°8′07″ N, 3°21′71″ O). La población se sitúa en un bosque abierto dominado por *Pinus sylvestris* con algunos ejemplares en las inmediaciones de *Crataegus monogyna* y *Quercus ilex* de menor porte. El matorral está dominado en el estrato arbustivo por *Erinacea anthyllis*, *Vella spinosa*, y *Berberis hispanica* (Figura 2a), y en menor medida *Rosa canina*, *Astragalus granatensis* y *Salvia lavandulifolia subsp. vellera*, junto con especies de menor porte como *Thymus mastichina*, *Teucrium aureum subsp. angustifolium* o *Artemisia campestris* entre otros. Entre las especies de porte herbáceo cuya floración solapa en algún momento con la de *E. mediohispanicum*, encontramos parches de *Phlomis crinita*, *Cerastium gibraltaricum* y *Polygala boissieri* entre otras.

Cada año marcamos 100 individuos reproductores que representaron alrededor del 85 % del total de individuos en flor. Con el objetivo de controlar todas las interacciones de los polinizadores con la especie de estudio, se eliminaron los escapos florales de los individuos no marcados dentro de la parcela experimental y en una banda de 10 metros alrededor de esta. La localización espacial de cada planta se realizó en relación a la parcela y a nivel de centímetro (Figura 2b). Para ello, dividimos la parcela en cuadrantes de 1 m^2 y usamos una cinta métrica para localizar dentro del cuadrante cada individuo.

En cada planta medimos una serie de variables que podrían influir en la atracción de los polinizadores. Estas variables describen la arquitectura y forma floral, así como los recursos florales (ver sección 'Fenotipado' mas abajo). Además, caracterizamos las condiciones microambientales del sitio donde cada planta crecía, midiendo variables edáficas y lumínicas (ver sección 'Caracterización ambiental').

Durante toda la floración, periódicamente medimos la fenología de floración de cada planta anotando su estado de floración y contando de forma esporádica el número de flores abiertas. Además, diariamente cuantificamos la abundancia, diversidad y comportamiento del conjunto de insectos que interactuaron con las flores de cada planta (ver sección 'Interacciones planta-animal').

Finalmente genotipamos cada una de las plantas monitorizadas así como una muestra de su progenie (ver sección 'Genotipado').

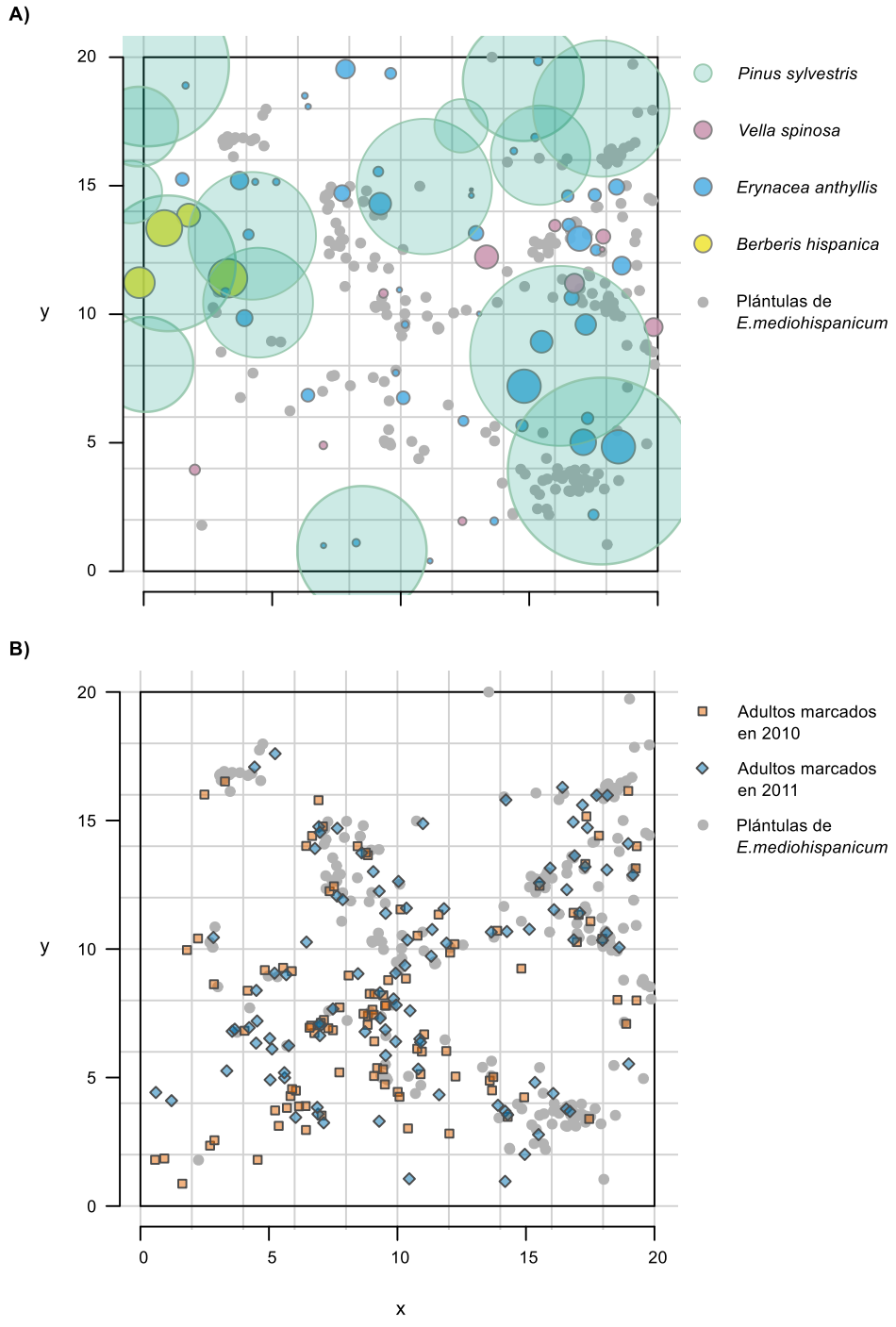


Figura 2: *Parcela de la población Em21. A) Estructura espacial de la vegetación acompañante más relevante, mostrando los individuos que componen el dosel arbóreo y el estrato arbustivo. El tamaño de los círculos representa el diámetro de estos individuos. B) Localización espacial de los individuos de Erysimum mediohispanicum marcados en cada año.*

Parcelas experimentales

En el año 2012, instalamos dos parcelas experimentales en el Jardín Botánico de Hoya de Pedraza, situado a 1950 m.s.n.m (37°6′41″ N, 3°26′13″ O). Cada parcela consistió en 48 plantas provenientes de semillas de plantas de la población Em21 florecidas en el año 2010. Estas semillas se sembraron y mantuvieron durante el primer año de vida en los invernaderos de la Universidad de Granada (UGR), y se trasladaron al jardín botánico durante el segundo año (2011) con el objetivo de aclimatarlas. En las parcelas, las macetas de plantas se enterraron en un marco de plantación de 0,5 x 0,5 metros en el que instalamos una sistema de riego por goteo.

Cada maceta contenía el mismo sustrato, y recibía la misma cantidad de agua diaria, homogeneizando las condiciones edáficas entre plantas. Además, la ausencia de arbolado aseguró las mismas condiciones lumínicas en todas las plantas. Debido a esto, en esta parcela no caracterizamos las condiciones ambientales.

Al igual que en la parcela Em21, para cada planta caracterizamos su fenotipo, y a lo largo de toda la floración, su fenología de floración y las interacciones que mantenía con los polinizadores.

Fenotipado

Para cada planta tomamos medidas de la altura máxima del escapo floral (cm) con una cinta métrica. En caso de que la planta presentase más de un escapo, se midió el de mayor altura. De las flores tomamos diferentes medidas de su arquitectura y forma floral, y cuantificamos el número de granos de polen producido por flor. Esta caracterización se realizó para todas las plantas utilizadas en la presente tesis doctoral.

Arquitectura floral

Con la ayuda de un calibre digital obtuvimos las siguientes medidas: longitud del tubo de la corola (mm); diámetro de la flor, tomada como la distancia entre los extremos de pétalos opuestos (mm); tamaño del pétalo (mm); y apertura del tubo de corola (mm). Además, guardamos en alcohol al 70 % una flor por planta, de las cuales se fotografiaron las diferentes estructuras florales con una cámara PCE-MM200 (PCE instruments). Usando el software ImageJ (Schneider *et al.*, 2012), de estas fotografías medimos la longitud de estambres cortos y largos (mm), longitud de estilo y longitud de sépalos (Figura 3a).

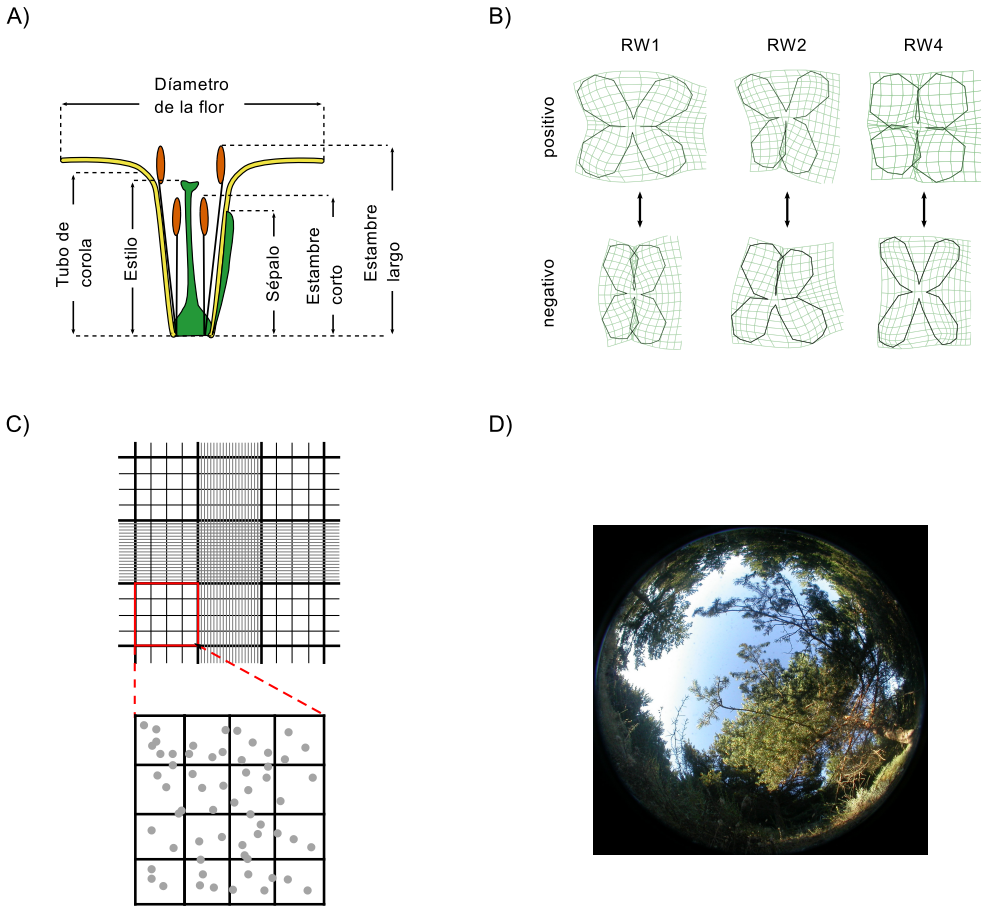


Figura 3: *Fenotipado y microambiente lumínico. A) Variables de la arquitectura floral de la flor. B) Variación en la morfología floral causados por los partial warps 1, 2 y 4. C) Cámara de Neubauer y la zona utilizada para el conteo de polen. D) Fotografía hemisférica utilizada para la caracterización del microambiente lumínico.*

Forma floral

La forma floral de cada planta se obtuvo a partir de la foto de una flor en antesis y tomada en posición frontal (Walker, 2000; Gómez y Perfectti, 2010), de forma similar a cómo un polinizador en vuelo la percibiría. Sobre las fotografías aplicamos un método de análisis de morfometría geométrica. Con este método determinamos la forma de la flor descomponiéndola en una serie de vectores ortogonales ('partial warps', *RW*, Figura 3b), que se asocian a diferentes aspectos de su forma. Para ello, sobre la imagen de la flor se definen 32 puntos de referencia, que se corresponden con estructuras conservadas, en nuestro caso nerviaciones de los pétalos en la mayoría de los casos. Con la

posición relativa de estos puntos, el método usado consiste en llevar a cabo un análisis de Procrustes del que se obtiene una matriz de covarianza. Sobre dicha matriz se realiza un análisis de componentes principales del cual se obtienen los 'partial warps'. Estos análsis se realizaron con los programas TPSDIG v. 1.4 y TPSRELW v. 1.11 (`http://life.bio.sunysb.edu/morph/morphmet.html`).

Producción de polen

La producción de polen se estimó usando una cámara de Neubauer (Figura 3c). Para ello, recolectamos flores justo antes de la antesis que se conservaron en alcohol al 70 %. De estas aislamos un estambre corto y dos largos en 1 mL de alcohol al 70 % y se sonicaron en un baño de ultrasonidos durante 30 minutos para separar los granos de polen de las enteras. La cuantificación se llevó a cabo en la cámara de 0,1 μL a partir de una gota de la muestra previamente homogeneizada. Este conteo se realizó sobre cuatro gotas de cada muestra para así tener una mejor estima de los granos de polen producidos por flor y planta.

Caracterización ambiental

Microambiente lumínico

Las condiciones lumínicas de cada planta se caracterizaron a partir de fotografías del dosel arbóreo (Figura 3d) usando una cámara digital (Coolpix 995, Nikon) y una lente hemisférica (FCE8, Nikon, Tokio, Japón). Las fotografías las tomamos durante el amanecer o el atardecer para evitar artefactos resultantes de la sobre-exposición a la luz directa del sol. Mediante la ayuda de un saco de tela relleno de garbanzos y un nivel, situamos la cámara a 20 cm del suelo apuntando al cénit, y mediante una brújula, direccionamos la cámara al norte. Posteriormente analizamos las fotografías con el software Hemiview v.2.1 (1999, Delta-T Devices Ltd, Cambridge, Reino Unido). Este software modela la trayectoria del sol a partir de la especificación de la latitud, longitud, altitud y la orientación a la que fue tomada la fotografía, además de diversos parámetros de deformación de la imagen producida por la lente. De estos análisis derivamos la proporción de radiación solar directa en relación a la esperada sin ningún obstáculo ('Direct Site Factor', DSF, Anderson, 1964).

Condiciones edáficas

Tomamos muestras del suelo adyacente a cada planta que comprendieron los primeros 10 cm de suelo. Estas se secaron y tamizaron con una malla de luz < 2 mm. A partir de esa tierra, determinamos su textura usando el método

de la pipeta de Robinson, y además cuantificamos la humedad a capacidad de campo −33 KPa y en el punto de marchitamiento a −1500 KPa (Cassel y Nielsen, 1986), y el pH en agua destilada (1 : 2, 5). Las bases de cambio (Na^+, K^+, Ca^{2+}, Mg^{2+}) se extrajeron con NH_4O_{Ac} 1 N, y medimos la capacidad de intercambio catiónico mediante saturación en sodio. Ca^{2+} y Mg^{2+} se cuantificaron mediante espectrofotometría de absorción atómica, mientras que Na^+ y K^+ por espectrofotometría de emisión de llama (SpectrAA 220 FS, Varian). Para cuantificar el carbono orgánico, el N^+ total y el $CaCO_3$, molimos y tamizamos la muestras en un tamiz de 0,125 mm. El carbono orgánico se midió por oxidación con dicromato (Tyurin, 1951), el N^+ total con el método de Kjeldahl (Bremner, 1965) y el $CaCO_3$ usando el método de Barahona *et al.* (1984). Finalmente, el fósforo se cuantificó por el método de Olsen y Sommers (1982). Estas medidas se realizaron para las plantas de la parcela Em21 en el año 2010. Los valores para 2011 se infirieron mediante el método de interpolación 'Kriging'.

Interacciones planta-animal

Frecuencias de interacción

Durante toda la floración se caracterizó el gremio de visitantes florales de cada planta. Diariamente, realizamos de uno a cuatro censos por planta. Los censos consistieron en cinco minutos de observación durante los cuales se anotaron las especies de insectos que contactaron las flores de *E. mediohispanicum*. Para cada interacción se anotó si era legítima -cuando el acceso al recurso floral se realizaba a través de la apertura de la corola, contactando el estigma- o ilegítima -cuando el acceso se hacía a través de la aperturas existentes entre los sépalos sin contactar con el estigma-. La identificación de las especies de insectos se realizó con la ayuda de una colección de referencia y mediante la fotografía de toda especie de dudosa identificación. Estas especies las agrupamos en grupos funcionales basándonos en características morfológicas y comportamentales. Para una descripción detallada de los grupos funcionales usados y de los principales taxones consultar el apéndice 'Functional groups'.

Patrones de vuelo

Determinamos los patrones de vuelo entre plantas de *E. mediohispanicum* realizados por las especies más frecuentes. Para ello realizamos el seguimiento de individuos de insectos seleccionados al azar, mientras pecoreaban sobre las flores de *E. mediohispanicum*, anotando qué plantas visitaban así como el número de flores contactadas por planta. Los seguimientos se realizaron hasta que el insecto abandonaba la parcela o se perdía de vista.

Genotipado

Obtención de material vegetal

Recolectamos al menos cuatro hojas de cada planta marcada en la población Em21 cada año (2010 y 2011) y de las utilizadas en la parcela HP y las preservamos en gel de sílice. Obtuvimos además material vegetal de aproximadamente 30 plántulas de la progenie de cada planta marcada en Em21. Para ello, parte de las semillas recolectadas de cada planta se germinaron en semilleros ubicados en las instalaciones de la UGR. Las plántulas resultantes se recolectaron y conservaron en gel de sílice. Sin embargo, la mayoría de la progenie se obtuvo en las instalaciones del Leibniz Institute of Plant Genetics and Crop Plant Research en Gatersleben, Alemania (IPK). En este centro, las semillas se esterilizaron utilizando hipoclorito sódico, y posteriormente, se germinaron en placas de petri con un medio Murashige-Skoog situadas en cámaras de cultivo a $\sim 20°C$ y con $\sim$ 18 horas de luz.

Extracción

Extrajimos ADN a partir de al menos 60 mg de hojas desecadas, o de hojas frescas según el caso. Para ello utilizamos el kit de extracción GenElute Plant Genomic DNA Miniprep (Sigma-Aldrich), en las instalaciones de la UGR, o bien utilizamos el kit Agencourt® CholoropureTM (Beckman Coulter), en las instalaciones del IPK. Este último es un método que utiliza microesferas magnéticas para la retención del ADN, y que fue automatizado con la asistencia del robot de pipeteo automatizado Biomek 3000 (Beckman Coulter) durante parte del proceso.

Espaciador cloroplastidial *trn*L-*trn*F

De las plantas de Em21 y de HP, genotipamos el espaciador intergénico cloroplastidial *trn*L-*trn*F. Siguiendo a Abdelaziz *et al.* (2011), las reacciones de PCR se llevaron a cabo usando los cebadores tabC y tabF (Taberlet *et al.*, 1991). Cada uno se usó a 0,2 μM con 5 ng de ADN en 25 μL de volumen de reacción. Este incluía 0,5 unidades de ADN Taq polimerasa, un tampón de reacción 2,5 μL 10× con $MgCl_2$ a 1,5 mM y nucleótidos a 0,2 mM cada uno (todos los reactivos de New England BioLabs).

Las PCRs se llevaron a cabo en las instalaciones de la UGR utilizando un termociclador Gradient Master Cycler Pro S (Eppendorf) con el siguiente programa: 35 ciclos con 15 segundos de desnaturalización a $94°C$, 30 segundos de alineamiento a $58°C$, 90 segundos de elongación a $72°C$, y 3 minutos de elongación final a $72°C$.

Microsatelites nucleares

Las plantas de Em21 y de HP, así como la progenie obtenida de las primeras, se genotiparon para 10 marcadores microsatelites nucleares previamente descritos por Muñoz-Pajares *et al.* (2011) en esta especie (Tabla 1). Las reacciones de PCR se realizaron en 14 μL con 0,3 unidades de Taq polimerasa, 1,4 μL de tampón de reacción 10× con 1,5 mM de $MgCl_2$, 0,16 mM cada nucleótido, 0,33 μM cada cebador y 2,5 ng de ADN.

Las PCRs se llevaron a cabo en las instalaciones del IPK utilizando varios termocicladores Gradient Master Cycler Pro S (Eppendorf) con el siguiente programa: una etapa de desnaturalización inicial de 3 minutos a 94 $°C$, seguido de 35 a 40 ciclos de extensión que consistieron en 15 segundos de desnaturalización a 94 $°C$, 30 segundos de hibridación a una temperatura específica para cada locus (ver Tabla 1), y una etapa de elongación de 90 segundos a 72 $°C$. Finalmente, una etapa de elongación de 3 minutos a 72 $°C$ (Figura 4). Para facilitar el trabajo, programamos el robot de pipeteo automatizado Biomek 300 (Beckman Coulter) para preparar alícuotas de ADN en placas de 98 pocillos para la amplificación. Los amplicones obtenidos se enviaron a MACROGEN para su caracterización electroforética en un analizador de fragmentos ABI 3730XLs.

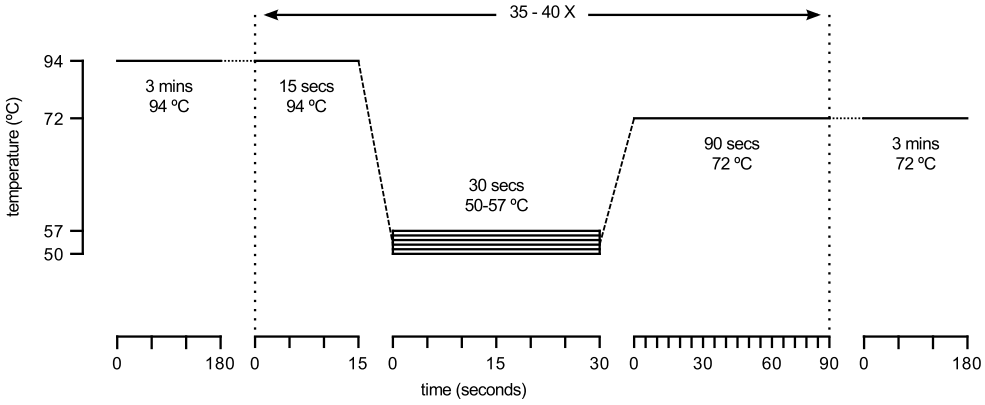


Figura 4: *Programa de PCR usado para la amplificación de los marcadores microsatelites.*

Tabla 1: *Caracteristicas de los 10 marcadores microsatélites. Adaptado de Muñoz-Pajares* et al. *(2011).*

Locus	Primer sequence (5'- 3')	Repeat motif	Product size (*bp*)	$T_a(^\circ C)$
C5 (JF766210)	F: TCTTTCTTCTGCGGTTTATTC R: CGTTTTTGTTGTTGTTCTGG	CCA_8	164 – 182	56
D2 (JF766211)	F: ACGGAAGATGACGATGATCGACTG R: CAATGTCCCTAATTGGTCAATGG	CAT_{23}	117 – 189	54
D4 (JF766212)	F: TAAGGTGTTACCGGATTGTC R: GTGACGATTCGCTCCTTG	ATC_7	200 – 215	57
E4 (JF766213)	F: CCTTCCTCCGACTACTCTCC R: TGAGCGACTGATGATGATTC	CT_{20}	145 – 178	57
E8 (JF766214)	F: AGCTCACAGCCGTCGATGTTTGC R: GAGGTGAAATACACGTAGAACCT	CT_{50}	157 – 229	50
D11 (JF766215)	F: TCCAGGGTCTGAGTCAATATG R: TTACCACTCCTTGCTTCTGAA	TCA_{14}	179 – 197	53
E6 (JF766216)	F: CTTGTAACCGAGCCACTCA R: ATACGGAGAAGAAAGCGAATC	TC_{14}	131 – 159	53
D10 (JF766217)	F: ACTGCCATCAAACGACCTC R: TTGGTTGGAAAAGGGATTG	TCA_{12}	166 – 185	53
E5 (JF766218)	F: TCCATTTACACAATCCGTTCAT R: CCAACCTGACATCTTTGCTTC	GA_{13}	167 – 195	50
E3 (JF766219)	F: TTCCTCCAGATGAAACTACACAGG R: ACTTACATCGGATCGGTTGAG	GA_{17}	215 – 253	56

Para cada locus mostramos el código de acceso de GenBank (entre paréntesis), los cebadores directos (F) y reversos (R), el motivo de repetición, rangos de tamaños alélicos, y la temperatura óptima de hibridación (T_a).

Eficacia de polinización

Para cada grupo funcional de visitantes florales cuantificamos la calidad de la interacción (probabilidad de que de ésta resulte en una semilla viable). Además también cuantificamos la diversidad del polen aportado por visita mediante el genotipado de la progenie resultante.

Calidad de las visitas

Cuantificamos la contribución de cada uno de los grupos funcionales en la producción de semillas tras la visita a una flor virgen de *E. mediohispanicum*. Para eso, durante los años 2012 y 2013 utilizamos 53 plantas provenientes de semillas de la parcela Em21 de los años 2010 y 2011 respectivamente. Estas semillas fueron germinadas en maceta en las instalaciones de la UGR, y en el segundo año de vida las plantas se trasladaron al Jardin Botánico Hoya de Pedraza para su aclimatación. Durante la floración, las plantas se

mantuvieron aisladas en tiendas de exclusión hechas a mano o en tiendas de exclusión de nylon con una malla de 160 μm de luz (BugDorm-2120, MegaView Co., Ltd.). Diariamente las flores vírgenes de estas plantas se expusieron a los polinizadores. Cuando un insecto se acercaba a forrajear sobre una planta se le permitía visitar un máximo de tres flores. De cada interacción con una flor anotamos el tiempo de la interacción y fotografiamos al insecto para su posterior identificación y además para facilitar la localización de la flor visitada. Las flores contactadas se codificaron mediante hilos de color y seguidamente cortamos sus pétalos para evitar otras visitas. Adicionalmente realizamos tres controles sobre las plantas experimentales. Un control de alogamia, en la que a un número de flores se le añadía polen de tres plantas diferentes; un control del tratamiento, en la que se le cortaban los pétalos a flores vírgenes; un control de autogamia facilitada, en la que a la flor se le añadía polen de sus propias anteras; y un control de autogamia espontánea en la que la flor se dejaba sin ser visitada ni manipulada.

De cada flor visitada cuantificamos el éxito de fructificación y el número de semillas viables, las semillas abortadas y el número de óvulos sin fecundar. Finalmente, parte de las semillas se sembraron en semilleros individuales y determinamos su tasa de germinación y establecimiento.

Genotipado

Genotipamos parte de la progenie producida tras la visita de cuatro grupos funcionales: abejas grandes de trompa larga, abejas grandes de trompa corta, abejas medianas de trompa corta y bombílidos. Para esto, usamos las plántulas del experimento del éxito de germinación previamente descrito. Para cada grupo funcional, de una serie de frutos producidos tras su visita, escogimos al azar cuatro plántulas por fruto. Estas plántulas y sus respectivas plantas madre se genotiparon en las instalaciones del IPK utilizando los 10 marcadores microsatelites antes descritos.

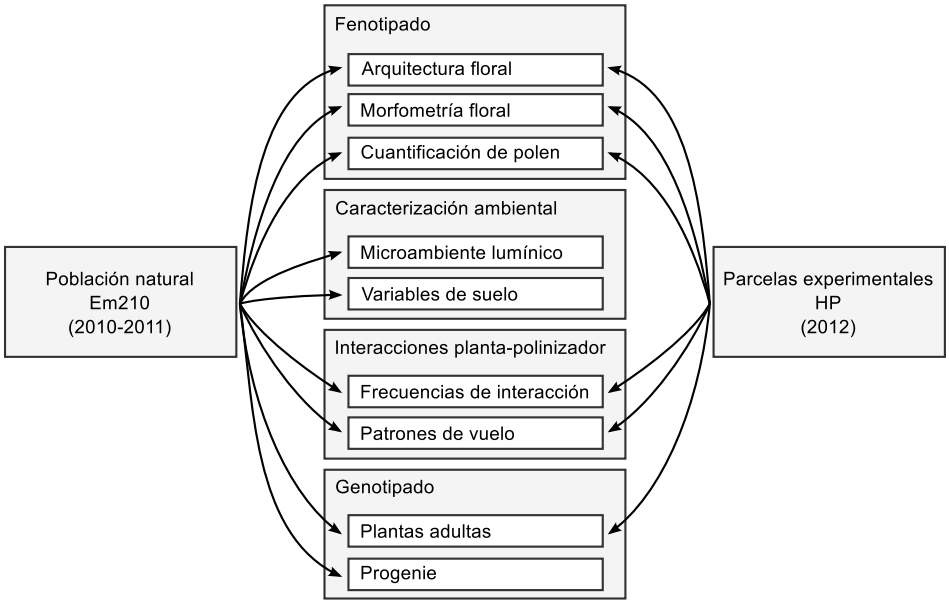


Figura 5: *Diagrama de los datos recogidos en cada parcela.*

Part II

Capítulos principales | Main chapters

1

Inter-annual maintenance of the fine-scale genetic structure in a biennial plant

Abstract

Within plant populations, space-restricted gene movement, together with environmental heterogeneity, can result in a spatial variation in gene frequencies. In biennial plants, inter-annual flowering migrants can homogenize gene frequencies between consecutive cohorts. However, the actual impact of these migrants on spatial genetic variation remains unexplored. Here, we used 10 nuclear microsatellite and one plastid genetic marker to characterize the spatial genetic structure within two consecutive cohorts in a population of the biennial plant *Erysimum mediohispanicum* (Brassicaceae). We explored the maintenance of this structure between consecutive flowering cohorts at different levels of complexity, and investigated landscape effects on gene flow. We found that cohorts were not genetically differentiated and showed a spatial genetic structure defined by a negative genetic-spatial correlation at fine scale that varied in intensity with compass directions. This spatial genetic structure was maintained when comparing plants from different cohorts. Additionally, genotypes were consistently associated with environmental factors such as light availability and soil composition, but to a lesser extent compared with the spatial autocorrelation. We conclude that inter-annual migrants, in combination with limited seed dispersal and environmental heterogeneity, play a major role in shaping and maintaining the spatial genetic structure among cohorts in this biennial plant.

Introduction

Spatial Genetic Structure (SGS) is the non-random spatial distribution of genotypes, a pattern occurring in most plant populations (Vekemans and Hardy, 2004). It results from an equilibrium among space-restricted gene movement, genetic drift and local selection (Wright, 1943; Epperson, 1993). SGS is most of the times the result of isolation by distance (IBD, Wright, 1943), in which local mating or short-distance seed dispersal generates a pattern such that kinship among individuals decreases with distance. The scale at which these processes act varies among species (Vekemans and Hardy, 2004), and recently, several studies have demonstrated that it can also happen at very fine scales (Hardy *et al.*, 2006; Volis *et al.*, 2014; Rouger and Jump, 2015; Lara-Romero *et al.*, 2016). The extent and strength of SGS depends on seed and pollen dispersal strategies as well as on mating system (Hamrick *et al.*, 1993; Vekemans and Hardy, 2004; Hardy *et al.*, 2006). Moreover, dispersal can be directionally biased, resulting in differences in IBD intensity at different spatial directions(Born *et al.*, 2011; Rhodes *et al.*, 2014; DiLeo *et al.*, 2014; Wang *et al.*, 2016) that promote spatial asymmetric SGS, a pattern also occurring at fine-scales. This spatial asymmetry usually responds to environmental heterogeneity (Born *et al.*, 2011; Rhodes *et al.*, 2014). At-site variability in ecological conditions can create differences in habitat suitability and, therefore, affect genetic connectivity via seed or pollen dispersal (Holderegger *et al.*, 2010; DiLeo *et al.*, 2014). Regarding the latter, an increasing number of studies are analysing environmental heterogeneity to understand fine-scale SGS and its effects on population dynamics and evolution .

Studies dealing with the temporal scale of SGS have been mostly aimed at disentangling how demography affects SGS by comparing among age-classes (Kalisz *et al.*, 2001; Jones and Hubbell, 2006; Zhou and Chen, 2010; Berens *et al.*, 2014). In perennials, age-classes coexist in the same spatio-temporal context, implying that individuals initially from different cohorts classes may interbreed. In contrast, in strict biennials, i.e., those plants completing their life cycle in two years, individuals plants from consecutive cohorts are reproductively isolated because they do not overlap in their flowering year (Kelly, 1989). However, this presumption is far from reality because only a few species are strictly biennial (Hart, 1977; Silvertown, 1984; Klinkhamer *et al.*, 1987a). Several processes promote variation in biennial life span, permitting the existence of inter-annual migrants that bridge years ('facultative biennials') (de Jong *et al.*, 1987; de Jong and Klinkhamer, 1988). These inter-annual migrants favour gene flow between cohorts, leading to homogenisation of their allelic frequencies citepKoniger2012. However, little is known regarding

how inter-annual migrants affect the similarities in SGS among cohorts of a biennial, particularly for small plant populations where temporal changes in population size could dramatically vary their genetic composition (Ellstrand and Elam, 1993). We think that the spatial location at which inter-annual migrants arrive to the next cohort may affect SGS. Particularly, in plants presenting limited seed dispersal, the localized arrival of inter-annual migrants may have predictable effects on the SGS, equalizing the spatial variation in allelic frequencies between consecutive cohorts and balancing population size fluctuations.

We have studied two consecutive cohorts of the biennial plant *Erysimum mediohispanicum* (Brassicaceae) to explore fine-scale SGS and to investigate the temporal maintenance of SGS between cohorts. *E. mediohispanicum* is a biennial plant endemic to the Iberian Peninsula (Nieto-Feliner, 2003), where a highly diverse assemblage of pollinators visits their flowers. Although slightly self-compatible, this species needs pollinators for a complete seed set (Gómez *et al.*, 2008a), whose dispersal occurs at very short distances due to its barochorous dispersal strategy (Gómez, 2007). Populations of this species are patchily distributed and formed by tens to several hundreds of individuals (Gómez and Perfectti, 2010). These populations vary largely in size due to climate fluctuations, especially linked to drought years.

We have analysed the temporal maintenance of SGS between cohorts of *E. mediohispanicum* using 10 nuclear microsatellite and one plastid DNA marker. We also assess to what extent spatial environmental variation shapes the observed SGS. We first explore if cohorts were genetically differentiated, followed by characterization and comparison of SGS between cohorts at different levels: 1) by using spatial principal component analysis (sPCA) to assess global genetic structure; 2) by testing for isotropic SGS using kinship-distance correlograms; 3) by using bearing correlograms to visualize SGS asymmetry (anisotropy). Finally, we assess associations among the spatial variation in genotypes and environmental factors using autoregressive spatial models.

Materials and methods

Sampling design

In years 2010 and 2011 we set up a 20 x 20 meter plot delimiting a population of *E. mediohispanicum* situated at 1723 m.a.s.l. at the Sierra

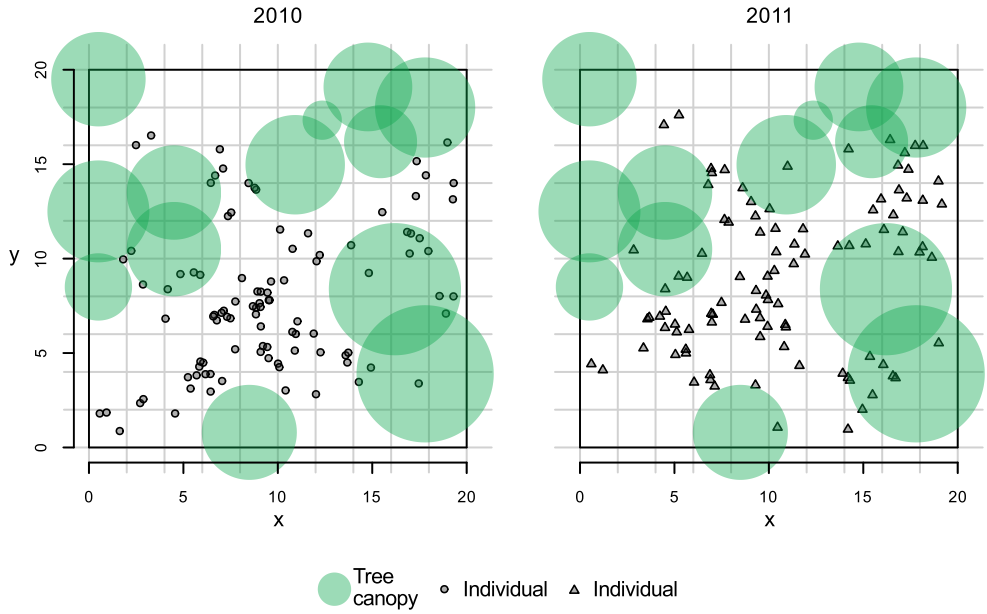


Figure 1.1: *Spatial location of the marked plants. Tree canopy is represented with green circles to evidence the fine-scale environmental heterogeneity. Scale is in meters.*

Nevada Protected Area (SE Spain; 37º 8.07' N 3º 21.71' W). Each year we randomly selected 100 flowering plants and mapped their spatial location at the centimeter level (Figure 1.1). These plants represented 85% of the total individuals comprising a reproductive cohort. We characterized light and soil conditions for each individual plant. Light availability, measured as Direct Site Factor (DSF), was derived from hemispherical photographs taken at the ground level using a Nikon coolpix 4500 and subsequently analysed by applying the Hemiview software (Delta-T Devices, Cambridge, UK). Soil was characterized by means of cations (Na^+, K^+ and Mg^{2+}) and anions (total nitrogen and phosphorous), and moisture content at field capacity as a reliable measure of the soil's ability to retain water (see Supplementary Information S1). To decrease variable dimensionality we ran a PCA over cations and anions independently. We found two components explaining cation variability: the first (cations 1) explaining 58% and was associated to Mg^{2+} and K^+ and the second (cations 2) explaining 33% and was associated to Na^+ (Figure 1.S5). Anions were synthesised in a variable explaining 70% of the variability and equally associated to nitrogen and phosphorous.

Genetic characterization

We genotyped each plant using nuclear and plastid molecular markers. We isolated DNA using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-

Aldrich) on 60 mg of plant material previously crunched in liquid nitrogen.

We amplified the plastidial *trn*L-*trn*F spacer (∼1300 bp) using the tabC and tabF primers (see Abdelaziz *et al.*, 2011, and Supplementary Information S2). PCR products were mixed with 0.15 volume of 3 M sodium acetate, pH 4.6, and 3 volumes 95% (v/v) ethanol and subsequently precipitated after centrifuging at 4 °C. Amplicons were then sent to Macrogen (Geumchun-gu, Seoul, Korea) for sequencing in both directions, using the respective PCR primers. Chromatograms were reviewed and contigs were produced using Geneious v.7 (Kearse *et al.*, 2012) (Biomatters, `http://www.geneious.com/`). Sequences were uploaded to Genbank (accession numbers KX641272 to KX641276).

From each plant we amplified 10 unlinked nuclear microsatellites loci (SSR) described in Muñoz-Pajares *et al.* (2011) (Supplementary Information S2). We additionally genotyped 500 offspring in order to detect and correct genotyping errors (e.g., allele dropout or null alleles) in the parental plant genotypes. Electropherograms were analysed and genotypes called using PeakScanner v.2 (Applied Biosystems) and exhaustive eye-inspection. We characterized each microsatellite locus within cohorts by computing observed and expected heterozygosity, number of alleles, and inbreeding coefficient. For this later we tested if deviated significantly from zero using 999 bootstrap replicates. We tested for Hardy-Weinberg equilibrium using an exact test based on Monte Carlo permutations for each locus and for all loci together. These tests were performed using the 'pegas'(Paradis, 2010) and 'diveRsity' (Valtueña *et al.*, 2013) packages in the open source software R v. 3.2.2.

Inter-annual genetic structure

We evaluated the amount of genetic differentiation between cohorts using Wright's FST statistic. The significance of this statistic was obtained by comparing with a null distribution obtained after 500 random permutations of genotypes. We also performed an AMOVA between cohorts using Chord's distances among genotypes (Excoffier and Smouse, 1992). Moreover, we used Bayesass v.1.3 (Wilson and Rannala, 2003) to infer rates of recent inter-annual migrants. This software uses Markov chain Monte Carlo techniques to estimate the posterior probabilities of recent individual immigrants. We ran three independent MCMC runs for 10^7 iterations with a thinning of 2000 and a burn-in of the first 10% of the iterations. Delta parameters of allele frequency, migration, and inbreeding were set as 0.15.

Spatial variation of allele frequencies

We checked for the occurrence of fine-scale SGS within each cohort by conducting a spatial principal component analysis (sPCA) (Jombart *et al.*, 2008)

on the nuclear markers. This multivariate ordination technique tests for the existence of global structures (such as spatial clines) or local structures (genetic differences between neighbours). sPCA integrates principal component analysis and Moran's autocorrelation index to reduce the multidimensional nature of genotype data into a set of highly informative orthogonal vectors that differentiate spatial patterns of genetic variation. To accomplish this analysis, a connection network depicting spatial weights among individuals has to be previously defined. We used the inverse of the spatial distances among pairs so that all plants were considered neighbours while accounting for the spatial cost distance. With the resulting sPCA principal components, we assessed spatial structures by representing the scores of the two main vectors in space. We only used the two first components (sPC_1 and sPC_2) to reduce the burden of the computation to the most informative sPCA principal components. We evaluated the significance of global and local structures using the test proposed by Jombart *et al.* (2008).

Isotropic spatial genetic structure

We explored the extent and intensity of SGS to evaluate if relatives were spatially clumped as a result of isolation by distance. For each cohort and for each nuclear (SSR) and plastid marker, we calculated Nason's kinship coefficients (Loiselle *et al.*, 1995), which are standardized to a population's allele frequencies and are highly robust to HWE deviations (Vekemans and Hardy, 2004). When calculating this coefficient using the plastid marker, we treated haplotypes as alleles from a haploid organism. To explore the spatial extent of SGS we calculated the average kinship coefficient for a set of distance classes (F_D) spanning 0.5 meters, and plotted them against distance (Hardy and Vekemans, 1999). We subsequently measured the intensity of SGS by calculating two gene dispersal parameters: F_1, the average kinship coefficient for the first distance class (< 0.5 meters), and the Sp coefficient1, defined as $-bF/(1 - F_1)$, where bF is the slope of the regression of kinship coefficients on the natural logarithm of the spatial distance among individuals within an optimized range of distances. The range of distances for which bF is calculated is related to the average parent-offspring distance and its approximation follows an iterative procedure1 implemented in the software SPAGeDi 1.5a (Hardy and Vekemans, 2002). Standard errors for all parameters were calculated jackknifing over SSR loci (Vekemans and Hardy, 2004; Hardy *et al.*, 2006), except in the case of cpDNA haplotypes, where only a marker was available. For SSR Sp, and each SSR F_D we obtained a null distribution of expected values under the hypothesis of no SGS by permuting genotypes among individuals 9999 times, and compared them with the observed values to obtain a significance value.

We investigated the maintenance of the isotropic SGS between cohorts by plotting kinship coefficients against spatial distance as explained before, but restricting the comparisons to individuals belonging to different cohorts. Then, we compared the intensity of the SGS between cohorts by a t-test using the average and standard errors obtained by jackknifing over loci. We also calculated bF, F_1 and Sp dispersal parameters restricting the comparisons to individuals belonging to different cohorts.

Anisotropic spatial genetic structure

We explored the anisotropy of SGS by testing whether its strength varied in different spatial directions using bearing correlograms (Rosenberg, 2000), an analysis that uses a Mantel test to correlate a genetic similarity matrix with a transformed distance matrix for a set of spatial directions, measured as the angles formed with the Y-axis (θ). For a given direction, the transformed distance matrix is obtained by first calculating the natural logarithm of the original distance matrix, followed by weighting its values by the squared cosine of the clockwise bearing angle depicted by each pair of individuals and the fixed spatial direction. For this analysis we used the obtained kinship matrix and a set of 128 equidistant bearing angles. The significance of the correlations was obtained using a permutation test (999 permutations). Through this analysis we were able to find the bearing angle denoting the strongest (θ_S, minimal Mantel correlation) and weakest SGS directions (θ_W, maximal Mantel correlation). Next, we calculated the strength of SGS at θ_S and θ_W for each cohort. To perform this, and to augment the number of paired kinship coefficients, we included the plants inside a range of 30° around θ_S and θ_W (Born *et al.*, 2011). For each cohort and θ_S and θ_W , we computed Sp and $F1$ and obtained their significance as explained before. These analyses were performed using SPAGeDi 1.5a61 and personalized scripts in R (Supplementary Information S3). As the temporal maintenance of anisotropy is determined by the congruence and strength of θ_S and θ_W between cohorts, we therefore compared the gene dispersal parameters Sp and $F1$ by means of a t-test.

Environment-genotype correlations

We evaluated the contribution of environmental factors to the spatial genotypic variation beyond isolation by distance. We used lagged simultaneous autoregressive models using the first two components of the sPCA as response variables (Robinson *et al.*, 2012). These models assume the inherent spatial autocorrelation occurring in the response variable and are fairly robust to type I errors (Kissling and Carl, 2007; Beale *et al.*, 2010). The models take the form:

$$G_i = \rho W_{ij} G_j + \beta X + \epsilon$$

In this equation, the typical ordinary least squares regression ($G_i = \beta X + \epsilon$) is modified by adding a term ($\rho W_{ij} G_j$) which controls for the inherent spatial autocorrelation which is assumed to occur in the response variable. The sPC scores of all other individuals (G_j) are weighted by the parameter ρ, which accounts for the lack of independence among individuals, and by the cost-distance-weighting matrix (W_{ij}). We computed W_{ij} using the inverse spatial distances among individuals because it approximately emulates a spatial autocorrelation under IBD (Robinson *et al.*, 2012; Kierepka and Latch, 2016). By using site-based measures we were able to determine the environmental factors associated with genetic structure (Jombart *et al.*, 2009).

From all possible combinations of models we selected those with ΔAIC $<$ 2 from the best model. Following, we model-averaged parameter estimates and calculated their relative importance following Burnham and Anderson (2002). Moreover, the selected models were checked for remaining autocorrelation in residuals by using a Moran's I test. Model performances and selection were calculated using the R packages 'spdep' (Bivand *et al.*, 2011) and 'AICcmodavg' (Mazerolle, 2016).

Results

Genetic characterization

We successfully characterized 200 sampled individuals (100 per cohort, Figure 1.1). The highest proportion of missing data (3%) was found in the locus E3 in 2011. Allelic richness was 11.7, ranging between four and 22 per locus (Table 1.1). Most loci were at Hardy-Weinberg equilibrium, with only one locus in 2010 and two in 2011 presenting a deficit of heterozygotes. The analysis of the plastid *trn*L-*trn*F spacer yielded five haplotypes with an uneven occurrence across the population, with 95.5% of individuals bearing one of the two most frequent haplotypes.

Table 1.1: *Population genetic parameters per locus and year (cohort). For each locus and cohort: allelic richness (R_S), percentage of missing data ($\%MD$), inbreeding coefficient (F_{IS}), observed heterozygosity (H_O), departure from the expected heterozygosity under Hardy-Weinberg equilibrium (H_O/H_E), and their corresponding chi-squared values are shown. Significant values are indicated in bold*

	2010						2011					
Locus	R_S	$\%MD$	F_{IS}	H_O	H_O/H_E	χ^2	R_S	$\%MD$	F_{IS}	H_O	H_O/H_E	χ^2
C5	10	0	-0.024	0.756	1.024	21.67	10	0	0.043	0.766	0.957	25.373
D4	8	0	0.027	0.558	0.973	11.625	8	0	-0.025	0.588	1.025	45.185
E4	14	1	0.053	0.794	0.946	148.649	15	0	0.064	0.816	0.936	**92.432**
D2	22	0	0.053	0.912	0.947	200.625	21	0	**0.076**	0.925	0.924	211.69
E3	4	0	0.094	0.244	0.906	7.963	6	3	0.062	0.287	0.938	14.71
E6	13	0	-0.057	0.694	1.057	47.827	12	0	0.03	0.684	0.969	40.239
E8	15	0	**0.192**	0.758	0.808	**204.151**	16	0	**0.17**	0.751	0.83	166.314
E5	16	0	0.052	0.869	0.948	117.717	14	1	**0.123**	0.857	0.877	**143.413**
D10	5	0	-0.022	0.531	1.022	5.928	5	1	-0.043	0.506	1.043	13.18
D11	6	0	-0.043	0.52	1.043	9.327	5	1	0.079	0.529	0.921	7.506

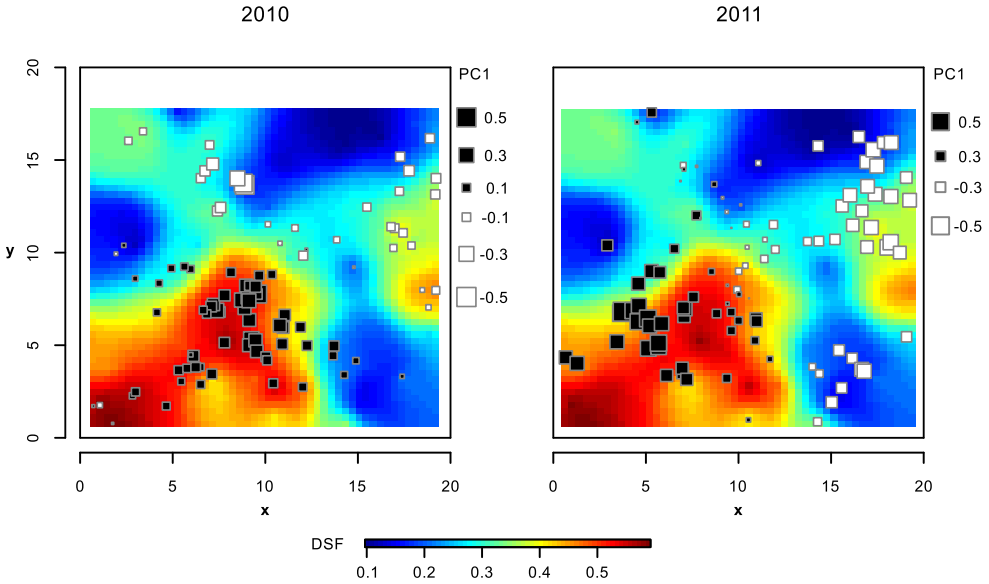


Figure 1.2: *Spatial representation of the first vector from the sPCA for both cohorts and light availability (DSF). Individuals tend to be surrounded by other individuals with similar score values, indicating local aggregation of related genotypes. Scores are represented with solid black squared symbols when positive and empty when negative. Square size is relative to the score value.*

Inter-annual genetic structure

All loci exhibited extremely low values of the genetic variance component related to inter-annual differences ($F_{ST} < 0.006$, $p > 0.05$ for all loci), similar to what we found for the multilocus value ($F_{ST} < 0.001$, $p = 0.226$). These low values were consistent with the lack of significance found after an AMOVA ($\sigma^2 = 0$; $p = 0.765$). Moreover, the proportion of inferred migrants between cohorts was high (0.272).

Spatial variation of allele frequencies

Tests for spatial structure indicated the existence of global spatial structures within cohorts ($max_{(t)} = 0.019$ for both cohorts, $p = 0.016$ and 0.036 for 2010 and 2011 respectively), but no local structures, e.g., genetic differentiation among close neighbours, ($max_{(t)} = 0.020$ for both cohorts, $p = 0.460$ and 0.126 for 2010 and 2011). The sPCA analyses showed that most of the data structure was explained by the first three principal components (sPC) in 2010 and by the first two in 2011 (Figure 1.S1a). The eigenvalues of these $sPCs$ were 0.045, 0.030 and 0.025 in 2010, and 0.040 and 0.027 in 2011, with the remaining eigenvalues showing lower values (Figure 1.S1b). For environment-genotype association studies, we retained the first two $sPCs$, as these stand out in terms of genetic variance and spatial autocorrelation (Figure 1.S1a). The global structure represented by the first sPC (sPC_1) from the sPCA, revealed similar spatial genetic patterns for both cohorts, extending as a cline along the X-axis of our study plot (Figure 1.2), while the second (sPC_2) did not revealed a clear spatial pattern (Figure 1.S2b).

Isotropic spatial genetic structure

Correlograms performed using nuclear markers showed positive significant values only at short distances ($< 2.5m$; Figure 1.3a). The significant positive values for the first distance class (0.063 ± 0.013 in 2010; 0.071 ± 0.020 in 2011) and the negative slope of the relationship between kinship and the natural logarithm of spatial distance (-0.011 for both cohorts) lead to the same significant value of intensity of SGS ($Sp = 0.012 \pm 0.001$; Table 1.2). The analysis of the plastid haplotypes Showdown similar results, with positive and significant values below 0.5 and $1m$ in 2010 and 2011 respectively, in addition to occasional significant values at further distances (Figure 1.3b). The F_1 and Sp coefficients depicting SGS intensity showed significant values for both cohorts ($F_1 = 0.467$ and 0.627; $Sp = 0.207$ and 0.019 in 2010 and 2011 respectively; $p < 0.05$).

Table 1.2: *Isotropic SGS strength within and between cohorts. b_F: kinship-log spatial distance regression slope within an optimized range of distances. F_1: average kinship for the first distance class. Sp: slope of the regression of kinship on the logarithm of the spatial distance, it equals to $-b_F/[1-F_1]$. Between cohorts parameters were obtained restricting the comparisons to individuals belonging to different cohorts. Significant values are indicated in bold. P-values of the t-tests comparing between cohorts are also shown.*

Marker	Cohort	bF	F1	Sp
SSR	2010	**-0.011 ± 0.001**	**0.064 ± 0.013**	**0.012 ± 0.001**
	2011	**-0.011 ± 0.001**	**0.072 ± 0.020**	**0.012 ± 0.001**
	Between	**-0.011 ± 0.001**	**0.063 ± 0.016**	**0.012 ± 0.001**
t-test between cohorts		1	751	1
cpDNA	2010	**-0.110**	**0.467**	**0.207**
	2011	**-0.007**	**0.627**	**0.019**
	Between	**-0.083**	**0.624**	**0.220**

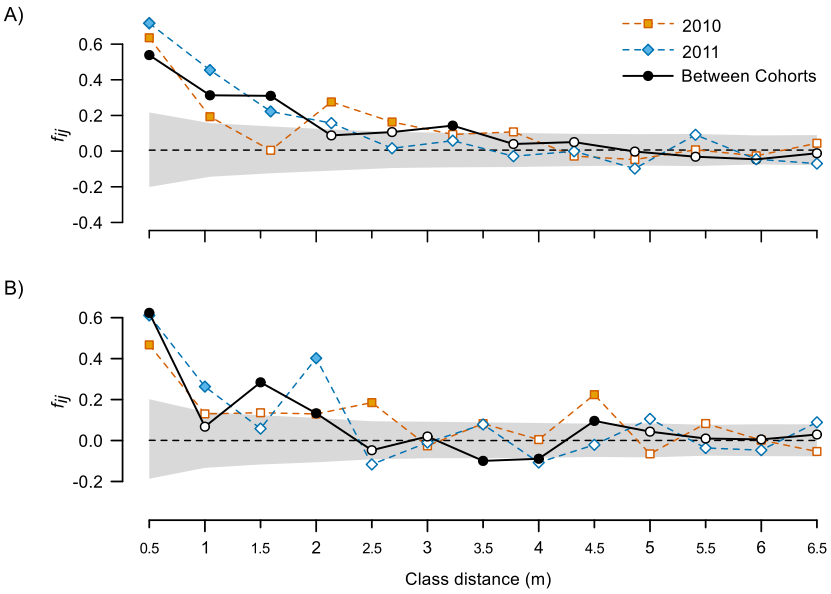


Figure 1.3: *Isotropic distograms.For nuclear (A) and plastid (B) markers, the average kinship at each distance class are plotted with differing symbols depending on the comparison: orange squares (2010), blue diamonds (2011) and black circles (comparisons between cohorts). Filled symbols denote significance of the value when compared with the null hypothesis of no isolation by distance. Grey filled areas represents 95% confidence intervals for the null hypothesis of no spatial structure in the between-cohort comparisons.*

We found similar patterns for the isotropic SGS between cohorts. When plotting the kinship coefficients of plants belonging to different cohorts against distance we observed that the pattern was maintained for both types of marker. We obtained significant positive values of average kinship coefficient below $1.5m$ for the nuclear markers (Figure 1.3a) and below $2m$ for the plastid marker (Figure 1.3b). Moreover, there were non-significant between-cohort differences in F_1 and Sp (Table 1.2).

Anisotropic spatial genetic structure

The correlation between kinship and spatial distance varied with compass directions for each cohort and marker, indicating the occurrence of anisotropy in SGS (Figure 1.4). This variation coincided between cohorts such that there was congruence in the bearing angles with the strongest and weakest SGS for each marker. For the nuclear markers, both cohorts presented the strongest correlation along the X-axis of the study plot ($r = -0.050$, $\theta_S = 90^\circ$ in 2010; $r = -0.059$, $\theta_S = 87^\circ$ in 2011; Table 1.3; Figure 1.4a), and the weakest correlation at relatively close angles ($r = -0.036$, $\theta_W = 171^\circ$ in 2010; $r = -0.007, \theta_W = 154^\circ$ in 2011). Plastid markers showed the same bearing angle with the strongest correlation ($\theta_S = 56^\circ$, $r = -0.112$ and -0.051 in 2010 and 2011; Table 1.3; Figure 1.4b) similar to the bearing angle with the weakest correlation ($\theta_W = 146^\circ$, $r = 0.033$ and 0.013 in 2010 and 2011 respectively).

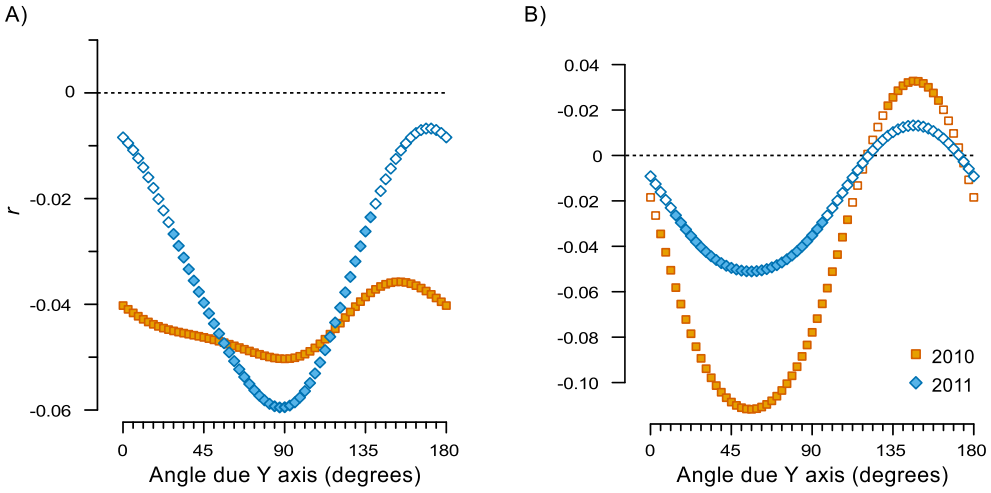


Figure 1.4: *Bearing correlograms. For a series of bearing angles from 0 to* 180° *due the Y-axis of the plot, the Mantel correlation coefficient between genetic similarity and transformed distance matrix is plotted for both cohorts and for the nuclear (A) and plastidial (B) markers. Orange squares denote values for the cohort in 2010; blue diamonds values for the cohort in 2011. Significance after permutation is represented with filled symbols.*

Table 1.3: *SGS strength within cohorts at the bearing angles with the strongest and weakest kinship-distance correlations. For the compass directions with the strongest (θ_S) and weakest (θ_W) kinship-distance correlations, we show their bearing angles (θ) and SGS strength parameters b_F, F_1 and Sp. P-values of the t-tests comparing between cohorts are also shown.*

bearing angle	marker	cohort	θ	b_F	F1	Sp
θ_S	SSR	2010	90°	**-0.013 ± 0.003**	-0.001 ± 0.001	**0.013 ± 0.003**
		2011	87°11′	**-0.013 ± 0.005**	0	**0.013 ± 0.005**
	t-test between cohorts			0.962	0.782	0.962
	cpDNA	2010	56°15′	**-0.104**	**0.029**	**0.107**
		2011	56°15′	-0.032	0.005	0.032
θ_W	SSR	2010	154°42′	**-0.020 ± 0.006**	0.000 ± 0.001	**0.020 ± 0.006**
		2011	171°34′	0.007 ± 0.003	-0.001 ± 0.001	-0.007 ± 0.003
	t-test between cohorts			0	0.426	0
	cpDNA	2010	146°15′	-0.059	**-0.020**	0.058
		2011	146°15′	0.011	**-0.011**	-0.011

For the nuclear markers the strength of the correlation at θ_S showed the same values ($Sp = 0.012$ and 0.013 in 2010 and 2011, respectively), while at θ_W these were different ($Sp = 0.020$ and -0.007). The plastid marker showed differing values of strength at both angles ($\theta_S : Sp = 0.107$ and 0.032; $\theta_W : Sp = 0.058$ and -0.011).

Environment-genotype correlations

The lagged autoregressive models performed on sPC_1 and sPC_2 presented low values of Moran's I index in the residuals (< 0.099), indicating a good performance of these models (Supplementary Table 1.S1), while ρ, the parameter associated with the inherent spatial autocorrelation term, was the most important parameter with values consistently above 0.8 (Table 1.4). The averaged parameters from the selected models indicated some environment-genotype associations. Light availability (DSF) was strongly associated in both cohorts with sPC_1 ($w^+ = 1$ and 0.585 for 2010 and 2011; Table 1.4; Figure 1.2) and in a lesser extent with sPC_2. Anions were strongly associated with sPC_1 in 2011 ($w^+ = 0.681$; Figure 1.S2a) and sPC_2 in 2010 ($w^+ = 0.479$), whereas cations had the strongest effect on sPC_2 both years ($w^+ = 0.721$ and 0.438 for 2010 and 2011 ; Figure 1.S2b).

Table 1.4: *Model-averaged parameter estimates after model selection. Regression model parameters with estimates, standard errors, and relative importance values (w^+) resulting from the model selection. ρ denotes the parameter associated with the inherent spatial autocorrelation term. The w^+ values of ρ equals to 1 because this is a structural parameter of the lagged simultaneous autoregressive models.*

		2010			2011		
		Estimate	SE	w^+	Estimate	SE	w^+
PC1	ρ	0.883	0.078	1	0.874	0.087	1
	Light availability (DSF)	0.158	0.041	1	0.036	0.042	0.585
	Anions (N and P)	-0.004	0.019	0.135	-0.06	0.059	0.681
	Cations 1 (Mg^{2+} and K^+)	-0.049	0.05	0.632	0.046	0.063	0.498
	Cations 2 (Na^+)	0.003	0.013	0.14	-0.001	0.014	0.078
	Field capacity	-0.054	0.042	0.807	-0.001	0.021	0.079
PC2	ρ	0.899	0.068	1	0.927	0.051	1
	Light availability (DSF)	-0.035	0.04	0.596	-0.004	0.018	0.217
	Anions (N and P)	-0.034	0.045	0.479	-0.001	0.021	0.158
	Cations 1 (Mg^{2+} and K^+)	-0.053	0.049	0.721	-0.022	0.041	0.438
	Cations 2 (Na^+)	-0.125	0.028	1	-0.008	0.026	0.244
	Field capacity	0.008	0.02	0.268	0.014	0.038	0.326

Discussion

We did not find genetic differentiation between two consecutive cohorts of the biennial plant *E. mediohispanicum*, a result indicating that these cohorts belong to the same gene pool and behave cohesively. Furthermore, fine scale SGS was congruent between cohorts, indicating recurrent seed dispersal limitation among years. Additionally, we found an association between the microenviroment and the spatial genetic variation. Below, we discuss the SGS found within the studied population and the causes that may be inducing it. We end by discussing how the absence of genetic differentiation and the maintenance of the SGS may be driven by inter-annual migrants between cohorts.

The spatial distribution of genotypes deviated each studied cohort from what it is expected under a random distribution of alleles, indicating the existence of SGS. Particularly, the genotypes were more similar at short distances, fitting a pattern of solation by distance even at this fine spatial scale. These results are supported by the global structure found using the sPCA and by the Mantel correlograms for both plastid and nuclear markers. This pattern emerges from restricted gene flow driven by the local dispersal of seeds or pollen, whose contributions to the final SGS may differ (Freeland *et al.*, 2012).

Limited seed dispersal promotes siblings to germinate and establish locally, creating aggregations of relatives and therefore structuring genotypes at the fine-scale (Volis *et al.*, 2010; Lara-Romero *et al.*, 2016). This is likely to have happened in our study population because the distances at which the significant positive correlation vanished ($< 1-2.5$ meters) are consistent with the reported distances of seed dispersal in *E. mediohispanicum* ($0-0.38$ m) (Gómez, 2007). However, limited seed dispersal may not be acting alone. The congruent SGS pattern showed by the nuclear and plastidial markers suggest that pollinators moving pollen at long distances are not blurring the SGS. In addition, we think that the spatial aggregation of individuals (Figure 1.S3) together with the prevalence of floral visitors that tend to forage at short distances (Pérez-Collazos *et al.*, 2015; Valverde *et al.*, 2016b), may facilitate mating events among nearby relatives, causing biparental inbreeding, and reinforcing the observed SGS. Nevertheless, even under random mating, SGS can occur as a byproduct of limited seed dispersal (Hamrick *et al.*, 1993; Kalisz *et al.*, 2001), and therefore further analyses are needed to disentangle the relative role of pollen and seeds dispersal as drivers of the observed pattern.

We found asymmetry in SGS for both cohorts, indicating directionally biased gene flow. Kinship-distance correlation varied periodically along the spatial directions, revealing higher resistances to gene flow at 90° and 87° due the Y-axis of the plot (Figure 1.4). Directionally biased gene flow has been related to spatial gradients on environmental factors (Wang *et al.*, 2016), and has been reported in plant species where topography or environmental factors reinforce SGS in particular directions (Austerlitz *et al.*, 2007; Born *et al.*, 2011; Rhodes *et al.*, 2014; Wang *et al.*, 2016). If these gradients are maintained over years, the predominance of gene flow along some axes may leave their signature in the SGS. However, we did not identify any environmental gradients because the analysed environmental factors showed a marked patchy heterogeneity. Several factors acting locally could produce the observed SGS asymmetry. For example, the non-random movement of pollinators driven by preferences for plant traits, or micro-environmental factors, may produce heterogeneous patterns of gene flow (DiLeo *et al.*, 2014). These environmental factors could also affect seed germination and seedling establishment, contributing to directionally biased SGS (Segarra-Moragues *et al.*, 2016). Additionally, directional genetic flow from other populations could contribute to the observed pattern, but at the present we have no evidences for this to be considered as an important factor shaping SGS asymmetry.

Site-specific ecological conditions mediate genetic connectivity (Dyer and Sork, 2001). In our study, the spatial variation in allele frequencies showed by the sPC_1 matched the spatial variation in understory light availability (Figure 1.2). This pattern could be produced by light modulating the suitability of spaces for seed germination and seedling establishment (Isselstein *et al.*, 2002),

and therefore contributing to a kind of fine-scale isolation-by-environment (Wang and Bradburd, 2014). In addition, this pattern could be reinforced by the influence of light on the identity and behaviour of the pollinators visiting the plants (Herrera, 1995b). Under this scenario, plants sharing the same light conditions would be visited by similar pollinators and therefore may exhibit higher genetic connectivity. This hypothesis, however, needs to be tested by further analyses of paternity on the offspring to relate pollen flow with understory light conditions and pollinators.

We also found that SGS was associated with some soil variables. Effect of soil heterogeneity on the pattern of genetic variation has been reported in several plant species. For example, Segarra-Moragues *et al.* (2016) have recently shown significant genetic differentiation between rosemary plants (*Rosmarinus officinalis*) growing in siliceous vs. calcareous soils. In our case, the observed fine-scale soil-SGS association could be caused by the differential performance (establishment, survival, growth) of some genotypes in slightly different edaphic conditions. However, it is noteworthy to highlight the high values of ρ (> 0.867) in our models, indicating that, at this scale, spatially limited dispersion prevails over these site-specific environmental factors. In this sense, more evidence is necessary to disentangle the importance of the genotype-environment interactions in shaping SGS at this fine scale.

Prudence is cautioned in interpreting spatially lagged regressions because little is known about their performance when applied to landscape genetic analyses (Kierepka and Latch, 2016). However, we are confident of our results for a number of reasons. It has been shown that these models are robust to type I errors (Kissling and Carl, 2007; Beale *et al.*, 2010) and do not fail when using sPCA principal components as response variables (Robinson *et al.*, 2012; Kierepka and Latch, 2016). In addition, ρ was consistently high in our models, and the residuals showed little to no spatial autocorrelation (Table 1.S1), indicating that these models accounted adequately for the spatial autocorrelation. Nevertheless we are aware that the associations found here have to be interpreted cautiously when inferring the causal mechanisms leading to these environment-genotype associations.

Plants flowering in different years did not differ in their allelic frequencies, indicating that the two studied cohorts belong to the same gene pool. This is confirmed by the low values of F_{ST} and the non-significance of the AMOVA. In small populations of biennial plants, such as *E. mediohispanicum*, recurring and catastrophic declines of population size could conduce to genetic differentiation even among cohorts, since these fluctuations in population size are expected to produce intense genetic drift in small populations (Ellstrand and Elam, 1993). But even in the case of large demographic events, the occurrence of gene flow between cohorts may counterbalance genetic differentiation. Gene flow is surely due to the high amount of inter-annual migrants, up to 17% according

to our Bayesass estimates, a high value when compared to most studies using this same algorithm (Meirmans, 2014). Several factors may promote between-cohort gene flow via different pathways in *E. mediohispanicum* (Figure 1.S4). Seed dormancy can be activated by unfavourable weather conditions (Baskin and Baskin, 2014) and leads to mating between the descendants of different cohorts (Königer *et al.*, 2012). In addition, the arrival of inter-annual migrants can take other different paths. Firstly, via the repeated flowering of some individuals in the oncoming year. This could be caused by delayed reproduction or via a reduced reproductive success during a year followed by subsequent resource allocation, permitting a new reproductive event in the following year (Klinkhamer *et al.*, 1987b). In our study species, several factors such as herbivory (Gómez, 2003) or pollen limitation (Gómez *et al.*, 2010) may trigger the existence of this type of inter-annual migrants. Additionally, delayed or precocious flowering can occur as a response to climatic suitability to plant growth (Klinkhamer *et al.*, 1987b), a likely candidate factor considering the climatic unpredictability of Mediterranean ecosystems (Gómez *et al.*, 2004). Whatever the reasons, our results demonstrate that inter-annual migrants prevent the genetic differentiation among cohorts, contributing to population cohesion.

Between-cohort gene flow, besides homogenizing allelic frequencies, also reinforced SGS. Firstly, kinship-distance correlations showed a similar pattern within and between cohorts, with the same significant positive values spanning up to 1.5 and 2 m for nuclear and plastid markers respectively. Along with this, SGS intensity did not differ significantly between cohorts, emphasizing the similarities in isotropic SGS. These findings confirm that plants growing nearby are genetically more similar independently of whether they belong to the same flowering cohort or not. In addition, SGSs were similar in their spatial asymmetry, with coinciding bearing angles for the strongest and weakest correlations. The maintenance of the SGS between cohorts is likely due to the limited dispersal of seeds. The integration of all individual seed shadows determines the starting template for the spatial genetic patterns of future flowering plants (Jones and Hubbell, 2006; Shimono *et al.*, 2006). Therefore, an inter-annual migrant stands as a representative of this spatial template in the new cohort. From this localized migration spot, the oncoming processes of gene flow through pollination and seed dispersal will spread the migrant genes in the new cohort similarly to what would occur in its parental cohort. In this sense, the environmental factors will similarly favour or restrict gene flow in some directions, and therefore migrant alleles will flow asymmetrically. These recurrent migration events will, in the long term, match the spatial genetic variation among cohorts, leading to the observed congruence in SGS. Furthermore, between-populations migration could also contribute to produce the observed SGS. However, biased and recurrent migration, probably

mediated by long-distance pollination events, would be needed to explain the observed pattern. As these pollination events do not contribute to the plastidial SGS, the importance of recurrent spatial migration in the temporal maintenance of the SGS is, at least, doubtful.

We have demonstrated the existence of congruent fine-scale spatial genetic variation in two consecutive cohorts of a natural population of the biennial plant *E. mediohispanicum*. This SGS was consistently associated with environmental factors, such as light availability and soil composition. More importantly, there was always a strong spatial autocorrelation in SGS, suggesting that SGS was mostly caused by the spatial pattern of limited seed dispersal in distance rather than by any environment-genotype association. The two studied cohorts had homogenized allelic frequencies, indicating the absence of genetic isolation and the existence of inter-annual migrants. These migrants, due to spatial limited seed dispersal exhibited by *E. mediohispanicum*, imprint a spatially localized genetic signature on the new cohort, which ultimately results in a between-cohort matching of SGS.

Supplementary information

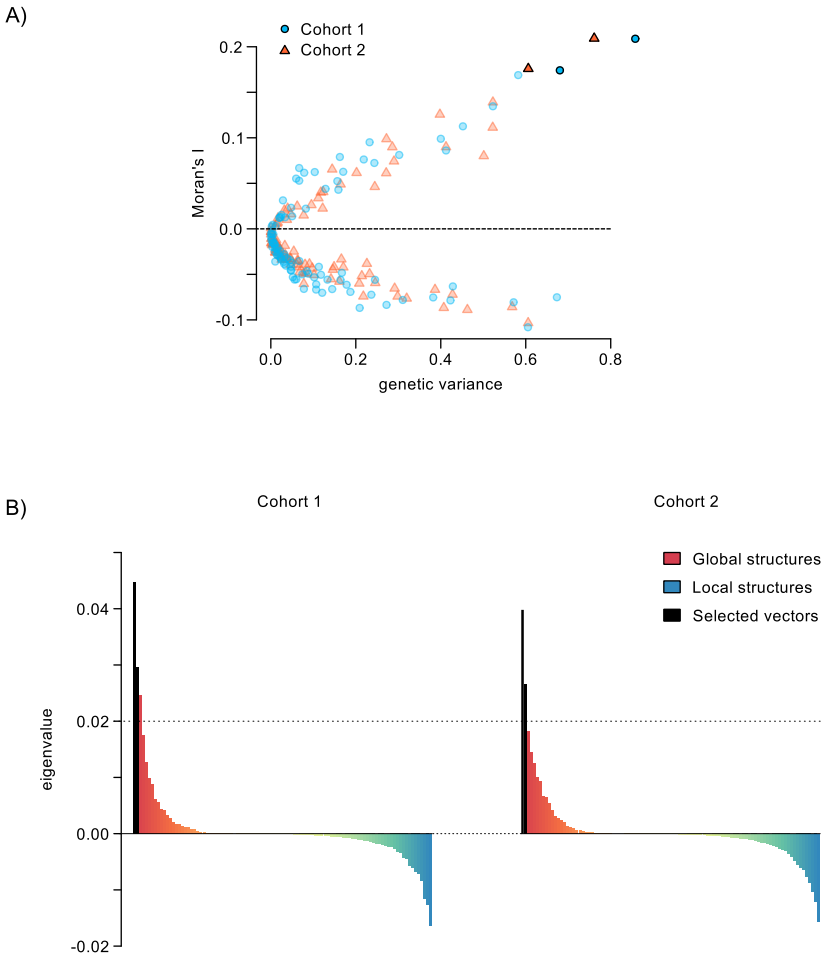


Figure 1.S1: *Eigenvalues of sPCA for cohorts 2010 and 2011. A) Eigenvalues decomposition into the genetic variance explained and Moran's I values. B) Barplot of eigenvalues showing positive (global structures) and negative (local structures) axes. In both, the selected sPCs for the spatial analyses are highlighted.*

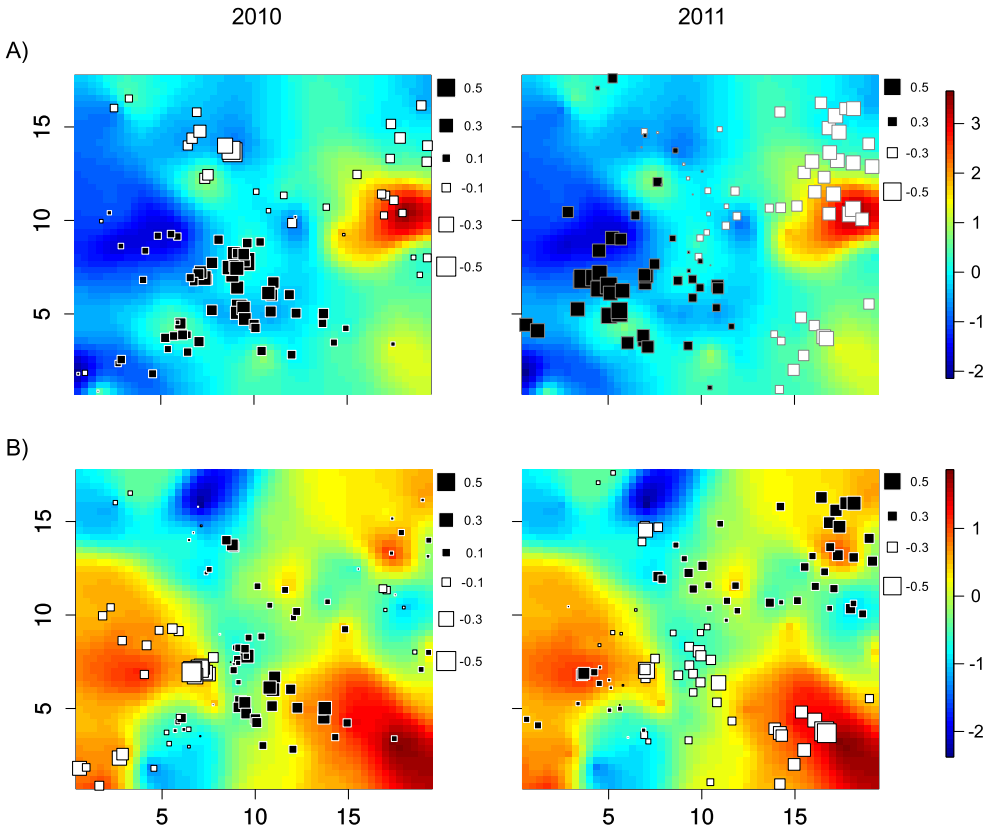


Figure 1.S2: *Lagged principal scores and inferred values of environmental variables. A) sPC_1 and anions. B) sPC_2 and cations 1.*

Table 1.S1: *Model selection for spatially lagged autoregressive models for sPC1 and sPC2. Best-ranked models are listed in ascendant AIC showing parameter estimates and values of Moran's I test over the residuals.*

PC1	Light availability	Anions (N, P)	Cations 1 (Mg^{2+},K^{+})	Cations 2 (Na^{+})	Field capacity	ρ	Moran's I	AIC_c	LogLik.	w_i
2010	**0.165**		**-0.075**		**-0.075**	**0.888**	**0.025**	91.27	-40.316	0.357
	0.169		-0.074	0.019	**-0.074**	**0.883**	**0.023**	93.144	-40.12	0.14
	0.144					**0.88**	**0.03**	92.509	-43.129	0.192
	0.143				-0.042	**0.875**	**0.027**	92.693	-42.136	0.175
	0.173	-0.026	-0.089		**-0.07**	**0.886**	**0.025**	93.222	-40.159	0.135
2011	0.069	**-0.1**				**0.869**	**0.026**	86.77	-39.175	0.232
			0.117			**0.878**	**0.032**	86.996	-40.373	0.207
	0.038		**0.101**			**0.866**	**0.028**	88.219	-39.899	0.112
		-0.099				**0.905**	**0.044**	88.202	-40.976	0.113
		-0.044	0.082			**0.879**	**0.031**	88.56	-40.07	0.095
	0.059	-0.079	0.032			**0.861**	**0.024**	88.788	-39.075	0.084
	0.068	-0.089			-0.019	**0.865**	**0.025**	88.922	-39.142	0.079
	0.068	**-0.102**		-0.009		**0.868**	**0.026**	88.951	-39.156	0.078

PC2	Light availability	Anions (N, P)	Cations 1 (Mg^{2+},K^{+})	Cations 2 (Na^{+})	Field capacity	ρ	Moran's I	AIC_c	LogLik.	w_i
2010		-0.072	**-0.098**	**-0.127**		**0.898**	**0.045**	70.497	-29.93	0.2
	-0.07			**-0.125**		**0.904**	**0.06**	70.81	-31.195	0.171
	-0.057		-0.041	**-0.125**		**0.893**	**0.052**	71.235	-30.298	0.138
	-0.04	-0.057	-0.079	**-0.131**		**0.893**	**0.047**	71.541	-29.319	0.119
		-0.083	**-0.089**	**-0.127**	0.03	**0.901**	**0.049**	71.888	-29.492	0.1
	-0.069			**-0.123**	0.03	**0.903**	**0.061**	71.735	-30.548	0.108
			-0.055	**-0.117**		**0.901**	**0.052**	71.803	-31.691	0.104
	-0.041	-0.068	-0.069	**-0.13**	0.031	**0.896**	**0.052**	72.915	-28.849	0.06
2011			-0.049			**0.927**	**0.098**	74.035	-33.893	0.173
					0.052	**0.928**	**0.095**	74.354	-34.052	0.147
				-0.037		**0.927**	**0.093**	74.954	-34.352	0.109
	-0.024					**0.927**	**0.092**	75.17	-34.46	0.098
		0.018				**0.927**	**0.093**	75.431	-34.59	0.086
			-0.046	-0.031		**0.927**	**0.097**	75.726	-33.653	0.074
		-0.035	-0.076			**0.929**	**0.101**	75.775	-33.677	0.072
				-0.029	0.047	**0.927**	**0.094**	76.111	-33.845	0.061
	-0.009		-0.045			**0.927**	**0.097**	76.15	-33.864	0.06
			-0.039		0.014	**0.927**	**0.097**	76.174	-33.876	0.059
	-0.019				0.049	**0.927**	**0.095**	76.199	-33.889	0.059

Abreviations: AIC_c, second order AIC; w_i, Akaike weight of each model. Significant values of parameters estimates and of Moran's I are in bold.

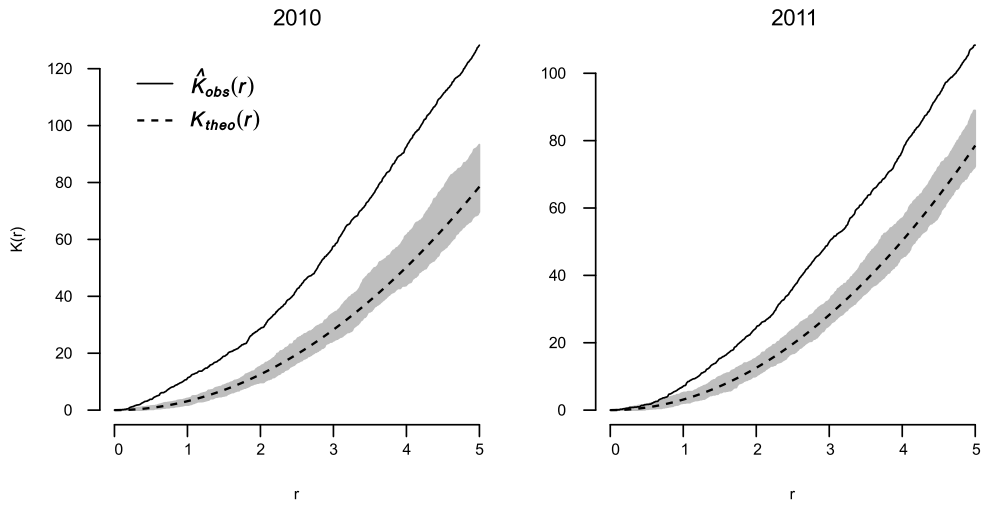


Figure 1.S3: *Ripley's K functions for both cohorts.*

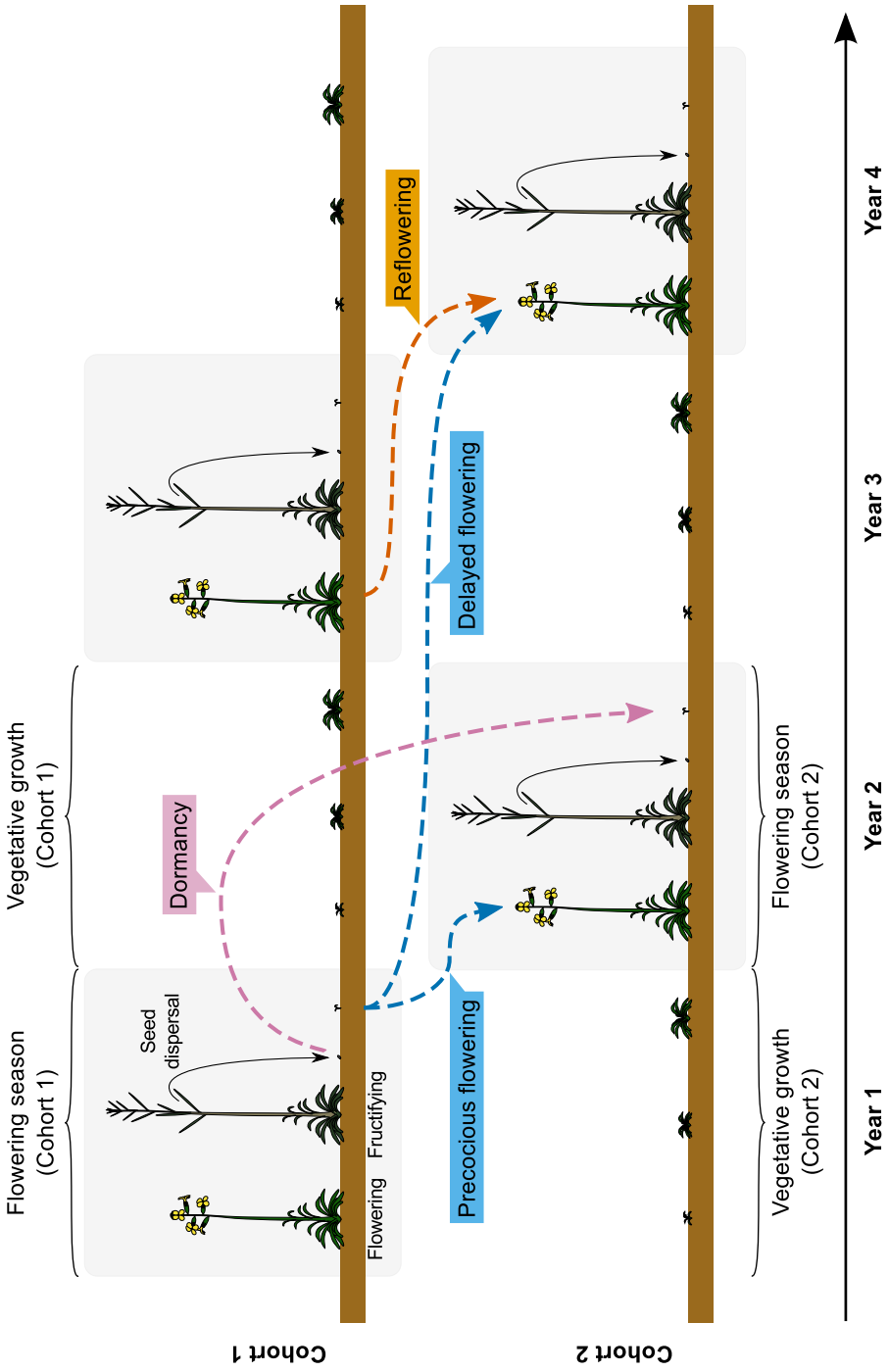


Figure 1.S4: *Pathways of inter-annual migration. Life cycle of two flowering cohorts (cohort 1 and cohort 2) are shown along four years (year 1 to 4). Dashed lines define the different hypothetical pathways taken by an inter-annual migrant from the flowering season of the cohort 1 to that of cohort 2.*

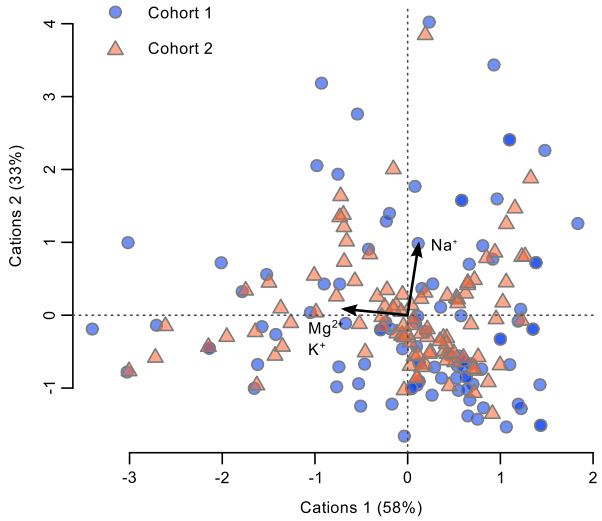


Figure 1.S5: *Biplot of the PCA performed over the soil cations. Circles and triangles depict the scores of the observations on the principal components 1 and 2 (cations 1 and cations 2). The coefficients of the variables are marked with arrows.*

2

The structure of individual plant-pollinator interactions

Abstract

In plants with a generalist pollination system, individuals often present varying levels of generalization. This variation can result from differential preferences of pollinators on plant traits or to spatio-temporal turnovers of the interactions. We characterized the individual generalization in a population of *Erysimum mediohispanicum* in two consecutive years, and decomposed it on three descriptors: niche breadth, overlap and identity. Following, we correlated these with plant phenotype, microenvironment and phenology. We found a high variation among individuals in the three descriptors. Niche breadth was affected positively by flowering duration, while niche overlap by light availability. Finally, we found that plant could be grouped in five clusters based on sharing similar pollinators. These clusters were not consistently related to any plant trait among years. We conclude that flowering phenology and light microenvironment structure the level of generalization within populations of *E. mediohispanicum*, and thus could be important driving factors for this plant evolution at a small spatial scale.

Introduction

Individual variation is on the basis of any evolutionary process (Hallgrímsson and Hall, 2005). In plants with a generalist pollination system, all plants in a population are assumed to interact with random subsets of pollinators as a consequence of sampling effects. However, several studies have demonstrated individual spatio-temporal variations in the floral visitors, mostly as a consequence of contrasting responses them to certain plant traits. For example, Herrera (1995a) found that the insect visiting the flowers of *Lavandula latifolia* responded to microenvironmental changes in light conditions, causing a variation in the pollinator assemblages at a fine spatial scale. This same author claimed that the variation in individual generalization among plants of *L. latifolia* was comparable in magnitude to the variation found among populations (Herrera, 2005). When variations like this occur, it is expected that mating among plants deviate from random. In this sense, other studies have found assortativity in the mating events among plants flowering at the same time (Weis *et al.*, 2005; Elzinga *et al.*, 2007; Ison *et al.*, 2014), having similar phenotypes (Jones and Reithel, 2001) or sharing same microenvironmental conditions (Dyer *et al.*, 2012; Kamm *et al.*, 2010). These studies evidence differences among plants in the pollinator visitation frequencies.

The level of generalization of a plant population is the consequence of integrating the generalization levels of all individuals. If all individuals interact with the same pollinator assemblage, each single individual will be representative of the overall population generalization. If, otherwise, different individuals interact with different subsets of the pollinator fauna, the pollinator assemblage of the population results from the integration of the individual pollinator assemblages. Under the latter conditions, we consider the generalization to be structured. Using the population assemblage as a baseline, individual generalization can be partitioned in several descriptors that may help to disentangle how generalization is structured. For example, niche breadth, refers to the variety of resources used, and can be measured as its number or diversity. This descriptors can vary among individuals (Araújo *et al.*, 2008) including plants referring to their pollination niche (Gómez *et al.*, 2011; Dupont *et al.*, 2014). Niche overlap, refers to the overlap in relation to that of the population (Bolnick *et al.*, 2002). Finally, niche identity alludes to the assignation to an assemblage of differentiated pollinators (Johnson, 2010; Pauw, 2013), and can be used to group individual plants sharing similar pollinators (Valverde *et al.*, 2016b).

This structuration of a resource use has been widely studied in animals (Bolnick *et al.*, 2003), but it also in individual-plant-pollinator interactions

(Dupont *et al.*, 2014). For example, in the highly generalist species *Erysimum mediohispanicum* (Gómez *et al.*, 2009b, 2014a), pollinators show preferences for some phenotypic traits (Gómez *et al.*, 2006b, 2008b; Gómez and Perfectti, 2010). Gómez *et al.* (2011) showed that these preferences give rise to individual-based networks that present relevant architectural properties, demonstrating that generalization can be structured among individuals in plant populations. More interesting, these authors found that this structure leads to important consequences for plant reproductive output (Gómez and Perfectti, 2012) suggesting assortative mating.

Recently, Valverde *et al.* (2016a) described the fine-scale spatial genetic structure (SGS) of a population of *E. mediohispanicum*. In this study, they showed that genotypes were distributed, at least to some extent, following a spatial gradient on light availability. They argued that this type of SGS could be the result of an assortative pattern of pollen flow. We hypothesize that, for this to happen, the population must present what we call a structured generalization. Structured generalization refers to an structured pattern of interaction among the plant and its pollinators that results in non-random differences in the generalization levels among individuals.

In this study we assess the individual variation in the generalization level occurring in the population of *E. mediohispanicum* studied by Valverde *et al.* (2016a). We explore if this variation responds to spatio-temporal factors and/or to among-plant variations in phenology or environmental conditions. To do so, we decompose the degree of generalization of each plant in its niche breadth, overlap and identity. Following, we correlate these descriptors with flowering phenology, light microenvironmental conditions and phenotypic traits. With this study we aim to set the groundwork to explore the evolutionary consequences of a structured generalization occurring at a fine spatial scale.

Materials and methods

Data sampling

During 2010 and 2011, we set up a 20 × 20 m plot in a natural population of *E. mediohispanicum* at the Sierra Nevada protected area. Each year we marked 100 flowering individuals and located them at the centimetre level. We removed the flowering stalks of the remaining individuals within the population and in a buffer diameter of 10 m width. Along the flowering

season we daily performed one to four observations of 5 minutes per plant in which we recorded all insect species interacting with the flowers. Insects were clumped in 23 functional groups (see Appendix 'Funcional groups') thought to exert similar selective pressures on *E. mediohispanicum* (in the sense of Fenster *et al.*, 2009). hen For each plant we measured some variables that might influence its pollinator assemblage. First, we characterized each plant phenotype measuring eight traits: stalk height (cm), flower tube length (mm), flower diameter (mm), the amount of pollen grains per flower using a Neubauer chamber, and four orthogonal variables describing different aspects of the corolla shape (partial warps, RWs). The latter were obtained after a Generalized Procrustes Analysis performed on 32 landmarks located on planar front-view pictures of the flowers in anthesis (Walker, 2000; Gómez and Perfectti, 2010). For that, we used the softwares TPSDIG v. 1.4 and TPSRELW v. 1.11 (`http://life.bio.sunysb.edu/morph/morphmet.html`).

We characterized the microenvironmental light conditions at each plant by means of hemispherical pictures taken with a fish-eye lens (FCE8, Nikon, Tokyo, Japan) and Coolpix 995 digital camera (Nikon). We processed these pictures with the Hemiview v. 2.1 software (1999, Delta-T Devices Ltd, Cambridge, UK) and extracted the Direct Site Factor, a variable that denotes the proportion of radiation at the site in relation to an open area (Anderson, 1964).

We measured the degree of spatial isolation using the area defined by the polygons resulting after a Voronoi tessellation (Bender *et al.*, 2003; Krebs, 1989). And finally, we characterized the flowering duration of each plant by daily surveying its flowering status (see Valverde *et al.*, 2016b).

Descriptors of individual generalization

For each plant we quantified the individual generalization by means of its niche breadth, niche overlap and niche identity. The following indices were used as estimators to characterize each of these descriptors of individual generalization:

1. Niche breadth was quantified using the following indices:

   a) Richness of functional groups visiting each plant.

   b) Diversity of functional groups using the Hurlbert's probability of interspecific encounter (PIE, Hurlbert, 1971). This index expresses the probability that insects chosen at random visiting an individual belong to different functional groups:

$$PIE = \frac{N}{N-1} \times \left(1 - \sum_{i=1}^{S} p_i^2\right)$$

where N is the sample size, and p_i^2 is the proportional abundance of the functional group i.

2. Niche overlap, was estimated using the Czekanowski's proportional similarity index (PS_i) (Feinsinger *et al.*, 1981; Schoener, 1968), adapted for a comparison with the whole population (Bolnick *et al.*, 2002), and calculated as:

$$PS_i = 1 - 0.5 \times \sum_j |p_{ij} - q_{.j}|$$

here, p_{ij} denotes the frequency at which the individual i interacts with the pollinator j, and $q_{.j}$ the total frequency of interaction of the pollinator j in the population. This index gets values from 0 to 1 (complete overlap with the population niche). We calculated this index using the package 'RInSp' in R (Zaccarelli *et al.*, 2015).

3. Niche identity was determined using the QuanBiMo algorithm implemented in the 'bipartite' R package (Dormann and Gruber, 2008). This algorithm considers individual plants and functional groups a nodes of a bipartite network and uses a simulated annealing-Monte Carlo procedure to find the groups (modules) of plant and pollinators interacting with a higher frequency than expected at random. To do so, this algorithm optimizes the modularity index (Q) of the network:

$$Q = \frac{1}{2 \times m} \times \sum_{ij} \left(A_{ij} - \frac{k_i \times k_j^T}{2 \times m}\right) \times \sigma(c_i, c_j)$$

where m denotes half of the links in the network. A_{ij} the weight of the link between the plant individual i and the functional group j. The term $k_i \times k_j^T / 2 \times m$ is the expected weight between i and j under a null model (Newman and Girvan, 2004). k_i is the marginal sum of row i while k_j is the marginal sum of column j. c_i and c_j are the modules assigned for the individual i and for the functional group j respectively, such that $\sigma(c_i, c_j)$ equals 1 if $c_i = c_j$ and 0 if $c_i \neq c_j$.

Because Simulated annealing can fall into local optima, and thus yield different modular configurations among runs (Guimerà and Amaral, 2005a), we performed 500 runs and kept the configuration with the highest modularity.

The robustness of the QuanBiMo algorithm was checked by exploring the concordances among the partitions of plants in modules yielded by each run. To do so, for each pair of runs we calculated the mutual information index developed by Danon *et al.* (2005):

$$I_{AB} = \frac{2 \times \sum_{i=1}^{N_M^A} \sum_{j=1}^{N_M^B} n_{ij}^{AB} \times log\left(\frac{n_{ij}^{AB} \times S}{n_i^A \times n_j^B}\right)}{\sum_{i=1}^{N_M^A} n_i^A \times log\left(\frac{n_i^A}{S}\right) + \sum_{j=1}^{N_M^B} n_j^B \times log\left(\frac{n_j^B}{S}\right)}$$

Being A and B the two partitions compared, S denotes the total number of nodes, n_i^A the number of nodes in module i of the partition A, n_j^B the number of nodes in module j of the partition B, and n_{ij}^{AB} the number of nodes in both modules i and j.

The distribution of the mutual information values was then compared with that obtained under a random assignation of modules in each run.

Correlates of individual generalization

We investigated the effects that flowering phenology, phenotype and microenvironment had on each of the indices used to estimate the descriptors of individual generalization. Those variables were standardized and we calculated their pairwise correlation in order to test for the absence of substantial correlations.

To explore the correlation with the quantitative indices (richness, diversity and proportional similarity) we constructed generalized least squared models. These models allowed us to take into account the spatial autocorrelation by incorporating a spatial correlation structure in the models. To explore the correlations with the module assignation, we used Generalised Linear Mixed Models using Markov chain Monte Carlo techniques (MCMCglmm) (Gómez *et al.*, 2014a; Hadfield and Nakagawa, 2010) using the package 'MCMCglmm' in R (Hadfield, 2010). These models are capable to handle multivariate response variables (module) and take into account spatial autocorrelation by incorporing the spatial distance in the variance structure. In addition, in order to be consistent with the latter analyses, we also analyzed the quantitative indices using MCMCglmm models.

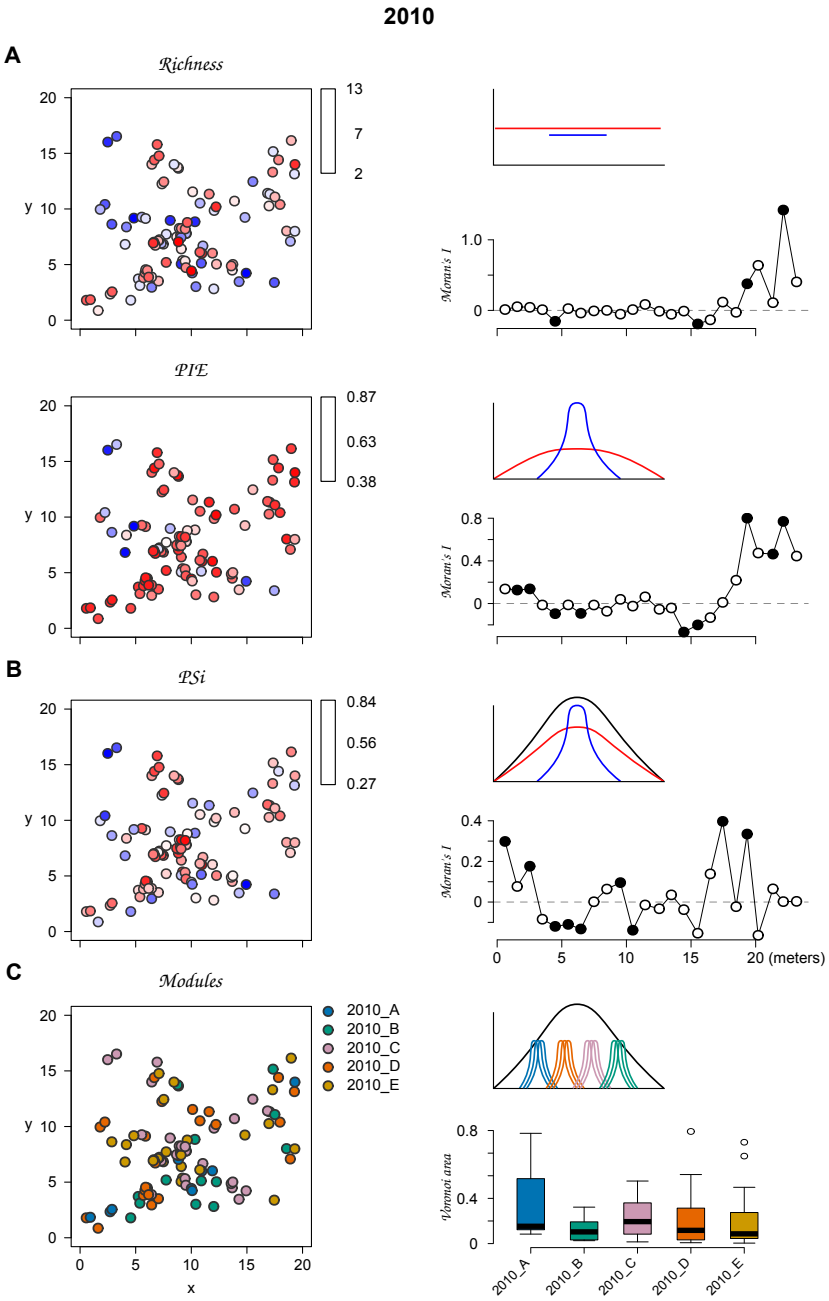
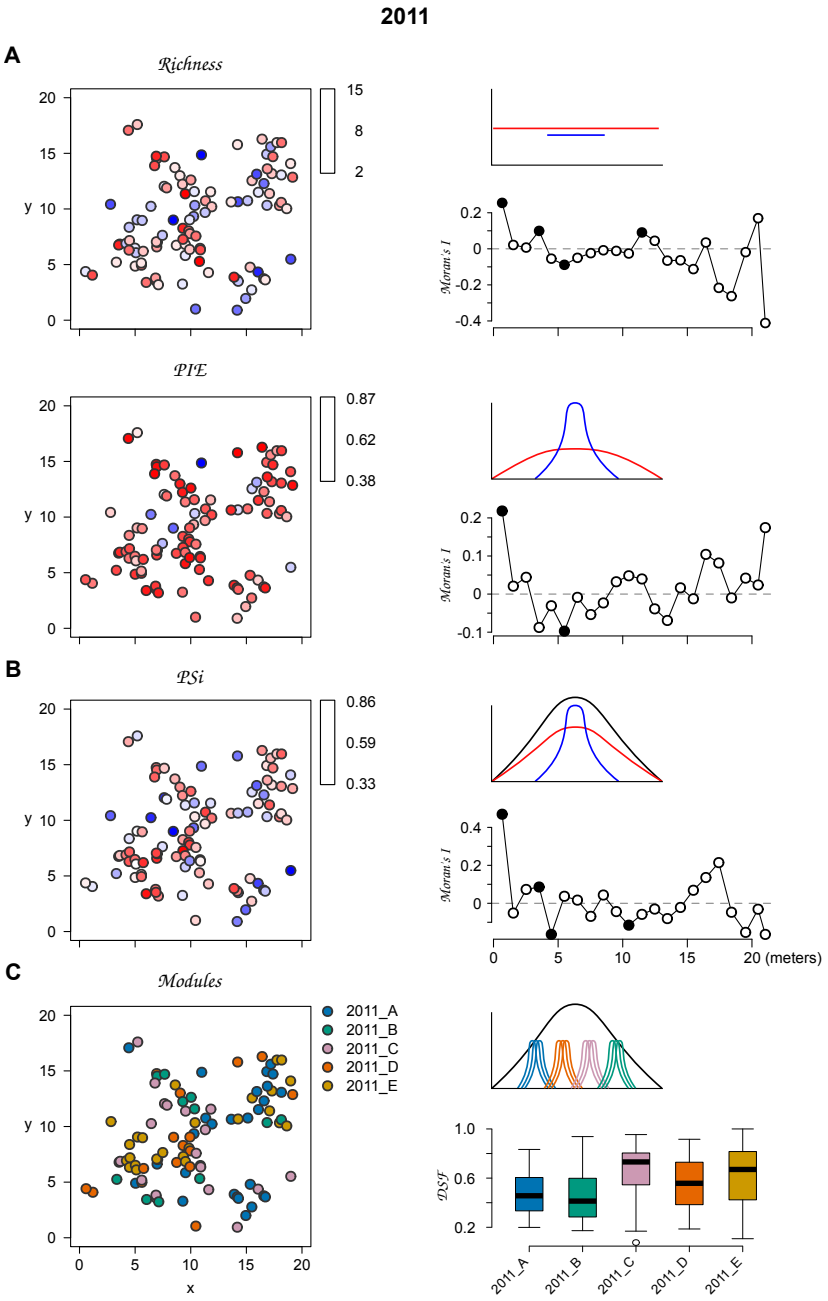


Figure 2.1: *Individual generalization in years 2010 (left page) and 2011 (right page). A) Niche breadth: richness and diversity (PIE). B) Niche overlap: proportional similarity index (PS_i). C) Niche identity: module assignation. Each panel contains the spatial location of plants and their value and an ideogram of the meaning of the descriptor with black lines indicating the population level and colors the different individual values. Panels within A and B contain a Moran's I distogram, while panel in C contains module values of Voronoi area in 2010 and DSF in 2011.*


2011
A
Richness
15
8
2
Moran's I
PIE
0.87
0.62
0.38
Moran's I
B
PSi
0.86
0.59
0.33
Moran's I
0 5 10 15 20 (meters)
C
Modules
2011_A
2011_B
2011_C
2011_D
2011_E
DSF
x
y

Results

Descriptors of individual generalization

Plants varied in their niche breadth. Richness ranged from 2 to 15 functional groups (7.81 ± 2.55 in 2010, 9.23 ± 2.89 in 2011). This index showed little spatial autocorrelation in 2010 but was autocorrelated below 1 meter in 2011 (Figure 2.1a). PIE, showed almost the same variation in both years, ranging from 0.37 to 0.87 ($average = 0.75 \pm 0.11$ in 2010, $average = 0.76 \pm 0.09$ in 2011).

Niche overlap, estimated with the PS_i index, also presented a wide variation among plants in both years ($average = 0.61 \pm 0.12$ in 2010, $average = 0.63 \pm 0.12$ in 2011), which was spatially autocorrelated below 3 meters (Figure 2.1b).

Regarding niche identity, we found 5 niches (modules) in each year. The analysis of modularity generated partitions with values of modularity ranging from 0.14 to 0.21 in 2010 (0.19 ± 0.01) and from 0.13 to 0.20 in 2011 (0.18 ± 0.01), bearing a number of modules between 2 and 5 (Figure 2.S1). The average mutual information index among runs was 0.38 for both years (95% CI $[0.12, 0.72]$ in 2012, 95% CI $[0.13, 0.71]$ in 2011), and deviated from that expected under a random module assignation ($\bar{I}_{AB(EXPECTED)} = 0.03$, 95% CI $[0, 0.07]$ for both years), indicating a good concordance among runs in the module assignation.

Correlates of individual generalization

The indices used to describe the individual generalization significantly correlated to some plant traits (Figure 2.2). However, most of these correlations occurred only for one year. Regarding niche breadth, richness showed significant positive correlations with flowering duration, which was consistent among years (model estimate = 6.552 ± 1.253 for year 2010, and 9.968 ± 2.375 for year 2011, Table **??**). This index also showed a significant negative correlation with Voronoi area in 2010 and positive with stalk height in 2010 and DSF in 2011. Contrary to richness, PIE did not show any significant correlations being consistent among years. This index showed significant positive correlations with DSF and flowering duration in 2010 and corolla tube and RW2 in 2011 (Table 2.S2).

In relation to niche overlap, PS_i showed in both years significant positive correlations with light availability (DSF; 0.134 ± 0.059 in 2010, and

0.127 ± 0.046 in 2011). Additionally, this index showed a significant negative correlation with Voronoi area and number of pollen grains in 2010, and positive with stalk height in 2010 and with flowering duration in 2011.

Finally, for niche identity, module assignation did not show consistence among years in the significances of the correlations found with plant traits (Figure 2.2). In 2010, module assignation correlated negatively with Voronoi area in 2010 ($posterior\ mean = -77.429,\ CI\ [-179.211,\ 6.644]$), with plants belonging to module A presenting higher Voronoi areas than those belonging to module B (one-side Wilcoxon rank sum test; $Z = 1.887,\ p = 0.030$; Figure 2.1c). In 2011, module assignation correlated positively with *DSF* ($posterior\ mean = 116.85,\ CI\ [-0.772,\ 116 - 608]$, see Table 2.S3). Plants from module C had higher values of *DSF* than plants from module B ($Z = 2.178,\ p = 0.015$; Figure 2.1c).

Discussion

In this chapter we demonstrate that within a population of *E. mediohispanicum* plants vary in their level of generalization. We have found this variation to happen for individual niche breadth, overlap and identity. In addition, our analyses have revealed that some of the individual plant traits, specially light microenvironment and flowering duration, can consistently affect to these descriptors of individual generalization.

The variation found among plants in their level of generalization indicates disparities in the insects visiting the studied plants. Typically, species presenting a generalist resource use are compounded of individuals presenting a varying range in their level of generalization (Araújo *et al.*, 2011; Bolnick *et al.*, 2002, 2003; Costa *et al.*, 2015). In entomophilous plants several studies have demonstrated that the individual niche of pollinators can vary among individuals (Dupont *et al.*, 2015; Gómez *et al.*, 2011; Gómez and Perfectti, 2012; Herrera, 1995a, 2005; Thompson, 2001). This variation may respond to pollinator preferences on plant phenotype such as floral resources (Leiss and Klinkhamer, 2005; Real and Rathcke, 1991), flower display (Eckhart, 1991; Benitez-Vieyra *et al.*, 2006), flower color (Malerba and Nattero, 2012) or flower shape (Gómez *et al.*, 2006a). But also to preferences on environmental factors such as light availability (Herrera, 1995a).

In our study, niche breadth was positively correlated to flowering duration. Specifically, we found significant effects of flowering duration on the richness of functional groups visiting the flowers of *E. mediohispanicum*. This finding

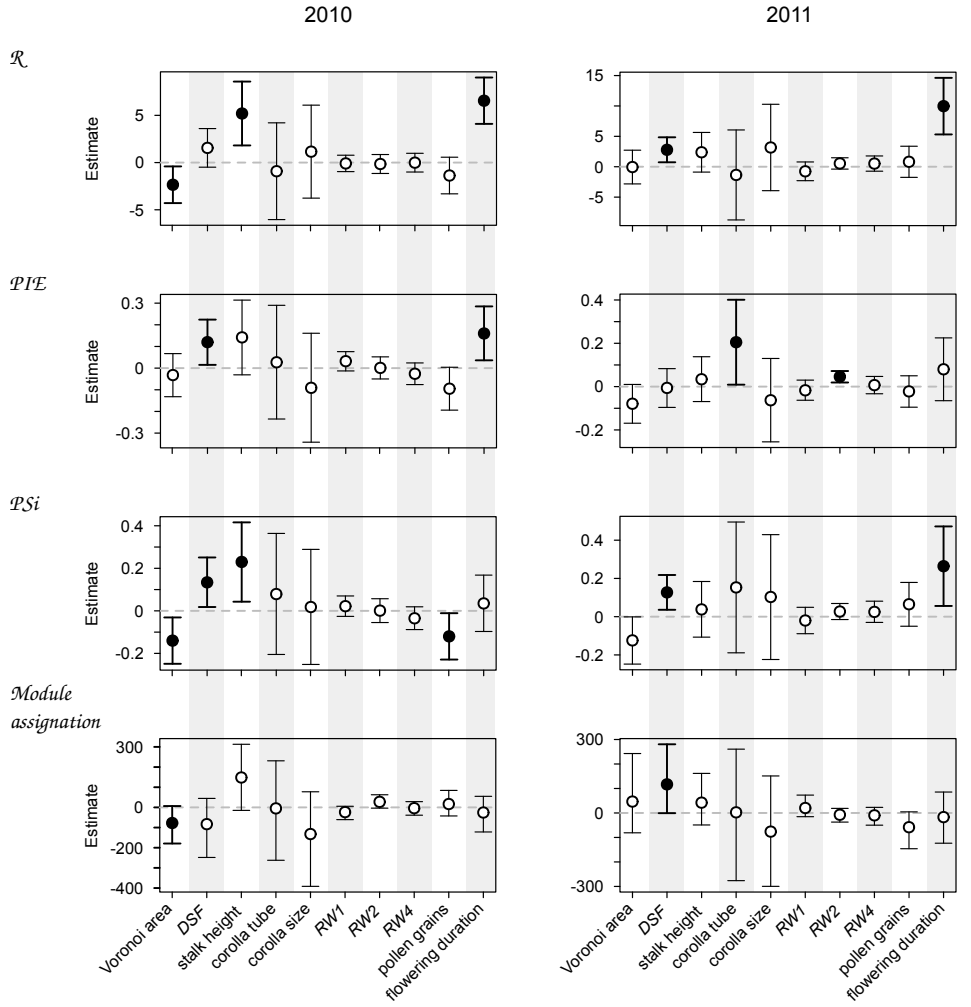


Figure 2.2: *Models estimates of the different factors affecting individual generalization.*

suggests that a temporal change of the pollinator fauna may exist in the studied population. Within a flowering season, pollinators may present a temporal turnover (Ashman and Stanton, 1991; Herrera, 1988; Olesen *et al.*, 2008; Tur *et al.*, 2015), affecting to the visitation frequency within a plant population (Elzinga *et al.*, 2007; Herrera, 1988). Under this scenario, if a plant flowers for a longer period it should interact with more species, thus presenting a wider niche breadth. In addition to this, we think that the temporal variation in pollinator assemblage together with differing flowering schedules may affect to the plant-pollinator interactions in such a way that plants flowering at the same time will present higher similarities in their pollinator fauna. If so, these coflowering plants should have higher chances of mate. Several studies have

demonstrated that flowering synchrony among conspecifics facilitates mating (Ison *et al.*, 2014; Ison and Wagenius, 2014; Weis, 2005). We think that in this population this may be an important factor determining the mating patterns occurring within population.

We found that niche overlap was affected by light availability. Plants growing in microenvironments with higher light availability had higher values of proportional similarity index, reflecting a preference of insects for open areas. Open areas in woodlands are known to attract a more diverse assemblage of pollinators (Herrera, 1995a,b; Lee *et al.*, 2001; Norgate *et al.*, 2010). Moreover, the difference in pollinator fauna between shaded and open areas affects the reproductive output of plants (Kilkenny and Galloway, 2008) as also their mating patterns. Regarding this, several studies have demonstrated assortativity in the mating patterns driven by the light conditions (Dyer *et al.*, 2012; Kamm *et al.*, 2010). We think that in our study population this may be also happening. In a former study, Valverde *et al.* (2016b) discovered that in this same population the distribution of adult plant genotypes followed a light gradient. These authors suggested that this could be due to an assortative mating occurring due to similarities in light availability. Our findings supports this hypothesis. However, further research is needed to demonstrate the functionality of the disparities in pollinator fauna in affecting pollen flow.

Finally, niche identity did not show consistent relationships with any plant trait. We think that this inconsistence may be due to the flowering phenology of plants. If both plants and pollinators show a temporal turnover, the scenario of interaction may also change over time. The integration of all interaction over time will thus mitigate any possible structure of the interactions happening in a short time period. To test this hypothesis further analyses are needed to disentangle the effects of time in the interactions occurring at the individual-plant level.

This study demonstrates that the individual generalization can vary at a fine-scale, indicating a structure in the interactions among individual plants and their pollinators, and that these differences among individuals in plan traits and flowering phenology are responsible of much of this structure. With these results we set the foundations for a deeper understanding of the evolutionary consequences of structured generalization in *E. mediohispanicum*.

Supplementary information

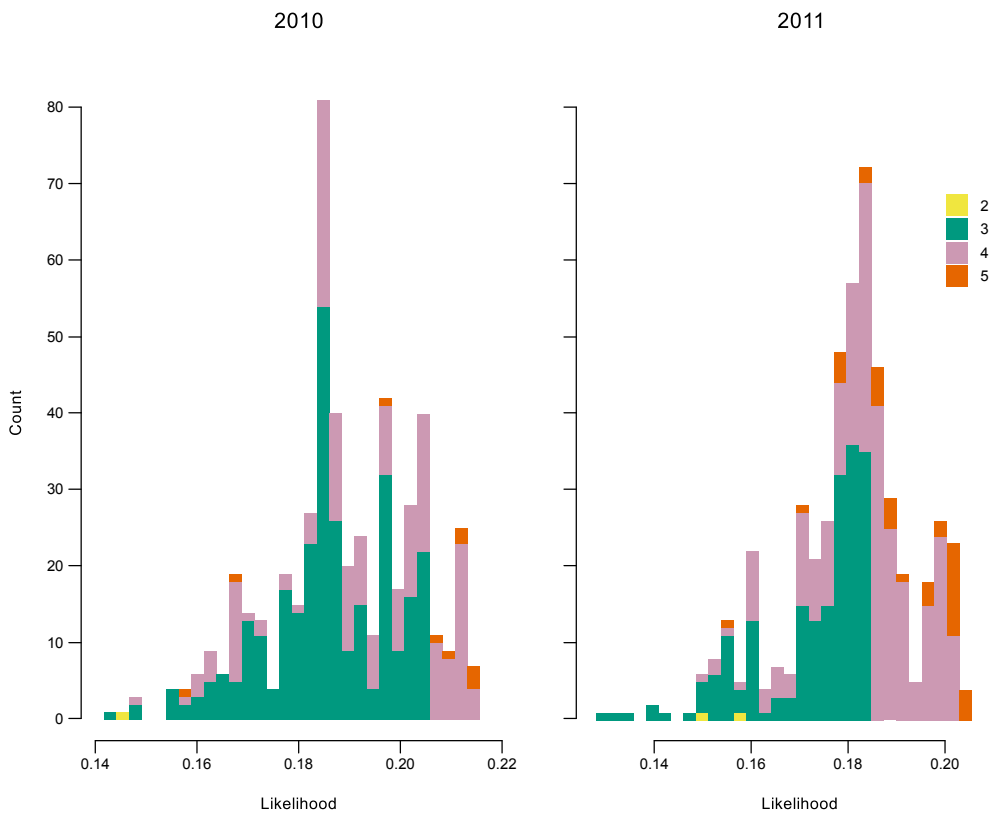


Figure 2.S1: *Likelihood (Modularity) distributions of all the QuanBiMo optimal configurations. The histogram stacks the counts bearing equal number of modules.*

Table 2.S1: *Correlation matrix among plant traits. Pearson's correlation for each pair of standardized variables is shown. Bold values denote its two tailed probability. Correlations for the year 2010 are shown below the diagonal, while for the 2011 are shown above the diagonal*

	Voronoi area	*DSF*	stalk height	stalk number	corolla tube	corolla size	*RW1*	*RW2*	*RW4*	pollen grains	flowering duration
Voronoi area	-	-0.17	0.022	-0.158	0.109	0.002	-0.057	0.084	0.057	-0.02	0.007
DSF	**-0.42**	-	-0.015	**0.29**	0.067	-0.021	0.021	0.06	0.069	-0.106	0.007
stalk height	0.095	0.049	-	-0.122	**0.34**	**0.245**	0.161	0.088	**-0.237**	-0.025	**0.495**
stalk number	-0.183	0.174	0.058	-	-0.043	0.048	-0.02	-0.046	0.064	0.007	-0.145
corolla tube	0.006	0.077	**0.276**	-0.053	-	**0.61**	0.114	0.001	0.022	0.121	**0.224**
corolla size	**0.208**	-0.161	**0.248**	-0.053	**0.451**	-	0.143	0.149	-0.079	-0.074	0.217
RW1	-0.111	-0.07	0.048	-0.144	-0.034	0.081	-	0	0	-0.006	0.183
RW2	**0.245**	0.022	0.154	-0.126	-0.019	0.151	0	-	0	**-0.232**	0.066
RW4	-0.097	0.08	0.05	0.091	0.146	**-0.199**	0	0	-	0.079	-0.109
pollen grains	0.184	0.111	**0.177**	-0.148	**0.248**	**0.273**	0.159	0.003	-0.042	-	0.088
flowering duration	0.004	0.066	0.396	0.051	-0.01	0.131	-0.07	0.148	-0.089	-0.115	-

Table 2.S2: *Generalized least squares models for the niche breadth and overlap. The columns denoted as 'estimates' show the values of the Akaike Information Criterion (*AIC*) and the estimate ± the standard deviation of the coefficients.*

		2010			2011		
		estimates	t-value	p-value	estimates	t-value	p-value
R	*AIC*	395.391			435.137		
	Intercept	1.371 ± 2.287	0.600	0.550	-1.472 ± 2.521	-0.584	0.561
	voronoi area	-2.349 ± 0.991	-2.370	0.020	-0.052 ± 1.414	-0.037	0.971
	DSF	1.555 ± 1.043	1.491	0.140	2.787 ± 1.049	2.658	0.009
	stalk height	5.197 ± 1.725	3.012	0.003	2.38 ± 1.664	1.430	0.156
	corolla tube	-0.915 ± 2.614	-0.350	0.727	-1.351 ± 3.777	-0.358	0.721
	corolla size	1.157 ± 2.512	0.461	0.646	3.167 ± 3.626	0.874	0.385
	RW1	-0.089 ± 0.443	-0.202	0.841	-0.755 ± 0.786	-0.961	0.339
	RW2	-0.151 ± 0.512	-0.294	0.769	0.542 ± 0.476	1.138	0.258
	RW4	-0.009 ± 0.506	-0.017	0.986	0.519 ± 0.64	0.811	0.420
	pollen grains	-1.371 ± 0.99	-1.385	0.170	0.812 ± 1.306	0.622	0.536
	flowering duration	6.552 ± 1.253	5.231	< 0.001	9.968 ± 2.375	4.198	< 0.001
PIE	*AIC*	-116.310			-145.703		
	Intercept	0.605 ± 0.117	5.150	0.000	0.603 ± 0.09	6.708	0.000
	voronoi area	-0.032 ± 0.051	-0.638	0.525	-0.079 ± 0.046	-1.740	0.085
	DSF	0.12 ± 0.053	2.242	0.028	-0.006 ± 0.046	-0.142	0.887
	stalk height	0.142 ± 0.088	1.611	0.111	0.034 ± 0.053	0.650	0.517
	corolla tube	0.027 ± 0.134	0.204	0.839	0.205 ± 0.1	2.053	0.043
	corolla size	-0.091 ± 0.128	-0.707	0.481	-0.063 ± 0.098	-0.637	0.526
	RW1	0.032 ± 0.023	1.392	0.167	-0.017 ± 0.024	-0.705	0.483
	RW2	0.001 ± 0.026	0.036	0.971	0.046 ± 0.013	3.405	0.001
	RW4	-0.026 ± 0.026	-1.022	0.310	0.007 ± 0.021	0.346	0.730
	pollen grains	-0.095 ± 0.051	-1.873	0.064	-0.022 ± 0.037	-0.604	0.547
	flowering duration	0.16 ± 0.063	2.535	0.013	0.08 ± 0.074	1.083	0.282
PS_i	*AIC*	-102.345			-99.768		
	Intercept	0.395 ± 0.128	3.095	0.003	0.188 ± 0.112	1.679	0.097
	voronoi area	-0.14 ± 0.056	-2.507	0.014	-0.124 ± 0.063	-1.980	0.051
	DSF	0.134 ± 0.059	2.262	0.026	0.127 ± 0.046	2.735	0.008
	stalk height	0.23 ± 0.095	2.413	0.018	0.038 ± 0.074	0.516	0.607
	corolla tube	0.079 ± 0.145	0.547	0.586	0.153 ± 0.174	0.876	0.384
	corolla size	0.018 ± 0.138	0.134	0.894	0.103 ± 0.167	0.618	0.538
	RW1	0.022 ± 0.025	0.908	0.367	-0.02 ± 0.035	-0.578	0.565
	RW2	0.001 ± 0.029	0.026	0.979	0.027 ± 0.021	1.276	0.205
	RW4	-0.035 ± 0.027	-1.271	0.207	0.025 ± 0.029	0.892	0.375
	pollen grains	-0.12 ± 0.056	-2.150	0.034	0.065 ± 0.059	1.104	0.273
	flowering duration	0.035 ± 0.068	0.523	0.602	0.264 ± 0.106	2.492	0,015

Table 2.S3: *MCMC generalized mixed models for the niche identity. The columns denoted as 'estimates' show the value of the Deviance Information Criterion (*DIC*).*

	2010			2011		
	estimates	95% CI	MCMC p-value	estimates	95% CI	MCMC p-value
DIC	243.921			253.554		
Intercept	153.835	[-101.461 to 432.864]	0.178	19.677	[-246.284 to 292.63]	0.846
voronoi area	-77.429	[-179.211 to 6.644]	0.049	46.639	[-81.023 to 242.707]	0.516
DSF	-83.113	[-248.3 to 44.538]	0.187	116.850	[-0.772 to 280.53]	0.020
stalk height	148.294	[-14.821 to 312.999]	0.095	42.182	[-48.98 to 161.608]	0.405
corolla tube	-4.95	[-262.317 to 231.004]	0.968	2.552	[-276.448 to 260.616]	0.893
corolla size	-132.235	[-391.637 to 77.425]	0.143	-76.110	[-299.696 to 151.16]	0.439
RW1	-23.416	[-60.742 to 5.536]	0.141	20.283	[-15.167 to 72.97]	0.305
RW2	27.56	[-3.746 to 62.599]	0.070	-7.267	[-37.245 to 18.756]	0.615
RW4	-3.861	[-38.729 to 28.483]	0.868	-9.693	[-49.837 to 22.903]	0.618
pollen grains	16.69	[-42.449 to 84.181]	0.560	-58.112	[-146.05 to 4.539]	0.082
flowering duration	-25.417	[-121.862 to 54.849]	0.555	-16.888	[-123.456 to 85.456]	0.726

3

Variación intraestacional en los visitantes florales de *Erysimum mediohispanicum* en Sierra Nevada

Resumen

Las interacciones planta-polinizador han sido tradicionalmente un tema de gran interés en el área de la ecología y la biología evolutiva, tanto por los servicios ecosistémicos que ofrecen como por la complejidad que presentan como sistema. La comunidad de visitantes florales de una especie vegetal puede ser muy diversa en plantas generalistas pero muy reducida en plantas especialistas, siendo los sistemas generalistas una realidad más común de lo que tradicionalmente se ha pensado. Además, esta comunidad puede presentar importantes fluctuaciones espacio-temporales en composición y abundancia. Esta variabilidad está sujeta a factores intrínsecos y extrínsecos a la planta, y podría afectar notoriamente a diversos aspectos poblacionales. En el presente trabajo estudiamos la variación temporal de la comunidad de visitantes florales en composición, abundancia y diversidad en dos parcelas experimentales de *Erysimum mediohispanicum* Polatschek (Brassicaceae), una especie típicamente generalista. Para centrarnos en las variaciones temporales en la comunidad hemos homogeneizado la distribución espacial y las condiciones microambientales de las plantas. Durante el periodo de floración censamos diariamente los insectos a nivel de morfoespecie, agrupándolos en 16 grupos funcionales basándonos en características morfológicas y comportamentales. Nuestros resultados muestran una gran diversidad en la comunidad de visitantes florales para ambas parcelas así como importantes fluctuaciones temporales en composición y abundancia relativa tanto para morfoespecies como para grupos funcionales. Esta variación temporal en abundancia de los visitantes florales de *E. mediohispanicum* puede tener importantes implicaciones ecológicas y evolutivas.

Plant-pollinator interactions have traditionally been a hot topic in ecology and evolutionary biology due to both the ecological services it offers and its complexity as a system. Plants generally show a high diversity of floral visitors, making generalism a more common condition than expected. Furthermore floral visitor community seems to have spatio-temporal fluctuations in diversity and composition. Those variations respond to plant intrinsic and extrinsic factors and can affect to evolutionary and ecological aspects of plant populations. Here we assess the temporal variation in the floral visitor assemblage of the generalist plant *Erysimum mediohispanicum* Polatschek (Brassicaceae). We set up two experimental plots homogenizing for spatial and microenvironmental conditions to ascertain only the temporal variations in the floral visitors assemblage. During the flowering season we did daily censuses of the floral visitors, determining them to morphospecies level and clumped them in 16 functional groups based on morphological and behavioral characteristics. We found a high diversity for the whole assemblage in both experimental plots with high temporal fluctuations during the flowering season. Our results suggest an important species turnover with high fluctuations in relative abundance for some functional groups, which may turn into important ecological and evolutionary consequences.

Introducción

Las interacciones entre las plantas y sus polinizadores han sido abundantemente estudiadas por ecólogos y biólogos evolutivos (Mitchell *et al.*, 2009). El interés en estos sistemas no solo radica en el atractivo intelectual que despierta su estudio sino también en los importantes servicios ecosistémicos que proveen los polinizadores (Costanza *et al.*, 1997; Kearns *et al.*, 1998) y más recientemente en la creciente preocupación por la actual "crisis de polinizadores" (Potts *et al.*, 2010; Bartomeus *et al.*, 2013) y sus efectos sobre la economía global (Archer *et al.*, 2014). El papel como vectores de flujo genético que juegan muchos animales ha moldeado la diversificación de las angiospermas y la percepción del mundo como lo conocemos hoy en día (Willmer, 2011). Por otro lado, la reciente adopción de herramientas matemáticas de la teoría de grafos (Bascompte y Jordano, 2007) ha incrementado el interés científico y social de los estudios en este campo.

Los ecólogos tradicionalmente han analizado los sistemas de polinización considerando que las plantas consiguen atraer e interaccionar con un grupo reducido de polinizadores eficientes (Faegri y van der Pijl, 1979; Proctor *et al.*, 1996; Burkle y Alarcón, 2011). Sin embargo, múltiples estudios han demostrado que la mayor parte de las plantas interaccionan con un gran número especies animales que actúan o pueden actuar como polinizadores con mayor o menor eficacia (Waser *et al.*, 1996). Puesto que el papel de los polinizadores como agentes selectivos de muchos caracteres reproductivos de las plantas está en la actualidad universalmente aceptado por la comunidad científica (Schemske y Bradshaw, 1999), es necesario caracterizar la comunidad de éstos para entender la evolución de las plantas. Y esto requiere la consideración no sólo de los polinizadores que se ajustan a los síndromes de polinización prefijados, sino de todos los visitantes florales que puedan afectar a la eficacia biológica de las plantas (Waser *et al.*, 1996; Brody, 1997; Cane *et al.*, 2005).

Las poblaciones de una especie vegetal pueden estar sujetas a variaciones en la composición y abundancia del conjunto de visitantes florales con los que interacciona. Esta variación obedece tanto a características intrínsecas de la planta (tamaño poblacional, agregación, fenotipo, etc.) como extrínsecas a ella (abundancia local de polinizadores, dinámica temporal de los mismos, factores climáticos locales, plantas acompañantes, etc.) (Herrera, 1995b). Todos estos factores pueden también afectar a la diversidad de polinizadores, que a su vez puede tener un efecto importante en el éxito reproductivo de la planta (Kremen *et al.*, 2002; Klein *et al.*, 2003; Gómez *et al.*, 2007; Perfectti *et al.*, 2009). Así, el conocimiento de la composición, abundancia y diversidad de los visitantes

florales es crucial para comprender la coevolución mutualista de las plantas y sus polinizadores (Thompson, 1994; Cane *et al.*, 2005).

Las interacciones entre las plantas y sus visitantes florales no son constantes ni en el espacio ni en el tiempo, pudiendo presentar variaciones notables (Herrera, 1988; Horvitz y Schemske, 1990; Herrera, 1995b; Aizen y Feinsinger, 2003; Price *et al.*, 2005; Petanidou *et al.*, 2008). La dimensión espacial ha sido ampliamente estudiada a diferentes escalas (eg, Herrera, 1988, 1995b; Traveset y Sáez, 1997; Minckley *et al.*, 1999; Gómez *et al.*, 2007, 2014a). La variación temporal, por otro lado, ha sido también estudiada a diversas escalas temporales, incluyendo la variación estacional y la plurianual (eg, Herrera, 1988; Wolfe y Barrett, 1988; Schemske y Horvitz, 1989; Horvitz y Schemske, 1990; Ashman y Stanton, 1991; Eckhart, 1992; Vaughton, 1992; Cane y Payne, 1993; Herrera, 1995b; Traveset y Sáez, 1997). Sin embargo, el conocimiento de la variación temporal a escala estacional en las interacciones planta-polinizador es aún limitada, especialmente en comunidades muy diversas donde ha sido difícil determinar la identidad y el grado de reemplazamiento temporal de los visitantes florales (Fang y Huang, 2012).

En este trabajo exploramos la dinámica temporal de los visitantes florales de *Erysimum mediohispanicum* (Brassicaceae) a lo largo del periodo de floración, analizando su abundancia, composición y diversidad, mediante censos intensivos. Para minimizar la variación espacial hemos establecido dos parcelas experimentales donde hemos homogeneizado la distribución espacial de las plantas y las condiciones ambientales. Los objetivos de este trabajo son (1) caracterizar la composición del conjunto de visitantes florales; (2) cuantificar la abundancia y diversidad de los mismos; y (3) analizar su dinámica temporal, para así obtener una visión más rigurosa de la dimensión temporal en la interacción entre *E. mediohispanicum* y sus visitantes florales.

Material y métodos

Especie de estudio

E. mediohispanicum es una herbácea bienal o perenne, generalmente semélpara (monocárpica), con cepa simple y que se distribuye por todo el centro y sureste de la península ibérica (Nieto-Feliner, 2003). Las semillas se dispersan por autocoria durante todo el otoño e invierno y las plántulas emergen a principio de primavera. Durante el primer verano y a veces durante el segundo verano crecen como roseta, entrando en fase reproductiva el segundo

o tercer verano. Durante la reproducción, cada individuo produce entre 1 y 9 escapos florales donde se desarrollan las flores en una inflorescencia corimbiforme que pasan a racimos en la fructificación. Las flores son amarillas, hermafroditas y ligeramente protándicas, con androceo tetradínamo. Cada flor posee dos nectarios laterales que rodean la base de los estambres laterales. El desarrollo de la inflorescencia es acropétalo, con una onda de desarrollo que viaja a lo largo del tallo florífero desde la parte basal a la apical, de tal forma que los primordios florales inferiores se desarrollan antes que los superiores. Tras el período de reproducción la mayoría de los individuos mueren. *E. mediohispanicum* es hipotetraploide ($n = 26$) en la mayoría de las poblaciones peninsulares, aunque las plantas de Sierra Nevada parecen ser diploides ($n = 14$, Nieto-Feliner, 2003). *E. mediohispanicum* presenta un sistema reproductor parcialmente autocompatible (Abdelaziz, 2013), aunque necesita del concurso de polinizadores para maximizar la reproducción (Gómez, 2005).

Diseño experimental

En la primavera del 2012 instalamos dos parcelas experimentales (A y B) de 9 m^2 separadas $15m$ entre sí en el Jardín Botánico de Hoya de Pedraza, situado a 1950 metros de altitud en Sierra Nevada (Granada, España). Justo antes del periodo de floración, trasplantamos 49 plantas a cada parcela experimental, distribuyéndolas en una cuadricula de 7 x 7 plantas distanciadas entre si $0.5m$. Las plantas utilizadas se obtuvieron a partir de semillas de 58 plantas de una población natural de Sierra Nevada, éstas crecieron durante los dos años previos al experimento en el mismo Jardín Botánico. Antes del trasplante, las parcelas y sus alrededores se roturaron para evitar interferencias con otras especies de plantas en la atracción de visitantes florales. Ambas parcelas estuvieron sometidas a similares condiciones lumínicas. En las parcelas instalamos un sistema de riego por goteo que aseguró las mismas condiciones hídricas para cada planta. Este diseño experimental nos permitió reducir la heterogeneidad espacial y microambiental. Las plantas se mantuvieron excluidas de visitantes florales mediante una estructura metálica con malla de $1.5mm$ de luz, excepto durante los periodos de observación.

El periodo de observación de visitantes florales se extendió desde el 23 de mayo hasta el 20 de junio de 2012, coincidiendo con la floración de *E. mediohispanicum* en las parcelas experimentales. Se efectuaron entre uno y cuatro censos por día de observación (con un total de 57 censos en la parcela A y 58 en la parcela B), en cada uno de los cuales se anotó el número e identidad de los insectos que visitaron las flores de cada planta durante cinco minutos. Debido a cierta asincronía floral entre los individuos, cada censo por parcela incluyó, dependiendo de los individuos en flor, de 115 a 245 minutos de

observación, totalizando 24700 minutos de observación. Los censos en ambas parcelas se realizaron aproximadamente a las mismas horas del día y los mismos días. Además, anotamos periódicamente el número de flores abiertas en cada parcela.

Los visitantes florales fueron determinados a nivel de morfoespecie visualmente *in situ* y por medio de fotografías, gracias a la experiencia previa del grupo y la colaboración de especialistas en los diferentes grupos de insectos. Los insectos se agruparon en 16 grupos funcionales en función del tamaño, longitud de la probóscide, ajuste morfológico con la flor y comportamiento (Tabla 3.1).

Análisis de datos

La composición del conjunto de visitantes florales fue comparada entre parcelas mediante análisis de contingencia y el índice de disimilitud de Chao-Jaccard. Este índice, acotado entre 0 y 1, está basado en la probabilidad de encontrar una pareja de individuos (uno de cada comunidad) pertenecientes al grupo de especies compartidas. Se trata de un estimador basado en las abundancias relativas y es poco sensible a la presencia de especies raras (Chao *et al.*, 2005). Para el total de censos efectuados durante la floración y para cada parcela experimental calculamos dos parámetros descriptores de la diversidad de visitantes florales: riqueza y probabilidad de encuentro interespecífico (Hurlbert, 1971). La riqueza (S_{OBS}, a partir de ahora) se midió como el número total de especies que visitaron las flores. La probabilidad de encuentro interespecífico (PIE, a partir de ahora) es un estimador de la equitatividad de la comunidad y se define como

$$PIE = \left(\frac{N}{N-1}\right) \times \left[1 - \sum_{i=1}^{S} \left(\frac{n_i}{N}\right)^2\right]$$

donde S es el número de especies, n_i el número de individuos de la especie i y N el número total de individuos. Este índice estima la probabilidad de que dos individuos muestreados pertenezcan a grupos diferentes, en nuestro caso morfoespecies. Estos índices ofrecen una información complementaria de la diversidad y tienen una interpretación biológica directa (Gotelli y Graves, 1996). Ambos índices fueron calculados tanto para morfoespecies como para grupos funcionales. Debido a las diferencias en los tamaños muestrales, la diversidad se comparó entre parcelas mediante técnicas de rarefacción basada en individuos (Gotelli y Graves, 1996). Tras 500 rarefacciones pudimos obtener la media y desviación estándar del estadístico rarefactado al menor de los números de individuos censados.

Exploramos la dinámica temporal de la diversidad de visitantes florales mediante gráficas de suavizado (Sheehy, 2010), utilizando ventanas temporales solapantes de tres días. Con el objeto de minimizar los errores debido a diferencias diarias en esfuerzo de muestreo, la diversidad en cada ventana temporal se estimó mediante remuestreo. Para ello calculamos la media y el intervalo de confianza de los dos estimadores de diversidad considerando todas las combinaciones de dos censos realizados en dicha ventana.

Hemos cuantificado además la autocorrelación temporal de morfoespecies y grupos funcionales mediante correlogramas de Mantel, transformando la composición de la comunidad mediante la transformación de Hellinger (Legendre y Gallagher, 2001; Rao, 1995). Finalmente exploramos visualmente la dinámica temporal en abundancia de los diferentes grupos funcionales mediante diagramas de huso. Este tipo de visualización permite comparar de forma gráfica los patrones de recambio en la comunidad de visitantes florales.

Todos los análisis se realizaron mediante el programa estadístico *R* (R Core Team, 2013). Algunos análisis de diversidad se realizaron mediante el paquete 'vegan' de esa plataforma (Oksanen *et al.*, 2014) y otros mediante scripts desarrollados propios.

Resultados

El número de flores abiertas disminuyó progresivamente en ambas parcelas experimentales. Así, la abundancia de flores abiertas en las parcelas experimentales fue máxima al inicio de la floración (683 flores, día 4 en la parcela A; 940 flores, día 6 en la parcela B), y mínima al final (79 flores, día 28 en la parcela A; 174 flores, día 28 en la parcela B), mostrando un patrón de floración explosivo posiblemente explicable por la homogenización ambiental que produjo el diseño experimental.

Durante todo el periodo de muestreo contabilizamos 3611 visitas (1670 en la parcela A, 1941 en la parcela B; Tabla 3.2) efectuadas por 88 morfoespecies (75 en la parcela A y 63 en la parcela B), pertenecientes a seis ordenes y 33 familias. El orden Hymenoptera fue el más abundante (70,0%), seguido por Diptera (24.0%) y Coleoptera (3.4%) (Figura 3.1). Las hormigas representaron la mayor parte del total de visitas (34.6%), seguidos por abejas grandes de trompa larga (principalmente *Anthophora spp.*; 16.9%; AGTL a partir de ahora), bombílidos (16.0%), abejas medianas de trompa corta (8.3%; AMTC) y abejas grandes de trompa corta (8.0%; AGTC). El resto de grupos funcionales presentaron abundancias por debajo del 6% del total de visitas, destacando en

Table 3.1: *Descripción de las características principales de los grupos funcionales en los que se ha clasificado a los visitantes florales de* Erysimum mediohispanicum. Characteristics of the main functional groups of flower visitors of *Erysimum mediohispanicum*.

Grupo funcional	Tamaño	Recurso	Rol*	Ordenes	Familias y géneros representativos
Abejas grandes de trompa larga	>10 mm	Néctar Polen	Polinizador	Hymenoptera	Hembras y machos de Apidae. Fundamentalmente *Anthophora spp., Apis mellifera* y varias especies de *Bombus spp.*
Abejas grandes de trompa corta	>10 mm	Polen Néctar	Polinizador	Hymenoptera	Hembras de Halictidae (*Lasioglossum spp., Halictus spp.*), Megachilidae (*Osmia spp.*), Colletidae (*Colletes spp.*) y Andrenidae (*Andrena spp.*).
Abejas medianas de trompa corta	5 - 10 mm	Polen Néctar	Polinizador	Hymenoptera	Hembras de Halictidae (*Lasioglossum spp., Halictus spp.*) y Andrenidae (*Andrena spp.*).
Abejas pequeñas de trompa corta	<5 mm	Polen Néctar	Polinizador Ladrón	Hymenoptera	Hembras de Halictidae (*Lasioglossum spp.*), Colletidae (*Hyleaus spp.*), Andrenidae (*Andrena spp.*), Apidae Xylocopinae (*Ceratina spp.*) y Apidae Nomidinae (*Nomada spp.*)
Hormigas	variable	Néctar	Polinizador Ladrón	Hymenoptera	Formicidae (*Formica, Camponotus, Proformica, Plagiolepis* y *Leptothorax*)
Avispas grandes	>7 mm	Néctar	Polinizador	Hymenoptera	Superfamilias Vespoidea y Chrysidoidea
Avispas pequeñas	usualmente <3 mm	Néctar	Polinizador Ladrón	Hymenoptera	Avispas parasíticas de las superfamilias Ichneumonoidea y Chalcidoidea
Bombílidos	variable	Néctar Polen	Polinizador	Diptera	Bombyliidae (*Bombylius spp.*) y Nemestrinidae
Sírfidos	variable	Polen Néctar	Polinizador	Diptera	Sirfidae y Bombyliidae de trompa corta (*Villa spp.*)
Moscas grandes	>5 mm	Polen Néctar	Polinizador Ladrón	Diptera	Muscidae, Calliphoridae, Tabanidae, Scatophagidae y Anthomyiidae
Moscas pequeñas	<5 mm	Néctar	Polinizador Ladrón	Diptera	Muscidae, Anthomyiidae, Micetophyliidae, Empididae, Bibionidae, Drosophilidae y Stratiomyidae
Escarabajos	variable	Polen Néctar	Polinizador Ladrón Herb. floral	Coleoptera	Melyridae (Malachidae y Dasytidae), Cleridae, Oedemeridae, Nitidulidae, Elateridae, Bruchidae, Buprestidae, Phalacridae y Chrysomelidae
Mariposas	variable	Néctar	Polinizador	Lepidoptera	Rhopaloceros de las familias Nymphalidae, Pieridae. Y Sphingidae diurnos.
Polillas	usualmente <7 mm	Néctar	Polinizador Ladrón	Lepidoptera	Adelidae y Incurvariidae
Chinches	variable	Néctar	Polinizador Ladrón	Hemiptera	Lygaeidae y Pentatomidae
Otros	variable	Polen Néctar	Polinizador Ladrón Herb. floral	Orthoptera, Dermaptera, Raphioptera, y Thysanoptera	Orthoptera, Raphidioptera, Thysanoptera y Dermaptera

orden descendente en porcentaje de contactos: sírfidos, escarabajos, moscas, abejas pequeñas de trompa corta (APTC) y mariposas (Figura 3.1).

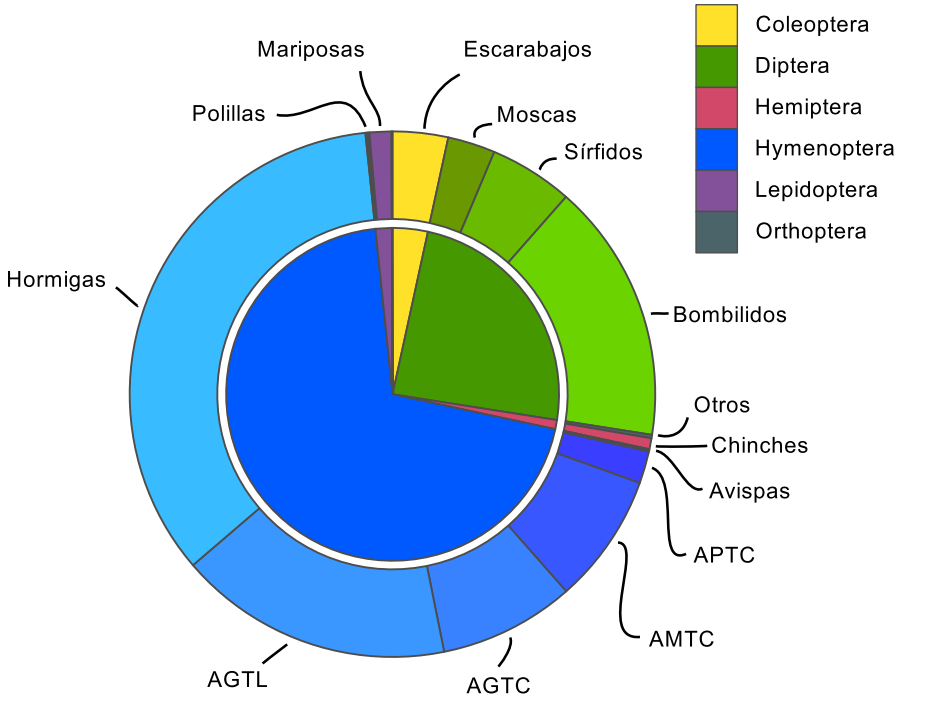


Figure 3.1: *Abundancias relativas de los visitantes florales en ambas parcelas. El círculo interno muestra las abundancias a nivel de orden, el círculo externo muestra las abundancias a nivel de grupo funcional. APTC = abejas pequeñas de trompa corta; AMTC = abejas medianas de trompa corta; AGTC = abejas grandes de trompa corta; AGTL = abejas grandes de trompa larga.*

Relative abundance of flower visitors in both plots. Inner cicle shows the abundances at order level, the inner circle shows the abundances at the functional group level. APTC = short-tongued small bees; AMTC = short-tongued medium-sized bees; AGTC = short-tongued large bees; AGTL = long-tongued large bees.

Las parcelas experimentales mostraron diferencias significativas en composición específica ($\chi^2 = 496.71$; $g.l. = 85$; $p < 0.001$). No obstante, el bajo valor del índice de disimilitud de Chao (0.10 ± 0.001) indicó una muy baja diferenciación entre parcelas. De hecho, las parcelas compartieron un 57% de las morfoespecies y un 86.60% de los grupos funcionales. Los mismos análisis para los grupos funcionales mostraron igualmente diferencias entre parcelas ($\chi^2 = 106.62$; $g.l. = 14$; $p < 0.001$) pero valores aún menores para la disimilitud entre parcelas ($Chao = 0.00 \pm 0.0006$). Estos resultados reflejan que las diferencias encontradas entre parcelas se debieron fundamentalmente a especies de ocurrencia baja.

Table 3.2: *Parámetros descriptivos de la diversidad de visitantes florales en las parcelas estudiadas. S = Riqueza; PIE = probabilidad de encuentro interespecífico.* Diversity of flower visitors at the experimental plots. Sobs = Richness; PIE = probability of interspecific encounter Parcelas

Nivel	Parcela	Num. censos	Num. contactos	S_{OBS}	$S_{RAREF.}$	PIE_{OBS}	$PIE_{RAREF.}$
Morfoespecies	A	57	1671	73	-	0.91	-
	B	58	1941	62	60.17 ± 1.25	0.93	0.93 ± 0
	Total	112	3611	87	-	0.93	-
Grupos funcionales	A	57	1671	14	-	0.83	-
	B	58	1941	14	13.97 ± 0.15	0.78	0.78 ± 0
	Total	112	3611	15	-	0.81	-

Los descriptores de diversidad estudiados (S_{OBS} y PIE) mostraron valores muy elevados. La riqueza de morfoespecies fue significativamente diferente entre parcelas, pero no así para la riqueza de grupos funcionales (Tabla 3.2). Las diferencias entre parcelas para el índice PIE fueron menores a nivel de morfoespecie ($PIE_{OBS} = 0.92$ para la parcela A ; $PIE_{RAREF.} = 0.93 \pm 0.001$ para la parcela B) que a nivel de grupos funcionales ($PIE_{OBS} = 0.83$ para la parcela A; $PIE_{RAREF.} = 0.78 \pm 0.001$ para la parcela B). La dinámica temporal de la riqueza para ambas parcelas fue similar (Figura 3.2a). La riqueza específica fluctuó alcanzando su máximo entre los días 5 y 10 en ambas parcelas (Parcela A $S_{OBS} = 18$; Parcela B $S_{OBS} = 21$). Sin embargo, la riqueza en grupos funcionales mostró mayor estabilidad temporal (Figura 3.2a). La diversidad medida como PIE mostró el mismo patrón tanto para morfoespecies como para grupos funcionales en ambas parcelas, con un máximo entre los días 5 y 15 y una progresiva disminución durante los últimos días de la floración (Figura 3.2b).

El correlograma de Mantel mostró la existencia de una marcada autocorrelación temporal en ambas parcelas, tanto para morfoespecies como para grupos funcionales. La correlación entre censos fue positiva a intervalos temporales pequeños y negativa a intervalos grandes (Figura 3.3). Esta variación temporal queda puesta de manifiesto en los diagramas de huso (Figura 3.4). Esta figura muestra como hubo diferencias entre grupos funcionales en el patrón temporal de abundancia. Así algunos grupos se mantuvieron abundantes durante toda la floración, como por ejemplo las hormigas, mientras que otros grupos, como AGTL o AGTC, presentaron marcadas fluctuaciones en su abundancia. Por último otros grupos, como mariposas o chinches, solo aparecieron durante ciertos momentos de la floración de las plantas.

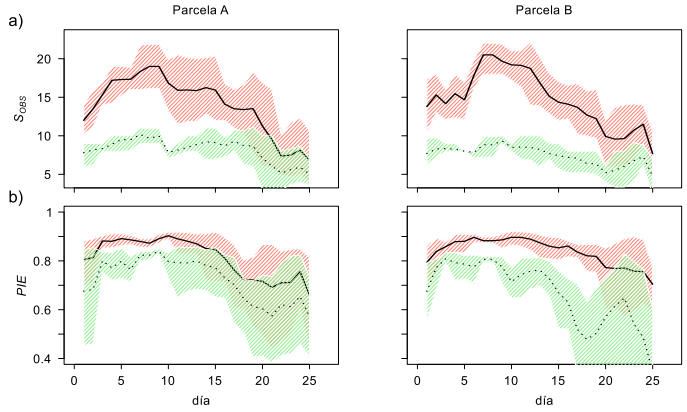


Figure 3.2: *Dinámica temporal de la riqueza (a) y diversidad medida como PIE (b) obtenido con el método de ventana solapantes de dos días. El nivel de morfoespecie aparece en rojo y el nivel de grupo funcional en verde. Los sombreados representan los intervalos de confianza del* 95% *obtenidos mediante remuestreo.*
Temporal dynamics of flower visitor's richness (a) and diversity, mesured as PIE, (b) obtained with a sliding window of two days. Values for mor- phospecies are depicted in red and for functional groups in green. 95% confidence intervals obtained by resampling are shown as coloured shadow.

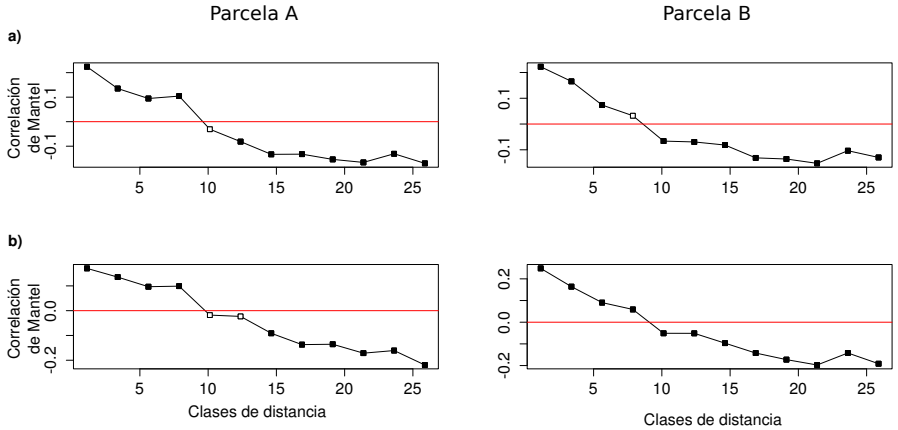


Figure 3.3: *Correlogramas de Mantel mostrando la autocorrelación temporal diaria entre pares de censos para ambas parcelas (parcela A a la izquierda, B a la derecha). a) Correlograma de Mantel para las morfoespecies; b) Correlograma de Mantel para los grupos funcionales. En ambos casos, los puntos negros indican valores significativos.*
Mantel correlograms showing the temporal autocorrelation between pair of censuses for the two plots (plot A, left; plot B, right). Mantel correlogram for morfoespecies (a) and for functional groups (b). Filled squares indicate significant values.

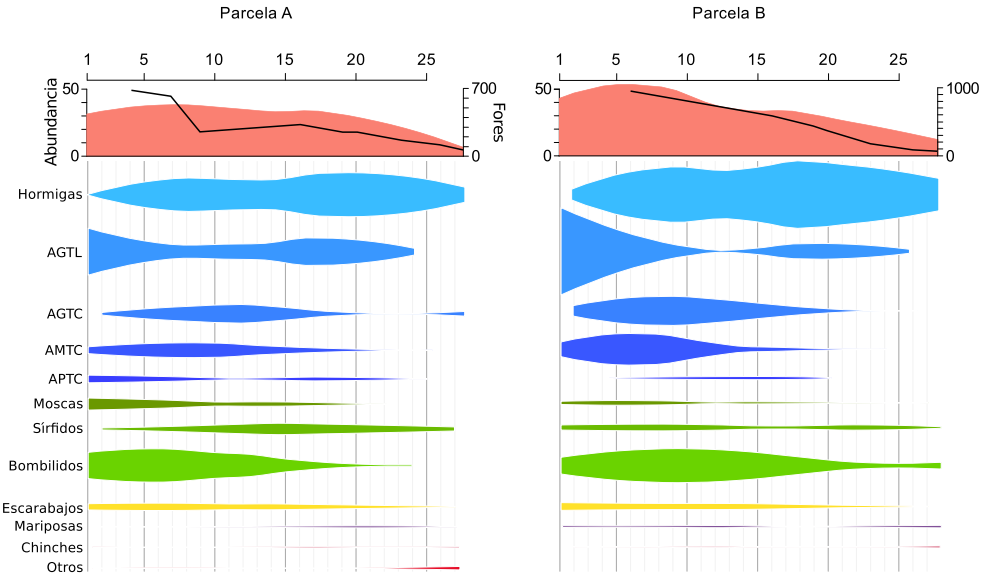


Figure 3.4: *Diagrama de huso de la evolución temporal de las abundancias absolutas de cada grupo funcional. La amplitud del uso de cada grupo funcional es proporcional a su abundancia absoluta en cada momento. En la parte superior del diagrama se muestra, la abundancia total de visitas (área) así como el número total de flores (línea) APTC = abejas pequeñas de trompa corta; AMTC = abejas medianas de trompa corta; AGTC = abejas grandes de trompa corta; AGTL = abejas grandes de trompa larga.*
Spindle diagrams showing the temporal dynamics of the absolute abundances of each functional group. Spindle amplitude is proportional to the absolute abundance at each temporal point. The upper part of the graph shows the total number of flower visitor contacts (area) and the number of open flowers (line). APTC = short-tongued small bees; AMTC = short-tongued medium-sized bees; AGTC = short-tongued large bees; AGTL = long-tongued large bees.

Discusión

Nuestros resultados ponen de manifiesto importantes fluctuaciones temporales en la composición y abundancia relativa de los visitantes florales, tanto para morfoespecies como para grupos funcionales, en un sistema de polinización generalista. Además apreciamos una marcada autocorrelación temporal en diversidad y abundancia de visitantes florales.

La comunidad de visitantes florales en estas parcelas experimentales fue muy numerosa tanto a nivel de morfoespecies (88) como de grupos funcionales (15), como corresponde al sistema de polinización "megageneralista" propio de la especie objeto de estudio (Gómez *et al.*, 2014a). Tanto la identidad

de morfoespecies como de grupos funcionales fue similar a la detectada en poblaciones naturales próximas (< 5 km) (Gómez *et al.*, 2007, 2014a). Sin embargo, la abundancia relativa de visitantes florales mostró diferencias con las encontradas en poblaciones naturales. Así, el grupo funcional de las hormigas fue el visitante más abundante en las parcelas experimentales pero no así en las poblaciones naturales cercanas muestreadas en diferentes hábitats y años (Gómez *et al.*, 2007). Esta singularidad puede deberse a la baja disponibilidad de recursos florales alternativos debido al tratamiento de roturación efectuado en las parcelas experimentales. No obstante, no es extraño que las hormigas sean visitantes florales frecuentes en otras especies del género *Erysimum* (Lay *et al.*, 2013, observación personal de los autores) o en otras crucíferas que conviven en la zona de estudio (Gómez y Zamora, 1992). Las hormigas actúan mayoritariamente como ladronas de néctar en *E. mediohispanicum*, al acceder al néctar a través de las aberturas existentes entre sépalos. Sin embargo, ocasionalmente pueden ser visitantes florales legítimos, como se ha observado en las parcelas de estudio y en poblaciones naturales (Gómez *et al.*, 2007). Los siguientes grupos funcionales en abundancia fueron AGTL y los bombílidos, dos tipos de polinizadores muy frecuentes en la mayoría de las poblaciones naturales de Sierra Nevada (Gómez *et al.*, 2009a). Estos grupos funcionales suelen actuar como visitantes florales legítimos, contactando con los órganos reproductores de la flor durante casi todas las visitas y actuando como polinizadores eficientes de la planta (Gómez *et al.*, 2011). El resto de grupos funcionales de visitantes florales representó menos del 33% de las visitas. Exceptuando las chinches, principalmente robadores de néctar, y algunos visitantes florales que actuaron como herbívoros florales (véase Tabla 3.1 para más detalles), el resto de los visitantes florales presentaron comportamiento dual, actuando como visitantes legítimos y ladrones de néctar a tiempo parcial (Gómez *et al.*, 2007, 2009a). Por último, cabe destacar la baja abundancia de escarabajos censados en este estudio en comparación con las poblaciones naturales circundantes (Gómez *et al.*, 2006b, 2009a). Nosotros pensamos que esta diferencia en abundancia puede deberse a las peculiaridades del sitio donde realizamos el experimento. Mientras que *E. mediohispanicum* crece de forma natural en pequeños claros de bosque y en zonas de matorral sobre sustrato rocoso, las planta experimentales de este estudio estuvieron ubicadas en una parcela abierta, bien irrigada y con suelo profundo donde la vegetación dominante era herbácea.

La composición específica del conjunto de visitantes florales puede variar a escalas espaciales pequeñas (eg., Herrera, 1988; Traveset y Sáez, 1997; Moeller, 2005; Price *et al.*, 2005) y responder tanto a factores intrínsecos como extrínsecos a las plantas (Herrera, 1995b). Las dos parcelas experimentales mostraron diferencias significativas en la composición de visitantes florales a pesar de estar separadas tan solo 15 m, aunque estas diferencias fueron pequeñas tal y como refleja el valor del índice de Chao, tanto para morfoespecies

(0.10) como para grupos funcionales (0.002). La selección de microhábitats (Beattie, 1971), la filopatría (Yanega, 1990) o la limitada área de movimiento de algunos polinizadores (Gathmann y Tscharntke, 2002) pueden contribuir a estas diferencias. Por último, un gran número de especies fue detectado a muy baja frecuencia, introduciendo diferencias entre parcelas. El papel de éstas en la biología reproductiva de *E. mediohispanicum* probablemente sea irrelevante, ya que la contribución de las especies infrecuentes al éxito reproductivo de las plantas es mínimo, independientemente de su efectividad polinizadora (Moeller, 2005; Perfectti *et al.*, 2009).

Hemos encontrado una muy elevada diversidad específica ($S_{OBS} = 88$, $PIE = 0.93$) en el conjunto de las dos parcelas experimentales. La riqueza de visitantes florales encontrada en 56 poblaciones de *E. mediohispanicum* fue como promedio 30 ± 9 especies (Muñoz-Pajares, 2013a), muy inferior a lo encontrado aquí. Esta diferencia puede ser debido a un menor esfuerzo de muestreo (en términos de censos y contactos entre flores e insectos) en esas otras poblaciones (Muñoz-Pajares, 2013a), ya que la riqueza específica es un parámetro muy sensible al esfuerzo de muestreo y a la duración de los mismos (Magurran, 2004). Por otra parte, los valores de diversidad PIE fueron similares a los encontrados en las 56 poblaciones ($PIE = 0.847 \pm 0.128$ Muñoz-Pajares, 2013a). Estos dos parámetros de diversidad mostraron diferencias cuando se comparó entre parcelas experimentales. Mientras que hubo diferencias en riqueza de especie entre parcelas, no las hubo para PIE. Las diferencias pueden achacarse a la presencia de especies raras que contribuyen a elevar la riqueza y la diferenciación entre parcelas pero no tienen apenas efecto sobre PIE. Cabe destacar que puesto que el índice PIE es un buen estimador del generalismo en la interacción entre plantas y polinizadores (Gómez y Perfectti, 2010), las poblaciones naturales y las aquí analizadas muestran una alta homogeneidad en su grado de generalismo.

Los valores de diversidad han fluctuado a lo largo de la floración, poniendo de manifiesto la existencia de una dinámica temporal en la interacción con los visitantes florales. El momento de máxima diversidad coincidió con los primeros días de floración, aunque la riqueza máxima fue posterior al máximo de floración en ambas parcelas (Figura 3.2). Diversos estudios han mostrado que en sistemas generalistas la abundancia de polinizadores, y por tanto la riqueza de especies, está asociada a la abundancia poblacional de flores abiertas (Heiathus *et al.*, 1982; Elzinga *et al.*, 2007). Sin embargo, no siempre hay una sincronización perfecta entre abundancia de polinizadores y momento del pico de floración, debido a que diferentes polinizadores muestran dinámicas temporales diferentes (Thompson, 2001) y a que éstos pueden ser atraídos por otras plantas en flor que coinciden en el mismo sitio (Cane y Payne, 1993; Filella *et al.*, 2013). El índice PIE, sin embargo, mostró menores fluctuaciones y mantuvo valores altos durante los dos primeros tercios de la floración,

implicando que el grado de equitatividad en la distribución de visitantes florales es poco sensible a las fluctuaciones en la riqueza de especies observada en este sistema. Tan solo al final de la floración, y coincidiendo con la disminución en el número de flores abiertas, se produjo un descenso en el valor del índice PIE. Por otro lado, al contrario que para morfoespecies, la riqueza en grupos funcionales fue más estable a pesar de la disminución en el número de visitas, manteniendo unos valores prácticamente constantes alrededor de $8 - 9$ grupos funcionales. Este patrón de depauperación en abundancia y riqueza de visitantes florales a final de la floración ya se ha observado en otros sistemas (Herrera, 1988) y cabe esperar que afecte a los servicios de polinización a medida que la floración avanza. No obstante, harán falta análisis más detallados que consideren tanto el número de visitantes florales por flor como la efectividad polinizadora de éstos para poder evaluar con precisión los efectos en el éxito reproductivo de la planta.

Hemos detectado la existencia de una dinámica temporal a nivel intra-estacional para la comunidad de visitantes florales. El gráfico de husos (Figura 3.4) es muy ilustrativo para visualizar esta dinámica a nivel de grupos funcionales. Se puede destacar la sincronía en la variación en abundancias así como coincidencias en los patrones de aparición/desaparición de algunos grupos funcionales. Por ejemplo, la disminución en AGTL fue coincidente en ambas parcelas con el aumento de AGTC, AMTC y bombílidos. La desaparición sincrónica de las abejas de trompa corta, tanto pequeñas como medianas y grandes, se produjo aproximadamente en la tercera semana de muestreo. Sin embargo, otros grupos como las hormigas o los sírfidos mantuvieron gran constancia temporal. Otros trabajos, aunque a niveles taxonómicos de especie, han encontrado al igual que nosotros variabilidad temporal en la abundancia e identidad de los polinizadores (Herrera, 1988). Esta variabilidad en los visitantes florales explica que se pierda la autocorrelación entre censos separados entre si más de diez días, tal y como ponen de manifiesto los correlogramas de Mantel (Figura 3.3). Esto implica que para obtener una imagen representativa de la comunidad de visitantes florales sea necesario realizar censos durante toda la floración, puesto que, como se ha demostrado para otros sistemas, diseños de muestreo basados en unos pocos censos concentrados temporalmente podrían sesgar la caracterización de la comunidad (Schmidt, 1985; Bried *et al.*, 2012).

La variabilidad en las abundancias de los grupos funcionales pueden deberse a diversos aspectos de la historia natural del sistema de estudio (Herrera, 1988). De este modo, podemos distinguir entre factores intrínsecos, como aquellos dependientes de la planta, y extrínsecos, todos aquellos dependientes del contexto, incluyendo factores tanto bióticos como abióticos (Thompson, 1994; Freitas, 2013). Así, factores intrínsecos a la planta como su propia fenología y sincronía floral a nivel de población pueden influir en la variación temporal de

flores y la consecuente atracción diferencial de distintos polinizadores (Elzinga *et al.*, 2007). Factores extrínsecos como las características microambientales pueden también afectar a la atracción de algunos grupos funcionales. Existen algunos estudios que demuestran que diferentes factores microambientales, como las condiciones lumínicas o la temperatura, condicionan la fauna local de polinizadores (Herrera, 1995b; Norgate *et al.*, 2010). Por otro lado, el solapamiento temporal en la floración a nivel de comunidad (Potts *et al.*, 2003), en conjunción con el carácter poliléctico de la mayoría de polinizadores (Herrera, 1996; Waser *et al.*, 1996), pueden afectar a los patrones de abundancia temporal de los polinizadores (Handel, 1985; Thomson, 1988; Klinkhamer *et al.*, 1989; Klinkhamer y De Jong, 1990; Feinsinger *et al.*, 1991; Eckhart, 1992; Kunin, 1993). Así, la presencia efímera en las parcelas experimentales de algunos grupos funcionales como por ejemplo las mariposas, puede explicarse por la variación en el mercado de la polinización (Filella *et al.*, 2013), entendido como la disponibilidad de polinizadores. Por otro lado, otros factores inherentes a la biología de los polinizadores pueden influir en la dinámica espacio-temporal observada. En ecosistemas de alta montaña la fase reproductiva de muchos insectos comienza rápidamente tras el deshielo, por lo que es esperable un pico de actividad al principio de la floración (Price *et al.*, 2005), tal y como hemos observado en nuestras parcelas experimentales. Conforme avanza la floración y disminuyen los recursos florales, los visitantes de mayor movilidad pueden desplazarse altitudinalmente en busca de nuevos recursos. De este modo, a medida que se pierden estos visitantes florales, la población focal se enriquece en insectos de menor movilidad como las hormigas. Además la diversidad puede variar tanto espacial como temporalmente debido a los diferentes patrones de pecoreo que muestran distintas especies de abejas (Gathmann y Tscharntke, 2002; Greenleaf *et al.*, 2007).

La variabilidad temporal en la composición, abundancia y diversidad de la comunidad de visitantes florales puede tener importantes implicaciones en la biología reproductiva de las plantas. Puesto que los visitantes florales difieren en su importancia (efectividad total) como polinizadores, fruto de sus diferencias en frecuencia de interacción y efectividad por visita floral (Herrera, 1987; Sahli y Conner, 2007; Perfectti *et al.*, 2009; Rodríguez-Rodríguez *et al.*, 2013), su variación temporal puede afectar a diversos aspectos de la reproducción de la planta y condicionar el éxito reproductivo si la sincronía floral de las plantas individuales no es perfecta. De este modo, si algunas plantas florecen cuando los polinizadores más abundantes sean aquellos menos eficientes se pueden producir eventos de geitonogamia o limitación de pólen (González-Varo *et al.*, 2009). Nuestros datos muestran que, por ejemplo, un grupo de visitantes florales poco eficiente como las hormigas fue relativamente más abundante al final de la estación, lo que puede afectar negativamente al éxito reproductivo de aquellas plantas que florezcan principalmente al

final de la estación. Además, la dinámica temporal de los visitantes florales y las diferencias fenológícas de las plantas también pueden incrementar la probabilidad de cruzamientos no aleatorios entre plantas (Herrera, 1988; Weis y Kossler, 2004; Weis, 2005; Weis *et al.*, 2005).

La diversidad, abundancia y persistencia de los visitantes florales puede tener profundas implicaciones en la coevolución entre plantas y polinizadores (Cane *et al.*, 2005). Puesto que la comunidad de visitantes florales puede mostrar importantes variaciones espacio-temporales, tal y como se pone de manifiesto en el caso de la variación temporal intra-estacional en *E. mediohispanicum*, es necesario analizar estas variaciones en la comunidad de polinizadores para así poder entender la coevolución entre plantas y sus polinizadores.

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4

The temporal dimension of individual-based plant-pollinator networks

Abstract

The pollination success of animal-pollinated plants depends on the temporal coupling between flowering schedules and pollinator availability. Within a population, individual plants exhibiting disparate flowering schedules will be exposed to different pollinators when the latter exhibit temporal turnover. The temporal overlap between individual plants and pollinators will result in a turnover of interactions, which can be analyzed through a network approach. We have explored the temporal dynamics of individual-based plant networks resulting from pairwise similarities in pollinator composition. During two flowering seasons, we surveyed the phenology and pollinator fauna of the individual plants from a population of *Erysimum mediohispanicum* (Brassicaceae). We analyzed the topology of these networks by means of their modularity, clustering, and core-periphery structure. These metrics are related to network functional properties such as cohesion, transitivity and centralization respectively. Afterwards, we analyzed the influence of each pollinator functional group on network topology. We found that network topology varied widely over time as a consequence of the differences in plant phenology and the idiosyncratic and contextual effect of pollinators. When integrating all temporary networks, the network became cohesive (non modular), transitive (locally clusterized), and centralized (core-periphery topology). These topologies could entail important consequences for plant reproduction. Our results highlight the importance of considering the entire flowering season and the necessity of making comprehensive temporal sampling when trying to build reliable interaction networks.

Introduction

Phenology is crucial to understand plant-pollinator interactions (Elzinga *et al.*, 2007). Most plant populations are composed of individuals showing different flowering schedules (Primack, 1980; Marquis, 1988) which vary in flowering intensity, timing, and duration (Augspurger, 1983; Mahoro, 2002). These individual flowering schedules entail important effects on plant reproduction (Munguía-Rosas *et al.*, 2011). For example, flowering synchrony among conspecific can influence seed siring (Herrera, 1992), or create intrapopulational assortativity in mating (Weis *et al.*, 2005; Elzinga *et al.*, 2007; Ison *et al.*, 2014) as mating probabilities are related to the level of synchrony (Hendry and Day, 2005; Devaux and Lande, 2008). Pollinators also display specific phenological patterns (Cane *et al.*, 2005), which create temporal variations in pollinator availability (Herrera, 1988). These variations in pollinator availability may cause differences in the subset of pollinator species interacting with those individual plants differing in flowering schedules. Consequently, and similarly to what occurs at the community level (Olesen *et al.*, 2008; Rasmussen *et al.*, 2013), a turnover of interactions is expected to emerge when individual plants flower at different moments and interact with different pollinators.

Plant-pollinator interactions have been widely analyzed during the last decades using network tools (Bascompte and Jordano, 2013). In pollination networks, links depict pollinator visits to plant reproductive organs. Accordingly, the occurrence and intensity of these links would be affected by plant and pollinator phenologies, since they can restrict pollination events to certain time windows (Olesen *et al.*, 2011a). Despite some overall network properties appearing to be constant among years (Olesen *et al.*, 2008; Petanidou *et al.*, 2008), recent studies have evinced the turnover of interactions within a flowering season, emphasizing the necessity of considering the seasonal dynamics to obtain a better understanding of these networks (Baldock *et al.*, 2011; Simanonok and Burkle, 2014). In this sense, the temporal dynamics of these interactions have been shown to influence some network properties. For example Olesen *et al.* (2008) found that the complexity of an arctic pollination network increased through time to reach a maximum before the network collapsed at the end of the season. Moreover, compartmentalization in Mediterranean pollination networks increased due to the mismatch of species phenophases (Bosch *et al.*, 2009; Martín González *et al.*, 2012), an effect that has been demonstrated to be enhanced when downscaling to pollinator individuals (Tur *et al.*, 2015).

Most pollination studies using network tools have focused on the community level. These studies do mostly use bipartite networks, which are composed

of two sets of nodes: plant and pollinator species (Bascompte and Jordano, 2013). Currently, there is a growing interest in downscaling the study of pollination networks to the individual level. This downscaling can be done for either plants or pollinators separately (Gómez *et al.*, 2011; Gómez and Perfectti, 2012; Tur *et al.*, 2014), or even both simultaneously (Dupont *et al.*, 2011, 2014). Contrasting with classic ecological networks, individual-based networks are able to reflect processes taking place at the population level. Some of these processes are even better analyzed when bipartite individual-based networks are projected into unipartite networks, a type of networks characterized by links connecting individuals of the same set and widely used to analyze social interactions. Interesting functional properties emerge in unipartite individual-based plant networks since links can serve as a proxy of mating probabilities among plants (Gómez *et al.*, 2011). However, studies considering individual-based unipartite networks are scarce (but see Gómez *et al.*, 2011; Gómez and Perfectti, 2012; Dáttilo *et al.*, 2014), even though this approach could produce novel insights about how interactions are structured within populations. For example, similarly to species in pollination network at the community level (Olesen *et al.*, 2007), individual plants can be organized into cohesive groups or modules by having similar pollinator assemblages. Moreover, if plants with similar flowering schedules are structured in clumps, a modular structure may appear only at some temporal periods. In individual-based pollination networks high values of clustering, a metric measuring transitivity (Newman, 2003b), could indicate a structure in the interaction with pollinators and the presence of groups of individuals that tend to mate frequently among themselves (Gómez *et al.*, 2011). Other metrics are related to the centralization of the networks (Newman, 2003a). Centralized networks are characterized by a core of nodes highly connected surrounded by a periphery of less connected nodes. We think that a core-peryphery structure may emerge as a consequence of the differences in pollinator sharing. This network topology may be due in part to differences in flowering synchrony among plants, resulting in differences in the number of links per plant and, therefore, in their position in the center or periphery of the network (Gómez *et al.*, 2011).

In this study we address, during two flowering seasons, how the interplay between individual-plant phenology and pollinator availability shape the topology of the individual-based plant network in a population of *Erysimum mediohispanicum* (Brassicaceae). This species is highly generalist, pollinated by a myriad of insect species belonging to disparate taxonomical and functional groups (Gómez *et al.*, 2014a). The interaction with pollinators vary spatially at different scales, such as between regions, between populations, and even between co-occurring individuals (Gómez *et al.*, 2009b). The presence of interindividual variation in pollinators has been successfully investigated using individual-based networks (Gómez *et al.*, 2011; Gómez and Perfectti, 2012).

Another feature of the pollinator assemblage of *E. mediohispanicum* is that it is composed of insects differing in their foraging periods. This generates an important seasonal turnover of the composition of *E. mediohispanicum* pollinator assemblages (Valverde *et al.*, 2014). Taking into account this preliminary information, in this study we aim to (1) explore the phenology of individual plants and their pollinators, (2) assess the between-plant differences in pollinator assemblage due to the temporal pollinator turnover, (3) determine the temporal shifts in the individual-based network topology described by modularity, weighted clustering and assortative mixing, three metrics related to network cohesion, transitivity and centralization, respectively (Borgatti *et al.*, 2013), and (4) estimate the influence of different types of pollinators on the topology of these networks. The main goal of this study is to explore how intra-seasonal variation in plant and pollinator phenologies determines the temporal variation in individual-based plant networks.

Materials and methods

Study system

Erysimum mediohispanicum (Brassicaceae) is a biennial, semelparous herb endemic to the Iberian Peninsula (Nieto-Feliner, 2003). They grow vegetatively for 2-3 years and then develop 1-8 flowering stalks bearing up to several dozens of flowers. The flowering season occurs from May to the end of June. Flowers display a bright-yellow corolla with separate petals that facilitate pollinator access. Flowers are hermaphrodite and partially self-compatible, although they need the assistance of pollinators to produce full seed set (Gómez, 2005). The pollination system of this species is extremely generalist, having several hundreds of pollinator species (Gómez *et al.*, 2009b, 2014a).

Sampling design

During 2010 and 2011, a 20 x 20 m plot was set up in a population of *E. mediohispanicum* at 1723 m.a.s.l. in a pine forest in the Sierra Nevada (SE Spain; $37° \ 8.07' \ N$, $3° \ 21.71' \ W$). Each year, one hundred plants were randomly selected within the plot, which represented the 80-90% of the total flowering plants in the population. To avoid interferences with other individuals, remaining individuals were removed or excluded from pollinators by bagging their flowering stalks.

Table 4.1: *Functional groups considered in this study, indicating a description of resource use (NC: nectar consumer, PC: pollen consumer, P: pollen collector) and the legitimacy of their interaction (Leg: legitimate visitor, Ileg: ilegitimate visitor). Fulfillment of the conditions are indicated with a + symbol*

Functional Group	Acronym	Size (mm)	Taxons	Resource			Legitimacy	
				NC	PC	P	Leg	Ileg
Long-tongued large bees	LtLB	>10	Hymenoptera: Anthophoridae (*Anthophora, Amegilla, Eucera*), Apidae (*Apis, Bombus*)	+			+	
Short-tongued large bees	StLB	>10	Hymenoptera: Halictidae, Andrenidae	+		+	+	
Short-tongued medium-sized bees	StMB	5 – 10	Hymenoptera: Halictidae (*Lasioglossum, Halictus*), Megachilidae (*Osmia*), Andrenidae (*Andrena*), Apidae (*Ceratina*)	+		+	+	+
Short-tongued small bees	StSB	<5	Hymenoptera: Halictidae (*Lasioglossum*), Colletidae (*Hylaeus*), Andrenidae (*Andrena*), Apidae (*Ceratina*)	+		+	+	+
Large ants	LA	>2	Hymenoptera: Formicidae (*Formica, Camponotus, Proformica, Cataphyphis, Lasius*)	+			+	+
Small ants	SA	<2	Hymenoptera: Formicidae (*Plagiolepis , Leptothorax*)	+			+	+
Pollen wasps	PW	variable	Hymenoptera: Vespidae. Masarinae (*Ceramius*)	+		+	+	
Small nectar-collecting wasps	SncW	<3	Hymenoptera: Ichneumonidae	+			+	+
Long-tongued beeflies	LtBf	variable	Diptera: Bombyliidae (*Bombylius*), Nemestrinidae	+	+		+	
Short-tongued beeflies	StBf	variable	Diptera: Bombyliidae (*Anthrax, Villa*)	+	+		+	
Large Hoverflies	LH	>5	Diptera: Syrphidae (Eristalini)	+	+		+	
Small Hoverflies	SH	<5	Diptera: Syrphidae (Syrphini, Merodontini, Bacchini)	+	+		+	+
Large flies	LF	>5	Diptera: Muscidae, Calliphoridae, Tabanidae, Scatophagidae, Anthomyiidae	+	+		+	+
Small flies	SF	<5	Diptera: Muscidae, Anthomyiidae, Empididae, Bibionidae, Drosophilidae.. among others	+	+		+	+
Large Beetles	LB	>7	Coleoptera: Lagridae, Mylabridae, Allecuninae		+		+	+
Small beetles	SB	<7	Coleoptera: Melyridae, Cleridae, Oedemeridae, Elateridae, Bruchidae, Buprestidae, Chrysomelidae		+		+	+
Small diving beetles	SDB	<3	Coleoptera: Nitidulidae, Dermestidae, Phalacridae	+	+		+	+
Butterflies	Btfly	variable	Lepidoptera: Pieridae, Nymphalidae, Lycaeidae, Hesperiidae	+			+	+
Large Moths	LM	>3	Lepidoptera: Crambidae, Noctuidae	+			+	
Small moths	SM	<3	Lepidoptera: Adelidae	+	+		+	+
Hawkmoths	Hwk	>7	Lepidoptera: Sphingidae	+			+	
Bugs	Bugs	variable	Hemiptera: Miridae, Lygaeidae, Pentatomidae (*Eurydema*)	+			+	+
Others	Oth	variable	Orthoptera, Raphidioptera, Neuroptera, among others.		+		+	

We monitored the flowering phenology of each plant during the two study years. For this we recorded daily the flowering status of plants and periodically counted their open flowers (16 times in 2010 and 28 times in 2011). In addition, we counted the insects visiting the flowers of each plant during five minutes intervals, performing between one to four surveys per day, totaling 58 surveys in 2010 and 62 in 2011. Floral visitors were clumped into functional groups (FG herein), groups of species presumably exerting similar selective pressures on floral phenotype (Fenster *et al.*, 2009). We considered only those insects contacting legitimately the plant reproductive organs at least during some foraging bouts. Based on morphological characteristics such as body size, proboscis length, floral fit or foraging behavior, we considered 23 FGs (see Table 4.1 for a description of FGs). Ants are not good pollinator in this system, but we decided to include them in the analyses not for their per-visit effectiveness but for their high abundance.

Plant phenology and flowering synchrony

We described the phenology of the studied plants by means of flowering curves, estimated using local polynomial functions (Appendix S1). Flowering pairwise synchrony of each individual plant with the rest of conspecifics was determined by means of two indices: (1) Unweighted synchrony ($X_{i,i}$; Eq.1 in Appendix S2). This index measures the overlap in flowering and was calculated using Augspurger (1983) index considering the number of co-flowering days. (2) Weighted synchrony (J; Eq.2 in Appendix S2). This index weights the flowering overlap by the number of open flower and was calculated using the Jaccard-type Chao dissimilarity index (Chao *et al.*, 2005) over the flowering curves. This index ranges from 0 to 1 and can be interpreted as the probability of two flowers chosen at random from two individuals being open the same day.

Temporal variation in pollinators

We analyzed the temporal variation in richness and diversity of FGs, calculated as the Hurlbert's PIE ($D_{Hurlbert}$ Hurlbert, 1971) (Eq. 3 in Appendix S2), by pooling all surveys within 3-days overlapping temporal windows (see Appendix S3). We visualized the temporal turnover in the FGs using spindle diagrams (Valverde *et al.*, 2014). Here, turnover refers to the temporal change in the frequency of the interactions of pollinators and *E. mediohispanicum* plants, independent of the local abundance of pollinators. To check the occurrence of this temporal turnover we calculated for each pair of surveys the dissimilarity in FG composition using the Morisita-Horn index (S_{M-H}; Eq. 4 in Appendix S2), and compared these distances with the temporal distances, in days, using a Mantel test. We complemented this analysis by constructing a

Mantel correlogram using the same distance matrices. This analysis allowed us to detect the minimum temporal distance at which the correlation in pollinator species composition dissapear. Using this temporal distance we partitioned each flowering season in discrete non-overlaping temporal windows (t_1,...t_n, herein). Each temporal window spanned a number of days higher than that minimum and contained an equitable number of surveys. Finally, we tested whether the composition in pollinators varied more between- than within-years using a PERMANOVA (Anderson, 2001), nesting temporal windows within year and using days as replicates.

Between-plant differences in pollinator composition

We calculated the dissimilarity between plants in the composition of pollinator faunas using the Morisita-Horn index. The resulting distance matrices were compared with the flowering schedules distance matrices, which were calculated using the Jaccard-type Chao distance index. The comparisons were made by means of Mantel tests based on Spearman correlation index. We also performed a Mantel correlogram using the compositional dissimilarity as a response variable to identify correlation trends of pollinator composition with asynchrony classes. With this procedure we assessed if there was plant-plant isolation in pollinator composition due to their flowering asynchrony.

Temporal shifts in the topology of the individual-based plant networks

Each study year and temporal window resulted in an adjacency matrix of individual plants-FGs, showing the number of visits of each FG per individual plant. As our aim is to describe the individual-based plant networks, for each temporal window (t_1,...,t_n) we projected the plants-FGs adjacency matrix into a plant-plant matrix using the pairwise similarity in pollinator assemblages. Similarity was calculated as 1 - Morisita-Horn index. Links in these networks indicate the probability of two plants sharing the same pollinators. Due to the high sampling effort (see Results below), no single plant was unlinked, resulting in a massive network that may obscure some network properties (May, 2006). To overcome this problem, we used percolation theory to find the simplest network while maintaining its percolation capacity (Rozenfeld *et al.*, 2008). From a starting network, links are sequentially removed starting from the weakest, until the step before the network breaks down into isolated subgraphs. This is the so-called percolation threshold, beyond which the network breaks down into disconnected sub-networks. Percolation is a useful tool to simplify networks' complexity while maintaining the important connections and nodes involved in information flow (Muñoz-Pajares, 2013b). With the resulting

percolated networks we made a topological description in terms of their modularity, weighted clustering and assortative mixing by degree.

Modularity (Q; Eq. 5 in Apendix S2) measures to what extent a network is organized into modules. Modules are defined as groups of nodes with a higher link density within the group than among groups (Newman, 2004). This metric measures the deviation from a random link distribution by calculating the observed fraction of links within each group minus the fraction expected at random (Newman, 2004). We used the heuristic walktrap community detection method based on random walks (Pons and Latapy, 2006) to find the most modular network structure. Here, modularity will give information on how the population is structured in groups of plants sharing similar pollinators.

Weighted clustering coefficient (C_w; Eq. 6 in Appendix S2), a metric measuring transitivity, was estimated as the extension proposed by Opsahl and Panzarasa (2009) of the global clustering coefficient (Newman, 2003b). While the clustering coefficient informs about the proportion of groups of three nodes (triad) fully interconnected, C_w weights each triad with the geometric mean of the links forming it. This metric measures the tendency of nodes to cluster together into tightly connected local groups. If node A shares a link with nodes B and C, in a network with a high value of C_w, nodes B and C will also share a link between them with a higher probability than with a node picked at random (i.e., high transitivity Junker and Schreiber, 2008). High values of this metric are expected in individual-based networks built on pollinator similarity. High values indicate that individual plants share the same pollinators producing a very compact network, while low values indicate sparse networks formed by plants visited by different subsets of the entire fauna of pollinators.

Assortative mixing by node degree (r; Eq. 7 in Appendix S2) measures to what extent nodes tend to be connected with nodes of similar degree. This metric works as a correlation parameter among degree values of interconnected nodes (Newman, 2003a). This metric takes a value of 1 if there is a perfect assortative mixing, 0 if there is no assortative mixing and close to -1 when the mixing is disassortative. Values close to -1 describe star-like networks while values close to 1 describe networks with a core of highly connected nodes against a periphery of nodes with lower degree (Newman, 2010). In the context of our work, positive values will denote a network topology formed by a core of plants highly similar in pollinator composition and a periphery of less connected plants.

For all measured network metrics we analyzed their deviations from what is expected under the null hypothesis of pollinators foraging randomly. We constructed 500 random adjacency matrices maintaining the marginal values of the FGs per survey (i.e., total number of contacts). Through this type of randomization, we maintain FGs overall abundances and restrict plants

sampling only in their flowering days. After this, we obtained the individual-based plant network as described previously (random networks herein) and calculated the mean and the 0.025 and 0.975 percentiles of the network metrics. We chose 500 replicates because beyond 400 replicates the mean and standard deviations of the metrics stabilized.

Influence of different FGs on network topology

We assessed the differential effect of each FG on network topology by comparing the empirical network with simulated networks where only insects belonging to a particular FG foraged randomly. For each bipartite matrix (total and temporary), the visits of a given FG were reshuffled among plants while maintaining the observed visitation distribution of the remaining FGs. The resulting matrix was percolated and network metrics were obtained as described before. We repeated this procedure 500 times for each FG and empirical matrix to obtain a distribution of simulated values for each network metric. We report the standardized effect sizes (SES herein) of each FG (Eq. 8 in Appendix S2). The sign and strength of SES indicates how the behavior of a given FG modifies the network metrics, with values close to 0 denoting similar effects to those produced when a FG forages randomly.

All analyses were performed under the R statistical analysis platform (R Core Team, 2013), using the packages 'vegan' (Oksanen *et al.*, 2014), 'igraph' (Csardi and Nepusz, 2006), 'network (Butts *et al.*, 2014), 'tnet' (Opsahl, 2009), and forraGEO (Valverde et al. in prep.). We developed personalized codes modified from Muñoz-Pajares (2013b) for some of the network analyses (see appendix S4). Figure 4.S1 resumes the workflow followed to perform the network analyses.

Results

We did 120 pollinator surveys of the whole population (58 in 2010 and 62 in 2011), distributed over 70 flowering days (32 in 2010 and 38 in 2011), and totaling 43 975 minutes of observation. We recorded 7421 pollinator-flower interactions in both years (2949 visits, 21 735 minutes in 2010; 4472 visits, 22 240 minutes in 2011). We recorded 23 FGs, with a predominance of large ants (24% of total visits), long tongued beeflies (21%), small beetles (13%), and small ants (12%). The rest of functional groups did not exceed 10% of the overall visits.

Plant phenology and flowering synchrony

Individual plants differed in their phenologies (Figure 4.1; Table 4.S1). Unweighted synchronies showed intermediate values ($X_{i,j} = 0.62 \pm 0.18$ in 2010, $X_{i,j} = 0.69 \pm 0.14$ in 2011), which decreased when considering flowering intensity ($J = 0.25 \pm 0.21$ in 2010, $J = 0.14 \pm 0.14$ in 2011).

Temporal variation in pollinators

Pollinator richness and diversity showed similar temporal trends in both years (Fiure 4.S2). The number of functional groups ranged from a minimum of seven at the onset of 2011 flowering season to 16 in the middle of the 2011 flowering season. Hurlbert's ranged from 0.18 in 2010 to 0.86 in 2011. Maximum values of diversity were found in the same temporal interval both years (140-155 julian days). However, temporal changes in richness were less congruent between years. Spindle diagrams showed contrasting patterns as a consequence of the temporal replacement of pollinators (Figure 4.1b). This replacement was evidenced by a significant positive correlation between temporal distance among surveys and dissimilarity in pollinator composition (2010: $r = 0.014$, $R^2 = 0.35$, $df = 1651$, $p < 0.001$; 2011: $r = 0.011$, $R^2 = 0.22$, $df = 1828$, $p < 0.001$; Figure 4.S3). There was a positive and significant correlation between compositional dissimilarity and temporal distance across surveys ($r_M = 0.597$, $p = 0.001$ in 2010; $r_M = 0.458$, $p = 0.001$ in 2011). Mantel correlograms indicated positive and significant correlation at the first distance classes (Figure 4.2). However, this autocorrelation was lost beyond an interval of 5 - 6 days suggesting the occurrence of temporal isolation of the community of pollinators at this time span. Consequently, we grouped the surveys in periods of six days, resulting in a partition of six temporal windows (t_1 to t_6) each year. Variation in pollinator composition was higher within year than between years (PERMANOVA, $SSbetweenyears = 0.506$, $p = 0.008$; $SSamongtemporalwindowswithinyear = 7.483$, $p = 0.001$; $SSwithintemporalwindows = 8.841$).

Between-plant differences in pollinator composition

The correlation between flowering asynchrony and pollinator dissimilarity was significant for both years (2010: $r_M = 0.079$, $p = 0.033$, 2011: $r_M = 0.21$, $p = 0.001$). Moreover, when analyzing the correlation patterns of pollinator dissimilarity along asynchrony classes using Mantel correlograms, we found a significant positive correlation for the two first distance classes ($asynchrony \leqslant 0.1$; 4.3). In 2010 this correlation was lost from the third distance class while, in 2011, the correlation was shifted to a significant negative one from the fourth distance class on ($asynchrony \geqslant 0.23$).

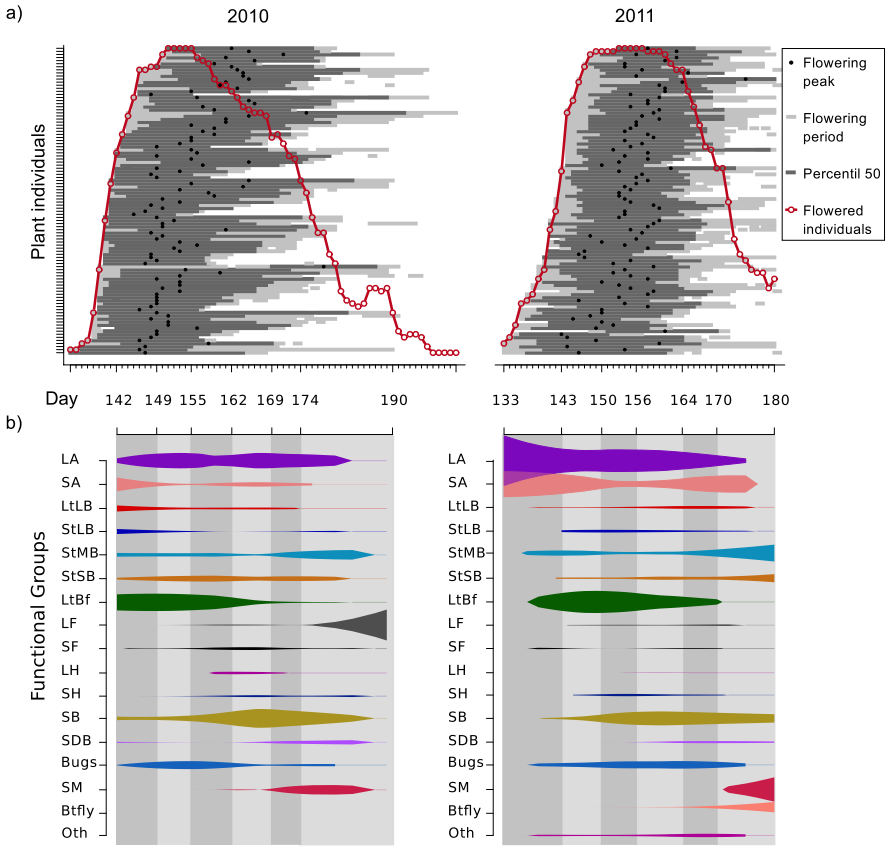

Figure 4.1: *Flowering schedules and pollinator visits through time. (a) phenogram showing for each individual its flowering span (light gray bars), time period within the 25 - 75% cumulative open flowers (darker bars), and flowering peak (black dots). The number of flowering plants per day (red circles and lines) is also shown. (b) bundance of pollinator functional groups. The width of the spindle diagrams denotes the relative abundance of each pollinator group (see Table 4.1 for acronyms).*

Temporal shifts in the topology of the individual-based plant networks

The total networks were composed of 95 and 89 connected plants in years 2010 and 2011 respectively (Figure 4.4). However, the temporary networks varied widely in size (42 to 96 connected plants in 2010; 35 to 93 connected plants in 2011). Some plants were removed from some networks during the percolation process because they were visited by none or just a few insects (Table 4.2).

Modularity varied widely in both years (Figure 4.5). In 2010, this metric showed higher and significant values only at the fourth temporary (t_4) network ($Q = 0.39$, $p < 0.05$, Table 4.2). In 2011, modularity only reached marginally

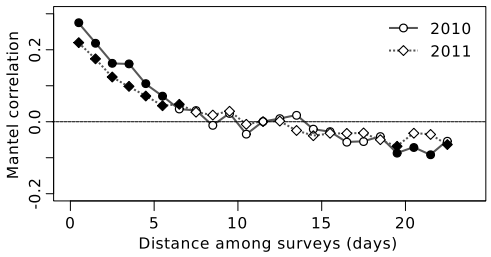

Figure 4.2: *Temporal turnover of functional groups in both years. Mantel correlogram of Morisita-Horn dissimilarity index over temporal distances among surveys. Filled circles and diamonds indicate significant values ($p < 0.05$) of the Mantel correlation tests.*

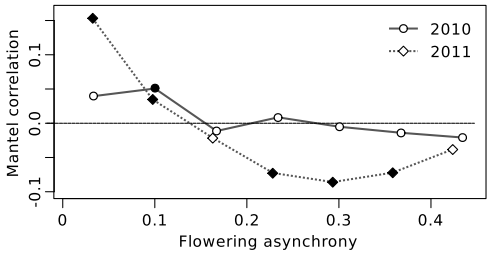

Figure 4.3: *Mantel correlograms of pairwise dissimilarities in pollinator composition (Morisita-Horn dissimilarity index) against flowering asynchrony (Jaccard-type Chao distance index). Significance values ($p < 0.05$) obtained after 999 simulations are pointed out as filled circles and diamonds.*

significant values at t_5 and t_6 networks ($Q = 0.41$ and 0.40, $p < 0.1$). By contrast, weighted clustering exhibited a marked positive deviation from the null model, reaching significance at the t_4 network in 2010 ($C_w = 0.86$, $p < 0.05$, Table 4.2) and marginal significance at the t_4 network in 2011 ($C_w = 0.68$, $p < 0.1$). This metric showed a marginal significant value for the 2010 total network ($C_w = 0.61$, $p < 0.1$), reaching significance in 2011 ($C_w = 0.61$, $p < 0.05$). Finally, assortative mixing by node degree exhibited positive deviations through the whole time period, reaching marginal significance at the t_4 network in 2010 ($r = 0.43$, $p < 0.1$, Table 4.2) and significance at the t_4 network in 2011 ($r = 0.45$, $p < 0.05$). This metric showed positive and significant values for this metric in both total networks ($r = 0.51$ and 0.48, $p < 0.05$ in 2010 and 2011 respectively).

Influence of different FGs on network topology

Only eight functional groups (long-tongued beeflies, large ants, small ants, bugs, small beetles, small moths, short tongued medium-sized bees and short-

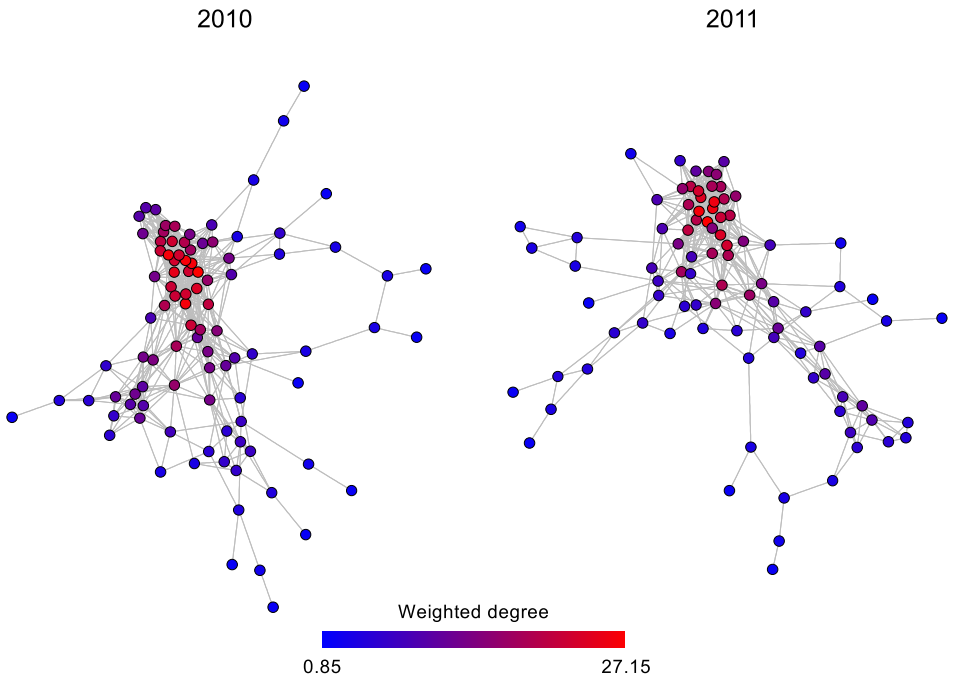

Figure 4.4: *Total networks for both years. Each node represents an individual plant. Links among plants indicate similarities in pollinator composition. Colors of each node represent its weighted degree calculated as the sum of all link weights pertaining to that node. Note the core-periphery topology denoted by the distribution of node degrees.*

tongued small bees) showed a relative frequency higher than 0.10 in at least one network. Short tongued small bees did not exceed this threshold in 2011, therefore its effect sizes are only reported for year 2010. The effects of these functional groups on network metrics were not temporally consistent, neither in size nor in sign (see Figure 4.5 for details). For example, modularity was inconsistently affected by small ants which exerted significant effects of different sign at some temporary networks ($SES = -1.53$ and 2.84 at t_4 and t_6 networks in 2011). For weighted clustering, effect sizes also shifted in sign through both seasons. Only large ants, bugs and small beetles showed significant effect sizes on this metric in some networks, particularly large ants which exerted significant positive effect sizes on two temporary networks ($SES = 3.16$ and 2.77 at t_2 and t_5 networks in 2010) and at both total networks ($SES = 3.11$ and $SES = 3.66$ in 2010 and 2011, respectively). Likewise, the effects of the functional groups on assortative mixing by degree shifted in sign through both years (Figure 4.5). The functional group large ants stands out, again, showing significant positive effect sizes at one temporary network ($SES = 2.00$ at t_2 network in 2011) and at both total networks ($SES = 3.88$ and 2.58 in 2010 and 2011, respectively).

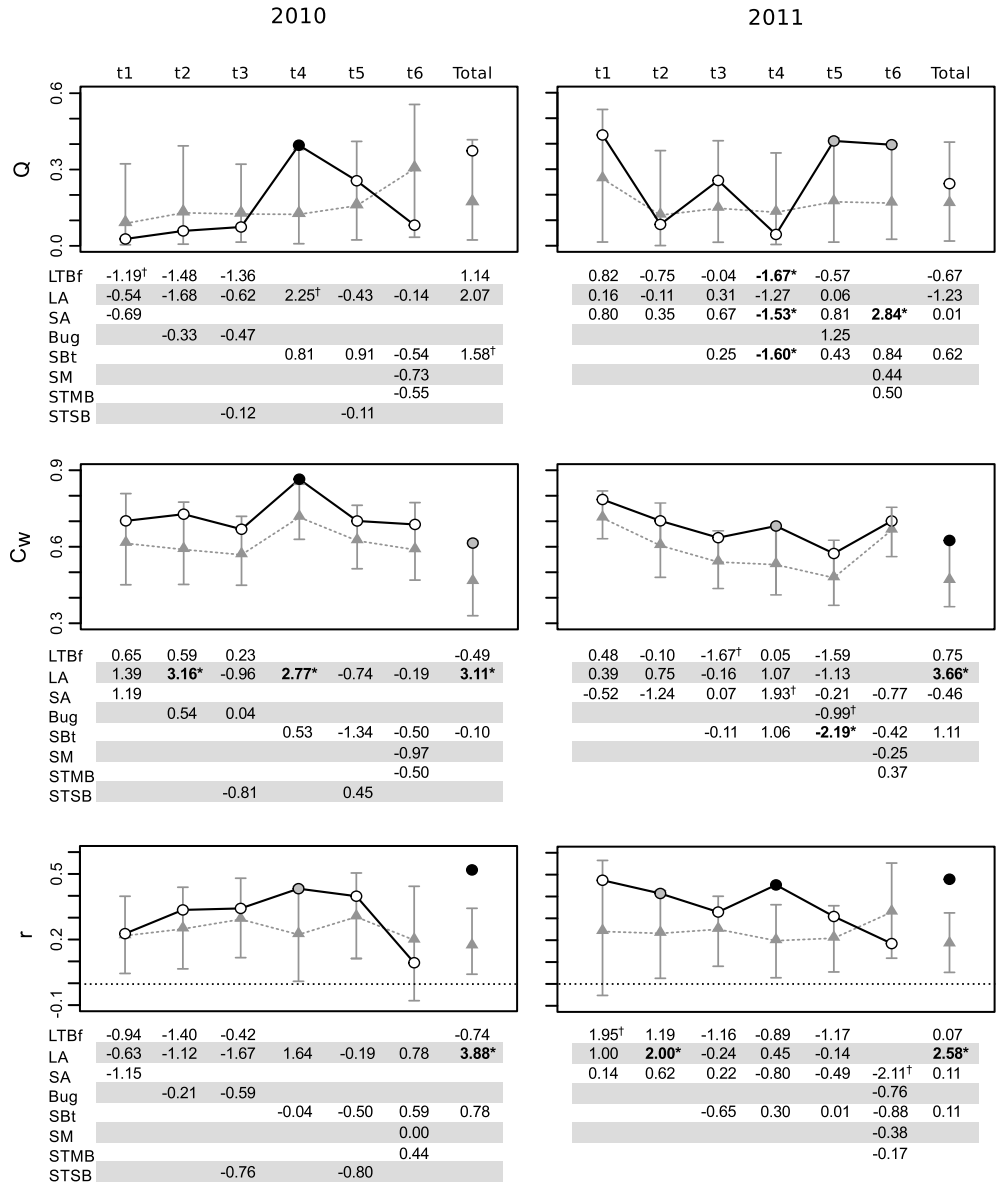

Figure 4.5: *Intra-seasonal variation in network metrics (Q, modularity; C_W, weighted clustering; r, assortative mixing by node degree) and effectsizes for the most important pollinator functional groups. For each temporary (t_1 to t_6) and total network, empirical values (dark circles and lines) and expected mean and 95% confi dence intervals obtained from the null model of pollinators behaving randomly (gray triangles and lines) are drawn. Black filled circles indicate significance ($p < 0.05$) and gray fi lled circles marginal signifi cance ($p < 0.1$). Values of eff ect sizes are denoted as $*$ when signifi cant ($p < 0.05$) and † when marginally signifi cant ($p < 0.1$).*

Table 4.2: *Sampling effort and topological metrics for each network and year. Network metrics are: Q, modularity; C_W, weighted clustering; r, assortative mixing by node degree. Network size includes the number of flowering plants for a time period (flowering), the number of plants visited by pollinators (visited) and the connected plants in the similarity network after the percolation process (connected). 1-threshold denotes the lower limit of link weights remaining in each network after percolation.*

Network		Sampling effort			Network size				Network metrics		
Year	Time lap	Days	Surveys	Visits	flowering	visited	connected	1 - threshold	Modularity (Q)	Weighted clustering (C_W)	Assortative mixing (r)
2010	142 - 147	6	8	691	93	88	88	0.56	0.03	0.7	0.23
	149 - 154	5	11	873	100	98	96	0.62	0.06	0.73	0.33
	155 - 159	5	10	630	100	92	92	0.66	0.07	0.67	0.34
	162 - 168	5	9	297	90	67	66	0.57	**0.39***	**0.86***	0.43†
	169 - 173	5	9	274	72	62	59	0.52	0.25	0.7	0.4
	174 - 189	6	11	162	61	42	42	0.40	0.08	0.69	0.09
	Total	32	58	2927	100	100	95	0.85	0.34	0.61†	**0.51***
2011	133 – 142	7	8	149	49	36	35	0.66	0.43	0.78	0.47
	143 - 149	6	11	212	100	94	93	0.75	0.08	0.70	0.41†
	150 - 155	6	8	844	100	96	91	0.75	0.26	0.64	0.33
	156 - 163	6	12	1178	100	94	93	0.70	0.04	0.68†	**0.45***
	164 - 169	6	14	858	96	84	74	0.72	0.41†	0.57	0.31
	170 - 180	6	8	207	66	50	49	0.40	0.40†	0.70	0.18
	Total	37	61	4448	100	100	89	0.88	0.23	**0.61***	**0.48***

Discussion

Exploring the phenology of individual plants and their pollinators

Erysimum mediohispanicum exhibited intermediate values of flowering synchrony during both years of study when calculated using the Augspurger's index. These values are similar to those found in montane plants (Gómez, 1993; Crimmins *et al.*, 2013) and correspond with the higher values observed for other species (Primack, 1980; Buide *et al.*, 2002; **?**; **?**). Nevertheless, the variation in flower intensity among individuals resulted in low values of weighted synchrony. Under these circumstances we expect that the probability of interacting with the different pollinators will vary among plants. These findings emphasize the need of considering flowering intensity to obtain more accurate estimates of pairwise synchrony (Freitas and Bolmgren, 2008) and individual plant-pollinator interactions (Encinas-Viso *et al.*, 2012). Several factors may influence flowering phenology, including genetic variation (Mitchell-Olds, 1996; Leinonen *et al.*, 2013) and microenvironmental heterogeneity (Herrera, 1988). Mediterranean open woodlands and shrublands, where populations of *E. mediohispanicum* grow, exhibit high microenvironmental variability (Gómez *et al.*, 2004; Valladares and Guzmán, 2006). Specifically, the plot maintains different microenvironments mainly related to the shaded areas that some pine trees produce. These microenvironmental differences occur at meter scales and translate in variation in resources and specially in humidity. We have observed that plants located in shaded areas flower for more time than exposed plants. We think that this heterogeneity may explain at least in part the observed variability in flowering and pairwise synchrony, and therefore could be a factor influencing plant-pollinator interactions.

The insects visiting the flowers of *E. mediohispanicum* also showed temporal changes in the relative occurrence of their visits, resulting in a turnover of interactions. This was reflected in the observed temporal variation in richness and diversity. Temporal changes in pollinator fauna have been reported mainly among years (Cane *et al.*, 2005; Price *et al.*, 2005; Dupont *et al.*, 2009) and between populations (Price *et al.*, 2005; Petanidou *et al.*, 2008; Dupont *et al.*, 2009). Moreover, temporal variations in pollinator fauna have also been recorded intra-annually for plant species having extended flowering (Herrera, 1988; Ashman and Stanton, 1991). In our case we have found a high intra-seasonal pollinator turnover in a plant without extended flowering. This fine-grain temporal variation has been previously detected at community level (eg, Olesen *et al.*, 2008; Baldock *et al.*, 2011; Simanonok and Burkle, 2014), for

specific plants (eg, Hurd, Paul D. and Linsley, 1975), and previously reported in *E. mediohispanicum* (Valverde *et al.*, 2014). Remarkably, this turnover was even stronger than the inter-annual variation, as could be expected for an extremely generalist plant when pollinator availability changes throughout the flowering season. Several intrinsic and extrinsic factors can influence pollinator turnover. Some important intrinsic factors are changes in insect population dynamics and emergence date Ellwood *et al.* (2011); Kudo (2013), altitudinal migration (Stefanescu, 2001; Gutiérrez and Wilson, 2014), and temporal changes in their feeding habits (Faegri and van der Pijl, 1979). On the other hand, extrinsic factors can include displacement by competition with other pollinators (Brosi and Briggs, 2013), or changes in local plant composition that may promote shifts in pollinator preferences (Cane and Sipes, 2006). Regardless of the specific reasons, they ultimately affect the composition of pollinator assemblages visiting a plant species and thus the pollinating scenario within a population (Herrera, 1988; Horvitz and Schemske, 1990).

The interplay among plant and pollinator phenologies affects the likelihood of their interaction (Kudo, 2013). Our results show that the variability in the phenologies of co-occurring individual plants and the turnover of pollinator visits lead to among-plant dissimilarities in their pollinator assemblages. Plants exhibiting disparate flowering schedules will be visited by different pollinators, resulting in a decoupling of interactions (Memmott *et al.*, 2007; Hegland *et al.*, 2009). Moreover, as pollination effectiveness can vary among pollinators (Sahli and Conner, 2007), and even temporally within the same species (Fishbein and Lawrence Venable, 1996; Rafferty and Ives, 2012) this decoupling of interactions can entail variation in plant fitness (Schemske, 1977; Mahoro, 2002). Since in *E. mediohispanicum*, pollinators vary widely in effectiveness (Valverde et al., unpublished data), we hypothesize that plants flowering when the most effective pollinators are available may have their reproductive fitness enhanced. On the other hand, because plant fitness has been positively correlated with pollinator diversity (Klein *et al.*, 2003; Gómez *et al.*, 2007; Perfectti *et al.*, 2009), plants flowering during the period of maximum diversity of pollinators may have their fitness enhanced. Further research should incorporate pollination performance to produce a more realistic view of how the interplay of plant and pollinator phenologies affects plant reproduction.

Temporal shifts in the topology of the individual-based plant networks

The patterns of node linkage can result in a non-random network topology (Newman, 2010). Shared characteristics among nodes can partition the network into cohesive modules, a common feature of pollination networks (Lewinsohn *et al.*, 2006; Olesen *et al.*, 2007; Martín González *et al.*, 2012). In

individual-based pollination networks, plants having similar pollinator assemblages would be more connected and thus will group together into modules. For this to happen, individual differences in pollinator assemblages are required, a common phenomenon occurring in generalist systems (Herrera, 1995b; Thompson, 2001). However, the presence of abundant shared pollinators will make these groups fuzzy. We have not found significant differences in modularity between the total empirical networks and the random networks, suggesting the absence of well defined groups of plants. Although modularity was significant in some temporary networks, most of them showed non-significant values. The high variation in the value of this metric suggests that the groups of plants sharing pollinators are temporally labile, meaning that network modules are not static, but divide and merge through time (Stanoev *et al.*, 2011). In this sense, the integration of the temporary networks in the total network is surely canceling out the formation of modules. We believe that this pattern will be common in generalist plants visited by many disparate pollinators that vary temporally in their frequency of interaction.

Weighted clustering values, although not significant, were high in all temporary networks and consistently above the mean values obtained in the random networks. Because this metric measures the proportion of closed triplets in a network (Opsahl and Panzarasa, 2009), our results indicate that the temporary networks were compact and enriched in closed triplets. Plants flowering at the same time will have higher likelihood of forming local clusters because of their higher probabilities of interacting with the same set of pollinators. This pattern is reinforced in the total network, where the temporary interactions between individual plants and pollinators are integrated. As a consequence, weighted clustering reached significance in the total network (Table 4.2; Fig 5). Under these circumstances, we would expect non-random mating among individual plants as a consequence of the pattern of pollinator sharing. High values of weighted clustering have been related to family aggregation in social networks (Fowler *et al.*, 2011). Detailed genetic studies, however, are needed to corroborate this idea.

Assortative mixing by node degree indicates whether there exists a pattern of node linkage based on node degree (Newman, 2002). The positive values found for this metric in our system indicate that temporary networks had a core-periphery topology that is significant in the total networks (Table 4.2, Figs. 4 and 5). We think that the core-periphery structure found in the total network could be in part due to the overlapping of temporary networks, in a similar way as Yang and Leskovec (2014) demonstrated for overlapping communities. In this sense, the network core would be enriched with plants flowering during the entire season and thus appearing in most temporary networks. These plants are more likely to be visited by a higher diversity of pollinators and therefore have high multiple similarities in pollinator

composition with other plants. On the other hand, the periphery will be mostly composed of plants visited by singular pollinator assemblages, reducing their multiple similarities (Jurasinski *et al.*, 2012), and decreasing the probabilities of being connected with more plants. Because being visited by a highly diverse pollinator assemblage enhances fitness (Klein *et al.*, 2003; Gómez *et al.*, 2007; Perfectti *et al.*, 2009), we presume that core plants will have high values of fitness. Moreover, because core plants are highly inter-connected, they would receive more diverse pollen loads and would also donate pollen to many other plants (Gómez and Perfectti, 2012), implying that their progenies will have higher genetic diversity. These mating patterns and the evolutionary consequences however should be verified using paternity analyses.

We have explored the effect that the non-random foraging behavior of pollinators may have on the topology of the networks. We did not find any consistent effect, measured as standardized effect sizes, for the temporary networks. Only for the total networks the effects of some type of pollinators became consistent (Figure 4.5). Large ants, although varying the sign of their effect through the temporary networks, significant and positively affected weighted clustering and assortativity metrics in the total networks. However the lack of a consistent effect for the rest of pollinators suggests that the foraging pattern of pollinators varies temporally. Changes in pollinator behavior can be due to changes in feeding habits (Faegri and van der Pijl, 1979), but also to the response to other pollinators' behavior. The arrival of new pollinator species can displace preexisting ones or change their foraging behavior as a result of competitive interactions (Morse, 1982; Brosi and Briggs, 2013). Moreover, aggressive resource consumers like ants (Blüthgen and Fiedler, 2004), might deplete floral resources and thus may provoke important changes in the intensity of interaction of this plant species with other pollinator types. We think that the observed pollinator turnover supports that the effects of different pollinators on network topology are context-dependent, i.e., the effects of a pollinator type on network topology depends on the abundance of other pollinator types. This context-dependence has been previously suggested as an important driver of ecological interaction networks (Poisot *et al.*, 2014) and could also be an important factor when downscaling to individual-based pollination networks.

Conclusions

Our study suggests that the interplay between plant phenology and pollinator availability shape the topology of the individual-based plant networks based on similarities in pollinator composition. Our networks were cohesive (non modular), transitive (locally clusterized), and centralized (core-periphery topology). These particular topologies could entail functional consequences for

the persistence and evolution of plant populations. Nevertheless, our study shows that the network properties changed over time, indicating that the effects of different types of pollinators on network topology are contextual. This finding demonstrates the importance of considering the entire flowering season and highlights the necessity of making comprehensive temporal sampling when trying to build reliable interaction networks.

Acknowledgements

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Supplementary information

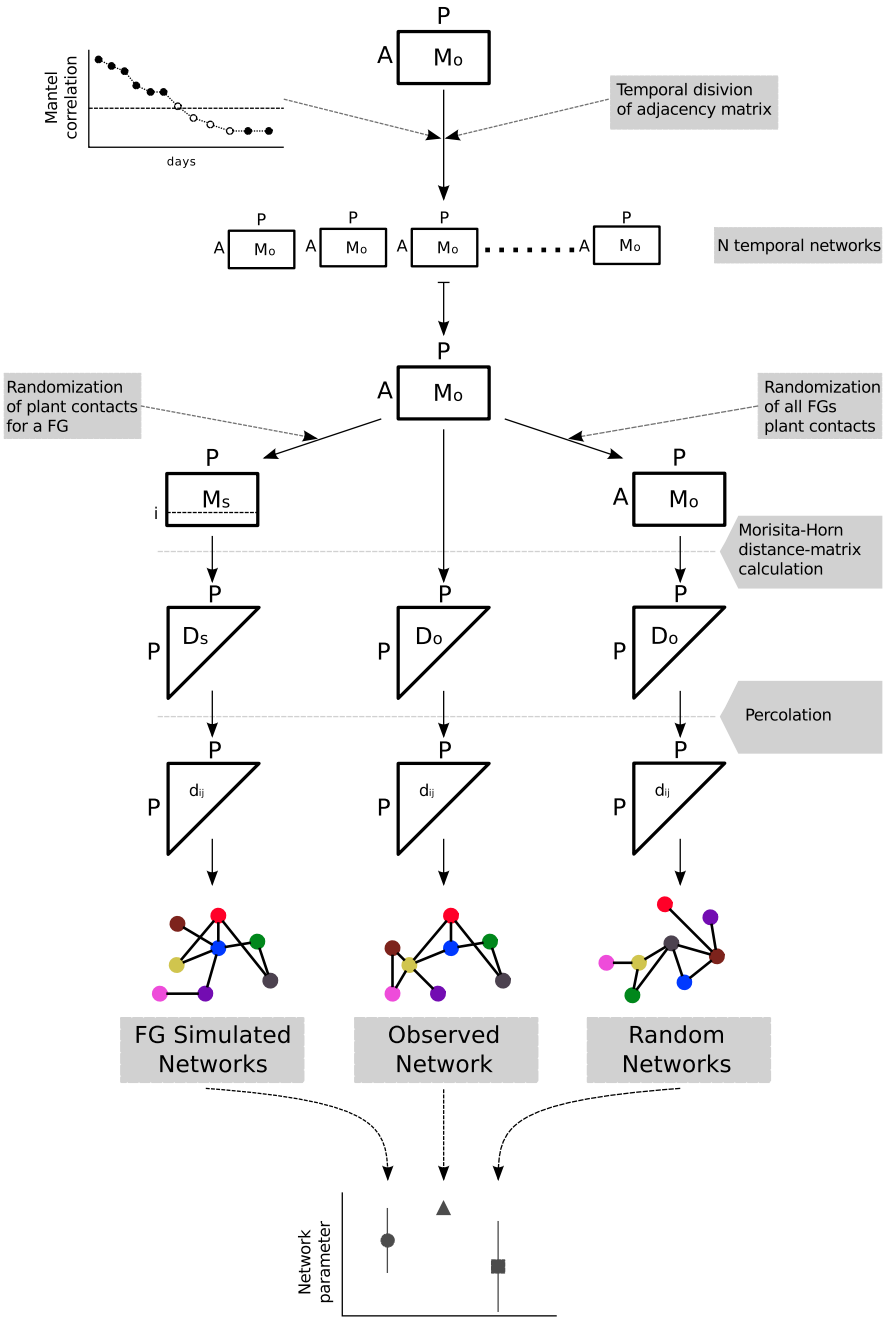

Figure 4.S1: *Workflow followed to perform the network analyses.*

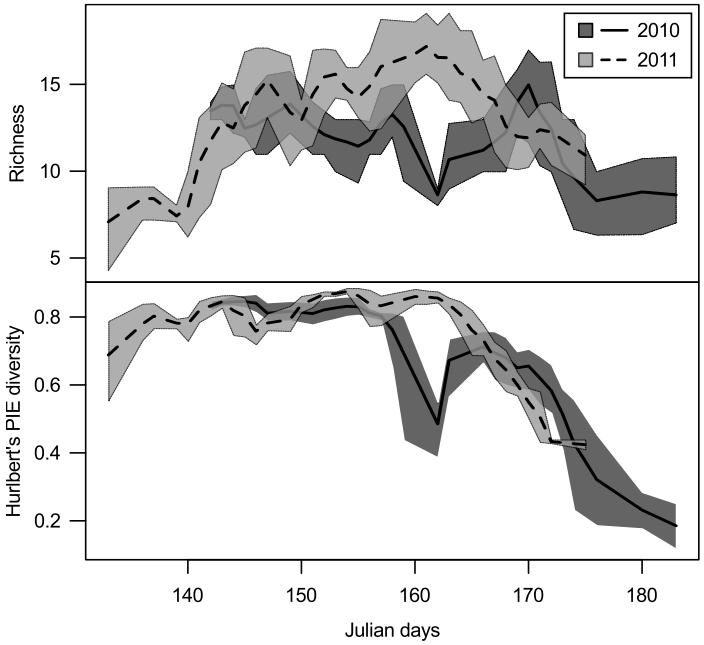

Figure 4.S2: *Intraseasonal temporal evolution of functional group richness and diversity measured as Hurlbert's PIE index. Dashed lines denote values for year 2010 while continuous lines values for year 2011. Envelopes indicate 95% confidence intervals. Values correspond to overlapping rolling windows spanning 3 days. See appendix S3 for a detailed explanation.*

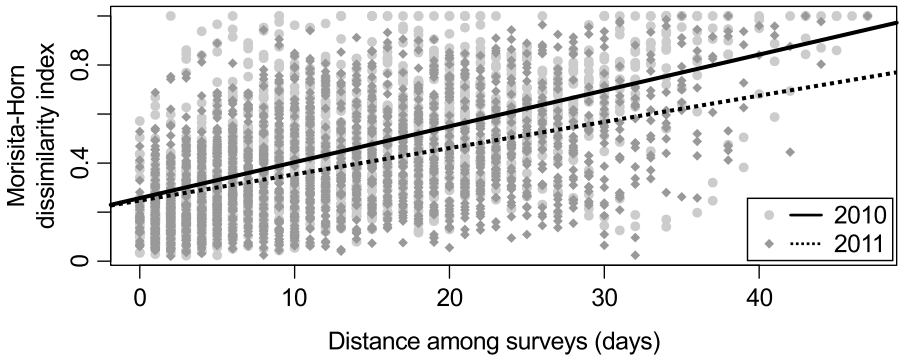

Figure 4.S3: *Linear regression of Morisita-Horn dissimilarity index over temporal distance among surveys for both years.*

Table 4.S1: *Flowering phenological parameters for each year.*

Year	Flowering days	Flowers at flowering peak	Flowering peak		Pairwise synchrony	
			Population level	Individual level	Augspurger X_{ij}	Jaccard type Chao index J
2010	34.15 ± 10.33	10.14 ± 6.92	153	154.82 ± 6.87	0.62 ± 0.18	0.25 ± 0.21
2011	30.71 ± 5.81	14.90 ± 10.89	156	155.16 ± 5.08	0.69 ± 0.14	0.14 ± 0.14

Flowering curve estimations

To estimate the individual flowering curves we fitted a polynomial function for the number of open flowers per day. These functions take the form:

$$f(x) = a_n x^n + a_{n-1} x^{n-1} + ... + a_1 x + a_0$$

Where each a is an independent real number, and n the degree of the function (i.e., the highest power of x in its expression). This kind of functions allows for multimodality in the flowering patterns.

Equations used on this manuscript

Eq1. Augspurger synchrony index (X_{ij})

Augspurger synchrony measures the flowering synchrony of a plant with the rest of the population:

$$X_i = \frac{1}{n-1} \times \frac{1}{f_i} \times \sum_{j \neq i}^{n} e_j$$

In this equation, n denotes the number of individuals in the population, f_i the number of flowering days and e_j the number of co-flowering days of individuals i and j. When making pairwise comparisons the index turns asymmetric in the sense that the values obtained differ depending of which plant is been compared with the other. This is because the flowering duration of each plant differs. To overcome this, we used a modified version of the Augspurger metric which corrects for this asymmetry:

$$X_{i,j} = \frac{e}{f_i + f_j - e_j}$$

In this modified version of the index, f_j indicates the number of flowering days of individual j. Note that the denominator defines the number of days where either one, another, or both plants are flowering.

Eq2. Jaccard-type Chao dissimilarity index (J)

We used this index proposed by Chao *et al.* (2005) as a proxy for a weighted flowering synchrony among pairs of plants:

$$J = \frac{U \times V}{U + V - U \times V}$$

Here U and V express the relative abundances of flowers belonging to the coflowering days for plant 1 and 2 respectively. This index ranges from 0 to 1, where 0 means no temporal overlap among a pair of individuals, and 1 complete temporal overlap.

Eq3. Hurlbert's PIE diversity index ($D_{Hurlbert}$)

We used this algorithm to study the pollinator visits along each flowering season. This index was proposed by (Hurlbert, 1971) as a modification of the Simpson diversity index. We chose this index because it is insensitive to sample size:

$$D_{Hurlbert} = 1 - \sum \left(\frac{n_i}{N}\right) \times \left(\frac{n_i - 1}{N - 1}\right)$$

Here, n_i refers to abundance of the i_{th} species, and N is total abundance.

Eq4. Morisita-Horn dissimilarity index (S_{M-H})

In order to study the seasonal turnover of the pollinator community by comparing temporal distances among all pairs of surveys with their community dissimilarity, we chose the Morisita-Horn dissimilarity index (Morisita, 1959; Horn, 1966). This index has a low sensitivity to rare species allowing us to eliminate the effect of relative meaningless flower contacts by some FGs:

$$S_{M-H} = \frac{2 \times \sum_{i=1}^{S} p_{i1} \times p_{i2}}{\left[\sum_{i=1}^{S} p_{i1}^2 + \sum_{i=1}^{S} p_{i2}^2\right]}$$

Here, S is the total number of species in both compared assemblages, p_{i1} and p_{i2} denotes the relative abundance of species i in assemblage 1 and 2 respectively.

Eq5. Modularity index (Q)

This metric measures the degree to which a network is structured in subgroups of highly connected nodes (modules) (Newman, 2004). It measures the observed fraction of links within groups minus that expected from a random distribution of the links:

$$Q = \frac{1}{2 \times m} = \sum_{ij} \left(A_{ij} - \frac{k_i \times k_j^T}{2 \times m} \right) \times \sigma(c_i, c_j)$$

For a given structure in modules, A_{ij} denotes the connexion among nodes i and j taking the value of 1 if these are connected, and 0 if not. k_i and k_j are the linkage degree, m is the number of links in the graph, c_i and c_j the modules into which nodes i and j are respectively assigned, and $\sigma(c_i, c_j)$ is a function which value is 1 when $c_i = cj$, and 0 otherwise (Newman, 2004; Newman and Girvan, 2004).

Eq6. Weighted Clustering Coefficient (C_W)

This metric, proposed by Opsahl and Panzarasa (2009) as an extension of the Global clustering coefficient (Newman, 2003b) was used to measure network transitivity. It measures the tendency of nodes to cluster together into tight groups using the proportion of closed triads of nodes over the overall number of all triads (open and close) in the network. A triad is a group of three nodes interconnected. Depending on how these are connected they are closed triads: if all of the three nodes are fully interconnected; or open triads: if two of the nodes are not inter-connected. The weighted extension used here weights each triad with the geometric mean of all links forming it.

$$C_W = \frac{\sum_{\tau_\Delta} \omega}{\sum_{\tau} \omega}$$

In this formula ω are the link weights forming all tripets (τ) and the subset of closed triplets (τ_Δ).

Eq.7 Assortative mixing by node degree (r)

This metric was introduced in graph theory by Newman (2003a). It is a measure of network centralization and denotes to what extent nodes tend to be connected with nodes of similar degree. For undirected networks this metric is defined as:

$$r = \frac{\sum_{jk}(e_{jk} - q_j \times q_k)}{\sigma_q^2}$$

Where e_{jk} is the fraction of nodes that connect nodes of degree j with nodes of degree k. q_j are the degree distributions of nodes with degree j attached to nodes of degree k and vice-versa for q_k. These distribution are related to e_{jk} through:

$$\sum_k e_{jk} = q_{j.}$$

σ_q^2 is the standard deviation of the distribution of qk. This metric r takes value of 1 if there is a perfect assortative mixing, 0 if there is no assortative mixing and close to -1 when is disassortative. Values close to 0 describe star-like networks while values close to 1 describe networks with a core of highly connected nodes against a periphery of nodes with lower degree (Newman 2010).

Eq8. Standarized effect size (SES)

$$SES = \frac{o - \tilde{e}}{std(e)}$$

Here o denotes the empirical value, e the expected distribution (obtained by reshufling), and $\tilde{e}$ the median of the expected distribution. We used the median instead of the mean due to the lack of normality in the expected distributions.

Visualization of the temporal variation of richness and diversity

To visualize temporal trends in richness and diversity of FGs, we used an overlapping rolling window spanning three days. For each combination of two surveys contained in a given window, we calculated the richness and diversity of FGs. Therefore, for each window we were able to calculate an average and 95% confidence interval. See Figure 4.S4 below. Here, the variation in sampling effort per day depended mainly on weather conditions.

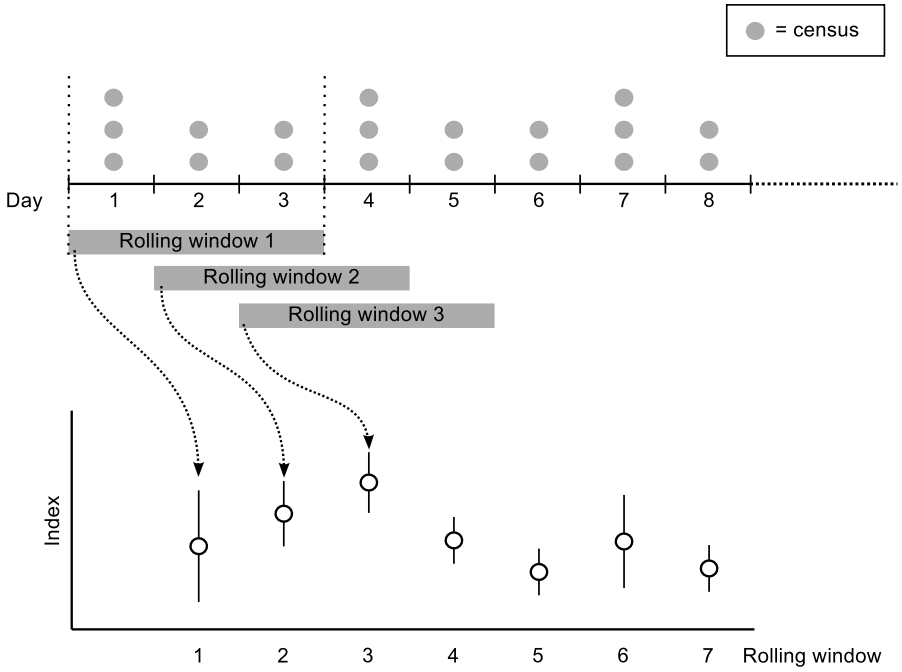

Figure 4.S4: *Rolling windows used to visualize temporal trends.*

5

Effectiveness of a generalist plant pollinator assemblage

english

Abstract

In generalist plant species, insects often vary in their effectiveness as pollinators, measured as the product of the frequency (quantity) and quality of their visit. In addition, insects can also vary in their ability of transferring pollen from different conspecifics. We studied the pollinator effectiveness of most of the functional groups visiting the flowers of *Erysimum mediohispanicum* by quantifying the visitation frequency occuring in a natural population and the per-visit performance measured as the reproductive output of the plant. We also measured the diversity of pollen transfered per flower visit of the four most important functional groups. Pollinators showed contrasting effectiveness. These were attributable to differences in either their visitation frequency or their per-visit performance, depending on the case. We found differences among two pollinator functional groups in the diversity of pollen transferred after a single visit to a flower. These results reveals that in *E. mediohispanicum* a great amount of its reproductive success depends on a subset of its pollinator assemblage, and that few pollinators contribute differentially to the genetic diversity of the ofspring. Our findings emphasize that assessing the pollinator effectiveness is necessary to obtain realistic perceptions of the intensity and sign of the plant-pollinator interactions.

Introduction

The reproductive success of most flowering plants relies on the service provided by flower visitors in transferring pollen among conspecifics (Ollerton *et al.*, 2011). The performance of this service, known as pollinator effectiveness (PE; Ne'eman *et al.*, 2010; Rodríguez-Rodríguez *et al.*, 2013; Armbruster, 2014), usually varies among flower visitors along a continuum between very effective pollinators and non-pollinating species (Herrera, 1987; Maloof and Inouye, 2000). This is especially true for generalist plant species (Waser *et al.*, 1996; Ollerton *et al.*, 2007), where the species visiting their flowers present a wide variety of visitation rates (Sahli and Conner, 2007) and floral fidelities (Brosi and Briggs, 2013). In generalist plants, overlooking the effectiveness of the flower visitors can lead to an incorrect characterization of their function as pollinators and thus to misinterpret as true pollinator species merely acting as free loaders or robbers. Therefore it is important to assess the variations in pollinator effectiveness to obtain realistic insights of the plant-pollinator interactions and evolution, as for to assess the relative importance of each pollinator as selective agents (Van Der Niet *et al.*, 2014). From a community perspective, we can reduce the uncertainty on the degree of interdependence between pairs of plants and pollinators, improving the reliability of plant-pollinator networks (King *et al.*, 2013). Finally, we can assess the contribution of each floral visitor in defining the structure of mating events within a plant population, an aspect of great impact on the resulting spatial genetic structure and in the fate of the populations (DiLeo *et al.*, 2014).

Pollinator effectiveness is composed of two components, a quantity component (QNC) and a quality component (QLT). The QNC of effectiveness is defined as the number of interaction events between populations of plants and their pollinators. This component has largely been used alone to measure PE, although, it has been proven to be a bad proxy for that (King *et al.*, 2013), but see Vázquez *et al.* (2005). The QLC of effectiveness is the probability that a single interaction event will produce a new adult plant (Schupp et al. in press). This component embraces the per visit effects on the reproductive success for both the male (number of offspring sired) and female (percentage of ovules being fertilized) functions of a plant species. Together, the product $QNC \times QLC$ conforms the PE of a pollinator and its position in the PE landscape (Schupp *et al.*, 2010; Rodríguez-Rodríguez *et al.*, 2013).

The hability of pollinators in transferring pollen from different conspecifics may also vary among species (Matsuki *et al.*, 2008; Brunet and Holmquist, 2009; Hasegawa *et al.*, 2011). This is mainly due to differences in both their

capacities to transfer pollen from a male-donor flower to a series of successive female-phase flowers (pollen carryover; Marshall and Ellstrand, 1985), but also because of their movement patterns (Aizen and Harder, 2007). These pollinator capacities can contribute differentially to the genetic composition of the offspring (Barthelmess *et al.*, 2006; Hasegawa *et al.*, 2015) resulting in important consequences for plant fitness derived from the opposing positive and negative effects of outcrossing and inbreeding on the offspring (Herlihy and Eckert, 2004; Waller, 2015). In spite of its importance, the contribution of pollinators to next generation genetic composition has been traditionally overlooked. We think that its inclusion as a postzygotic component of pollinator effectiveness can contribute to improve its estimate.

In this study we explore the variation in effectiveness among the flower visitors of a highly generalist plant species, *Erysimum mediohispanicum* (Brassicaceae). Besides being partially self-compatible, this plant needs pollen vectors to full seed set (Abdelaziz *et al.*, 2014). The flowers of this plant species are visited by more than 500 insect species across its entire geographic range (Gómez *et al.*, 2014a). In the study area the pollinator assemblage can be classified in 23 functional groups (see Valverde *et al.*, 2016b, and appendix 'Functional groups' for a detailed description of those). We hypothesize that PE may vary among floral visitors in both QNC and QLC. Several studies on this species have demonstrated an uneven distribution of visitation frequencies (Gómez *et al.*, 2009b; Valverde *et al.*, 2014, 2016b), suggesting a variation in the QNC. Additionally, the high variety in foraging behaviors and functional traits occurring among floral visitors advocates for differences in the QLC (Gómez *et al.*, 2008b,a, 2009b). These differences in PE may have important implications for the population dynamics and evolution of *E. mediohispanicum*, and yet, there are no studies assessing the direct contributions of the different types of pollinators to the reproductive success of this plant. Here, we experimentally determine the quantity and quality components of the PE of most of the functional groups visiting *E. mediohispanicum*. Furthermore, for some functional groups, we also determine their contribution to the genetic diversity of the offspring, as an additional attribute of their quality as pollinators.

Materials and methods

Sampling design

This study was conducted between 2010 and 2013 at the Sierra Nevada natural park (south-east Spain), in a medium-high mountain Mediterranean-

type ecosystem where *E. mediohispanicum* grows. The experiments were performed in two areas: a natural population situated in an open woodland (1723 m a s l, 2010 and 2011), and at the Botanical Garden of Hoya de Pedraza (SI1872m.a.s.l., 2012 and 2013). We tried to characterize the *PE* of the entire set of functional groups visiting the flowers of *E. mediohispanicum* in the study area by accounting their quantity and quality components as well as their contribution to the genetic diversity of the resulting offspring. However, we obtained enough information for only 10 functional groups, and consequently performed all the statistical analyses with this subset. These functional groups were also the major visitors of *E. mediohispanicum* all over its entire distribution (Gómez *et al.*, 2014a).

Quantifying the pollinator effectiveness

The quantity component

We quantified the *QNC* as the frequency of flower-pollinator interaction. For this we considered three subcomponents: visitation frequency at the plant level, number of contacted flowers per visited plant and proportion of legitimate visits to a flower. We calculated the visitation frequency by means of 5 minutes surveys on 200 focus plants (100 per year in 2010 and 2011). Surveys were performed from 1 to 4 times per day along the whole flowering season in order to integrate the temporal variation in the pollinator assemblage (Valverde *et al.*, 2014, 2016b). To obtain the number of contacted flowers per visited plant we randomly selected insects foraging on plants of *E. mediohispanicum* and divided the number of contacted flowers by the total number of flowers actually open per plant. Finally, we obtained the percentage of legitimate visits to a flower by recording the pollinator handling behavior. Additionally to the later, we added observational data from previous years.

The quality component

We quantified the *QLC* as the probability that an individual interaction event produces a new adult ($\frac{fruit}{visit} \times \frac{seeds}{ovules} \times \frac{seedling}{seed}$). Between 2011 and 2013, we selected several flowering plants grown in nurseries from seeds proceeding from plants in 2010. We isolated these plants using hand-made tents and nylon isolation tents with 160 μm mesh aperture (BugDorm-2120, MegaView Co., Ltd.). Daily, we exposed the virgin flowers and waited for a visit to occur. Flower visitors were allowed to contact up to three flowers. For each event we recorded the handling time and photographed the interaction to correctly locate the visited flower and identify the insect species. The contacted flowers were marked with a color code using cotton threads and their petals

cut off to prevent further visits. Additionally, we performed three controls on the experimental plants: Allogamy, in which allogamous pollen from three individuals was added to a virgin stigma; Autogamy, in which flowers were left unpollinated; and a Procedural Control, were flowers were left unpollinated and its petals cut off.

We recorded the fruit set and counted the number of unfertilized and fertilized ovules per fruit. For the latter, we distinguished between viable seeds and seeds aborts. Finally, for a certain number of fruits produced per FG we sown all seeds and assessed its germination success. With this data we obtained four quality subcomponents. Fruiting probability ($\frac{fruit}{visit}$), proportion of ovules fertilized ($\frac{fertilized\ ovules}{oules}$), proportion of ovules successfully fertilized ($\frac{seeds}{ovules}$), and the germination success ($\frac{seedling}{seed}$).

Pollen diversity

We assessed the contribution of some FGs to the genetic diversity of the offspring. For this experiment we used the same plants used for the *QLC* experiments performed in 2012 and the seedlings resulting from the germination experiment. That year, plants were exposed in an experimental plot consisting on 98 plants that served as potential pollen donors. We chose four seedlings per fruit from a set of fruits resulting from single visits of long-tongued beeflies, long-tongued large bees, short-tongued large and short-tongued medium-sized bees. We chose these functional groups because of their importance in visitation frequency and because of their ability to produce seeds after visit to a virgin flower.

We extracted the DNA of these seedlings, their corresponding maternal plants and the 98 potential pollen donors. For this we used the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) on at least 60 mg of plant tissue. Following, we genotyped each individual by means of 10 unlinked nuclear microsatellites loci (Muñoz-Pajares *et al.*, 2011) using a Gradient Master Cycler Pro S (Eppendorf, Hamburg, Germany). Amplification programs and primer information are given in Appendix 2. We used Peak Scanner Software version 2.0 (Applied Biosystems) and posterior eye inspection for genotype callings.

From the offspring genotypes, we characterized the alleles belonging to the siring plant by elimination of the known maternal ones. Moreover, we performed paternity analyses on each seedling using the categorical allocation method implemented in the Cervus 3.0 software (Marshall *et al.*, 1998). The parentage assignations were obtained based on critical likelihood values of 95% and 80% after 10 000 simulations and using an underlying allele frequencies calculated from the adult plant genotypes. The parameters set for the analysis were: minimum number of matching loci = 6; error rate = 0.01; proportion

of sampled candidates = 0.9; proportion of loci typed = 0.7. We used a personalized script to handle genotyping errors (see appendix 'Handling of genotyping errors'). Unsuccessful assignations were assigned to a same dummy parent because we considered those alleles coming from outside the study plot or either from a non-genotyped adult plant.

Data analysis

We compared each QNC subcomponents among functional groups by means of generalized mixed models. To analyze the visitation frequency subcomponent we constrained our data to those plants that had more than 5 visits, and fitted a model using a zero-inflated Poisson distribution using plant as a random factor. For the contacted flowers subcomponent we used a Poisson distribution to fit the model. For these analyses, we clumped data from years 2010 and 2011 because of the low inter-annual variation in the pollinator assemblages (Valverde *et al.*, 2016b) and corrected by the number of open flowers by setting its natural logarithm as an offset variable. To asses the number of open flowers each day, we estimated each plant's flowering curve using local polynomial functions (Valverde *et al.*, 2016b). Finally, to study the handling time we used a negative binomial distribution. All models were performed using the package 'glmmADMB' in R (Fournier *et al.*, 2012). Post hoc comparisons among FGs on the three subcomponents where made using a Tukey's range test and a bonferroni correction with the tools implemented in the 'multcomp' package (Hothorn *et al.*, 2008).

We analyzed QLC subcomponents using generalized mixed models. We used plant identity as a random effects variable and relativized the number of fertilized ovules and viable seeds by the per-plant average number of available ovules per flower. We fitted the models using a binomial distribution for the fruiting probability and a quasi-binomial and logit link function for both the proportion of fertilized ovules and the proportion of ovules successfully fertilized. The abortion rate resulting from the later two subcomponents was explored using generalized models and quasibinomial distribution. Post hoc multiple comparisons among functional groups where performed using Tukey's range test and a Bonferroni correction.

We obtained the pollinator effectiveness of each FG multiplying the QNC and the QLC. Because all subcomponents here result from independent set of observations and experiments, we used a bootstrap-based methodology proposed by Goodman (1960, 1962) and adapted to pollination ecology by Reynolds and Fenster (2008) to estimate their mean and 95% confidence interval (Rodríguez-Rodríguez *et al.*, 2013). To derive the quantity component ($\frac{pollinator-flower\ interaction}{5\ minutes\ observation}$), we multiplied the visitation rate of a FG on a plant and the numbers of flowers visited. The visitation rate was obtained

dividing the total number of plant contacts by the number of censuses in the population, so that we avoided overestimating pollination events occurring in plants flowering for few days. Because we based this component in 5 minutes, we limited the maximum number of contacted flowers to that a given FG may contact in five minutes in order to limit our estimates to this time range. To obtain the quality component ($\frac{fruit}{visit} \times \frac{seeds}{ovules} \times \frac{seedling}{seed}$), we multiplied the fruiting probability, the proportion of ovules successfully fertilized, and the proportion of seeds germinating into a seedling. Both components were plotted in a PE landscape following Schupp *et al.* (2010).

Finally, we analyzed the contribution of each FGs to the genetic diversity of the offspring. For each pool of sampled seeds per fruit, we obtained the diversity of paternal alleles and of pollen donors. For the genetic (allelic) diversity, we calculated the unbiased heterozygosity because this index is robust to the inequality in the number of genotyped alleles per locus. The diversity of pollen donors was calculated using the Hurlbert probability of interspecific encounter (PIE) because of its similarity with the heterozigosity measure. Following, we performed paired comparisons among FGs for both diversity measures. Because of the small data size after clumping genotypes by fruit, and due to the non-Gaussian data distributions, we performed the comparisons using the two-sample permutational Wilcoxon test in the 'perm' package in R (Fay and Shaw, 2010).

Results

Quantity subcomponents

We recorded a total of 2022 plant visits of the functional groups (889 visits, 21 735 minutes of survey in 2010; 1133 visits, 22 240 minutes of survey in 2011). Most of the visits were performed by long-tongued beeflies species (27.45%), followed by small beetles (23.62%), and short-tongued small bees (15.86%). When comparing the per-plant visitation rate (5 minutes census) among FGs, long-tongued beeflies were significantly the most common plant visitors (0.14; Figure 5.1a; Table 5.1), followed by small beetles (0.07) and short-tongued large bees (0.05).

By contrast, the number of flowers contacted per visit was more evenly distributed among functional groups, being highest for long-tongued beeflies (3.88; Figure 5.1b) but not significantly different from the following, long-tongued large bees (3.66) and short-tongued large bees (3.52).

Handling time showed that the four groups visiting the most number of flowers per visit were also the fastest in handling the flowers of *E. mediohispanicum* reflecting the negative correlation between both variables ($r = -0.734$, $p = 0.015$). Particularly, long-tongued large bees were the fastest in handling time, followed by long-tongued beeflies, and short-tongued large and medium-sized bees (Figure 5.1c).

Quality subcomponents

We obtained data from 765 single visits to virgin flowers pertaining to 53 plants. Our results showed that the allogamy control performed better for all the three subcomponents studied (fruit set = 0.956 ± 0.206, proportion of fertilized ovules = 0.431 ± 0.219, seed set = 0.345 ± 0.204; Table 5.1), being significantly better than all FGs for the proportion of fertilized and for the proportion of viable seeds ($p < 0.05$ for all comparisons). On the other hand, the procedural and the spontaneous autogamy controls showed the lowest values in all the subcomponents (fruit set < 0.29, proportion of fertilized ovules < 0.09, seed set < 0.07), only comparable to the values obtained for some FGs. Long-tongued large bees, short-tongued large and medium-sized bees and long-tongued beeflies stand out in quality. The differences on fruit set among FGs was very gradual (Figure 5.1d), with short and long-tongued large bees together with large hoverflies showing the highest values (0.899 ± 0.303, 0.863 ± 0.345, 0.857 ± 0.378, respectively) and with no significant differences with the hand-pollination treatment. By contrast, for the percentage of fertilized ovules, large hoverflies presented very low values (0.059 ± 0.030) while short and long-tongued large bees remained as the most effective FGs (0.265 ± 0.211 and 0.283 ± 0.215, respectively; Figure 5.1e). The values found for the seed set presented a similar pattern with the later subcomponent, with short and long-tongued large bees producing the most seeds per flowers visit (0.224 ± 0.191 and 0.231 ± 0.183, respectively; Figure 5.1f). These data reflect the rates of abortion of fertilized ovules in which we found no significant differences between any functional group and the hand-pollinated flowers, except for large hoverflies which showed high and significant values of seed abortion (Figure 5.2).

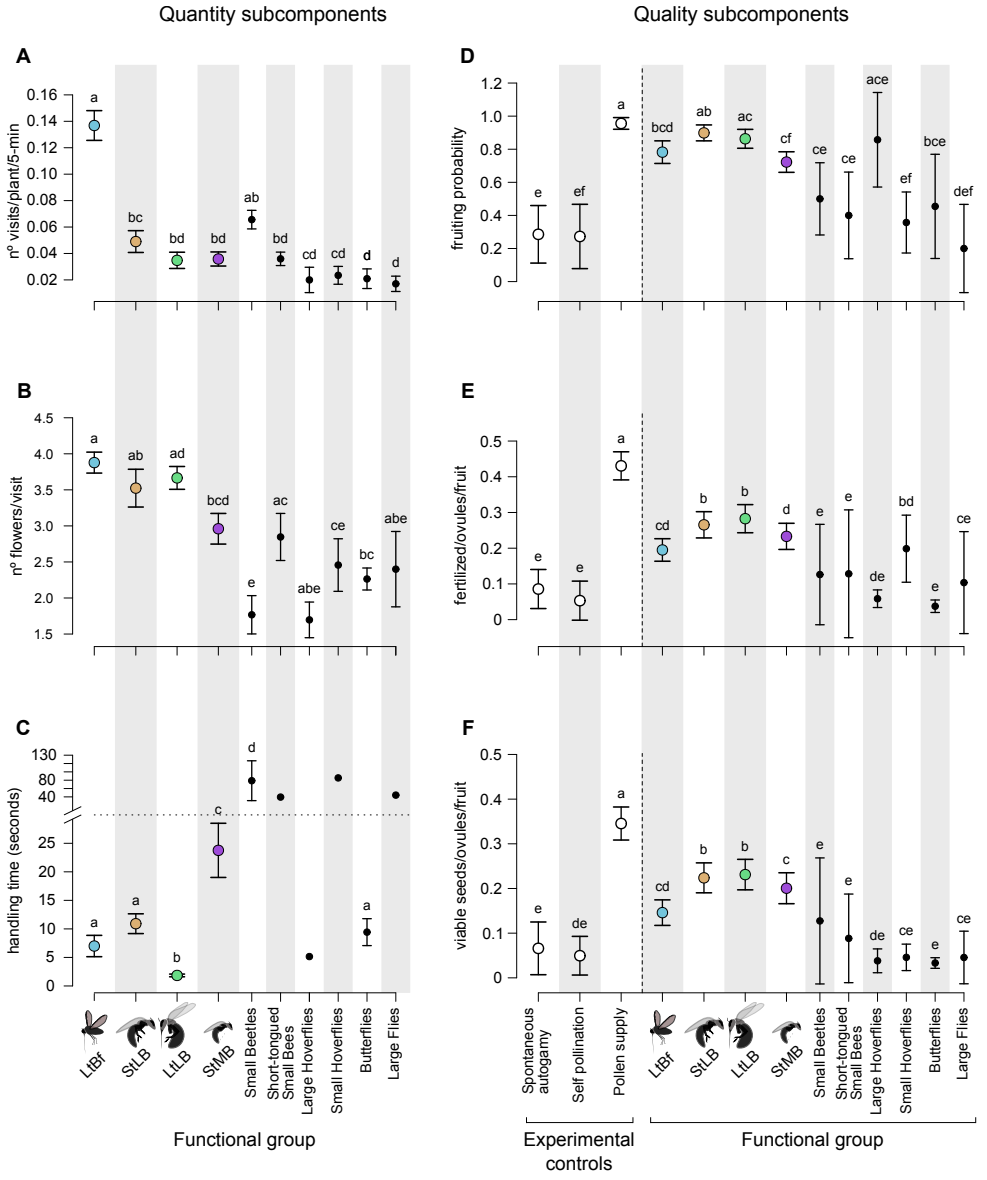

Figure 5.1: *Quantity and quality subcomponents of the pollinator effectiveness. Quantity subcomponents: A) Visitation frequency; B) Number of flowers contacted per visited plant; C) Handling time. Quality subcomponents: D) Fruit set, calculated as the proportion of visited flowers developing a fruit; E) Proportion of fertilized ovules; F) Seed set. Functional groups are ordered following their final effectiveness estimates. Values for the four groups presenting the highest values of effectiveness are highlighted (LtBf: long-tongued beeflies; StLB: short-tongued large bees; LtLB: long-tongued large bees; StMB: short-tongued medium-sized bees). Values are mean ± SE.*

Table 5.1: *Quantity and quality subcomponents of the pollinator effectiveness. Between parenthesis the sampling size, for the quality subcomponents the first number denotes the number of flowers and the second the number of plants.*

		Quantity subcomponents				Quality subcomponents		
	Treatment	$\frac{n_{VISITS}}{plant}$	$\frac{n_{FLOWERS}}{visit}$	legitimate visits	handling time	fruit set	fertilized ovules	seed set
FGs	Long-tongues beeflies	0.137 ± 0.419 (147)	3.878 ± 3.618 (2344)	1 (667)	4.416 ± 9.451 (308)	0.782 ± 0.414 (147 / 33)	0.195 ± 0.161 (105 / 29)	0.146 ± 0.14 (96 / 29)
	Short-tongued large bees	0.049 ± 0.241 (147)	3.524 ± 3.491 (655)	1 (200)	7.301 ± 6.995 (136)	0.899 ± 0.303 (158 / 26)	0.265 ± 0.211 (132 / 25)	0.224 ± 0.191 (130 / 25)
	Long-tongued large bees	0.035 ± 0.192 (147)	3.666 ± 3.623 (1995)	1 (298)	1.378 ± 1.045 (176)	0.863 ± 0.345 (146 / 27)	0.283 ± 0.215 (119 / 26)	0.231 ± 0.183 (115 / 25)
	Short-tongued medium-sized bees	0.036 ± 0.193 (147)	2.960 ± 2.577 (530)	0.851 (377)	16.766 ± 21.094 (150)	0.722 ± 0.449 (209 / 41)	0.233 ± 0.202 (122 / 36)	0.201 ± 0.186 (115 / 36)
	Small beetles	0.066 ± 0.283 (147)	1.767 ± 1.514 (73)	0.935 (278)	62.48 ±94.991 (25)	0.500 ± 0.512 (22 / 12)	0.126 ± 0.211 (9 / 6)	0.127 ± 0.199 (8 / 5)
	Short-tongued small bees	0.04 ± 0.216 (147)	2.847 ± 2.534 (215)	0.813 (168)	28.667 ± 27.062 (3)	0.400 ± 0.507 (15 / 8)	0.128 ± 0.200 (5 / 5)	0.088 ± 0.111 (5 / 5)
	Large hoverflies	0.02 ± 0.14 (147)	1.696 ± 0.989 (56)	1 (12)	3.734 ± 2.526 (7)	0.857 ± 0.378 (7 / 5)	0.059 ± 0.030 (6 / 4)	0.038 ± 0.033 (6 / 4)
	Small hoverflies	0.023 ± 0.171 (147)	2.457 ± 2.078 (116)	1 (73)	49.81 ± 52.947 (7)	0.357 ± 0.488 (28 / 15)	0.199 ± 0.115 (6 / 5)	0.046 ± 0.036 (6 / 5)
	Butterflies	0.021 ± 0.143 (147)	2.263 ± 1.786 (482)	1 (96)	5.931 ± 8.188 (122)	0.455 ± 0.522 (11 / 5)	0.038 ± 0.020 (5 / 4)	0.033 ± 0.013 (5 / 4)
	Large flies	0.017 ± 0.129 (147)	2.400 ± 1.653 (30)	0.985 (194)	44.24 (1)	0.200 ± 0.422 (10 / 6)	0.104 ± 0.101 (2 / 2)	0.046 ± 0.042 (2 / 2)
Controls	Allogamy	-	-	-	-	0.956 ± 0.206 (136 / 47)	0.431 ± 0.219 (124 / 47)	0.345 ± 0.204 (122 / 46)
	Procedural control	-	-	-	-	0.273 ± 0.456 (22 / 14)	0.053 ± 0.061 (5 / 5)	0.05 ± 0.043 (4 / 4)
	Spontaneous autogamy	-	-	-	-	0.286 ± 0.46 (28 / 21)	0.086 ± 0.072 (7 / 7)	0.066 ± 0.066 (5 / 5)
Other FGs	Large moths	0.016 ± 0.128 (200)	2.214 ± 2.665 (14)	-	-	0.500 ± 0.707 (2 / 2)	0.690 (1 / 1)	0.690 (1 / 1)
	Pollen wasps	0.016 ± 0.128 (200)	-	-	-	1.000 ± 0.000 (3 / 2)	0.273 ± 0.068 (3 / 2)	0.236 ± 0.108 (3 / 2)
	Small diving beetles	0.017 ± 0.137 (200)	-	-	-	0.667 ± 0.577 (3 / 3)	0 (3 / 3)	0 (3 / 3)

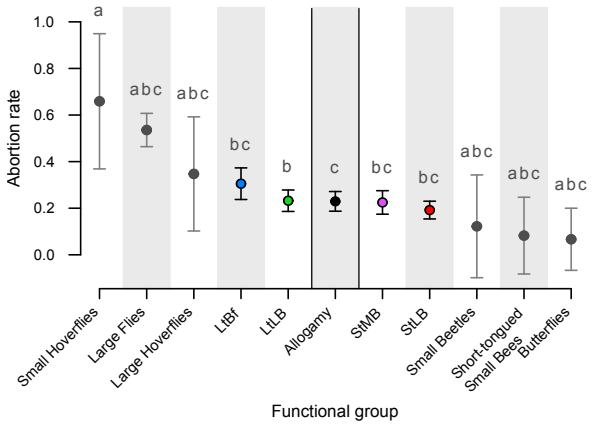

Figure 5.2: *Rates of abortion of fertilized ovules.*

Table 5.2: *Estimates of the quantity (QNC) and quality (QLC) components of the pollinator effectiveness (PE). Values are average with standard deviations. Values in parenthesis denote the contribution to the plant's overall reproductive output due to each component.*

Functional group	*QNC*	*QLC*	*PE*
Long-tongues beeflies	0.683 ± 0.034 (53%)	0.075 ± 0.009 (14%)	0.051 ± 0.007 (55%)
Short-tongued large bees	0.081 ± 0.008 (6%)	0.167 ± 0.014 (30%)	0.013 ± 0.002 (14%)
Long-tongued large bees	0.081 ± 0.007 (6%)	0.157 ± 0.015 (28%)	0.013 ± 0.002 (14%)
Short-tongued medium bees	0.129 ± 0.010 (10%)	0.081 ± 0.009 (15%)	0.010 ± 0.001 (11%)
Small beetles	0.165 ± 0.022 (13%)	0.023 ± 0.015 (4%)	0.004 ± 0.003 (4%)
Short-tongued small bees	0.108 ± 0.010 (8%)	0.013 ± 0.009 (2%)	0.001 ± 0.001 (1%)
Large hoverflies	0.003 ± 0.001 (0%)	0.028 ± 0.011 (5%)	<0.001 (0%)
Small hoverflies	0.0258 ± 0.003 (2%)	0.003 ± 0.002 (1%)	<0.001 (0%)
Butterflies	0.009 ± 0.002 (1%)	0.007 ± 0.004 (1%)	<0.001 (0%)
Large flies	0.014 ± 0.003 (1%)	0.002 ± 0.002 (0%)	<0.001 (0%)

Pollinator effectiveness

Our estimates of the pollinator effectiveness shows that long-tongued beeflies were the most effective FG with an average value of 5.1×10^{-2} (Figure 5.3). This superiority was mainly due to the quantity component, which was eight times bigger than the second in the PE rank. Following, short and long-tongued large bees showed the same PE values (1.3×10^{-2}) and similar values for both components, and short-tongued medium-sized bees (1.0×10^{-2}). The rest of FGs exhibited lower values of effectiveness, especially butterflies, small and large hoverflies and large flies ($PE < 0.001$, $QNC < 0.01$ and $QLC < 0.03$).

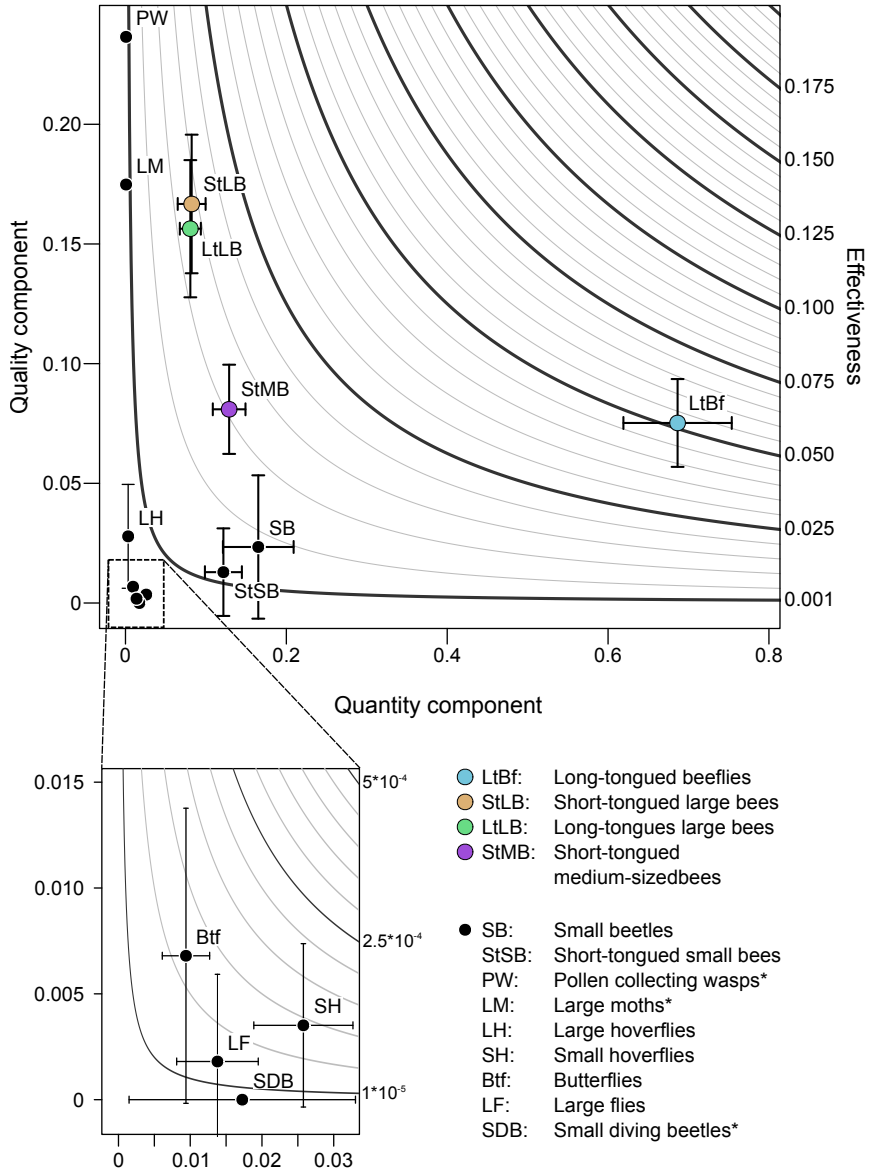

Figure 5.3: *Pollinator effectiveness landscape of the pollinator assemblage of* E. mediohispanicum. *The upper panel shows the positioning of all functional groups in the landscape. Isoclines depict PE values (QLC × QNC). The lower panel zooms into the functional groups presenting the lowest values of PE. Values are mean ± SD. Functional groups marked with '*' only show the average value.*

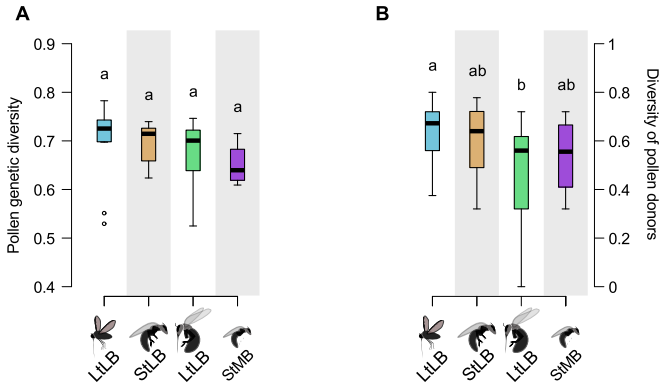

Figure 5.4: *Pollen diversity. Boxplots denote pollen's allelic diversity (A) and diversity of pollen donors (B) found in a fruit after a single visit to a virgin flower.*

Table 5.3: *Pollen diversity. We show: the number of fruits and seeds genotyped, the mean ± SD of allelic diversity, sires number and diversity within each fruit.*

Functional group	Fruits	Seeds	Allelic diversity	Sires	
				Number	Diversity
Long-tongues beeflies	10	50	0.696 ± 0.069	0.710 ± 0.229	0.621 ± 0.152
Short-tongued large bees	7	38	0.692 ± 0.046	0.600 ± 0.177	0.594 ± 0.166
Long-tongued large bees	19	100	0.677 ± 0.064	0.540 ± 0.174	0.475 ± 0.193
Short-tongued medium-sized bees	4	20	0.644 ± 0.057	0.550 ± 0.173	0.538 ± 0.171

Pollen diversity

We genotyped a total of 317 individuals (219 offspring, 20 known mothers and 78 potential fathers). The frequencies of alleles per locus deviated from what expected at Hardy-Weinberg equilibrium only for the E4, E8 and D2 loci, showing an excess of homozygotes. Null alleles were present in relatively high proportions for E3, E4, and E8 (0.241, 0.189 and 0.134 respectively). We used 208 offspring for the paternity analyses ($\leqslant$ 5 loci genotyped) from which 78% were correctly assigned to a candidate father while 22% kept unassigned and therefore assigned to a dummy father. The per-fruit averaged level of allelic diversity (heterozygosity) found in the paternal alleles of the sampled seeds, ranged from 0.696 for long-tongued beeflies to 0.644 for short-tongued medium-sized bees (Table 6.4), and showed no significant differences between pairs of groups (Figure 5.4a). However, when examining the diversity of pollen donors, long-tongued beeflies had the highest average diversity (0.621), significantly higher than the values found for long-tongued large bees (0.475; Figure 5.4b).

Discussion

In this study we characterize the pollinator effectiveness of most of the functional groups visiting the flowers of *E. mediohispanicum*. Our survey resulted in contrasting values of this effectiveness among all the functional groups that revealed that in spite of the high diversity of functional groups visiting the flowers of this species, a great amount of the final reproductive success was attributable to just four functional groups: long-tongued beeflies and large bees, and short-tongued large and medium-sized bees. These disparities were attributable to the quantity or quality subcomponents depending on the case, and were of great importance when assessing the final rank in PE. Additionally, we assessed the contribution to the genetic diversity of the offspring by the four most efficient functional groups. We found that the pollen loads transferred by long-tongued beeflies were composed of a higher diversity of pollen donors than those transferred by long-tongued large bees. Our findings emphasize the need to assess both components of the PE for obtain fair estimates of pollinator services, and remarks the necesity of improving PE estimates by including the contribution to the genetic diversity of the offspring.

Our analyses show a wide variation in PE in the pollinator assemblage of *E. mediohispanicum* (Figure 5.4). This disparity resulted from different contributions of the quantity and quality components and their respective subcomponents. Regarding the most effective functional group, long-tongued beeflies stood out from the rest of pollinators, mainly due to the quantity component. These insects were the most frequent visitors, visiting individual plants almost twice times more than the second functional group in the quantity rank. Whereas they were the fourth functional group in the rank defined by the qualitative component. Species of this functional group are present in most of the populations of *E. mediohispanicum* (Gómez *et al.*, 2014a), standing as important pollinators in terms of visitation frequency and having important effects on plant populations. For example, their presence affect plant reproductive success (Gómez *et al.*, 2009b) and influences the patterns of individual plant-pollinator networks (Gómez *et al.*, 2011; Valverde *et al.*, 2016b). Specifically, because of their indiscriminate movement among plants within a population, they act as network hubs, increasing connectivity and decreasing functional specialization among individual plants (Gómez *et al.*, 2011). With our results, these previously reported findings gain strength by confirming the importance of this functional group as pollinators of *E. mediohispanicum*. Following the rank of PE, short and long-tongued large bees had similar values of PE resulting from nearly the same values in both

components. However, long-tongued large bees were the fastest functional group in handling flowers. This may be because they forage mainly for nectar while short-tongued bees spend more time foraging also for pollen, but also because the former have faster between-flowers movements than the latter (personal observation). Contrasting to long-tongued beeflies, most of the contribution to their PE was due to their quality as pollinators (Table 6.4). These differences surely reflects the local conditions of the population studied. In our study species the composition and visitation frequency of its flower visitors are known to change widely among populations (Gómez *et al.*, 2008b, 2009b, 2010, 2014a), a characteristic that may be due to the local pollinator composition but also due to extrinsic influences of neighboring plants (Mitchell *et al.*, 2009; Ogilvie and Thomson, 2016). This indicates that PE may be sensitive to the spatial variation in the pollinator assemblage due to simultaneous changes in the weighting effects of the quantity component. Moreover, the disparities between quantity and quality components in the PE ranking and the resulting values of PE, adds to the many examples demonstrating that QNC and QLC can be decoupled (Schemske and Horvitz, 1984; Mayfield, 2001), and emphasizes the need of characterizing both components when assessing the pollinator effectiveness (Sahli and Conner, 2007; Rodríguez-Rodríguez *et al.*, 2013; King *et al.*, 2013). The rest of functional groups had considerably lower values of PE, mainly due to the QLC, indicating that although an ecological generalist, *E. mediohispanicum* relies on a few insect taxa for reproduction.

This shrinkage of the degree of ecological generalization to a narrower functional generalization seems to be common in plant species (Herrera, 1989; Gómez and Zamora, 1999; Watts *et al.*, 2012), and has been previously reported in other Brassicaceae species (Sahli and Conner, 2007; Robertson and Leavitt, 2011). Flowers of generalist plants are expected to have an averaged tuned morphology resulting from the conflicting selections exerted by its different flower visitors (Aigner, 2001; Strauss *et al.*, 2005). We think that flowers of *E. mediohispanicum* have a compromised architecture to the marginal fitness associated to each functional group phenotype selection, and that this architecture may explain, at least in part, the divergences found in PE. For example, the deep and narrow corolla tube may favor a legitimate access to nectar to those species presenting a long-tongued proboscis (Harder, 1985). This seems to happen here with long-tongued large bees (Figure 5.5a) and beeflies (Figure 5.5b), both found to be very effective. As a consequence, this architecture may restrict other visitors proper access to nectar but also to most of the pollen. Our observations revealed that this is happening for some functional groups such as flies, hoverflies (Figure 5.5c) or even some short-tongued small bees (Figure 5.5d), all of them groups with low PE. This restriction, however, is selective. Sepals in this species permit the aperture

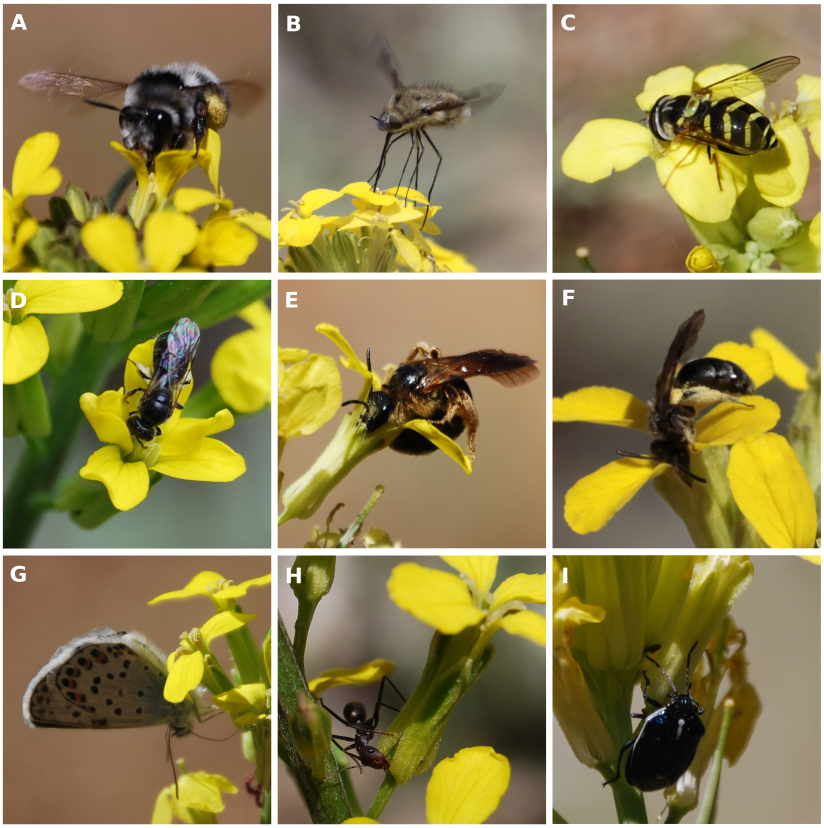

Figure 5.5: *Examples of some species visiting the flowers of* E. mediohispanicum. *Legitimate visits: A)* Anthophora leucophaea *(long-tongued large bees) feeding on nectar; B)* Bombylius major *(long-tongued beeflies); C)* Dasysyrphus sp. *(small hoverflies) feeding on pollen; D)* Lasioglossum sp. *(shot-tongued small bees) collecting pollen; E)* Lasioglossum xanthopus *(short-tongued large bees) feeding on nectar; F)* Andrena sp. *(short-tongued medium bees) feeding on nectar. Non-legitimate visits robbing nectar: G)* Polyommatus amandus *(butterflies); H)* Cataglyphis sp. *(ants); I)* Eurydema oleracea *(bugs). Photos A and C to I by Javier Valverde, except B by Francisco Perfectti.*

of the corolla to bigger sized bee species, facilitating the access to floral resources even to short-tongued species (Figure 5.5e and f). On the other hand, this latter trait allows non-legitimate access to nectar through the space between sepals by butterflies (Figure 5.5g) and other functional groups, such as ants (Figure 5.5h), bugs (Figure 5.5i) or eventually short-tongued small bees (Figure 5.5j). This counterpoint may represent a cost for plant fitness because these foraging behaviors consume floral rewards with little or no benefits in return (Bronstein, 2001), secondarily influencing the behavior of legitimate pollinators (Heinrich and Raven, 1972; Roubik, 1982; Maloof and Inouye, 2000).

These findings support the idea that the evolution of generalization entails some costs to the system (Palaima, 2007), and adds to the literature showing that opportunistic behaviors and step differences in the per-visit pollinator effectiveness are common in generalist plant species (Ollerton *et al.*, 2007; Sahli and Conner, 2007).

We also analyzed the contribution of the four most effective functional groups to the genetic diversity of the offspring. We found a variation among these functional groups in the per-fruit diversity of sires after a single visit to a virgin flower, indicating differential abilities to transfer pollen from multiple pollen donors. Specifically, long-tongued beeflies transferred pollen from significantly more pollen donors than long-tongued large bees. These disparate contributions to the diversity of stigmatic pollen loads may entail important consequences for plant reproductive success. Diverse pollen loads increase the chances of fertilization by more genetically different pollen (Marshall *et al.*, 2000; Bernasconi, 2003; Kron and Husband, 2006), and promote postpollination sexual selection on pollen from different pollen donors (Skogsmyr and Lankinen, 2000; Swanson *et al.*, 2016) or even within pollen from the same donor (Lankinen *et al.*, 2009). The differences found among functional groups could be attributable to some prepollination factors affecting the diversity of sires in natural populations, such as plan isolation (Krauss, 2000), or microenviromental conditions (Herrera, 1995a). However, because all plants in the Hoya de Pedraza study site were equally distant and under homogeneous environmental conditions, these differences surely reflects intrinsic pollen carryover abilities of the functional groups (Marshall and Ellstrand, 1985; Waser, 1988; Galen and Rotenberry, 1988). Steep carryover curves negatively affect to pollen genetic diversity (Campbell, 1998). This pattern is related to some pollinator behavioral features such as grooming (Wilson and Thomson, 1991; Castellanos *et al.*, 2003), or to morphological features such as body hairs (). Whatever the reasons leading to the variation among flower visitors in their contribution to the genetic diversity of the offspring, its mere existence demonstrates the need to be incorporated to the estimate of PE.

This study may lack of considering some pieces of the puzzle. First, we did not assessed the male component of the PE. Some studies have estimated this component as the amount of withdrawn pollen from the anthers after a visit (Rodríguez-Rodríguez *et al.*, 2013) . However pollen collecting and pollen deposition on stigmas are not always correlated (Adler and Irwin, 2006), suggesting that this approach simplifies the male component as it neglects the different fates that pollen removed from anther may take (Inouye, 1994). Genetic tools are needed to track the diversity and number of female sired by male-phase flowers in order to improve the estimation of male fitness (Brunet, 2009). Second, we used fruit and seed set to characterize the QLC.

This approach has the inconvenience that can confound prezygotic resource allocation processes, such as those affecting fruit or seed production depending on the flower position within an inflorescence (Ashman and Hitchens, 2000; Wesselingh, 2006) or those favoring earlier fertilized flowers and flowers with higher pollen loads (Delph *et al.*, 1998), or more diverse offspring (Collin *et al.*, 2009). However, we located the hand-pollination control flowers along the flowering stalks to embrace possible changes in resource allocation due to flower position, finding little to no differences between the proportion of fertilized ovules and the seed set. Therefore, resources constrains in seed filling can be disregarded. Third, we are aware that our estimates of the genetic diversity were based on a small sample size (number of fruits), and therefore we have to be cautious in interpreting our results. Regards of this, our findings points to differences in the ability of transferring pollen from different donors and we think that increasing the sample size will surely reveal even bigger differences among groups.

The information provided by this study will surely contribute qualitatively in further studies on this plant species. For example, it will help in shed some light on the evolutionary dynamics leading to the geographical mosaic of selection found in this species (Gómez *et al.*, 2009b). Using the PE and the previous information of flower trait selections by the different functional groups (Gómez *et al.*, 2008b, 2009b) we could also clarify some questions related to the evolution of flowers shape towards an ecological generalist system (Aigner, 2005; Ollerton *et al.*, 2007). Or, by weighting the plant-insect interactions with the QLC found here, we could give a more reliable functional sense to plant-plant networks previously analyzed in this plant species (Gómez *et al.*, 2011; Gómez and Perfectti, 2012; Valverde *et al.*, 2016b) and give us pertinent information to analyze the mating probabilities among plants due to pollinator sharing.

Aknowledgements

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6

Mating networks in a generalist plant species

english

Abstract

Contemporary pollen flow in animal-pollinated plants results from the foraging movements of pollinators. These movements can be influenced by pollinator's preferences on different plant phenotypes and microenvironments. As a consequence, non-random matings among plants may arise and thus affect to plant population spatial genetic structure. The determination of these patterns and their relationship is therefore necessary to understand the importance of plant-pollinator interactions on the evolution of plant populations. In a population of the generalist plant *Erysimum mediohispanicum* (Brassicaceae) we characterized the phenotype and microenvironment of the plants flowering in each of two consecutive years. For each year we analyzed the mating patterns occurring among these plants, as well as the patterns of pollinator visitation and foraging movements. We found that matings mainly occurred among nearby plants presenting synchronous flowering times. Additionally, plants growing in microenvironments with similar light availability mate more frequently. As a consequence of these patterns, the study of the resulting mating network revealed that plants tended to mate with the most promiscuous ones and that plants also tended to mate in clusters. The structure of the mating network fitted the predictions of the foraging movements of pollinators. We conclude that the foraging movements of pollinators constrained by spatial distance and differing preferences on light availability are the responsible of the mating patterns occurring within the population. Our study is the first linking individual-based plant networks with contemporary pollen flow and demonstrates that in a generalist plant species the interactions with pollinators, and thus pollen flow, can be structured.

Introduction

In animal-pollinated plants, the interaction between the foraging movement of pollinators and the plant's mating system are the responsible of the patterns of effective pollen flow (i.e. the amount of pollen flow that results in reproductive output). A common pattern of effective pollen flow is that occurring more likely among nearby conspecifics (Fortuna *et al.*, 2008; Buehler *et al.*, 2012). This results as a consequence of the optimal foraging strategies of pollinators, in which movements at short distances are the rule of thumb (Waser, 1982; Ohashi and Thomson, 2009; Lysenkov, 2014). Additionally, plant populations are often composed of individuals flowering at different times (Elzinga *et al.*, 2007). Flowering schedules together with the pollinator foraging phenologies modulate plant-pollinator interactions (Valverde *et al.*, 2016b), and regulates the temporal windows available to mate (Hendry and Day, 2005; Gérard *et al.*, 2006; Ison *et al.*, 2014; Ison and Wagenius, 2014) constraining the pollen flow to synchronous plants (Fox, 2003; Elzinga *et al.*, 2007; Weis *et al.*, 2014). Since flowering synchrony can erode or strengthen the spatial constrains to pollen flow (Ison and Wagenius, 2014), the integrated analysis of these two factors is necessary to understand the genetic structure of plant populations and, consequently, their co-evolutionary dynamics.

Beyond the latter spatio-temporal constrains, the disparate preferences of pollinators on some abiotic and biotic factors (Herrera, 1995b; Thompson, 2001) can also contribute to pollen dispersal (McRae, 2006; Dyer *et al.*, 2012; Dileo *et al.*, 2013). Among these factors, the spatial distribution of plants (Dyer and Sork, 2001; Meagher and Vassiliadis, 2003), certain landscape features such as habitat heterogeneity (Lander *et al.*, 2013) or light availability (Kamm *et al.*, 2010; Dyer *et al.*, 2012), or the phenotype of plants (Jones and Reithel, 2001; Kobayashi *et al.*, 2010; Van Der Niet *et al.*, 2014) are known to mediate the frequency of mating between plants (García *et al.*, 2005). As a result, the mating network defined by the pattern of effective pollen flow may exhibit some structural properties. For example Fortuna *et al.* (2008) found that within a population of the tree *Prunus mahaleb*, matings where structured in groups of plants interbreeding more frequently than expected at random. Their study completed the previous study by García *et al.* (2005) that showed how tree density and ecological neighborhood affected effective pollen flow. These results demonstrate that the analysis of the mating network can unveil hidden information about the structure of gene exchange among individual plants.

The study of plant-pollinator interaction networks at the individual level may discern structural patterns hidden within their complexity (Gómez *et al.*, 2011; Pires *et al.*, 2011; Gómez and Perfectti, 2012; Tur *et al.*, 2014; Dupont

et al., 2014; Valverde *et al.*, 2016b). Some studies have projected these interactions into an plant-plant network based on pollinator sharing (Dupont *et al.*, 2011; Gómez *et al.*, 2011; Gómez and Perfectti, 2012; Valverde *et al.*, 2016b). In these networks plants sharing some of their floral visitors are connected, and the strength of the connection is relative to the similarity in their pollinator assemblage. It has been argued that these networks could be a proxy, albeit imperfect, of the mating network, since plants that share most pollinators could mate more likely. Studies performed on the generalist plant *Erysimum mediohispanicum* demonstrated that these networks exhibited a well-defined architecture (Gómez *et al.*, 2011; Gómez and Perfectti, 2012; Valverde *et al.*, 2016b). These findings suggest that plant-pollinator interactions may be structured in generalist plants, and that consequently, assortative mating may even occur within generalist plant populations (Gómez *et al.*, 2011). However, it is necessary to know the directionality of the pollinator flights, i.e., the polarity of the edges in the network, to predict effective pollen flow (Fang and Huang, 2016). In this sense, Dupont *et al.* (2014) went further and constructed plant-plant networks based on the movements of individually marked insects foraging on the thistle *Cirsium palustre*. They found that the small-scale movements of insects promoted a compartmentalized plant-plant network. In addition, pollination effectiveness usually varies widely among pollinators in generalist plant species (Sahli and Conner, 2007). This supposes that the contribution to the effective pollen flow may also vary (Herrera, 1987; Proctor *et al.*, 1996), and therefore this information should be taken into account when constructing plant-plant networks. Finally, although these studies aimed to use plant-pollinator interactions as a proxy for effective pollen flow, there is no evidence so far of their reliability and no study has linked these networks to actual mating networks.

In the present study we aim to test if the mating events within a population of a generalist plant occur assortatively and thus result in a structured mating network. We also aim to study to what extent this is due to non-random interactions among individual plants with their pollinators. To do so, we monitored a population of the generalist plant *E. mediohispanicum* (Brassicaceae) during two flowering season. At each season we characterized the phenotype and microenvironmental conditions of each flowering plant. Additionally, we surveyed the individual plant-pollinator interactions and genotyped the flowering plants as well as part of their offspring. Using this information, we aimed to answer the following questions: i) Are the mating events within the population constrained by spatial distance and/or flowering time? Are phenotype and/or abiotic factors also influencing to these matings? ii) Is the mating network structured? iii) Are networks based on plant-pollinator interactions a good proxy of the mating network?

Materials and methods

Data collection and preparation

Sampling design

During 2010 and 2011 we demarcated a natural population of *E. mediohispanicum* with a plot of 20 x 20 m at the Sierra Nevada Natural park (37°8′07″ N, 3°21′71″ W; 1723 m a s l). Each year we selected 100 plants from the population and removed the flowering stalks of the remaining individuals inside the plot and in a buffer perimeter of 10 m width. We measured the spatial distance and estimated the flowering asynchronies among the whole set of plants. Spatial distances were calculated using the euclidean distances among plants based on a plot template established at the centimeter level. To calculate the pairwise flowering asynchrony, we periodically recorded the number of open flowers per plant (16 times in 2010 and 28 times in 2011) and used the Jaccard-type Chao dissimilarity index (Chao *et al.*, 2005) as described in (Valverde *et al.*, 2016b).

Phenotype characterization

Apart from the flowering time, we also characterized other phenotype traits potentially influencing pollinator visitation rate. Among these, we measured plant stalk height (cm), flower tube length (mm), and flower diameter (mm). We also extracted four orthogonal variables quantifying different aspects of corolla shape (relative warps, RWs). These correspond to the principal components of the covariance matrix resulting from a Generalized Procrustes Analysis performed on 32 landmarks located on planar front-view pictures of flowers in anthesis (Walker, 2000; Gómez and Perfectti, 2010). We obtained the RWs using the softwares TPSDIG v. 1.4 and TPSRELW v. 1.11 (`http://life.bio.sunysb.edu/morph/morphmet.html`). Finally, we counted the number of pollen grains produced per a non-visited flower in each plant using a Neubauer chamber.

Micro-environment characterization

We quantified light availability at each flowering plant growing site using the Direct Site Factor (DSF), the proportion of direct radiation relative to that outside the canopy (Anderson, 1964). For this, at each plant location we took a hemispherical photograph with a fish-eye lens (FCE8, Nikon, Tokyo,

Japan) using a digital camera (Coolpix 995, Nikon). Photographs were taken at dawn or dusk at 20 cm from the ground, leveled to the zenith and directed towards north. Images were analyzed using the software Hemiview v. 2.1 (1999, Delta-T Devices Ltd, Cambridge, UK).

In addition, we characterized the degree of spatial isolation of each plant. For this, we calculated the area of the polygons resulting from a Voronoi tessellation (Krebs, 1989; Bender *et al.*, 2003). We corrected for border effects using a toroidal shift, a method that maintains most of the spatial structure of the data (Fortin and Dale, 2005).

Pollinator surveys

We determined the assemblage of floral visitors contacting legitimately the plant reproductive organs by means of 5-minutes surveys on each plant. Pollinator observations were conducted from approximately 11:00 to 18:00, the active period for pollinators of *E. mediohispanicum*. In order to capture the daily and seasonal turnover in the pollinator assemblage, we performed 1-4 surveys per day along the whole flowering period. All insects were identified at the lower taxonomic level (from family to species, depending on the insect). These were grouped in 23 functional groups based on their morphology, floral fit and foraging behavior, and which are thought to exert similar selective pressures on floral phenotype (Fenster *et al.*, 2009)(see Appendix 'functional groups' for a detailed description of these).

Additionally, we recorded the trajectories of pollinators among plants in the population. To do so, we followed insects foraging on the marked plants and recorded the sequence of plants consecutively visited until the insect was lost or leaved the plot.

Genotyping and paternity analyses

After fructification, we randomly collected 30 seeds per plant for genotyping. Seeds were sown in individual nurseries in a greenhouse (at the University of Granada) or were sown in petri dishes under a Murashige and Skoog medium after seed sterilization and stored in growing chambers (at the Leibniz Institute of plant genetics and crop plant research, IPK).

We isolated DNA from adult plants using dry leafs and from the offspring using fresh leaves. We used at least 60 mg of leaf tissue on the Agencourt® CholoropureTM kit (Beckman Coulter), a procedure that uses magnetic beads to isolate nucleic acids. For all individuals we amplified 10 microsatellite markers specifically developed for this plant species (Muñoz-Pajares *et al.*, 2011) using a Gradient Master Cycler Pro S termocycler (Eppendorf, Hamburg,

Germany). The amplified fragments were analyzed on a ABI 3730XLs by MACROGEN (South Korea). Fragment scoring was assessed manually using the Peak Scanner v. 2.0 software (Applied Biosystems) and a 400HD ROX size standard. In order to accelerate the DNA isolation and genotyping process, we programmed a series of automatized protocols in the Biomek 3000 pipetting robot (Beckman Coulter) that helped in the DNA extraction process and in preparing DNA aliquots for microsatellite amplification.

Paternity analyses were performed using the categorical allocation method implemented in the software CERVUS v. 3.0 (Marshall *et al.*, 1998). We ran CERVUS using an underlying allele frequencies framework calculated from the adult plant genotypes. For each assignation we obtained critical likelihood values using 95% and 80% confidence in assignments after 10,000 simulations with the following parameters: minimum number of matching loci = 6; error rate = 0.01; proportion of sampled candidates = 0.9; proportion of loci typed = 0.7. In addition, we ran a tailored script in R to locate mother-offspring and father-offspring mismatches and classify them as mismatches due to null alleles, allelic dropouts or real mismatches (see Appendix 'Handling of genotyping errors' for a detailed description of the procedure). This helped us to discriminate true fathers among alleged candidates.

Plant-plant networks

From the paternity assignments and the pollinator surveys, we developed for each year of study four plant-plant networks, a mating network and three pollinator-based networks (see figure 6.S1a):

- Mating network (MNet): This is a weighted directed network that depicts the mating events occurring within our study population. Edges goes from each siring father to the sired mother plant, and the strength represents the number of mating events (seeds).
- Similarity network (SNet): This is an undirected one-mode weighted network representing the pollinator sharing among plants. Edges depict the similarities in pollinator composition between two plants calculated using the 1 - Morisita-Horn index (Valverde *et al.*, 2016b).
- Weighted similarity network (WSNet): This is an undirected one-mode weighted network. Its construction is identical to the former, but previoulsy, all plant-pollinator interactions are weighted by the average quality component of the pollinator effectiveness (the probability of producing a new plant individual after visiting a flower, **?**, ; Valverde et al. (in prep.)).

- Foraging Network (FNet): This is a directed weighted network representing the pollinators movement patterns among visited plants. The edges depict the trajectories of flights. We distinguished between the FNet constructed pooling all functional groups movements, and the FNets' obtained from each functional group.

I) Factors influencing the effective pollination

Spatial isolation and timing of flowering

We obtained the distribution of spatial distances and flowering asynchronies associated to the realized matings. These distributions were then compared with random mating models using a Kolmogorov-Smirnov test. These models were defined as the distribution of pairwise distances and flowering asynchronies among all the plants in the population. Additionally, we performed a more detailed analysis of the mating patterns occurring at spatial intervals of 1 meter and at asynchrony intervals of 0.1. For each interval, we calculated the mean and standard error of the per-mother proportion of sires falling within that interval. Similarly, we obtained the distribution of expected random matings by calculating the per mother mean $\pm$ 95% confidence interval of all the potential pollen donors falling within each interval. The observed and expected distributions were represented against spatial distance and flowering asynchrony, and the statistical significance of their comparisons calculated (for a synthesized scheme of these analyses, see Figure 6.S1b).

We also studied how the interaction between spatial isolation and flowering time affected the probability of mating among plants. Following Ison and Wagenius (2014), we modeled these effects using generalized linear models at two levels. First, we modeled the probability of mating using a binomial response variable (mating or not). Following, we modeled the proportion of sired seeds using the proportion of seeds of each plant sired by each alleged father. Model selection was based on the Akaike Information Criterion. Finally, we explored how different scenarios of flowering asynchrony (absence, average and full asynchrony) modulate the relationship between mating and distance.

Phenotype and abiotic factors

We studied whether phenotypic and environmental variables were related to the effective pollen flow while taking into account the spatial constraints to the pollen movement. For this analysis, we transformed the mating network (i.e., adjacency matrix) into a symmetrical matrix containing the shared offspring which was calculated as:

$$\frac{m_{ij} + m_{ji}}{n_i + n_j}$$

where $m_{ij} + m_{ji}$ denote the number of shared offspring between the plants i and j, and $n_i + n_j$ the total number of genotyped offspring from plants i and j. The shared offspring ranges from 0 to 1, where 1 denotes exclusive mating among the pair of individuals.

We used partial Mantel tests using the standardized phenotypic similarity (1 - standardized euclidean distance) and the standardized spatial proximity as covariate matrix. Following, we compared the observed correlations with those expected under three null models: a model of complete random mating, a model of complete assortative mating, and a spatial model. The random mating model was constructed by randomly assigning a sire parent to each offspring. The assortative model was constructed by assigning a sire parent with a probability based on the phenotypic similarity. The spatial model was constructed by assigning a sire parent with a probability equal to 1/(spatial distance). The three models maintained the total number of genotyped offspring per plant. Each model was run 500 times. Additionally, we calculated the spatial autocorrelation of each of the variables by means of the Moran's I autocorrelation coefficient using the package 'ape' (Paradis *et al.*, 2004).

II) Structure of the mating network

Degree distribution

We calculated the cumulative distribution of node degrees for the male (out-degree) and the female (in-degree) reproductive functions, and explored whether the data fitted to an exponential, power-law or truncated power-law using the 'bipartite' package (Dormann and Gruber, 2008).

Modularity

We identified the occurrence and identity of groups of individuals (modules) interbreeding with more frequency than expected at random. Due to the hermaphrodite nature of this species, this network can be treated as a bipartite network, where male and female sexual functions represent each set of the network. We therefore applied the QuanBiMo algorithm, developed for weighted bipartite networks (Dormann and Strauss, 2014). This algorithm uses a simulated annealing-Monte Carlo approach to find the modules maximizing a function called modularity (Newman and Girvan, 2004; Barber, 2007). Simulated annealing can fall into suboptimal modular configurations, yielding

different classifications of nodes into modules among runs (Guimerà and Amaral, 2005a). We therefore performed 500 runs and tested the robustness of the QuanBiMo algorith by measuring the concordances among runs in module assignation using the mutual information index developed by Danon *et al.* (2005). From the 500 runs, we selected the configuration bearing five modules with the highest modularity (figure 6.S2a).

III) Pollinators as drivers of the effective pollen flow

We explored to what extent realized matings were related to the plant-pollinator interactions. To do so we explored how the three pollinator-based networks (SNet, WSNet and FNet) explained the mating network and its architechture.

Relationship with the overall effective pollen flow

We performed Mantel tests using the Spearman's rank correlation between the shared offspring matrix (resulting from the Mnet, see section I) and the three pollinator-based networks. Because FNet is not symmetrical, we transformed FNet as we did to calculate the shared offspring.

Relationships with the mating network structure

We explored whether the node degree of the MNet correlated with those from the pollinator-based networks by means of linear models. Due to the differing number of offspring genotyped per plant, we used the out-degree of the mating network as response variable. Because SNet and WSNet are highly connected networks, with no single plant unlinked, we used the generalized degree centrality measure developed by Opsahl *et al.* (2010). This degree measure allows to tune the relative importance of link weights:

$$c_D^{w\alpha} = k_i \times \left(\frac{S_i}{k_i}\right)^{\alpha}$$

here k_i is the unweighted degree (i.e. the number of adjacencies of the node i) and S_i is the node strength (i.e. the sum of its weights). α is a tuning parameter that determines the relative importance of the $\frac{Si}{k_i}$ relation. For the comparison with the foraging network we used the out-degree, were k_i and S_i only consider the edges originating from that node (Opsahl *et al.*, 2010). We explored the result under four different values of α: 0 ($= k_i$), 0.5, 1 ($= S_i$) and 1.5.

Following we explored to what extent plant-pollinator interactions matched the modular configuration defined by the mating network. To do so, for each pollinator-based network (SNet, WSNet and FNet) we calculated its modularity when nodes were assigned to the modules obtained from the mating network. For SNet and WSNet, we calculated the 1-mode modularity described in. This modularity informed us to what extent plant-plant similarities in pollinator assemblage were structured in well defined clusters corresponding to the mating modules. For FNet, we calculated the 2-mode (bipartite) modularity described before, and it informed us if pollinator movements were resulting in a pollen flow mostly occurring among plants pertaining to the same mating module.

Because stochastic (Guimerà *et al.*, 2004) and spatially limited processes (Dupont *et al.*, 2014) may produce network structures, we generated some null models for each type of pollinator-based networks to which compare the observed modularity values.

- Random model for the similarity networks (SNet and WSNet) where interactions occurred at random. In this model the interactions where randomized among plants flowering the days of the recorded pollinator activity while maintaining the total abundance of each pollinator.
- Models for the foraging networks (FNet). These consisted in simulating each of the observed insects movements maintaining the number of flights per insect individual and using the first plant of each flight as a starting point.
    - Random model where the pollinators moved randomly among the plants in the population.
    - Spatial model where movements were chosen following a prior distance probability. This probability was obtained from the empirical distribution of flight distances among visited plants

The observed values were compared with 999 values resulting from the networks constructed under the previous null models.

Results

We successfuly genotyped 2517 and 2450 offspring in 2010 and 2011 respectively, with most of the individuals being genotyped for at least 6 loci (98% in 2010 and 94% in 2011). The assignment success was of 91% in 2010 (2088 individuals) and 97% in 2011 (2370 individuals). From these, 16% in 2010 and 19% in 2011 were assigned to immigrant pollen.

An interactive visualization of the mating networks obtained for each year can be found here:

- network for the 2010 mating events:

  `http://www.ugr.es/~evoflor/strugen/mating_network_2010.html`

- network for the 2011 mating events:

  `http://www.ugr.es/~evoflor/strugen/mating_network_2011.html`

I) Factors influencing the effective pollination

Spatial isolation and timing of flowering

The realized matings were produced at significantly shorter distances than those expected by random ($D = 0.435$ and 0.555 for 2010 and 2011 respectively, $p < 0.05$). Matings occurred at significantly higher rates below two meters in 2010 and below three meters in 2011 (Figure 6.1a). Similarly, the values of flowering asynchrony associated to the realized matings differed from the pairwise flowering asynchronies among plants in the population ($D = 0.136$ and 0.164 for 2010 and 2011 respectively, $p < 0.05$). However, within the studied intervals of pairwise asynchronies, mating events occurred at the rates expected by the pairwise distribution of flowering asynchronies among plants in the population (Figure 6.1b).

Models including the interaction between spatial distance and flowering asynchrony explained better both the mating probability and the proportion of sired seeds (table S1). In both models, coefficients of the spatial distance and flowering asynchrony showed significant negative values (Table 6.1). Simulations of different scenarios of asynchrony showed that values close to complete flowering asynchrony (0.9) were necessary to erode the effect of spatial distance on the response variables (figure 2).

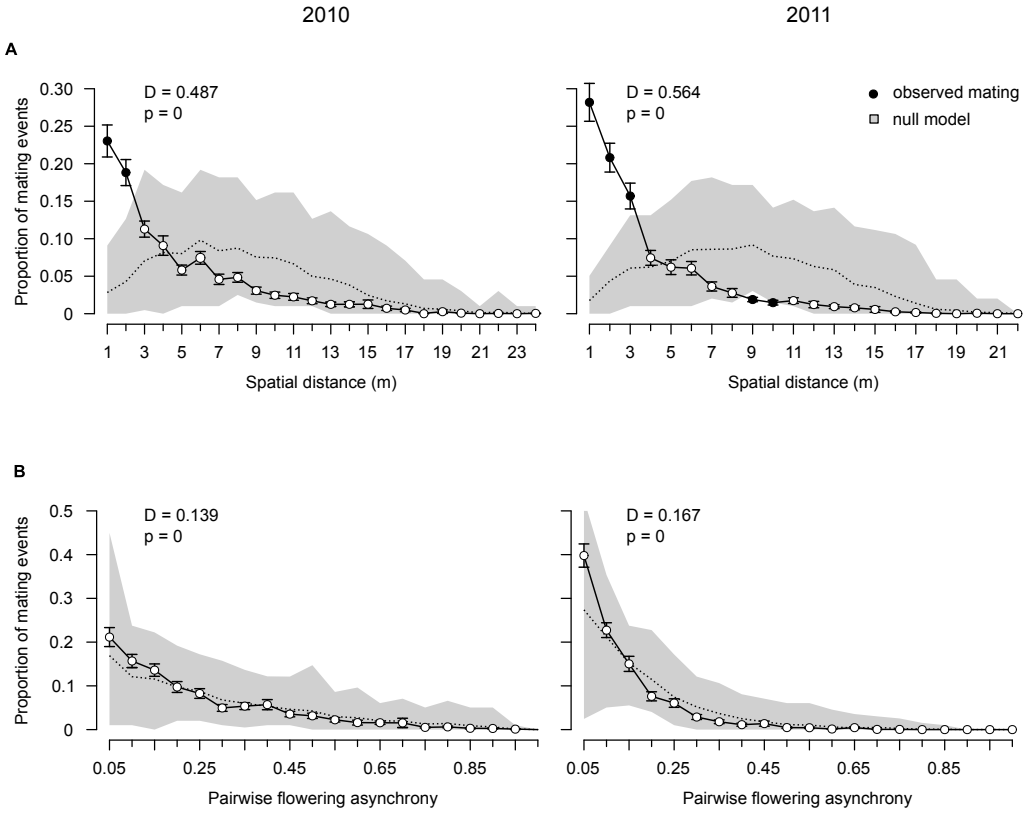


Figure 6.1: *Effects of spatial distance and flowering asynchrony on the effective pollen flow. The proportion of mating events are plotted in relation to the spatial distance (A) and the flowering asynchrony (B). Dots denote the average and standard error of the observed values, filled area represents the 95% CI of the expected values under a random mating*

Table 6.1: *Spatio-temporal effects on matings. The table shows coefficient estimates, z-statistic and significance for the selected models explaining the mating events in terms of mating probability and proportion of sired seeds.*

Model	Coefficient	2010			2011		
		estimate	z	p value	estimate	z	p value
Mating probability	intercept	0.039 ± 0.095	0.409	0.682	0.618 ± 0.098	6.311	<0.0001
	Asynchrony	-2.024 ± 0.339	-5.966	<0.0001	-3.457 ± 0.606	-5.703	<0.0001
	Distance	-0.296 ± 0.017	-17.897	<0.0001	-0.400 ± 0.018	-22.178	<0.0001
	interaction	0.187 ± 0.054	3.46	0.0005	0.278 ± 0.104	2.66	0.00781
Proportion sired	intercept	-2.269 ± 0.058	-39.239	<0.0001	-1.791 ± 0.054	-33.178	<0.0001
	Asynchrony	-2.065 ± 0.227	-9.092	<0.0001	-3.148 ± 0.387	-8.143	<0.0001
	Distance	-0.438 ± 0.014	-30.665	<0.0001	-0.519 ± 0.015	-35.057	<0.0001
	interaction	0.298 ± 0.470	6.382	<0.0001	0.285 ± 0.090	3.177	0.002

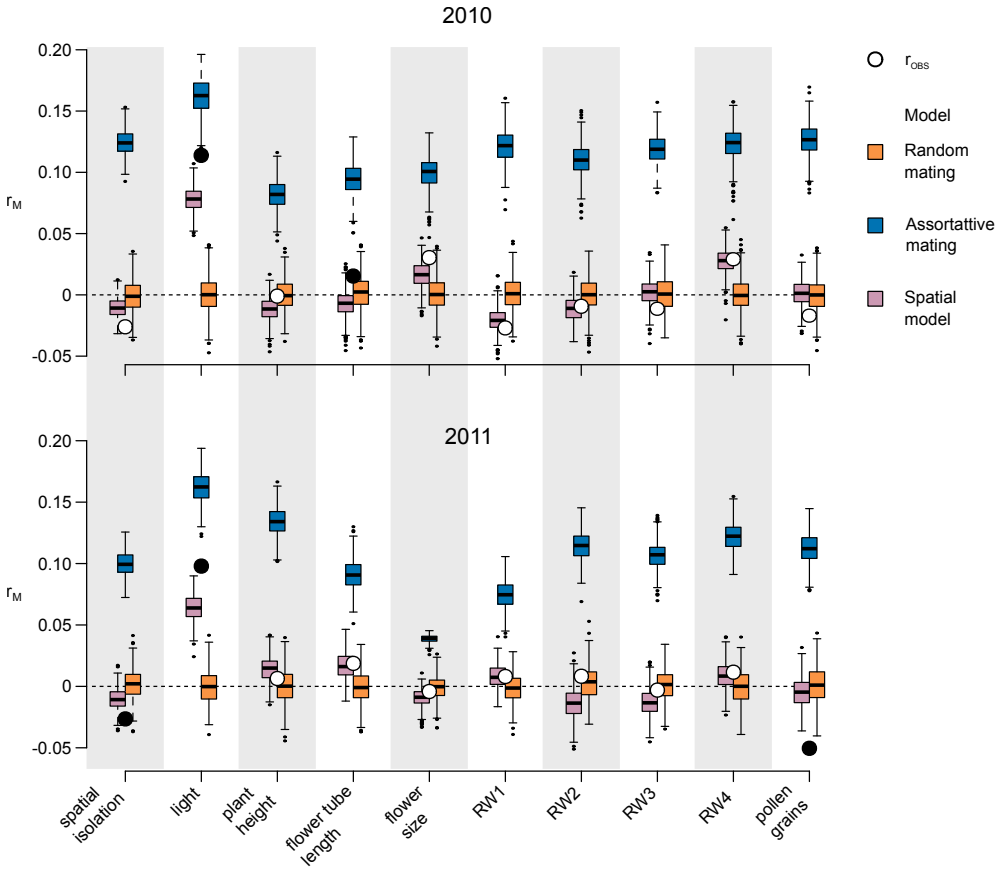


Figure 6.2: *Phenotypic and abiotic effects on the effective pollen flow. Boxes represent the distribution of null Mantel correlations obtained from the random and assortative mating models. Significant deviations of the observed values from the spatial model are depicted with filled symbols.*

Phenotype and abiotic factors

All the studied variables presented negative Moran's I coefficients ($-0.005-0.128$; table S2). However, only light conditions ($Moran's I = -0.128$ and -0.120 in 2010 and 2011 respectively) and spatial isolation ($Moran's I = -0.079$ and -0.023 in 2010 and 2011 respectively) presented a significant autocorrelation in both years. Additionally, flower diameter ($Moran's I = -0.030$) and $RW4$ ($Moran's I = -0.031$) presented significant autocorrelations in 2010.

Beyond the spatial constrains to pollen flow, the mating patterns related to similarities in some phenotypic and environmental variables (Figure 6.2). Matings frequencies deviated in both years from what was expected under the spatial constrained model, implying assortative mating based on plant similarities in light availability ($r_{OBS} = 0.113$ and 0.098). We also found a

disassortative mating driven by dissimilarities in spatial isolation in both years ($r_{OBS} = -0.027$, $p > 0.05$ and -0.029, $p = 0.052$). Additionally, pollen flow seemed to happen more frequently among plants with similar flower tube length in 2010 ($r_{OBS} = 0.014$), whereas in 2011 it occurred more often among plants with disparate number of pollen grains per flower ($r_{OBS} = -0.051$).

II) Structure of the mating network

Degree distribution

The cumulative distributions of the in and out-degrees (female and male function respectively) in both years followed a truncated power law distribution that explained above the 96% of the variation in the data (table 6.2, Figure 6.3). The correlation among in and out-degrees was positive in both years. This was significantly different from what expected under random mating in 2010 ($observed = 0.13$; $expected = 0 \pm 0.023$) whereas it was not significant in 2011 ($observed = 0.039$; $expected = 0 \pm 0.023$).

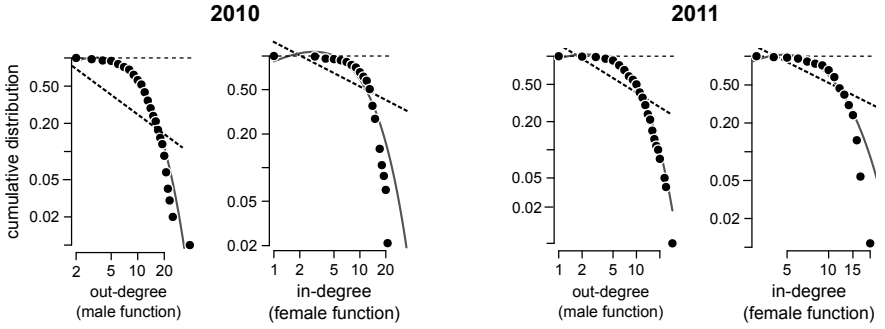


Figure 6.3: *In and out-degree distributions of the mating network. Dashed line represents the fit to an exponential function, while the solid to a truncated power-law*

Table 6.2: *Function fits to cumulative in and out-degree distributions.*

	2010				2011			
out-degree (male)	estimate	SE	R^2	AIC	estimate	SE	R^2	AIC
exponential	0.107	0.008	0.973	-44.98	0.108	0.007	0.977	-46.547
power law	0.697	0.093	0.872	-9.39	0.517	0.077	0.838	-4.17
truncated power law	-0.7	0.067	0.996	-90.678	-0.4	0.034	0.997	-95.237
in-degree (female)								
exponential	0.087	0.012	0.92	-14.421	0.107	0.014	0.932	-14.465
power law	0.399	0.093	0.726	6.266	0.724	0.141	0.837	-1.626
truncated power law	-0.479	0.116	0.968	-28.859	-1.388	0.241	0.985	-35.593

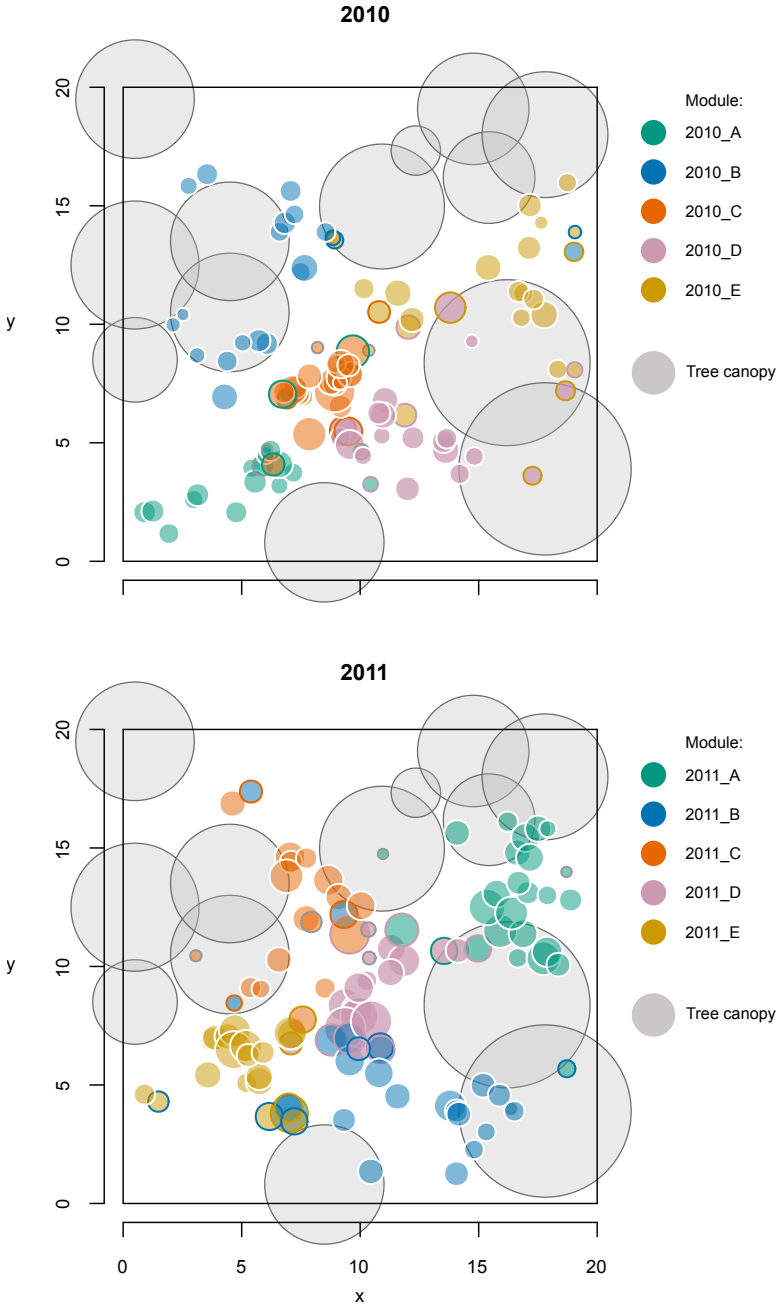


Figure 6.4: *Modular structure of the mating network. For each of study we show the spatial location of each plant and its module assignation. Node color denotes the module, when two colors are present, inner color refers to male and outer color to female functions. Node size is relative to the out-degree.*

Modularity

Modularity analyses resulted in configurations bearing four to nine modules. All of them deviated significantly from a random network ($z = 79.758 - 106.097$ and $115.429 - 159.649$ in 2010 and 2011 respectively, $p < 0.05$), and showed modularity coefficients ranging from 0.343 to 0.429 in 2010 and from 0.404 to 0.527 in 2011, with a median of 0.390 in 2010 and 0.487 in 2011 (Figure 6.S3). There was a significant higher concordance among runs than what it is expected under random module assigantion ($I_{AB\ (male)} = 0.429 \pm 0.093$, $I_{AB\ (female)} = 0.462 \pm 0.097$, in 2010; $I_{AB\ (male)} = 0.543 \pm 0.105$, $I_{AB\ (female)} = 0.567 \pm 0.103$, in 2011). The chosen configurations presented modularity values of 0.424 in 2010 and 0.518 in 2011 (Figure 6.4).

Because of the absence of genotyped offspring from some plants, the female function of five plants in 2010 and seven plants in 2011 were not assigned to any module. Among the rest of plants, for both years, we found a high concordance in module assignation among sexual functions. 81% and 75% of the fully assigned plants in 2010 and 2011 respectively, were assigned to the same module for both sexual functions. Suggesting that they reproduced with the same partners even when they behaved as male or female.

III) Pollinators as drivers of the effective pollen flow

Relationships with the overall effective pollen flow

The mating networks did not show a high correlation with any of the pollinator similarity matrices in any year (Table 6.3). However they did with the foraging networks ($r = 0.398$ and 0.425, for 2010 and 2011 respectively; $p < 0.001$).

Table 6.3: *Fit of the pollinator-based networks with the mating networks (MNet). For each pollinator-based network we show: 1) The Mantel correlation (r_M) with the MNet. 2) The correlation among node weighted degrees (ρ_{DEGREE}) at different tunning values of α (Opsahl* et al.*, 2010). Values in parenthesis depict the R^2. 3) The modularity when nodes are assigned to the modules found for the MNet. Bold values in r_M and ρ_{DEGREE} depict significant correlations.*

year	network	r_M	ρ_{DEGREE}				Q
			$\alpha = 0$	$\alpha = 0.5$	$\alpha = 1$	$\alpha = 1.5$	
2010	SNet	**0.112**	1.183 (0.015)	0.214 (0.059)	**0.147 (0.058)**	**0.132 (0.058)**	0.06
	WSNet	**0.121**	**0.678 (0.051)**	**0.157 (0.072)**	**0.125 (0.078)**	**0.12 (0.083)**	0.055
	FNet	**0.396***	**0.931 (0.399)**	**0.58 (0.432)**	**0.309 (0.401)**	**0.153 (0.348)**	0.521
2011	SNet	**0.117**	0.509 (0.001)	**0.311 (0.144)**	**0.215 (0.151)**	**0.194 (0.157)**	0.057
	WSNet	**0.146**	0.173 (0.032)	**0.128 (0.088)**	**0.124 (0.111)**	**0.126 (0.126)**	0.056
	FNet	**0.425***	**0.961 (0.403)**	**0.608 (0.485)**	**0.334 (0.502)**	**0.17 (0.48)**	0.545

Table 6.4: *Modularities from the FNets of some functional groups. For each group the observed modularity is shown, obtained from the observed plant-plant flights and the mating module assignations. Modularities for random flights and for the spatial distance constrained model are also shown.*

year	Functional group	insects	flights	Q_{OBS}	Q_{EXP}	
					random flights	distance constrained
2010	Long-tongued beeflies	154	740	0,521	**0** **(-0.03 - 0.031)**	**0,369** **(0.324 - 0.41)**
	Long-tongued large bees	39	207	0,477	**-0,002** **(-0.054 - 0.054)**	**0,299** **(0.224 - 0.378)**
	Short-tongued large bees	36	199	0,462	**-0,003** **(-0.061 - 0.052)**	**0,315** **(0.227 - 0.405)**
	Short-tongued medium bees	58	243	0,493	**-0,001** **(-0.05 - 0.048)**	**0,376** **(0.304 - 0.448)**
	Short-tongued small bees	44	86	0,689	**-0,004** **(-0.08 - 0.081)**	**0,593** **(0.509 - 0.672)**
	Butterflies	10	57	0,412	**-0,009** **(-0.107 - 0.096)**	**0,19** **(0.036 - 0.34)**
	Small beetles	31	43	0,401	**-0,001** **(-0.114 - 0.113)**	**0,21** **(0.067 - 0.348)**
	All groups	380	1604	0,522	**0** **(-0.02 - 0.021)**	**0,361** **(0.331 - 0.389)**
2011	Long-tongued beeflies	145	631	0,524	**0** **(-0.033 - 0.031)**	**0,426** **(0.375 - 0.471)**
	Long-tongued large bees	74	428	0,609	**0** **(-0.039 - 0.038)**	**0,392** **(0.33 - 0.452)**
	Short-tongued large bees	27	85	0,531	**-0,003** **(-0.086 - 0.085)**	**0,426** **(0.325 - 0.525)**
	Short-tongued medium bees	11	24	0,474	**-0,012** **(-0.155 - 0.167)**	0,343 (0.14 - 0.531)
	Short-tongued small bees	17	44	0,648	**-0,007** **(-0.118 - 0.117)**	**0,44** **(0.295 - 0.565)**
	Butterflies	27	185	0,345	**-0,001** **(-0.06 - 0.059)**	0,271 (0.175 - 0.362)
	Small beetles	10	11	0,421	**-0,006** **(-0.19 - 0.232)**	0,299 (0.099 - 0.52)
	All groups	333	1491	0,545	**0** **(-0.02 - 0.022)**	**0,406** **(0.375 - 0.437)**

Relationships with the mating network structure

The out-degree distribution of the foraging network explained 40% of the variation in the out-degree of the mating network in both years (Table 6.3, Figure 6.5), with almost a 1:1 relationship between variables (0.931 and 0.961 in 2010 and 2011 respectively).

All pollinator-based networks matched the modular configuration of the mating networks. When nodes were assigned to the modules found in the mating networks, SNet and WSNet presented low values of modularity, although significant when compared to those generated by the random model (0.06 and 0.055 for SNet and WSNet respectively in 2010; 0.057 and 0.056 in 2011; Table 6.4). On the other hand, FNet responded to that modular

configuration with higher values of modularity (0.521 and 0.545 in 2010 and 2011 respectively). These values deviated from what expected for both, the random foraging models and the distance-constrained foraging models (Table). Finally, the FNet of each functional group also responded to the modular configuration of the mating network with modularity values that deviated significantly from what expected for the random foraging model. However, they were close to what expected under the null hypothesis of flights occurring at short distances, being not significant for short-tongued medium bees (0.474), butterflies (0.345) and small beetles (0.421; Table 6.4) in 2011.

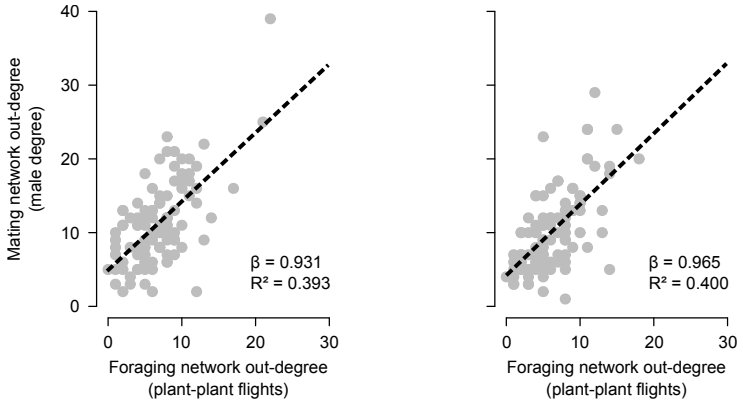


Figure 6.5: *Linear fit among inter-plant flights and mating out-degree*

Discussion

Factors influencing the effective pollination

In this study we demonstrate that in a population of *E. mediohispanicum* the realized matings occur mostly a short distances, mainly due to the spatially restricted movement of the most effective pollinators. We have shown how these matings are constrained among coflowering plants and how the flowering schedules can modulate the effects of spatial distance on the mating patterns. In addition, we have explored how these matings could be described as individual-based networks and analyzed some of their emerging properties.

Spatial isolation and timing of flowering

Plants can modulate their mating pattern through the response of pollinators to plant spatial arrangement and density (Handel, 1983; Oddou-Muratorio

et al., 2006; Llorens *et al.*, 2012). For example, high local densities are positively related to shorter distances of pollen dispersal (Goodell *et al.*, 1997; Robledo-Arnuncio *et al.*, 2004). We think that this is happening in our study population, where plants presented a clumped spatial distribution and inter-plant flying distances occurred mainly among close plants (Figure 6.S6). We have found that the effective pollination of *E. mediohispanicum* mostly occurred at short distances, at least in the studied population. Thus, half of the siring parents were located at less than 4.20 meters from the mother plant. Pollen flow at short distances is a common pattern found in insect pollinated plants (Fortuna *et al.*, 2008; Buehler *et al.*, 2012) that results from pollinator mainly moving among nearby plants (Ohashi and Thomson, 2009; Lysenkov, 2014).

Effective pollination from distant plants occurred more frequently than what expected by the solely distribution of direct inter-plant flights. Thus, flying distances showed a more left-skewed distribution than those of the mating distances (Figure 6.S6). This lack of fit between both distributions might be attributable to various factors. For example, pollen carryover is known to increase pollen dispersion distance (Karron *et al.*, 1995; Bernasconi, 2003). The mating system may also influence this misfit. In populations presenting a marked genetic structure, such as *E. mediohispanicum* (Valverde *et al.*, 2016a), pollinator foraging at short distances will deposit a mixture of pollen with an elevate proportion of pollen from close relatives (Harder and Barrett, 1996). It is possible that *E. mediohispanicum* plants be rejecting this pollen as incompatible, or even whether this pollen fecund the ovules, these be aborted because of inbreeding depression after biparental inbreeding (Uyenoyama, 1986). In addition, pollen competition or other process producing cryptic incompatibility could also contribute to increase the siring of more distant plants (Cruzan and Barrett, 1993). Last, we are not excluding the possibility that the distribution of flying distances be affected by an incomplete sampling of pollinator flights, implying that more observations should be gathered to obtain a better estimation of the flight distribution. However, we think that our sampling of flights (1604 in 2010 and 1491 in 2011; Table 6.4) is robust, at least for the most important functional groups.

Spatially-restricted gene flow promotes the appearance of spatial genetic structures (SGS) (Wright, 1943; Epperson, 1993). In this same population, Valverde *et al.* (2016a) showed that the spatial distribution of genotypes followed an isolation by distance pattern, with kinship decreasing with spatial distance. This pattern occurred for both nuclear and chloroplastidial markers, indicating that seed dispersal occurs at short distances in accordance to their barocorous dispersal strategy (Gómez, 2007). This limited seed dispersal can generate SGS even under random mating (Hamrick *et al.*, 1993; Kalisz *et al.*, 2001). However, as our results demonstrate, matings mostly occur among

nearby individuals, implying that actual pollen flow should be strengthening the SGS created by the limited seed dispersal.

Some pollination events also occurred at long distances. We have found that above 15% of the offspring was sired by parents outside the population. The relative low size of studied population (100 individuals), may contribute to this proportion of immigration pollen, such as has been described for other plants (White *et al.*, 2002). This pollen should be carried by insects capable to flight long distances, such as butterflies, beetles or large bees (Waser, 1982; Herrera, 2000; Lysenkov, 2014). In our study system, butterflies and beetles were not very frequent (**?**Valverde *et al.*, 2016b), and, additionally, they have been proven to be bad pollinators for this plant (Figures 5.1 and 5.3, Valverde et al. in prep.). In contrast, long-tongued bees, particularly those of the genera *Anthophora* could be the main vectors promoting gene flow among populations of this species. Our personal observations showed that individuals of this genus, in contrast to the other pollinators, mainly arrived to the population from outside the plot and leaved it after visiting a few plant individuals. To proven this hypothesis further research is needed as, for example, catch and release experiments (e.g., Decourtye *et al.*, 2011) or the analysis of other-population's marked pollen in pollinator's bodies.

Flowering phenology also played a major role in the distribution of mating events. First, our results showed that pollen flow was restricted to coflowering plants. The distribution of matings events fitted the predictions defined by the distribution of flowering synchronies in the population, demonstrating that the probability of mating is proportional to the temporal window at which plants coflower, as it is necessarily expected (Wagener, 1976; Kirkpatrick, 2000; Fox, 2003; Weis and Kossler, 2004; Weis *et al.*, 2005; Ison *et al.*, 2014). Second, similarly to the findings of Ison *et al.* (2014), we found that flowering asynchrony modulated the probability of mating based on spatial distance. These authors found that both distance and flowering affected the pollination patterns in *Echinacea angustifolia*, with mating events increasing with spatial proximity. Our simulations were similar to their findings in that values close to complete flowering asynchrony (0.9, i.e. little flowering overlap) were necessary to erode the observed pattern of matings occurring at short distances. We think that the spatial autocorrelation found in flowering time (Figure 6.S6) may contribute to this pattern. When asynchrony is low, nearby plants tend to be coflowering and, therefore, nearby plants tend to mate frequently among them. However, when population asynchrony is high, coflowering plants tend to be situated far in the plot, implying that mating events will occur at longer distances. These findings, however, result from simulations and we are aware that experimental data obtained with controlled phenologies are needed to corroborate this.

Phenotype and abiotic factors

Beyond the spatial and phenological constrains, matings also occurred more frequently among nearby plants sharing similar light conditions. Correlations among mating plants for this variable were higher than those expected under a random mating and in the case for preference for short-distance matings. These findings suggest that mating events, even preferentially occurring among nearby plants, were frequently occurring among those plants sharing similar light conditions. Light availability as been reported to influence gene flow, either promoting assortative reproduction among plants (Dyer *et al.*, 2012) or increasing genetic diversity of pollen arriving to open areas (Kamm *et al.*, 2010). The heterogeneity found in the light microenvironment () in addition to the higher similarities in pollinator assemblages among plant sharing the same light environment (), points to that these patterns result from a partitioning of the pollinator assemblage as a response to open/shaded areas (Herrera, 1995b; Kilkenny and Galloway, 2008). Valverde *et al.* (2016a) found that this population showed a non-homogeneous distribution of genotypes with a spatial genetic structure that varied in intensity with compass directions. Given the heterogeneous microenviroment were plants grow, the preferential mating among plants with similar light microenvironment probably contributes to this non-homogeneous distributed SGS. The heterogeneity found in the light microenvironment (Valverde *et al.*, 2016a), in addition to the higher similarities in pollinator assemblages among plant sharing the same light environment (), points to that these patterns result from a partitioning of the pollinator assemblage as a response to open/shaded areas (Herrera, 1995b; Kilkenny y Galloway, 2008).

Structure of the mating network

At each year of study, the effective pollen flow occurring within the population produced a mating network with a defined structure. Particularly, we found that the mating networks built up following a truncated power-law growing mechanism, and that matings occurred more frequently among well-defined groups of plants.

Degree distribution

Plants in this population did not reproduce randomly nor with all their conspecifics. Contrary, the node degree (number of reproductive partners) of both sexual functions in the mating network fitted a truncated power-law distribution. Power-law distributions are the generality in other bipartite networks such as in plant-pollinator or plant-frugivores networks (Jordano

et al., 2003), and also in unipartite networks belonging to as different realms as the neuronal (Amaral *et al.*, 2000) and protein (Jeong *et al.*, 2001) interaction networks of the worm *Caenorhabditis elegans*, the World Wide Web (Albert *et al.*, 1999) or the movie-actor collaboration network (Barabási and Albert, 1999). This scale-free distribution is produced when a network grows by the new nodes attaching preferentially to nodes presenting high degrees. For a population of plants, this is equivalent to that individual plants tend to mate with those most promiscuous. A reiterative visitation pattern of pollinators to highly connected plants seemed to be in part responsible of this. The mating out-degree was positively correlated with the out-degree of the foraging network (FNet) in a nearly 1:1 relation with a relatively good adjustment ($\beta > 0.93$, $R^2 > 0.39$). In addition, this pattern could be produced if plants with extended phenology are attracting more and more diverse pollinators and, therefore, mating with more conspecifics. In our system, the probability of mating was proportional to the coflowering temporal window (see above), therefore, increasing the probability of reach a higher degree.

The highest degree in these networks was 39, which explains the truncated power-law distribution of these mating networks. This distribution implies that the probability of finding plants with higher degree (high multiple paternity) decreases with an exponential cutoff. This decay usually results from constrains to the addition of new edges (Amaral and Ottino, 2004). We think that this pattern could be explained by the limitations imposed by the flowering synchrony and also by the effective pollinators being foraging at a small-local scale. These processes imply that the most isolate plants (both phenologically and spatially) will also present lower degree. Similar such a pattern has been found in other plant species, such as *Oenothera harringtonii* (Onagraceae) where spatial isolation imposes important reductions in multiple patternity (**?**). Truncation has been associated to dramatic effects on the dynamics of complex networks, specially when nodes with high degree has special roles (Mossa *et al.*, 2002). For example, the reduction in maximum degree in this type of networks could reduce the probability of infection spreading or other process of information spreading (e.g., Pastor-Satorras and Vespignani, 2001). The implications of these network structures in the spreading of genetic innovation both in a pure mutation-drift equilibrium or submitted to positive selection deserved consideration and should be analytically explored.

Modularity

The modularity analyses revealed that plants tend to mate in groups. This kind of structure has being previously reported for other plant species. Fortuna *et al.* (2008) studied the mating network of *Prunus mahaleb*, and found a modular structure that was highly influenced by the spatial distribution of

plants. Similarly, in our study, the plants belonging to the same module were nearby, in such a way that modules formed well defined patches in space (Figure 6.3) As discussed in the previous section, the pollen dispersal kernel was affected by the spatial arrangement of plants, a pattern previously reported (Handel, 1983; Oddou-Muratorio *et al.*, 2006; Llorens *et al.*, 2012). And more important, we empirically demonstrated the effects of among-plant similarities in light environment in shaping the patterns of pollen flow. We think that the interplay of these two factors, mediated by the foraging movements of pollinators are determining the clustering of the mating events. This idea is supported by the pollinators' foraging network (FNet). FNet showed high modularity values when plants were assigned to the modules found in the mating network. These values where even higher than those expected for the spatial model, in which the movement of pollinators was spatially constrained. In other words, pollinators in our study population moved among nearby plants, but preferentially among those presenting similarities in some factors such as light availability. These foraging patterns result in communities of plants preferentially mating among them. This pattern also explains the truncated distribution of node degrees, since confining interactions to nodes inside these communities will limit the number of reproductive partners.

The high concordance found among runs of the QuanBiMo algorithm demonstrates its robustness in finding modules in weighted networks. However the runs yielded different partitions of plants in modules and varying number of these. This suggests that modules in these mating networks are not discrete, but rather fuzzy entities with some plants in between of two or more modules. In fact, following the cartography of node roles proposed by Guimerà and Amaral (2005a,b), some of the plants presented relatively high participation coefficients, i.e., how well distributed their links are among modules (see Figure 6.S5). In addition, some nodes pertained to different modules depending on the sexual function considered, indicating its labile assignation to a specific module.

The fuzzy nature of the modules of these mating networks is a consequence of plants reproducing with an elevate number of partners, although they usually did with plants of the same module. In fact, most plants could be ascribed to a peripheral role (R2 in the classification of Guimerà and Amaral (2005b) for both male and female functions because most of their links were with those plants belonging to their same module. However, an important proportion ($> 30\%$ for some modules and year; see figure 6.S5) of plants in each module behaved as non-hub connector nodes, i.e., nodes with many links to other modules, what contribute to increase the fuzziness of these networks. It is noteworthy that only a few plants did have an authentic hub role and acted as main connectors between modules.

The modular nature of these networks is in accordance with the idea that

the population could be structured in genetic neighborhoods. Valverde *et al.* (2016a) showed that a genetic structure exist in this same population. Moreover, this structure was maintained among years, which implies that reproduction or/and dispersal was spatially limited. We have now demonstrated that mating mainly occurred between plants of the same module, contributing to the development of (fuzzy) groups of plants. Similar genetics neighborhoods have been reported in other organisms, although usually at much bigger scales (Boshier *et al.*, 1995; Ruckelshaus, 1996; Fortuna *et al.*, 2008). Small neighborhoods could be an important factor in the accumulation of deleterious mutations and in the increasing risk of population extinction (Kawata, 2001). In *E. mediohispanicum*, in addition, small population size and increase biparental inbreeding (Abdelaziz, 2013) together with spatially limited reproduction (our present data) could accentuate this risk. However, purge of genetic load after recurrent inbreeding (Husband and Schemske, 1996; Byers and Waller, 1999; Crnokrak and Barrett, 2002) may operate in *E. mediohispanicum* populations (Abdelaziz *et al.*, 2014) and contribute to the temporal persistence of this population. Moreover, the important incoming of genes from other populations (16% - 19%) surely contribute to increase the genetic diversity of this population and, definitely, to their temporal persistence.

Pollinators as drivers of the effective pollen flow

We explored to what extent plant-plant networks based on plant-pollinator interactions could explain the patterns of the actual pollen flow. Our results showed that those networks based in sharing pollinators, i.e., similarity networks, where not the best proxies of the mating networks. However, and as could be expected, the explanatory power of the similarity networks increased when the pollinator's effectiveness is incorporated. But it was the network based on the foraging movements of pollinators which explained the most of the actual pollen flow patterns.

Former studies have emphasized the need to downscale networks to the individual level for a higher resolution of the mechanisms involved in the structuration of these interactions (Araújo *et al.*, 2008, 2010; Gómez *et al.*, 2011; Gómez and Perfectti, 2012; **?**; Dupont *et al.*, 2014; Tur *et al.*, 2014; Valverde *et al.*, 2016b). However, the use of networks gains relevance when these express a function. In this sense, directed networks may offer a more complete understanding of network functioning as edge directionality expresses information flow. Our results show that when studying plant-plant networks using animal movements, link directionality can predict pollen flow among plants better than networks merely based on pollinator sharing. The directionality of pollinator movements have been previously used to construct plant-plant networks (Dupont *et al.*, 2014), and have been even used to explain

patterns of pollen deposition (Fang and Huang, 2016). However, so far, this is the first study in putting toghether patterns of pollinator's plant visitation and actual pollen flow.

Within the framework of the Structured Generalization hypothesis, the similarity and the foraging networks represent different layers of information. These networks, together with the mating network and the spatial genetic structure, synthesize this hypothesis. The similarity network resumes how pollinators are partitioned among plants and thus offers us information about the selective pressures that each plant might be subject to. The foraging network represents how individual pollinators forage within their preferences, and thus express how resources are partitioned among pollinator individuals. These pieces of information materialize in a structured mating network that, together with the seed dispersal, governs the movement of genes in space to give place to a fine-scale spatial genetic structure.

Supplementary information

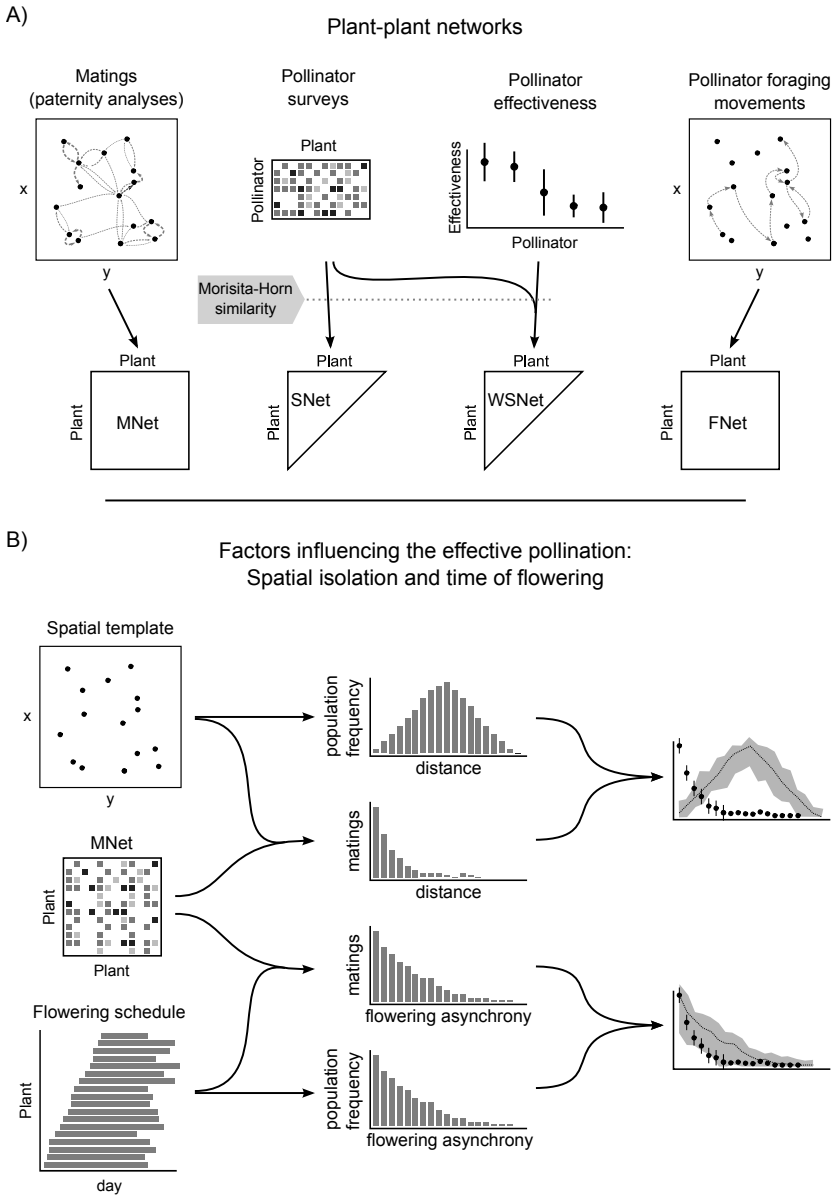


Figure 6.S1: *Workflow followed to: A) construct the plant-plant networks: mating network (MNet), similarity network (SNet), weighted similarity network (WNet) and foraging network (FNet); B) analyze the spatial and phenological isolation in the patterns of effective pollen flow.*

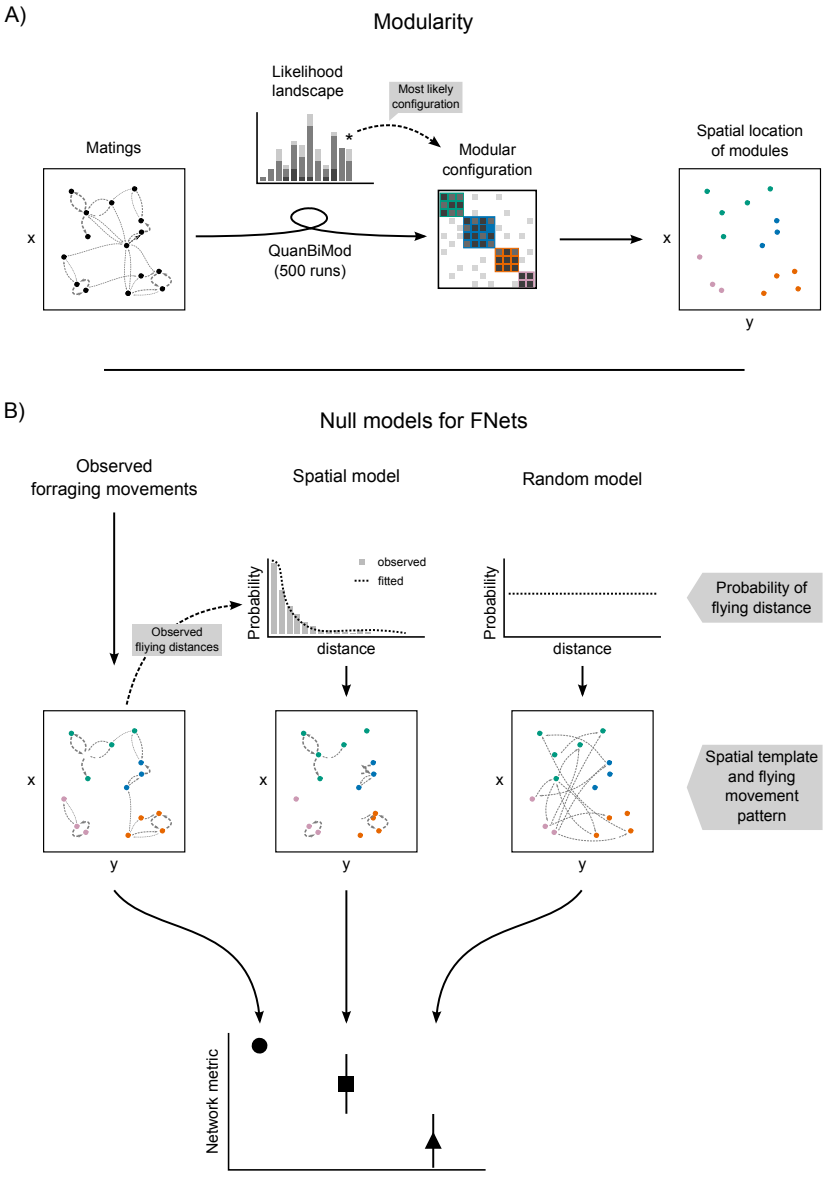


Figure 6.S2: *Workflow followed for: A) finding the most likely modular configuration; B) construction of the spatial and random null models for comparisons with the FNet network values. In the latter we show the example for modularity, note module assignation of each plant in the spatial template.*

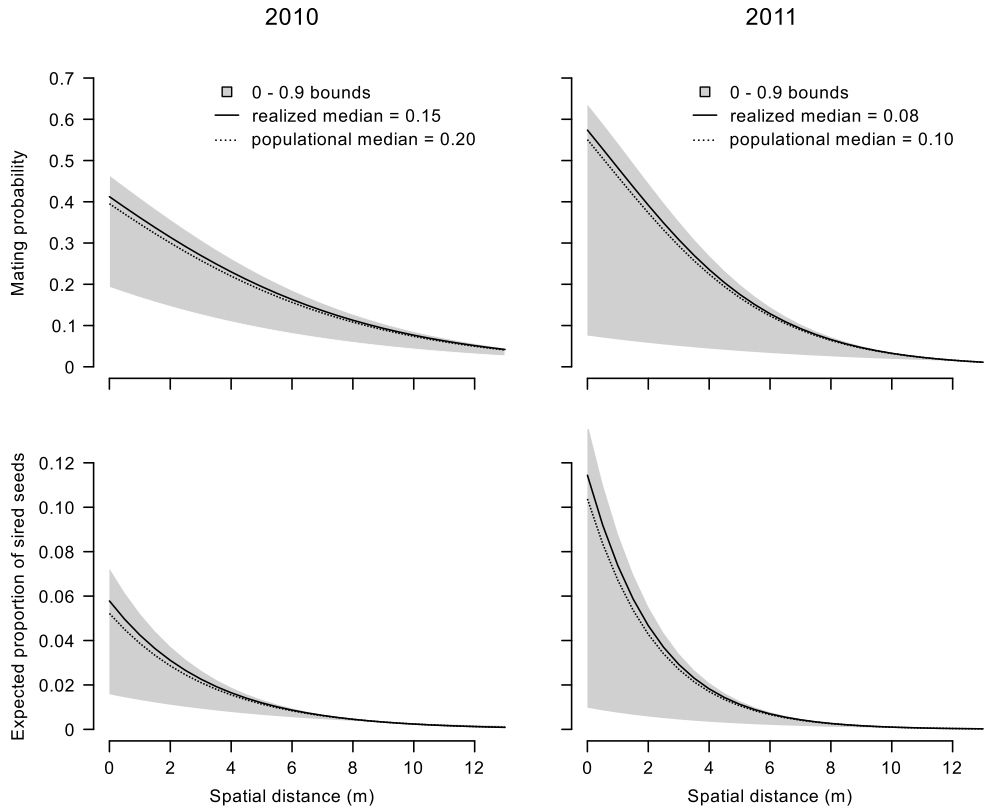


Figure 6.S3: *Interaction between spatial and phenological isolation. Panels show the model predicted curves of mating probability (upper panels) and proportion of sired seeds (lower panels) in relation to the spatial distance. Predictions for different values of phenological asynchrony are shown, realized median, populational median, and 0 and 0.9 bounds.*

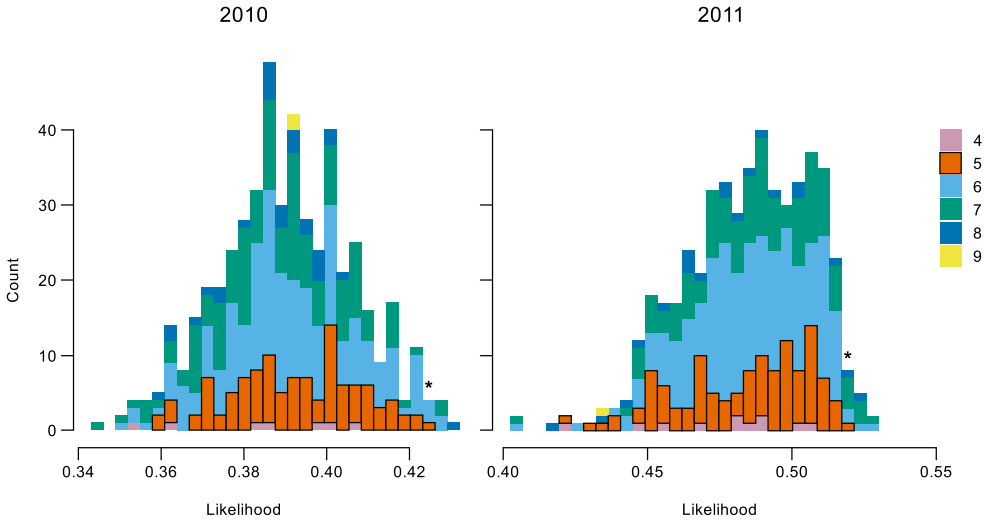


Figure 6.S4: *Likelihood (Modularity) distributions of all the QuanBiMo optimal configurations. The histogram stacks the counts bearing equal number of modules. Asterisk (*) indicate the bin where the chosen configuration is located.*

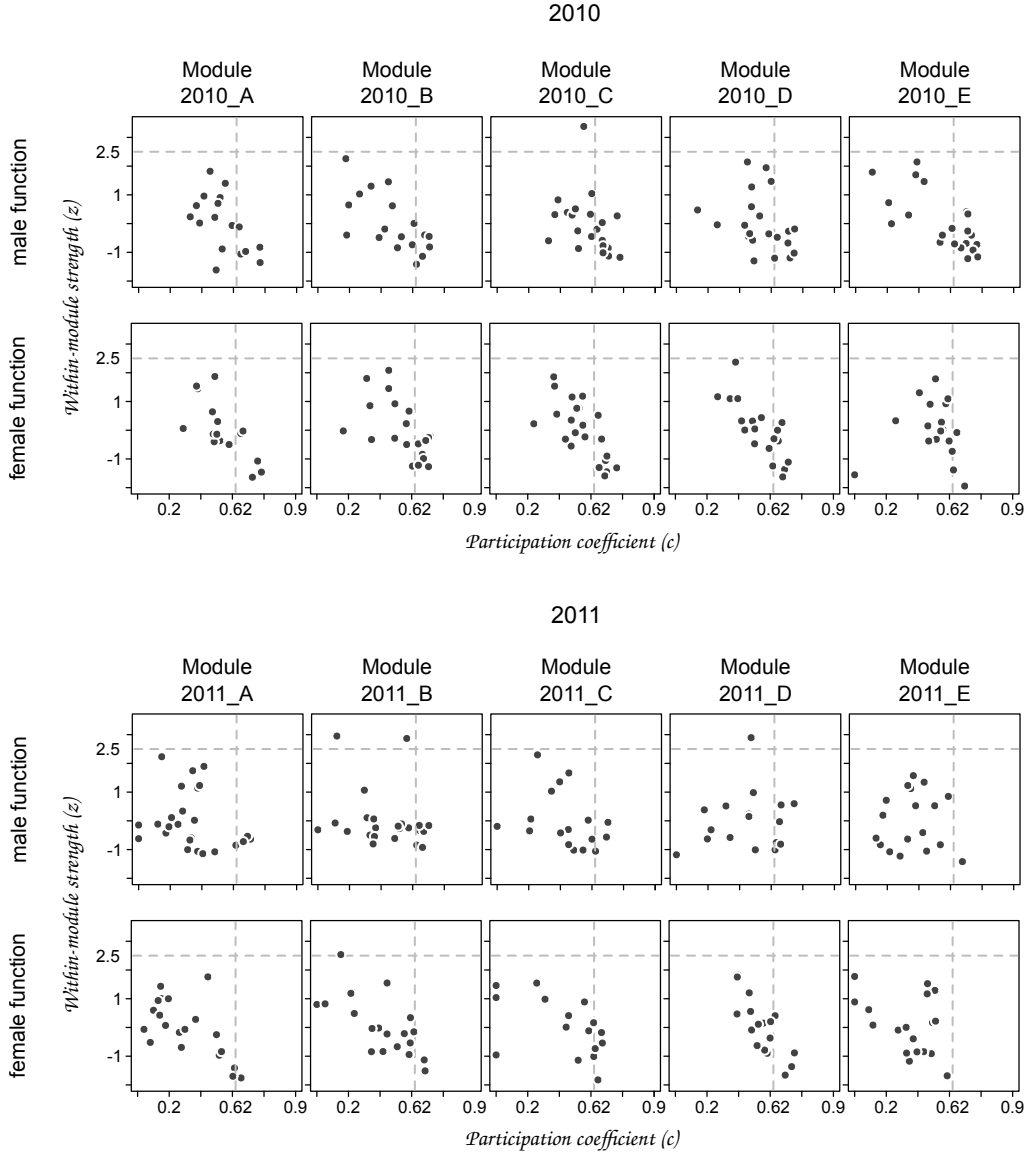


Figure 6.S5: *Within module degree (c) and participation coefficient (z) for plants in the mating networks. We calculated the weighted version of Guimerà's c and z (2005) as:*

$$z_i = \frac{k_{iS} - \bar{k}_S}{\sigma_{KS}} \qquad and \qquad c_i = 1 - \sum_{S=1}^{N_M} \left(\frac{k_{it}}{k_i}\right)^2$$

In these equations, k_{iS} is the strength of node i within its module, and $\bar{k}_S$ and σ_{K_S} are the average and standard deviation of the strengths of all plants in module S. k_i is the total strength of i and k_{it} is the strength of i to nodes of module t.
Discontinuous lines delimit the role-specific regions of Guimerà and Amaral (2005a) ($c = 0.62$ and $z = 2.50$).

Bipartite modularity

The modularity index is calculated as:

$$Q = \frac{1}{2 \times m} \times \sum_{ij} \left(A_{ij} - \frac{k_i \times k_j^T}{2 \times m} \right) \times \sigma(c_i, c_j)$$

in this index, m is the half of the links present in the network. A_{ij} represents the observed weight of the link between the male function of the plant i and the female function of the plant j (i.e. number of seeds on plant j sired by plant i). The term $k_i \times k_j^T / 2 \times m$ depicts the expected weight between i and j under a null model (Newman and Girvan, 2004). k_i is the marginal sum of row i, and corresponds to the number of sired offspring by the individual i. k_j is the marginal sum of column j, and corresponds to the total number of genotyped offspring of the individual j. c_i and c_j corresponds to the module assignation for the male function of i and the female function of j respectively. The term $\sigma(c_i, c_j)$ gets the value of 1 if $c_i = c_j$ and 0 if $c_i \neq c_j$. Simulated annealing can fall into suboptimal modular configurations, yielding different classifications of nodes into modules among runs (Guimerà and Amaral, 2005a). We therefore performed 500 runs and selected the configuration bearing five modules with the highest modularity (figure 6.S2a). We chose five modules in order to ease the comparison of both years.

Mutual information index

This index, developed by Danon *et al.* (2005), compares two partitions (A and B):

$$I_{AB} = \frac{2 \times \sum_{i=1}^{N_M^A} \sum_{j=1}^{N_M^B} n_{ij}^{AB} \times log\left(\frac{n_{ij}^{AB} \times S}{n_i^A \times n_j^B} \right)}{\sum_{i=1}^{N_M^A} n_i^A \times log\left(\frac{n_i^A}{S} \right) + \sum_{j=1}^{N_M^B} n_j^B \times log\left(\frac{n_j^B}{S} \right)}$$

here, S is the total number of nodes, n_i^A is the number of nodes in module i of the partition A, n_j^B is the number of nodes in module j of the partition B, and n_{ij}^{AB} the number of nodes that are in modules i and j. The distribution of values was then compared with those obtained under a random assignment of modules to plants.

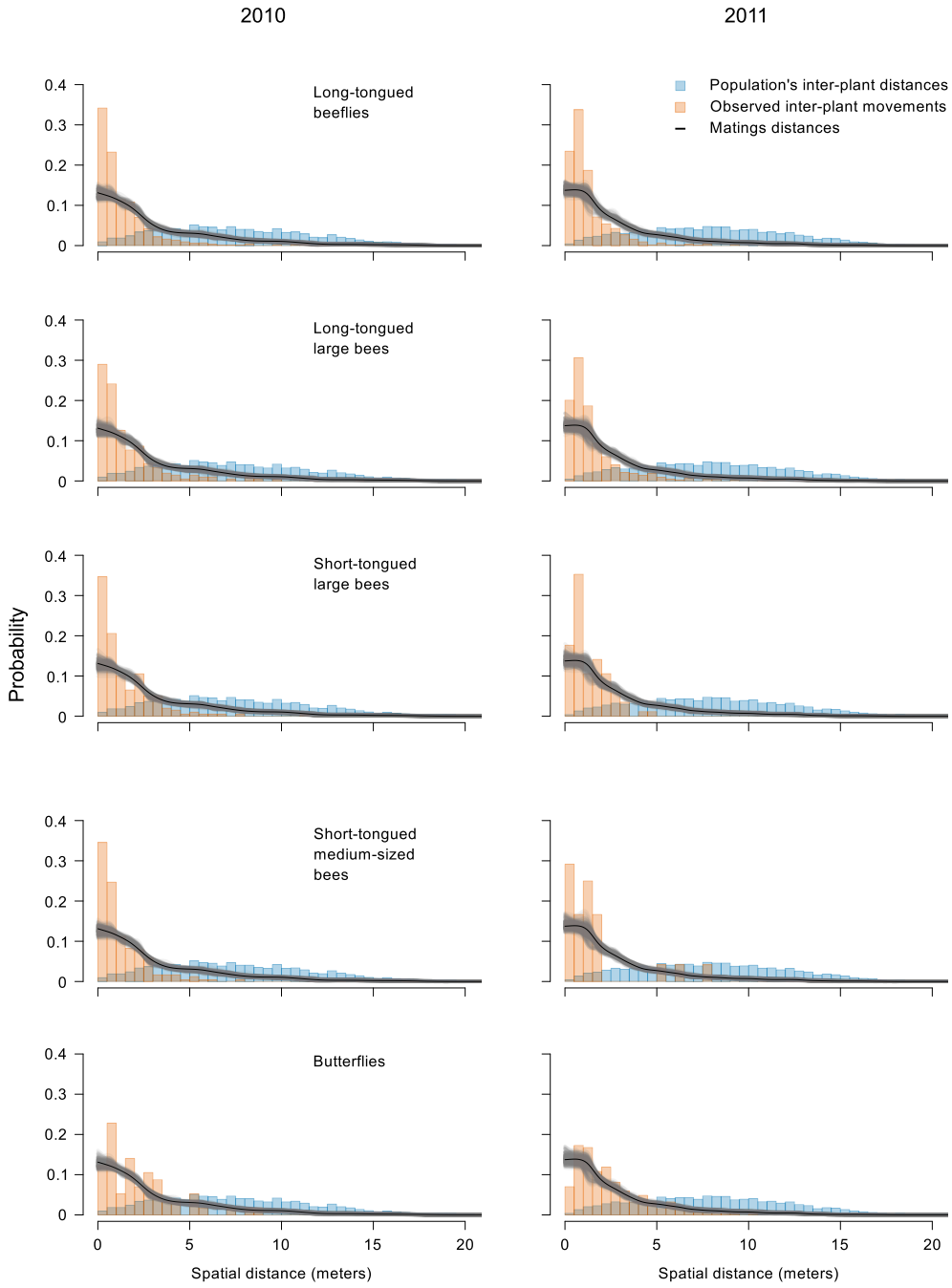


Figure 6.S6: *Patterns of flying distances and effective pollen flow. For a set of examples of pollinator functional groups we show the probability distribution of observed fights among plants (orange bars; 0.5 meters bining). Expected distances are pairwise distances among plants in the population (blue bars). We show non-parametric spline fits on the effective pollen flow (black lines), and on bootstrapped data (grey lines).*

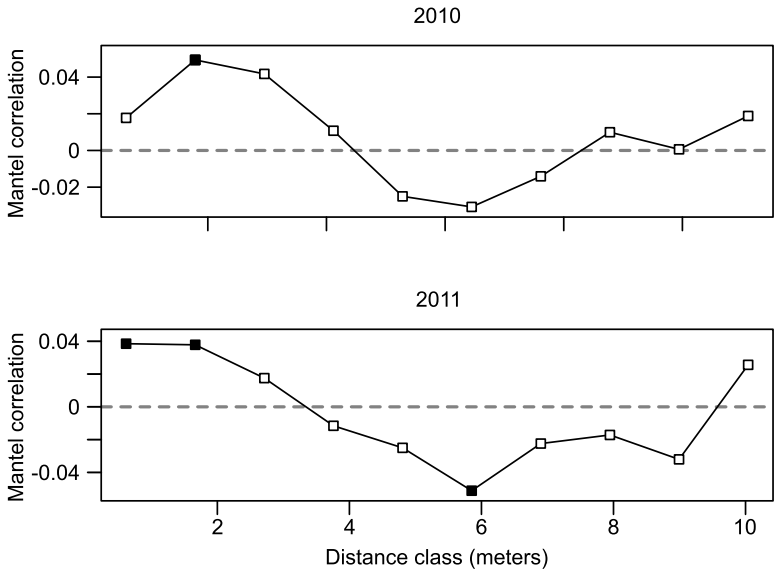


Figure 6.S7: *Spatial autocorrelation of flowering time. Panels show the correlogram of the Mantel correlation of pairwise flowering asynchronies at different spatial distances.*

Part III

Conclusiones | conclusions

Conclusiones generales

1. La población de *Erysimum mediohispanicum* estudiada presenta una estructura genética a escala espacial fina. En esta población las plantas de diferentes cohortes de floración presentan dicha estructura. Los genotipos se distribuyen siguiendo un patrón de aislamiento por distancia que es además congruente entre cohortes de años consecutivos, indicando una dispersión de semillas a corta distancia. Además, en cada cohorte los genotipos se asocian a un gradiente de luz. Estos resultados sugirieren que la luz influye en el movimiento de genes en el espacio, bien sea afectando a la dispersión y establecimiento de semillas o al flujo de polen entre plantas.

2. Las plantas en esta población son visitadas por diferentes conjuntos de polinizadores, haciendo que el grado de generalización individual varíe. La generalización individual está asociada a algunos factores fenotípicos y ambientales. Por ejemplo, la amplitud de nicho de polinizadores se relaciona con el tiempo de floración, indicando un recambio temporal de los polinizadores. Además, aquellas plantas situadas en zonas abiertas del dosel arbóreo muestran una composición de polinizadores más afín a la población en su totalidad. La variación en el grado de generalización individual y su relación con algunos factores fenotítipos y ambientales confirman una de las asunciones centrales de la hipótesis de la generalización estructurada.

3. Los polinizadores de *E. mediohispanicum* presentan un recambio temporal incluso en ausencia de heterogeneidad ambiental a escala local y de estructuración genética espacial. La acción conjunta de este recambio y de la fenología de floración provoca que a lo largo del tiempo la estructura de las redes de compartición de polinizadores varíe. Además, la integración temporal de todas la interacciones confiere propiedades emergentes a estas redes: cohesión, transitividad y centralización. Esto demuestra la importancia de integrar toda la floración para construir redes de interacción más fiables. En el contexto de la hipótesis de la generalización estructurada este recambio en las interacciones es uno de los factores gobernantes de los eventos de cruzamiento.

4. Los insectos que visitan las flores de *E. mediohispanicum* varían en su capacidad de transferir polen de forma efectiva. Esto se debe tanto a diferencias en la frecuencia de visita como en la habilidad de producir semillas por visita. De este modo, cuatro de los 23 grupos funcionales de polinizadores son responsables del 94% del éxito reproductivo de las plantas en la población natural demostrando que el grado de

generalización disminuye cuando se considera la funcionalidad de los visitantes florales como polinizadores. Además estos insectos difieren en la diversidad del polen que transfieren, un componente que debería incorporarse en los estudios de eficacia de polinización.

5. Los movimientos de pecoreo de los polinizadores más efectivos son responsables de los patrones de flujo de polen en *E. mediohispanicum*. Este flujo ocurre principalmente entre plantas cercanas y de floración sincrónica, además de entre aquellas creciendo en ambientes lumínicos similares. Como resultado la topología de la red de cruzamiento sigue una ley de potencia truncada, mostrando una gran disparidad en el grado de promiscuidad de las plantas. Además estos patrones mostraron ocurrir de forma compartimentada en comunidades de plantas que puede conllevar importantes consecuencias eco-evolutivas, como intensificar la estructura genética espacial.

6. Hemos demostrado que el fenotipo y el ambiente pueden determinar el grado de generalización individual. Las diferencias en el grado de generalización generan una reproducción clasificada determinada por el movimiento y preferencias de los polinizadores más eficaces. Finalmente, inferimos que este mecanismo es responsable en parte de generar la estructura genética espacial de la población. De este modo, demostramos parte de los pilares en los que se fundamenta la hipótesis de la generalización estructurada.

General conclusions

1. In the studied population of *Erysimum mediohispanicum*, genotypes present a fine-scale spatial genetic structure. In this population, plant genotypes are distributed following an isolation by distance. This is maintained among years, demonstrating a seed dispersal at short distances. Moreover, the associating among genotypes and light availability, suggests this factor to influence either seed or pollen dispersal.

2. Plants in this population present differing individual generalization which is associated to some phenotypical and environmental factors. For example, pollinators niche breadth augments with flowering duration, and plants growing in shades are visited by a more singular composition of pollinators. Suggesting a temporal turnover of pollinators and an important role of light in shaping plant-pollinator interactions.

3. Pollinators of *E. mediohispanicum* present a temporal turnover even under the absence of fine-scale spatial heterogeneity and any spatial genetic structure. The joint action of this turnover and flowering phenologies provoke a temporal variation in the architecture of the plant-plant networks based on pollinator sharing. In addition, the integration of the interactions occurring throughout the flowering season confers emerging network properties: network cohesion transitivity and centrality. These findings emphasizes the importance of considering the entire flowering season to build reliable interaction networks. In the context of the hypothesis of structured generalization, the temporal turnover of the interactions is one of the factors governing the mating events.

4. The insects visiting the flowers of *E. mediohispanicum* vary in their ability to transfer pollen efficiently. This results from differences in the visitation frequency and in th per-visit ability to produce seeds. In this way, four out of 23 functional groups are responsible of the 94% of the overall reproductive output in the natural population. These results demonstrates that the degree of generalization shrinks when considering insects functionality as pollinators. Moreover, insects also vary in the diversity of the pollen transferred, a component that should be incorporated in studies about pollination effectiveness.

5. Foraging movements of most effective pollinators are responsible of the patterns of pollen flow in *E. mediohispanicum*. This flow mainly occurs among nearby plants with synchronous flowering times and among those

plants growing in similar light microenvironments. As a result the topology of the mating network showed followed a truncated power-law showing a great disparity in the degree of promiscuity of plants. In addition matings occurred more often among well defined groups of plants. This pattern may imply important eco-evolutive consequences as intensifying the spatial genetic structure

6. We have demonstrated that phenotype and environment determine de individual generalization. We also show that as a consequence, a biased pollen flow emerges influenced by the preferences and movements of the most effective pollinators. Finally we inferred the effects of this mechanism in shaping the population spatial genetic structure. All these finding validate some of the basic foundations of the hypothesis of the structured generalization.

Part IV

Apendices | Appendices

Functional groups

In pollination ecology, functional groups are taxonomically independent organisms exerting similar selective pressures on floral phenotype (Fenster *et al.*, 2009). In *E. mediohispanicum*, floral visitors have been clustered based on resource use (pollen, nectar), flower handling, body size and morphological fit. This lead to classify insects into eight functional groups (Gómez *et al.*, 2008a,b, 2009a,b, 2011; Ortigosa and Gómez, 2010). Nevertheless, pursuing a more detailed clasification, this number have eventually been split into nine (Gómez *et al.*, 2010; Gómez and Perfectti, 2010), 16 (Valverde *et al.*, 2014; Gómez *et al.*, 2014a), 19 (Gómez *et al.*, 2014a), 20 groups (Gómez *et al.*, 2014b), and 23 (Valverde *et al.*, 2016b). Table A.1 resumes these classifications. In the present thesis we use 23 functional groups (Table A.2, also 4.1), except for chapter 2 were we use 16 (Table 3.1 in chapter 3).

Table A.1: *Different functional groups used to clump the species visitting* E. mediohispanicum*. Empty rectangles depict the divisions used in each classification.* L.*,* S. *and* XS. *indicates large, small and extra-small respectively*

Minimal functional division	Number of functional groups					
	8	9	16	20	19	23
Long-tongued L. bees						Long-tongued L. bees
Short-tongued L. bees						Short-tongued L. Bees
Short-tongued medium-sized bees						Short-tongued medium-sized Bees
Short-tongued S. bees						Short-tongued S. bees
Short-tongued XS. bees						
L. pollen wasps						Pollen-collecting wasps
L. nectar-collecting wasps						
Long-tongued beeflies						Long-tongued beeflies
Short-tongued beeflies						Short-tongued beeflies
L. hoverflies						L. hoverflies
S. hoverflies						S. hoverflies
Florivorous beetles						L. beetles
L. beetles						
S. beetles						S. beetles
S. diving beetles						S. diving beetles
Butterflies						Butterflies
Hawkmoths						Hawkmoths
L. moths						L. moths
S. moths						S. moths
Others						Others
Grasshoppers						
Thrips						
Bugs						Bugs
S. nectar-collecting wasps						S. nectar-collecting wasps
L. flies						L. flies
S. flies						S. flies
L. Ants						L. ants
S. Ants						S. ants

Table A.2: *Functional groups considered in this study, indicating a description of resource use (NC: nectar consumer, PC: pollen consumer, P: pollen collector) and the legitimacy of their interaction (Leg: legitimate visitor, Ileg: ilegitimate visitor). Fulfillment of the conditions are indicated with a + symbol*

Functional Group	Acronym	Size (mm)	Taxons	Resource			Legitimacy	
				NC	PC	P	Leg	Ileg
Long-tongued large bees	LtLB	>10	Hymenoptera: Anthophoridae (*Anthophora, Amegilla, Eucera*), Apidae (*Apis, Bombus*)	+			+	
Short-tongued large bees	StLB	>10	Hymenoptera: Halictidae, Andrenidae	+		+	+	
Short-tongued medium-sized bees	StMB	5 – 10	Hymenoptera: Halictidae (*Lasioglossum, Halictus*), Megachilidae (*Osmia*), Andrenidae (*Andrena*), Apidae (*Ceratina*)	+		+	+	+
Short-tongued small bees	StSB	<5	Hymenoptera: Halictidae (*Lasioglossum*), Colletidae (*Hylaeus*), Andrenidae (*Andrena*), Apidae (*Ceratina*)	+		+	+	+
Large ants	LA	>2	Hymenoptera: Formicidae (*Formica, Camponotus, Proformica, Cataphyphis, Lasius*)	+			+	+
Small ants	SA	<2	Hymenoptera: Formicidae (*Plagiolepis , Leptothorax*)	+			+	+
Pollen wasps	PW	variable	Hymenoptera: Vespidae. Masarinae (*Ceramius*)	+		+	+	
Small nectar-collecting wasps	SncW	<3	Hymenoptera: Ichneumonidae	+			+	+
Long-tongued beeflies	LtBf	variable	Diptera: Bombyliidae (*Bombylius*), Nemestrinidae	+	+		+	
Short-tongued beeflies	StBf	variable	Diptera: Bombyliidae (*Anthrax, Villa*)	+	+		+	
Large Hoverflies	LH	>5	Diptera: Syrphidae (Eristalini)	+	+		+	
Small Hoverflies	SH	<5	Diptera: Syrphidae (Syrphini, Merodontini, Bacchini)	+	+		+	+
Large flies	LF	>5	Diptera: Muscidae, Calliphoridae, Tabanidae, Scatophagidae, Anthomyiidae	+	+		+	+
Small flies	SF	<5	Diptera: Muscidae, Anthomyiidae, Empididae, Bibionidae, Drosophilidae.. among others	+	+		+	+
Large Beetles	LB	>7	Coleoptera: Lagridae, Mylabridae, Allecuninae		+		+	+
Small beetles	SB	<7	Coleoptera: Melyridae, Cleridae, Oedemeridae, Elateridae, Bruchidae, Buprestidae, Chrysomelidae		+		+	+
Small diving beetles	SDB	<3	Coleoptera: Nitidulidae, Dermestidae, Phalacridae	+	+		+	+
Butterflies	Btfly	variable	Lepidoptera: Pieridae, Nymphalidae, Lycaeidae, Hesperiidae	+			+	+
Large Moths	LM	>3	Lepidoptera: Crambidae, Noctuidae	+			+	
Small moths	SM	<3	Lepidoptera: Adelidae	+	+		+	+
Hawkmoths	Hwk	>7	Lepidoptera: Sphingidae	+			+	
Bugs	Bugs	variable	Hemiptera: Miridae, Lygaeidae, Pentatomidae (*Eurydema*)	+			+	+
Others	Oth	variable	Orthoptera, Raphidioptera, Neuroptera, among others.		+		+	

Catalogue of floral visitors

We show 115 morphospecies corresponding to the most representative taxa of each functional group. Because of the difficulty of identifying each insect to the species level, for each specimen we indicate the lowest taxon level reached. '*' indicates tentative taxa, and the most likely when doubts exists between two taxa.

Long-tongued large bees

Anthophora sp. ♀
leucophaea
or *aestivalis*

Anthophora sp. ♀
*retusa**
or *plumipes*

Anthophora
retusa ♂

*Amegilla sp.** ♀

Apis
mellifera

Bombus
terrestris

Bombus
*pascuorum**

Eucera sp. ♀

Short-tongued large bees

Andrena agilissima
♀

Andrena
nigroaenea ♀

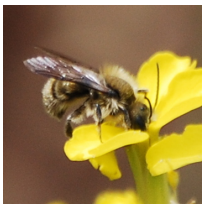
Andrena
sp. ♀

Halictus
scabiosae ♀

Halictus
simplex ♀

Lasioglossum
xanthopus ♀

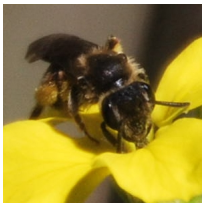
Osmia
brevicornis ♀



Osmia
brevicornis ♂

Short-tongued medium-sized bees

Andrena marginata ♀

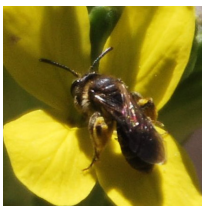


Andrena sp. ♀ *nana** or *alfkenella*

Andrena nitidula ♀

Andrena sardoa ♀

Andrena sp. ♀



Andrena sp. ♀

Andrena sp.

Halictus subauratus ♀

Halictus sp. ♀

Lasioglossum sp. ♀

Halictidae ♀ *Lasioglossum sp.**

Ceratina sp.

Ceratina cucurbitina ♀

Ceratina cucurbitina ♂

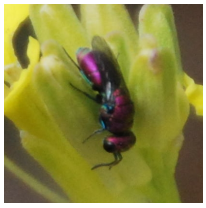
Ceratina cucurbitina ♀ + ♂

Shot-tongued small bees

Andrena sp. ♀

Halictidae ♀ *Lasioglossum sp.**

Hylaeus sp.



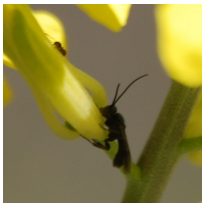
Chrysura sp.

Pollen wasps

Ceramius lusitanica.

Small nectar-collectiong wasps

Athalia sp.



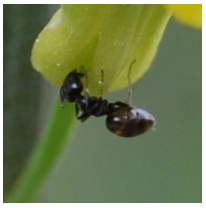
Braconidae (Cheloninae)

Large ants

Cataglyphis sp.

Lasius sp.

Small ants

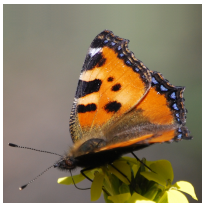


Plagiolepis sp.

Butterflies

Polyommatus amandus

Polyommatus bellargus



Aglais urticae

Argynnis pandora

Issoria lathonia

Lassiomata megera

Melitaea sp.

Iphiclides podalarius

Colias croceus

Euchloe crameri

Pieris brassicae

Hawkmoths

Macroglossum stellatarum

Titanio pollinalis

Large moths

Omia cyclopea

Chamaesphecia mysiniformis

Small moths

Adela sp.

Long-tongued beeflies

Bombylius major

Bombylius fimbriatus

Large hoverflies

Erystalis tenax ♀

Erystalis tenax ♂

Erystalis arbustorum ♀

Merodon sp.

Small hoverflies

Chrysotoxum intermedium ♀

Dasysyrphus albostriatus

Eupeodes corollae ♀

Eupeodes corollae ♂

Melanostoma sp.

Paragus sp.

Platycheirus sp.

*Platycheirus sp.**

Small hoverflies (cont.)

Scaeva albomaculata ♀

Sphaerophoria scripta ♀

Syrphus ribesii ♀

Large flies

Empis sp.

Milichia sp.

group Calyptrata

Muscidae

Small flies

Agromizidae

Bibionidae

Myopa sp.

Thaumatomyia sp.

Large beetles

Certallum ebulinum

Lachnaia sp.

Proctenius granatensis

Ragonycha fulva

Stenopterus sp.

Lagridae

Small beetles

Anthaxia sp.

Melyridae
*Dasytes sp.**

*Cantharis sp.**

Cardiophorus sp.

Malachius lusitanicus

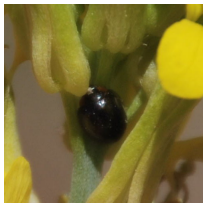
Psilothrix sp.

*Trichoides apiarius**

Thea vigintiduopunctata

Anthicidae

Bruchidae



Coccinelidae
*Chilocorus sp.**

Melyridae
*Allotarsus sp.**

Mordellidae
*Mordellistena sp.**

Small diving beetles

*Anthrenus festivus**

Attagenus trifasciatus

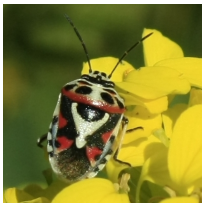
Meligethes sp.

Phalacridae

Bugs

Dolycoris baccarum

Eurydema oleracea



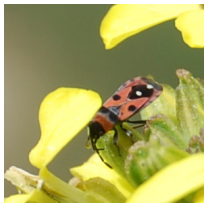
Eurydema ornata

Hadrodemus m-flavum

Hadrodemus noualhieri

Hadrodemus sp. nimph

Rhynocoris cuspidatus



Lygaeidae

Tingidae

Hemiptera

Others

Neuroptera *Chrysopa sp.*

Orthoptera

Orthoptera

Orthoptera

Orthoptera

Raphidioptera *Raphidia sp.*

Handling of genotyping errors

Microsatellite markers are prone to suffer from genotyping errors (Marshall *et al.*, 1998; Hoffman and Amos, 2004), that affect allele scoring. Some of them, such as an unusual number of alleles in a locus may be obvious, however there are other criptic sources of error. In parentage analysis, these errors can cause misassignments, and lead to erroneous interpretations of the data (Marshall *et al.*, 1998; Wang, 2010; Pompanon *et al.*, 2005; Kalinowski *et al.*, 2007). Their identification is therefore of great importance for accurate paternity assignment.

There are several types of genotyping errors:

I) Extra alleles. Genotype scoring can result in loci with more alleles than expected (more than two for diploids). This anomaly may be due to DNA contamination, either during DNA extraction or fragment amplification. Extra alleles can also originate as a consequence of tandem duplications within the genome. These duplications, with time, evolve to differing fragment sizes and thus appear as a new alleles. In this case, the two copies are linked, behaving and segregating like a single allele.

II) Null alleles and allelic drop-outs. Null alleles appear as a consequence of a mutation in the sequences flanking the marker where the primers bind. This derives into a poor primer annealing and a PCR failure for that allele. Allelic drop-outs occur when amplification of one allele in a heterozygous individual outcompetes the amplification of the other allele. Depending on the mutation, it is possible to differentiate between both types of errors. Null alleles refers to those unable to amplify at all during the polymerase chain reaction, while allelic drop-outs can amplify or not depending on the circumstances. In heterozygotes, the latter may result in an apparent homozygote and thus affect to paternity assignment (Carlsson, 2008; Dakin and Avise, 2004).

In our study we genotyped the totality of alleged parents inside the population and genotyped next to 30 offspring from plant. This allowed to correct adult genotypes by directly comparing them with those of its offspring. But most important, it allowed us to detect genotyping errors occurring either in the offspring of in the alleged parent.

To do so, we first completed the genotyping from the adult plants by comparing the matching of each mother-offspring pair. Second, we performed a paternity assignment using the categorical allocation method implemented in the Cervus 3.0 software (Marshall *et al.*, 1998) using the followgin parameters:

- critical likelihood = 95% and 80% confidence.
- number of simulations = 10,000.

- minimum number of matching loci = 6.
- error rate = 0.01
- proportion of sampled candidates = 0.9
- proportion of loci typed = 0.7

Finally, we developed an R script capable to locate mother-offspring and father-offspring mismatches. This script uses the assignments output from Cervus and the raw genotypes to compare the loci of the offspring with those of their mother and alleged father. The script locates parent-offspring mismatches and uses a decision tree to classify the possible cause of the mismatch as follows:

Offspring-mother mismatches

To follow the decision tree to locate and classify these mismatches see Figure A.1. The script starts questioning if the offspring is homozygous. If it does, and when the mother has a known null allele (C_{NULL}) then the mismatch is considered to be due to that null allele (solution A). Otherwise, it considers that a drop-out exists for one of the mother alleles (solution B). If, on the other hand, the offspring is heterozygous, there are two possible solutions. When the mother is homozygous, the mismatch should be due to an unknown maternal null allele (solution C). Otherwise, when the mother is heterozygous then the mismatch is considered a true mismatch (solution D).

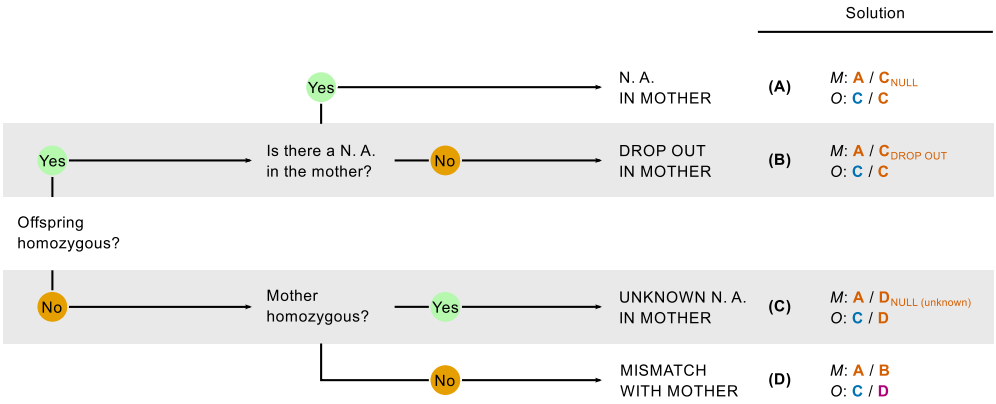


Figure A.1: *Decision tree to solve offspring-mother mismatches. Solutions show mother (M) and offspring genotypes (O). Alleles in orange belong to the studied mother, blue are from the father and purple represent misassigned maternal alleles. N.A. stands for a missing allele in the initial genotyping procedure.*

Offspring-alleged father mismatches

To follow the decision tree to locate and classify these mismatches see Figure A.2. The script starts questioning whether the offspring is homozygous or heterozygous. If homozygous, and if the alleged father does not have a known null allele in that locus, then depends on whether the latter is homozygous or heterozygous. When the alleged father is homozygous, the mismatch could be considered to be due to an unknown father null allele in the alleged father (solution B). If the alleged father is heterozygous, then it implies a drop-out in the offspring (solution C). On the other hand, if the offspring is heterozygous the script asks for the number of unassigned alleles in the offspring. When two alleles are unassigned, then a real mismatch with the mother also exists in regards of the misassignments with the father (solution H). When only one allele is unassigned, and the alleged father does not present any known null allele, then the script questions whether the father is homozygous. If it is so, then either there is a real mismatch with the father (solution E) or either this presents an unknown null allele (solution F). If, on the other hand, the alleged father is heterozygous, then a real mismatch exists with the offspring (solution G).

Once corrected for the first mismatches, we iteratively make the whole procedure up to three times and finally evaluated the most probable father among the set of alleged fathers taking into account the real source of each mismatch and the total progeny of each plant.

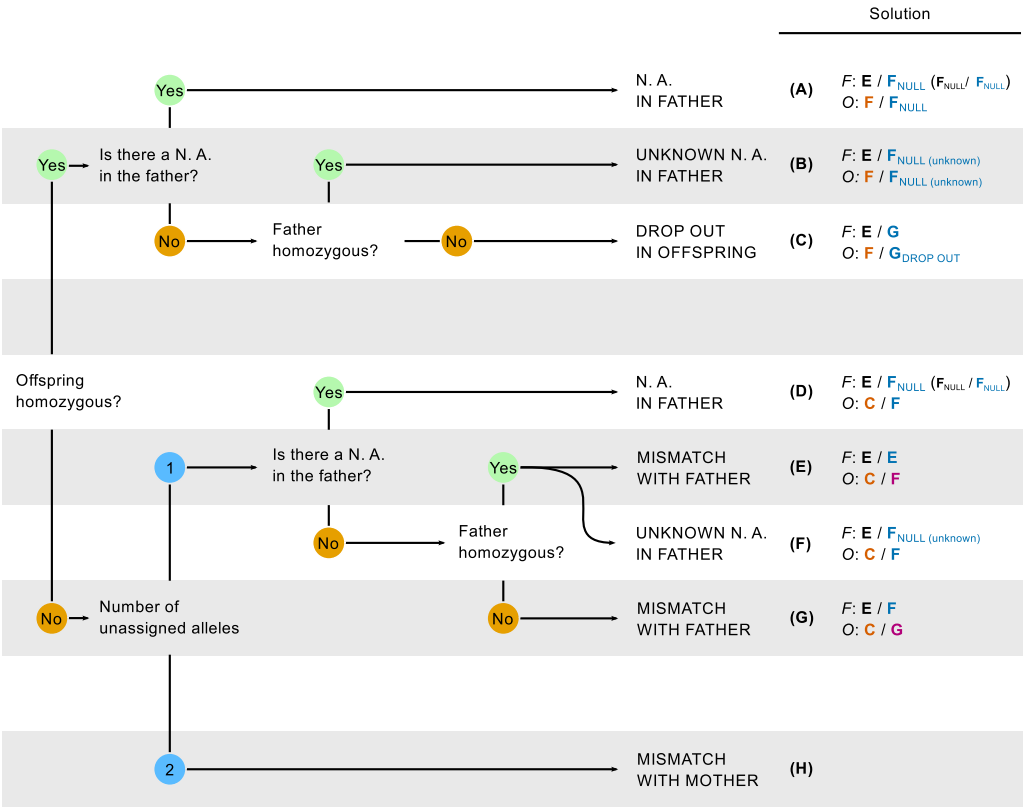


Figure A.2: *Decision tree followed to solve offspring-alleged father mismatches. Solutions show the respective mother (M) and offspring genotypes (O). Alleles from the mother are in orange, in blue or black those from the alleged father, and in purple the misassigned paternal alleles.*

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