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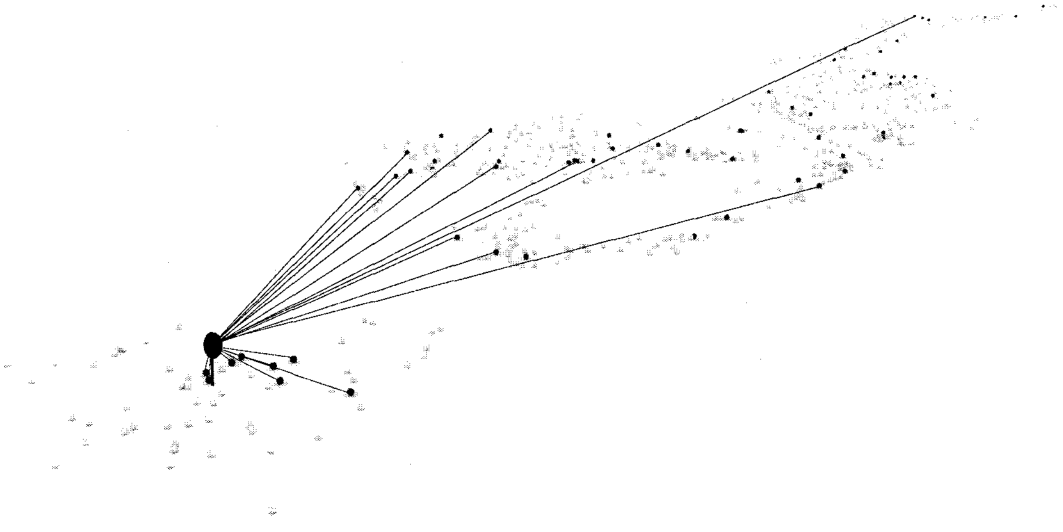
TESIS DOCTORAL

Patrones de dispersión de polen y semillas asistida por animales en una población de *Prunus mahaleb* (Rosaceae)

Cristina García



Estación Biológica de Doñana (CSIC) - 2006

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Integrative Ecology Group

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Memoria presentada por la Licenciada Cristina García Pérez para optar al título de Doctor en Biología por la Universidad de Sevilla

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La semilla que ha dado lugar a esta Tesis fue la colaboración entre mis directores de Tesis el Dr. Pedro Jordano y el Dr. Jose Antonio Godoy. La calidad y originalidad de sus trabajos anteriores, el interés de las preguntas que propusieron entonces (y siguen proponiendo), junto con su maravillosa intuición y acierto al incorporar las técnicas moleculares a los estudios de dispersión asistida por animales, hizo que dispusieran de la beca de formación predoctoral (FPI) concedida por el Ministerio de Ciencia y Tecnología. Gracias a esta beca puede desarrollar la Tesis Doctoral en la Estación Biológica de Doñana, cuyas instalaciones y personal hacen que el trabajo de Tesis Doctoral sea estimulante en todas las fases, campo, lab, escritura, discusión. En especial quiero agradecer a todos los miembros del Integrative Ecology Group (IEG) su ayuda durante todo este tiempo. Previamente, el director de mi tesina, el Dr. Fernando Valladares y algunos profesores muy admirados de la Facultad de Biología de la Universidad Complutense de la Facultad de Biología de la Universidad Complutense de Madrid, como el Dr. Jose Luis Tellería y el Dr. Luis Balaguer, me enseñaron aspectos fascinantes de la Biología y de la investigación científica. Mi agradecimiento a ellos, y por extensión, a todos los buenos docentes de las Facultades de Biología que despiertan y estimulan en sus clases el interés por la Biología y por la Ciencia.

Las técnicas moleculares aplicadas en esta Tesis se han realizado en el Laboratorio de Ecología Molecular (LEM). Su equipo está formado por doctorandos y técnicos cuya experiencia y profesionalidad me han enseñado mucho. En primer lugar, mi agradecimiento infinito a Juan Miguel Arroyo (Juanmi), el técnico del grupo que ha trabajo con nosotros durante años. Hemos sido muchos los que hemos aprendido de su profesionalidad, paciencia, generosidad, y constancia. Esta Tesis no hubiera sido posible, o hubiera sido peor sin duda, sin su ayuda. También me gustaría reconocer el esfuerzo de Cristina Rigueiro, actual técnico de laboratorio del grupo, su trabajo y esfuerzo por aprender técnicas nuevas y sacar adelante todo el trabajo en los últimos meses. Cuando el trabajo se acumuló, la ayuda de Pilar Bazaga y María González fue muy bienvenida. Muchas gracias a ellas y a todos los miembros del LEM con los que he compartido muchas horas de trabajo llenas de buen humor y compañerismo.

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Las estancias breves en *l'Institut National de la Recherche Agronomique* (2002) con la Dra. Sophie Gerber y en la *Universidad de California Los Ángeles* (UCLA) con Victoria Sork (2003) sirvieron para empezar a construir la historia que había detrás los datos que iban proporcionando el campo y el laboratorio. Muchas gracias a Dra. Sophie Gerber y a todo su equipo por enseñarme las técnicas de paternidad y sus aplicaciones. Se trata de uno de los laboratorios de genómica forestal más interesantes en los que cubren rango de técnicas y preguntas que favorecen la aproximación integrativa de la ecología. Por otro lado, la contribución de Victoria Sork ha sido muy fructífera. Gracias a ella pude contactar con Peter Smouse, cuya clarividencia con las técnicas de análisis de datos moleculares fue de una gran ayuda.

En el laboratorio, durante las estancias breves y en el campo se acumula mucha información sobre la que luego hay que pensar y estructurar en un manuscrito. Este proceso puede ser largo y muy arduo sin la ayuda de los directores de Tesis y de un grupo de personas que te ayudan a ver lo que tienes entre manos. Entre ellos, muchas gracias a Daniel García, Victoria Sork, Sophie Gerber, Katrin Böhning-Gaese, Alfredo Valido, Eva Albert, Jordi Bascompte, Jofre Carnicer, Abelardo Aparicio, Paco Rodríguez,

I am just now begining to discover the difficulty of expressing one's ideas on paper. As long as it consists solely of description it is pretty easy; but where reasoning comes into play, to make a proper connection, a clearness and a moderate fluency, is to me, as I have said, a difficulty of which i had no idea. (Charles Darwin)

Miguel Ángel Fortuna, Arndt, Rubén Sanz, Juanjo Arnuncio-Robledo. Además Peter Buston (Pete) corregió pacientemente mi pobre escritura inglesa nos ha compartido con nosotros alguna de las claves para escribir un buen artículo. Muchas gracias a Xavier Picó por proponerme escribir un revisión sobre las técnicas moleculares aplicados al estudio de la dispersión. Aprendí mucho durante el proceso y de su visión integradora de la Ecología. Tengo que agradecer a Richard Schnabel, Ran Nathan y Douglas Levey, Remy Petit y Victoria Sork (y a otros muchos referees anónimos) su labor constructiva como editores o referees de los manuscritos que hemos enviado. Afortunadamente la EBD recibe un gran número de visitas de otros investigadores que han sido muy útiles a la hora de discutir algunos puntos interesantes de la tesis. Gracias en especial a Eugene Shupp (Gino) y Janis (Manolita), Jens Ollensen, Paulo Guimares (Pauliño), Susan Kalisz, Kimberly Holbrook, Santiago González.

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1

Introducción General: Contexto ecológico y evolutivo del estudio de la dispersión en plantas

1. Una aproximación evolutiva al estudio de la dispersión de polen y semilla

La gran variedad de mecanismos que utilizan las plantas para dispersar el polen y las semillas ha interesado siempre a los naturalistas, que han reconocido su importancia en la formación y distribución de las especies vegetales (Fig. 1)(Darwin 1859, Darwin 1862, Wallace 1880, Carlquist 1966). Las grandes expediciones de los siglos XVIII y XIX a lo largo del mundo documentaron una amplia diversidad geográfica de formas florales, especies de polinizadores, tipos de frutos y modos de dispersión de las semillas (Hill 1883 a,b, Ridley 1930, van der Pijl 1969). A partir de entonces, cómo y por qué cambian espacial y temporalmente los procesos de dispersión de polen y semillas y sus consecuencias ecológicas y evolutivas han sido preguntas fundamentales de diferentes disciplinas biológicas.

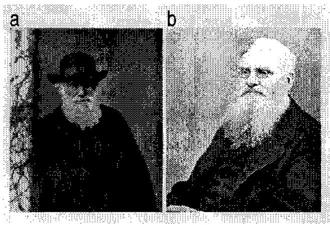

Figura 1. Charles Darwin (a) y Alfred R. Wallace (b) son los padres del paradigma evolutivo bajo el cual se estudian los patrones y procesos actuales e históricos que han dado lugar a la diversidad biológica. Ambos autores destacaron en sus obras el papel de la dispersión de polen y de semillas en la configuración de las especies y en su distribución geográfica.
Imágenes:
a. http://www.nhm.ac.uk/
b.http://pages.britishlibrary.net/charles.darwin/texts/more_letters/mletters2_fm.html

Los trabajos pioneros de Daniel H. Janzen (1970), Joseph Connell (1971) y John L. Harper (1967, 1977) integraron el estudio de la dispersión en un marco conceptual articulado entorno a dos aproximaciones: una ecológica y otra evolutiva. La aproximación ecológica identifica qué factores bióticos y abióticos interacionan con las plantas determinando los procesos de dispersión y cuáles son las consecuencias dinámicas de la dispersión para una especie y para la comunidad (cuál es el efecto la dispersión en el ciclo de reclutamiento de una especie y en el ensamblaje de las especies que componen una comunidad vegetal). La aproximación evolutiva dilucida y cuantifica las ventajas de dispersar el polen y las semillas para las plantas (en qué medida las plantas que dispersan su polen y/o sus semillas incrementan su *fitness* o eficacia biológica).

La dispersión del polen determina dos aspectos fundamentales de la biología de la reproducción: i) el tipo de sistema de cruzamiento (*mating system*); y b) los patrones de polinización (que determinan la identidad y diversidad de indivuduos que polinizan la progenie de una planta). El tipo de sistema de cruzamiento se define por el origen de la semilla (autopolinización, polinización cruzada o apomixis) (Barrett & Harder 1996). La cuantificación de la proporción de eventos de autopolinización (*selfing*) y los factores intrínsecos y extrínsecos que lo determinan han sido una de los cuestiones más importantes en biología de la reproducción (Darwin 1892, Barret 2003). Una frecuencia alta de selfing implica un vecindario genético espacial y númericamente reducido que se traduce en una una alta estructuración genética a pequeña escala (Loveless & Hamrick 1984, Nason *et al.* 1998) que puede dar lugar a divergencias morfológicas (pj. diseño floral) y funcionales (pj. fenología) entre diferentes poblaciones, o incluso, entre diferentes grupos de plantas dentro de una población. Por otro lado, desde el punto de vista del árbol productor de polen, los patrones de polinización determinan el número de eventos de polinización en los que participa un individuo (*male fertility*) que constitiye un componente importante de su fitness. Así, junto con la identidad genética, la localización espacial de los padres determinan el grado de intercambio genético (*gene flow*) via polen entre poblaciones. El estudio de la dispersión de polen ha contribuído ampliamente a la dilucidación de los factores ecológicos que determinan el sistema de cruzamiento y los patrones de polinización en las poblaciones de plantas (Godt & Hamrick 1993, Harder & Willson 1998). Finalmente, la dispersión de polen influye en la siguientes etapas demográficas afectando a la dispersión de semillas (Herrera *et al.* 1998, Davis *et al.* 2004); cuantificar y caracterizar esta interacción y conocer sus consecuencias para el reclutamiento de las plantas es uno de los objetivos principales de la ecología actual.

De forma análoga, el paradigma evolutivo atribuye a la dispersión de semilla un efecto positivo neto para el árbol madre. Janzen (1970) y Connell (1971) propusieron un modelo para explicar la gran diversidad de especies observadas en bosques tropicales, así como su baja densidad específica y distribución espacial no agregada (Fig. 2). Este modelo predice que la densidad de plántulas alcanza un valor máximo a cierta distancia de la madre (donde las semillas han sido depositadas tras la dispersión); a distancias más cortas la acción de herbívoros/patógenos específicos impediría la germinación exitosa, mientras que a distancias mayores la densidad de semillas sería demasiado baja como para asegurar un número de semillas reclutadas. Janzen (1970) también reconoció otra ventaja de dispersar semillas: el incremento de la oportunidad de colonizar nuevos sitios no ocupados por las plantas, aumentando las posibilidades de germinar y establecerse (Schupp 1988).

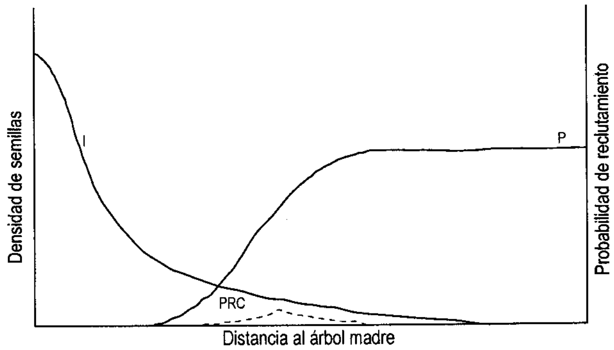

Figura 2. Esta figura ilustra el modelo propuesto por D. Janzen (1970). La densidad de semillas dispersadas disminuye exponencialmente con la distancia al árbol madre (I) mientras la actividad de los patógenos específicos que atacan las semillas dispersadas depende positivamente de la densidad. Por tanto, la probabilidad de reclutamiento de una semilla (P) es mayor al alejarse del árbol madre. Esto resulta en una mayor probabilidad de germinación exitosa (PRC), que es máxima a cierta distancia del árbol madre.
(Modificado de Janzen 1970).

Así, al intentar comprender la alta diversidad de especies de plantas que componen los bosques tropicales, Janzen (1970) situó la dispersión de semillas en una posición central del ciclo de reclutamiento. Es más, su importancia sólo se puede comprender conociendo la relación espacial entre procesos pre-dispersivos (producción de semilla y depredación de semillas no dispersadas), dispersión y procesos post-dispersivos (depredación y germinación de semillas y supervicencia de plántulas). Trabajos posteriores han confirmado esta hipótesis (Augspurger 1984, Schupp 1988), aunque otros han limitado su validez a unas pocas especies y a corta distancia del árbol madre (Condit *et al.* 1992). La importancia de los procesos dependientes de la distancia y de la densidad propuestos por el modelo de Janzen y Connell no se limita al ciclo de reclutamiento de una especie, sino que trabajos posteriores han extendido su efecto a la generación de comunidades hiperdiversas como los bosques tropicales (Connell *et al.* 1984, Hubbell *et al.* 1990, Harms *et al.* 2002).

El marco conceptual establecido tras estos trabajos mejoró la comprensión de los efectos de las interaciones planta-animal en el ciclo demográfico de las plantas con la integración de estudios de frugivoría. Estos trabajos incluían información detallada de la dieta de los frugívoros, de su patrón de movimiento y sus características fisiológicas (Snow 1971, Howe 1977). Estudios posteriores documentaron un incremento en las tasas de germinación de semillas dispersadas por vertebrados frente a no dispersadas, presumiblemente debido a un tratamiento químico/ mecánico durante la digestión (Howe & Smallwood 1982). Es decir, la dispersión por vertebrados frugívoros no sólo implica el traslado de las semillas desde la copa a un lugar a cierta distancia, donde la densidad de conespecíficos es menor, sino que el paso por el tracto digestivo del animal puede estimular la germinación (Traveset 1998). Además, estos frugívoros pueden trasladar gran cantidad de semillas a sitios donde la germinación para una planta es favorable, como las aperturas de los bosques tropicales (dispersión dirigida, Venable & Brown 1993, Wenny & Levey 1998, Wenny 2001). Los sistemas de dispersión de semillas planta-animal, por tanto, empezaron a considerarse resultado de un proceso de ajuste recíproco de las estrategias de la planta (dirigidas a la atracción de dispersores eficientes) y de los frugívoros (dirigidas a maximizar la obtención de nutrientes durante la búsqueda de alimento). Esta circunstancia ofreció la posibilidad de incorporar modelos de coevolución a los sistemas mutualistas de dispersión de semillas (McKey 1975), previamente aplicados al estudio del papel de las interacciones planta-animal en la diversificación y co-especiación en plantas e insectos fitófagos (Ehrlich & Raven 1964). Bajo esta perspectiva, el estudio del conjunto de interaciones planta-animal implicados en la dispersión de semilla planteó el paradigma especialista-generalista (Gauthier-Hion *et al.* 1985, Howe 1993). Esta hipótesis predice que las especies de plantas dispersadas por un conjunto pequeño de frugívoros especializados que realizan una dispersión favorable para la planta, producen frutos altamente nutritivos, mientras que aquellas especies dispersadas por frugívoros oportunistas producen frutos de menor valor nutritivo. Es decir, el conjunto de frugívoros que dispersan una especie no sólo afecta a la lluvia de semillas (*seed rain,* la distribución espacial de las semillas dispersadas respecto al árbol fuente y otros conespecíficos) y posteriores fases del ciclo demográfico, sino que determina las características morfológicas, estructurales y químicas de sus frutos en respuesta a los requerimientos de los frugívoros. Janzen (1970) incluso llega a argumentar que la dispersión por viento (anemocoria) podría ser una adaptación a la escasez de frugívoros que actúen de dispersores. Los datos de campo no siempre han confirmado la hipótesis del especialista-generalista (Howe 1993) ya que las consecuencias de las interacciones planta-animal son complejas y contexto dependientes. Esto es, se trata de sistemas que no están en equilibrio ni desde el punto de vista espacial (Jordano 1993, Thompson 1994), ni temporal (Herrera 1998), ni evolutivo (Jordano 1995).

2. De la regeneración de una especie a la formación de un bosque: los aspectos dinámicos de la dispersión

La ecología teórica ha prestado mucha atención a los mecanismos de dispersión de las plantas así como a sus consecuencias dinámicas para la comunidad, que enlazan con los trabajos de Janzen y Connell. Las hipótesis más relevantes han surgido a partir de la biogeografía de islas (MacArthur & Wilson 1967) y de la ecología de comunidades (Grubb 1977, Hubbell 2001, Harms *et al.* 2002). La biogeografía insular explica por qué el número de especies presentes en islas es menor que áreas continentales del mismo tamaño. MacArthur & Wilson (1967) proponen un modelo dinámico basado en el equilibrio entre procesos de colonización de nuevas especies y de extinción de especies locales. La colonización (que implica dispersión y establecimiento de individuos) depende fundamentalmente del grado de aislamiento del fragmento (a mayor distancia al continente, menor probabilidad de colonización de la isla o fragmento) y la extinción dependen negativamente del área (a menor área, mayor probabilidad de extinción). Por otro lado, la ecología de comunidades ha centrado parte de sus trabajos en dilucidar los mecanismos que explican la coexistencia de especies en comunidades hiperdiversas. Las dos propuestas más interesantes giran entorno al concepto de *nicho ecológico* (una comunidad ecológica consiste en un conjunto limitado de especies que coexisten en equilibrio demográfico y adaptativo alcanzado mediante un estricto reparto de los recursos limitantes, Levin 1970) y de *limitación de la dispersión* (una comunidad ecológica es un sistema abierto, en constante cambio y alejado del equilibrio, donde la presencia, abundancia o ausencia de las especies resulta de procesos de dispersión acoplados a un procesos de especiación, deriva ecológica y extinción, Hubbell 2001). Estas dos aproximaciones no son mutuamente excluyentes sino que se complementan; el reto actual no consiste en demostrar la validez de una frente a la otra, sino en establecer un marco conceptual que las unifique (Hubbell 2001).

La biogeografía de islas y la ecología de comunidades convergen en uno de los aspectos más intrigantes y relevantes desde el punto de vista ecológico de la biología de plantas: la dispersión a larga distancia, es decir, aquella en la que los propágulos se dispersan entre poblaciones distantes entre sí. Los eventos de colonización en islas median a través de la dispersión a larga distancia mientras que la baja frecuencia de este tipo de eventos conduce a una limitación de la dispersión que favorece la presencia de especies diferentes incrementando así la riqueza específica. Cuando observamos ambos escenarios desde un punto de vista histórico, la dispersión a larga distancia explica, por ejemplo, rápida expansión postglacial de algunas especies forestales y la distribución espacial de su diversidad genética (Cain *et al.* 2000, Hewitt 2000, Petit *et al.* 2003).

La gran relevancia de los eventos de dispersión a larga distancia, unida a la dificultad intrínseca de detectarlos y cuantificarlos por medios directos, ha hecho que la caracterización de la curva de dispersión (la curva que describe la frecuencia de eventos de dispersión en función de la distancia) sea uno de los objetivos más importantes de la ecología vegetal en las últimas décadas (Nathan 2006a). Hasta la llegada de técnicas moleculares basadas en marcadores hipervaribles, las aproximaciones indirectas han sido las más exitosas. El ajuste de datos de densidad de semillas dispersadas o de las plántulas germinadas mediante modelos fenomenológicos proporciona estimas de dispersión real y effectiva, respectivamente (Fig. 3). Estas curvas de dispersión han puesto de manifiesto que la frecuencia de eventos de dispersión a larga distancia es mayor de la que hasta entonces se suponía en poblaciones de plantas (Portnoy *et al.* 1993, Ribbens *et al.* 1994, Clark *et al.* 1999, Jones *et al.* 2005, Hardesty *et al.* 2006). La dificultad de ajustar curvas de dispersión consiste en encontrar un modelo que estime correctamente tanto el primer tramo de la curva como la cola. Aunque las curvas de dispersión están sesgadas a la derecha (lo cual implica una mayor frecuencia de eventos a corta distancia que lo esperado respecto a una curva normal), los modelos mecanicistas tienden a sobre-estimar el número de eventos a corta distancia. Sólo aquellos de cola larga (*"fat-tailed"*), como los modelos exponenciales o el modelo 2Dt, son capaces de estimar correctamente la frecuencia de eventos a larga distancia.

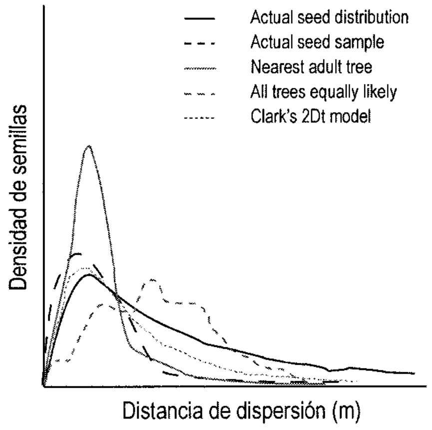

Figura 3. Diferentes distribuciones de la frecuencia de las distancias de dispersión (curvas de dispersión). Cuando no es posible obtener de forma directa las distancias de dispersión se puede recurrir a modelos mecanicistas para ajustar la distribución de la densidad de semillas. La precisión de las distancias estimadas depende de la calidad del diseño de muestreo y del modelo utilizado. Además sólo aquellos que presentan una cola larga son adecuados para estimar eventos de dispersión a larga distancia, como el modelo 2Dt de Clark (Clark *et al.* 1999). Otros factores pueden añadir complejidad a la curva de dispersión, como son la heterogeneidad espacial, la disposición relativa de los árboles y de los puntos de muestreo o el movimiento no aleatorio (selectivo) de los frugívoros que dispersan las semillas.
(Modificado de Nathan & Muller-Landau 2000)

Por otro lado los modelos mecanicistas modelizan el comportamiento del agente de dispersión proporcionando una aproximación causal del patrón de dispersión de polen y semillas (Okubo & Levin 1989, Nathan *et al.* 2002, Westcott *et al.* 2005). El reto pendiente, sin embargo, es la obtención de curvas de dispersión por aproximaciones directas, esto es, basadas en el seguimiento directo de los propágulos, que cuantifiquen de forma fiable el número de eventos de dispersión a larga distancia (Ouborg *et al.* 1999, Cain *et al.* 2000, Godoy & Jodano 2001, Nathan 2006a).

3. La dispersión de polen y semilla desde otras aproximaciones: la genética de poblaciones

La genética de poblaciones estudia los procesos actuales e históricos que han determinado las características genéticas de una población y el efecto de factores ecológicos que actúan sobre ellos. Entre estos procesos está la dispersión de individuos que da lugar un flujo de genes que determina (junto con la colonización, los cuellos de botella, el efecto fundador y la deriva génica) la variabilidad genética de las poblaciones naturales y su distribución espacial (Heywood 1991). En las plantas, el flujo génico tiene lugar durante la polinización y la dispersión de semillas, por lo tanto, es importante conocer ambos procesos e integrarlos junto con el aspecto espacial y demográfico (o númerico) de la dispersión. Estas tres dimensiones de la dispersión han sido raramente combinadas en un mismo estudio (Jordano & Godoy 2002), en parte porque falta un marco de conceptual común a ecólogos y genéticos que permita unificar ambas aproximaciones (Sork *et al.* 1999, Cain *et al.* 2000, Nathan & Muller-Landau 2000, Wang & Smith 2002). La contribución de la genética de poblaciones es doble (y necesaria): aporta herramientas que permiten el seguimiento de los eventos de dispersión (incluídos aquéllos a larga distancia), y por tanto, permite caracterizar de forma detallada los procesos de dispersión; además, incorpora modelos genéticos y estadísticos que permiten evaluar los patrones espaciales que resultan de los procesos de dispersión (Wright 1943, Slatkin 1985, Epperson 2003).

4. Limitaciones técnicas del estudio de la dispersión

Ciertamente, una de las preocupaciones comunes en los trabajos sobre dispersión de polen y de semilla, ha sido las limitaciones técnicas que han impedido trazar el movimiento de cada evento de dispersión de polen y de semillas, especialmente cuando se trata de dispersión de propágulos entre poblaciones (Bossart & Prowell *et al.* 1998, Ouborg *et al.* 1999, Cain *et al.* 2000, Nathan 2006a). La introducción de avances técnicos para trazar el movimiento e identificar el origen del polen y de las semillas ha contribuido a dilucidar los patrones de dispersión y caracterizar el destino de las semillas después de ser dispersadas. Así, el uso de marcaje fluorescente (Levey & Sargent 2000), radiotransmisores y marcadores radioactivos (Primack & Levy 1988, Vander Wall 1992) y, últimamente, los marcadores moleculares (Godoy & Jordano 2001) han posibilitado la obtención de datos que resuelven preguntas largamente planteadas en los estudios de dispersión. Entre ellas, cabe destacar la caracterización de la sombra de semillas en dirección y distancia (*seed shadow*, Janzen 1970, Willson 1993), la asignación de eventos de dispersión a la población de origen (Godoy & Jordano 2001), la cuantificación de la variación espacial y temporal de los patrones de dispersión (Kalisz *et al.* 1999) y las consecuencias a corto y largo plazo de cada mecanismo de dispersión en la dinámica poblacional (Álvarez-Buylla & García-Ramos 1991, Clark *et al.* 1999, Petit *et al.* 2003). Las técnicas moleculares, por tanto, han impulsado los estudios de dispersión en plantas integrando diferentes los aspectos ecológicos, biogeográficos y genéticos de la dispersión (Hewitt 2000).

5. ¿Qué sabemos actualmente de la dispersión de polen y semilla?

El marco conceptual previamente expuesto y el conjunto de las técnicas y métodos de análisis adecuados nos ha proporcionado una extensa información sobre la dispersión de polen y semillas en poblaciones naturales. Los aspectos fundamentales mejor resultos son:

I. La dispersión de polen y de semillas son dos procesos centrales en el ciclo de reclutamiento de las plantas, especialmente la dispersión de semillas que conecta numérica, espacial y genéticamente las etapas de reproducción con las de reclutamiento (Janzen 1970, Augspurger 1984, Clark & Clark 1984, Estrada & Fleming 1986, Murray 1986, Schupp 1992, Fleming & Estrada 1993, Howe 1993, Schupp & Fuentes 1995, Jordano & Herrera 1995).

II. La interacción de procesos predispersivos y postdispersivos es compleja. Por ejemplo, la dispersión de semillas no tiene un único destino, (germinar tras ser depositada por un frugívoro desde el árbol madre), sino que varios procesos pueden ocurrir hasta la germinación: dispersión secundaria, dormición, depredación (Forget *et al.* 2005).

III. La frecuencia de ocurrencia de estos procesos es contexto dependiente y variable entre años. Por ello, es necesario que, en conjunto, los estudios de dispersión abarquen diferentes ecosistemas, escalas espaciales y temporales y diferentes especies de plantas y de dispersores (Fleming & Estrada 1993, Levey *et al.* 2002, Forget *et al.* 2005).

IV. El seguimiento directo de los eventos de dispersión de semillas ha mostrado una compleja interacción de procesos predispersivos, dispersivos y postdispersivos, que a veces resultan desacoplados (Schupp 1995, Jordano & Herrera 1995, Forget *et al.* 2005).

V. El carácter idiosincrático de los efectos de las interacciones planta-animal que implican dispersión de polen y semilla hace que no puedan ser interpretadas desde la dicotomía especialista-generalista. Es necesario un análisis que diseccione el componente cualitativo y cuantitativo del efecto de cada frugívoro sobre los patrones de dispersión y sobre el ciclo de reclutamiento de la población, ilustrando un continuo entre los extremos del gradiente de generalización (Schupp 1993).

VI. Las consecuencias de las interacciones mutualistas para un ecosistema sólo se pueden abordar estudiando las interacciones *per se*, y no sus componentes por separado (estrategias de las plantas *vs.* estrategias de los frugívoros) (Bronstein 1994, Jordano *et al.* 2003, Westcott *et al.* 2005).

VII. La evidencia empírica sugiere que los mutualismos planta-animal, incluídos aquellos implicados en la dispersión de polen y semillas, participan en la formación y estructuración de la diversidad biológica, aunque el mecanismo todavía está muy discutido. Actualmente, la aproximación más completa, integradora y mejor articulada explica que la intensidad y tipo de interacciones planta-animal varían temporal y espacialmente al cambiar contexto ecológico (Thompson 1994). Estas interaciones, como las poblaciones donde se encuentran, están sujetas a dinámicas de colonización-extinción y dispersión (flujo génico) entre diferentes núcleos de población (dinámica metapoblacional). Este paisaje heterogéneo donde tienen lugar las interacciones da lugar a un espacio multidimensional donde las consecuencias de las interaciones planta-animal no son únicas sino un subconjunto de las muchas posibles, que finalmente se estructuran espacialmente en forma de mosaico geográfico. (Thompson 1994, 2005).

En definitiva, el estudio de la dispersión de polen y semillas, especialmente aquella asistida por animales, nos ha ayudado a comprender el ciclo de reclutamiento de las poblaciones de plantas y su complejidad (Fig. 4a), a explicar cómo se regenera un bosque y qué factores y procesos determinan la coexistencia de las especies vegetales en el tiempo y espacio (Fig. 4b), y a entender el papel ecológico y evolutivo de la dispersión y de las interaciones planta-animal en la formación y distribución de la diversidad biológica (Fig. 4c).

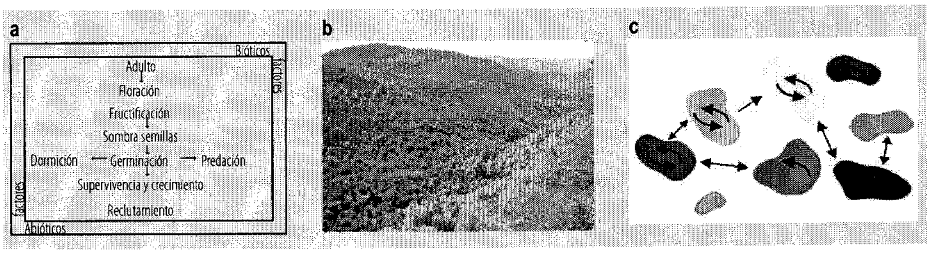

Figura 4. El estudio de la dispersión de polen y semilla, especialmente aquélla asistida por animales, ha contribuido a entender preguntas clave en Ecología y Biología Evolutiva. Esta figura ilustra tres de ellas: a) el ciclo demográfico de las plantas, en el que la dispersión es una de las etapas limitantes de la regeneración de las poblaciones; b) los procesos espaciales y temporales que conducen a la formación de sistemas multiespecíficos; y c) cómo se produce y organiza espacialmente la diversidad biológica. a, Modificado de Schupp & Fuentes (1995); b, Imagen, Sierra de Cazorla, Segura y las Villas; c, Modificado de Thompson (2005).

6. Patrones de dispersión de polen y semilla en una especie polinizada y dispersada por animales, *Prunus mahaleb* (L.)

El contexto ecológico y evolutivo descrito anteriormente enmarca el presente trabajo de tesis doctoral y, por tanto, comparte con él conceptos, preguntas, objetivos y métodos. El objetivo general de este trabajo es el estudio de los patrones de dispersión de polen y semilla generados por animales y sus consecuencias en el flujo genético entre y dentro de poblaciones. Este trabajo representa el primer paso hacia el objetivo más amplio y ambicioso: comprender los patrones de reclutamiento en poblaciones de plantas aisladas y ecológicamente heterogéneas, dispersadas por animales. Para ello, debemos conocer primero la contribución relativa de cada etapa demográfica en el reclutamiento final así como las interaciones entre ellas (Schupp & Fuentes 1995, Nathan & Muller-Landau 2000, Wang & Smith 2002). Este trabajo incluye algunos de los avances recientes en los estudios de dispersión de plantas y se estructura en los siguientes capítulos, que corresponden a trabajos publicados o en vías de publicación:

Capítulo 2: An unified framework for the study of dispersal in plant populations. Este capítulo intenta unificar las perspectivas ecológicas y evolutivas en el estudio de la dispersión. Se exponen las tres ideas fundamentales que articulan un espacio común junto con los principales métodos utilizados para abordar cuestiones de interés para las dos aproximaciones. Este capítulo nace de la reflexión de que los aspectos espaciales, demográficos y genéticos están íntimamente ligados a través de la dispersión. A diferencia de trabajos previos (Lande 1988), este capítulo propone una aproximación integradora al estudio de la ecología de poblaciones que nosotros hemos centrado en la dispersión. Este trabajo está en revisión en *Evolutionary Ecology* como *Invited Review Paper*

Capítulo 3: Mating patterns, pollen dispersal, and the ecological neighbourhood in a *Prunus mahaleb* (L.) population. En este capítulo nos centramos en una de las etapas previas a la dispersión de semilla en el ciclo de reclutamiento: la dispersión de polen. Mediante técnicas genéticas y análisis multivariante intentamos dilucidar cómo varían los patrones de dispersión de polen entre individuos de una misma población caracterizada por su heterogeneidad ambiental. El vecindario materno, esto es, la estructura del paisaje alrededor del árbol madre, determinó significativamente las características del *pool* de polen que recibió cada árbol madre. Este trabajo está publicado en *Molecular Ecology* (2005) 14, 1821-1830.

Capítulo 4: Contemporary pollen and seed dispersal in a *Prunus mahaleb* population: patterns in distance and direction. Una de las preguntas más difíciles de responder hasta la fecha se refiere a la cuantificación de la contribución relativa del polen y de la semilla al flujo génico total que recibe una población. En este trabajo lo hemos cuantificado por métodos directos. Además, los patrones de dispersión locales (dentro de población) han sido caracterizados en distancia y dirección, dos aspectos poco documentados (Willson 1993).

Trabajos previos con especies dispersadas polinizadas por viento y sin mecanismos especiales para dispersar sus semillas han encontrado un alto porcentaje de flujo génico vía polen. Nuestro trabajo, con una especie polinizada por insectos y dispersada por vertebrados (endozoócora) muestra un patrón contrario: los genes entran en la población fundamentalmente vía semilla. Este trabajo está en prensa en *Molecular Ecology* (2006) **15**, 000-000.

Capítulo 5: Spatial and maternal source tree correlations in a frugivore-generated seed rain. Los aspectos genéticos de la dispersión han sido poco explorados en la literatura, no así los numéricos y espaciales. La íntima relación entre las tres dimensiones de la lluvia de semillas (*seed rain*, la distribución espacial de las semillas dispersadas respecto al árbol fuente y otros conespecíficos) reclama el estudio de las correlaciones genéticas entre semillas dispersadas en paisajes heterogéneos por frugívoros. Nuestros datos muestran un proceso de limitación genética por el cual los árboles maternos no acceden a todos los sitios disponibles en el paisaje. Este proceso de limitación completa otros procesos de limitación asociados a la dispersión, como el de limitación numérica y espacial (Jordano & Godoy 2002). Por otro lado, el la distribución de los genotipos maternos que resulta de la dispersión por frugívoros, captura la heterogeneidad de la población. Es decir, existe una asociación no aleatoria entre genotipo materno y microhabitat donde es depositado. Esta asociación está relacionada con las características del vecindario materno. La interacción entre frugivoría, heterogenidad del paisaje y origen materno de las semillas crea un lluvia de semillas genéticamente estructurada con tres patrones claramente distinguibles: i) aquellos caracterizados por una alta riqueza de madre, baja correlación maternal y niveles bajos de parentesco materno; ii) aquellos con una baja riqueza de madres, alta correlación materna y parentesco materno; iii) aquellos con una alta riqueza de madres, pero que registran valores altos de relatedness materno debido a eventos de dispersión a larga distancia desde árboles cercanos. Actualmente este trabajo está en revisión.

Capítulo 6: Differential contribution of frugivores to complex seed dispersal systems. La gran cantidad de sistemas de dispersión estudiados han puesto de manifiesto su complejidad; en la mayoría de ellos participan diferentes vectores de dispersión (Nathan 2006b). Cuando las semillas son dispersadas por diferentes especies de frugívoros, la curva de dispersión de semillas (*seed dispersal curve*, la frecuencia de eventos de dispersión respecto a la distancia al árbol madre) resulta de la suma de cada una de las curvas de dispersión de semillas generada por cada especie de frugívoro. La literatura que documenta la contribución relativa de cada frugívoro a la sombra de semillas es escasa, a pesar de ser un aspecto clave para evaluar la importancia relativa de cada especie de dispersor sobre la planta dispersada. Nuestro objetivo por tanto, se centra estimar la contribución relativa de cada tipo de frugívoro a la curva de dispersión de semillas del *P. mahaleb*, incluyendo eventos de dispersión a larga distancia. Actualmente este trabajo está en revisión.

Capítulo 7: Discusión general y conclusiones. En este capítulo se sintetizan y discuten en un marco más amplio los resultados y conclusiones de cada uno de los capítulos anteriores.

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The contribution of molecular markers to the study of dispersal in plant populations:

Recent advances and perspectives

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Abstract

Pollen and seed dispersal are two key processes involving genetic, demographic and spatial changes in plant populations. The incorporation of highly polymorphic molecular markers based on DNA along with the parallel improvement of analytical methods dealing with the genetic information have provided new valuable insights into the study of dispersal processes in plants. Here I review the most successful applications of molecular markers for reliable assessment of dispersal, the consequences of dispersal processes, the mechanisms of dispersal, and the identification of factors shaping dispersal. Among the merits of highly polymorphic molecular markers are: i) the ability to work directly with the genotype instead of phenotypes; ii) providing unique multilocus genotypes for each individual; iii) allowing the estimation of relatedness among individuals, groups or populations; iv) ability to assess neutral variation, since some types of molecular markers are located in non-coding regions. Molecular markers have greatly improved our understanding of contemporary pollen and seed dispersal processes by providing reliable estimates of dispersal curves, including long distance dispersal events. Moreover, the pollen and seed contributions to total gene immigration can be dissected. Hence, the genetic dimension of the seed rain (i.e., how do the maternal genotypes distribute progeny over the landscape?) can be addressed. The estimation of direct measures of pollen dispersal patterns and relatedness among individuals contributed valuable insights on mating system variation. Hence, by characterizing dispersal processes, we can assess their effect on recruitment patterns, an issue especially interesting when dispersal involves mutualistic interactions between plants and animals. I overview the main techniques and analytical methods applied to the study of dispersal processes, with an emphasis on AFLP, SSRs, and SNPs, the molecular markers more frequently used. Discussion of these techniques and methods within an ecological framework leads to an enumeration of the main drawbacks for molecular markers to address some relevant questions dealing with dispersal. Some widely recognized limitations are: the difficulty of getting high quality and reliable data sets, the lack of accurate statistical support to address some key aspects involving dispersal (and especially those involving long distance dispersal), and the need of highly time- and money-consuming lab protocols.

Conceptually, I highlight dispersal as a demographic, spatial and genetic process that should be referred to the whole recruitment cycle and approached with spatially-explicitly methods. Molecular markers already have a central place within this framework and they are likely to continue improving our understanding of the relevance of dispersal processes in plants in combination with other different technical, analytical, and conceptual efforts. Finally, I outline some future venues that along with molecular markers will contribute to impulse dispersal studies.

Key words: AFLPs, dispersal assessment, gene flow, long distance dispersal, mating system, microsatellites, pollen dispersal, recruitment patterns, scoring errors, seed dispersal, SNPs,

1. The study of dispersal in plant populations

The ample variety of plant dispersal structures and mechanisms have largely amazed biologists, who recognized the ecological and evolutionary importance of dispersal processes, especially those mediated by seeds (Darwin 1859, Ridley 1930, MacArthur & Wilson 1967, Janzen 1970). Dispersal stems the genetic, demographic and spatial characteristics of plant populations. By increasing the probability of recruitment, seed dispersal determines the fitness of fruiting trees within a population (Janzen 1970, Harper 1977). It also plays a relevant role in determining the recruitment patterns in populations (Augspurger 1983, Condit *et al.* 1992, Schupp 1993, Harms *et al.* 2002), the genetic composition of plant populations and their spatial distribution (Hamrick *et al.* 1993), the species range distribution (Hewitt 2000, Petit *et al.* 2003), the assemblage of species in species-rich communities (Hubbell 2001), and the geographic dynamics of plant-animal coevolution (Thompson 1994, Thompson 2005). On the other hand, pollen dispersal defines mating systems by determining the number and genetic identity of siring fathers (Levin 1988). Hence, dispersal is central to most of the leading ecological and evolutionary frameworks in the last century aiming at understanding how biodiversity is formed, maintained and spatially distributed.

Dispersal studies have greatly benefited with the incorporation of molecular markers by providing a highly useful set of tools to track contemporary and historical dispersion and migration in plants and by integrating plant population genetics theory into an ecological framework (Berry *et al.* 1992, Hamrick & Nason 1996, Sork *et al.* 1999, Bullock *et al.* 2002, Jordano & Godoy 2002, Wang & Smith 2002). Three characteristics make highly polymorphic markers a suitable tool for dispersal studies in plant populations: i) their high polymorphism allows unique multilocus genotypes for each study individual to be obtained; ii) since they are located in the genome, genetic variation can be accessed directly; additionally, their inheritance from parents to offspring allows the assessment of genetic relatedness among populations or even among individuals within populations; iii) some of them are subject to selection whereas others are neutral, allowing an accurate dissection of how local selection and dispersal processes set spatial genetic patterns.

Probably, the aspects better understood by the application of molecular markers refer to the estimation of dispersal distances, the quantification of pollen and seed immigration (Dow & Ashley 1996, Nason *et al.* 1998, Hamilton 1999, Streiff *et al.* 1999, Godoy & Jordano 2001, Sork *et al.* 2002, Oddou-Muratorio *et al.* 2005, Grivet *et al.* 2005, Robledo-Arnuncio & Gil 2005) and their relative contribution to overall gene flow among populations (McCauley 1994, Ennos 1994, Latta *et al.* 1998, García *et al.* 2006a). Moreover, reproductive biology has greatly benefited by the possibility of identifying the pollen source tree to characterize mating systems in detail (who mates whom) and to quantify male reproductive success and the factors determining its variation (Brown 1990, Broyles & Wyatt 1991, Meagher 1991, Snow & Lewis 1993, Burczyk *et* *al.* 1996, Kaufman *et al.* 1998). The direct estimation of both pollen and seed dispersal helps elucidating the frequent correlations between the type of mating system, dispersal mode and the spatial genetic structure (Govindaraju 1988, Hamrick *et al.* 1993). Finally, the possibility of assigning dispersal events to their source popualtion yields contemporary rates of dispersal to explain the colonozation dynamics (Levin 1988, Olivieri *et al.* 1990, Waser & Strobeck 1998), and the historical routes leading to recolonization processes following glaciations (Hewitt 2000, Liepelt *et al.* 2002, Petit *et al.* 2003).

The success of molecular markers so far in elucidating long-standing questions in dispersal studies and setting a common ground for ecologists and geneticists to meet has been highlighted by some authors (Cruzan 1998, Ouborg *et al.* 1999, Avise 2004), whereas others tend to consider it as still awaiting for major contributions (Nathan 2006a) and for an actually unified framework to emerge (Silvertown 2001). Both views of the current state of the art are actually valid: it depends on whether we consider the glass half-full or half-empty. Thus, it is time to review the most successful applications of molecular markers to dispersal studies in plant populations, the main conceptual contributions that bridged plant ecology and plant genetics along with the drawbacks that prevent the great potential of molecular techniques to fully come along.

2. The half-full glass: what have molecular markers taught us about dispersal in plant populations?

Molecular markers have provided valuable insights into several aspects related with dispersal processes in plant populations namely: a) reliable assessment of dispersal; b) causes of dispersal; c) consequences of dispersal; and d) ecological factors shaping dispersal.

Reliable assessment of dispersal

Reliable assessment of dispersal distances has been tough to achieve, especially for long distance dispersal events (Oubourg *et al.* 1999, Cain *et al.* 2000, Nathan 2003). By providing unique multilocus genotypes for each individual in the population, molecular markers allow tracking pollen and seed dispersal events. This involves two main advances in dispersal studies: the reliable quantification dispersal curves at the popoulation level, in terms of distance and direction (Godoy & Jordano 2001, Grivet *et al.* 2005, Jones *et al.* 2005, Robledo-Arnuncio & Gil 2005, García *et al.* 2006a, Jordano 2006), and the straightforward characterization of individual seed shadows and the frequency of long-distance dispersal events. Thus, by inferring the population source of each dispersal event, we can estimate the percentage of immigrants and quantify the relative contribution of pollen and seed vectors to overall immigration (García *et al.* 2006a). Thus, contemporary pollen and seed dispersal patterns within and among populations can be assessed in detail. These studies have evidenced so far that:

i. Dispersal curves are fat-tailed and complex: By providing unique multilocus genotypes of the source trees and the propagules in a mapped population we can obtain dispersal curves (i.e., the frequency distribution of all dispersal distances) and directional histograms depicting pollen and seed dispersal patterns. Most previous studies considered gene flow in plant populations as highly restricted (Levin & Kerster 1974, Harper 1977), but the first studies applying molecular markers (allozymes) showed that both pollen and seed dispersal (the vectors of gene flow) can reach much longer distances than previously assumed (Schaal 1980, Handel 1983, Ellstrand *et al.* 1989, Schnabel & Hamrick 1995). Moreover, the application of highly polymorphic molecular markers based on DNA provides reliable seed and pollen dispersal curves (Dow & Ashley 1998, Godoy & Jordano 2001, Grivet *et al.* 2005, Robledo-Arnuncio & Gil 2004, Oddou-Muratorio *et al.* 2005, García *et al.* 2006a). As expected, these curves are highly leptokurtic (markedly biased towards short distance dispersal events), but they also show that dispersal events at certain distance from the source tree (e.g., above 100 m) are not infrequent and that extreme distance events can occur. Interestingly, as more data are available, a wide spectrum of dispersal curves becomes evident, besides the usually reported exponentially decaying frequency of dispersal events with increasing distance from the source tree. Rather, dispersal curves might present additional peaks involving a high frequency of dispersal events far away from the source trees. Thus, García *et al.* (2006a) reported a seed dispersal curve with a second peak located at 225 m from the source tree in a *Prunus mahaleb* population, a fleshy-fruited species dispersed by frugivorous vertebrates. The highly non-random foraging patterns are likely to yield complex dispersal curves since dispersal patterns are strongly influenced not only by distance, but also by the arrangement of the different patches and source trees within the population. Previous studies showed complex seed dispersal curves based on direct observations of frugivores feeding patterns and postfeeding movements (Gómez 2003, Wescott *et al.* 2005); however, tracking dispersal events by direct observations is not always feasible in natural populations and it does not provide an estimate of the frequency of immigrant propagules (dispersed from other populations). By applying molecular markers, reliable dispersal curves can be obtained for a wider range of ecological scenarios, and thus, a more comprehensive understanding of dispersal patterns can be obtained.

ii. Dispersal curves present marked directions: Tracking the dispersal events within a spatially-explicit approach yields 2D-dispersal curves (i.e., combining distance and direction). This provides highly valuable information since dispersal is an intrinsically spatial process occurring, at least, in two spatial dimensions (Ennos 2001). Additionally, dispersal agents (either biotic or abiotic) rarely distribute propagules evenly in all directions. Thus, pollen dispersal by wind resulted highly anisotropic (variable with direction) in an isolated population of *Pinus sylvestris* (Robledo-Arnuncio & Gil 2004) due to the uneven distribution of the adult trees and the difference on male reproductive success. Similarly, a marked directionality was observed in the previously mentioned study with *P. mahaleb*, where an increased number of pollen dispersal events along the NS axis of the population and a high frequency of seed dispersal events following SE-SW directions were reported (García *et al.* 2006a). Marked directionalities set by pollen and seed dispersal might persist through different demographic stages and have a lasting signal in the local genetic structure, as reported by Dutech *et al.* (2005) that found a spatial genetic structure in the adult population of *Quercus lobata*, highly consistent with prevailing wind-directions.

iii. Dispersal among populations is not infrequent and might be driven by seed rather than pollen dispersal: The ability to identify long distance dispersal events has demonstrated a non-negligible frequency of pollen and seed immigration events (dispersal events among populations) (Nason *et al.* 1998, Hamilton 1999, Cordeiro & Howe 2003, Jones *et al.* 2005, Hardesty *et al.* 2006, Jordano 2006). Working with genomes differing in the mode of inheritance (paternal, such as the chloroplastic genome in gymnosperms; maternal, such as the mitochondrial genome; or biparental, such as the nuclear genome) allows estimating the relative effective contribution of pollen dispersal *vs.* seed dispersal given a model based on F_{st} values of population differentiation (Ennos 1994). Pollen has been reported to be the main vector connecting fragmented populations for anemophilous species with no special devices for seed dispersal, or with distance-limited dispersal (e.g., rodent caching), such as *Quercus* spp., *Pinus* spp., and *Fagus* spp. (Ennos 1994, Latta *et al.* 1998, Sork *et al.* 2002). However, immigration might be mainly driven in endozoochorous species by large- to medium-sized frugivores that can move seeds over kilometers (Fragoso 1997, Holbrook *et al.* 2002), allowing an extensive interchange of genes via seed (García *et al.* 2006a, Pairon *et al.* 2006). Recent studies have reported extensive seed dispersal among populations in fragmented scenarios of fleshy-fruited species by the agency of frugivores (Cordeiro & Howe 2003, Bacles *et al.* 2004, Oddou-Muratorio *et al.* 2005). The use of microsatellites to identify the pollen donors and seed source trees has shown a higher contribution of seed dispersal events to actual gene immigration (García *et al.* 2006a) and to effective gene immigration (Bacles *et al.* 2006). The levels of seed and pollen immigration determine the demographic and genetic connectivity among fragmented populations, having pervasive consequences on the recruitment dynamics and the genetic structure of isolated populations. Molecular makers are, up to date, the most accurate tools we have for its study (Sork & Smouse 2006).

The consequences of dispersal processes

i. Pollen dispersal patterns result in a wide variation of mating system traits: Different mating systems show variable proportions of mating events and individual fertility among adult trees and their fine identification is required to gain insights into one long-standing evolutionary question: why are there so many different mating systems? (Darwin 1896, Barrett & Harder 1996, Bernasconi 2004). The retrospective study of mating systems (i.e., the reconstruction of mating events based on the maternal progeny) is only possible with molecular markers, since that allows the estimation of relatedness among individuals (Brown 1990, Barrett & Harder 1996). The study of mating systems with molecular markers has a long tradition (reviewed in Brown 1990, Snow & Lewis 1993). The application of allozymes resulted useful when comparing mating systems among species and related them with life history traits such as floral morphology or pollination syndromes (see Cruzan 1998, Snow & Lewis 1993). The exploration of mating system variability among individuals within populations due to ecological factors was not actually possible until the incorporation of highly polymorphic and neutral molecular markers providing multilocus individual genotypes. As pointed out by Ritland (2002), mating systems are highly dynamic and subject to the influence of ecological factors such as pollinator abundance or density of conspecifics (Stacy *et al.* 1996), resulting in frequent extensive individual variation, observed even at the flower level. By allowing a direct estimation of selfing (within individual mating events), inbreeding (mating among relatives), and correlated paternity (frequency of maternal half-sib progeny resulting from the same siring tree) at different biological scales (among species, populations, individuals or flowers) molecular markers allow to explore the ecological factors shaping mating aspects (Cruzan 1998).

Non-random pollen dispersal is one of the ecological factors causing wide individual mating variation, both for wind- and insect pollinated species (Broyles & Wyatt 1991, Schnabel & Hamrick 1995, Burczyk *et al.* 1996, Stacy *et al.* 1996, Kaufman *et al.* 1998, Robledo-Arnuncio *et al.* 2004, García *et al.* 2005). Hence, molecular markers link the study of pollen dispersal patterns with the study of mating systems, an elusive task although urgently demanded to fully understand the evolutionary consequences of ecological factors shaping matting patterns (Barrett & Eckert 1999). When incorporating pollen dispersal patterns to mating studies, it becomes evident that restricted or strongly anisotropic pollen movements result in non-random mating among individuals within a population, generating ample variation in selfing rates and male reproductive success (Broyles & Wyatt 1991, Meagher 1991, Godt & Hamrick 1993, Burczyk *et al.* 1996, Dow & Ashley 1998, Kaufman *et al.* 1998, Stacy *et al.* 1996, He & Smouse 2002, Ritland 2002, Vassiliadis *et al.* 2002). Finally, molecular markers are also useful to assess spatial genetic structure in the adult population. Interestingly, several studies correlate pollen dispersal patterns and mating traits with the level of spatial genetic structure, evidencing that pollen dispersal has pervasive consequences in plant populations (Hamrick & Loveless 1987, Govindaraju 1988). Therefore, mating studies gain valuable insights due to the ability of molecular markers to: i) track pollen dispersal events by identifying the source tree (Meagher 1986); and ii) characterize mating patterns both at population and individual level based on the relatedness among maternal progenies (Clegg 1980, Ritland 2002).

ii. The unraveled aspects of the seed rain: Since the seminal works by Janzen (1970) and Connell (1971) seed dispersal has been considered as a key process within the recruitment cycle. Subsequent studies reported the numeric and spatial patterns observed in the seed rain as a consequence of dispersal (Debussche *et al.* 1994, Kollman & Pirl 1995, Loiselle *et al.* 1996, Nathan & Muller-Landau 2000, Jordano & Schupp 2000, García-Castaño 2001, Schupp *et al.* 2002, Russo & Augspurger 2004). Dispersal also involves the movement of genes, but the genetic aspect of the seed rain remained elusive until the arrival of molecular markers. Recent advances in the application of DNA-based genotyping of maternally-derived tissues in dispersed seeds have expanded the possibilities to fully understand the genetic dimension of dispersal (Godoy & Jordano 2001). The distribution of the maternal genotypes in the seed rain as a result of dispersal process is frequently unknown in spite of its presumably role in determining pervasive aspects in the recruitment cycle such as density- and distance-dependent recruitment dynamics (Janzen 1970, Álvarez-Buylla & García-Barrios 1991, Augspurger & Kitajima 1992) and kin selection (Willson *et al.* 1987, Ingvarsson & Giles 1999, Koelewijn 2004). García *et al.* (2006b) reported a non-random distribution of maternal genotypes in the seed rain as a result of vertebrate frugivores activity, driven by the type of microhabitat in the deposition site and the characteristics of the maternal tree neighbourhood. Hence, the study of seed dispersal can incorporate the genetic, spatial and numeric aspects linking dispersal with postdispersal processes to evaluate their role in the recruitment cycle.

iii. Dispersal shapes metapopulation dynamics: Local processes (colonization/extinction) strongly depend on dispersal among populations and that in turn influences regional processes of metapopulation dynamics and regional persistence (Hanski 1998), genetic structure (McCauley 1991, Giles *et al.* 1998, Ouborg & Eriksson 2004) and, ultimately, the evolution of dispersal traits (Olivieri *et al.* 1995). Molecular markers have also contributed to metapopulation theory by providing empirical evidence about dispersal patterns and their consequences. Thus, Giles & Goudet (1997) found a kin-structure within populations of Silene dioica that resulted from an extensive seed colonization and restricted local dispersal after establishment. Therefore, dispersal can act both as a driver of colonization processes and as a cohesive force in fragmented settings, influencing connectivity among patches (Urban & Keitt 2001). The application of molecular markers has been a key addition to the available tools to approach these problems.

iv. Dispersal as an evolutionary force: Different equilibrium and non-equilibrium models have been proposed to interpret current population genetic patterns in terms of historical migration, selection and drift (Wright 1943, Levin 1988, Slatkin 1995, Harrison & Hastings 1996, Epperson 2003) but very scarce empirical evidence has been available until the arrival of molecular markers. For example, the above mentioned study with S. dioica illustrates the combined action of founder effects (colonization by a reduced number of individuals), restricted pollen flow, and local adaptation in shaping genetic spatial structure. Actually, these forces might act sucessively in the recruitment cycle enhancing or erasing the pattern previously set by dispersal. This was evidenced by Kalisz *et al.* (2001) with Trillium grandiflorum where seedling populations resulted more structurated than samplings and adults. Currently one of the major topics under study refers to the dynamic consequences of climate warming trends observed in the last century. The response of the plant species will depend on their adaptative potential and migration ability to new sites (and less available patches due to increasing fragmentation). Both aspects can be assessed with molecular markers since the adaptive potential can be estimated from markers subject to natural selection, while migration rates can be estimated from neutral markers (Jump

& Peñuelas 2006, Hanski & Saccheri 2006).

v. Dynamic consequences of dispersal in plant communities: dispersal also plays a central role in determining the recruitment patterns in plant populations (Janzen 1970, Clark & Clark 1984) and in doing so, it influences the assembly of species in species-rich communities (Dalling 1999, Hubbell 2001, Harms *et al.* 2002). Moreover, the seed rain patterns are reported to be spatially clumped and numerically heterogenous, with most seeds dispersed into a few deposition sites (Jordano & Schupp 2000, Schupp *et al.* 2002, Russo & Augspurger 2004). This is especially true for animal-dispersed species, although anemochorous species (dispersed by wind) also fit this pattern (Jones *et al.* 2005). Then, distance and density dependent processes modulate the final recruitment pattern as explained by the Janzen-Connell model (Janzen 1970, Connell 1971). This model holds that recruitment probability increases at a certain distance from the source tree as a result of the interaction between a decreasing seed density and an increased survivorship to specific pathogens with increasing distance from the source tree. By providing accurate estimates of seed dispersal, molecular markers are contributing a better understanding of distance and density effects in recruitment dynamics.

Dispersal mechanisms

i. Dispersal patterns and dispersal mechanisms: a pending task in dispersal studies is the link between dispersal mechanisms and dispersal patterns to fully understand the mechanistic role of dispersal. However, methods for tracking dispersal, such as molecular markers, frequently do not provide information on the dispersal agent, whereas methods explicitly considering the dispersal agent (e.g., radiotracking) hardly provide accurate data on dissemination sites (Jordano 2006). Moreover, dispersal is usually assisted by several dispersal agents resulting in highly complex patterns (Higgins *et al.* 2003, Forget *et al.* 2005, Nathan 2006b). Thus, the combination of molecular markers and techniques providing information of the dispersal agent, such as mechanistic models (Nathan *et al.* 2002, Westcott et al 2005), will allow dissecting the contribution of different dispersal vectors to the total dispersal kernel (Nathan 2006b). Thus, Jordano *et al.* (2006) reported the relative contribution of different frugivorous species to the seed dispersal curve of *P. mahaleb*, including long distance events, demonstrating that most of the long distance dispersal events were contributed by medium- to large- sized frugivores vertebrates, resulting in frequent seed immigration. This type of information is indispensable to evaluate the effect of the loss of frugivorous species on plant recruitment, especially in highly defaunated ecosystems (Galetti *et al.* 2006).

ii. Complex mechanisms of dispersal: genetic markers have also revealed subtle mechanisms of dispersal and colonization with pervasive consequences for plant populations. Pollen dispersal involves hybridization when pollen grains from one species fertilize the ovules of another species, usually, a closely related and sympatric taxon. Directional hybridization between two sympatric species accomplished by directional backcrossing has been invoked to explain the genetic composition and distribution of two oak species *Quercus* petrea and Q. robur (Petit *et al.* 2004). Moreover, this is likely to be a mechanism for increasing gene flow and distribution range among sympatric and genetically related species with restricted seed dispersal (Potts & Reid 1988, dePamphilis & Wyatt 1989). Thus, molecular markers not only contribute to the study of contemporary dispersal processes, but also to unravel the complexity of historical dispersal mechanisms in plant communities.

Factors shaping dispersal

i. Landscape features shape dispersal patterns with contrasting outcomes among different study systems: molecular markers not only have contributed to know where, how, and why individuals are dispersed, but to identify the ecological factors influencing dispersal dynamics and to quantify their effect. The spatially-explicit consideration of the genotypes in the population and the incorporation of the relevant traits in the landscape such as vegetation type, the spatial distribution of the fruiting trees, the location of barriers to gene flow, etc., provide useful information within the framework named landscape genetics (Sork *et al.* 1999, Manel *et al.* 2003, Holderegger & Wagner 2006). Since dispersal processes and their outcomes are markedly context-dependent (Ennos 2001), it is necessary to identify the key aspects for each population influencing dispersal. Distance is one of the obvious factors limiting dispersal, but in heterogeneous landscapes dispersal processes might respond to landscape traits such as the arrangement of different vegetation patches, forest edges, etc., obscuring the relevance of distance. Hence, García *et al.* (2006b) reported the spatial distribution of the maternal genotypes in a frugivore-generated seed rain. The maternal genotypes reaching different microhabitats correlated with the characteristics of the maternal neighbourhood rather than with distance. Thus, complexities in the genetic make up of the seed rain in animal-dispersed species are likely to emerge as a result of foraging preferences of the frugivores. Additionally, genetic data spatially treated have been proved to be very useful to evaluate the effect of human-induced disturbances on genetic connectivity among populations (Sork & Smouse 2006). However, the results are not conclusive since some evidence a decrease of gene flow whereas others find no effect or even increasing levels of gene flow (Fuch *et al.* 2003). The question, thus, is to know why gene flow is affected in different ways under similar regimes of landscape alteration, and molecular markers are a valuable tool to answer it.

3. An overview of techniques to study dispersal in plant populations based on molecular methods

Among available molecular techniques, we can distinguish those based on DNA (genetic techniques) from those based on proteins (Avise 2004). Certainly, the former has already displaced the latter ones. Molecular techniques based on DNA present several characteristics that make them suitable to be used in dispersal studies. First of all, DNA-based markers involve working directly with the genotype instead of the phenotype of the individuals, i.e., we access straightforwardly to the DNA variation. Secondly, they are highly polymorphic, allowing the identification of individuals within populations. Thirdly, they not only provide a measure of individual variation but they also provide accurate assessment of genetic relatedness among individuals, groups or populations (Rousset 2002). Additionally, we can choose among markers located in coding regions and others located in non-coding regions depending on our interest for neutral or selective processes. These three characteristics explain the great contribution of molecular markers to ecology and evolutionary biology. Additionally, we can choose among markers located at coding regions (subject to selection) and non-coding regions, such as microsatellites, showing neutral variation. Moreover, DNA-based markers allow working with DNA located at different organules in the cell: nuclear DNA, cytoplasmic DNA, and mitochondrial DNA that present a variable range of mutation rates yielding different levels of polymorphism. Thus, DNA-based molecular markers allow to jump from individuals to populations, and from the purely description of genetic diversity to the inference of contemporary and historical processes originating current patterns at different spatial scales in plant populations. Several reviews detail and explain the differences among molecular techniques in depth, with some practical considerations before choosing the right genetic marker for a specific problem (Cruzan 1998, Parker *et al.* 1998, Ouborg *et al.* 1999, Bernatchez & Duchesne 2000, Avise 2004, Schlötterer 2004, Selkoe & Toonen 2006).

Unquestionably, among DNA-based markers those based on polymerase-chain reaction (PCR) amplification are currently most widely used. This DNA amplification technique simplified, improved and reduced the costs and time of lab protocols allowing its application to non-model species and opening the possibility of working with different tissues, even those containing low quantity or highly degraded DNA (Schlötterer 2004). With time, these methods become more affordable and less time consuming (Selkoe & Toonen 2006). Among all available PCR based techniques, dispersal studies have greatly benefited from AFLP and SSRs markers, whereas SNPs is still a promising technique (Table 1). For an introductory review on the rationale of different techniques see Avise (2004) and for a thorough explanation of the lab procedures when dealing with molecular techniques see Hoelzel (1998).

AFLPs (Amplified Fragment Length Polymorphisms) result from the selective amplification of a subset of fragments obtained when digesting the DNA with restriction enzymes. Thus, a priori knowledge of the primer sequence is required. This technique yields a high number of bands that show absence/presence variation. Hence, the polymorphism per locus is low, but it can be compensated by increasing the number of loci sampled. The reliability of the technique depends on selecting a consistent set of bands, which rely very much on the experience of the researcher. The analytical ground is well developed for AFLPs to perform paternity analysis gene flow estimation, population allocation, and relatedness assessment (Streiff *et al.* 1999, Duchesne

& Bernatchez 2002, Hardy 2003). However the mutational mechanisms are unknown, making difficult the assessment of the reliability of the results (Schlötterer 2004).

SSRs or microsatellites are short tandem repeated sequences with repeating motifs of 2-6 pairs of bases (bp). They present length polymorphism (i.e., the alleles differ in the length of the fragment), Mendelian inheritance, a high mutation rate (10^{-4}), they are abundant over the genome, and they are located in non-coding regions. Alleles are expressed in codominant mode, and each locus can present dozens of alleles, so the amount of information per locus provided by SSRs is higher in comparison with AFLP and SNPs (see below). These characteristics make them the suitable marker for tasks demanding detail genetic information to discriminate among individuals even with high level of inbreeding such as paternity analysis, contemporary gene flow estimation, and relatedness assessment among inbred individuals. Analytical methods to perform those analyses are widely available. Among their shortcomings, they require a priori knowledge of the marker sequence, and SSRs are yet more time and money demanding than AFLPs. However, these techniques are becoming quickly affordable and feasible for a wider range of natural species (Selkoe & Toonen 2006). Additionally, despite their extended use, SSRs present serious drawbacks since they are error-prone (Pemberton *et al.* 1995). An accurate performance of the PCR is required but this is not always easy to reach; poor DNA template yields allele peaks difficult to score that might introduce a high frequency of errors and a non-negligible bias in the data analysis stage (Bonin *et al.* 2004, Broquet & Petit 2004, Hoffman & Amos 2005, Pompanon *et al.* 2005 for a comprehensive and practical appraisal on genotyping errors). However, software packages are already available to assess genotyping reliability or to evaluate the effect of different error rates on the performance of individual identification or paternity analysis (Jones & Arden 2003, Pompanon *et al.* 2005). When the identification of the loci associated to the error is not possible, the researcher should at least evaluate the effect of the estimated (or assumed) error rate when performing data analysis, such as individual identification or assignment (Valière 2002). Finally, the reliability of the data yielded by any marker depends on the knowledge of its mutation mechanism, not yet fully understood for SSRs, although approximate models are used (Schlötterer 2004).

SNPs are Single Nucleotide Polymorphisms distributed all over the genome, so a large amount of loci sites are potentially available. They present codominant expression and an allele can have four possible states: A, G, C, and T. SNPs have a low mutation rate (10^{-8}-10^{-9}) compared to SSRs. Hence, they are less informative than SSRs per locus but a larger number of loci can be screened over the genome. The screening of the SNPs basically consists on genotyping large genome fragments searching for variable positions. However, these positions are usually invariant within a population, thus, screening usually involves a large number of samples and different populations and a priori knowledge of the allelic variation is required (or highly desirable). This information is not always available for non-model species, and additionally, natural populations sometimes consist of few individuals. These reasons make SNPs to be confined to model organisms such as Arabidopsis thaliana (Jackson *et al.* 2002), although they are quickly being incorporated into studies dealing with natural populations (Picó *et al.* 2002). Some strategies can be applied to facilitate the location of SNPs for non-model organisms, such as sequencing AFLPs (Morin *et al.* 2004). Given the lower amount of information provided per locus, a higher number of SNPs than SSR is required to obtain similar resolution (Schlötterer 2004). This is actually an advantage since it involves working with a higher number of markers distributed all over the genome, decreasing the probability of linkage among loci. The main drawback of SNPs is the ascertainment bias that results when the selected individuals or loci are not representative of the population polymorphism. This tends to overestimate population variability and introduce a strong bias in the estimation of population parameters. However, this bias can be corrected analytically (Morin *et al.* 2004). Thus, for questions involving historical demographic events SNPs are more reliable that SSR whereas the last ones are recommended for paternity and assignment methods (Brumfield *et al.* 2003, Schlötterer 2004). Robust algorithms are already developed to perform likelihood-based parentage on a few tens of SNPs, even in situations involving thousands of candidate parents, which would be unfeasible by using SSRs markers (Wilson & Rannala 2003, Morin *et al.* 2004, Ben-Ari *et al.* 2005, Anderson & Garza 2006).

Analytical methods

The large amount of information provided by using highly polymorphic genetic markers has lead to the development of analytical procedures used both to characterize dispersal processes (dispersal assessment) and to identify and quantify ecological factors shaping dispersal patterns (landscape genetics, see Manel *et al.* 2003). Next, the main analytical methods are reviewed.

Dispersal assessment is achieved by tracking dispersal events in mapped populations and inferring the distance between the source tree and the deposition site (Godoy & Jordano 2001), by identifying immigrants and assigning them to the source population, or by estimating immigration rates among populations (Wilson & Rannala 2003). Methods available to attempt these goals can be divided into direct and indirect methods (Table 2). The direct methods are based on the comparison of the multilocus genotypes of a set of individuals of interest (maternal progeny, dispersed seeds, seedlings, or adults) with the multilocus genotypes of the candidate source trees or populations. By applying direct methods we can identify the most likely father/mother/parents of the study individuals (paternity/maternity/parentage analysis), estimate the male or female reproductive success of the trees in a population whereas estimating the amount of gene flow (mating models), and assign individuals to their source population to estimate migration rates (assignment to population). Indirect methods are focused on modelling functions that yield parameters indirectly related with the variables defining dispersal; thus, spatial-genetic or spatial-demographic patterns are used to infer the dispersal processes that generated them. They include models based on population genetic structure (TWOGENER, KINDIST), and those extended to spatial autocorrelation (Sokal & Oden 1978, Epperson 2003, Hardy *et al.* 2006).

On the other hand, the distributions of the allelic frequencies can be used to infer dispersal patterns and to to identify ecological traits shaping them by using spatially explicit models capturing the landscape heterogeneity. This is the rationale behind most of the studies focused on ecological genetics (Hamrick & Allard 1972, Endler 1973) and, more recently, landscape genetics (Manel *et al.* 2003). Thus, landscape genetics offers a set of combined genetic and geostatistical tools to characterize the distribution of the genotypes in a population and to infer its relationship with dispersal patterns. Thus, an increasing difference in the genetic make up of the populations with increasing distances might be the consequence of restricted gene flow among populations (isolation by distance). Additionally, other statistical techniques can be used to identify the factor shaping the dispersal patterns, such as river systems (Piertney *et al.* 1998, Guillot *et al.* 2005) or vegetation type (Geffen *et al.* 2004). In the following lines I briefly go over all these methods applied to assess and characterize dispersal processes in plant populations.

Dispersal assessment

Direct methods

i) Paternity/maternity/parentage analysis: these procedures allow each dispersed propagule to be assigned to its source tree based on the genetic compatibility of the offspring (seed or seedling) with the candidate parents, assuming Mendelian inheritance (Meagher 1986, Devlin & Ellstrand 1990). Statistical inference is commonly approach with maximum likelihood, although Bayesian inference is starting to be applied (Hadfield *et al.* 2006).

Paternity analysis aims at searching for the pollen source tree to estimate pollen dispersal distance and the amount of pollen immigration. Maternal progenies with known mother trees are sampled and genotyped, so analytically the problem consists on searching for the multilocus genotype compatible with the pair mother-offspring among all candidate fathers (Meagher 1986). The assignment of a given candidate father can be made by exclusion methods (i.e., all candidate fathers are excluded but the compatible one). However, frequently more than one candidate father result compatible. Then, maximum likelihood (ML) methods can be applied to grant all candidate fathers with a likelihood of siring the offspring (a LOD score), and exclude all but one most likely putative father. Statistical support for the assignment can be inferred by simulations (Marshall *et al.* 1998, Gerber *et al.* 2003). If all candidate fathers in the population are excluded, then, the pollen donor tree is assumed to be located outside the focal population, i.e., it involves an immigration event (Devlin & Ellstrand 1990). Thus, pollen dispersal distance curves including long distance events can be reliably assessed (Vassiliadis *et al.* 2002, Oddou-Muratorio *et al.* 2003, García *et al.* 2005, Robledo-Arnuncio & Gil 2005). Assignment can be also performed by fractional assignment weighting all non-excluded candidate fathers with a

Table 1. A summary of the main characteristics of the most frequently used PCR-based molecular markers in dispersal studies, AFLPs (amplified fragment length polymorphisms), SSRs (microsatellite simple sequence repeats), and SNPs (single-nucleotide polymorphisms).

	AFLP Amplified fragment length polymorphism	**SSRs** Short tandem Repeated Sequences	**SNPs** Single Nucleotide Polymorphisms
Molecular characteristics			
Type of alleles	Dominant	Codominant	Codominant
Location in the genome	All over the genome	Non coding regions	Coding regions and non coding regions all over thte genome
Distribution over the genome	Moderate abundance over the genome	Abundant, specially in certain regions of the genome	Abundant all over the genome
Source of polymorphism	Presence/Absence	Length polymorphism	Four potential states (A,G,C,T)
Rate of mutation		10^{-4}	10^{-8}-10^{-9}
Information provided			
Amount of information per locus	Low	High	Moderate
Relibility of the data/drawbacks	To be tested DNA- Methylation might affect band patterns	Error prone (scoring errors, null alleles and allelic drop out)	Ascertainment bias
Advantages			
Cost/information provided	High	Moderate	Still low
Best potential applications in terms of accuracy	Gene flow studies in large scale studies and comparative studies involving a large number of different species	Paternity/Maternity/Parentage analyses, dispersal assessment, and assignment studies involving small- to medium-size populations	Small- to medium-size populations and studies involving dispersal and adaptive processes. Still restricted to model species
Example studies	Gerber *et al.* (2000) Hardy *et al.* (2006)	Streiff *et al.* (1999) García *et al.* (2006) Hardesty *et al.* (2006), Bacles *et al.* (2006)	Picó *et al.* (2002) Bakker *et al.* (2006)

probability proportional to their LOD-likelihoods (Devlin *et al.* 1988, Jones & Ardren 2003). Recently Bayesian methods have been incorporated to paternity analysis, increasing the power of parentage assignment (Haldfield *et al.* 2006).

Parentage analyses can estimate both pollen and seed flow based on the multilocus genotypes of recruited seedlings or samplings. Following the same rationale than the paternity analysis, parentage analysis involve two sequential stages: i) searching for the first compatible parental; and ii) searching for the second parental compatible with the pair putative parental-offspring pair (Dow & Ashley 1996, Ennos 2001). Parentage, thus, is more challenging since we ignore which genotype is inherited from the mother tree and which genotype comes from the father (Meagher & Thompson 1987). For species with limited seed dispersal, it is usually assumed that seed dispersal is more restricted than pollen dispersal, and therefore, the nearest parental tree is assumed to be the maternal tree (Dow & Ashley 1996), though this is a tricky assumption for many plant species with evidences for restricted pollen flow (Aizen & Feinsinger 1994, Davies *et al.* 2004, Jump & Peñuelas 2006).

With an analogous rationale, *maternity analysis* searches for the source fruiting tree of dispersed seeds to infer seed dispersal distances and the amount of seed immigration. Traditionally, maternity analyses have been approached applying mating models accounting for female fertility to fit the observed distribution of the seedling's genotypes (Schnabel *et al.* 1998) (see below mating models). However, regardless female fertility, reliable seed dispersal distance can be obtained by applying maternity analysis when dispersed seeds present maternally-derived tissues that are genotypically identical to the source tree (Godoy & Jordano 2001). This is the case for endocarps in most drupe-like fruits, mesocarps for acorns and gametophytes in gymnosperms. Hence, a straightforward tracking of the seed dispersal event can be made by unambiguously identifying the source tree for a sample of seeds obtained from seed traps or other direct collection of seeds (Godoy & Jordano 2001, Zieghangen *et al.* 2003, Robledo-Arnuncio *et al.* 2004, Grivet *et al.* 2005, Jones *et al.* 2005, García *et al.* 2006a, Jordano *et al.* 2006).

Paternity/maternity/parentage analyses can be applied to obtain pollen and seed dispersal curves based on a set of highly polymorphic markers providing unique genotypes for each candidate parent in a mapped population. Note, however, that these analyses have been frequently based on established seedlings (e.g., Aldrich & Hamrick 1998, Schnabel *et al.* 1998, Sezen *et al.* 2005, Bacles *et al.* 2006, Hardesty *et al.* 2006), which provide estimates of effective dispersal distance (i.e., recruited dispersal events). Only dispersal estimates based on dispersed seeds can provide actual dispersal distances resulting from the action of dispersal agents (either biotic or abiotic). Certainly, effective dispersal is the important genetic and demographic outcome, but we need to dissect the relative contribution of dispersal per se and postdispersal processes (differential loss of seeds to seed predators and pre-germination pathogens) to overall recruitment patterns to fully understand the role of dispersal in plant populations (Cain *et al.* 2000, Nathan *et al.* 2003).

In spite of the accuracy of these methods for getting dispersal distances, some drawbacks make them unfeasible under certain circumstances. They require an exhaustive sampling of the focal population to avoid an overestimation of the gene flow parameters: if pollen dispersal is of interest most (if not all) pollen donors should be sampled; if seed dispersal is to be assessed, all potential maternal trees should be included in the analysis. However, as the number of candidate parents increase, the probability of unambiguous father/parent pair assignment decreases, so the number of progeny sampled per mother tree needs to be higher than the number of candidate fathers (Sork *et al.* 1999). Thus, an exhaustive (and likely expensive) sampling of large populations might yield a disappointing low proportion of assignments to one single father/parent pair. In such cases, fractional assignments, mating models or pollen structure-based methods are more accurate. Maternity analyses are not so problematic since they are not based on ML assignment but in searching for matching genotypes. The ideal scenario to apply paternity/parentage (and to a certain extent, maternity) to assess dispersal distance consists on small- to medium-sized populations with clear geographical edges where all adult trees are mapped and genotyped for several highly polymorphic markers (Adams *et al.* 1992).

ii) Mating models: they consider female and male reproductive success and account for the amount of pollen and seed flow. The most popular is the NEIGHBORHOOD model (Adams *et al.* 1992, Burczyk *et al.* 1996) that estimates the likelihood for the candidate parents located within a circle set around the source tree to sire embryos from sampled progenies. The extent of the circle is decided by the researcher based on previous knowledge or logistic strategies. The parameters of the neighbourhood models are estimated by ML model fitting to the multilocus pollen gametic arrays of maternal progenies (Burczyk *et al.* 1996); allele frequencies of the background (patch of forest outside the circle) are required, although the model can estimate them independently from the mating parameters (Burczyk & Chybicki 2004). Mating parameters such as selfing rates, proportion of pollen coming from outside the circle, mean pollen dispersal distance within the circle and their correlates with phenotypic traits are provided (see Burczyk & Chybicki 2004 for an assessment of the statistical properties). Additionally, the NEIGHBORHOOD model can be extended to assess both seed and pollen immigration on the basis of naturally regenerated seedlings (Burczyk *et al.* 2006). The strength of the method lies in its feasibility for large or continuous populations, and in the integration of predispersal (reproductive success and factors shaping it) and postdispersal information for a given natural population, an interesting task when dealing with recruitment forest dynamics. The reliability of the estimates provided by the NEIGHBORHOOD model will depend on the accuracy of both the curve used to fit seed and pollen dispersal distributions and the estimates of the allele frequencies in the surrounding population. Exponential curves are frequently chosen since empirical evidence has supported it for some species (Austerlitz *et al.* 2004), whereas some others has yielded more complex dispersal curves, especially when dispersal is assisted by vertebrates (Wenny & Levey 1998, Godoy & Jordano 2001, Gómez 2003, Westcott *et al.* 2005, Jordano *et al.* 2006, Morales & Carlo 2006).

iii) Assignment methods: the source population can be identified for a set of individuals based on their multilocus genotypes and the allele frequencies of the potential source populations by applying any of the methods included as assignment methods. The accuracy of different molecular markers for determining the source population of individual genotypes has been reviewed by Smouse & Chevillon (1998) and the assignment methods have been reviewed by Manel *et al.* (2005). Basically, they estimate the likelihood of a given multilocus genotype to occur in each of the candidate populations given their allele frequencies; the individual is assigned to the population yielding the highest likelihood (Paetkau *et al.* 1995). Besides the requeriments of Hardy-Weinberg and linkage equilibrium, this model assumes that all potential source populations are sampled (and thus, they are defined in advance) and that the allele frequencies estimated from the subsampling are the actual ones. Exclusion methods are based on the genetic distance between the individual genotype and the source populations. They take the allele frequencies of each population to generate a genotype distribution that functions as the distribution of the genotypic likelihood values to calculate the probability of each individual to be produced in a given population with a given confidence level; the individual is assigned to the non-excluded population (Cornuet *et al.* 1999). Thus, by excluding unlikely source populations with a statistical support, exclusion methods are a useful extension of the assignment methods when the source population has not been sampled and equilibrium conditions are not met. Alternatively, Rannala & Mountain (1997) identified immigrant individuals (or immigrant ancestry up to two generations) even with a slight genetic differentiation among populations. They used a Bayesian approach to obtain a probability density of allele frequency of each population and Monte Carlo simulations to obtain statistical support for the individual assignments. This method does not assume Hardy-Weinberg equilibrium but linkage equilibrium among loci is required. Thus, an estimation of the proportion of immigrants can be derived with these methods once we have identified migrant individuals. Wilson & Rannala (2003) proposed a Bayesian method for explicitly assessing non-symmetrical migration rates among populations. Additionally, this new Bayesian multilocus genotyping method allows incorporating an inbreeding coefficient for each population.

Note that all previous methods require an a priori delimitation of the source populations, a condition not always feasible in natural populations. When source populations are not previously delimited, a cluster-approach based on a Bayesian clustering method can be used to both define structured populations and to assign individuals to K defined populations, regardless of the previous knowledge of the K number (Pritchard *et al.* 2000). More recently, Huelsenbeck *et al.* (2006) have improved this method to explore different outcomes with variable number populations (thus K is not fixed). Originally, this method required both Hardy-Weinberg and linkage equilibrium, but it has been extended to allow for admixture linkage disequilibrium (Falush *et al.* 2003). Finally, an interesting Bayesian method proposed by Guillot *et al.* (2005) incorporates a landscape genetic thinking for the identification of boundaries and geographical discontinuities based on individual georreference multilocus genotypes while identifying structuring populations and assigning individuals to them.

Indirect methods

i) Genetic structure of pollen pools: A combination of paternity analysis and genetic structure approach was proposed by (Smouse & Peakall 1999) to infer population genetic parameters related to dispersal. The model, known as TWOGENER, relies on different maternal seed progenies to infer the array of paternal gametes by subtracting the maternal contribution (Smouse *et al.* 2001, Smouse & Sork 2004). A genetic distance matrix among the N male gametes is calculated to later dissect the genetic variation due to within and among maternal pollen pools (AMOVA analysis, Excoffier *et al.* 1992). By doing so, an intra-class correlation of male gametes measuring the divergence among maternal pollen pools is estimated (ϕ_{ft}), i.e., the level of non-overlapping sampled males. From ϕ_{ft}, an effective number of pollen donors (N_{ep}) can be inferred by fitting a pollen dispersal curve (Austerlitz *et al.* 2004) along with an estimation of an effective mating area (δ) and dispersal distance. This approach has recently been extended to the study of seed dispersal (Grivet *et al.* 2005). Additionally, it has been improved to become independent of the effective adult density (a parameter not always available) in a model named KINDIST (Robledo-Arnuncio *et al.* 2006). The TWOGENER approach focuses the sampling effort on the maternal trees rather than on the progeny sample size allowing extensive populations to be studied, thus it represents a useful tool to address dispersal studies (both via seed and pollen) at the landscape level, and to compare different ecological scenarios such as fragmentation levels or management strategies (Sork *et al.* 2002). The methodological approaches mentioned above have been compared and discussed by Smouse & Sork (2004) (direct and TWOGENER approaches), Burczyk & Koralewski (2005) (NEIGBOURHOOD *vs.* TWOGENER approaches), and Burczyk & Chybicki (2004) (genotypic exclusion vs. NEIGBOURHOOD).

ii) Autocorrelation methods: spatial genetic structure (SGS, i.e., the non-random spatial distribution of genotypes within a population) can be assessed based on molecular markers by applying autocorrelation methods. A pairwise genetic correlation coefficient is estimated for all pairs of individuals sampled at progressively increasing interval distances (Sokal & Oden 1978, Hardy & Vekemans 1999, Ennos 2001, Kalisz *et al.* 2001, Epperson 2003). Assuming the absence of genetic drift, historical SGS emerges if dispersal is spatially restricted (Heywood 1991, Epperson 2003); under this scenario, two nearby individuals are expected to present higher genetic correlation among them than two individuals randomly drawn from the same population (Epperson 2003, Hardy & Vekemans 1999). Smouse & Peakall (1999) extended SGS to a multiallelic and multilocus approach reducing the variation among alleles. On the other hand, indirect estimates of historical gene flow can be obtained from SGS based on drift-dispersal equilibrium, derived from the slope of the regression of pairwise kinship coefficient on spatial distances (Hardy *et al.* 2006). This approach has been used by Hardy *et al.* (2006) to assess and compare the historical gene dispersal distances for 10 tropical tree species. Their dispersal distance estimates ranged between 150 m and 1200 m. The authors concluded that high genetic polymorphism, increased sample sizes, and reliable effective density estimates are required to obtain accurate estimates of gene dispersal.

Identifying factors shaping dispersal patterns

When molecular markers are assumed to be neutral (i.e., not subject to local selection), we can relate the distribution of the allelic frequency with dispersal patterns and identify ecological factors influencing such distribution. Thus, highly restricted dispersal would yield strongly structured populations; moreover, some specific life-history traits (mating system or seed dispersal vector) are reported to correlate with specific spatial genetic patterns (Loveless & Hamrick 1984). Thus, the integration of dispersal assessment, genetic patterns at landscape level, and ecological information can provide a complete understanding of the role of dispersal in plant populations.

Among the multiple factors shaping dispersal, distance is likely the most widely recognized since Wright (1943) proposed the isolation by distance model. Statistical correlations between a matrix with some coefficient measuring the genetic distance among populations and a geographic distance matrix can be tested with a Mantels test; significant positive correlations involve a correlation between genetic and geographic distance, frequently interpreted as decreased gene flow with increasing distance among populations. Partial Mantel tests allow a third matrix with ecological data to be incorporated to the analysis, so a second factor, besides the geographical distance can be tested (Smouse *et al.* 1986). When dispersal patterns are not homogenous over the landscape, bearing correlograms provide a method to test for directional dispersal patterns (directional spatial autocorrelation techniques, Rosenberg 2000, Dutech *et al.* 2005). The previously mentioned autocorrelation methods are also a useful tool to assess the role of dispersal distance on shaping genetic patterns at very fine scales. Finally, algorithms connecting populations on the basis of spatial and genetic information are valuable tools to visualize the spatial and genetic data (see Manel *et al.* 2003), identify geographical boundaries, and test for the role of dispersal limitation vs. vicariance for populations currently isolated (Dyer & Nason 2004).

Multivariate ordination techniques such as Canonical Correspondence Analysis (CCA) and Redundancy Analysis (RdA) have also proved to be useful to detect spatial genetic structure in plant populations related with the scale of environmental heterogeneity and seed flow (Volis *et al.* 2004). On the other hand, the scores obtained from a Principal Component Analysis (PCA) can be projected on a map of the study area to detect geographical barriers to gene flow as zones of maximum slopes of PC1 scores (Piertney *et al.* 1998). Lastly, Gaggiotti (2002) has reported the utility of hierarchical Bayesian models for integrating genetic, demographic, and environmental data to infer colonization patterns in natural populations.

4. The half-empty glass: limitations of molecular markers to study dispersal

Most recent reviews dealing with dispersal highlight the great potential of molecular markers to elucidate long-standing questions (Cruzan 1998, Ouborg *et al.* 1999, Cain *et al.* 2000, Wang & Smith 2002). Actually, the contribution of molecular markers is already a reality. However, some key questions that can be approached with molecular markers are still under-investigated or the data remain insufficient to have a complete picture of the relevance of dispersal for plant populations (Jordano 2006, Nathan 2006b). Molecular markers and their associated analytical methods present a number of difficulties that limit their efficient application. Recently, several reviews have approached the main drawbacks and obstacles related with molecular techniques and their analytical methods (Bonin *et al.* 2004, Broquet & Petit 2004, Hoffman & Amos 2005, Pompanon *et al.* 2005, Selkoe & Toonen 2006).

i. The accuracy of molecular markers depends on the biological system: despite being obvious, the versatility of the molecular markers, specially those based on DNA, gave the wrong impression that they were equally applicable for any biological system. Some organisms are technically more difficult to deal with, as for example, those with no previous genetic information, or with difficulties to develop microsatellites (see Selkoe & Toonen 2006). On the other hand, if dispersal is to be assessed by applying paternity/parentage analysis, medium- to small-populations are preferable (see paternity analysis section). Thus, for extensive populations, paternity analysis quickly becomes unfeasible or with important limitations. Hence, if the development of the microsatellites becomes fraught and the statistical analysis requires large sample sizes, the actual applicability of molecular markers to track dispersal events is drastically reduced. When the delimitation of different populations is not obvious a priori, we can define the number of populations by using genetic data applied to models detecting genetic structure. These procedures used to require unrealistic assumptions such as Hardy-Weinberg and linkage equilibrium, but as stated above, Bayesian approaches have been recently incorporated to develop non-equilibrium models (Wilson & Rannala 2003). Hence, exhaustive sampling efforts will allow having accurate assignment results to infer immigration events in spite of lacking of all adult trees sampled, allowing the application of molecular markers to a wider range of biological systems (Beaumont & Rannala 2004).

ii. High quality and reliable data sets are difficult to obtain: all molecular markers present serious shortcomings that hamper the production of good quality data sets. The reliability of AFLPs is still questioned for some applications (Schlötterer 2004), SSR are error prone due to frequent allelic drop out, null alleles and scoring errors that can introduce non-negligible bias in the data analysis (Bonin *et al.* 2004), and the isolation of SNPs involves a serious ascertainment bias that might affect the estimation population parameters (Morin *et al.* 2004, Schlötterer 2004). Recent reviews report specific protocols to be applied with different molecular marker types. As a general rule, extreme caution in the lab-protocols is required. Working with microsatellites requires multiple repetitions per sample, estimation on the error rate for each locus, and consistent scoring criteria (Broquet & Petit 2004, Hoffman & Amos 2005, Selkoe & Toonen 2006). For SNPs, the genotyping of different regions in the genome, large sample sizes involving a large number of individuals and populations is desirable for avoiding ascertain bias (Syvänen 2001, Morin *et al.* 2004). Error estimations are rarely reported in final papers, and thus we frequently ignore the reliability of the data, and therefore the validity of the results and conclusions. Hence, in spite of the increasing ease to produce multilocus genotypes based on molecular markers, high quality and reliable data sets are scarce yet.

iii. Accurate and robust analytical methods are not always available: the possibility of having detailed genetic information has been feasible at least from the late 60's (allozymes), early 90's (microsatellites) and late 90's (SNPs); however robust and accurate analytical methods are not always available (Bohonack *et al.* 1998, Bossart & Prowell 1998). In fact, the drawbacks of molecular tools are usually more related with the analytical procedures than with the lab procedures (Bossart & Prowell 1998, Bohonack *et al.* 1998, Ouborg *et al.* 1999, Withlock & McCauley 1999, Nathan *et al.* 2003). Recent advances have improved our ability to use molecular markers to infer dispersal processes (Luikart & England 1999, Pearse & Crandall 2004). Most of the authors claim for a change of the analytical framework for a more accurate application with current molecular markers such as AFLPs, SSR, and SNPs (Bossart & Prowell 1998, Whitlock & McCauley 1999, Cain *et al.* 2000, Pearse & Crandall 2004). This requires a change from a single locus dialleleic model to a multilocus and multiallelic approach (Smouse & Peakall 1999). More challenging, we need to jump from the static description of population structure to a hypothesis testing approach to infer the role of different processes operating under different (non-equilibrium) ecological scenarios and their dynamic consequences. Here, population landscape and coalescence theory are likely to provide realistic and accurate tools to go beyond the classical Fst estimations (Pearse & Crandall 2004). Therefore, the delay in the development of robust, accurate and friendly statistical procedures to deal with non-equilibrium systems and complex biological models might partly explain why molecular markers have not still fulfilled the expectations that ecologists had a decade ago.

Table 2. This table presents the analytical methods for studying dispersal in plant populations discussed in the text. The software implementing each approach and the reference explaining their analytical basis are indicated. Some special features and requirements are also pointed. HWE refers to Hardy –Weinberg equilibrium and LE indicates linkage equilibrium. The URL with the software freely available is provided.

Analytical methods	Software	Special features	URL
Paternity/Maternity/ Parentage methods	CERVUS 2.0 (Marshall *et al.* 1998)	An estimation of an error rate and the proportion of sampled population can be introduced Matching genotypes can be identified -score to assign the putative parental HWE required	http://helios.bto.ed.ac.uk/evolgen/cervus/cervusregister.html
	GIMLET (Valière 2002)	Construction consensus genotypes and estimation of the error rate from the data Matching genotypes can be identified HWE required	http://pbil.univlyon1.fr/software/Gimlet/gimlet.htm
	FAMOZ (Gerber *et al.* 2003)	Nuclear dominant, codominat, or cytoplasmatic can be used Error rate can be introduced HWE required	http://www.pierroton.inra.fr/genetics/labo/Software/Famoz/
Mating models	NEIGBOURHOOD (Burcyk *et al.* 1996) (Burcyk *et al.* 2006)	An integrative approach that asses pollen and seed gene flow on the basis of predispersal (mating system) and postdisperal information (from regenerated seedlings) The multilocus genotypes of all individuals within the defined area is required Seed and pollen dispersal is assumed to be exponential	On request from the author

Tabla 2/2

Assignment to populations and migration rates	Immanc (Rannala & Mountain 1997)	Source populations are delimited *a priori* Bayesian approach required	http://www.rannala.org/labpages/software.html
	MIGRATE (Beerli 2006)	Source populations are delimited *a priori* Estimation of effective population sizes and past asymmetric migration rates among populations with different subpopulation sizes. ML or Bayesian approach LE required Sequence data, microsatellites and SNPs	http://popgen.csit.fsu.edu/
	GENECLASS (Cornuet *et al.* 1999)	Source populations are delimited *a priori* Non equilibrium conditions required	http://www.montpellier.inra.fr/URLB/
	BayesAss+ (Wilson & Rannala 2003)	Source populations are delimited *a priori* Bayesian approach Identify immigrants and immigrant ancestry over several generations Estimation of asymmetric migration rates Non equilibrium conditions required	http://www.rannala.org/labpages/software.html
	GENECLASS2 (Paetkau *et al.* 2004)	Source populations are delimited *a priori* Identify first generation migrants Non equilibrium conditions	http://www.montpellier.inra.fr/URLB/GeneClass2/
	STRUCTURE (Pritchard *et al.* 2000)	Populations are not delimited *a priori* Bayesian clustering approach Definition of *K* populations and assignment of individuals to them HWE and LE required Codominant and dominat markers	http://pritch.bsd.uchicago.edu/software/structure.html
	STRUCTURAMA (Huelsenbeck & Andolfatto 2006)	Populations are not delimited *a priori* Bayesian clustering approach Definition of *K* populations, where *K* can be variable LE required	http://www.structurama.org/index.html
	GENELAND (Guillot *et al.* 2005)	Populations are not delimited *a priori* Bayesian clustering approach Definition of *K* populations, where *K* can be variable HWE required	http://cran.r-project.org/doc/packages/

Tabla 2/3

Pollen pool structure	GENER (Dyer 2005)	Based on TWOGENER approach (Smouse *et al.* 2001) Significance of ϕ_{ft} is tested by permutations The maternal trees can be set by strata if differences among different groups are of interest. The sampling of the pollen donors is not required Codominat and dominant genetic markers are allowed	http://dyerlab.bio.vcu.edu
	KINDIST (Robledo-Arnuncio *et al.* 2006)	Essentially the same approach of TWOGENER but the effective density is not required	Available upon request from the author
Autocorrelation	SGS (Degen *et al.* 2001)	Codominant and dominant markers can be used as well as nuclear and cytoplasmic Genetic structure can be related with phenotypic structure Deals with both fine and broad scales	http://software.bfh-inst2.de/download3.html
	SPAGeDi (Hardy & Vekemans 2002)	Estimates several genetic relatedness statistics for individuals or populations Haploid and autopoliploid individuals are allowed	http://www.ulb.ac.be/sciences/ecoevol/spagedi.html
	GeneAlex (Peakall & Smouse 2005)	A multiallelic and multilocus approach is used to reduce the variance	http://www.anu.edu.au/BoZo/GenAlEx/

5. Perspectives for the integration of molecular markers in dispersal studies

Molecular techniques are bound to continuing the integration of genetics and ecological approaches in the future. Future work on dispersal will require both an exhaustive analytical and synthetic effort towards the elucidation of complex dispersal processes (Jordano *et al.* 2006, Nathan 2006b) and the understanding of the emergent dynamics driven by dispersal in fragmented populations (Urban & Keitt 2001).

Dissecting complex dispersal systems

The dispersal systems described up to date tend to consider only the dispersal vector that mobilizes most of the seeds from the source tree. This simplified view implicitly ignores two main aspects reported to be relevant for dispersal in plant populations: secondary dispersal processes, i.e., the dispersal of seeds away from the first deposition site (Vander Wall 2004), and the role of non-standard means of transport as dispersal agents that can mediate long distance dispersal events (Higgins *et al.* 2003). Most plant species are likely to be dispersed in a sequential fashion from the source tree to the final deposition site and multiple biotic and abiotic dispersal vectors tend to be involved. Empirical data encompassing all these aspects are extremelly scarce in the literature. Nathan (2006b) has reviewed and evidenced the complexity of dispersal systems and the urgent need of quantifying and modelling them to obtain reliable dispersal curves. For example, the contribution of frugivore species to the long-distance section of the dispersal curve needs not to be correlated with their quantitative contribution in terms of overall fruit removal. Thus, specific frugivores might disproportionately contribute the long-distance dispersal events or the seed delivery to optimal patches for recruitment. Even more, we should go beyond the accurate description of the overall dispersal curve by decomposing the contribution of each dispersal vector at each interval distance. Actually, this is a first step towards the evaluation of the differential contribution of different dispersal vectors to recruitment dynamics.

Studies assessing dispersal by applying mechanistic models are useful to provide information on the effect of a given vector on dispersal patterns (Box 1). Molecular markers rarely consider dispersal from the vector point of view, but from the source tree point of view (Nathan *et al.* 2003, Pairon *et al.* 2006). Thus combining molecular markers with phenomenological models provide a valuable tool to test the reliability of the data yield by these models; moreover, the effect of the sampling design (seed trap), and landscape tratis such as the arrangement of the adult trees can be tested. Additionally, mechanistic models can be applied to test hypothesis regarding the effect of a given dispersal vector such as wind (Nathan *et al.* 2002) or an array of frugivorous vertabrates (Westcott *et al.* 2005). Therfore, only the combination of molecular markers with different analytical tools will allow us dissecting source of complexity in dispersal systems (Jordano 2006, Nathan 2006b).

Box 1. Other techniques aiming at assessing dispersal in plant populations

Two kinds of models can be used to study dispersal (Levin *et al.* 2003). Phenomenological models aim at fitting curves that reproduce the observed dispersal patterns regardless the characteristics of the dispersal vector (Austerlitz *et al.* 2004). They usually yield highly leptokurtic distributions that account for short distance dispersal events. For the inclusion of long distance dispersal events, "fat-tailed" kernels (with a decay drop off with distance less rapid than the negative exponential functions, Clark *et al.* 1999) and mixed-kernels (combining two different functions accounting for both short and long distance dispersal events, Clark *et al.* 1999, Higgins *et al.* 2003) are more accurate. Fat-tailed models are reported to be consistent with the speed of invasion of plant species after glaciations (Clark *et al.* 1999), and mixed-models are accurate when short and long distance dispersal modes differ, which is likely to be a more common situation, although more empirical evidence is required (Higgins *et al.* 2003).

On the other hand, the mechanistic approach models the parameters characterizing the dispersal vector, such as wind speed, to yield reliable predictions on dispersed seeds (Nathan *et al.* 2002a,b). This approach has provided a better understanding of the processes driving dispersal as well as the role of landscape traits shaping the dispersal patterns. Nathan *et al.* (2002a) proposed a mechanistic model considering the turbulent transport processes coupling with seed released by wind that showed that uplifting is enough to account for the long distance dispersal events. This conclusion has been validated by other studies dealing with dispersal by wind. Westcott *et al.* (2005) successfully applied mechanistic models for estimating seed dispersal distances by animals. They reported mean seed dispersal distance of 239 m after incorporating data on feeding behaviour and foraging daily movements by the cassowary (*Casuarius casuarius*), the main seed dispersers in Australian rainforest. This kind of studies highlight the complexities associated to processes driving by plant-animal interactions, and thus, dispersal assisted by animals is only fully explained consediring the interface among frugivore guild-plant populations-landscape structure (Nathan 2006).

The consequences of dispersal assessed under a network approach

At a landscape level, few studies have incorporated the network thinking to the study of dispersal in heterogeneous populations (Proulx *et al.* 2005). Under this approach, populations can be envisioned as a set of nodes connected by pollination or seed dispersal events (links), obtained from direct assessment based on genetic and spatial information. This allows exploring the functional and structural characteristics of the metapopulation emerged due to dispersal at the whole system level (Barabási 2002). Urban & Keitt (2001) extended the network approach to a spatially explicit context by exploring the structure of the network emerged when incorporating the probability of dispersal among patches along with their size and spatial location. Another interesting utility of the network approach was illustrated by Dyer & Nason (2004). They characterized the topology of the network composed by a set of populations of Lophocereous schottii in Sonora desert based on the genetic composition and the spatial location of the populations. The authors concluded that the current genetic structure of the metapopulation is due to isolation by distance (involving a restriction to seed dispersal) rather than vicariance events. This rationale is implicit in recent efforts to understand complex geographic mosaics of coevolving species (Thompson 2005). Besides testing for different evolutionary scenarios, the network perspective offers an integrative approach suitable for studying dispersal in heterogeneous populations; it summarizes the landscape traits covering different spatial scales and dealing simultaneously with the structure and the function of the ecological systems (Urban & Keitt 2001) in a way analogous to other complex ecological networks (Bascompte & Jordano 2006). Additionally, networks can undergo structural or functional changes such as node removal or modifications in dispersal flows to evaluate the contribution of certain nodes or motifs (subnets with 3-5 connected nodes) to overall recruitment and to identify source-sinks dynamics. The dynamic consequences of non-randomly assembled networks (i.e., those with links connected following a non-random processes such as preferential attachment) are well known in the literature: fast propagation of information, resilience to random failures, high susceptibility to loss of the most connected nodes (Albert *et al.* 2000, Strogatz 2001).

Dispersal assisted by frugivores in plant population is a markedly non-random process that might give rise to this type of networks where a few patches (or trees) contribute most dispersal events (Aldrich & Hamrick 1998, Sezen *et al.* 2005) due to the selective feeding behaviour of the frugivores (Jordano & Schupp 2000). We have evidence that most of the source trees in a *P. mahaleb* population contributed with few (if any) seeds to a one-year seed rain, whereas a few ones contributed with a larger number of seeds (García *et al.* 2006b). Thus, a thorough understanding of the network structure emerged from dispersal dynamics assisted by animals provides valuable insights to fully understand the differences among species in historical migration rates (Clark *et al.* 1999) or large-scale distribution of the genetic diversity (Petit *et al.* 2003). In spite of being a powerful approach, we only will make the most of these techniques when obtaining dispersal processes-based measures for the whole landscape and the individual node or patch (Hanski 1998). Thus, network models should be based on reliable estimates of the dispersal processes among demes, (ideally dissecting the contribution of different vectors, as stated above) and incorporate detailed ecological data to elucidate the role of dispersal shaping recruitment dynamics.

Definitely, a unified framework with common concepts, metrics, and analytical methods to address dispersal in plant populations has emerged. This common framework widens the approachable array of topics dealing with dispersal; new questions arise while getting accurate answers to the old ones, insufficiently solved solely under an ecological or genetic perspective. More interestingly, the emergence of new common spaces requires the accuracy of established concepts under the ecological and the genetic paradigms to be evaluated (see Waples & Gaggioti 2006 for a review on the concept of population under the ecological paradigm and the evolutionary paradigm). The forthcoming goal involves fulfilling the puzzle with ecological data and genetic models to fully understand the ecological and evolutionary role of dispersal in plant populations.

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3

Mating patterns, pollen and the ecological maternal neigbourhood in a *Prunus mahaleb* (L.) population

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Abstract

Gender polymorphism, plant-animal interactions, and environmental heterogeneity are three important sources of variation in mating system and pollen dispersal patterns. We used progeny arrays and paternity analysis to assess the effects of gender type and density level on variation in mating patterns within a highly isolated population of *Prunus mahaleb*, a gynodioecious species. All the adult trees in the population were sampled and located. The direct estimate of long-distance insect-mediated pollination events was low (< 10 %). Gender expression deeply influenced the mating system, decreasing the outcrossing rates (t_m) and the pollen pool diversity in hermaphrodite trees. Long intermate distances (> 250 m) were significantly more frequent among female mother trees. Variation in local tree density also affected pollen pool diversity and intermate distance, with a higher effective number of fathers (k_e) and longer intermate distances for female trees in low-density patches. A canonical correlation analysis showed significant correlations between mating variables and the maternal ecological neighbourhood. Only the first canonical variable was significant and explained 78% of variation. Outcrossing rates tended to decrease, and the relatedness among the fathers to increase, when mother trees grew in dense patches with high cover of other woody species and taller vegetation, away from the pine forest edge. We highlight the relevance of considering maternal ecological neighbourhood effects in mating system and gene flow studies since the maternal trees act simultaneously as receptors of pollen and as sources of the seeds to be dispersed.

Key words

Mating system, pollen dispersal, paternity analysis, environmental heterogeneity, canonical correlation analysis.

Introduction

A long-lasting question in plant reproductive biology relates to mechanisms influencing the evolution of mixed-mating systems and their contribution to genetic diversity within a population (Charlesworth & Charlesworth 1978). Accurate, fine scale measures of the mating system can be obtained with molecular markers, and most previous studies have estimated outcrossing rates as population averages (Ritland 2002). A wide range of variation has been found, however, at the individual plant level in *Cavanillesia platanifolia* (Murawski & Hamrick, 1992) where outcrossing rate estimates varied between 0 and 0.95 depending on the density of flowering trees around the maternal tree. Thus, the mating system is subject to maternal ecological correlates that differ among individuals within a population, especially in heterogeneous landscapes (Schnabel & Hamrick 1995, O'Connell *et al.* 2004). An additional step is needed to elucidate the structure and strength of the interaction between mating and ecological variables within a population. This goal needs to be approached under a spatial-explicit framework incorporating the environmental heterogeneity of the natural populations, i.e. extending the approach of Sork *et al.* (1999) for the study of pollen flow from the landscape level to the local (population) scale.

We assessed variation in both mating system and maternal ecological neighbourhood in order to identify the maternal correlates shaping mating and pollen dispersal patterns in a population of *Prunus mahaleb* (L.), a gynodioecious species (*i.e.,* having hermaphrodite and functionally female trees). We used microsatellite markers and a mixed-mating model developed by Ritland (1989) to estimate outcrossing rates (t_m). For studies aiming to test the impact of one or more male-specific features on relative reproductive success, a fractional assignment procedure is recommended (Devlin *et al.* 1988). Moreover, male reproductive success or ongoing pollen flow studies are usually carried out in large populations where pollen flow is extensive and the genotyping of all potential pollen donors is not feasible (Smouse & Sork 2004). In such cases, mating models that incorporate prior information influencing male relative success are fitted to multilocus genotypes from mother-offspring arrays (Burczyk *et al.* 1996). However since we deal with a highly isolated population where all trees have been sampled, and our molecular markers reach a high exclusion probability (EP), we apply a direct approach based on paternity analysis and maximum likelihood methods to assign paternity to progeny arrays (Meagher 1986, Devlin *et al.* 1988, Adams *et al.* 1992). A low percentage of pollen immigration is expected due to a marked physical isolation and to non-overlapping phenology with surrounding populations.

The maternal reproductive trees in a population (i.e. functionally female trees) are key elements in the demography and natural regeneration cycles. They act as pollen targets during pollination, provide the environment for progeny growth, and become sources for seed dispersal. Therefore, understanding variation in female success as pollen receptors and as sources of dispersing seeds is a central issue in gene flow studies (Ennos 2001). There are three potential factors determining mating and pollen dispersal traits within the *P. mahaleb* study population: i) the gender type, since female and hermaphrodite (and autocompatible) trees coexist; ii) pollinator foraging behaviour, as it is an insect-pollinated species; and iii) the maternal ecological neighbourhood in a highly heterogeneous landscape. Selfing has a pervasive influence through decreasing pollen pool diversity (number of siring trees, their contribution and the genetic relatedness among them) and intermate distance for the progeny of hermaphrodite trees. Moreover, female and hermaphrodite trees can differ in reproductive resource allocation or floral biology (Gibson & Diggle 1997) and pollinators are sensitive to these variations (Murawski 1987). Jordano (1993) recorded higher visitation rates in female trees and significant differences between gender types in the pollinator assemblages in the study population, due to significant differences in the nectar volume secreted per flower and per day. Consequently, the differential activity of the pollinators on female and hermaphrodite flowers would shape the mating and pollen dispersal patterns beyond the foreseeable effects of selfing. On the other hand, the spatial arrangement of the conspecific trees determines pollen dispersal distance, as shown by Handel (1983), who found restricted pollen dispersal when trees were clumped instead of following a regular arrangement. Additionally, the maternal ecological neighbourhood differs individually in ecological characteristics such as vegetation structure, and this might increase or decrease mating variation depending on the ecological requirements of the pollinator community (Godt & Hamrick 1993).

According to van Treuren *et al.* (1993), outcrossing rates among individuals of animal-pollinated species vary due to plant density and gender polymorphism, mainly because of related changes in flower production. Nonetheless, the effects of tree density and gender type on pollen pool diversity still remain unclear. These two factors would be expected to modulate pollen pool diversity and, presumably, intermate distance. Previous analyses show that no single factor explains variation in mating success among male (Smouse *et al.* 1999) or female trees (Schnabel & Hamrick 1995). Currently, multivariate techniques are useful tools to quantify the relative contribution of multiple individual characteristics acting simultaneously to shape variation in mating patterns on a fine scale (Angers *et al.* 1999, Gram & Sork 2001). Here, we use canonical correlation analysis (CCA) to relate mating system variation among mother trees (outcrossing rates, number and diversity of distinct fathers, and distance to the pollen sources) with a set of variables defining tree characteristics and the ecological maternal neighbourhood.

In this study we analyze two major factors influencing mating system and pollen dispersal patterns: gender expression and density of conspecifics. Additionally, the maternal and ecological correlates that determine mating variation within the population are explored. Specifically, the aims are to: i) estimate the proportion of pollen immigration; ii) test the effect of gender type and density level on outcrossing rates; iii) evaluate the effect of these two factors on pollen pool diversity in terms of number of fathers (k), effective number of fathers (k_e) and relatedness among the fathers siring a progeny (r) ; iv) assess the effect of gender type and density level on intermate distances; and v) study the multivariate relationship between mating system variation and the characteristics of the mother trees, and their ecological neighbourhood in heterogeneous landscapes.

Material and methods

Species characteristics and study site

The study species is *Prunus mahaleb* (L.), a rosaceous treelet that in SE Spanish populations is gynodioecious, with some individuals producing hermaphrodite flowers and others with androsterile flowers which behave as functional females (Jordano 1993). In the southern Iberian Peninsula this species flowers between mid-May and mid-June at high elevations (over 1300 m) and insects, mainly bees (Hymenoptera: Andrenidae, Apidae) and flies (Diptera: Calliphoridae, Syrphidae), act as pollen vectors (Jordano 1993) (Fig. 1). *P. mahaleb* produces fleshy fruits (drupaceous) with 1 seed per fruit. In late July, fleshy fruits are produced and consumed by frugivorous animals that disperse the seeds until late August or early September. *P. mahaleb* is widely distributed through Central, Southern, and Eastern Europe and West-central Asia (Webb 1968). In the southeastern Iberian Peninsula it thrives both on deep soil and exposed rocky slopes The study population is located in Nava de las Correhuelas, (Parque Natural de Sierra de Cazorla, Segura y las Villas, Jaén province, 37°59'N, 2°54'W), at 1615 m elevation. The site is dominated by grasslands with scattered patches of deciduous vegetation, gravelly soil or rock outcrops covered by shrubs or small isolated trees. The rocky slopes are dominated by open pine forest (*Pinus nigra* subsp. *salzmannii*) (Valle *et al.* 1989). Our population consists of 104 hermaphrodite and 92 female trees, with a scattered and patchy distribution as shown in Fig. 2. Populations of *P. mahaleb* are typically found in this region as small, isolated patches of trees, frequently with < 100 trees. The nearest *P. mahaleb* population is located at 1.5 km.

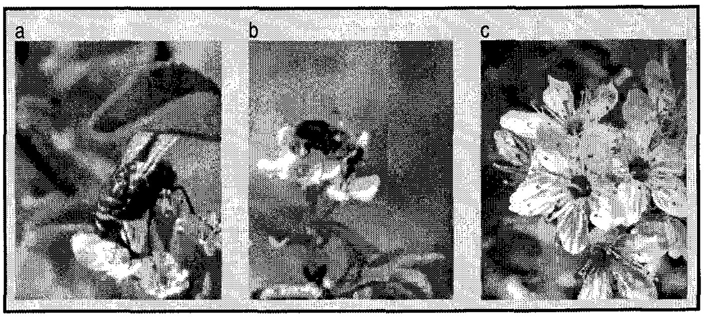

Figure 1. Flies and bees are the main pollinators of *P. mahaleb* flowers. a. Diptera: Calliphoridae, Syrphidae; b. Hymenoptera: Andrenidae, Apidae; c. Flower of *P. mahaleb*

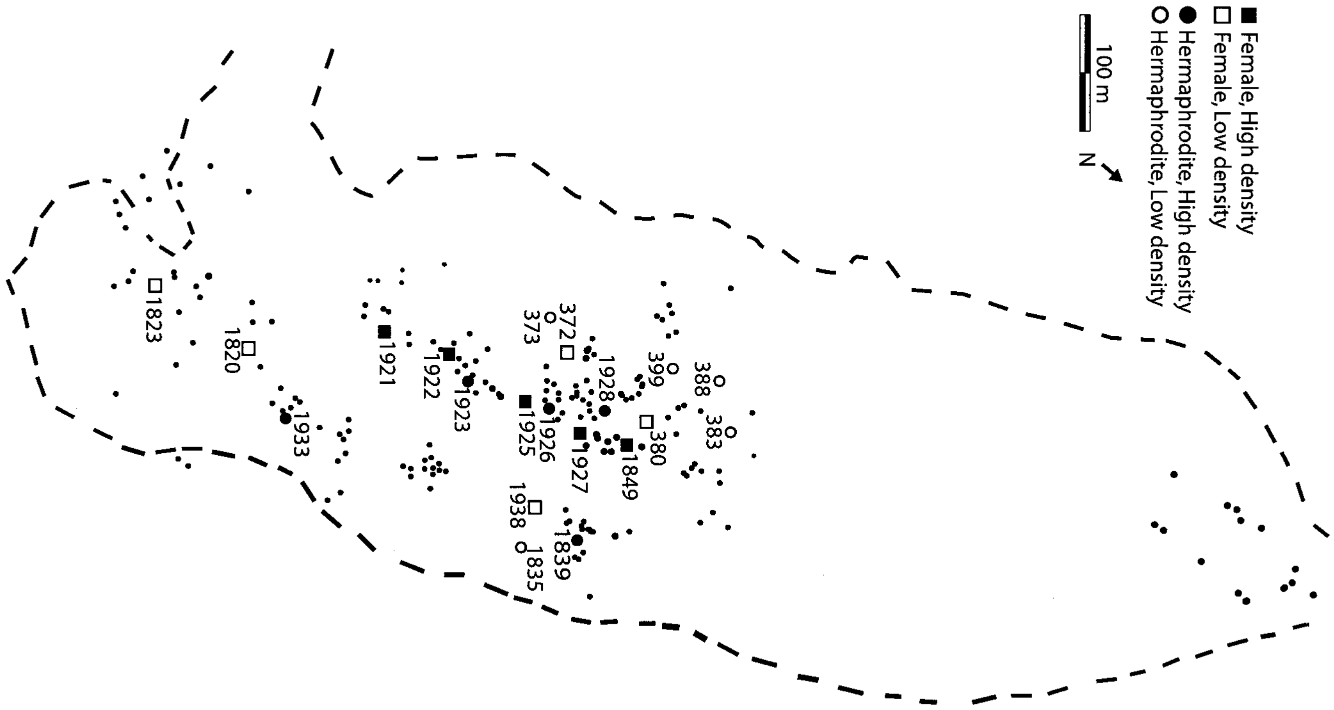

Figure 2. Spatial distribution of all adult trees (N= 196) over the study site. Small dots indicate the locations of all the reproductive trees. The mother trees included in this study are indicated with squares (female mother trees) or circles (hermaphrodite mother trees). Density situations are indicated as solid (high density) and open symbols (low density). Numbers are the identifier for each tree. The dashed line indicates the approximate location of the pine forest edge.

Sampling design

The study population was previously genotyped and mapped by Godoy & Jordano (2001) with a few trees added to this previous sample; the additional trees grow in a small patch 300 m N of Nava de las Correhuelas and have been included in the population sample. This represents a thorough sample of the whole population and we are confident that it includes all the reproductive trees (Fig. 2). A random selection was made of 20 mother trees stratified by gender type and local density of adult conspecifics. Local density levels were defined for each tree based on the number of conspecifics within a 15 m radius. This threshold value was determined from an analysis of nearest neighbour distances. Trees in high density had at least 4 conspecifics within this distance and low density trees had 1-2 conspecifics. Finally there were four groups with *N*= 5 mother trees per group in four combinations of gender type x density: female, low density (FL); female, high density (FH); hermaphrodite, low density (HL); and hermaphrodite, high density (HH). Ten fruits were collected at random from each mother tree at the start of the ripening period with 50 fruits analysed per group and a total number of 200 seeds sampled.

Six variables characterizing the maternal tree and its ecological neighbourhood were measured: area of the vertical projection of the canopy (CNPY); mean height of woody shrubs beneath the canopy (UCHGT); the number of species beneath the canopy (UCSP); the percentage of woody cover beneath the canopy (UCWC); under canopy rocky cover (UCOVR); and distance to the pine forest edge (DFOREST, see Fig. 2.). All variables were measured at 4 regularly-located points in the 4 compass directions from the main trunk of the tree (see Jordano & Schupp 2000).

Molecular typing

Each harvested seed was separated from the pulp, and the embryo carefully extracted from the seed endocarp and the seed coat. DNA was extracted from all embryos using a SIGMA Gen Elute^{TM} Plant Genomics DNA Kit, with 5-10 μL used as a template for polymerase chain reaction (PCR) in a final volume of 20 μL (see Godoy & Jordano 2001 for protocol details). All embryos were genotyped using 10 microsatellite markers previously used to type the adult trees (Godoy & Jordano 2001). Amplified fragments were analysed using an ABI 310 capillary electrophoresis system (Applied Biosystem) and fragment sizes were assessed with GenScan Analysis ® 3.1.2 and Genotyper ® 2.5 software using TAMRA 350 (Applied Biosystem) as a size standard. The multilocus genotypes of all trees differed in at least one allele per locus. Each marker presented between 2 and 21 alleles with an estimated mean number of alleles per locus of 7.6 and a paternity exclusion probability (i.e. the probability to exclude a randomly chosen non-father, based on allele frequencies) of 0.996. Three embryos were excluded from the analysis due to several mismatches with the mother tree. The incidence of null alleles was discarded, as mismatching occurred at several heterozygous loci. Moreover, in previous work by Godoy & Jordano (2001) where progeny from diallelic crosses of known sires and dams were compared with parental genotypes, no evidence was found for the occurrence of null alleles. Allele scoring was carefully checked several times by different people in order to minimize the scoring error rate.

Data analysis

Paternity analysis

Paternity analyses (the assignment of a putative father for a genetically known mother-offspring pair) were performed using CERVUS 2.0 (Marshall *et al.* 1998), a software program based on maximum likelihood methods (Meagher 1986). In addition, 10,000 simulations were run with the same sample size and allele frequency as the study population to assign confidence levels to paternity analysis (95% or 80% by default). CERVUS uses five parameters set by the user: i) the minimum number of matching loci (8 loci), ii) error rate (0.1%), iii) number of candidate parents (104, including all the hermaphrodite trees within the population), iv) proportion of candidate parents sampled (0.95 as a conservative estimate) and v) proportion of loci typed (0.99). In this work the relaxed confidence level of 80% was used, since we are less interested in identifying particular mates than in drawing a general picture of pollen movement within the population. Following the criteria proposed by Slate *et al.* (2000), the most likely father assigned at an 80% confidence interval was accepted when its LOD score was ≥ 3, and rejected when its LOD score was ≤ -3. When the LOD score was between 3 and –3, no most likely father was assigned. In spite of the high exclusion probability reached, a fraction of the offspring assigned to one most likely father may be erroneously assigned to a putative father within the population, whereas the true father is outside the population. This defines cryptic gene flow (CGF) (Devlin & Ellstrand 1990) and was estimated here with FAMOZ software (Gerber *et al.* 2003). A simulated population was built with the following parameters: simulated offspring (N= 200), error rate (0.1%), number of mothers (N= 20), number of contributing fathers (N= 104).

Mating system and pollen dispersal distances

Outcrossing rates (t_m) were estimated using MLTR 3.0 software based on the method of moments, a more accurate procedure than maximum likelihood when dealing with small progeny size (Ritland 2002). Both single locus and multilocus outcrossing rates were obtained (t_s and t_m, respectively), but only t_m data is reported, as they yielded similar values. The result from paternity analysis with CERVUS 2.0 was then used to obtain the minimum number of fathers siring each mother tree (k) and the effective number of fathers (k_e) was estimated according to Nielsen *et al.* (2003), who provide an unbiased estimator of the effective number of fathers for small sample sizes, k_e:

$$k_e \simeq \frac{(n-1)^2}{\sum_{i=1}^{k_0} \hat{p}_i^2 (n+1)(n-2)+3-n}$$

with an approximate variance:

$$var[k_e] = \frac{2k^2(k-1)(n-2)^2(n-1)n(n+1)^2}{(2k+(n-2)(n+1))^4}$$

where n is the number of embryos analyzed, k is the number of distinct fathers, and p_i^2 is the frequency of each different father. Using SPAGeDi (Hardy & Vekemans 2002), an estimation was also made of the relatedness (r, Queller & Goodnight 1989) among fathers siring each mother tree, defining 20 groups (one per mother tree), each containing the fathers siring each progeny. Pairwise comparisons were restricted to within groups regardless of the spatial location of the trees, and using N= 10,000 permutations.

Significant differences among groups were tested by using a two-way GLM model with Poisson-distributed errors and a log link function for k and k_e; otherwise normal errors and an identity link were used, as in usual ANOVA (Venables & Ripley 2002). The post-hoc differences were checked with a Tukey-Kramer HSD test (Zar 1999). Two separate sets of analyses were performed, one involving the whole progeny sample of the hermaphrodite trees and a second one excluding the selfed progeny. We report the estimates of mating parameters for both sets of samples, although only the complete set (including the selfed progeny) reflects the real mating pattern in nature. All analyses were carried out with the R package (R Development Core Team 2003).

Measurements were made of the distance between the mother and the putative father (intermate distance) when one most likely father was assigned, given the geographic coordinates (x, y) in a GIS-referenced map in ArcView 3.0a software (Mitchell 1999). In addition, the distances between each mother tree and all hermaphrodite trees (potential intermate distance) were also measured to determine if the observed intermate distance (d) was influenced by the spatial arrangement of the adult trees. A comparison was made of the skewness (g_1) and the kurtosis (g_2) of the frequency distributions of both distances. Within each group of gender and local density combination, the observed and potential frequency distribution distances were compared using a Wilcoxon matched pair test (Zar 1999). The effect of gender type and density level on intermate distance was tested using a GLM analysis (R Development Core Team 2003). All analyses were carried out with both inclusion and omission of the selfed progeny of the hermaphrodite trees.

Canonical correlation analysis

Relationships between mating measurements and ecological variables were examined by means of canonical correlation analysis (CCA). The mating pattern for each tree was defined by mating variables estimated from the progeny genotyping: median intermate distance (*d*), minimum number of distinct fathers (*k*), effective number of fathers (k_e), multilocus estimate of the outcrossing rate (t_m, Ritland 2002), and relatedness among fathers siring the sampled progeny (*r*, Queller & Goodnight 1989). The ecological setting for each tree was defined by the six environmental variables previously described. The canonical correlation analysis (CCA) aims at generating composite variables (canonical variables) that maximize correlations among the sets of mating and ecological variables. These can be envisioned as mating and ecological vectors (Angers *et al.* 1999, Gram & Sork 2001) that define a gradient of variation among trees in a mating system and in characteristics of the growing site. Each original variable has a correlation with the new canonical variables, which define a canonical structure that can be used to interpret them. The canonical correlation squared (R^2) is the percentage of variance in the dependent set explained by the independent set of variables along a given dimension. In order to asses whether this relationship is significant the CCA uses a Wilk's lambda test (Everitt & Dunn 1991). We used PROC CANCORR (SAS Institute 1998) to carry out the canonical correlation analysis.

Results

Paternity analysis and pollen immigration

A single most likely father was assigned to 160 embryos (81.21 %), all of them with a LOD score ≥ 3. Several compatible fathers, but no one most likely father was found for 24 embryos (12.29%), all of them with a LOD score between 3 and –3. For 13 embryos (6.50%), the most likely father assigned was rejected as it had a LOD score value ≤ -3. Every time a most likely father was rejected, all candidate fathers had more than 3 mismatches with the embryo. When the estimated cryptic gene flow (CGF) is added to the estimate of apparent gene flow, the percentage of pollen immigration is 9.5%. Given the high exclusion probability (EP= 0.996) and exhaustive sampling of the adult trees, it can be assumed that this percentage represents a robust estimate of the percentage of pollen immigration in our population.

Gender type and density level effects on mating system

Results shown in Table 1 confirm that all pollination events recorded in female trees were outcrossed ($t_m \approx 1$) whereas the hermaphrodite mother trees had considerably lower levels of outcrossing ($t_m = 0.37 - 0.46$), significantly different from $t_m = 1$ ($\chi^2 = 2.83$, df= 9, $P < 0.05$). Thus, more than half of the progeny from hermaphrodite trees were selfed, with an average

Table 1. Mean (± 1SD) values for mating system and pollen dispersal variables. Mating system was characterized by t_m (mean multilocus outcrossing rate), k (number of distinct fathers), k_e (effective number of fathers), and r (relatedness among the fathers siring each progeny). We also report the mean, the first quartile (Q_{25}), the third quartile (Q_{75}), and the minimum (Min) and maximum (Max) intermate distances recorded for each group of trees. The number of seeds sampled per group is n. Two series of values are reported for the hermaphrodite mother trees: those for the complete sample and those excluding the selfed progeny (indicated by "-0" suffix).

Gender type	Density	n	t^1_m	k^1	k^1_e	r^1	Mean [Q25-Q75]^1	Min-Max
Female	Low	50	1.01 (0.02) $^{a-1}$	7.9 (1.5) $^{a-1}$	5.7 (0.4) ^{a}	0.1 (0.1) $^{a-1}$	125.2 [53.1-158.3] $^{a-1}$	6.7-548.1
Female	High	48	1.01 (0.04) $^{a-1}$	7.2 (1.4) $^{a-1}$	4.1 (0.4) ^{b}	0.31 (0.1) ^{a,b}	72.0 [17.7-76.8] $^{b-2}$	1.6-362.7
Hermaphrodite	Low	44	0.41 (0.2) ^{b}	3.9 (1.8) ^{b}	2.6 (0.4) ^{b,c}	0.51 (0.1) ^{b}	91.4 [0.0-29.5] ^{b}	0.0-252.9
Hermaphrodite	High	44	0.49 (0.3) ^{b}	3.1 (1.1) ^{b}	1.6 (0.4) ^{c}	0.59 (0.2) ^{b}	75.1 [0.0-123.3] ^{b}	0.0-278.7
Hermaphrodite -0	Low	15	0.98 (0.2) ^{1}	3.5 (0.8) ^{2}	-	0.45 (0.1) ^{2}	132.9 [55.8-182.8] ^{1,2}	23.1-252.9
Hermaphrodite -0	High	18	0.99 (0.1) ^{1}	2.1 (0.6) ^{2}	-	0.41 (0.1) ^{2}	116.9 [55.6-138.6] ^{1,2}	10.7-278.7

[1] Superscripts with the same letter indicate non-significant differences for the whole sample; superscripts with the same number indicate non-significant differences for the restricted sample excluding the selfed progeny of hermaphrodite trees ($P< 0.05$, Tukey-Kramer HSD test). k_e was not estimated for the Hermaphrodite-0 group due to insufficient progeny size.

estimate of outcrossing of $t_m = 0.41$ for HL and $t_m = 0.49$ for HH. The components of the pollen pool diversity also differed among gender types. When considering full progenies, those from female mother trees had a higher number of fathers (k), (F= 23.26, df= 1, 16, P< 0.001). When excluding selfing, low values of k and large variances were obtained for hermaphrodite families, indicating that self-pollen tended to dominate the pollen pool, although to a variable degree (Hermaphrodite-0; Table 1). Gender type also affected the effective number of fathers (k_e), and female mother trees presented almost double the k_e of hermaphrodite trees (F= 78.20, df= 1, 16, P< 0.001). Additionally, progeny from FL had significantly higher k_e compared to progeny from FH, indicating that a high density of conspecifics around the mother trees lowered k_e (F= 6.67, df= 1,8, P< 0.01). When excluding selfing, the progeny sample per tree was too small to estimate k_e by applying the Nielsen *et al.* (2003) algorithm, since it is advisable to have at least 10 offspring per mother tree for this purpose. The third component of the pollen pool diversity, the relatedness among fathers siring a progeny (r), also varied between gender types (F= 6.95, df= 1, 16, P= 0.02). Among outcrossed pollination events, the offspring from female mother trees were less correlated than the offspring from hermaphrodite mother trees, with the lowest mean r value found in FL mother trees (Table 1). The interaction term between gender type and density level was not significant for r (F= 0.59, df= 1, 16, P= 0.45).

Gender type and density level effects on pollen dispersal distance

The frequency distribution of observed intermate distances (d) had higher skewness (g_1= 2.1) and kurtosis (g_2= 5.4) than the distribution of potential intermate distances (g_1= 1.1; g_2= 0.4, respectively) (both t> 4.1, P< 0.05). Observed and potential intermate distances differed for all mother tree groups (Wilcoxon matched pairs test, all comparisons Z> 2, P< 0.05); the median

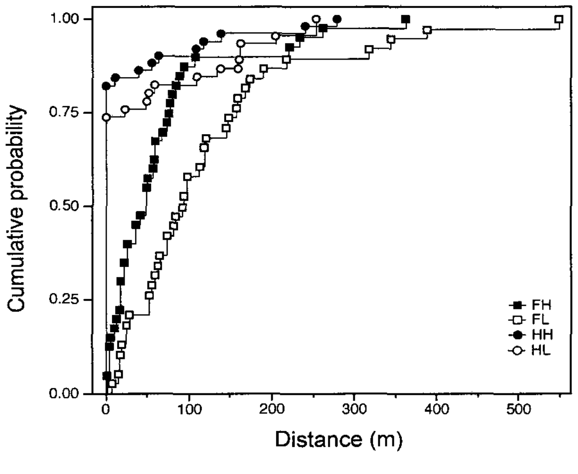

Figure 3. The cumulative probability curves for the frequency distributions of intermate distances (distance between the maternal tree and the putative father). Maternal trees are grouped as female mother trees (squares) and hermaphrodite mother trees (circles), located in high density (solid) and low density (open) situations. A zero intermate distance represents self-pollination events.

observed intermate distance was always lower than the median potential distances. Gender type and density level had significant effects on *d* when including selfed crosses (F= 15.81, df= 1, 99, P= 0.0001 for gender, and F= 4.32, df= 1, 98, P= 0.04 for density; Table 1). When considering outcrossed pollination events, only the density effects were significant (F= 6.11, df= 1, 88, P= 0.015). As expected, intermate distances < 50 m were significantly more frequent among hermaphrodite mother trees (Z= 8.80, P< 0.01), due to high frequency of selfing (d= 0.0 m, by definition) (Fig. 3). More interestingly, the cumulative frequency curve showed significant differences between gender types at distances beyond 250 m (Z= 2.06, P= 0.047), with a higher frequency of long-distance pollination events for isolated, female trees (Fig. 3.). Density had a significant effect on female mother trees, and FL mother trees presented longer *d* than FH mother trees. There were no observed differences between HL and HH in mean intermate distance when including or excluding selfing (Table 1).

Canonical correlations of mating and ecological neighbourhood

Variation in mating variables among individual trees was significantly related to differences in their ecological setting, as evidenced by the significant CCA (Wilk's λ= 0.0172, F= 2.657, df= 30, 18, P= 0.042). Only the first canonical variables (Mating1 and Ecol1) were significant, with a canonical correlation of R^2= 0.939 (F= 2.66, P= 0.041); the best fit model with these two canonical variables explained 78.1% of variation. The first mating canonical variable (Mating1) was negatively associated with outcrossing rate (t_m) and increased relatedness among fathers

Original variables	Canonical variables
A.	
	Mating 1
d	- 0.0664
t_m	- 0.4563
k	0.1019
k_e	0.1995
r	0.4651
	Ecol1
CNPY	0.0362
UCHGT	0.4617
UCSP	0.5781
UCWC	0.5283
UCOVR	0.4927
DFOREST	-0.4759
B.	
	Ecol1
d	-0.0643
t_m	-0.4627
k	0.0987
k_e	0.1932
r	0.4506

Table 2. Results of canonical correlations analysis involving mating variables and ecological measurements of trees and growing sites. Only the first canonical variable was significant. A. The canonical structure is described by the correlations between the original mating measurements and the canonical mating variable (Mating1); and by the correlation between the original ecological variables with the canonical ecological variable (Ecol1). B. Correlations between the mating variables and the canonical ecological variable, Ecol1. Bold numbers > 0.40; P< 0.05, df= 18.

(r) (Table 2A). The ecological variables describing the growing site of the trees determined Ecol1, the ecological canonical variable. This variable defines a gradient of increasing cover of woody shrubs (UCWC), height of vegetation beneath the canopy of the trees (UCHGT), higher number of woody species close to the tree (UCSP), and higher rock cover (UCOVR); the distance to the pine forest edge (DFOREST) was negatively correlated with this trend (Table 2A). Trees in dense clumps with high cover of other woody species and taller vegetation, and growing away from the pine forest edge had positive scores in this canonical variable. The mating variables which showed a more marked response to this ecological gradient were outcrossing rate (t_m)and relatedness among siring trees (r) (Table 2B). Outcrossing rate was negatively correlated with Ecol1 while r was positively correlated with it (Table 2B). Thus t_m tends to decrease, and r among the fathers tends to increase, when trees grow in dense patches of high vegetation cover, usually away form the pine forest edge.

Discussion

Despite the importance of pollen flow as a process shaping genetic structure in natural plant populations, little direct evidence of long distance events has been reported (Chase *et al.* 1996, White *et al.* 2002, Gaiotto *et al.* 2003). Previous work based on artificially arranged, experimental plantations suggests that pollen dispersal patterns within and among populations are more complex than expected when the distance component only is considered (Richards *et al.* 1999). Our results with *P. mahaleb* confirm this by showing a marked influence not only of distance effects, but also of the complex landscape surrounding the reproductive trees. Evidence is given for low pollen immigration, with less than 10 % of the seeds being sired by trees outside the population. While the area was exhaustively sampled, the presence of some isolated trees between our study population and the nearest one cannot however be discounted. The amount of pollen immigration reported here is quantitatively low, but substantial in terms of its potential implications, as it might represent consistent long-distance insect-mediated pollination events given that the nearest population is located at 1.5 km. Our estimate is similar to previously reported direct estimates, ranging from one to several kilometres for *Swietenia humilis* (White *et al.* 2002), in one of the few studies focusing on a strictly isolated population. When geographic isolation involves differences in elevation and phenology, gene flow by pollen can be severely limited (Chase *et al.* 1996, Jordano & Godoy 2000).

The effect of phenology is illustrated in our dataset by the fact that a single pollination event, of the 200 embryos analysed, came from a small group of trees (*N*= 17) located 300 m N of the main population site. These trees tend to flower earlier (P. Jordano pers. obs.), and this appears to limit pollen flow with the rest of the trees due to their largely non-overlapping phenologies. Distance alone can not explain the low levels of pollen immigration in our study area, as insects may easily transport pollen over long distances (Janzen 1971, Chase *et al.* 1996, Nason *et al.* 1998, Fuchs *et al.* 2003). Chapman *et al.* (2003) documented that workers of *Bombus terrestris* and *B. pascuorum* colonies - both pollinators of *P. mahaleb* - travelled kilometres from their colonies. However they are more likely to promote short distance pollen movement, particularly in high density and clumped situations such as our study site (Levin & Kerster 1969, Harder 1990). Therefore, patchy regional distribution with populations separated over a kilometer, together with non-overlapping phenology due to altitudinal differences and a clumped arrangement of the adult trees, might favour locally restricted foraging of the pollinators and limit the number of long distance pollination events.

Mating system variation was pervasively influenced by gender expression, as variable selfing levels translated into significant differences in t_m, k, k_e and r. The estimated values of outcrossing for hermaphrodite trees were slightly lower but congruent with the indirect estimate obtained previously, based on seed mass data and other values for self-compatible species (Jordano 1993, and references therein). Outcrossing rates were highly variable among hermaphrodites, as shown by their high standard deviation for HL and HH groups. Despite the potential limitations of the progeny sample, the data reveal robust differences. However larger progeny arrays would be needed to assess variation at the tree level. The low outcrossing rates found in hermaphrodite trees (0.37-0.46) yielded low values of k and k_e. We also found that fathers siring hermaphrodite trees were more related among themselves than fathers siring female trees. However, this cannot be attributable to selfing alone, as r values were significantly higher when excluding selfing in hermaphrodites. Rather, variation between the two genders might be influenced by the local spatial genetic structure of the adult trees, since adults located within 35 m distance are genetically more similar than randomly expected (Jordano & Godoy 2000). As female mother trees had a wider range of intermate distance, the relatedness among siring trees was lower than the relatedness of trees siring hermaphrodites. Thus, hermaphrodite mother trees received pollen from a lower number of fathers, contributing less evenly and being more related among them than the fathers siring female trees. This effect appears as a direct consequence of the spacing patterns in the population. Previous studies have stated that, all else being equal, the larger the size of the paternity pool, the higher will be the fitness of the mother plant (Levin 1988). The combined effects of selfing, biparental inbreeding and a less diverse pollen pool can explain the differences in fitness associated with gender type found by Jordano (1993) in the same studied population, where reduced fruit production and smaller seed mass were reported for hermaphrodite trees.

From an evolutionary point of view, hermaphrodite trees disperse their genes twice (both at pollination and seed dispersal stages), and since they do not depend on an external pollen source for successful fruit set, pollen limitation during unfavourable weather and low pollinator activity is less likely to occur (Jordano 1993 and references therein). Thus, a higher reproductive fitness of female trees can be invoked to explain their maintenance in the population. In our study area, almost 50% of the adult trees were female. The present results, combined with previous analyses, indicate that the pollen cloud of female trees renders a highly diversified progeny that can result in higher relative fitness, especially when pollinator activity is not limiting. However, additional field data relating variation in mating patterns to variation in fitness due to gender expression is needed to fully understand the evolution of the mixed-mating system and the persistence of the gynodioecious condition.

Gender type also influenced intermate distance, as selfing implies $d = 0$ m by definition. We thus found striking differences between gender types with respect to frequency of long distance pollination events, with female mother trees receiving pollen more frequently from hermaphrodites located at 250 m or further. This interesting result implies important differences between genders in the provenance of the pollen cloud beyond the obvious consequences of selfing. To our knowledge, differences in pollen dispersal distance between gender types have not been previously reported, although we do know that the species composition of the pollinator assemblage and visitation rates differed between female and hermaphrodites in the study population (Jordano 1993). Female flowers were significantly more visited than hermaphrodite flowers and their pollinator assemblage was dominated by calliphorid flies. On the other hand, hermaphrodite flowers were visited by solitary bees and syrphid flies (pollinivorous) in a similar proportion. Despite the dominance of calliphorid flies, female trees might be benefiting from the greater effectiveness of solitary bees in terms of pollen removal, flight distances and visitation rates (see e.g., Herrera 1987). Reliable visitation of female trees by solitary bees would assure pollen receipt from hermaphrodites even outside the denser patches.

The spatial arrangement and local density of the adult trees also shapes pollen dispersal patterns. Handel (1983) found that, all other things being equal, a clumped arrangement of the trees favoured shorter intermate distances compared with more regular distributions. This is also illustrated in our study, as the observed intermate distance frequency distribution is more skewed and leptokurtic than expected from the potential intermate distances. Presumably pollinator foraging movements are enhancing pollen transfer among nearby trees, as was previously reported for entomophilous species where the pollen pool tended to be dominated by immediate neighbours when the trees grew in clumped patches (Broyles & Wyatt 1991, Murawski & Hamrick 1991, Stacy *et al.* 1996). Moreover, we found that isolated locations of the female mother trees (FL) tended to promote longer pollen dispersal distances, a more even contribution of the siring trees and a lower relatedness among them. Therefore, variations in mating system and pollen dispersal pattern due to gender expression and conspecific density might set a range of genetic variation among progenies: from highly selfed progeny of hermaphrodites (HL, HH) with a low number of siring trees (most of them highly correlated and located close to the mother tree), to the outcrossed progenies of females (FH and, especially, FL) with a high number of fathers contributing evenly and sometimes located far away from the mother tree. The importance of this variation might be related to environmental conditions during the pollination phase. A cold and stormy spring would decrease pollinator activity, yet fruits can be set from selfing, avoiding the collapse of the demographic cycle due to pollinator limitation. Sunny periods would favour pollinator activity and increase the outcrossed progeny and the contribution of isolated trees, thus increasing the effective number of trees at the population level. In this context, only a long-term study would elucidate the ecological and evolutionary consequences of variation in mixed-mating species by examining the interactions between weather conditions, pollinator activity and mating patterns.

Not only the density of conspecifics, but the ecological characteristics of the maternal neighbourhood determined mating pattern variation in our population. Thus the canonical correlation analysis showed that dense maternal neighbourhoods (in terms of vegetation cover, height and number of woody species) were associated with lower outcrossing rates and higher relatedness among the siring trees. The negative correlation of the mating canonical variable with distance to the pine forest reflects the spatial distribution of the adult trees in the population, since open neighbourhoods with low vegetation cover tend to be located in the periphery of the population, near the pine forest edge (Fig. 2). Trees located in a more central position within the population, with a denser neighbourhood, tend to have progenies with lower t_m and higher r values. Most *P. mahaleb* pollinators are generalist, and a dense vegetation cover around a mother tree would enhance locally-restricted foraging (Cresswell 1997), and thus promote short intermate distances. Interestingly, our results show only a small influence of canopy size on the mating system, whereas previous works have emphasized the tree size/canopy size as an important factor influencing outcrossing rates (Burczyk *et al.* 1996, O'Connell *et al.* 2004). As we selected only adult trees, variation in the vertical projection of the canopy was small and consequently did not explain much variation in mating system. Individual variation in mating patterns is therefore the result of a complex interaction between gender expression, pollinator foraging, and the patchy environmental setting of complex landscapes.

Finally, consideration should be given to the dual role of functional female trees, both as targets for pollen receipt and as sources of dispersing seeds, especially in animal-dispersed species. In fleshy-fruited species, mating variation of seed-bearing trees also influences the subsequent stages of seed dispersal, because the maternal tree acts as a source of dispersed seed. Our results indicate that outcrossed and less-related seeds tend to be produced by trees located closer to the pine forest edges, and that less diversified progeny is expected in dense patches. Additionally, we know that a fraction of variation in seed mass among trees is attributable to the breeding system, as outcrossed seeds are significantly heavier, and that frugivores show significant selection effects on fruits that will influence fruit removal success (Jordano 1993, 1995). Moreover, different frugivore species show marked differences in habitat preferences and tree visitation, and we expect a highly variable dispersal success of this heterogeneous progeny. Evidence from direct observation of frugivores indicates that isolated trees are more likely to benefit from long-distance dispersal by efficient frugivores compared to trees growing in dense patches (Jordano and Schupp 2000; C. García & P. Jordano, pers. obs.). These sequential effects illustrate the relevance of maternal trees as key demographic elements in plant populations, simply because gene flow patterns (via pollen and seeds) pivot on their individual effects. A thorough view of animal-mediated effects on total gene flow patterns would necessarily consider on one hand the combined influences of variation in mating patterns and in seed dispersal ecology, and on the other hand, the assessment of the male and female fitness functions and their ecological correlates. Our analysis shows that both pollen- and seed-related processes are linked through the mother tree, its ecological environment, and the complex landscape where pollinators and frugivores forage. The simultaneous use of progeny genotyping and spatially-explicit analysis of environmental variables, including the outcomes of plant-animal interactions, will be key elements for an expanded approach to gene flow analysis considering dispersal via pollen and seeds.

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4

Contemporary pollen and seed dispersal in a *Prunus mahaleb* (L.) population:

Patterns in distance and direction

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Abstract

Pollination and seed dispersal determine the spatial pattern of gene flow in plant populations, and for those species relying on pollinators and frugivores as dispersal vectors animal activity plays a key role in determining this spatial pattern. For these plant species, reported dispersal patterns are dominated by short distance movements with a non-negligible amount of immigration. However, the contribution of seed and pollen to the overall contemporary gene immigration is still poorly documented for most of plant populations. In this study we investigated pollination and seed dispersal at two spatial scales in a local population of *Prunus mahaleb* (L.), a species pollinated by insects and dispersed by frugivorous vertebrates. First, we dissected the relative contribution of pollen and seed dispersal to gene immigration from other parts of the metapopulation. We found high levels of gene immigration (18.50%), due to frequent long distance seed dispersal events. Secondly, we assessed the distance and directionality for pollen and seed dispersal events within the local population. Pollen and seed movement patterns were non-random and with skewed distance distributions: pollen tended moved up to 548 m along a close to N-S direction, and seeds were dispersed up to 990 m frequently along the SW and SE axes. Animal-mediated dispersal contributed a substantial amount of gene immigration into the local population and had a markedly non-random pattern within the local population. Our data suggest that animals can impose distinct spatial signatures in contemporary gene flow, with the potential to induce significant genetic structure at local scales.

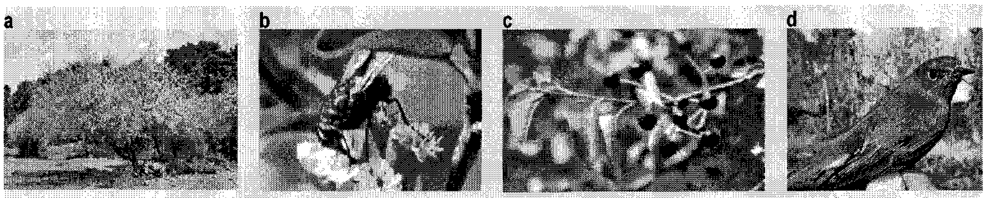


Figure 1. In species dispersed by pollinators and frugivore animals a large amount of gene immigration and non-random dispersal patterns are expected due to animal feeding patterns in heterogenous landscapes. When *Prunus mahaleb* trees are in blossom (a) pollinators such as flies (b) dispers their pollen, and their fleshy-fruits (c) are dispersed by vertebrates such as birds (d, *Phoenicurus ochruros*)

Key words

Dispersal distance, dispersal direction, gene immigration, microsatellites, pollination, seed dispersal.

Introduction

Pollination and seed dispersal are two critical stages in plant recruitment cycles, frequently involving the interaction with one or more animal species providing dispersal services for pollen or seeds (Howe & Westley 1988). The feeding and postfeeding movements of pollinators and animal frugivores, thus, influence the distance, direction, and magnitude of pollen and seed dispersal that ultimately determine the spatial pattern of gene movement within and among plant populations (Nathan & Muller-Landau 2000). Pollinators and frugivores can remove large amounts of pollen and fruits, a fraction of which might be deposited several hundreds of meters away from the source tree (Howe 1986, Harder & Barrett 1996, Nason et al. 1998, Schnabel et al. 1998, Godoy & Jordano 2001, Hardesty et al. 2006), thereby connecting fragmented populations and favouring the colonization of new patches (Cain et al. 2000, Tewksbury et al. 2002). In spite of having demographic and genetic consequences for plant populations, the relative contribution of contemporary pollen and seed dispersal to overall gene immigration is still poorly reported.

Ideally, we should be able to characterize both pollen and seed dispersal movements (the processes) in order to understand their role in shaping the adult spatial distribution and genetic structure in plant populations (the patterns) (Bossart & Prowell 1998). Dispersal usually involves an array of different vectors (biotic and abiotic) that distribute pollen and seeds non-randomly over all available target sites, either receptive flowers (pollination) or deposition sites in the landscape (seed dispersal). When animals are the dispersal vectors, the distribution of propagules is aggregated and heterogeneous due to selective feeding movements (Fragoso 1997, Wenny & Levey 1998, Nason et al. 1998, Jordano & Schupp 2000, Schupp et al. 2002, Russo & Auspurger 2004). The source of the dispersed propagules is usually unknown due to the difficulty of tracking pollen and seed movement (Cain et al. 2000). This information, however, is highly relevant since it would provide reliable estimates on dispersal distance and directionally required to assess the role of dispersal processes in shaping the recruitment patterns and genetic structure in plant populations. Moreover, the ability to identify pollen and seed sources allows dissecting the relative contribution of both gene vectors into a local population, an issue addressed by indirect estimates so far (Ennos 1994). Both aspects of contemporary pollen and seed dispersal are required to fully understand the ecological outcomes of plant-animal interactions for plant species.

The combination of genetic markers yielding individual multilocus genotypes with the spatial locations of both adult trees and dispersed propagules (either seed and pollen) provides a spatially-explicit framework for the study of seed and pollen dispersal and their long lasting consequences in plant populations (Sork et al. 1999, Jordano & Godoy 2002). Assignment methods allow to track local pollen and seed dispersal events in terms of distance and directionality while identifying pollen and seed immigrants, i.e., those propagules collected in the study area but produced in another population, thus inferring the relative contribution of seed and pollen to overall gene immigration. Here, we used highly variable molecular markers (SSRs) to identify the source tree of a sample of dispersed seeds by genotyping the woody seed endocarps (a tissue of maternal origin) and comparing them with all adult trees within the population (see also Ziegenhagen *et al.* 2003, Grivet *et al.* 2005, Jones *et al.* 2005). The percentage of immigrant seeds can thus be estimated as the percentage of seeds with no matching genotype within the population, when the sampling is exhaustive (see Godoy & Jordano 2001 for details). Our general goal was to estimate seed immigration in our study population and combine this estimate with previous results on pollen dispersal (García *et al.* 2005) in order to dissect the potential contribution of pollen and seed dispersal to total gene immigration. Secondly, we described and compared the seed and pollen dispersal patterns within the population in terms of distance and directionality from the source plants. If dispersal patterns of pollen and seeds essentially capture the foraging patterns of pollinators and frugivores, we expected a strong non-random and spatially aggregated pattern of dispersal (Jordano & Schupp 2000, García-Castaño 2001).

Material and Methods

Study species

The study species is *Prunus mahaleb* (L.), a gynodioecious rosaceous tree. In any given population there are individuals with hermaphrodite flowers (functional hermaphrodites) and others with androsterile flowers (functional females; Jordano 1993). In the southern Iberian Peninsula flowers are produced between mid-May and mid-June at high elevations (over 1300 m). Bees (Hymenoptera: Andrenidae, Apidae) and flies (Diptera: Calliphoridae, Syrphidae) are the main pollen vectors (Jordano 1993). Between late July and mid-August, fleshy fruits are produced and consumed by frugivorous birds, such as blackbirds (*Turdus merula*), mistle thrushes (*Turdus viscivorus*), blackcaps (*Sylvia atricapilla*), and black redstarts (*Phoenichurus ochruros*) (Jordano & Schupp 2000) (Fig. 1). Jordano (1995) reported seed dispersal by lizards (*Lacerta lepida*) and Herrera (1989) and J.L. García-Castaño, pers. comm., reported seeds being dispersed by red foxes (*Vulpes vulpes*), badger (*Meles meles*) and stone marten (*Martes foina*). See Jordano & Schupp (2000) and García *et al.* (2005) for additional details.

Study site and sampling design

The study population is located in Nava de las Correhuelas (Parque Natural de Sierra de Cazorla, Segura y las Villas, Jaén province, Spain, 37°59'N, 2°54'W) at 1615 m elevation. Populations of *P. mahaleb* in this region are typically isolated and small (<100 trees). Vegetation is dominated by grasslands with scattered patches of deciduous vegetation, gravely soil or rock

Figure 2. Map of the study population showing the spatial location of all adult *Prunus mahaleb* trees (white dots). The area is highly heterogeneous, dominated by a pine forest (*Pinus nigra* subsp. *salzmannii*) at the edge of the *P. mahaleb* population, a central open grassland area, and rocky soils (white areas).

outcrops covered by shrubs or small isolated trees. The rocky slopes are dominated by open pine forest (Pinus nigra subsp. salzmannii). Our study population consists on a total of 104 hermaphrodite and 92 female trees distributed as shown in Fig. 2. The next nearest *P. mahleb* population is found at 1.5km, and 8 more are found within 20km.

We analyzed two distinct seed samples, one for the study of pollen dispersal patterns (see García *et al.* 2005) and the other for seed dispersal. To study pollen dispersal, we used 200 fruits harvested when ripe, before the start of consumption by frugivores, from the canopy of 20 mother trees previously used to characterize the mating system of *Prunus mahaleb* (L.) (for details on sampling design see García *et al.* 2005). To study seed dispersal we collected all dispersed seeds (N= 914) from seed-traps located at 613 sampling points all over the study area, each consisting of two seed-traps randomly located within a microhabitat type (for further details see García-Castaño 2001). We genotyped a random subsample (N= 549) of the dispersed seeds collected in the seed traps. As some amplifications failed for several markers, we finally successfully obtained the multilocus genotype for at least 8 markers for 465 endocarps.

Microsatellite genotyping

To obtain direct estimates of seed and pollen dispersal distances we obtained the genotype at 10 microsatellite loci of all adult trees within the population, along with the multilocus genotype of the endocarp of dispersed seeds and of the embryos extracted from the canopy-harvested seeds. The study population was previously genotyped and mapped by Godoy & Jordano (2001), and a few trees were added to this previous sample by García *et al.* (2005). Briefly, endocarps were opened and separated from the embryo tissue and immersed in liquid Nitrogen before being grounded in a ball-mill (Retsch Mixer Mill MM 200). DNA was extracted following Cheung *et al.* (1993) and finally resuspended in 80µl l of TLE buffer (10mM Tris-HCl, 0,1 mM EDTA). 5 µl of extract was used as template for polymerase chain reaction (PCR). Amplified fragments were analysed using an ABI 310 Genetic Analyser (Applied Biosystems). The genotyping of adult trees was performed similarly from leaf tissue. For further details on genotyping protocols see Godoy & Jordano (2001). On the other hand, embryos from fruits harvested directly from the mother tree were carefully separated from the endocarp and the seed coat. DNA was extracted from all embryos using a SIGMA GenEluteTM Plant Genomics DNA Kit, with 5-10 µL used as a template for PCR in a final volume of 20 µL. Amplified fragments were analysed using an ABI 310 capillary electrophoresis system (Applied Biosystem). For full details on genotyping protocols for embryos see García *et al.* (2005).

Data analysis

Pollen immigration and paternity analysis

In a previous study we used paternity analysis to describe the mating system of this *P. mahaleb* population by assigning a most likely father to each pair of mother-offspring (paternity analysis) (for the detailed analysis procedure, see García *et al.* 2005). By doing so, we estimated the apparent pollen immigration as the percentage of embryos not assigned to a candidate tree within the population (Devlin & Ellstrand 1990). We also used the set of unassigned (*i.e.,* apparent gene flow) embryos along with the cryptic gene flow estimation to assess total (actual) pollen immigration. The cryptic gene flow (CGF) is defined as the fraction of the offspring assigned to a putative father within the population, whereas the true father is outside the population (Devlin & Ellstrand 1990). We used CERVUS 2.0 to perform the paternity analysis (Marshall *et al.* 1998) following the criteria proposed by Slate *et al.* (2000) to define the scoring threshold and FAMOZ software (Gerber *et al.* 2003) to estimate the pollen CGF. Here, we compared the estimate of actual pollen immigration provided in our previous paper (García *et al.* 2005) with the estimates for dispersed seeds. Additionally, we used the set of assigned embryos to characterize pollen dispersal patterns in terms of distance and directionality within the population.

Seed immigration and the assignment of the source tree for dispersed seeds

In order to identify the source tree for each dispersed seed (maternity analysis), we searched the matching genotypes between each endocarp and the candidate adult trees for a complete set of 8 microsatellite markers out of 10 loci, as some loci were excluded due to a high frequency of failed amplifications. We used CERVUS 2.0 (Marshall *et al.* 1998) to identify the endocarp-source tree matching pairs allowing 0 mismatches. Given that all adult trees in the population yielded a different multilocus genotype, we assumed that endocarps with no matching adult genotype came from other populations, and we considered the percentage of non-assigned endocarps an estimate of apparent seed immigration (Godoy & Jordano 2001). We estimated the cryptic seed flow (CSF) that in analogy to CPF represents the proportion of seeds assigned to a local source tree that have been produced outside the population. Based on Harju & Nikkanen (1996) we estimated the sum of the all expected probabilities for the local maternal genotypes to be produced in a nearby population (*i.e.,* the proportion of non-distinguible gametes, NDG). Assuming Mendelian inheritance, Hardy-Weinberg equilibrium, and no linkage disequilibrium, this can be estimated based on the allele frequencies of the eight nearest populations (for details on the nearby populations see Jordano & Godoy 2000). The actual seed flow is obtained by dividing the observed seed flow by NDG. Moreover, the simulations performed with CERVUS indicated that with genotyping error rates ranging between 0 and 0.1, an increment of 0.001 in the error rate increases the estimated seed immigration percentage in 2.5% (C.G. unpublished data).

Intrapopulation dispersal distances and directions

The identification of the source tree for each pollen and seed dispersal event allowed the estimation of pollen and seed dispersal distances and directionality (Godoy & Jordano 2001, García *et al.* 2005). The x, y coordinates for each adult tree and each seed trap were recorded in a GIS database and used to calculate the distance and azimuth between each adult tree (*i.e.*, inter-tree distance), each mother tree and their siring trees (*i.e.*, pollen dispersal events), and between each dispersed seed and its source tree (*i.e.*, seed dispersal events) with Arcview 3.2 (Mitchell 1999). We tested for differences in pollen and seed dispersal distance distributions by applying a Kolmogorov-Smirnov non-parametric test. Additionally, we used Oriana 2.0 (Kovach Computing Services, Wales, UK) to describe pollen and seed dispersal direction patterns. This software estimates the mean direction for both data sets. If we have n observations and θ_i is the direction of the n= *i* observation, we can calculate the mean vector direction as:

$$\theta = arctan(S/C)$$

and the mean length as:

$$\bar{R} = \sqrt{\frac{S^2+C^2}{n}}$$

where

$$S = \sum_{i=1}^{n} sin\theta_i$$

and

$$C = \sum_{i=1}^{n} cos\theta_i$$

Oriana 2.0 also provides statistical tests to assess if pollen and seed dispersal direction patterns differ from a uniform distribution (Rayleigh *Z*-test), and to compare pollen and seed dispersal direction distributions (Watson U^2-test for two samples). We expected the direction distributions to differ for pollen and seed dispersal since they result from the activity of different dispersal agents. To evaluate the influence of the adult hermaphrodite trees (pollen sources) arrangement in shaping pollen dispersal directions we estimated the potential pollen (PP) dispersal angle distribution in the population (angles between each sampled mother tree and each hermaphrodite in the population) and compared them with the actual pollen (AP) dispersal angle distribution (angle between each sampled mother tree and its siring hermaphrodite). Similarly, we compared the potential seed (PS) dispersal angle distribution (angle between each adult tree and each seed trap) and the actual seed (AS) dispersal angle distribution to evaluate the influence of the seed traps arrangement imposed by the sampling design on seed dispersal angle distribution. Both comparisons were performed by applying a Watson U^2-test for two samples (Zar 1999).

Results

Among population dispersal

Observed seed immigration was more than twice the documented pollen immigration within the study site (García *et al.* 2005). While we found 20.34% of the dispersed seeds as coming from other populations, the previously reported percentage of actual pollen immigration showed that only 9.5% of the embryos were fathered by trees located outside the population. Relaxing the assumption of correct genotyping by allowing 1 mismatch, we still obtained a high rate of seed immigration (18.61 %), *i.e.*, a conservative estimate between 19-20 % of seed immigration. The proportion of non-distinguishable gametes resulted extremely low, NDG= 10-7, and therefore the actual seed gene flow should be almost equivalent to the above reported percentage of seed immigration. In terms of gene immigration, this means that 189 gametes carried by seeds (465 seeds x 2 gametes x 0.2034) and 19.5 gametes carried by pollen (197 embryos x 1 gametes x 0.0950) came from other populations. Thus, 208.5 of the analysed gametes over a total of 1127 (seed: 465 x 2 + pollen: 200 x 1) were immigrant, which represents 18.50% of the total gamete pool.

Within population dispersal

Overall, pollen tended to move over shorter distances than seeds within the population, with a range of dispersal from 0 m (for self pollinations in hermaphroditic trees) to 548.0 m. On the other hand, frugivore-mediated seed dispersal distances ranged from 0 m (seeds collected beneath the source tree) to 990.2 m (Fig. 3). The frequency distributions of seed and pollen within-population dispersal distances differed significantly (Kolmogorov-Smirnov Test, Z= 5.71, P< 0.01). The pollen dispersal distances had a median value of 62.9 m with 22.5 m and 129.3 m as the first and third quartile, respectively. The median seed dispersal distance was 145.2 m, with 54.1 m and 267.3 m as first and third quartile, respectively (Fig 3.).

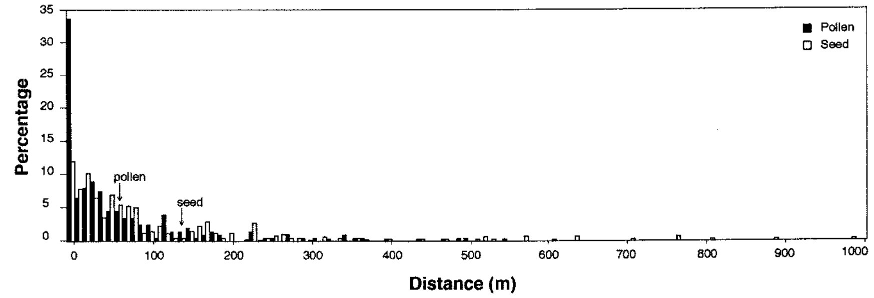
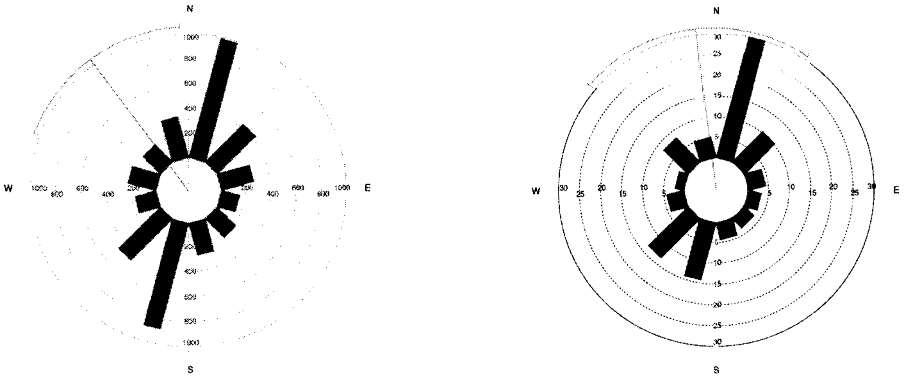


Figure 3. Frequency distribution of the pollen (black bars) and seed (white bars) dispersal distances in the study area. The high percentage of pollen dispersal events in the 0 m interval is due to selfing and the seed dispersal events in this interval mostly include seeds collected beneath the source tree canopy. The arrows indicate the median dispersal distance value.

Potential and actual pollen dispersal angle distributions (PP and AP) did not differ (U^2= 0.155, dfl= 100, df2= 2099, P> 0.05) (Fig. 4a and 4b), while potential and actual seed dispersal angle distributions (PS and AS) differed significantly (U^2= 4.26, dfl= 368, df2= 120343, P< 0.01) (Fig. 4c and 4d). All circular histograms shown in Fig. 4 depicting the potential and actual angle distribution for pollen and seed differed from the uniform distribution (U^2= 11.46, P< 0.01 for PP; U^2= 0.50, P< 0.01 for AP; U^2= 190.25, P< 0.01 for PS; and U^2= 0.43, P< 0.01 for AS). Actual pollen and seed dispersal directionality also differed (U^2= 0.595, df= 362, 100, P< 0.001), with an estimated mean vector θ= 352.76° and R= 0.187 for pollen and θ= 174.30° and R= 0.225 for seeds (Fig. 4b, Fig. 4d). Thus, pollen moved, on average, with a marked N-S directional component, but with wide variance from the NW and NE (Fig. 4b), whereas most of the dispersed seeds followed the SE-SW directions (Fig. 4d).

a. Potential pollen dispersal (PP)

b. Actual pollen dispersal (AP)



c. Potential seed dispersal (PS)

d. Actual seed dispersal (AS)

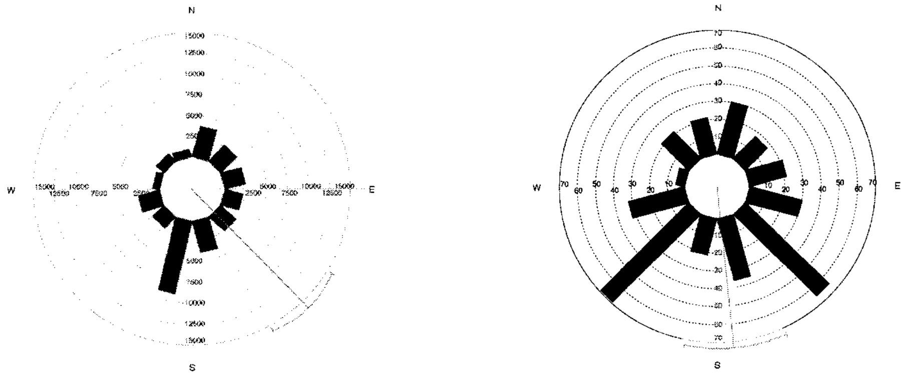


Figure 4. Circular histograms of the directions distribution for the potential pollen dispersal events (4a, PP), actual pollen dispersal events (4b, AP), potential seed dispersal events (4c, PS), and actual seed dispersal events (4d, AS). The mean direction and the associated standard error are indicated as an inner solid line direction and an external bar and the figures on the axis are the sample size (*N*). Note that the large difference on sample size between histograms containing potential dispersal events (4a, 4c) and actual dispersal events (4b, 4d) is due to the formers include all potential mother-father pairs and tree-seed trap pairs respectively, whereas the latters include only the observed data..

Discussion

Plant-animal mutualisms shape seed and pollen dispersal patterns in heterogeneous landscapes, having pervasive consequences on the genetic diversity, spatial distribution and recruitment patterns of plants (Nason *et al.* 1998, Schnabel *et al.* 1998, Hamilton 1999, Jordano & Schupp 2000, Oddou-Muratorio *et al.* 2001, Westcott *et al.* 2005). Our results indicate that pollinators and frugivores generate extensive gene migration into the study population, despite its relative geographic isolation: 18.50% of the observed gametes came from another population. This level of gene immigration might change quantitatively (amount) or qualitatively (population source) among years, but it is clear that a non-trivial amount of gene migration connects our study population with nearby populations located between 1.5 km and up to 20 km. The extensive gene flow observed is due mainly to a high percentage of seed immigration, which was double the pollen immigration in our population. This result is congruent with concurrent field work on seed dispersal ecology of *P. mahaleb* in the same study population showing that frugivores consistently remove a high fraction of the available fruit crop allowing for a wide-ranging dispersal (Jordano 1995, Jordano & Schupp 2000, García-Castaño 2001). In contrast, other studies have reported pollen reaching longer distances than seeds, specially in outcrossed-anemophilous species with no special devices for seed dispersal or with highly restricted seed dispersal around the mother tree by scatter-hoarding vertebrates (e.g., *Quercus*, *Fagus*, *Pinus*, etc.) (Ennos 1994, Nason *et al.* 1998). Recent evidence on vertebrate-dispersed species suggests that long-distance movements occur consistently and can account for a significant number of dispersal events, a likely pattern when large-bodied frugivores disperse the seeds (Fragoso 1997, Holbrook *et al.* 2002, Schupp *et al.* 2002, Bacles *et al.* 2004, Westcott *et al.* 2005). Pollinators are also able to fly long distances (Handel 1983) but, in the case of *P. mahaleb*, the patchy distribution of the adult trees coupled with their non-overlapping phenology appears to restrict pollen immigration in the study population (García *et al.* 2005). The present study along with recent publications dealing with fleshy-fruited species (Aldrich *et al.* 1998, Oddou-Muratorio *et al.* 2001, Bacles *et al.* 2004, Levey *et al.* 2005, Sezen *et al.* 2005) highlights the role that frugivorous vertebrates play in interchange of individuals and genes among fragmented patches or populations.

As pointed out by Hamrick & Nason (1996), the interpretation of spatial genetic patterns and their variation requires the consideration of both pollen and seed dispersal biology. Despite the evidence for not so infrequent long-distance dispersal events (the tail of the dispersal curve), our data revealed highly skewed pollen and seed dispersal distance distributions, with almost 30% of the seeds and 47% of the pollen (23% excluding self-pollination events) being dispersed within a 30 m radius from the tree source . Additionally, a previous study reported frequent selfing in the same *P. mahaleb* population (García *et al.* 2005). These figures suggest that both seed and pollen dispersal are locally restricted, increasing in the long-term the probability of spatial clustering of related individuals (Heywood 1991), and therefore the emergence of spatial genetic structure at fine scale. Moreover, by extending the study of dispersal from one dimension (distance) to two dimensions (directionality), our analysis shows that dispersal events are highly directional. This result suggests that the observed non-random and restricted pollen and seed dispersal patterns assisted by animals in heterogeneous landscapes set the initial conditions to generate a heterogeneous genetic spatial structure at fine scale.

As in previous studies, we found a close relationship between disperser movements and the seed and pollen shadow patterns relative to the sources (e.g., Chase *et al.* 1996, Nason *et al.* 1998, Wenny & Levey 1998, Jordano & Schupp 2000, Jordano & Godoy 2002, Schupp *et al.* 2002, Tewksbury *et al.* 2002). Pollen tended to move preferentially along a N-S direction following the spatial location of the adult trees, as confirmed by the lack of significant difference between the potential and actual pollen dispersal angles. Pollinators tended to fly from one *P. mahaleb* to the next one or to a nearby non-conspecific tree or shrub with an overlapping blooming, frequently located following the main N-S direction axis of tree arrangement. On the other hand, frugivores tended to forage tracking the location of *P. mahaleb* trees and using short flights to nearby perches or, less frequently, longer flights to pine trees (Jordano and Schupp 2000). These landscape effects on behavior would explain that frugivores tend to frequently contribute dispersal events following the SE and SW directions. The dominance of the S vs. the N directions in the seed dispersal movements can also be caused by a higher frequency of open rocky patches and a lower availability of the more preferred covered microhabitats in the Northern area of the population. Open substrates are rarely visited by frugivorous birds; only the black redstart (*Phoenicurus ochruros*) and mammals contribute to the scarce seed rain reaching open microhabitats (Jordano & Schupp 2000, García-Castaño 2001). These results indicate that the combination of a complex landscape and the non-random behaviour of pollinators and frugivores can modulate distance effects by imposing directionalities and non-random paths relative to the flowering and fruiting trees. Our data, thus, suggest that animal vectors influence the template of propagules that starts the recruitment cycle, and that ultimately might shape the spatial demographic and genetic structure of the plant population.

Additional fieldwork involving long term studies of marked individuals is required to assess the contribution of dispersal and postdispersal processes to recruitment patterns (Schupp & Fuentes 1995, Aldrich *et al.* 1998, Kalisz *et al.* 2001). Direct data will elucidate the role of dispersal vectors due to their recurrent and non-random movements. However, we still lack a precise understanding of the differential survival prospects for immigrant progeny compared to in situ-dispersed propagules, and of the relative contribution of post-dispersal mortality factors and local drift or selection modifying allele frequencies (Gram and Sork 2001). The usefulness of the direct approach for dissecting pollen and seed dispersal contribution to overall gene flow and describing dispersal patterns is that it provides a first step towards the elucidation of lasting consequences of plant-animal mutualisms in heterogeneous plant populations.

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5

Abstract

When frugivorous animals disperse seeds, the resulting seed rain combines propagules from source trees located all over the landscape. Spatial and genetic correlations in the seed rain arise from the contribution of distinct maternal trees to the dispersed seed in a given site (maternal richness); the proportion of maternal half-sib seeds within a site (correlated maternity); and the genetic relatedness among the maternal source trees (r_{iw}). These aspects remain virtually unexplored, despite their central effect on demographic and genetic structure in animal-dispersed plant populations. We used microsatellite genotypes obtained from endocarps, a tissue of maternal origin, to identify the seed source tree, and combined this with spatially explicit models considering microhabitat variation and the spatial location of the sample points. We analyzed variation across sample points in maternal richness, correlated maternity, and relatedness among the maternal genotypes. Sample points beneath *P. mahaleb* tree canopies showed reduced mean maternal richness, whereas higher mean values were recorded in covered sample ponits. Seed progenies were not randomly associated to specific microhabitats and those collected from the same sample point presented increased values of maternal relatedness. Mean correlated maternity ranged between 0.61 (under conspecific *P. mahaleb* canopy) and 0.16 (open microhabitats). Both estimators of maternal genetic relatedness were influenced by microhabitat type and adult tree clumping. Three outcomes of genetic combinations among dispersed seeds were observed in different microhabitats: sample points with high maternal richness and low relatedness (high seed density sites with frequent dispersal events coming from different mothers located all over the population); sample points with very low maternal richness and high relatedness (recurrent dispersal from a nearby, isolated source tree); and sample points with high maternal richness and high relatedness (long-distance dispersal events from several closely-growing source trees). Our results reveal fine-scale maternal genetic structure in the seed rain resulting from two main processes related with frugivores activity: a genetic dispersal limitation, *i.e.*, the fail of specific mother trees to cast seed genotypes to all available sites; and the non-random delivery of seed progenies due to influences of ecological heterogeneity on frugivore movements from the source trees.

Key words

Correlated maternity, frugivore activity, heterogeneous landscape, maternal progeny, microhabitat, microsatellites, *Prunus mahaleb*, seed dispersal limitation, seed endocarp, seed rain, spatial location.

5

Spatial and maternal source tree correlation in a frugivore-generated seed rain

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In review

Introducction

A seed shadow is the spatial pattern of dispersed seeds relative to their source tree and to other conspecifics (Janzen 1970) and the sum of all seed shadows in a population makes up the seed rain (Alcántara *et al.* 2000, Clark *et al.* 2005). The seed rain acts as the primary template for new recruitment events in plant populations thereby linking the end of the reproductive stage with the start of the recruitment or post-dispersal stages (Schupp and Fuentes 1995). In endozoochorous species seed rain patterns result from mutualistic interactions with frugivores dispersing seeds in ingested fruits (Jordano 2000). Hence, our fully understanding of the relevance of the seed dispersal processes in determining recruitment patterns requires an exhaustive characterization of the dispersal processes along with an integrated analysis of the seed rain patterns, *i.e.*, the spatial and maternal origin of seeds deposited across the landscape.

Seed dispersal process are reported to be numerically- and spatially- limited, *i.e.*, among all seeds produced, only a set is dispersed to few deposition sites of the available ones (Jordano & Godoy 2002). A dispersal-related limitation scenario might arise not only from low rates of seed dispersal, but also when propagules from specific maternal tree sources fail to reach available sites or are spatially clumped. The genetic patterns embedded in these dispersal-related limitation processes might shape the recruitment patterns by mediating habitat selection (Donohue 2003, Holt *et al.* 2004) and susceptibility to genotype-specific pathogens (Augspurger & Kitajima 1992, Schnable *et al.* 1998). Moreover, highly heterogeneous dispersal processes, where certain genotypes are recurrently dispersed to specific sites in the landscape, create strong spatial genetic patterns setting the potential for plant populations to diverge.

In spite of having pervasive demographic, genetic and spatial implications for plant populations at ecological and evolutionary time scale we unknown the spatial configuration of maternal progenies in the seed rain. This involves the number of contributing trees (maternal richness), the proportion of maternal half-sibs (correlated maternity), and the genetic relatedness among those contributing trees (maternal relatedness) observed in a seed clump (Table 1). Over the seed rain, these components are expected to vary due to spatial and ecological changes in the landscape, especially when dispersal processes response to the ecological heterogeneity. This is the case for endozoochorous species; when seeds are dispersed by the agency of animals, maternal progenies are deposited to those sites visited by frugivores for perching (Jordano and Schupp 2000), resting (Julliot 1997), feeding (Holbrook *et al.* 2002), or displaying (Westcott 1997). These highly non-random delivery patterns produce a spatially heterogeneous seed rain composed by only few sites with large seed clumps combining propagules from various distances and source trees located (Schupp *et al.* 2002).

In this study we analyzed the distribution of maternal genotypes in the seed rain of *Prunus mahaleb* (L.), an endozoochorous species inhabiting a heterogeneous landscape. We considered explicitly two levels of heterogeneity, namely the microhabitat level (shaped by the different physiognomic units over the landscape) and the deposition site level (involving the spatial locations of the seed sample points). The use of highly informative molecular markers (Short Sequence Repetiton, SSRs) allowed us to identify the source tree of each dispersed seed (Godoy and Jordano 2001), and to estimate the genetic relatedness among the contributing trees. Our specific objectives were: i) to quantify the contribution of source trees to different microhabitats; ii) to evaluate the role of spacing patterns and variation in the ecological maternal neighborhoods driving the differential contribution of the source trees. We also examined the extend of the seed dispersal limitation: i) as genetically limited (only a few genotypes are widely dispersed over the landscape), ii) as spatially limited (fruiting trees fail to dispers to most of available sites), and iii) as ecologically limited (fruiting trees fail to disperse to most of the available microhabitats). We tested if contributing trees to the same site genetically more related than those contributing to different sites, trying to elucidate if it is the consequence of the spatial genetic correlation present in the adult population (Jordano & Godoy 2000). We finally tested for the effect of spatial and ecological factors in shaping the genetic structure in the seed rain.

Table 1. Main components of the spatial genetic structure in the seed rain related to the contribution of distinct maternal source trees to the dispersed seeds, and potential factors influencing it.

Component	Definition	Influencing factors
Maternal richness	R : Number of maternal trees contributing with dispersed seeds in the seed rain, a sample point, or a microhabitat	Foraging behavior Number of fruits ingested per bout Number of foraging trees Gut retention time Frugivore guild Number and type of frugivores Frequency of visits
Correlated maternity	Number of maternal trees contributing with dispersed seeds in the seed rain, a sample point, or a microhabitat	Foraging behavior Number of fruits ingested per bout Number of foraging trees Gut retention time
Maternal relatedness	r_{in}: Genetic relatedness (Queller and Goodnight 1989) among the maternal trees reaching the same deposition site r_{out}: Genetic relatedness (Queller and Goodnight 1989) among the maternal trees reaching different deposition sites	Foraging behavior Number of fruits ingested per bout Number of foraging trees Gut retention time Distance among foraging trees Frugivore guild Number and type of frugivores Spatial genetic structure in the adult tree population

Material and Methods

Species and study site characteristics

Our study species, *Prunus mahaleb* (L.) (Rosaceae), is a tree producing fleshy-fruits ingested by frugivores that disperse their seeds after regurgitating or defecating them. This species is frequently visited during July to mid-August by small- and medium-sized birds (Jordano and Schupp 2000) and carnivorous mammals have also been reported to feed on *P. mahaleb* (García-Castaño 2001).

P. mahaleb presents a patchy distribution at the regional scale with several isolated populations consisting of dozens to hundreds of trees. Our study population is located at 1615 m elevation in Nava de las Correhuelas, (Parque Natural de las Sierras de Cazorla, Segura y las Villas, Jaén province, 37°59'N, 2°54'W). The population is characterized by a high heterogeneity due to the mosaic-like distribution of different vegetation types that represents physiognomic units named here as microhabitats. The population consists on 196 adult trees distributed over an area of 5 ha as clumps of variable density (see García *et al.* 2006).

Seed rain sampling design

Seed rain was sampled using sample points distributed according to a random stratified design by microhabitat type (Kollmann and Goetze 1997). We arranged 614 sampling points, each one consisting of two seed-traps (for further details see García-Castaño 2001). Thus, methodologically the microsite is equivalent to the sampling point, and we will use sample point hereafter. We characterized six different microhabitats (MH) according to the type of plant cover above the sampling point. Three of them presented fleshy-fruited species: i) MH-*Prunus*, dominated by adult *P. mahaleb* trees; ii) MH-High shrub, dominated by high-shrub, fleshy-fruited, woody cover reaching over 1.5 m height (*Crataegus monogyna*, *Lonicera arborea*, *Rosa* spp.); and iii) MH-Low shrub, dominated by low-shrub species below 1.5 m height such as *J. communis*, *Berberis hispanica* and *J. sabina*. Three other microhabitats were dominated by non-fleshy-fruited species: iv) MH-*Pinus*, dominated by pine trees (*Pinus nigra* subsp. *salzmannii*); v) MH-*Acer-Quercus* dominated by *Acer granatensis* and *Quercus faginea* or *Q. ilex*, which presented a scattered distribution in the population; and vi) MH-Open, including rocky soil and grassland. We genotyped all dispersed seeds collected in all microhabitats and an exhaustive subsample in MH-*Prunus* and MH-High shrub (Table 2).

Genotyping material

All adult trees were previously genotyped with 11 polymorphic microsatellites markers (Godoy and Jordano 2001). We genotyped the endocarp, a tissue of maternal origin, of the dispersed seeds for 9 microsatellite markers in order to identify the source tree of the dispersed seeds (Godoy and Jordano 2001). Therefore, the identification of the source tree is unambiguous when all adult trees are genotyped with a polymorphic set of microsatellite markers, so a unique multilocus genotype can be obtained for each one. This technique was successfully used by Godoy and Jordano (2001) with the same species and study site (also see Ziegenhagen *et al.* 2003, Grivet *et al.* 2005, Jones *et al.* 2005 for similar approaches). Briefly, endocarps were split open, separated from the embryo tissue and immersed in liquid N before being grounded in an electric grinder. DNA was extracted following Cheung *et al.* (1993) and 5 µl was used to perform polymerase chain reaction (PCR) (increased up to 10 µl when the amplification failed). Amplified fragments were analyzed using an ABI 310 Genetic Analyzer (Applied Biosystems). The genotyping of adult trees was performed similarly, but with DNA extracted from leaf tissue. For further details on leaf and endocarp genotyping protocols see Godoy and Jordano (2001).

Data analysis

Tree source assignment

Since the endocarp is a maternal tissue, its multilocus genotype is identical to its source tree, thus we use this information to assign each endocarp to its source tree by comparing the genotypes (Godoy & Jordano 2001). Additionally, since the multilocus genotypes of adult trees differed at least for one locus, we can state that two seeds come from the same mother tree when the seeds' endocarp multilocus genotypes are identical. We used GIMLET software (Valère 2002) first to identify each different multilocus genotype obtained for the endocarps and second, to find the matching adult multilocus genotype for each endocarp with at least 8 loci successfully typed.

Contribution of maternal source trees to the seed rain

In order to characterize the distribution of the maternal progenies in a frugivore-generated seed rain and test for the ecological factors determining it, we: i) estimated the maternal richness, *i.e.*, the number of distinct maternal genotypes contributing to the seed rain; ii) assessed the frequency distribution of the maternal genotypes in each microhabitat; iii) tested the association between maternal genotypes and microhabitat type; and iv) tested for the effect of distance among source trees and similarity of the maternal neighborhoods in shaping the contributions of different maternal source trees in the seed rain.

i) Comparison of the maternal richness: the uneven seed sample size among microhabitats makes direct comparisons unreliable. To overcome this problem we performed a sample-based rarefaction analysis in order to obtain mean maternal richness values per microhabitat for a given sample size (see Hurlbert 1971 for a detailed description of the technique and Petit *et al.* 1998 for application in population genetics). We estimated the mean maternal richness after sampling 1000 times the seeds collected per microhabitat (Fig. 1). The mean richness value per microhabitat obtained after rarifying (R_s) were calculated with EcoSim 7.71 software (Gotelli and Entsminger 2005). We tested the effect of the microhabitat type on the R_s by using a 1-way ANOVA.

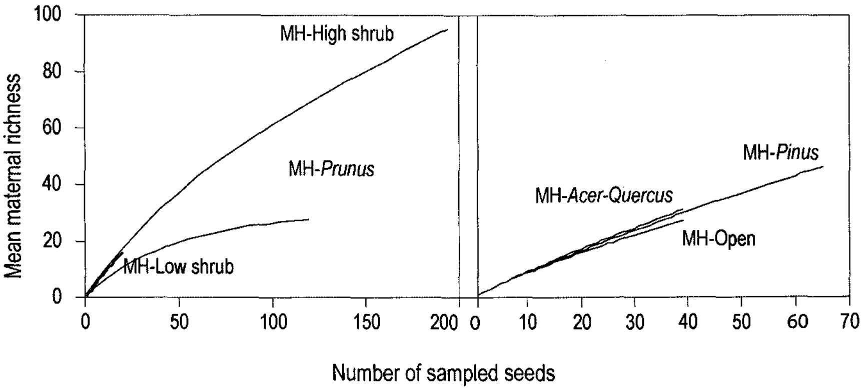


Figure 1. Rarefaction curves showing the increasing of mean richness of maternal genotypes per sampled seed from the total seed sample per microhabitat. We performed 1000 simulations in each microhabitat. To improve clarity, the curves obtained for sampling sites beneath fleshy-fruited and non-fleshy fruited microhabitats are plotted separately.

ii) Frequency distribution of the maternal genotypes per microhabitat: this informs us about the relative contribution of each source tree to an environmentally-structured seed rain. The comparison of the mean number of contributed seeds per source tree among microhabitats was performed by a non-parametric Kruskall Wallis's Test.

iii) Association between maternal source trees and microhabitats: we performed a Correspondence Analysis (CA) to visualize and test the association between the set of maternal genotypes and the microhabitat type. CA performs an ordination of the maternal trees seed shadows relative to their frequencies reaching different microhabitat types. In the context of correspondence analysis the inertia reflects the variation accounted for by the canonical dimensions (Everitt and Dunn 2001). The X^2-Pearson tests for null hypothesis of independence between the two sets of data, *i.e.*, the random association of maternal progenies with target microhabitats. CA analysis was performed with JMP software (SAS Institute, NC, USA).

iv) Ecological and spatial traits shaping configuration of maternal genotypes in the seed rain: in order to explain the non-random association observed in the CA we performed two Mantel's tests to test if this result is due to: a) a distance effect (nearby trees dispers to similar microhabitats); b) the influence of the maternal neighbourhood (trees with similar maternal neighbourhoods dispers to similar microhabitats, regardless the distance among source trees). Matrix A is a distance matrix based on the maternal genotype frequency in each microhabitat. Matrix B is a distance matrix with the raw geographic distances between each pair of maternal trees in the population. Matrix C is a distance matrix based on the frequency of different microhabitat types around the source trees within a 30 m radius circle. Matrix C, thus, describes how similar are the neighborhoods of the source trees. We set the radius of the neighborhood based on previous information (see Jordano and Schupp 2000 for details). The first Mantel's test tested the correlation between Matrix A and B. The second Mantel's test tested the correlation between Matrix A and C. All Mantel tests were performed with R (R Core development Team 2003).

Maternal genetic relatedness and correlated maternity

In order to assess if trees contributing to the same sample point are genetically closely related, we estimated the genetic relatedness among them, given that the endocarp of the seed contain the same multilocus genotype than source tree (relatedness *sensu* Queller and Goodnight 1989) We used SPAGeDi 1.2 (Hardy and Vekemans 2002) to perform the relatedness calculation. Note that when the maternal relatedness value is equal to 1, the pair endocarps compared belong to maternal half-sibs seeds, *i.e.*, they come from the same mother tree. We also calculated the correlated maternity that, analogous to correlated paternity (Ritland 1989), is defined as the proportion of seeds collected within the same sample point that are maternal half-sibs, *i.e.*, they were produced by the same adult tree.

Landscape variation in maternal genetic relatedness

The question of how the maternal genetic relatedness values are distributed over the landscape was addressed at two different levels: i) at the sample point level; ii) at the landscape level.

i. Sample point level: in order to elucidate if the source trees for seeds dispersed within the same sample point tended to be genetically more related than those for seeds deposited in different sample points, we estimated all r values for pairs of endocarps collected in the same sample points (r_{in}) and all r values for pairs of endocarps collected in different sample points (r_{out}). Then, we compared both mean values by applying a non parametric Kruskall-Wallis test (Zar 2003).

ii. Landscape level: we tested the effect of the microhabitat type and the spatial arrangement of the fruiting trees and sample points on the mean and mean correlated maternity values. The spatial arrangement was characterized by assessing the distance and the azimuth from each sample point to the nearest *P. mahaleb* tree, hereafter DNT and ANT, respectively. Previous studies show that seed dispersal patterns are strongly-non random with a high frecuency of short dispersal events and marked directionality (Jordano & Schupp 2000, García *et al.* 2006). Thus, we chosed DNT and ANT to test if as well as determining the spatial distribution of the seed rain, they influence the distribution of the maternal genotypes over the seed rain. Additionally, we tested the effect of the tree arrangement near each sample point on the genetic configuration of the maternal genotypes. We assessed the degree of clumping around each sample point by the Dirichlet tile area. By definition, the Dirichlet tile contains all points that are closer to the given tree than to any other tree in the population (Legendre and Legendre 1998). We calculate the Dirichlet tile area for ecah tree and we assigned this value to all sample points included in the area. Thus, each sample point present the Dirichlet tile area belonging to the nearest *P. mahaleb* fruiting tree. A sample point located within a large Dirichlet tile area is near an isolated *P. mahaleb*, whereas a sample point with a small Dirichlet tile area is located near a clump of *P. mahaleb* trees. The Dirichlet tile area associated with a sample point decreases with the increasing density of fruiting trees.

Spatially explicit models with linear mixed-effects

We performed two linear mixed-effect models fitted by REML (Residual Maximum Likelihood Estimation) to test the effects of ecological and purely spatial variables on seed rain characteristics. The first model considered that the residuals were independent and evenly distributed (Independent model), whereas the second model took into account the spatial structure of the residuals (autoregressive model or AR model) by including the spatial coordinates of each sampling point. For a thorough discussion on spatial non-independent models see Keitt *et al.* (2002). The best-fitting model was selected on the basis of the reduction in the Akaike's Information Criterion (AIC), along with the P value associated to the likelihood ratio test between the independent model and the AR model. The AIC has become a standard criterion to assess the quality of the fit, and thus, to select the best fitting models when dealing with autoregressive problems; the smaller the value of the AIC, the better the fitting of the model to the observed data (Crawley 2002). We used the R package (R Core development Team 2003) to estimate the Dirichlet tile area (library deldir), and to fit the Independent model and the AR model (library *nlme*).

Results

Contributions of maternal source trees to the seed rain

We identified 253 distinct endocarp genotypes among the 462 seeds genotyped, which unequivocally identify their maternal source trees. Among the 614 sample points, only 151 of them contained at least one dispersed seed. There was a general trend for maternal richness to increase with the number of sampled seeds in a given microhabitat, both overall and per sample point (R and $\bar{R}$, Table 2). After rarefying the seed sample (Fig. 1), we observed significantly different mean richness values (R_s) among microhabitats (Table 2, F= 183.43, d.f.= 5, P< 0.001): MH-*Prunus* and MH- High shrub yielded the lower and the higher R_s values, respectively (10.64 ± 0.04 and 15.83 ± 0.04) (Tukey's Test).

The number of seeds contributed per maternal tree varied significantly among microhabitats (Kruskall-Wallis, χ^2 = 18.36, d.f.= 5, P< 0.01, Fig. 1). Most of the source trees contributed with a single seed dispersal event to a given microhabitat (Fig. 2). Only under the canopy of conspecifics (MH-*Prunus*) we observed a decrease in the frequency of trees with a single contribution. The highest contribution per source tree was observed in MH-*Prunus* and MH-High shrub, where a few trees contributed up to 10 seeds, whereas MH-Open and MH-Low shrub presented the lower maximum contribution per tree (never above 3 seeds and 2 seeds per tree, respectively).

Table 2. Details of seed sampling and summary of genetic variation in *P. mahaleb* seed rain. For each microhabitat we indicate the number of sample points, the number of seeds sampled, the percentage of seeds genotyped over the total of seeds collected, and the mean number of seeds genotyped per sample point. Additional columns report the summary statistics for the maternal richness per microhabitat (R, the number of distinct maternal trees with dispersed seed), along with the mean richness ($\bar{R}$, the mean number of distinct maternal trees with dispersed seed per sampling point), mean maternal genetic relatedness (r_{in}, genetic relatedness among maternal trees with seeds dispersed to the same sampling point), and mean correlated maternity (proportion of seeds dispersed to the same sample point that are maternal half-sibs).

Microhabitat	No. sample points	No. seeds sampled	% of seeds genotyped	No. seeds/ sample point	Richness (R)	Mean richness ($\bar{R}$)	Mean maternal genetic relatedness (r_{in})	Mean correlated maternity
MH-*Prunus*	10	106	42.4	10.6 ± 1.8	38	3.40 ± 0.34	0.57 ± 0.07	0.61 ± 0.08
MH-High shrub	42	194	43.2	7.8 ± 0.6	95	5.6 ± 0.62	0.33 ± 0.04	0.28 ± 0.05
MH-Low shrub	11	19	100	1.7 ± 0.4	16	1.27 ± 0.19	0.18 ± 0.07	0.22 ± 0.19
MH-*Pinus*	31	65	100	2.1 ± 0.3	46	1.71 ± 0.21	0.42 ± 0.06	0.26 ± 0.07
MH-*Acer-Quercus*	32	39	100	1.7 ± 0.2	31	1.34 ±0.12	0.47 ± 0.09	0.53 ± 0.12
MH-Open	25	39	100	1.6 ± 0.2	27	1.32 ± 0.11	0.32 ± 0.09	0.16 ± 0.10

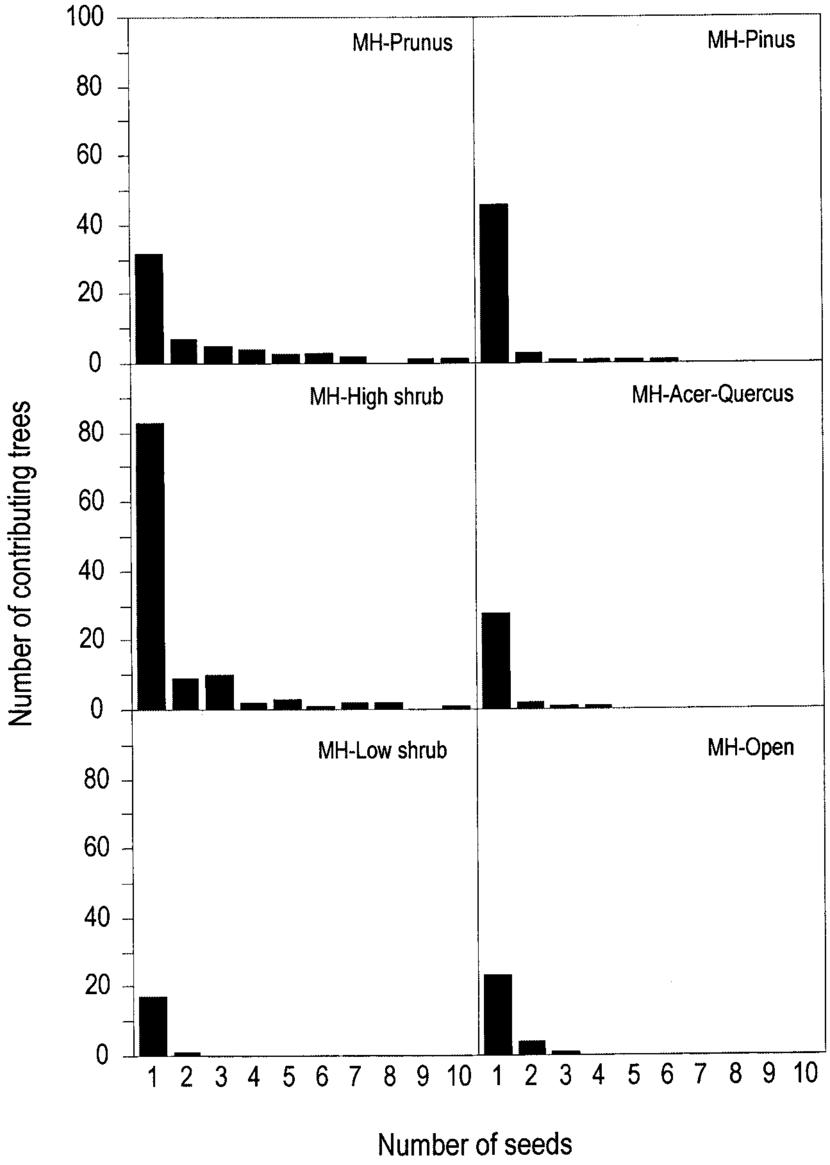


Figure 2. Frequency distribution of the number of seeds contributed per source tree in the seed rain at each microhabitat type. Note that most source trees contributed one seed, except in MH-*Prunus*, where the relative frequency of single trees contributing with at least one seed decreased.

The correspondence analysis showed a non-random distribution of maternal source trees according to their contribution of dispersed seeds among microhabitats (χ^2= 1173.88, d.f.= 1260, P< 0.05), with the axes C1 and C2 accounting for 81.85% of the variance. The maternal trees dispersing most progeny to microhabitats dominated by fleshy-fruited species fall in the negative side of axis C1, whereas the source trees with seeds reaching microhabitats with non fleshy-fruited species tend to fall on the positive side of the axis (Fig. 3). The MH-*Acer-Quercus* presented the more dissimilar array of maternal genotypes, whereas the set of maternal genotypes dispersed to the fleshy fruited-microhabitats were more similar among them than those dispersed to other microhabitats (Fig. 3). Similarly, the combination of maternal source trees in MH-Open and MH-*Pinus* is more similar relative to other microhabitats. Additionally, some trees were closely associated to a single microhabitat, (maternal genotypes indicated in boxes in Fig. 3). The arrangement of the maternal seed shadows according to microhabitat type (*i.e.*, the difference in location of the source trees on the canonical correspondence ordination, Fig. 3) did not correlate with the distance among their source trees (Mantel Test, *r*= -0.0055, P> 0.05), but with the difference in the ecological neighborhood of the source trees (Mantel test, *r*= 0.454, P< 0.001). In other words, the source trees of any two seeds randomly collected from a sample point with the same microhabitat are more likely to share an ecological neighborhood (similar growing site) than a spatial neighborhood (growing at close distance).

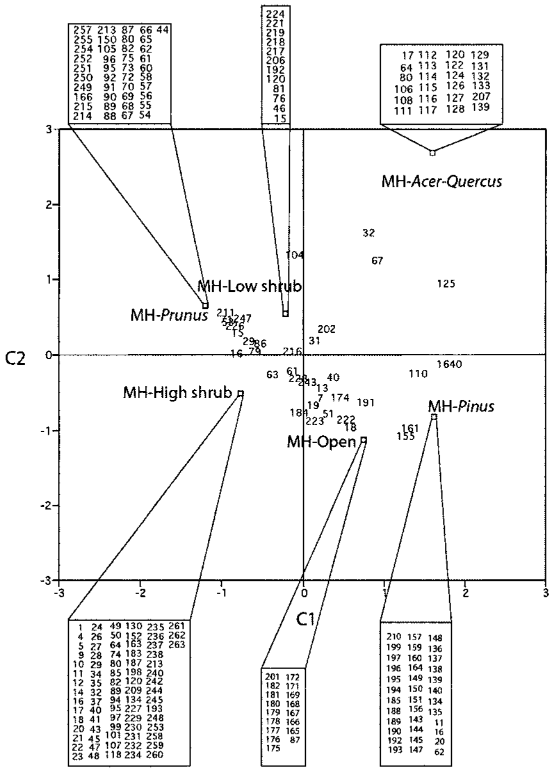


Figure 3. Results of the correspondence analysis on the distribution of seed progenies in the seed rain according to microhabitat type. The numbers identify different maternal tree genotypes contributing dispersed seed progeny. Boxes include those maternal genotypes with the same coordinates on C1 and C2. Microhabitat dominated by fleshy-fruited species fall in the negative side of the C1 axis, whereas microhabitats with non fleshy-fruited species fall in the positive side of the axis.

Maternal genetic correlations

We observed higher mean maternal relatedness values among maternal trees contributing dispersed seed to the same deposition site (r_{in}) than among maternal trees with seeds dispersed to different sites (r_{out}) (H= 19.11, d.f.= 1, P< 0.001). The $\bar{r}_{in}$ value doubled the $\bar{r}_{out}$ value ($\bar{r}_{in}$= 0.4047 ± 0.0005 and $\bar{r}_{out}$= 0.2273 ± 0.0046). Moreover, we found a higher frequency of r= 1.0 values among endocarps collected from the same sample point than from different sample points (18.54% vs. 0.89%) (Fig. 4), involving that seeds collected- at the same sample point have a higher probability to be maternal half-sibs than seeds collected from different sample points.

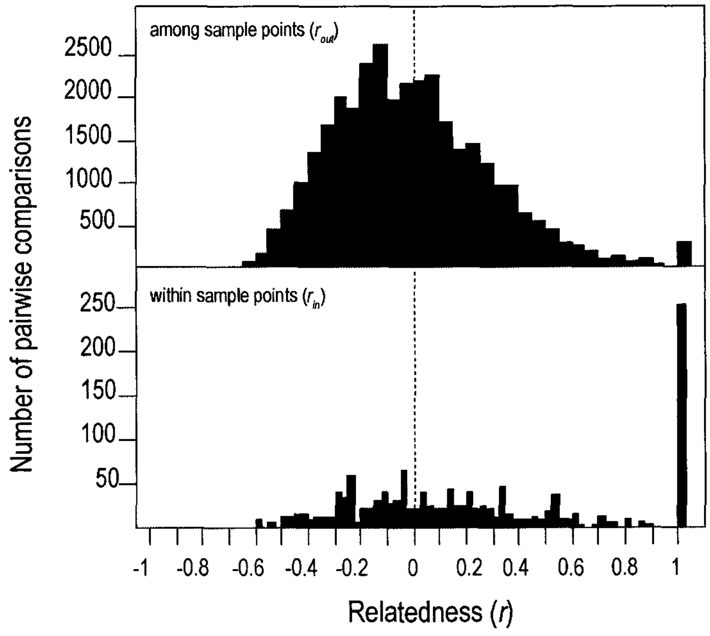


Figure 4. Frequency distribution of relatedness values between pairs of seeds collected from the same sample point (r_{in}) and pairs of seeds collected from different sample points (r_{out}). Note that for a given pair of endocaprs, r= 1 involves that they belong to maternal half-sibs seeds.

Mean maternal genetic relatedness differed among microhabitats (Kruskall-Wallis H= 13.61, d.f.= 5, P< 0.05) with the highest rin found in MH-*Prunus* and MH-*Acer-Quercus* (Table 2). Similarly, mean correlated maternity (percentage of maternal half-sibs seeds per deposition site) differed among microhabitats (Kruskall-Wallis H= 16.24, d.f.= 5, P< 0.01), exceeding 50% in MH-*Prunus* and MH-*Acer-Quercus*. Note that r_{in} values can increase due to: i) a high frequency of maternal half-sibs (involving high correlated maternity as in MH-*Prunus*, Table 2); ii) a strong genetic relatedness among maternal trees (high r_{in} values not involving correlated maternity).

When the adult popuulation present spatial genetic structure, nearby trees are closely geneticaly related. In our population, the r_{in} values negatively correlated with the distance between the maternal trees growing sites ($r = -0.224$, $R^2_{adj} = 0.403$, $P < 0.001$).

Spatial and ecological factors shaping the maternal genetic correlations in the seed rain

We found a highly significant effect of microhabitat type on the richness of maternal genotypes (F= 49.02, d.f.= 5, P< 0.01), the maternal genetic relatedness (F= 42.23, d.f.= 5, P< 0.01), and the correlated maternity (F= 13.04, d.f.= 5, P< 0.01). In contrast, the effects of the area of the Dirichlet polygons (Dirichlet area, a surrogate of the clumping level of the adult *P. mahaleb* trees near the sample point), and the distance and azimuth from the sample point to the nearest *P. mahaleb* tree (DNT and ANT) had no significant effects. The significantly lower AIC values yielded by the AR model compared to the values obtained with the independent model suggested a strong spatial signature in the data (Table 3). The AR model showed a negative and marginally significant effect of DNT on the correlated maternity values (F= 3.01, d.f.= 1, P= 0.086). Thus, correlated maternity tended to increase when the distance to the nearest *P. mahaleb* tree tended to decrease. After taking into account the spatial structure of the error terms (AR model), the negative effect of the Dirichlet area on the richness value resulted marginally significant (F= 3.08, d.f.= 1, P= 0.084), although small ($\beta = -0.017$). This result indicates that sample points located in areas with high clumping of trees (small Dirichlet area, high clumping of trees, and low DNT), tended to present a high richness of maternal genotypes irrespective of location effects.

Table 3. AIC values yielded after comparing the independent and the autoregresive model (AR model) for the three varibles considered to describe the distribution of the maternal genotypes in the seed rain.

Variable	AIC (Independent model)	AIC (AR model)	Likelihood Ratio Test	P-value
Maternal richness	724.53	719.47	4.41	0.05
Correlated maternity	158.58	76.421	17.78	0.05
Maternal relatedness	121.28	95.126	24.17	0.05

Discussion

The distribution of the maternal genotypes in a vertebrate-generated seed rain

By identifying the maternal origin of dispersed seeds we observed a non-random distribution of the maternal genotypes in the seed rain. This pattern is characterized by a low contribution of individual trees to the seed rain, except for sites dominated by *Prunus mahaleb*, and a strong association between microhabitat type and the maternal genotypes. Seed dispersal assisted by vertebrates has been reported to be limited when a fraction of the seed crop is not dispersed (numerical limitation) or when most of the dispersal events involve short distances and they are concentrated to few sites in the landscape (spatial limitation) (Augspurger 1983, Wenny & Levey 1998, Nathan & Muller-Landau 2000, Schupp *et al.* 2002). Our data suggest a third component of the dispersal limitation: a genetic limitation. It raises when the maternal genotypes fail to reach a wide range of sites and microhabitat types associated with the dispersal process. We, therefore, extend the consequences of the frugivore dispersal activity to the uneven distribution of the maternal progenies in the seed rain over an environmentally-structured landscape.

Genetic seed dispersal limitation in the seed rain: the ecological causes

Previous work in the present population has provided detailed information on the dispersal patterns assisted by frugivores (Jordano & Schupp 2000, García *et al.* 2006). The non-random seed delivery patterns characterized by a high frequency of short distance dispersal events and a marked directionality help us to to explain why maternal genotypes fail to reach all available sample points in the population: frugivore birds (the main vertebrate dispersers) visit selectively certain microhabitats and they flight consistently with a higher frequency to certain sites in the landscape, as those covered by high shurb and pine trees. Thus, the maternal richness value obtained per microhabitat mirror the relative frequency of visits by frugivore birds, higher under sites covered with high shrub. Moreover, the fact that maternal trees present a high seed contribution to sites located under their canopy also reflect the fondness of bird disperser for certain fruiting trees in the population (Jordano & Schupp 2000). Hence, since the maternal richness and the relative maternal contribution to each microhabitat are closely linked with the number of deposited seeds, the preference for certain microhabitats by frugivore birds are likely determining both of them. However, these preferences for certain microhabitats in the landscape do not explain why microhabitats with cover of fleshy-fruited species presented similar combinations of maternal genotypes, while more scattered microhabitats (MH-Acer-Quercus) had less similar maternal genotypes. The Mantel's test show that source trees with similar ecological neighborhood tended to disperse seed progeny to similar combinations of microhabitat types. Previous work reported the relevance of the maternal ecological neighborhood attracting frugivores (e.g., D. García *et al.*2001), affecting removal rates (e.g., Sallabanks 1993), and shaping the seed dispersal kernel (Carlo 2005), and here we extend the influence of the maternal neighborhood to the spatial distribution of the maternal progenies among microhabitats in the population. Thus, the distribution of the maternal progenies in the seed rain resulting from a seed dispersal genetic limitation by frugivore activity can only be fully explained by considering the interaction between the feeding behaviour of the dispersers and the landscape configuration at both the departure point (maternal neighbourhood) and at the deposition site.

Genetic seed dispersal limitation in the seed rain: the genetic patterns

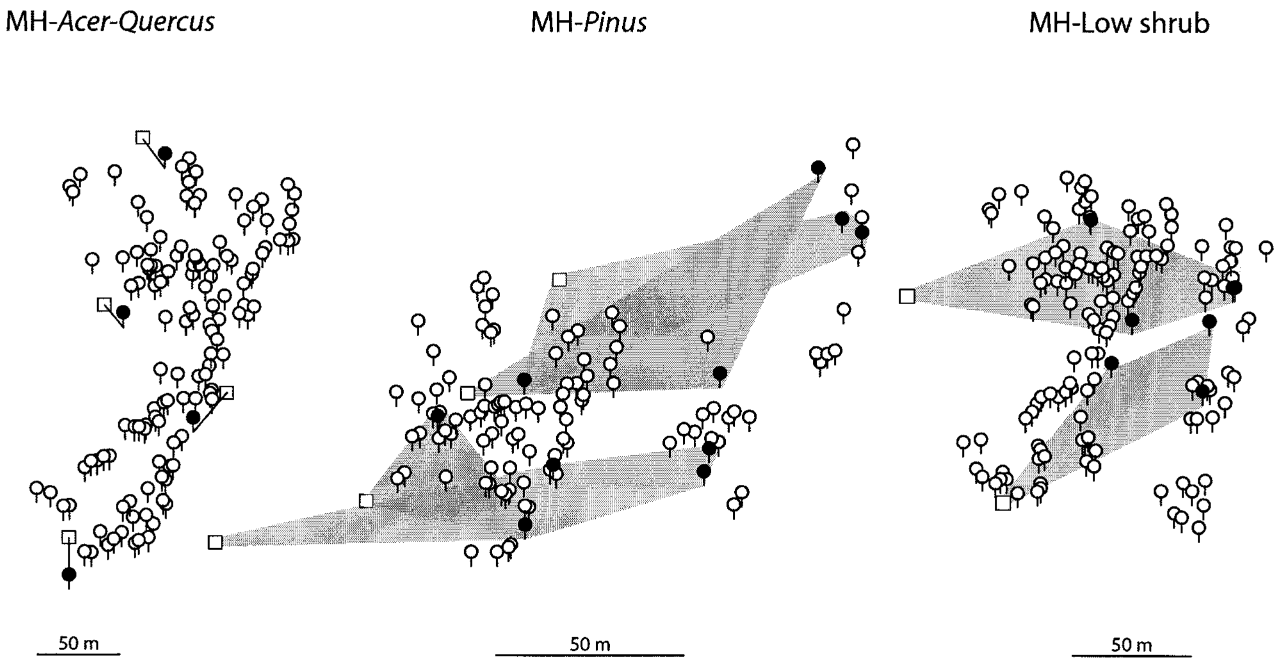
The genetic limitation associated with frugivore-seed dispersal translated into a fine-scale genetic structure present in the seed rain. This was observed in our population as increased rin values, *i.e.*, related maternal trees contributed to the same sample point. Previous studies have reported plant populations consisting in small groups of genetically related individuals at postdispersal stages of the recruitment cycle (Hamrick *et al.* 1993, Epperson & Alverez-Buylla 1997, Giles & Goudet 1997, Schnabel *et al.* 1998). The structure emerged in plant populations are usually explained as the result of mating patterns (high selfing rates or inbreeding depression), genetic drift (Levin 1988), and strong local selection pressures (Ingvarsson & Giles 1999). Hence, although widely recognized to be a relevant process shaping spatial genetic patterns (Hamrick & Nason 1996, Giles *et al.* 1998, Schnabel *et al.* 1998), direct evidences of the role of the seed dispersal in shaping genetic structured patterns are scarce so far, likely due to the inherent difficulty of tracking seed dispersal movements. Our study reports direct evidence that seed dispersal assisted by vertebrates promotes the aggregation of maternal progenies in the seed rain. Moreover, the level of maternal relatedness and aggregation (correlated maternity) is associated to the environmental heterogeneity (microhabitat type). This is because the components of the maternal genetic correlations varied among microhabitats coherently with dominant seed dispersal patterns. The increased values of maternal relatedness along with the low correlated maternity values and high mean maternal richness observed for MH-*Pinus* indicate repeated dispersal events from several closely-growing source trees (Fig. 3). This figure illustrates the receipt of frequent long distance dispersal events from about 100 m distance, that Jordano & Schupp (2000) attributed to the activity of mistle thrushes (*Turdus viscivorus*), the main frugivore dispersing seeds to this microhabitat. In contrast, sample points located in MH-*Acer-Quercus* presented low mean maternal richness, high mean maternal genetic relatedness, and increased correlated maternity. This genetic pattern suggests frequent dispersal events from the same source tree to the same sample point (Epperson and Alvarez-Buylla 1997). Finally, MH-Low shrub presented low maternal diversity (due to low frequency of dispersed seeds) and low relatedness and correlated maternity means. This pattern indicated that sites located under low shrubs collect endocarps from different source trees located at variable distances. MH-Low shrub patches are visited by several small passerine species (Jordano and Schupp 2000) whose feeding behavior favored the deposition of seeds from different mother trees that are not necessarily neighbors, and can be located at variable distances. Similarly, another microhabitat visit by a wide range of frugivore species is MH-High shrub that showed intermediate values of maternal relatedness and correlated maternity, and a high value of maternal richness due to a high frequency of visits. Finally, high correlated maternity values were expected in sampling points under *P. mahaleb* trees due to the effect of the source tree (Clark *et al.* 2005).

Therefore, highly non-random dispersal processes as those observed in endozoochorous species, and more generally in complex dispersal systems (those assisted by different biotic and abiotic vectors, Nathan 2006), are expected to arrange overlapping seed shadows shaping a heterogenous seed rain consisting in clumps of seeds with different degree of maternal correlation. This genetic variation set after dispersal and the ecological heterogenity underlies the outcomes of postdispersal events determining the final recruitment patterns. For instance, Augspurger & Kitajima (1992) shows that mixed arrays of genotypes increased the probability of germinating. Clumps of seeds genetically correlated are more prone to pathogens attack than not clumps of seeds composed by non-related individuals (Janzen 1970). On the contrary, kin selection might act at certain microhabitats favouring the recruitment of related progeny (Stevens *et al.* 1995, Ingvarsson & Giles 1999). However, our ignorance on the genetic structure set in the seed rain after dispersal and their influencing factors for most of plant species, prevent us of having a comprenhensive knowledge of the dynamic consequences of dispersal into the recruitment cycle.

Spatial explicit models for heterogenous landscapes

Although seed dispersal is an inherently spatial process (Ennos 2001, Keitt *et al.* 2002), dissecting environmental and spatial influences has been untraceable until the use of molecular tools within spatially explicit frameworks. Our data showed that models were best fitted when incorporating the spatial structure of the data, with some effects varying the significance level. The Dirichlet area effect on the richness of maternal genotypes represented in a given sample point became significant when taking into account the spatial structure of the error terms. This is due to the Dirichlet area around each tree being itself autocorrelated, since small tiles indicate clumped stands and large tiles correspond to peripheral, isolated trees. On the other hand, sample points in areas with high clumping of *P. mahaleb* tended to present a high richness of maternal genotypes irrespective of location effects. These high-cover stands are areas of high frugivore activity (Jordano and Schupp 2000) with high overlap of individual seed shadows and hence high richness values in comparison with other sites with microhabitats less favored by frugivores (as in MH-Acer-Quercus). This results in spatially variable genetic combinations of dispersed seeds including, e.g., high maternal richness and low relatedness (high seed density sites with progeny from maternal trees located all over the population); very low maternal richness and high relatedness (recurrent dispersal from a nearby, isolated source tree); and high maternal richness and high relatedness (seeds from long-distance dispersal events from several closely-growing source trees). Seed rain results from a biological process sensitive to ecological

Figure 3. Illustrative map examples of different dispersal patterns observed. The map sections of the study area show the locations of P. mahaleb trees (blank dots), and those that contribute seeds to the same sample point: left, in MH-Acer-Quercus, (dominated by Acer spp. and Quercus spp.); middle, in MH-Pinus (dominated by Pinus nigra subsp. salzmanii); and right, in MH-Low shrub (dominated by low shrub species). The grey shadow area connects the mother trees (filled dots) contributing dispersed seed to the same sample point (open squares). Endocarps dispersed in MH-Acer-Quercus tended to come from the very same, closely located, source tree (resulting in high mean correlated maternity); endocarps deposited in MH-Pinus tended to come from different, closely growing, maternal trees located far away from the sample point (resulting in high mean relatedness but low correlated maternity values); and endocarps deposited in MH-Low shrub tend to come from different and distant maternal trees (low mean correlated maternity and low mean relatedness values).



MH-*Acer-Quercus*

MH-*Pinus*

MH-Low shrub

heterogeneity, *i.e.*, seed dispersal assisted by frugivorous animals (Levey *et al.* 2005, Westcott *et al.* 2005). Our analysis with *P. mahaleb* shows evidence for non-overlapping seed shadows, but also for particular locations in the forest (sites) where seed shadow overlap is extensive due to a high attraction of frugivore activity. The elucidation of recruitment patterns resulting from dispersal of different arrays of maternal genotypes in different patches is a next step to fully understand the outcomes of mutualistic interactions in the recruitment cycle of plant populations.

Conclusions

New molecular techniques and the explicit consideration of the ecological complexity of the landscape in natural populations elucidate two key aspects of the seed rain generated by frugivores: it results from a genetic dispersal limitation processes, and it is driven by the ecological heterogeneity present both in the ecological maternal neighborhood (the point where frugivore arrive to feed and the point where the seed dispersal starts) and in the depositon site (the end of the dispesal process). Besides the previously reported numerical and spatial limitation associated to frugivore activity, we report a genetic limitation related with the failure of source trees genotypes to reach all available sites. This results in a highly genetic structured seed rain consisting on seed clumps of maternally related progenies. Additionally, the degree of relatedness and the level of mixing among maternal genotypes varies with the microhabitat type as a consequence of the different arrays of frugivore species visiting each microhabitat and their selective feeding behaviour. Therefore, the assembly of the maternal genotypes in a frugivore-generated seed rain retains the ecological heterogeneity of the landscape and the frugivore seed delivery patterns in a heterogenous landscape. Hence, complex seed rains are only accurately tackled when considering the spatial location of the available sites and the source trees, the ecological heterogeneity, along with previous information describing the foraging preferences by the major frugivores.

Our work adds empirical evidence to the branch of ecology studying the interplay among genetic patterns, plant-animal interactions, and ecological heterogeneity in natural populations fully understand ecological to fully understand the processes underlying the recruitment patterns in plant populations (Thompson 2005, Westcott *et al.* 2005). A forthcoming goal is assessing the dynamic consequences of seed shadow assembly, *i.e.*, the elucidation of recruitment patterns resulting from dispersal of different arrays of maternal genotypes in different environments and locations. Future analyses of the lasting consequences of plant-animal interactions on plant populations will require the integration of genetic, ecological.

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6

Differential contribution of frugivores to complex seed dispersal patterns

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En revisión

Abstract

Seed dispersal systems are increasingly recognized to be complex since they usually involve a combination of several biotic and abiotic vectors. However, no direct evidences are available about the relative contribution of each dispersal agent to distance and probability of seed dispersal among populations, two central aspects of the recruitment dynamics. We used DNA-based genotyping techniques on *Prunus mahaleb* seeds dispersed by frugivorous birds (small- and medium-sized passerines) and carnivorous mammals to infer the source trees, dispersal distances, and the probability for each seed to come from outside the study population. Small passerines contributed mainly to short-distance dispersal, with 71% of the dispersal events located <100 m from the source trees and <20% of the seeds coming from other populations. The long-distance dispersal events were disproportionately contributed by medium-sized birds (50% of the seeds dispersed >110 m), and carnivorous mammals (50% of the seeds dispersed >495 m). When the quantitative contribution to fruit removal was accounted for, mammals were responsible for 66.9% of the immigrant seeds, while birds accounted for 33.1%. Our results provide direct evidence that frugivores widely differ in their effects on long-distance dispersal patterns and to seed- mediated gene flow. Moreover, frugivores varied in their seed deposition patterns due to the selective used of microhabitat types. Since microhabitats provide diferent probabilities of germination, seed dispersal patterns assisted by each frugivore type would translate to postdispersal stages. Therefore, at local scale, critical long-distance dispersal and local recruitment might rely on very few species despite highly diverse coteries of mutualistic frugivores dispersing seeds. At regional level, population declines or extinction of key frugivore species should seriously impair seed-mediated gene flow and connectivity in fragmented landscapes by truncating the long-distance dispersal events.

Key words

Dispersal vector, frugivorous vertebrates, long-distance dispersal, seed dispersal curve, seed rain, heterogeneous landscapes

Introduction

The dispersal of seeds sets up the initial template for regeneration in natural plant populations, thereby influencing key demographic, genetic, and spatial patterning processes (1, 2). Successful dispersal consists in the displacement of seeds from the source tree to the site where they eventually germinate and establish. Different dispersal agents (both biotic and abiotic) can contribute to it and it is increasingly acknowledged that seed dispersal systems are poorly described when considering one single major dispersal vector (4, 5, 6, 7, 8). For instance, secondary hydrochory (reshuffling of seeds already dispersed by animal frugivores) can be a non-negligible component of many dispersal systems (9). By ingesting fruits at feeding trees and later regurgitating or defecating their seeds, frugivorous vertebrates distribute the seeds across available sites in the population, generating the seed rain (10). The combined seed rain thus results from the interaction between the feeding behaviour of each dispersal vector and the landscape structure, mediated by habitat preferences and the dynamics of digestion processes in animal-dispersed species that rely on diverse frugivore assemblages.

The contribution of each dispersal agent to seed rain patterns can vary enormously. Species-specific differences has been reported regarding quantitative aspects of visitation, fruit removal, or post-feeding behaviour, such as effects on germination (for reviews see 8, 13, 17, 18). Thus, large-sized mammals and birds can transport large quantities of seeds over long distances, thereby connecting distant populations (6, 19, 20, 21, 22, 23). In contrast, medium- to small-sized birds tend to concentrate seed dispersal events nearby the source tree, although dispersal distances reaching several hundreds of meters have been reported (13, 14). The qualitatively component, however, has rarely been assessed; this involves the relative contribution of each frugivore type to the seed dispersal curve (i.e., the frequency distribution of dispersal distances, or total dispersal kernel (5)) including the long-distance dispersal events, and to seed immigration (dispersal from other source populations). For example, twenty-five bird species and three mammal species have been reported to disperse the seeds of *Prunus mahaleb*, a fleshy-fruited species, with most dispersal events located near the source tree (13, 15). Interestingly, a single species, the mistle thrush (*Turdus viscivorus*), almost exclusively disperses seeds to a particular patch in the landscape: the pine forest. Mistle thrushes fly away from the feeding tree to pine trees located on the forest edge. In contrast, up to seven species deliver seeds beneath high shrubs, while mammals disperse seeds mostly to open patches (13, 15). Hence, frugivore species exhibit ample variation in the relative contribution to the seed rain in terms of seed dispersal distance, and type of deposition site. Similarly their relative contribution to seed immigration is expected to vary due to different dispersal ability associated to body size and feeding behaviour (2, 5, 11, 16), but this information is unknown for most dispersal systems. As a consequence, our current understanding of the effect of plant-animal mutualism on plant population dynamics is scarce and uncomplete in spite of their recognized relevance specially in fragmented landscapes (2, 33).

How can we determine which frugivore species contributed each dispersed seed in the seed rain and which tree was the source for that seed? This a central issue in seed dispersal studies that only recently has been successfully tackled. Analytical models applied to assess seed dispersal curves (both inverse modelling and mechanistic/phenomenological models) do not successfully capture all the complexities of the dispersal stage, especially the long-distance dispersal events (2). The direct tracking of each seed dispersal event by means of molecular markers remains the most accurate method to obtain reliable estimates of seed dispersal distances reached by different frugivores, specially those involving long distance dispersal events (16, 24). Ideally, both approaches could be combined to robustly assess dispersal patterns, as they emphasize differently the dispersal component (from a source tree perspective) and the arrival component (from a target site perspective) (5, 25).

Here, we aim at dissecting the relative contribution of large carnivorous mammals, medium-sized birds (*Turdus viscivorus* and *Corvus corone*), and small- sized birds (e.g., warblers) to the seed rain of *Prunus mahaleb* (Rosaceae). Our prediction is that variation in distance, contribution to seed immigration and and frequency of seed delivery to different microhabitats will be extensive in mutualistic dispersal systems like these, characterized by a high diversity and low specificity of the interactions. Specifically we i) assessed the contribution of each frugivore type to the seed dispersal curve; ii) estimate the contribution of each frugivore type to available microhabitats; and iii) quantified their contribution to seed dispersal immigration into the study population.

Results

Our data indicate that different types of frugivores indeed accounted for different proportions of seeds dispersed to each distance class (Fig. 1A). Whereas small-sized birds were by far the main seed dispersers up to 250 m, larger frugivores dispersed most of the seeds to distances up to 990.2 m. Medium-sized birds (*T. viscivorus* and *C. corone*) also contributed to short dispersal distances but they dispersed seeds mostly beyond 100 m, where small birds rarely dispersed. Seed dispersal distances by carnivorous mammals ranged from 0 m (i.e., under the source tree canopy) up to nearly 900 m, with a peak at 650-700 m. These distance intervals correspond to within-population dispersal events (seeds consumed in trees growing in the study population); but seeds can be moved longer distances when being dispersed from other populations (Fig. 1B).

Most immigrant seeds in the study population were dispersed by carnivorous mammals (Fig. 1B); their weighted contribution to the immigrant seed pool (considering both the fraction of immigrant seeds in their samples and their quantitative seed removal) was 66.9% while frugivorous birds accounted for the remaining 33.1%. Among these, 0.07% was contributed by *C. corone*, 21.5% by *T. viscivorus*, and 7.8% by the small bird species (Fig. 1). When considering each dispersal vector separately, we observed that 74.2% of the seeds dispersed by mammals were imported from outside the population, whereas 21.9% of the seeds dispersed by birds came from other populations (Table 1). Specifically, 20.6% of seeds dispersed by *T. viscivorus* and 56.5% of the seeds carried by *C. corone* were inferred to be immigrant seeds.

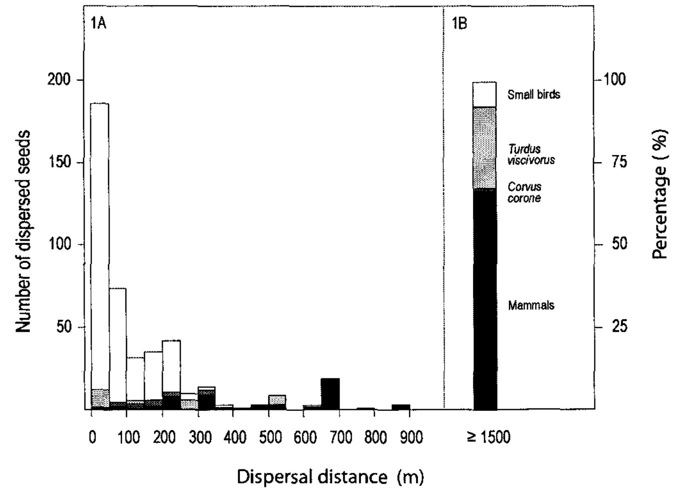

Figure 1. Frequency distribution of seeds dispersed over distance (seed dispersal curve, 1A) showing the relative contribution of major frugivore taxa at different distance classes: small- to medium-size frugivorous birds (white bars; including e.g., *Erithacus rubecula, Phoenicurus ochruros, Turdus merula, Sylvia* spp.); *Turdus viscivorus* (light grey); *Corvus corone* (dark grey); and carnivorous mammals (black bars; including *Vulpes vulpes, Martes foina*, and *Meles meles*). The bar in the right (1B) represents the weighted contribution of each dispersal vector to seed immigration to the study population (dispersal distances ≥1500 m), *i.e.*, fruits consumed in fruiting trees growing in other populations while the seeds being regurgitated or defecated in the study population. For each disperser group, the proportion of immigrant seeds in the genotyped sample was weighted by the overall contribution to fruit removal.

Vector	Sampling design		Source tree location		% Deposition per microhabitat (% Immigrant seeds, P_i^{out})					
	No. Seeds (N_i)	No. Sampling points	Within population (N_i^{in})	Outside population (P_i^{out})	*P. mahaleb*	High shrub	Low shrub	*Pinus*	*Acer-Quercus*	Open
Small-birds	292	143	234	58	23 (17.0)	42 (20.0)	5 (7.2)	14 (18.55)	8 (10)	8 (7.2)
T. viscivorus	173	38	137	36	18 (19.4)	2 (33.0)	-	66 (21.5)	7 (16.7)	7 (16.7)
C. corone	23	4	10	13	-	-	-	-	-	100 (56.5)
Mammals	167	20	43	124	24 (79.4)	-	-	-	-	76 (67.2)

Table 1. Description of the *Prunus mahaleb* seed rain generated by four different frugivore types in the study population: small- to medium- sized birds (SM-birds; including warblers Sylvia spp., redstarts Phoenicurus spp., and robins Erithacus rubecula), mistle thrushes (*T. viscivorus*), carrion crows (*C. corone*), and mammals. For each frugivore type we report the total number of genotyped endocarps (Ni), the number of sampling points, the number of seeds coming from source trees growing within (Niin) and outside (Niout) the population, and the percentage of seeds deposited in each microhabitat type, with the percentage of them coming from outside the population (Piout) in parentheses.

The frequency of seed deposition in different microhabitats differed significantly among dispersal vectors (χ^2 = 596.93, df= 15, P < 0.001). Whereas small-sized birds dispersed seeds mainly beneath the canopies of *P. mahaleb* and other fleshy-fruit trees or shrubs, mammals deposited dispersed seeds preferentially in open sites (rocky soils and open ground with little woody vegetation or grass cover) (Table 1). Medium-sized birds dispersed seeds mainly to open areas (*C. corone*) and beneath pine trees (*T. viscivorus*). The differential use of the microhabitats by different frugivores translated into a variable percentage of immigrant seeds received by each microhabitat (Table 1). Thus, almost 80% of the seeds dispersed by mammals under *P. mahaleb* canopy were immigrant seeds whereas this proportion dropped to 17% when small- to medium-sized birds were the dispersal vectors. Similarly, >50% of the seeds deposited in open sites by *C. corone* (56.5%) and mammals (67.2%) came from other populations, whereas immigrant seeds only represented 7.2% and 16.7% of the seeds dispersed by small-birds and *T. viscivorus*, respectively.

Discussion

Recent advances in the study of animal-mediated dispersal are evidencing the complexities of the seed dispersal curves resulting from the combination of diverse frugivore assemblages and heterogeneous landscapes (5, 8, 26). Actual effective dispersal usually involves more than one biotic or abiotic vector and different animal species that assist for primary and/or secondary seed movement (5, 7, 9). However, for most of these complex dispersal systems we lack field data to estimate the contribution of each dispersal vector to different intervals of the dispersal curve. With the help of theis information it would be possible to forecast the consequences of local extinctions of large frugivores and how they determine serious modifications of gene flow patterns via seed and loss of connectivity among fragmented populations.

By combining DNA-based genotyping methods and field observations we found that seed dispersal assisted by different types of frugivores involves a distinct contribution of different frugivores to each distance class and microhabitat type, with only a few species responsible for the long distance dispersal events. Frugivores differentially contributed to the seed dispersal curve with small to medium sized birds mainly accounting for most short distance dispersal and larger frugivores (both birds and mammals) mostly for long distance dispersal. Our results, are largely congruent with field observations of post-feeding flight patterns and distances (13). Variable shapes of seed dispersal curves have been reported by a series of field studies mainly using direct field observation and/or tracking of frugivores (14, 19, 21, 27, 28) particularly, in heterogeneous landscapes. All these previous studies illustrate the inherent heterogeneity of the seed dispersal systems assisted by frugivores, but fail to document the frequency of long-distance dispersal, especially in the case of immigrant seeds. Our data showed that the *P. mahaleb* dispersal curve presented two distinct parts: seed dispersal is mainly contributed by a diverse array of small sized frugivores over short distances, whereas carnivorous mammals are the ma jor dispersal agents beyond 300 m. Moreover, carnivorous mammals and large birds are responsible for most of the seed exchange among populations, involving dispersal events between 1.5 and 17 km (P. Jordano, pers. obs.). Hence, neglecting the role of the large-bodied dispersal vectors despite their relatively marginal quantitative relevance (13) would lead us to seriously underestimate the frequency of long distance dispersal events (both within and among populations). On the other hand, ignoring small-sized vectors would seriously underestimate the frequency of short distance dispersal events and overestimate dispersal distance. Thus, the analysis of the seed dispersal curve should be approached as a combined function assembled with the differential contribution of different frugivore types (the total dispersal kernel, *sensu* 5).

Interestingly, small-sized birds not only dominated the left section of the dispersal curve (short distance dispersal events), but almost 20% of the seeds they dispersed came from another population. This type of frugivores likely accounts for a non-negligible number of immigrant seeds, since they are responsible for most fruit removal (13), with consumption rates well above those by mammals. Our data indicate that they dispersed seeds to all microhabitats, whereas large birds and mammals selectively dispersed seeds to open microhabitats (*C. corone* and carnivorous mammals) and pine forest (*T. viscivorus*). More importantly, small birds are the main contributors to the seed rain beneath covered microhabitats (under *P. mahaleb*, and high and low shrubs), where seeds have higher chances to establish (29)(E.W. Schupp and P. Jordano unpublished data). Thus, reliable seed dispersal curves in connection with ecological information on the microhabitat type provided highly valuable information for a complete assessment of disperser effectiveness, i.e., the contribution each disperser makes to the future reproduction of a plant (*sensu* 29).

Mechanistic models involving direct tracking of frugivores and modeling gut passage dynamics have been used to infer dispersal distances. The main difficulty with these mechanistic models is the inference of the seed source (an identification of seed immigration events) and actual dispersal distance, due to imprecision of the location methods; however, they are quite precise identifying the dispersal agent (25). Using combined functions in inverse modeling approaches certainly has a sound biological basis. On the other hand, molecular-based techniques are powerful for source tree and distance estimation but rely on indirect methods (e.g., identification of the feces or regurgitations, or direct observation) for determining the dispersal agent that delivered the seed(s). Our work, additionally, unequivocally identified species-specific contributions to both the seed dispersal curve and realized seed immigration from other populations by combining molecular techniques and exhautive field observations on frugivore feeding behaviour.

Finally, our data highlight that seed dispersal curves emerge from the interaction of three main components (8): frugivore abundance (determining which species remove most fruits and disperse most seeds), frugivore feeding behaviour (feeding preferences and dispersal movement), and the structure of the landscape (the relative position of the fruiting trees and the deposition sites). As the frugivore community or the landscape change, the dispersal curve will also be modified. Thus, if carnivorous mammals go extinct in our study population, the movement of seeds among populations would strongly decrease as well as the arrival of seeds to open microhabitats, resulting in a significant truncation of dispersal distances and an increase of population isolation (30). We may also envisage sizeable changes in seed shadows and in the dispersal curve accompanying year-to-year variation in the frugivore assemblage (31). In addition, the reduction of the pine forest might shift the dispersal curve to the left, involving a strong restriction of the seed dispersal distance. These types of effects have broad implications for the conservation of frugivore species, where specific dispersers have disproportionate seed contributions to the safer sections of the seed shadows (e.g. 32, 28), and are thus generalizable to other dispersal systems. For instance, the fast-paced extintion of large tropical frugivores will not only reduce removal rates, a quantitative effect repeatedly documented (33), but will also truncate dispersal curves and severely limit seed-mediated gene flow. Overall, a comprehensive approach to seed dispersal in plant populations requires the incorporation of a new paradigm in seed dispersal systems: moving from simple to complex seed dispersal systems (5), and envisioning seed dispersal curves as an emergent property of plant animal interactions with context dependent outcomes.

Material and Methods

Species and study site characteristics

The study species, *Prunus mahaleb* (L.) (Rosaceae), is a shrub or small tree producing fleshy fruits ingested by frugivores that disperse their seeds after regurgitating or defecating them. This species is frequently visited during July to mid-August by small- and medium-sized birds (13) and carnivorous mammals (15). *P. mahaleb* presents a patchy distribution at the regional scale with scattered, isolated populations consisting of dozens to hundreds of trees. Our study population consists of 196 adult trees distributed over an area of ca. 6 ha in patches of variable density. Other populations exist as scattered patches of 10-150 trees, with some reaching > 1000 trees, within 20 km distance. The nearest population is 1.5 km away. Additional information on the study population is reported in (13) and (15).

Relative contribution of each dispersal vector to the seed rain

Here we differentiate four major frugivore types: large carnivorous mammals (such as foxes, badgers, and stone martens); two species of medium-sized frugivorous birds, mistle thrushes (*Turdus viscivorus*) and carrion crows (*Corvus corone*); and a pool of several species of small-sized frugivorous birds, including warblers, redstarts, and robins (13). These frugivores vary widely in their relative contribution to seed removal in the study population. While small birds and *T. viscivorus* account for a large fraction of the seed rain (up to 71.0 % and 13.8 %, respectively (31, 13, 15), and P. Jordano, pers. obs.), removal by *C. corone* is marginal (0.2 %, estimated from visit frequencies and consumption rates; (13), P.Jordano pers. obs.). The overall contribution of mammals, according to seed trap data and direct sampling of scats in the area, can be estimated in 15 % (15, 34, 31, 13, report data supporting this estimate). We combined these estimates with the direct assignment of dispersed seeds to their source tree based on molecular markers (see below) to dissect the relative contribution of each frugivore group in terms of distance, frequency of seed immigration from other populations, and distribution of immigrant seeds among microhabitat types. For each distance class, the contribution of each disperser group is simply the sum of seeds originating from its sample and identified reaching that distance. The contribution of a disperser group to seed immigration is the % immigrant seeds in the genotyped endocarp sample weighted by the overall quantitative contribution to fruit removal, estimated by direct watches and visitation records (31, 13).

Sampling of dispersed seeds

To estimate the relative contribution of each dispersal vector to seed dispersal curve and to seed deposition in different microhabitat types, we first collected dispersed seeds following different sampling schemes according to the dispersal vector. Seeds were collected in 1997-1999 and 2003-2005. These sampling schemes were as follows:

-. **Seeds dispersed by mammals:** we haphazardly collected 130 feces during the dispersal period and we recorded their spatial location and microhabitat. Feces contained 106.2 ± 66.2 seeds (mean ± SD). From the frequency distribution curve of the number of seeds per feces we randomly choose five feces from each quartile and we genotyped 10% of the seeds in each fecal sample. Overall, we genotyped 167 seeds from 20 feces. Samples included scats mostly from red fox, *Vulpes vulpes* and stone marten, *Martes foina*, as well as samples from badger, *Meles meles* (<10 samples) ((15), C. García and J.L. García-Castaño, pers. obs.).

-. *Seeds dispersed by* C. corone : Regurgitation pellets of this species can be visually identified. The species is a scarce visitor of fruiting *P. mahaleb* trees, but one of the few large-bodied frugivorous birds in the disperser assemblage. We used all the samples we had available for it. We collected a total of four pellets containing 54.5 ± 16.9 seeds per pellet (mean ± SD) and randomly chose

10% of the seeds to perform genetic analysis. A total of 23 seeds were analyzed. The spatial location and the microhabitat type for each pellet were recorded.

-. **Seeds dispersed by small birds:** We collected seeds dispersed in seed traps in a random stratified sampling by microhabitats (see 35, 15, for further details). We characterized six different microhabitats (MH) according to the type of plant cover at the sampling point. Three of them presented fleshy-fruited species: i) MH-*Prunus*, dominated by adult *P. mahaleb* trees; ii) MH-High shrub, dominated by high-shrub, fleshy-fruited, woody cover over 1.5 m height (*Crataegus monogyna*, *Juniperus phoenicea*, *Lonicera arborea*, *Rosa* spp., *Taxus baccata*); and iii) MH-Low shrub, dominated by low-shrub species below 1.5 m height such as *Berberis vulgaris*, *Juniperus communis*, and *Rhamnus saxatilis*. Three other microhabitats were dominated by non-fleshy-fruited species: iv) MH-*Pinus*, dominated by pine trees (*Pinus nigra*, subsp. *salzmannii*); v) MH-*Acer-Quercus* dominated by *Acer granatense* and *Quercus faginea* or *Quercus ilex*, which presented a scattered distribution; and vi) MH-Open, including rocky soil, very low (< 0.20 m) woody vegetation, and/or grassland. We genotyped the endocarp of all dispersed seeds collected in each microhabitat and a subsample of seeds in MH-*Prunus* and MH-High shrub (35), where seed density is frequently very high. This seed sample included seeds dispersed by small and medium-sized passerine species, such as *Phoenicurus ochruros*, *Turdus merula*, *Erithacus rubecula*, *Sylvia communis*, *Sylvia atricapilla*, etc. (13). Seeds in this samples also include a fraction dispersed by *T. viscivorus* (see below).

-. **Seeds dispersed by *T. viscivorus*:** Seeds collected in the *Pinus* microhabitat were all assigned to this species, according with our previous extensive feeding observations and foraging data (15, 13). In addition, most seeds in the seed traps in this microhabitat appeared in scats with 5-10 seeds, which matched the number expected for a single scat of this species. *T. viscivorus* also contributed seeds to the samples from seed traps described in the previous section, but visual identification was not possible. Therefore, from the seed sample genotyped for each microhabitat type, we estimated the percentage dispersed by *T. viscivorus* by extrapolating from the relative contribution of this species to the seed rain in each specific microhabitat, as decsribed in (13). Given N seeds in microhabitat i and a relative contribution p by *T. viscivorus*, we estimated $N_i *p$ seeds to be contributed by this species. The contribution of *T. viscivorus* to immigrant seeds in microhabitat i would be $N_i * p *(N^{Tvis}_{out} /N_{Tvis})$, where N^{Tvis}_{out} /N_{Tvis} is the proportion of immigrant seeds (N^{Tvis}_{out}) in the seed sample of *T. viscivorus* (N_{Tvis}).

Seed dispersal curve

The seed dispersal distance curve was obtained directly by measuring the distance between the seed deposition site (either feces, pellet, or seed trap) and the location of the source tree as determined by parentage analysis based on molecular markers (36). We genotyped all adult trees in the population (N = 196) along with the endocarp of the seeds collected from the seed traps

(N = 465), mammal scats (N = 167) and *C. corone* pellets (N = 23). Since the endocarp is a tissue of maternal origin, the multilocus genotype of a given endocarp and the genotype of its source tree are identical (36). We used a set of 10 polymorphic microsatellites (simple DNA sequence repeats, SSRs) to obtain the multilocus genotypes of both the adult trees (candidate source trees) and the sample of seed endocarps (for details see (36)). All adult trees in the population had a distinct multilocus genotype. Thus, an unambiguous assignment of each seed to its source tree can be made. When a full match between the endocarp genotype and any of the adult tree genotypes in the poplation was not possible, we assumed that the seed came from another population. To assess the effect of typing errors we re-examined the exclusion of genotypes due to a single locus mismatch, two loci mismatches, etc. At the analysis level, any exclusion of identity between a seed and a potential mother tree based on only 1 or 2 loci mismatching was rechecked. We used GIMLET software (37) first to find the matching adult multilocus genotype for each endocarp with 8 or more loci successfully typed. Because each seed belonged to one of the four groups of dispersers, we cloud thus derive the relative contribution of each frugivore group to different distance classes, microhabitats, and to seed immigration.

Contribution of each dispersal vector to seed immigration

Based on the total number of genotyped endocarps, we estimated for each frugivore type i the number of seeds coming from source trees growing within (N_i^{in}) and outside (N_i^{out}) the population. In addition, based on the percentage of seeds deposited per microhabitat type by each frugivore group, we estimated the fraction of seeds coming from outside the population (P_i^{out} for each microhabitat). The weighted contribution to overall seed immigration for each frugivore type (seeds coming from outside the population) was obtained as the product of the fraction of immigrant seeds in each frugivore type sample (N_i^{out} / N_i) by their proportional contribution to overall fruit removal. All these values are reported in Table 1.

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7

Discusion General y Conclusiones

1. La dispersión de polen y semilla: dos procesos y múltiples aproximaciones de estudio

La dispersión de polen y semilla ha sido abordada desde múltiples perspectivas científicas, generalmente de forma aislada e independiente. Así, para un genético, la dispersión da lugar a flujo génico entre poblaciones (Wright 1943); para un demógrafo, tiene una implicación fundamentalmente numérica (Harper 1977); y para un ecólogo de comunidades, la dispersión modifica la composición específica de una comunidad (Grubb 1977). La integración de los aspectos genéticos y ecológicos ya es una (tímida) realidad (Capítulo 2), pero sólo podremos entender el papel ecológico y evolutivo de la dispersión en las poblaciones de plantas bajo un marco conceptual coherente que unifique diferentes aproximaciones de estudio, dirigido a:

A. Conectar patrones y procesos: la dificultad de caracterizar los procesos de dispersión con métodos directos ha obligado a reconstruirlos a partir de métodos indirectos. La estructura genética espacial obtenida con marcadores nucleares (de herencia biparental) y marcadores citoplasmáticos (generalmente de herencia materna) observada en la población de individuos reclutados, plántulas o adultos (Ennos 1994) proporciona estimas históricas de flujo génico vía polen y vía semilla. Sin embargo, esta aproximación indirecta no excluye el efecto de procesos postdispersivos (Nathan & Muller-Landau 2000). Actualmente, la combinación de diferentes herramientas metodológicas y analíticas permite caracterizar en detalle los procesos de dispersión de forma directa, incluidos aquéllos que implican dispersión a larga distancia, diseccionando la contribución del polen y la semilla (Cap. 3). La combinación de estudios experimentales (Jordano & Godoy 2002), modelos espacialmente explícitos que consideren la heterogeneidad del paisaje a diferentes escalas espaciales (McCauley 1995, Clark *et al.* 1999, Sork *et al.* 1999, Urban & Keitt 2001) y de información sobre los patrones de movimiento de los agentes de dispersión como el viento (Nathan *et al.* 2002) o los frugívoros (Jordano & Schupp 2000) son necesarios para identificar la importancia de los procesos de dispersión actuales en el ciclo de reclutamiento.

B. Considerar simultáneamente la dimensión genética, demográfica y espacial de la dispersión. Aunque influidas por diferentes procesos, estos tres aspectos conectan durante la dispersión (especialmente durante la dispersión de semillas) (Janzen 1970, Willson 1992, Schupp & Fuentes 1995, Jordano & Godoy 2002). Es decir, la trayectoria demográfica de una población y su distribución espacial probablemente no sean independientes de su composición genética (Hanski & Saccheri 2006). A pesar de la amplia bibliografía sobre la influencia de procesos dependientes de la densidad y de la distancia al árbol madre en el reclutamiento de nuevos individuos (Schupp 1988, Harms *et al.* 2000), el componente genético ha sido ampliamente ignorado. El Capítulo 5 de este trabajo, muestra que cada punto del paisaje muestrea semillas de diferentes árboles madre y que la correlación materna entre las semillas que llegan a un mismo punto es muy variable, parcialmente influida por el microhabitat donde es depositada la semilla. Es decir, existe una interacción de la contribución maternal a cada punto y la heterogeneidad espacial. Sin embargo, desconocemos en qué medida esta interacción determina los patrones espaciales de reclutamiento.

Los avances en los estudios de la dispersión de polen y semilla junto con las nuevas posibilidades metodológicas y analíticas han creado un nuevo marco conceptual común donde se integran estudios de dispersión desde diferentes aproximaciones. Sólo así podremos llegar a comprender los mecanismos ecológicos implicados en la dispersión, dilucidar sus consecuencias evolutivas y replantear el significado de la dispersión en la dinámica y evolución de las poblaciones vegetales. Esta integración es especialmente relevante ante un escenario de cambio global con tendencias registradas hacia la depauperación de la fauna local que asiste en la dispersión (Cordeiro & Howe 2003, Galetti *et al.* 2006), el aumento de las especies introducidas que afecta a las interacciones locales (Richardson *et al.* 2000) y el incremento de fragmentación de las poblaciones que aísla las poblaciones, modificando su dinámica (Aldrich & Hamrick 1998, Higgins & Richardson 1999, Hamilton 1999, Fuch *et al.* 2003, Nason et al 1998, White *et al.* 2002).

2. El proceso de dispersión de polen y semilla asistido por animales en una población de *Prunus mahaleb*

Los capítulos 3, 4, 5 y 6 de este trabajo nos indican que la dispersión de polen y de las semillas es un proceso intrínsecamente espacial y dependiente del contexto (Willson 1992, Nathan & Muller-Landau 2000, Ennos 2001), es decir, influido por la estructura del paisaje a pequeña escala (microhabitat, Clark *et al.* 1999) donde los eventos de dispersión a larga distancia no son infrecuentes, especialmente aquellos que llegaron vía semilla.

A. Nuestros datos indican que la dispersión es un proceso restringido espacialmente, es decir, dominado por los eventos de dispersión a corta distancia, que es la característica común a todos los sistemas de dispersión en plantas; la frecuencia de los eventos de dispersión disminuye con la distancia a la fuente. Sin embargo, la frecuencia de los eventos a larga distancia es más variable entre sistemas, aunque éste es un aspecto mucho menos documentado y más discutido. La posibilidad de identificar y cuantificar los eventos de dispersión a larga distancia de forma directa demostró que éstos suponen un porcentaje no despreciable del total de eventos de dispersión (Schaal 1980, Ellstrand *et al.* 1989, Willson 1992), en contra de las conclusiones expuestas en las décadas anteriores (Ehrlich & Raven 1969). Los estudios de dispersión de polen en especies tropicales, donde la densidad específica es generalmente muy baja, demostraron que los eventos de polinización a larga distancia eran frecuentes (Bawa 1990, Nason *et* *al.* 1998). En poblaciones continuas de especies anemócoras (dispersadas por viento) de bosques templados también se han encontrado porcentajes elevados de polen procedente de otras poblaciones (Streiff *et al.* 1999, Sork *et al.* 2002, Robledo-Arnuncio *et al.* 2005). Nuestro trabajo, sin embargo, junto con otros publicados recientemente, resaltan el papel de los frugívoros movilizando semillas a larga distancia frente al papel de los polinizadores (Kollmann & Pirl 1995, Fragoso 1997, Alcántara *et al.* 2000, Schupp *et al.* 2002, Clark *et al.* 2005 Bacles *et al.* 2006). Probablemente, las especies endozóocoras dispersadas por vertebrados de tamaño grande y mediano y polinizadas por insectos no se ajusten al patrón anterior en el que el flujo génico llega fundamentalmente vía polen, sino que la vía fundamental de entrada de propágulos es la semilla, como el *P. mahaleb.*

B. La dispersión de polen y semilla está significativamente condicionada por la heterogeneidad ambiental. Los patrones de dispersión que hemos documentado resultaron marcadamente no aleatorios, no sólo en cuanto a dispersión, sino a direccionalidad, y a la frecuencia relativa de los árboles que dispersaron sus propágulos, bien polen o semilla. Los patrones de distancia y dirección reflejan la estructura heterogénea del paisaje donde diferentes microhabitats (nosotros hemos diferenciado cinco tipos fisionomicamente diferentes) se disponen en mosaico. Así, los eventos a larga distancia dentro de la población se deben fundamentalmente a movimientos frecuentes desde árboles fuente a rodales dominados por pinos que se localizan en la periferia de la población (Cap. 5 y 6). La frecuencia relativa de los árboles que dispersan propágulos (polen o semillas) en el pool de polen o en la sombra de semillas probablemente refleje, además, las preferencias de los dispersores visitando selectivamente ciertos árboles (García *et al.* en preparación). ¿Por qué unos árboles serían más visitados que otros (y, por extensión, dispersados con más éxito)? Probablemente resulte de una combinación entre los requerimientos específicos de cada frugívoro y las características del vecindario materno (Saracco *et al.* 2005) (Fig. 7.1). Los Cap. 3 y 5 demuestran que las características del vecindario materno determinan la composición del pool de polen de los árboles madre y de la sombra de semillas. Estudios basados en paternidad han encontrado diferencias individuales en la dispersión de polen correlacionadas con el tamaño y nivel de floración del árbol madre (Burczyk & Prat 1997, Burczyk *et al.* 2006, Schnabel *et al.* 1995, Kauffman *et al.* 1998) y la densidad específica (Stacy *et al.* 1996, Sork *et al.* 2002, Robledo-Arnuncio *et al.* 2004). De forma análoga, el vecindario materno modifica la sombra de semillas (distribución espacial de las semillas dispersadas desde un mimo árbol fuente) de especies dispersadas por frugívoros. La alta heterogeneidad espacial y numérica de la lluvia de semillas generada por frugívoros se ha explicado recurriendo a las características de los puntos de deposición de las semillas (Hoppes 1988, Gómez 2003, Schnabel *et al.* 1998, Jordano & Schupp 2000, Schupp *et al.* 2002) pero el vecindario materno posiblemente tenga un papel más relevante de lo reconocido hasta la fecha (Herrera & Jordano 1981, Sargent 1990, Carlo 2005).

El aspecto más interesante del vecindario materno, es que vincula los dos procesos de dispersión de propágulos (y sus genes); la llegada de polen y la salida de semillas. La llegada de las técnicas basadas en marcadores moleculares que identifican de forma no ambigua el árbol fuente hace posible profundizar en este aspecto y considerar simultáneamente los dos aspectos espaciales (origen y destino de las semillas) con las características de la lluvia de semillas. Los estudios sobre dispersión de polen y/o semilla deberían considerar tanto el vecindario materno como las unidades de paisaje que determinan la variabilidad intrapoblacional en los patrones de dispersión.

3. Replanteando el papel de la dispersión como proceso ecológico y evolutivo

Los patrones de dispersión mediada animales capturan, por tanto, los patrones de movimiento de los polinizadores y de los frugívoros, que responden a la heterogeneidad ambiental en función de sus requerimientos (Saracco *et al.* 2004). Es decir, son altamente heterogéneos cuantitativa (número de propágulos dispersados) y cualitativamente (distancia, dirección y frecuencia de los individuos dispersados). El movimiento de genes que conlleva la dispersión de polen y semillas, por tanto, será desigual, es decir, los genes de un individuo no tienen la misma probabilidad de llegar a todos los árboles maternos, vía polen, ni a todos puntos del paisaje, vía semilla. Además, aunque dominan los eventos de dispersión locales (dentro de la población), la dispersión entre poblaciones no es infrecuente incluso en poblaciones aisladas (Nason *et al.* 1996, White *et al.* 2002, Godoy & Jordano 2001).

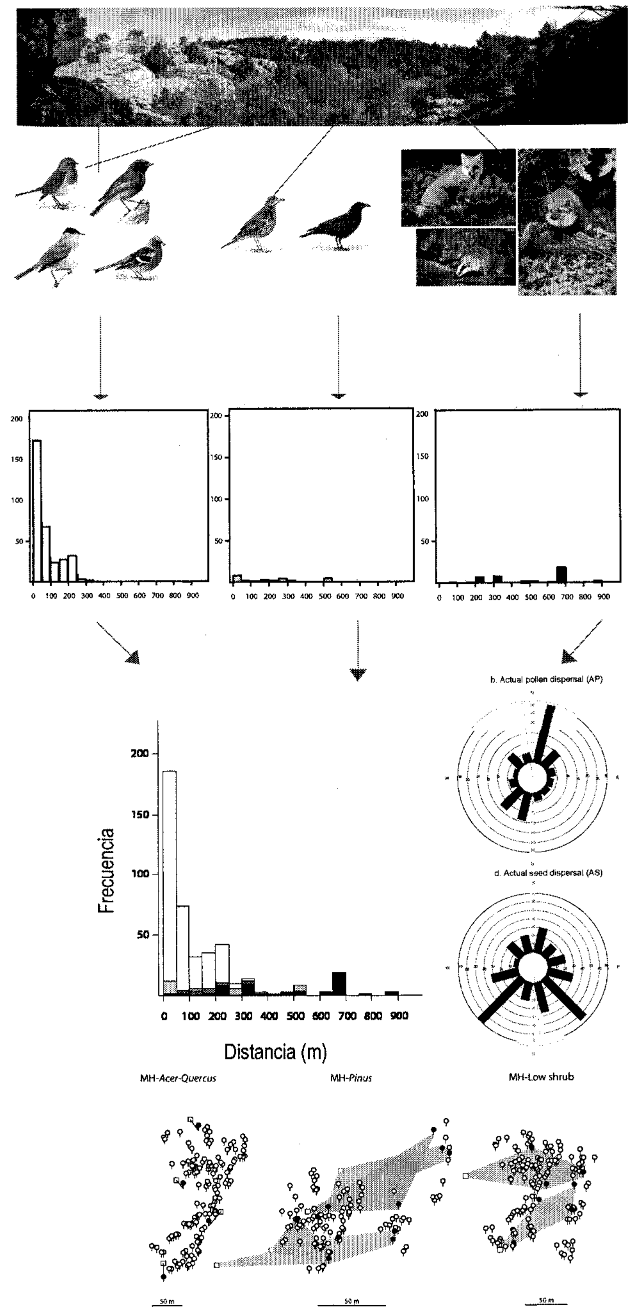
Estudios recientes han documentado la dominancia de unos pocos individuos adultos en el reclutamiento de las plántulas en fragmentos de bosques (Aldrich & Hamrick 1998, Sezen *et al.* 2005). Sorprendentemente, las plántulas encontradas en los bosques fueron producidas por árboles aislados localizados en pastizales adyacentes, es decir, necesitaron ser dispersadas hasta el bosque donde luego germinaron. Sin embargo, en este tipo de estudios los análisis se centran en plántulas y brinzales, con lo cual es difícil dilucidar el papel de la dispersión frente a la selección local en este patrón de reclutamiento. Durante la germinación y los siguientes procesos postdispersivos ciertos genotipos se reproducen más exitosamente que otros (Jordano & Herrera 1995, Kalisz *et al.* 2001). El efecto de la selección local (proceso por el cual se observa un cambio de las frecuencias alélicas en la generación siguiente debido a una mayor reproducción o supervivencia de algunos genotipos) y la deriva génica (el cambio en las frecuencias alélicas se produce de forma aleatoria) está ampliamente reconocido en la caracterización de la dinámica poblacional (Futuyma 1998). Sin embargo, el flujo génico a menudo se considera un proceso que homogeneiza las frecuencias alélicas de la población, contrarrestando los efectos de la selección natural, deriva génica y cuellos de botella (Slatkin 1985, Futuyma 1998); la ausencia de flujo génico conllevaría una estructuración de las poblaciones, generalmente debido a un aumento de la distancia (Wright 1943). Otros trabajos han destacado que la migración entre poblaciones también está sujeta a estocasticidad, lo cual disminuye su potencial homogeneizador de la composición genética entre poblaciones (Levin 1988). Esto ocurre, por ejemplo cuando el *pool* de individuos migrantes no es representativo de las frecuencias alélicas de la población, por ejemplo, porque los miembros de una familia tienden a migrar juntos (Ingvarsson & Giles 1999). Sin embargo, los resultados aquí expuestos muestran que no todos los individuos de una población se dispersan con la misma probabilidad y con la misma distribución espacial. Es decir, la dispersión (y el flujo génico que origina) no es un proceso esencialmente homogeneizador, que facilita la estructuración de las poblaciones cuando no actúan, sino que un intenso flujo génico puede generar y mantener diferencias genéticas (y fenotípicas) entre y dentro de poblaciones. Igualmente la dispersión asistida por animales en poblaciones de plantas, por tanto, no es homogénea para todos los individuos, sino que actúa como un primer filtro del pool de genes que se transmiten entre generaciones en una población; los genotipos de los individuos adultos se dispersan de forma diferencial en el paisaje. Sólo necesitamos un paisaje heterogéneo y un proceso biológico que responda a la heterogeneidad ambiental; por ejemplo, la dispersión asistida por polinizadores y frugívoros (Fig. 1). Este efecto no homogenizador de la dispersión puede producirse, por tanto, a pequeña escala espacial y temporal. Así, la dispersión diferencial es el mecanismo que explica las diferencias fenotípicas entre diferentes individuos de carboneros (*Parus major*) entre individuos que ocupan una población continua compuesta de diferentes parches de vegetación (Garant *et al.* 2005, Postma & Noordwijk 2005). La dispersión y el flujo génico que origina, está sujeta, por tanto, a estocasticidad y promueve por sí mismso la estructuración entre poblaciones a pequeña (Giles y Goudet 1997) y gran escala (Levin 1998). L a estocasticidad y la asimetría se han introducido en modelos mateméticos para explicar las consecuencias de la dispersión (Olivieri *et al.* 1990), o se han invocado *ad hoc* para interpretar los patrones observados (Levin & Krester 1974, Hamrick 1987). Sin embargo, los trabajos en los que se identifican los procesos y se cuantifican los efectos de los factores ecológicos que introducen estocasticidad en la dispersisón son escasos. El papel de la dispersión diferencial está poco explorado en poblaciones de plantas aunque probablemente explique parte la variabilidad fenotípica observada y de la estructura genética intra-poblacional, generalmente pequeña pero significativa (Loveless & Hamrick 1984).

4. El papel de la dispersión depende del contexto geográfico

El papel de la dispersión como motor de flujo génico, por tanto, no es único (contrarrestar los efectos de otros procesos demográficos y genéticos como la selección natural, la deriva génica y los cuellos de botella) sino que varía en función del contexto geográfico, esto es, número y tipo de dispersores, estructura del paisaje, grado de aislamiento, y distribución espacial de los individuos adultos que se dispersan. Este conjunto de elementos determinan las características del proceso de dispersión (Jordano & Godoy 2002): la extensión numérica (en qué medida se dispersan todos los propágulos de la población), la extensión genotípica (en qué medida se dispersan todos los genotipos de la población), la extensión espacial (en que medida la dispersión abarca un rango geográfico amplio), y la extensión ambiental (en qué medida hay dispersión exitosa a todas las unidades del paisaje o microhabitats). Es decir, la dispersión asistida por animales es un proceso dinámico, dirigido por las preferencias de los polinizadores y frugívoros, contexto dependiente y que estructura los genotipos movilizados en respuesta a la heterogeneidad ambiental.

Esta aproximación a la dispersión en plantas y sus consecuencias para el flujo génico replantean el papel de la dispersión y del flujo génico en las poblaciones de plantas tanto desde un punto de vista dinámico como evolutivo. Así, las dos aproximaciones que explican la coexistencia de especies en una comunidad tendrían un hilo conductor en los procesos de dispersión, al plantear la dispersión no como proceso aleatorio y equiprobable, sino heterogéneo y sujeto a variabilidad ambiental. Es decir, no se trata de estudiar la limitación (espacial) de la dispersión frente a la ocupación de nichos ecológicos, sino de evaluar en que medida la limitación de la dispersión de genotipos condiciona el ensamblaje multiespecífico entorno a una serie de nichos ecológicos. Finalmente, en el marco del mosaico geográfico, la dispersión de propágulos y el flujo génico como proceso contexto dependiente añade una dimensión más de complejidad al mosaico coevolutivo, ya que puede incrementar las diferencias entre poblaciones, no por aislamiento, sino precisamente por un flujo asimétrico de genotipos entre ellas.

Las preguntas nuevas que surgen de replantear el papel de la dispersión en plantas necesitan evidencias empíricas que nos permitan completar nuestro conocimiento del proceso de dispersión asistido por animales. La gran variedad de técnicas experimentales y de análisis actualmente disponibles nos ofrecen la oportunidad de estudiar la dispersión en plantas bajo un marco conceptual amplio que explique cómo se regenera una población, cómo se forma un bosque y finalmente, como se forma y estructura la diversidad biológica.



Landscape heterogeneity

1. Patches arrangement
2. Maternal neigbourhood (Cap. 3 y 5)

Complex frugivorous assemblage

1. Specific feeding behaviour (Jordano & Schupp 2000)
2. Diverse assemblage (Jordano & Schupp 2000)

Complex numeric patterns

1. Diferential relative contribution of frugivores to the seed dispersal curve (Cap. 6)

Complex spatial patterns

2. Distance (Cap. 4)
3. Direction (Cap. 4)

Complex genetic patterns

4. Pollen pool diversity (Cap. 3)
5. Correlated paternity in the pollen pool (Cap. 3)
6. Proportion of maternal genotypes (Cap. 5)
7. Maternal correlation (Cap. 5)

Figura 1. Esta figura ilustra llos factores más importantes que condicionan el proceso de dispersión de polen y semilla en poblaciones de plantas (heterogeneidad ambiental, estructura del paisaje y patrón de movimiento de los vectores de dispersión). La parte inferior de la figura ilustra los patrones de dispersión que resultan de este tipo de procesos (fuertemente agregados, espacialmente restrigidos y capturan la heterogeneidad ambiental).

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5. ¿Qué hemos aprendido de la dispersión de polen y semilla por animales?

Conclusiones

I. La dispersión de polen y semillas debe ser estudiada desde un marco conceptual que unifique las diferentes aproximaciones de estudio que actualmente se interesan por la dispersión. Este marco conceptual debe facilitar la integración de los patrones y procesos implicados en la dispersión así como la consideración explícita de la dimensión demográfica, genética y espcial de la dispersión. Sólo de esta forma conseguiremos entender completamente el papel ecológico y evolutivo de la dispersión en poblaciones de plantas.

II. La dispersión a larga distancia se ha atribuído generalmente a la dispersión de polen que conecta poblaciones aisladas geográficamente. Este modelo es habitual es especies de bosques templados anemócoras y que carecen de agentes eficentes que dispersen las semillas a larga distancia. Sin embargo, en especies polinizadas por insectos y productoras de frutos carnosos dispersados por vertebrados frugívoros la dispersión a larga distancia que diferentes fragmentos, se debe fundamentalmente a la dispersión via semilla.

III. Los trabajos actuales reconocen tres tipos de limitación asociados a la dispersión de semillas por frugívoros que afectan al reclutamiento de las poblaciones de plantas: i) la limitación cuantitativa; ii) la limitación en distancia; y iii) la limitación debida a la disposición agregada de la lluvia de semillas. Nuestro trabajo añade la limitación genotípica, es decir, la imposibilidad de los árboles maternos de dispersar a sus semillas a una variedad de puntos y microhabitas que cubran la heterogeneidad ambiental de la población.

IV. La dispersión de polen y semillas asistida por animales debe considerar explícitamente la heterogeneidad ambiental que condiciona los patrones de movimiento de los dispersores. Nosotros hemos detectado que el vecindario materno determina tanto la llegada de polen como la sombra de semillas de un árbol fuente en especies dispersadas por animales. Aunque la heterogeneidad debe ser considerada a varias escalas espaciales, el vencidario materno representa la unidad espacial que conecta el comportamiento de los frugívoros y ambos procesos de dispersión y debe por tanto, tenerse en cuenta en los estudios de dispersión.

V. Los sistemas de dispersión asistido por animales se caracterizan por ser complejos, es decir, están compuestos por varias especies y cada una de ella genera un patrón de dispersión de semillas específico. La disección de la contribución de cada uno de ellos a la curva de dispersión de semillas nos ayuda a evaluar la importancia de los frugívoros como dispersores de semillas y capacidad para conectar demográfica y genéticamente poblaciones geográficamente aisladas. Así, la dispersión a corta distancia dependió fundamentalmente de aves de tamaño pequeño y mediano, mientras que la dispersión a larga distancia y la inmigración de semillas se debió fundamentalmente a frugívos de mayor tamaño. El gran número de semillas movilizados por los frugívoros de pequeño tamaño, sin embargo, hace que su papel como dispersores a larga distancia sea relevante.

VI. El flujo génico a menudo se considera un proceso que homogeneiza las frecuencias alélicas de la población, contrarrestando los efectos de la selección natural, deriva génica y cuellos de botella. Sólo la ausencia de flujo génico estructuraría las poblaciones genéticamente. Nuestro trabajo, sin embargo, indica que la dispersión asistida por animales no es homogénea para todos los individuos, sino que actúa como un primer filtro del *pool* de genes que se transmiten entre generaciones en una población; los genotipos de los individuos adultos se dispersan de forma diferencial en entre diferentes unidades del paisaje heterogéneo. Esta aproximación a la dispersión en plantas y sus consecuencias para el flujo génico replantean el papel de la dispersión y del flujo génico en las poblaciones de plantas tanto desde un punto de vista dinámico como evolutivo.

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Reunido el tribunal en el día de la fecha, integrado por los abajo firmantes, para evaluar la tesis doctoral de D. Cristina García Pérez
titulada Patrones de dispersión de polen y semillas asistida por animales en una población de Prunus mahaleb (Rosaceae).
acordó otorgarle la calificación de

Sevilla, a 5 de Diciembre de 2006.

Vocal,

Vocal,

Vocal,

Presidente,

Secretario,

Doctorando,

Cristina García

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