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CERTIFICA

Que los trabajos de investigación desarrollados en la memoria de tesis doctoral, "Síndromes de polinización en *Silene*. Evolución de las interacciones polinizador-depredador con *Hadena*" son aptos para ser presentados por el Ldo. Samuel Prieto Benítez ante el tribunal que en su día se consigne, para aspirar al Grado de Doctor en el Programa de Doctorado de Conservación de Recursos Naturales por la Universidad Rey Juan Carlos de Madrid.

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TESIS DOCTORAL

Síndromes de polinización en *Silene*. Evolución de las interacciones polinizador-depredador con *Hadena*.

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A mi familia y a Sofía,
gracias por el apoyo y
el cariño que me dais.

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RESUMEN

ANTECEDENTES

Las relaciones simbióticas hacen referencia a interacciones estrechas entre dos organismos y que dependiendo del signo de la interacción pueden ser mutualistas (+,+), competitivas (-,-), parasitarias (+,-) o comensalistas (+,0) (Boucher et al. 1982, Douglas 2010). Las plantas establecen relaciones simbióticas con un sinfín de organismos que determinan en gran medida su establecimiento, desarrollo y éxito reproductivo. Un ejemplo clásico de mutualismo es la polinización mediada por animales (polinización zoófila). La polinización es la transferencia de polen desde la antera hasta el estigma, paso que precede a la fertilización del ovulo y formación de la semilla (Proctor et al. 1996). En la polinización zoófila la flor adquiere el polen y el polinizador alimento (néctar y/o polen) o alguna otra recompensa (aceites, esencias), si bien en ciertos casos muy concretos la interacción no resulta mutualista, pues el polinizador puede ser falsamente atraído sin beneficio alguno (polinización engañosa). Este mutualismo ha modelado la coevolución de las plantas zoófilas y de los animales polinizadores (Kiester et al. 1984). Los atributos de las flores han evolucionado para atraer a los polinizadores más eficaces, más frecuentes o ambas cosas, mientras que los rasgos morfológicos, fisiológicos y etológicos de los polinizadores han evolucionado para explotar mejor aquellas plantas que les ofrecen más recursos y/o de mejor calidad, o que les suponen menos esfuerzo en obtenerlos (Stebbins 1970; Faegri & van der Pijl 1979; Proctor et al. 1996). De acuerdo con esto los sistemas planta-polinizador deberían haber evolucionado hacia la especialización. Sin embargo numerosos estudios han demostrado que raramente ocurre esto y la tendencia general es que, a pesar de mostrar a veces fenotipos florales muy especializados, la mayoría de plantas son visitadas por un amplio y diverso grupo de especies animales (Waser et al. 1996; Herrera 1996; Aigner 2001), es decir que son plantas aparentemente generalistas (Armbruster et al. 1999; Armbruster et al. 2000). A pesar de esto, es fácil percibir cómo plantas no relacionadas taxonómicamente que son visitadas por los mismos grupos funcionales de polinizadores a menudo desarrollan fenotipos florales similares. Ello sugiere la existencia de procesos de selección fenotípica

que habrían derivado en convergencias evolutivas (Fenster et al. 2004; Jürgens 2004; Ollerton et al. 2009). Estos fenotipos florales, caracterizados por la correlación de diversos atributos relacionados con la atracción de un grupo específico de polinizadores (forma, color, emisión de esencias, producción de néctar, etc.), se conocen como síndromes de polinización (van der Pijl1960, Faegri 1979, Fenster et al. 2004). Las características florales que constituyen un síndrome de polinización pueden separarse en tres categorías: atracción (forma, color y olor), recompensa (néctar, polen, aceites, y en ocasiones las propias esencias) y eficiencia de la polinización (forma y posición de los órganos sexuales de la flor que afectan a la liberación, adherencia y deposición del polen) (Bradshaw et al. 1995). Las flores tienen color gracias a numerosos pigmentos, principalmente flavonoides, y lo usan como señal visual para atraer a los polinizadores. Los colores son percibidos de diferente manera según qué animales, y pueden atraer selectivamente a distintos polinizadores. Por ejemplo, el color rojo se ha sugerido que es más fácil de detectar por colibríes que por las abejas (Spaethe et al. 2001; Rodríguez-Gironés & Santamaría 2004; Lunau 2011). La composición, cantidad y momento de la emisión de las esencias florales también son esenciales para la comunicación entre plantas y polinizadores (Knudsen & Tollsen 1993; Knudsen et al. 2006). La mayor parte de las flores emiten compuestos orgánicos volátiles (VOCs), y se ha descrito una inmensa variedad de ellos. Son conocidos sobre todo los derivados de ácidos grasos, terpenoides, compuestos aromáticos, nitrogenados y sulfurados (e.g. Knudsen et al. 1993; Dudareva et al. 2000; Raguso 2001). Los estudios sobre la importancia de cada VOC como atrayente generalista o específico de polinizadores no son aún lo suficientemente abundantes, e impiden tener una visión de conjunto. El néctar, junto con el propio polen, es una de las mayores recompensas para los polinizadores (Simpson & Neff 1983; Proctor et al. 1996). Está compuesto mayormente de agua y carbohidratos pero también de aminoácidos, proteínas y algunos lípidos (Nicolson & Thornburg 2007). Incluso puede contener, al igual que el polen, terpenoides volátiles que pueden actuar como reclamo para los polinizadores (Raguso 2004, Dötterl & Jürgens 2005). El néctar puede variar en composición, en volumen y en su dinámica temporal de producción, y se ha comprobado cómo estas características están asociadas con las necesidades de los distintos polinizadores (Barker & Barker 1983, Cruden et al 1983, Nicolson 2007).

El concepto de síndrome de polinización ha sido criticado muchas veces porque, como se ha dicho previamente, la mayoría de las plantas son visitadas por múltiples polinizadores en potencia, y por lo tanto los supuestos síndromes no predicen satisfactoriamente los visitantes (Herrera 1996; Waser et al. 1996; Armbruster et al. 2000 Fenster et al. 2004; Ollerton et al. 2009). Sin embargo, no todos los visitantes florales tienen por qué ser polinizadores efectivos, por lo que una planta con un espectro de visitantes generalista puede volverse especialista al examinar la eficiencia de cada uno de ellos, y su síndrome volver a adquirir sentido. Además, hay que tener en cuenta que los síndromes de polinización son supuestamente el resultado de fuerza selectivas pasadas, y que el actual contexto ecológico de la planta puede haber cambiado (Ollerton et al. 1996). De esta forma, los polinizadores que deberían ser más importantes según el síndrome de polinización de una planta pueden haber desaparecido, o seguir formando parte del grupo de polinizadores incluso cuando haya otros que actualmente sean más importantes (Rosas-Guerrero et al. 2014).

Por otro lado, a parte del efecto selectivo de los polinizadores, la variación en los atributos florales también puede deberse a otras razones a como la presencia de otros agentes selectivos (depredadores, parásitos, robadores de néctar), o pueden deberse a restricciones filogenéticas (Raguso 2001; Nicolson & Thornburg 2007). Al fin y al cabo, los atributos que componen el síndrome de polinización están heredados de sus ancestros, y esta restricción filogenética puede imponer diferentes limitaciones a la variación en estos atributos. A pesar de estas posibles restricciones filogenéticas se ha observado que los cambios de síndrome dentro de un mismo grupo de plantas están muy extendidos, como por ejemplo el síndrome “colibrí”, que aparece de forma independiente al menos en diez ocasiones en Solanaceae (Knapp 2010). Estos cambios en los síndromes pueden deberse a que algunos atributos están muy correlacionados en su regulación. Por ejemplo, cambios en el color de las flores debidos a pequeños cambios genéticos (Hoballah et al. 2007; Wu et al. 2013), a su vez pueden afectar a las rutas bioquímicas de producción de determinadas esencias florales (Sheehan et al. 2012). Estos pequeños cambios genéticos pueden producir por tanto cambios drásticos de síndrome, y especiación por aislamiento reproductivo de una manera rápida (Bradshaw et al. 1995).

Dentro de la controversia que han generado los síndromes de polinización y la generalización tenemos un claro ejemplo en la tribu Sileneae L. (Caryophyllaceae) (Bittrich 1993; Harbaugh et al. 2010; Greenberg & Donoghue 2011). La tribu Sileneae se compone de los géneros *Silene*, *Lychnis*, *Agrostemma*, *Atocion*, *Viscaria*, y *Petrocoptis*, siendo *Silene* el más diverso con más de 700 especies (Oxelman et al. 2013). En Sileneae tradicionalmente se han descrito dos síndromes de polinización, diurno y nocturno (Lindman 1897; Greuter 1995; Jürgens et al. 1996). Las especies denominadas nocturnas tienen la corola blanca o color crema, la emisión de esencias es mayor durante la noche y la antesis de la flor se produce al atardecer o durante la noche. Además, en algunas especies, el limbo de los pétalos se abre y cierra enroscándose sobre sí mismo todos los días hasta que se produce la fecundación de la flor (nictinastia o fotonastia). La apertura de los pétalos facilita el posado de los polinizadores y hacen a la flor más atractiva visualmente. Las especies diurnas tienen pétalos de color rosa o rojo, no hay un cambio marcado en la producción de esencias entre el día y noche, y las flores permanecen siempre abiertas (Jürgens et al. 2002a; Jürgens 2006). La forma de las flores también es ligeramente distinta entre las especies diurnas y nocturnas en la subfamilia Caryophylloideae (*Agrostemma*, *Diantus*, *Saponaria*, *Silene* y *Vaccaria*). Las especies nocturnas poseen cálices más alargados, que implican mayor distancia entre la fuente de néctar y la zona de deposición y recogida del polen en el cuerpo del polinizador (Jürgens 2006). Además, la dinámica de producción de néctar y la forma floral parecen correlacionarse bien con los supuestos polinizadores según el síndrome. Por ejemplo las polillas nocturnas tienen su probóscide alargada, lo que les permite llegar al néctar producido de noche por las largas flores de síndrome nocturno (Witt et al. 1999; Jürgens 2006). Pero no todos caracteres florales en *Silene* y géneros relacionados se han estudiado en profundidad. Por ejemplo, aunque se sabe que muchas especies de *Silene* tienen pétalos nictinásticos, poco se sabe sobre si estos ciclos día-noche afectan también a otros caracteres funcionalmente relacionados. En un reducido grupo de especies se observó que las supuestamente nocturnas producían néctar solo de noche, mientras que la única especie diurna analizada (*S. dioica*) produjo néctar de manera continua (Witt et al. 1999). Por otro lado, la apertura de los pétalos puede también contribuir a la atracción olfativa, puesto que es conocido que los pétalos producen muchas de las esencias florales (Dobson et al. 1990; Bergström et al. 1995). Hasta ahora,

los estudios sobre las esencias florales en cariofiláceas se han centrado en la comparación entre especies asignadas *a priori* al síndrome diurno o nocturno, basándose en algunos de los caracteres antes mencionados y en la percepción humana de la fragancia floral (Jürgens et al. 2003, 2002b; Jürgens 2004). Estos estudios han revelado interesantes descubrimientos sobre la composición química de las esencias de este grupo tan diverso, pero están inevitablemente sesgados porque las muestras sólo se recogían durante el día en las especies diurnas y durante la noche en las especies nocturnas. En las pocas especies de *Silene* en las que se han estudiado las esencias de día y de noche, se ha encontrado presencia en ambos periodos, con cierta variabilidad tanto en cantidad como en composición (Castillo et al. 2014; Dötterl et al. 2012; Giménez-Benavides et al. 2007; Martinell et al. 2010; Waelti et al. 2008). Por ello, es necesario un estudio a mayor escala para caracterizar la variación día-noche y entender cómo se correlaciona con los demás caracteres florales que definen los síndromes. Por otro lado, a pesar de esta dualidad en los fenotipos florales, casi todas las especies de la tribu Sileneae tienen visitantes tanto diurnos (abejas, moscas, mariposas, incluso algunas colibríes) como nocturnos (polillas) (Jürgens 2004; Jürgens et al. 2002b; Kephart et al. 2006; Reynolds et al. 2009). En las pocas especies en las que se ha estudiado el efecto de cada tipo de visitante se ha comprobado que tanto las visitas diurnas como la nocturnas son efectivas, en distinto grado según la especie (Young 2002; Giménez-Benavides et al. 2007; Barthelmess et al. 2006; Reynolds et al. 2009; Martinell et al. 2010). Estos grupos de polinizadores podrían estar ejerciendo diferentes presiones selectivas sobre los distintos caracteres florales que definen los síndromes (Fenster & Dudash 2001). Además, otros agentes selectivos también pueden afectar a la evolución de los síndromes de polinización en Silene, como el hongo parásito de las anteras, *Mycobotrium violaceum,* que causa esterilidad masculina sobre unas 100 especies de cariofiláceas (Antonovics et al. 2002), o los polinizadores-depredadores de semillas.

Los polinizadores-depredadores de semillas (*nursery pollinators*) son aquellos polinizadores cuya progenie se alimenta con frutos o semillas de la misma planta a la que polinizan. Los sistemas de *nursery pollination* más conocidos son obligados, en los que ambos socios dependen del otro para reproducirse, como el establecido entre las yucas y las polillas de las yucas (Pellmyr 2003) o entre los *Ficus* y las avispillas Agaonidae

(Wiebes 1979). Algunos requisitos indispensables para el establecimiento de este tipo de mutualismos obligados son: a) una alta especificidad recíproca, b) una alta eficiencia del huésped como polinizador, y c) la presencia de algún sistema que limite en consumo de semillas por parte del huésped, para no incurrir en parasitismo (Dufay & Anstet 2003). En algunos de estos sistemas obligados se ha comprobado que existe un alto grado de congruencia filogenética entre los linajes de huéspedes y hospedadores, pudiendo hablar de la existencia de verdadera coevolución que ha derivado en coespeciación (Pellmyr 2003; Kato et al. 2003; Machado et al. 2005). Sin embargo, todos estos sistemas altamente específicos suelen ser en realidad la excepción a la norma (Herrera 2001; Herrera & Pellmyr 2002). La mayoría de sistemas polinizador-depredador no suelen ser tan recíprocamente específicos, y pueden ser facultativos debido a la presencia de otros polinizadores (Thompson & Pellmyr 1992), que hacen que la presencia del polinizador-depredador no sea esencial para la reproducción de la planta. Los sistemas de polinización-depredación facultativos eventualmente pueden oscilar entre el mutualismo y el parasitismo según el contexto ecológico de las poblaciones (Dufay & Anstett 2003, Thompson & Fernandez 2006). Estos sistemas facultativos son comunes entre las especies de *Hadena* Schrank. (lepidópteros nocturnos de la familia Noctuidae) y muchas especies del género *Silene* (Kephart et al. 2006). Los adultos de ambos sexos de estas polillas visitan y consumen el néctar de las flores de *Silene*, polinizándolas al mismo tiempo. Una vez realizada la cópula, las hembras ponen los huevos sobre o en el interior del cáliz de algunas de las flores visitadas (Brantjes 1976; Bopp 2003; Giménez-Benavides et al. 2007; Reynols et al. 2012). De este modo, las larvas emergen de los huevos e inmediatamente penetran en el fruto en desarrollo, donde permanecerán alimentándose durante varios estadios larvarios. Al completar los primeros estadios larvarios, las larvas se mueven del fruto inicial o primario, ya completamente depredado, a otros frutos en la misma o distinta planta para completar su desarrollo, pudiendo depredar también flores. El número de frutos depredados por larva para alcanzar el estado de pupa varía entre pares hospedador-huésped (por ejemplo, de 3-5 en S. latifolia- H. bicruris, de 17-47 en *S. vulgaris-H. perplexa*, de 5-12 en *S. ciliata-H. consparcatoides,* de 32-46 en *S. stellata-H. ectypa*) (Brantjes 1976; Peschken & Derby 1990; Giménez-Benavides et al. 2007; Reynols et al. 2012). Normalmente las hembras depositan un huevo por flor, e incluso uno o pocos huevos por planta, lo que asegura la

abundancia de alimento a cada larva, pues éstas suelen tener un comportamiento de canibalismo muy desarrollado (Brantjes 1976; Peschken & Derby 1990; Elzinga et al. 2002; Reynols et al. 2012). En cuanto a la presencia mecanismos que limiten en consumo de semillas, tan sólo se ha descrito en el par *S. latifolia-H. bicruris* cómo la planta puede abortar selectivamente algunos frutos parasitados para controlar la densidad de larvas (Burkhardt et al. 2009). Por tanto, dependiendo del balance neto entre flores polinizadas y frutos depredados por *Hadena*, la interacción fluctuará entre mutualismo y parasitismo, y los caracteres florales que utilice *Hadena* para la elección de plantas podrían ser positiva o negativamente seleccionados. Por ejemplo, se ha demostrado además que *Hadena bicruris* selecciona su planta hospedadora por color y olor (Page et al. 2014), y ovoposita selectivamente en individuos de *S. latifolia* con más flores y de mayor tamaño (Burkhardt et al. 2012).

Todo lo anterior convierte el sistema *Silene-Hadena* en un buen modelo donde estudiar la integración de los rasgos que definen los dos síndromes de polinización (y en particular la correlación de las esencias florales con el resto de caracteres), así como la especificidad y el contexto ecológico que puede hacer fluctuar este sistema del mutualismo al parasitismo. Tanto las especies de *Hadena* como el resto de polinizadores y depredadores, y en general cualquier organismo que interaccione con una especie de *Silene* podría ejercer de agente selectivo sobre los distintos caracteres florales. Además, este contexto ecológico puede variar en el tiempo y entre poblaciones, tal como postula la Teoría Coevolutiva en Mosaico Geográfico (Thompson 1994, 1999). Es por ello que para profundizar en el conocimiento de este sistema se necesita abordarlo desde una perspectiva espaciotemporal y taxonómica amplia.

A pesar de que la distribución y diversidad de ambos taxones no está lo suficientemente explorada, los datos disponibles muestran que la riqueza específica de *Silene* y *Hadena* a lo largo del Paleártico están fortísimamente correlacionadas (Figura 1A), lo que de algún modo refleja la historia evolutiva común que tienen ambos linajes (Ocaña 2015). La Región Mediterránea es uno de los centros de mayor diversidad y endemicidad tanto de *Silene* como de *Hadena* (Talavera 1990; Greuter et al. 2015; Hacker 1992, 1996, 1999) (Figura 1B). Este hecho la convierte en un espacio predilecto para estudiar este sistema. Sin embargo, prácticamente todos los estudios realizados hasta la fecha se han llevado a

cabo en Norteamérica y en el centro-norte de Europa, y en especial con el par *Silene latifolia-Hadena bicruris*. Mientras tanto, se desconoce cuál o cuáles son los hospedadores de la mayoría de especies del género *Hadena*, formado por 134 especies en la región Paleártica (Troubridge & Crabo 2002), o hasta qué punto son estas interacciones monoespecíficas o genéricas.

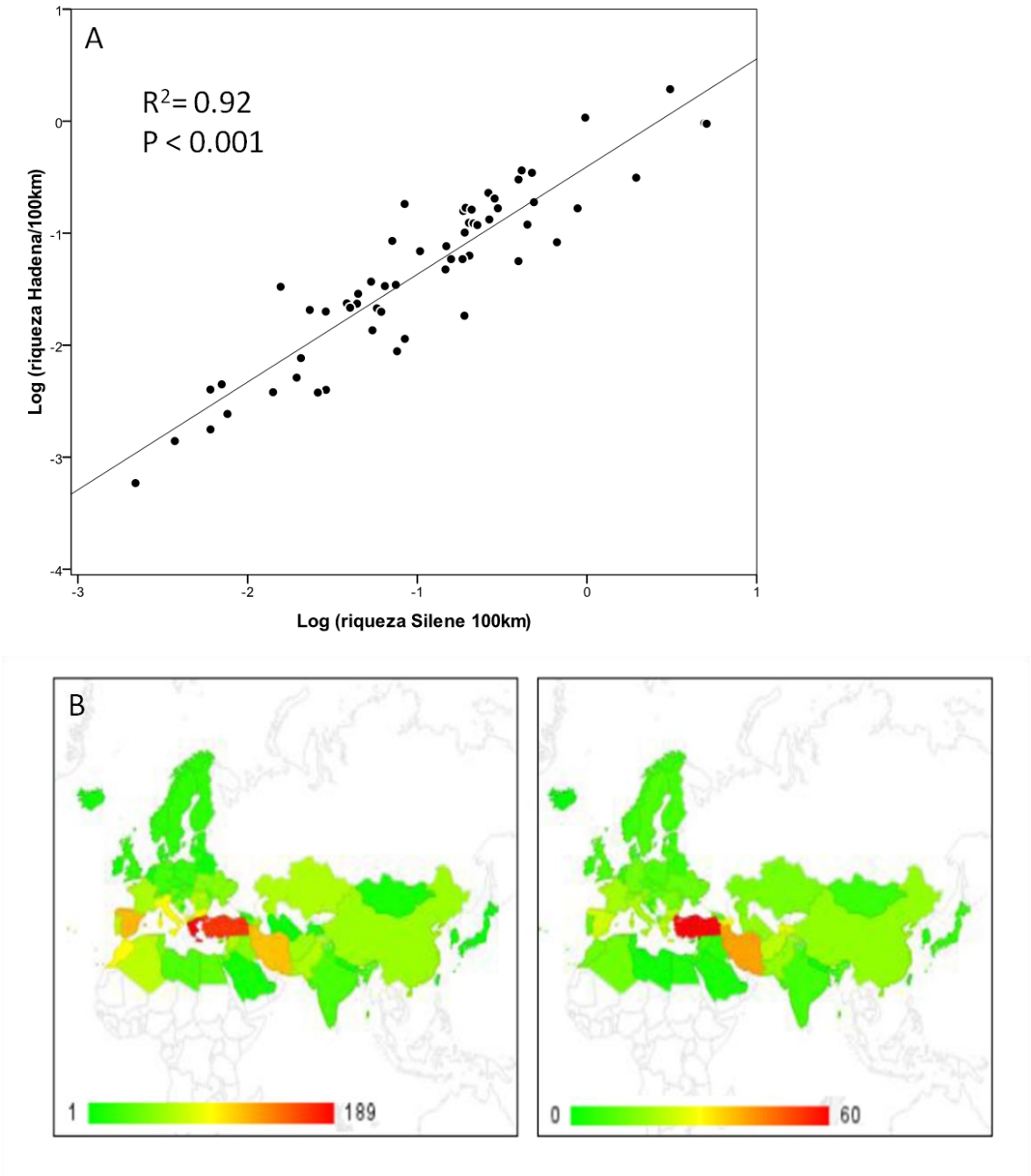

Figura 1. A) Diagrama de dispersión que muestra la alta correlación entre la riqueza específica/100Km^2 de *Silene* y *Hadena* por países del Paleártico. B) Mapas de riqueza específica *Silene* (izquierda) y *Hadena* (derecha) por países. Adaptado de Ocaña 2015.

OBJETIVOS

Los objetivos generales de esta tesis son obtener un mayor conocimiento sobre la variación entre en los caracteres florales que definen los síndromes de polinización en *Silene*, y comprobar cómo se ajusta esta variación en los atributos florales a los distintos visitantes florales, incluyendo a los polinizadores-depredadores del género *Hadena*. Para abordar estos objetivos generales se han desarrollado los siguientes objetivos específicos:

1) Revisar y actualizar el estado de conocimiento sobre la frecuencia y especificidad de los sistemas polinización-depredación entre Caryophyllaceae y *Hadena,* mediante la revisión bibliográfica y la incorporación de nuevos pares de especies localizados en España.
2) Explorar en un amplio grupo de especies de la tribu *Sileneae* si la variación cualitativa y cuantitativa en la producción de esencias florales entre el día y la noche se ajusta a la definición de su síndrome de polinización basada en otros caracteres florales.
3) Averiguar si existe congruencia entre las esencias florales de Silene y la filogenia del grupo, comprobando que compuestos están sujetos a restricciones filogenéticas y cuáles pueden ser consecuencia de fenómenos de convergencia evolutiva, como consecuencia de presiones selectivas similares.
4) Describir la dinámica día-noche de los distintos caracteres florales de una especie, *S. colorata,* cuyos atributos están a caballo entre el síndrome diurno y nocturno. Además, comprobar la coherencia funcional entre estos atributos florales y los polinizadores observados en sus poblaciones naturales.
5) Evaluar si el contexto ecológico en el que se desenvuelve la interacción entre S. *colorata* y su polinizador-depredador *Hadena sancta* influye en el balance neto de la relación, examinando la variación espaciotemporal del efecto de otros polinizadores, depredadores y robadores de néctar sobre el éxito reproductivo de *S. colorata*.

METODOLOGÍA

En cada uno de los capítulos de esta tesis se detalla la metodología específica para abordar cada uno de los objetivos. Aún así esta sección refuerza y resume algunos aspectos metodológicos generales de la tesis.

Revisión de los "nursery pollination systems"

En el **capítulo 1** se hace una revisión del estado de conocimiento de los sistemas polinización-depredación entre especies de la familia Caryophyllaceae y especies del género *Hadena*. Para ello se realizaron búsquedas en distintas fuentes bibliográficas y bases de datos. Además, para ampliar el conocimiento de estas interacciones en la región Mediterránea, se realizaron muestreos en distintos puntos de España. Para ello durante las primaveras de 2010 a 2013 se recolectaron frutos inmaduros de todas las especies de *Silene* y *Dianthus* localizadas. Los frutos se mantuvieron en incubación a temperatura ambiente hasta que las larvas fueron saliendo de las cápsulas primarias. Las larvas se alimentaron hasta la formación de las pupas, y los adultos que emergieron de éstas fueron identificados a nivel de especie. Una vez recopilados los datos se resumieron en una matriz de adyacencia cualitativa y se realizaron análisis de redes para buscar posibles patrones que expliquen las interacciones entre *Hadena*-Caryophyllaceae

Muestreo de esencias florales

En los **capítulos 2, 3 y 4** se estudiaron las esencias florales de *Silene* desde distintas perspectivas**.** Las especies muestreadas en el **capítulo 2 y 3** fueron obtenidas a partir de semillas de bancos de germoplasma y recolecciones propias. Las semillas fueron sembradas y crecieron en las mismas condiciones, dentro del invernadero de la Universidad Rey Juan Carlos. Las plantas de *S. colorata* usadas para la toma de esencias del **capítulo 4** fueron obtenidas a partir de semillas de las poblaciones naturales y crecieron dentro de una zona de exclusión de polinizadores instalada en las proximidades del mismo invernadero (Figura 3). Para la toma de esencias de todas las especies se seleccionaron dos momentos del día, de 11:30 a 13:00 y de 21:00 a 00:00, independientemente del síndrome de polinización que aparentemente presentara cada especie. Para ello, se embolsaron las inflorescencias en bolsas de polietileno sin separarlas de la planta madre, pues los daños en los tejidos vegetales pueden producir

compuestos volátiles defensivos que enmascaren las esencias florales. Se dejó concentrar el olor durante 5 minutos (Figura 2C), pasados los cuales se recogieron las esencias florales con ayuda de una pequeña bomba de vacío que hacía pasar el aire contenido en la bosa por un microvial durante 5 minutos (Figura 2A). El microvial de cuarzo (15 mm de longitud y 2 mm de diámetro interno) iba relleno de dos sustancias adsorbentes (3 gramos de mezcla 1:1 de Tenax-TA y Carbotrap) y taponado con algodón de vidrio para facilitar el flujo de aire. Después de tomar las muestras de esencias, los microviales fueron congelados hasta su análisis mediante cromatógrafía de gases acoplada a espectrometría de masas (GC-MS). Los microviales son directamente insertados en el inyector del cromatógrafo, donde son calentados a 250ºC para desorber los compuestos volátiles atrapados. Mediante un flujo de Helio, los VOCs pasan directamente a la columna cromatográfica sin ningún efecto de dilución, lo cual ocurre con otras técnicas que usan disolventes. En la columna los compuestos volátiles van siendo separados según su tiempo de retención, y pasan a continuación al espectrómetro de masas, donde se mide su relación carga/masa de iones (z/m). Finalmente, para identificar cada compuesto, estos son comparados con una base de datos de tiempos de retención y razones z/m de moléculas patrón (Dötterl et al. 2005).

Caracterización completa del síndrome de polinización

En el **capítulo 4** se estudió la dinámica diaria de varios rasgos florales de *S. colorata,* y si esta variación se ajusta a uno de los síndromes de polinización descritos tradicionalmente en *Silene*. Además de las esencias florales, se analizaron la variación día-noche en la producción de néctar, la dinámica de apertura y cierre de los pétalos y el éxito de la polinización en distintos momentos del día. Las plantas usadas permanecieron dentro una zona de exclusión para polinizadores (Figura 3). Para estudiar la variación de néctar se usaron microcapilares, que extraen todo el volumen de néctar almacenado en la base del cáliz mediante capilaridad. La dinámica de apertura y cierre repetida de los pétalos (nictinastia) se midió con un calibre digital en intervalos de media hora, y mediante la técnica fotográfica *time-lapse* a partir de fotogramas tomados cada 15 minutos. Además, mediante un experimento factorial con dos niveles de riego y de radiación solar, se estudió la influencia de estas variables ambientales sobre la emisión de esencias y la nictinastia. El éxito de la polinización a distintas horas del día (mañana,

tarde y noche) se evaluó mediante polinizaciones manuales. Este experimento se aprovechó también para evaluar el sistema de cruzamiento de *S. colorata* mediante cruzamientos geitonogámicos y xenogámicos a distintos niveles. Finalmente, se comprobó la coherencia funcional entre la dinámica de estos atributos florales y las tasas de visita y el éxito reproductivo de S. *colorata* en tres poblaciones naturales.

Evaluación de las interacciones bióticas que determinan el éxito reproductivo de *S. colorata* a distintas escalas espaciotemporales

En los **capítulos 4 y 5** se seleccionaron tres y diez poblaciones, respectivamente, del par hospedador-huésped *S. colorata-Hadena sancta*. En cada una de ellas se establecieron parcelas de 1x1 m en las que, durante dos años, se realizaron censos diurnos y nocturnos de visitantes florales, se estimó el éxito reproductivo de la planta y se calculó la abundancia del huésped y de otros depredadores de semillas. Los censos diurnos fueron presenciales, los nocturnos mediante cámaras de video de infrarrojos adaptadas a esta función por nosotros (Figura 4A y B). Las cámaras se situaban a la altura de las flores gracias a una estaca (Figura 4C y D) y se mantenían grabando durante toda la noche alimentadas con baterías de 12 V. (Figura 4D). Al final del periodo reproductivo, se recolectaron diez plantas por parcela, en las que se registraron la cantidad de flores y frutos totales producidos, así como la cantidad de flores atacadas por robadores de néctar y flores y frutos depredados.

Análisis estadísticos

En los diferentes capítulos se describen de forma detallada las técnicas estadísticas empleadas. Este apartado incluye sólo una breve descripción.

En el **capítulo 1** se utilizaron técnicas de análisis de redes bipartitas para describir la matriz de interacciones hospedador-huésped entre las distintas especies de *Hadena* y Caryophyllaceae y se determinó la modularidad de la red.

Para el análisis cualitativo y cuantitativo de las esencias usamos diversas técnicas multivariantes. En los **capítulos 2 y 4** usamos PERMANOVA y NMDS (*non-metric multidimensional scaling*), mientras que el **capítulo 3** usamos pPCA ("*phylogenetic principal components analysis*") para ver la congruencia entre las esencias y la filogenia. Para realizar el pPCA, se construyeron dos árboles filogenéticos, uno para las especies de

la tribu Sileneae de las que se disponían datos de esencias emitidas durante la noche y otro para las especies que se tenían datos de día. Las secuencias genéticas (ITS y rps16) para la construcción de los árboles filogenéticos fueron obtenidas del GenBank, o bien fueron obtenidas de nuestros ejemplares. Los árboles fueron construidos mediante los métodos de máxima verosimilitud e inferencia bayesiana. Además en este capítulo se realizaron reconstrucciones de estado ancestral de los principales compuestos volátiles presentes en *Silene*.

En el **capítulo 4** la dinámica día-noche de los distintos caracteres florales fueron analizados empleando modelos lineales simples (LM) o generalizados (GLM). En el **capítulo 5** también se usaron LMs y GLMs para analizar la relación de *Hadena sancta*, los demás polinizadores, depredadores de frutos y robadores de néctar con el éxito reproductivo de *S. colorata,* y para estimar su variación espaciotemporal. En este capítulo también se utilizaron modelos lineales mixtos (LMM) para construir un modelo d-sep de gráficos causales.

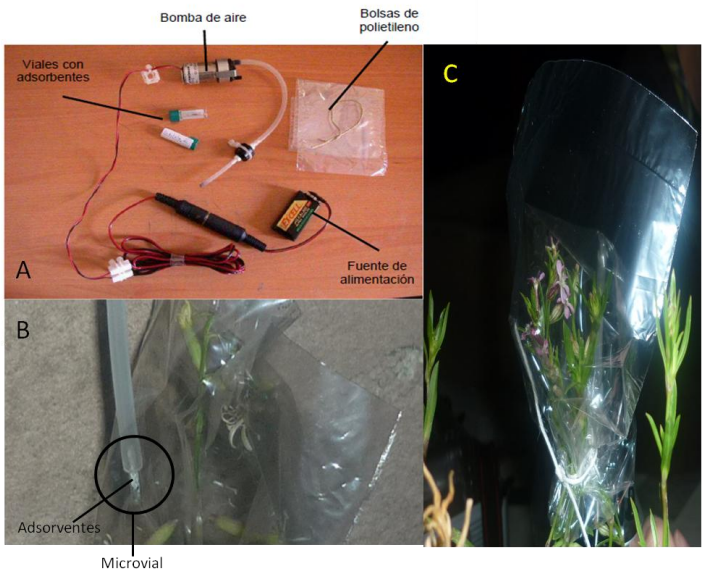

Figura 2. A, Material utilizado para la extracción de las esencias florales. B, microbial insertado en la bolsa. C, inflorescencia de *S. giraldii* embolsada.

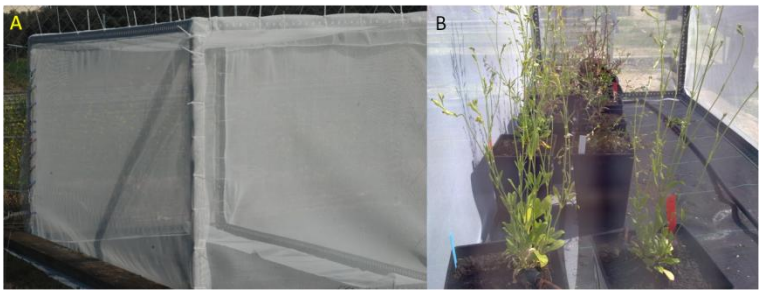

Figura 3. A, Zona de exclusión de polinizadores. B, interior de la estructura.

Figura 4. A y B, cámaras de video adaptadas, con una carcasa impermeable y conexión a batería. C y D, montaje de las cámaras grabando *S. ciliata* (C), con la ayuda de un ordenador portátil (encuadre), las estacas (fijación) y la batería (alimentación).

CONCLUSIONES

De los cinco capítulos en los que se ha estructurado esta tesis se pueden extraer las siguientes conclusiones generales.

1) Debido a su elevada diversidad específica, la región mediterránea y en concreto España alberga potencialmente una amplia diversidad de pares hospedador-huésped entre Caryophyllaceae y *Hadena*. La falta de especificidad de estas interacciones puede explicarse en gran medida por la distribución geográfica y el hábitat de las especies. Las especies generalistas comparten una distribución geográfica extensa o hábitats como alta montaña o pastos. También se dan casos de mayor especificidad como es el ejemplo *Dianthus-Hadena compta*, en que las especies de *Dianthus* interacciona casi exclusivamente con esta especie de polilla.

2) Tal como esperábamos, la mayoría de las especies de la tribu Sileneae emiten compuestos volátiles potencialmente atrayentes para polinizadores tanto de día como de noche, aunque con grandes diferencias cualitativas y cuantitativas entre especies y momentos del día.

3) En muchos casos, la variación día-noche en la producción de esencias no se correspondió con el resto de atributos florales que tradicionalmente definen los síndromes de polinización, en especial con el color de la corola. Esto podría explicar por qué los supuestos síndromes de muchas especies de *Silene* no predicen los gremios de polinizadores que realmente presentan en condiciones naturales, y resalta que los síndromes de polinización no son compartimentos estrictos que favorezcan la atracción de ciertos polinizadores excluyendo a los otros.

4) Un muestreo de esencias sesgado por una definición de síndrome basado sólo en algunos caracteres puede producir perdida de información esencial para comprender la comunicación planta-animal y, por lo tanto, la polinización de una especie.

5) Aunque se daba por hecho que las esencias florales eran evolutivamente demasiado lábiles para la inferencia filogenética, usando datos semicuantitativos es posible encontrar señal filogenética para algunos de los compuestos presentes en ellas.

6) La señal filogenética en las esencias de la tribu Silenae está principalmente marcada por la contraposición de dos monoterpenos, E-β-ocimeno y linalool. Parte de la composición de las esencias de Silenae se debe a restricciones filogenéticas, mientras que otros compuestos han surgido recientemente en Sileneae y producen diferencias en la composición de las esencias entre taxones relacionados.

7) En *S. colorata*, la dinámica de algunos caracteres florales (nictinastia, producción de néctar y esencias florales) está fuertemente controlada por el ritmo circadiano diario impuesto por los ciclos de luz y oscuridad, y definen un síndrome predominantemente nocturno. Sin embargo, durante la mañana las flores de *S. colorata* pueden seguir siendo atractivas a los polinizadores más o menos tiempo dependiendo de otras condiciones ambientales (disponibilidad de agua e intensidad de la radiación solar). Además, los cruzamientos manuales demostraron que la polinización por la mañana es tan efectiva como por la noche, y mayor que por la tarde. Todo ello, amplía el periodo de mayor atracción y receptividad para la polinización de *S. colorata* más allá de la noche. Esto encaja con la presencia de polinizadores tanto diurnos como nocturnos en sus poblaciones naturales y puede ser una alternativa para asegurar el éxito reproductivo frente a una baja disponibilidad de polinizadores nocturnos.

8) La tasa de visitantes florales diurnos y nocturnos de *S. colorata* varía notablemente entre poblaciones y años, así como la de su polinizador-depredador *H. sancta* y la de otros insectos depredadores de semillas y robadores de néctar. Esta variación tanto geográfica como temporal podría condicionar la intensidad y dirección de las presiones selectivas ejercidas sobre los caracteres florales de *S. colorata*, tal como predice la Teoría del Mosaico Geográfico de Coevolución.

9) Existe variación entre años y poblaciones en el efecto que los polinizadores diurnos y nocturnos tienen sobre el éxito reproductivo de *S. colorata*. Además, el efecto neto de la abundancia de *H. sancta* sobre el éxito reproductivo de *S. colorata* varió entre las poblaciones y años debido a la presencia de los demás agentes biológicos que interaccionan con ellas. El análisis en global sugiere que esta relación hospedador-huésped está fuertemente desviada al parasitismo.

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CAPÍTULO 1/CHAPTER 1

Revisión y actualización del estado de conocimiento de las relaciones polinización-depredación entre Caryophyllaceae y *Hadena* (Noctuidae).

RESUMEN

Los sistemas de polinización-depredación son comunes entre las especies de *Hadena* (Noctuidae) y Caryophyllaceae. La revisión anterior de este sistema está basada en datos principalmente del norte y centro de Europa, de especies hospedadores y huéspedes fundamentalmente de distribución muy amplia, no existiendo información de la región Mediterránea, siendo ésta uno de los centros de diversificación mundial de ambos grupos taxonómicos. El objetivo de este trabajo es revisar y actualizar el conocimiento general sobre las interacciones entre Caryophyllaceae y *Hadena*, aportando datos inéditos obtenidos en uno de los centros de diversificación de los dos taxones (España), y buscando patrones que puedan explicar el grado de generalismo de este sistema. En la revisión encontramos descritas interacciones entre 25 especies de noctuidos con 11 géneros 76 especies de cariofiláceas. De todas estás interacciones, 26 fueron nuevas en España, de los cuales 18 no se habían descrito antes. La red Caryophyllaceae-*Hadena* fue modular. En estos módulos se observa una relación muy estrecha entre *H. compta* y el género *Dianthus*. En el resto de módulos, sin haber tanta relación con la taxonomía del hospedador, las interacciones entre Caryophyllaceae-*Hadena* se explican por la distribución o el hábitat de las especies.

INTRODUCCIÓN

Los sistemas polinización-depredación, "*nursery pollination systems*", son aquellos en los que el polinizador alimenta a su progenie con las propias estructuras reproductivas de la planta a la que poliniza. Estos sistemas pueden ser obligados, como el establecido entre las yucas y las polillas de las yucas (Pellmyr 2003) o facultativos, debido a la presencia de otros polinizadores. Estos sistemas facultativos son interesantes para el estudio de la evolución y mantenimiento de los sistemas mutualistas porque están a caballo entre el mutualismo y el parasitismo (Dufaÿ & Anstett 2003). En una revisión realizada por Kephart y colaboradores en 2006 se mostró que los sistemas de polinización-depredación son comunes entre las especies de *Hadena* (Noctuidae) y ciertas Caryophyllaceae, especialmente *Silene*. El sistema *Silene-Hadena* está caracterizado por un cierto grado de especialización, ya que *Hadena* es muy específica tróficamente de *Silene* y géneros cercanos. Sin embargo, todas las especies de *Silene* estudiadas hasta la fecha tienen otros polinizadores además de *Hadena* (Petterson 1991; Giménez-Benavides et at. 2007; Reynolds et al. 2012). La revisión de este sistema encontró un total de 58 interacciones entre 14 especies de *Hadena* y 26 especies de cariofiláceas (14 de ellas *Silene*). El origen de estos datos es principalmente el norte y centro de Europa, y puntualmente algún caso aislado en Norteamérica, y comprende fundamentalmente hospedadores y huéspedes de distribución muy amplia, como *Silene latifolia*, *S. dioica*, *S. vulgaris*, *H. bicruris* y *H. perplexa*. Sin embargo, hasta la fecha no existía información del sistema Caryophyllaceae-*Hadena* en la región Mediterránea, siendo ésta uno de los centros de diversificación mundial de ambos grupos taxonómicos.

El género *Silene* cuenta en la Región Mediterránea con 504 especies, 372 de las cuales son endémicas. Sólo España cuenta con 104 especies (y subespecies), de las cuales 15 son endémicas (Talavera 1990; Greuter et al. 2015). El género *Hadena* pertenece a la familia Noctuidae, posiblemente la familia más diversificada de lepidópteros con aproximadamente 20.000 especies. *Hadena* posee una distribución Holártica con, aproximadamente 134 especies solo en la región Paleártica (Troubridge & Crabo 2002). En España *Hadena* cuenta con 24 especies, 5 de ellas endémicas (Hacker 1992, 1996, 1999; J. L. Yela, datos no publicados). Estos números pueden darnos una idea aproximada de la enorme diversidad de interacciones específicas totalmente desconocidas que pueden existir en la región, y del importante escenario coevolutivo

que puede subyacer. Aunque existen numerosos estudios que han explorado la interacción entre algunas especies particulares de *Silene* y de *Hadena* (Pettersson 1991; Elzinga et al. 2007; Giménez-Benavides et al. 2007; Labouche & Bernasconi 2010; Reynolds et al. 2012; revisado en Kephart et al. 2006), ninguno que ha estudiado este sistema a una escala taxonómica y geográfica amplia.

Al contrario que en los sistemas polinización-depredación obligados más conocidos (Yucca-Tegeticula, Ficus-Agaonidae, Phyllanteae-*Epicephala*) las interacciones entre *Hadena* y Caryophyllacea en general parecen poco especificas. Hay especies de cariofiláceas que interactúan con varias especies de *Hadena* y viceversa, una especie de *Hadena* interactuando con varias especies de plantas (Kephart et al. 2006). Este patrón parce alejarse mucho de aquellos sistemas coevolutivos estrechos en los que la relación entre ambos linajes es muy cercana y recíproca, pudiendo llegar a la coespeciación (de Vienne et al. 2013; Althoff et al. 2014). Sin embargo, los sistemas coevolutivos altamente específicos son en realidad una excepción a la norma. Nos muestran el grado máximo al que puede llegar la coevolución en casos muy extremos como algún hospedador-parásito, pero sin mostrarnos todas las situaciones de transición. Se ha sugerido que los casos en los que las interacciones son generalistas entre especies de *Hadena* y Caryophyllacea podrían deberse a que las especies con las que interactúa uno de los socios están muy emparentadas filogenéticamente (Kephart et al. 2006). Por otro lado, también la distribución geográfica de los socios podría explicar la especificidad o generalismo de las interacciones Caryophyllaceae-*Hadena* (Westerbergh 2004; Kephart et al. 2006).

La Teoría del Mosaico Geográfico de Coevolución propone una explicación más realista y menos idealizada del proceso coevolutivo que puede ayudarnos a entender este tipo de situaciones transicionales (Thompson 2005). Debido a las variaciones locales que poseen las poblaciones y las especies, la dirección e intensidad de las interacciones también pueden variar, generando sólo en algunas situaciones verdaderas fuerzas selectivas (los denominados coevolutionary hotspots, Thompson 1997). Además, la coevolución no tiene por qué darse estrictamente especie a especie, sino de una manera menos específica o incluso entre múltiples especies al mismo tiempo, en función del contexto ecológico de las poblaciones. A pesar de su laxitud, todo este mosaico geográfico de interacciones difusas puede contribuir también a la coespeciación (Thompson 2005).

Nuestra hipótesis es que las cariofiláceas y *Hadena* presentan un sistema de polinización-depredación coevolutivamente difuso, fuertemente influenciado por las relaciones de parentesco entre las especies, su rango geográfico y el contexto ecológico de las poblaciones. Desgraciadamente no existen filogenias bien resueltas para ninguno de los dos grupos taxonómicos, especialmente para *Hadena*, por lo que no es posible a día de hoy hacer análisis robustos de reconciliación filogenética. Sin embargo, si existen fuerzas coevolutivas difusas que modulen en cierta forma la diversificación de ambos grupos, cabría esperar cierta congruencia biogeográfica. Es decir, que compartan áreas de máxima riqueza y un rango de distribución similar.

El objetivo de este trabajo es revisar y actualizar el conocimiento general sobre las interacciones polinización-depredación entre Caryophyllaceae y *Hadena*, aportando datos inéditos obtenidos en uno de los centros de diversificación de los dos taxones, y buscando patrones que puedan explicar el grado de generalismo de este sistema. Para ello, llevamos a cabo una revisión bibliográfica de todos los pares de especies que se tiene constancia interaccionan, a la que incorporamos nuestros propios datos procedentes de muestreos en España. Con los datos actualizados se construyó una matriz bipartita de interacciones, que analizamos con técnicas de análisis de redes para describirla y extraer conclusiones relativas a la taxonomía, hábitat y distribución de las especies.

MATERIAL Y MÉTODOS

Revisión de las interacciones Hadena-Caryophyllaceae

Los adultos de *Hadena* visitan las flores de las cariofiláceas, polinizándolas, antes de que la hembra ponga los huevos sobre o el interior del cáliz en alguna de las flores visitadas para que la larva se alimente de los frutos en desarrollo (Brantjes 1976a; Bopp 2003; Giménez-Benavides et al. 2007; Reynols et al. 2012). Por esta razón se tomaron como buenos registros: 1) aquellos que daban cuenta de todo el proceso de polinización y depredación, y 2) las referencias que constataban sólo la fase de depredación, asumiendo que los progenitores habían polinizado previamente. Esta asunción es conservadora puesto que el tamaño de las *Hadena* en relación a la flor, y la manera de posarse para libar el néctar y/o ovopositar (sobre o en el interior del cáliz), hacen

prácticamente imposible que la polilla no toque los órganos sexuales. Las referencias y bases de datos utilizadas en esta revisión bibliográfica se encuentran en el Material suplementario 1.

Búsqueda de sistemas Caryophyllaceae-*Hadena* en España

La observación directa de visitas de *Hadena* a cariofiláceas resulta muy poco productiva para un estudio extensivo, pues la tasa de visitas nocturna es muy baja y la visibilidad reducida. Por ello, en la mayoría de los casos utilizamos sólo la fase de depredación como evidencia de la existencia de relación *nursery pollination*. Durante las primaveras de los años 2010 a 2012, se recogieron frutos de 20 especies de *Silene* y 1 especie de *Dianthus*. En la Tabla 1 se resumen las especies y poblaciones de origen. Los frutos recogidos en campo se metieron en bolsas de plástico con autocierre y se conservaron en un lugar fresco y sombrío hasta llegar al laboratorio. Una vez allí, los frutos se extendieron en recipientes de plástico y según emergían las larvas de los frutos, éstas fueron separadas en placas Petri para evitar el canibalismo (Brantjes 1976b; Peschken & Derby 1990; Elzinga et al. 2002). Puesto que la identificación de las larvas a nivel de especie es a menudo muy complicada, éstas fueron alimentadas con más frutos inmaduros hasta completar todas las fases larvarias (alimentación *ad libitum*). Las placas Petri se cubrieron de una fina capa de vermiculita para facilitar la formación de las pupas, pues las larvas de *Hadena* se entierran bajo las plantas nutricias. Algunos ensayos previos nos mostraron que la mayoría de especies (menos *H. bicruris*) son univoltinas y necesitan una diapausa en condiciones invernales para completar su desarrollo, por lo que las pupas se introdujeron en una cámara de incubación (Selecta Hotcold GL, Barcelona, España) a 15/10 ºC de temperatura y 12 horas de luz/12 horas de oscuridad de fotoperiodo durante 5 meses. Al inicio de la primavera siguiente los adultos emergidos se utilizaron para identificar las especies (Figura 1). Los nuevos pares de especies identificados se incorporaron a la matriz de interacciones procedente de la revisión bibliográfica.

Tabla 1. Localidades en las que se recogieron frutos verdes de *Silene* y *Dianthus* para buscar posibles "nursery pollination".

Provincia	**Localidad**	***Silene***
Asturias	Picos de Europa	*S. cialata, S. vugaris*
Badajoz	Herrera del Duque	*S. colorata, S. gallica*
	Zalamea de la Serena	*S. colorata*
Cáceres	Monfragüe	*S. colorata*
	Toril	*S. colorata*
Cádiz	Barbate	*S. littorea*
	Faro de Trafalgar	*S. littorea*
	Puerto Real	*S. vulgaris*
	Tejeda	*S. psammitis*
Cuenca	Belinchon	*S. colorata*
Granada	Sierra Nevada	*S. boryi*
Huelva	El Portil	*S. nicaensis*
	Punta Umbria	*S. ramosissima*
Lugo	San Pedro	*S. uniflora*
Madrid	Arganda	*S. colorata, S. conica, S.nocturna, S.vulgaris*
	Arroyomolinos	*S. colorata*
	Berzosa	*S. portensis, S. scabriflora*
	Brunete	*S. colorata, S. gallica, S. nocturna*
	Carabaña	*S. colorata*
	Colmenar del Arroyo	*S. colorata*
	El Nevero	*S. boryi*
	Embalse de Valmallor	*S. colorata*
	Gargantilla del Lozoya	*S. portensis*
	Hoyo de Manzanares	*S. colorata, S. conica, S. latifolia, S. nocturna, S. vulgaris*
	Morata de Tajuña	*S. vulgaris*
	Móstoles	*S. colorata, S. conica*
	Navacerrada	*S. nutans*
	Navalmedio	*S. portensis, D. lusitanus*
	Navas del rey	*S. colorata, S. mellifera, S. scabriflora*
	Peñalara	*S. boryi, S. cialata, D. lusitanus*
	Peralejo	*S.conica, S. gallica*
	Rascafria	*S. legionensis*
	San Agustin de Guadalix	*S. psammitis*
	Silla Felipe II	*S. nutans*
	Tielmes	*S.colorata, S.conica*
	Torrelodones	*S.latifolia, S. vulgaris*
	Valdemorillo	*S. colorata, S. gallica, S. scabriflora*
	Villaviciosa de Odón	*S.latifolia, S. colortata, S. mellifera*
	Zarzalejo	*S.gallica, S. nocturna*
Segovia	Segovia	*S. pendula*
Toledo	Illescas	*S. colorata*

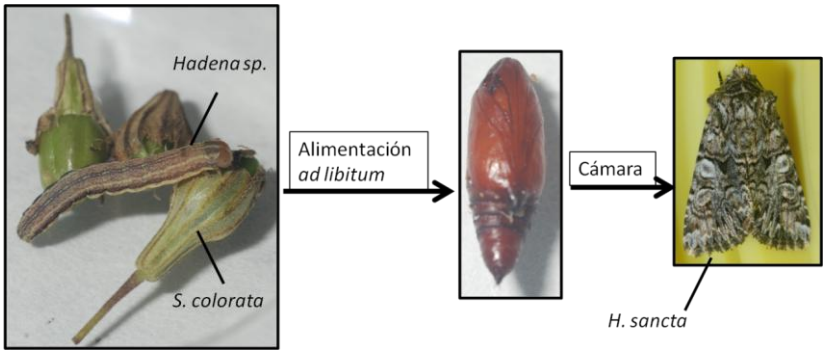

Figura 1. Esquema del proceso de identificación de una interacción *Silene-Hadena*.

Análisis de la red de interacciones

Para explorar las características del sistema *Hadena*-Caryophyllaceae, la matriz de interacciones fue analizada con técnicas de análisis de redes bipartitas. Mediante los paquete *vegan* y *bipartite* de R (Dormann et al. 2009; Oksanen et al. 2015) se construyó el grafo de la red y se calculó la modularidad de la red. Una red es modular cuando puede ser subdividida en grupos de especies (módulos) que comparten más interacciones con los miembros de un grupo y menos con los miembros de otros grupos que las esperadas por azar (Guierà et al. 2007). Para calcular la modularidad se simularon 100 redes compuestas de subredes aleatorias que tenían las mismas dimensiones, conectancia y totales marginales que la matriz original (Dormann et al. 2009; Oksanen et al. 2015). El modelo nulo se halló usando el método quasi-swap por tratase de datos de presencia-ausencia (Miklós & Podani 2004). La moduralidad se consideró significativa si el valor de *z-scores* fue mayor de 2 (Dormann et al. 2014).

RESULTADOS

En total, encontramos descritas interacciones entre 25 especies de noctuidos de la tribu Hadenini (24 de *Hadena* y 1 de *Aneda*) con 11 géneros (*Agrostema, Atocion, Dianthus, Gypsophila, Heliosperma, Lychnis, Petrorhagia, Saponaria, Silene, Spergularia, Viscaria*) y 76 especies de cariofiláceas, siendo *Silene* y *Dianthus* los géneros en los que más especies muestran al menos una interacción (40 y 18, respectivamente) y más número de interacciones suman (106 y 32, respectivamente) (Material suplementario 1). De todas estás interacciones el trabajo de campo nos ha permitido encontrar 26 nuevas interacciones hospedador-huésped en España, de los cuales 18 no se habían descrito antes (Tabla 2).

La red de interacciones muestra que en general entre estos dos grupos hay poca especifidad (Figura 2). Sin embargo, hay patrones muy destacables como que *H. compta* es huésped principalmente en especies del genero *Dianthus,* al contrario que el resto del género, que está más especializado en *Silene*. El resto de relaciones entre especies de Caryophyllaceae y *Hadena* no parecen seguir un patrón taxonómico claro.

La red Caryophyllaceae-*Hadena* fue significativamente modular (Modularidad = 0,25; *z-score* = 4.1), detectándose 11 módulos (Figura 3). En los módulos se asociaron varias especies de cariofiláceas con varias especies de *Hadena* (módulos 1, 3, 5, 6 y 7), o una especie de *Hadena* asociada a una o más especies de cariofiláceas (módulos 2, 4, 8, 9, 10 y 11) (Figura 3).

Tabla 2. *Silene-Hadena* "Nursery pollination systems" encontrados en España.

	S. vulgaris	*S. nutans*	*S. latifolia*	*S. mellifera*	*S. colorata*	*S. littorea*	*S. gallica*	*S. nocturna*	*S. portensis*	*S. psammitis*	*S. boryi*	*S. ciliata*	*S. stockenii*	*S. conica*	*S. scabriflora*	*S. legionensis*	*S. niceensis*
H. bicruris	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H. compta	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1*
H. confusa	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H. albimacula	-	-	-	1*	-	-	-	-	1*	-	-	-	-	-	-	1*	-
H. perplexa	1	-	-	1*	1*	-	-	-	-	1*	-	-	-	-	-	-	-
H. sancta	1*	-	-	-	1*	1*	1*	1*	-	-	-	-	1	1*	1*	-	-
H. consparcatoides	-	-	-	-	-	-	-	-	-	-	1*	1	-	-	-	-	-
H. clara	-	-	-	-	-	-	-	-	-	-	1*	-	-	-	-	-	-
H. wehrlii	1*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H. plebeja	-	-	-	-	-	-	-	-	-	-	1*	-	-	-	-	-	-

* Son "nursery pollination systems" inéditos.

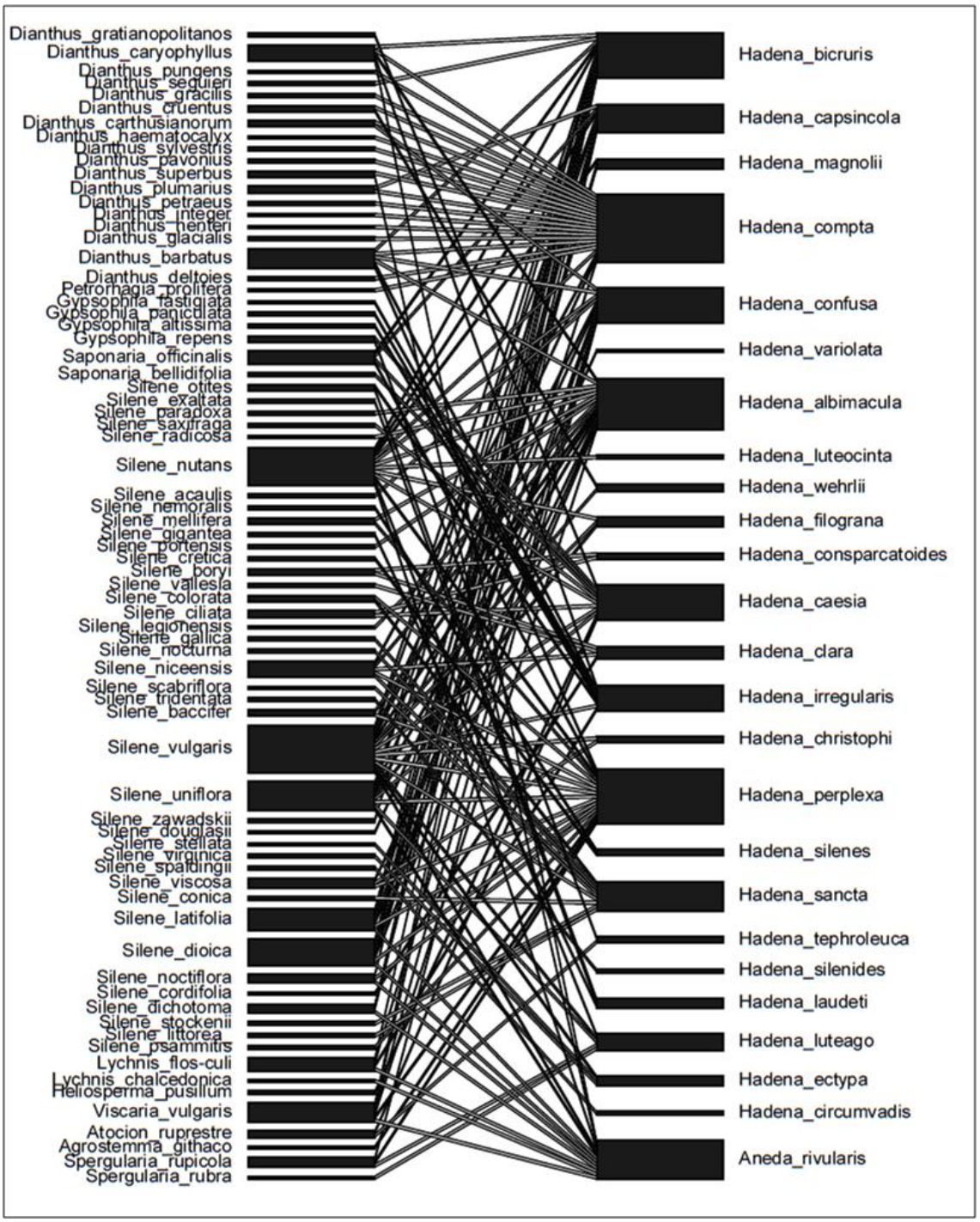

Figura 2. Red de “nursery pollination” Noctuidae-Caryophyllaceae. Las especies estas ordenadas siguiendo la taxonomía de los dos grupos.

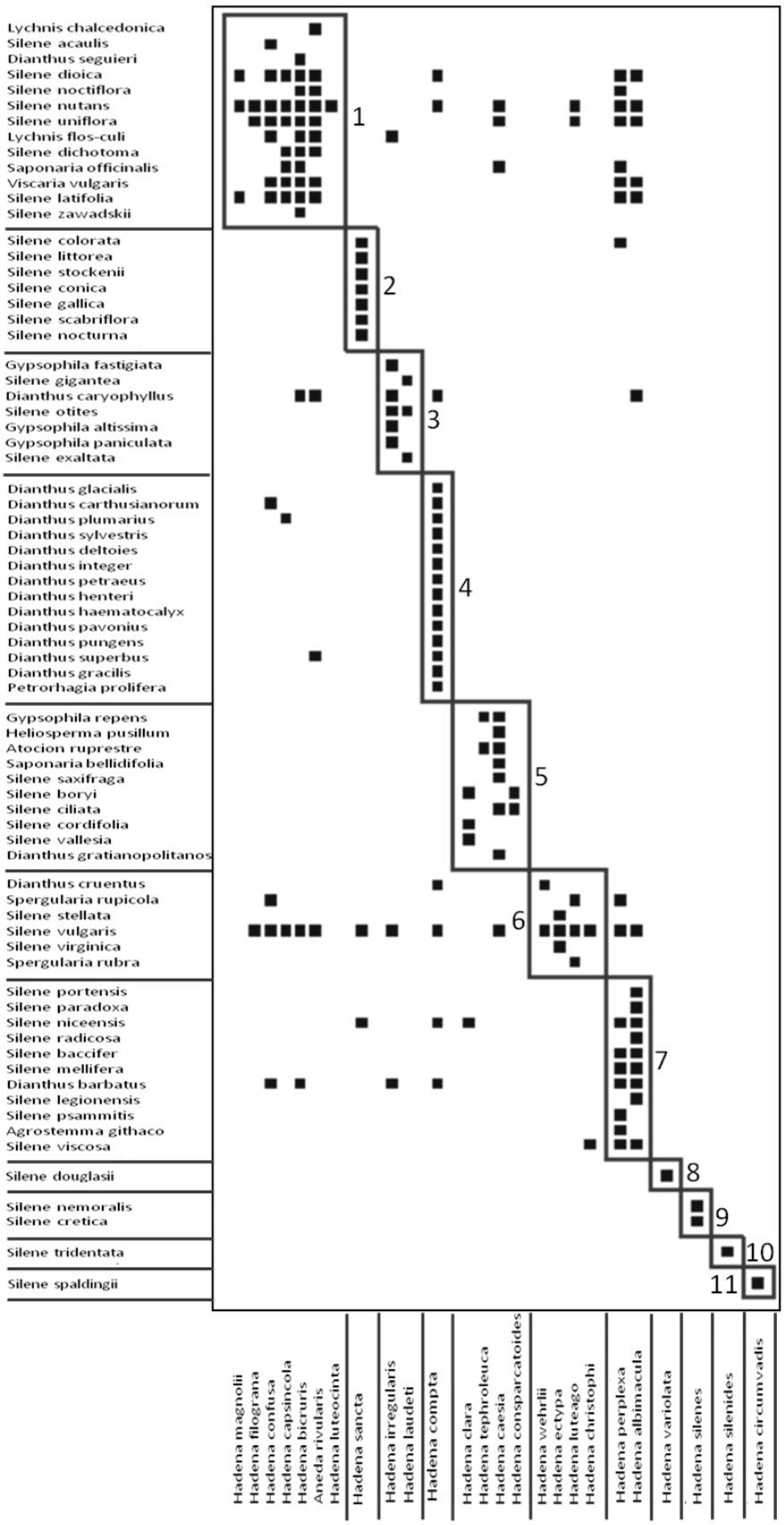

Figura 3. Clasificación de las especies de Caryophyllaceae y Noctuidae en distintos módulos de sistemas polinización-depredación. En uno de los lados de cada módulo está el número que el identifica.

DISCUSIÓN

La especie del genero *Aneda* (Noctuidae), *A. rivularis*, se ha descrito previamente como parte de las interacciones Hadena-Caryophyllaceas como *Hadena rivularis* (Kephart et al. 2006) y por eso se incluye en esta revisión. La revisión de los sistemas de polinización-depredación llevada a cabo a aumentado el número de especies de *Hadena* en 11 y de cariofiláceas en 50 con respecto a la revisión anterior de este sistema (Kephart et al. 2006), incrementando el número de interacciones que se conocían entre estos dos taxones en gran medida. Aun así, el estudio de las interacciones polinización-depredación entre Caryophyllaceae y Hadena está muy lejos de ser definitivo. El muestreo realizado en España, detectó contactos entre 21 especies de cariofiláceas y 10 de *Hadena*, siendo el mayor esfuerzo de muestreo en el centro de la península y de manera no sistemática, pues muchas especies de potenciales huéspedes no fueron muestreadas. Esto sugiere que probablemente quedan aun muchos pares hospedador-huésped por descubrir en la península ibérica. Más aun, otros enclaves de la cuenca mediterránea como Turquía albergan una diversidad y endemicidad de *Hadena* (60 especies, 12 endémicas) y cariofiláceas (181 especies, 31 endémicas) mucho mayor que la Península Ibérica (Greuter et al. 2015; José Luis Yela, datos no publicados) lo que sugiere que podrían albergar muchas más interacciones específicas. De todas formas, los datos aportados por esta revisión nos ayudan a entender la frecuencia de este sistema y su grado de especificidad a grandes rasgos.

En la red de interacciones se muestra que la muchas especies de noctuidos y cariofiláceas son generalistas y no sólo a nivel de género, pues las interacciones de algunas especies de *Hadena* suele abarcar más de un género de cariofiláceas. Sin embargo sí que se observa una relación más estrecha entre *H. compta* y el género *Dianthus*, que sugiere un salto de hospedador. Esta relación queda soportada estadísticamente por el análisis de modularidad, que genera un sólo módulo para *H. compta* y *Dianthus*. El resto de módulos, sin tener tanta relación con la taxonomía del hospedador, podrían explicarse por la distribución o el hábitat de las especies. Por ejemplo, el módulo 1 se compone de especies de amplia distribución, tanto huéspedes como *H. confusa*, *H. bicruris* o *H. magnolii*, como hospedadoras como *S. vulgaris*, *S. latofolia*, *S. nutans* o *Saponaria officinalis*. Algunas de estas plantas son cosmopolitas

debido a su carácter arvense, colonizando bordes de cultivos y zonas alteradas (Lainz & Garmendia 1990; José Luis Yela, datos no publicados; rbg-web2.rbge.org.uk/FE/fe.html). Sin embargo, el modulo 6 también tiene especies de cariofiláceas de amplia distribución pero asociadas a especies de *Hadena* con distribuciones más pequeñas, que sólo coinciden en algunos lugares de la distribución de sus respectivas cariofiláceas (Walters 1996; José Luis Yela, datos no publicados). En general, se puede decir que las especies de Caryophyllaceae de más amplio rango de distribución son capaces de establecer asociaciones con un mayor número de especies de Hadena, y viceversa, debido al solapamiento total o parcial de sus respectivas áreas. Las especies del módulo 2 comparten una distribución principalmente mediterránea (Walters 1996; Lainz & Garmendia 1990; José Luis Yela, datos no publicados). También hay módulos más específicos y geográficamente muy localizados, como el 8 y 11 que son de especies de distribución exclusivamente Neártica (Rabeler & Hartman 2005, http://pnwmoths.biol.wwu.edu/), el módulo 9 con especies del centro y sur de Europa y Asia (Lainz & Garmendia 1990; José Luis Yela, datos no publicados) y el módulo 10 del sur de Europa y norte de África (Lainz & Garmendia 1990; José Luis Yela, datos no publicados). Por otro lado, el modulo 5 está compuesto por especies propias de zonas de alta montaña, tanto huéspedes como hospedadores (Lainz & Garmendia 1990; Wagner 2015), mientras que el módulo 3 está formado por especies que viven en suelos arenosos o rocosos (Lainz & Garmendia 1990; Lu et al. 2004; Wagner 2015), y el módulo 7 por especies propias de zonas abiertas como pastos o zonas rocosas (Lainz & Garmendia 1990; Wagner 2015)

Con estos resultados preliminares, basados en una red de interacciones manifiestamente incompleta, tan sólo queremos mostrar el potencial de este sistema como modelo de estudio de las relaciones coevolutivas planta-animal a una escala biogeográfica amplia. Una vez de disponga de las dos filogenias bien resueltas, futuros análisis cofilogenéticos combinados con el estudio de la distribución geográfica de las especies nos permitirán profundizar un poco más en el origen y evolución de este sistema de interacciones.

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MATERIAL SUPLEMENTARIO 1. Matriz de interaccioes *Hadena-Caryophyllaceae*

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Silene vulgaris	2, 3, 4, 5, 19, 10, 10	3		3, 4, 5, 19,10	3, 4, 5, 19, 10,10		3, 4, 5, 19, 10	3, 19	3, 4, 5, 19	3, 4, 5, 6, 1, 19, 10	3	3	11		4, 5, 19, 10	4, 5, 10, 19			10						10	
Silene nutans	3, 4, 5, 19, 10, 10	3	3	3, 4, 19, 10	3, 4, 5, 19, 10		3, 4, 5, 8, 19, 10	3	3	3, 4, 5, 1, 4, 19, 10					19, 10	5							10			
Silene latifolia	2, 3, 5, 6, 4, 19, 10,10	3	10		3		3, 5			3, 4, 5, 1, 6, 19, 29						5, 4, 10, 19										
Silene dioica	2, 3, 4, 5, 19, 10	3		3	3, 5		3, 5			3, 4, 5, 19, 10						5, 19										
Silene noctiflora	3, 5									3, 5						5										
Silene uniflora	5,3	3			5, 3,19		5, 3, 19	19	5, 3, 19	5, 3, 10					5, 19	5, 10, 19										

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Silene zawadskii	3																									
Silene dichotoma	4, 19, 10	3														4, 10, 19										
Silene viscosa							3			3, 4, 10		3														
Silene otites											3, 4, 5, 19			11												
Silene stellata													4, 19													
Silene acaulis					5																					

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Silene paradoxa							9																			
Silene radicosa							9																			
Silene saxifraga									11																	
Silene spaldingii																						10, 19				
Silene cordifolia																					11					
Silene vallesia																					11					

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Silene exaltata														11												
Silene gigantea														11												
Silene nemoralis																	11									
Silene cretica																	10									
Silene tridentata																								19		
Sielne douglasii						19																				

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Silene virginica													19													
Silene baccifer							3			3, 10																
Silene mellifera							6			6																
Silene colorata										6									6							
Silene littorea																			7, 6							
Silene gallica																			6							

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Silene nocturna																			6							
Silene portensis							6																			
Silene psammitis										6																
Silene boryi																				8	6					25
Silene ciliata									11											8						
Silene stockenii																			8, 19							

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Silene conica																			6							
Silene scabriflora																			6							
Silene legionensis							6																			
Silene niceensis				29			2			1									5		1					
Agrostemma githaco										10, 3																
Spergularia rupicola					5					3, 5					4, 5											

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Spergularia rubra															5											
Saponaria officinalis	2, 3	3							4, 19	3, 19																
Saponaria bellidifolia									11																	
Dianthus barbatus	3, 4, 5, 19			3, 4, 5, 19, 10	3, 5		3, 5			3, 5	3															
Dianthus carthusianorum				3, 19	3																					
Dianthus caryophyllus	4, 19			3, 4, 19, 10			3, 5				3					4, 19										

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Dianthus deltoides				3, 4, 19, 10																						
Dianthus glacialis				3																						
Dianthus gracilis				3																						
Dianthus henteri				3																						
Dianthus pavonius				3																						
Dianthus petraeus				3																						

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Dianthus seguieri	3																									
Dianthus plumarius		3		3, 4, 19, 10																						
Dianthus pungens				3																						
Dianthus superbus				3												4, 10, 19										
Dianthus sylvestris				10, 19																						
Dianthus cruentus				10																					25	

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Dianthus integer				10																						
Dianthus haematocalyx				10																						
Dianthus gratianopolitanos									19																	
Petrorhagia prolifera				3																						
Lychnis flos-cuculi	3, 5, 19				3, 4, 5, 10						5					4, 5, 10, 19										
Lychnis chalcedonica																4, 10, 19										

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Viscaria vulgaris	10, 19	3			3, 19, 10		3, 19, 10			3, 11, 19, 10						10, 19										
Gypsophila altissima											3															
Gypsophila fastigiata											3															
Gypsophila paniculata											3															
Gypsophila repens									11									10								
Vaccaria dioica	4																									

	H. bicruris	H. capsincola	H. magnolii	H. compta	H. confusa	H. variolata	H. albimacula	H. filograna	H. caesia	H. perplexa	H. irregularis	H. christophi	H. ectypa	H. laudeti	H. luteago	H. rivularis	H. silenes	H. tephroleuca	H. sancta	H. consparcatoides	H. clara	H. circumvadis	H. luteocinta	H. silenides	H. wehrlii	H. plebeja
Atocion rupestre									11									11								
Heliosperma pusillum									11																	

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29. Francisco Balao (comunicación personal)
30. Red de polinización nocturna de Sierra Nevada URJC (Silvia Santamaría, Carlos Lara, Javier Morente, José María Iriondo, Marcos Méndez y Luis Giménez)

CAPÍTULO 2/CHAPTER 2

Diel Variation in Flower Scent Reveals Poor Consistency of Diurnal and Nocturnal Pollination Syndromes in Sileneae.

ABSTRACT

The composition of flower scent and the timing of emission are crucial for the chemical communication between plants and their pollinators; hence, they are key traits for the characterization of pollination syndromes. In many plants, however, plants are assigned to a syndrome based on inexpensive to measure flower traits, such as color, time of flower opening and shape. We compared day and night scents from 31 Sileneae species and tested for quantitative and semi-quantitative differences in scent among species classified a priori as diurnal or nocturnal. As most *Sileneae* species are not only visited by either diurnal or nocturnal animals as predicted by their syndrome we hypothesize that, even if flower scent may be preferentially emitted during the day or at night, most species also emit some scents during the opposing periods of the day. This phenomenon may contribute to the generalized assemblage of flower visitors usually observed in Sileneae species. We found that diel variations of scent were often not congruent with the syndrome definition, but can partially be explained by the taxonomy and sampling times. Most species emitted compounds with attractive potential to insects during both the night and day. Our results highlight the current opinion that syndromes are not watertight compartments evolved to exclude some flower visitors. For these reasons, important information may be lost when scents are either collected during day- or night-time, depending on the a priori classification of the species as diurnal or nocturnal.

INTRODUCTION

Pollination syndromes are defined as the suite of correlated floral traits associated with the attraction and utilization of specific groups of animals as pollinators (Fenster et al. 2004). This concept is based on the observation that unrelated plant taxa visited by the same functional group of pollinators often display similar floral phenotypes due to convergent evolution (Fenster et al. 2004; Jürgens 2004; Ollerton et al. 2009). The pollination syndrome concept has been criticized because empirical observations around the globe show that most plant species are visited by multiple potential pollinators, so the supposed syndromes do not successfully predict pollinator guilds (Armbruster et al. 2000; Fenster et al. 2004; Herrera 1996; Ollerton et al. 2009; Waser et al. 1996). However, recent work shows that when quantity and quality data are available to distinguish pollinators from visitors, pollination syndromes effectively predict the most effective pollinators (Rosas-Guerrero et al. 2014).

Among flower traits, the composition of flower scent and the timing of emission are important for chemical communication between plants and their pollinators, and hence, a crucial part of the characterization of pollination syndromes (Knudsen et al. 2006; Knudsen and Tollsten 1993). However, there is not always a match between scent characteristics and the type or time of activity of pollinators (Pichersky et al. 1994; Schlumpberger and Raguso 2008), and in some taxa, the scent traits are not consistent with other traits, such as the flower morphology (e.g., Schlumpberger and Raguso 2008). Currently, there is no survey with many species to test the correlation between pollination syndromes and flower scent. Thus, new data are essential for better characterization.

A good example of the controversy around pollination syndromes is the tribe Sileneae (Caryophyllaceae) (Bittrich 1993; Greenberg and Donoghue 2011; Harbaugh et al. 2010), which comprises *Silene*, *Lychnis*, *Agrostemma*, *Atocion*, *Viscaria,* and *Petrocoptis* (Oxelman et al. 2013). In Sileneae, two pollination syndromes have been traditionally described, nocturnal and diurnal (Greuter 1995; Jürgens et al. 1996; Lindman 1897). The nocturnal syndrome is characterized by the following correlated traits apparently devoted to nocturnal pollination by moths (phalaenophily and sphingophily): (1) white or pale corolla, (2) synchronized and repeated flower opening in the evening/night

(nyctinasty), and (3) intense scent emission in the evening/night as perceived by the human nose. The diurnal syndrome is the combination of the following flower traits apparently adapted to maximize generalist diurnal pollination: (1) pink or red corolla and (2) flowers continuously open during the day and night, without obvious changes in scent intensity. Despite this clear duality in flower phenotypes, pollination studies have revealed that almost every Sileneae species is visited by diurnal and nocturnal insects to some extent (Jürgens 2004; Jürgens et al. 2002b; Kephart et al. 2006). Flowers of nocturnal Sileneae are visited mostly by moths during the night (i.e., Noctuidae, Sphingidae, Geometridae and Crambidae) and by different groups of visitors during the day (i.e., Hymenoptera, Diptera and Lepidoptera) (Giménez-Benavides et al. 2007; Jürgens et al. 2002a, b; Martinell et al. 2010; Reynolds et al. 2009). Diurnal Sileneae are visited by similar assemblages of visitors during the day (and by hummingbirds (Reynolds et al. 2009)) and by some moths during the night (Jürgens et al. 2002a). These results reveal that Sileneae species may have evolved to maximize the visits of some pollinator suites without reducing the complete spectra of visitors. In addition to emitting scent during day and night, plants may change the scent composition in the course of the day as diurnal and nocturnal floral visitors likely have different olfactory abilities and scent preferences (Bergström et al. 1992; Gregg 1983; Miyake et al. 1998).

Some multi-species chemical-analytical studies on flower scent of Caryophyllaceae have centered on the comparison between species assigned to these syndromes (Jürgens 2004; Jürgens et al, 2003, 2002b) and revealed interesting insight in the chemical composition of scents emitted during day- and night-time in this ecologically diverse group of plants. However, based on the a priori classification, species classified as diurnal were sampled for scent analysis only during the day, and only at night for species classified as nocturnal. Generally, only rarely are temporal scent patterns analyzed by chemical-analytical tools and only in few species is the scent emission known at both day- and night-time. We believe that both composition and temporal dynamics of flower scent are key traits to predict the main pollinator group(s) of plant species. We hypothesize that flower scent in most Sileneae species is mainly emitted at day- or night-time, but some scent is also emitted at the opposing period of the day. This may contribute to attraction of the opposing visitor assemblages as revealed in the pollinator studies of some *Silene* species. To test this hypothesis, we collected and analyzed the

flower scent from a wide spectrum of Sileneae species from different sections during the day and at night. Then, we compared the intra- and inter-specific variations in scent amount and composition, and asked whether variation in scent patterns is congruent with the previous assignment of each species as a nocturnal or diurnal pollination syndrome. Finally, we also analyzed the possible influence of taxonomy (section) on scent variation.

METHODS AND MATERIALS

Plant Material

Floral scents were collected from 26 *Sileneae* species (Table 1) grown from seeds in the greenhouse of the Universidad Rey Juan Carlos (Móstoles, Madrid 40º20'02.85"N, 3º52'57.94"W, altitude 651 m). Seeds were obtained from field samplings or from scientific exchanges with seed banks and botanical gardens (Table 1). Seeds were sown in 5 cm seedling trays and after three months, plantlets were transferred to 2 l pots until flowering. Perennial species were sown in 2010 and flower scents were sampled in 2011, while annual species were sown and sampled in 2011. Scent analyses from another five species were obtained from published data (Table 1). Although the classification of the tribe Sileneae has been controversial (Desfeux and Lejeune 1996; Greuter 1995; Oxelman and Lindén 1995), we use the classification of Oxelman et al. (2013) (Table 1).

Scent Collection and Analysis

Collections of floral volatile organic compounds (VOCs) of the 26 species grown in our greenhouse were completed outdoors using the dynamic headspace method. Inflorescences were enclosed in polyethylene oven bags for 5 min and the emitted volatiles were then trapped for another 5 min in adsorbent tubes, the same method as described by Dötterl et al. (2005) and Dötterl and Jürgens (2005) with a 9 V battery-operated pump (Giménez-Benavides et al. 2007). For each individual, samples were taken both during the day (between 11:30 to 13:00) and during the night (between 21:00 to 00:00). The two time periods were chosen based on literature (activity of pollinators and flower opening) and our personal observations (times of flower opening), but we cannot rule out the possibility that we missed the time of (maximal)

scent emission. Diurnal and nocturnal flower scents from each species were sampled in 1-3 individuals, and we collected 118 samples overall. Because the number of replicates per species was small, scent data within species were pooled for each time period (mean of nocturnal and mean of diurnal samples). The number of flowers used for each sample was counted, and after sampling, flowers were removed and dried in an oven at 60ºC for 24 hours for determining the dry weight. Ambient air samples were taken as negative controls to distinguish between floral compounds and ambient contaminants. To control for the emission of green leaf volatiles (GLVs, Light et al. 1993; Visser et al. 1979), which would not necessarily help pollinators to accurately locate the flowers (Honda et al. 1998), samples from vegetative parts (leafs and stems) were taken from eight species (Table 1). We assumed that the GLVs detected in these samples (Supplementary Material 2) are similar among all of the study species. Then, we deleted the GLVs from the final matrix of flower scent compounds.

All samples were analyzed on a Varian mass spectrometer coupled to a gas chromatograph (GC-MS) using a 1079 injector that had been fitted with the ChromatoProbe kit (Dötterl et al. 2005). The adsorbent tube was loaded into the probe, which was then inserted into the modified GC injector. The column and settings used for the analyses were the same as those described in Dötterl et al. (2005) and Dötterl and Jürgens (2005). The GC-MS data were processed using the Saturn Software package 5.2.1. Compound identification was carried out using the NIST 05 mass spectral database or MassFinder 3, and confirmed by comparing retention times with published data (Adams 1995). The identification of some compounds was also confirmed by comparison of mass spectra and retention times with those of authentic standards. To determine the total absolute amount of scent collected, known amounts of monoterpenoids, aromatics and aliphatics were injected, and the mean peak area of these compounds was used for quantification (Dötterl et al. 2005). In each sample, we also determined the contribution (percentage) of each single compound to the total peak area, which was set to 100 % (relative data; Dötterl et al. 2012).

Assignment of Pollination Syndromes

The species were classified as nocturnal or diurnal independent of the levels of self-pollination because some species with high levels of self-pollination (facultatively) have

flower scents and flower visitors (Jürgens et al. 2002a). Classification occurred based on flower traits mentioned earlier in this paper (time and pattern of flower opening) (Table 1), according to Jürgens et al. (2012, 2002a). A species was treated as nocturnal if there was a synchronized flower opening or repeated flower opening at night/evening (nyctinasty) and diurnal if the first flower opening was not synchronized at night and the flowers remained open. Petal color was discard as a diagnostic trait because Jürgens (2004) and Jürgens et al. (2002b) found that some diurnal species have white corollas (*H. alpestre* and *A. rupestre*) and that some nocturnal species have pink corollas (*S. sericea* and *S. subconica*).

Statistical Analysis

For the species sampled in this study, we calculated the absolute amount of scent (ng) by flower (number), dry weight (grs) and time (min). To test if the diurnal species emitted more scent at day-time and nocturnal species at night, we calculated the ratio day:night of the total absolute amount of scent within each species. Then, we used a Mann-Whitney U Test to test for possible differences in this ratio between species classified a priori to the diurnal or nocturnal syndrome. To test whether scent composition differed between the day and night samples and among species, we carried out a global PERMANOVA analysis with *sampling time* and *species* as fixed factors. To explore the presence of phylogenetic pattern in the scent composition, we performed a second PERMANOVA including additionally the taxonomic sections (following Oxelman et al. 2013). *Sampling time* and *section* were used as fixed factors, and *species* nested in *section* as a random factor. A post-hoc test explored pairwise differences between the sections. A third PERMANOVA tested whether variation in scent pattern correlates with the a priori assignment of each species as nocturnal or diurnal. *Sampling time* and *syndrome* were used as fixed factors, and in addition to *sampling time*, we also controlled for s*ection* (fixed factor) as significant section effects were found in the previous analysis (see Results).

To test whether day and night samples differ in variability (dispersion), and if this difference might have been responsible for the significant effect of the *sampling time* (see Results), we performed a permutational analysis of multivariate dispersions (PERMDISP). PERMDISP analysis showed no differences in the variability (dispersion) of

the scent compositions between the day and night samples ($F_{1,55}$=2.2, *P*=0.17), indicating that the day samples were similarly variable compared with the night samples. The compounds most responsible for differences in scent between the day and night samples within a species were determined by a similarity percentages analysis (SIMPER; factors *species* and *sampling time*). Non-metric multidimensional scaling (NMDS) analyses were used to depict variation in floral scent composition among pollination syndromes and between the day and night emissions. All PERMANOVA and PERMDISP analyses were performed with 10 000 permutations. PERMANOVA, PERMDISP and NMDS were based on the Bray-Curtis pairwise similarities matrix (Clarke and Gorley 2006). Most of the species emitted a few highly abundant compounds and additionally minor compounds (Supplementary Material1). To prevent analyses from being largely influenced by the most abundant compounds, relative percent data were Fourth root transformed (Clark and Warwick 2001). All of these analyses were implemented in PRIMER 6.1.11.

Table 1. List of the species used. Number of samples per time (night/day) and pollination syndromes of each species are shown.

Taxa/Species	Night/Day samples	Traditional pollination syndrome	Reference	Source of seeds
Lychnis				
L. coronaria[a]	2/2	D	This study	BBGK
Silene				
Sect.Atocion				
S. aegyptiaca	3/3	D	This study	IGB
S. fraudatrix	3/3	D	This study	BGBM
Sect.Auriculatae				
S. boryi	1/1	N	This study	BGVA
S. linicola[b]	3/2	D	This study	FRANK
Sect.Behenantha				
S. fabaria[a]	3/3	N	This study	BBGK
S. fabarioides	1/1	N	This study	BBGK
S. holzmannii	1/1	D	This study	NKUA
S. uniflora	2/2	N	This study	UOV
S. variegata	2/2	N	This study	NKUA
Sect.Melandrium				
S. diclinis[a]	2/2	D	This study	UVAL
S. dioica	16/16	D	Waelti et al. 2008	
S. latifolia	20/20	N	Waelti et al. 2008	
Sect.Psammophilae				
S. cambessedesii	3/3	D	This study	UPO
S. littorea	3/3	D	This study	UPO
S. psammitis	3/3	D	This study	UPO & BGVA
Sect.Psysolychnis				
S. stellata	3/2	N	Castillo et al. 2014	FRANK
S. zawadzkii[a]	1/1	N	This study	
SubSect. Sclerocalycinae				
S. frivaldszkyana[a]				BGBM
S. saxatilis	3/3	N	This study	BGBM
Sect.Silene	1/1	N	This study	
S. ciliata			Giménez-Benavides et al. 2007	
	15/4	N	This study	
S. colorata[a, b]			This study	UVAL
S. fuscata	3/3	N	This study	IGB
S. giraldii	2/2	D	This study	KEW
S. scabriflora	2/2	D	This study	BGVA
S. tridentata	3/3	D		UVAL
	3/3	N		
Sect.Siphonomorpha				
S. flavescens	2/2	N	This study	BBGK

Taxa/Species	Night/Day samples	Traditional pollination syndrome	Reference	Source of seeds
S. longicilia[a]	3/3	N	This study	BGVA
S. sennenii	6/3	N	Martinell et al. 2010	
S. tatarica[a]	3/3	N	This study	BGBM
Viscaria				
V. vulgaris[c]	1/1	D	This study	BGBM

[a] Species sampled for green leave volatiles (GLVs).

[b] We considered *S. linicola* to be diurnal despite the earlier definition of nocturnal or self-pollinated (Jürgens et al. 2002a; Jürgens et al. 2012). In spite of its level of self-pollination, we observed that petals remain open the whole day. On the other hand, we found discrepancies with the *S. colorata* syndrome definition given by Jürgens et al. (2002a, 2012). We reassigned *S. colorata* to the nocturnal syndrome because, despite having pink petals, it has repeated flower opening at night.

[c] Scent in *V. vulgaris* was also studied by Jürgens (2004) but only during the day.

Sources of seeds: Israel Plant Gene Bank (IGB), Botanischer Garten und Botanisches Museum Berlin-Dahlem (BGBM), Banco de Germoplasma Vegetal Andaluz (BGVA), Frankfurt Botanical Garden (FRANK), The Balkan Botanic Garden at Kroussia Mountains (BBGK), National and Kapodistrian University of Athens (NKUA), University of Virginia (UOV), Jardin Botanico de la Universidad de Valencia (UVAL), Universidad Pablo de Olavide (UPO), and the Royal Botanic Gardens (KEW).

RESULTS

Congruency of Pollination Syndromes and Scent Production

The total absolute amount of scents (diurnal + nocturnal samples) per species was highly variable, and this variability was obvious in all of the species regardless of syndrome classification (Fig. 1). There were no differences in the ratio day:night scent production between syndromes (U=44, P>0.05). Of the 12 species ascribed to diurnal syndrome (Table 1), only five (41.7 %) produced more scent during the day. Of the 13 species described as nocturnal, ten species (76.9 %) produced more scent during the night. No scent was detected in two nocturnal species during the daytime (*S. colorata, S. flavescens*). The most unexpected results were that *S. littorea* and *S. cambessedesii*, both classified as diurnal, were among the species with the highest amount of scent emitted at night and almost no scent during the day (Fig. 1). *S. holzmannii* did not emit any flower-specific scents during the day, even though it was also described as diurnal. *S. saxatilis* and *S. tridentata,* both classified originally as nocturnal, produced greater

amounts of scent during the day than at night. *V. vulgaris* had no floral VOCs in any sample during the day or night and was not taken into account in further analyses.

Flower Scent Composition

The flower scent composition of the species was quite variable (e.g., novel species in Supplementary Material 1). Terpenoids represented 61 of the 155 VOCs detected, followed by 47 aromatic compounds, 17 N-bearing, and 28 unknown compounds. Prenyl acetate was the only C5-branched chain compound, and 2-ethyl-1,6 dioxaspiro[4.4]nonane was the only spiroacetal. The spiroacetal, 14 terpenoids, 23 aromatic compounds, and 8 N-bearing compounds were not found in previous Sileneae scent studies (Supplementary Material 1). Altogether, 148 compounds were emitted in the samples collected at night. Even though most species showed only a relatively weak fragrance during daytime to the human nose, 65 compounds were emitted during daytime, with the exception of the three species for which we could not detect any scent during the day. Most of the compounds emitted during daytime were also emitted during the night (58 of 65), but 90 compounds were exclusively found in the night samples. Differences in the relative amount of monoterpenes (e.g., (*E*)-β-ocimene, linalool) and aromatics (e.g., benzaldehyde, 2-phenylethanol) were mostly responsible for the ordination of samples along the two axes in a non-metric multidimensional scaling (Fig. 2). The scent composition did not create a significant pattern between diurnal and nocturnal species in the NMDS (*Pseudo-*$F_{1,56}$=1.04, P>0.05; Fig. 2). However, significant differences in scent composition were found according to the *sampling time* (*Pseudo-*$F_{1,56}$=2.62, P=0.023) and *species* (*Pseudo-*$F_{28,56}$=2.2, P<0.001). Fig. 3 shows the temporal changes between the scent patterns within species. Most obvious changes were owed to the relative amount of terpenoids, aromatics, and N-bearing compounds. Different species change their scents between the day and night in a variety of ways. This is true for the magnitude of changes (e.g., *S. dioica* and *S. littorea* had small and large dissimilarity, respectively) and for the compound classes most responsible for the day-night differences (e.g., *S. frivaldszkyana* produces mainly terpenoids during the day and N-bearing compounds at night, but in *S. linicola* the dissimilarity was owed to changes in the importance of different terpenoids (Supplementary Material 1)). Species that produce more amounts of scent at night than during the day also emitted different

scent compounds. *S. bory*, *S. littorea*, *S. cambessedesii*, *S. tatarica*, *S. uniflora*, *S. fricaldszkyana* and *S. psammitis* emitted high relative amounts of aromatics (e.g., benzaldehyde and benzyl acetate) or N-bearing compounds (e.g., 2-methylbutyl aldoxime and 1-nitropentane) at night, but large amounts of monoterpenes (e.g., (*E*)-β-ocimene and sabinene) during the day (Supplementary Material 1). Other species emitted high relative amounts of lilac aldehyde or linalool at night, but large amounts of bicyclic monoterpenes (e.g., camphene) during the day (*S. fabaria*, *S. fabarioides* and *S. variegata*; Supplementary Material 1). *S. fuscata*, *S. longicilia,* and *S. scabriflora* emitted a lower amount of scent during the daytime, but the main compound did not differ from the night samples (e.g., (*E*)-β-ocimene in *S. fuscata*). Some other changes were only followed by a single species, as from large amounts of lilac aldehyde at night to large amounts of 2-phenylethanol during the day (*S. stellata*). There were also some common patterns within the species that produce more scent during the day. For example, *S. saxatilis*, *S. linicola*, *S. aegyptiaca* and *S. tridentata* emitted large amounts of (*E*)-β-ocimene at night (99 % and 100 % of the nocturnal scent of *S. aegyptiaca* and *S. linicola,* respectively), but large amounts of bicyclic monoterpenes during the day (e.g. sabinene, β-pinene and camphene) (Supplementary Material 1).

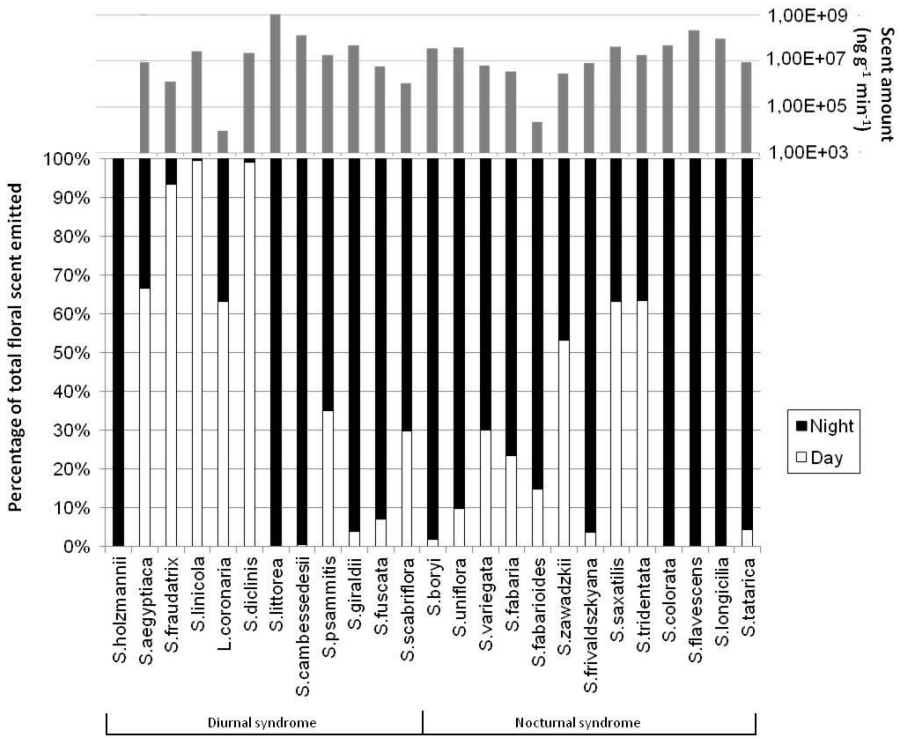

Fig. 1 Percentage (mean) of the total floral scent production emitted during the day and at night by the Sileneae species analyzed in this study. Above each bar is the (mean) total scent production (ng scent/gr flower * min; absolute total amount trapped in day + night samples). Species are grouped according to pollination syndrome. Note that the scent amount is on a Log10 scale. The weight of the dry flowers for standardization of *S. holzmannii* was unavailable. *S. littorea* and *S. longicilia* emitted a small amount of scent (0.05 % and 0.28 %, respectively) during the day.

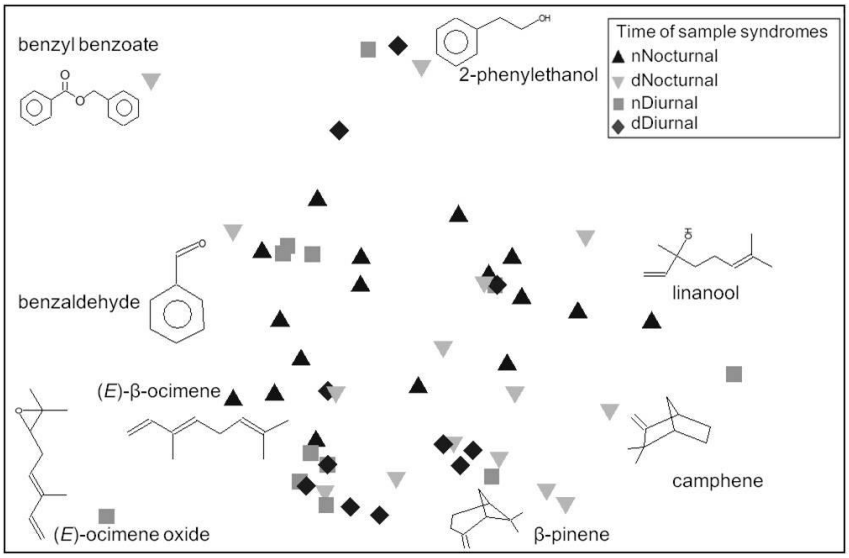

Fig.2 Non-metric multidimensional scaling (NMDS) of flower scent VOCs found in Sileneae species in relation to the sampling time (n, night and d, day) and the pollination syndrome (Nocturnal/Diurnal). 2D stress value was 0.15

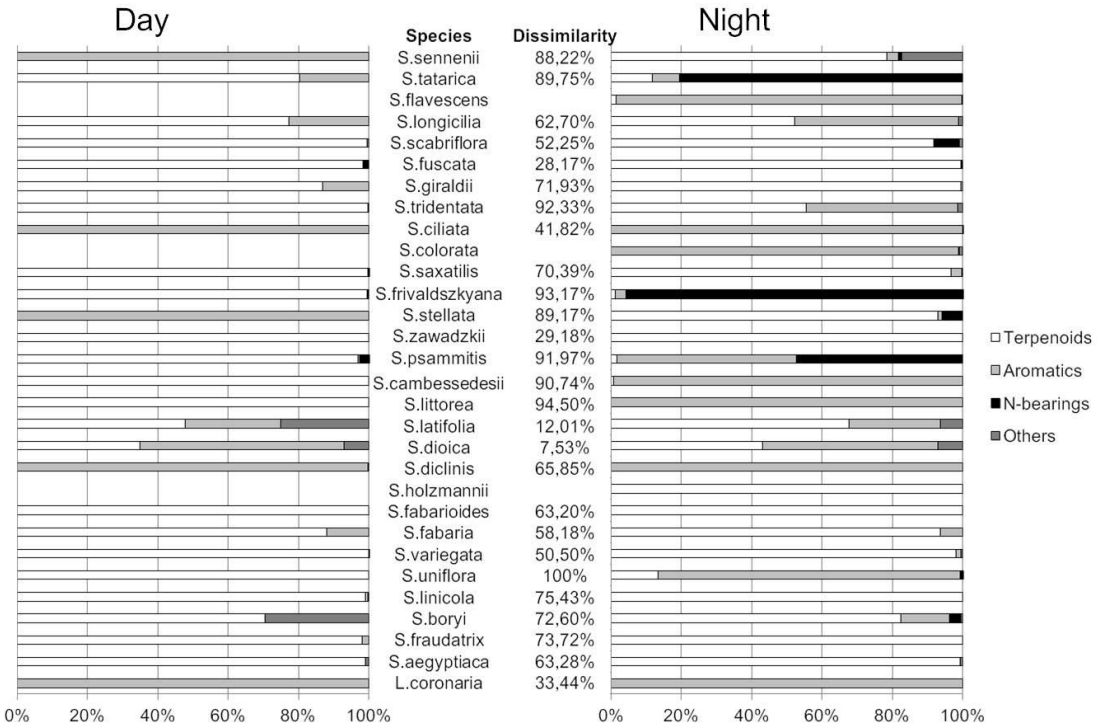

Fig.3 Within-species Bray-Curtis dissimilarity between day and night floral scent emissions. Bars denote the contribution of each compound class to the total scent emitted during the day and at night for each species. "Other" class compounds are the sum of C5-branched chain compound, unknown compounds and the spiroacetal. * *S. colorata*, *S. flavescens* and *S. holzmannii* have no dissimilarity values because no flower-specific scents were found in the day samples. Species are ordered according to taxonomy (see Table 1).

In the phylogenetic PERMANOVA, we found significant differences for *species* (*Pseudo-*$F_{20,56}$=1.84, P<0.001) and *taxonomic section* effects (*Pseudo-*$F_{9,56}$=1.79, P<0.001), but not for *sampling time* (*Pseudo-*$F_{1,56}$=2.24, P>0.05) and the *section*sampling time* (*Pseudo-*$F_{9,56}$=1.22, P>0.05) interaction. Therefore, the differences in scent composition owed to *sampling time* found in the global PERMANOVA decreased when controlling for *taxonomic section* (phylogeny) because the effect of *section* takes part of the variance explained for *sampling time*. The post-hoc pairwise analysis by *section* revealed that the scent composition was significantly different between 11 of the 45 (24.4 %) possible pairwise comparisons (Table 2). Behenanta was the section that differed most from other sections (6/9).

Table 2. Matrix of differences in scent composition between sections based on PERMANOVA. An (x) denotes sections that differs significantly and a (-) denotes no significant differences.

	Atoc	Auri	Behe	Lych	Mela	Phys	Psam	Scle	Sile	Siph
Atocion		-	X	-	-	-	-	-	-	-
Auriculatae			X	-	-	-	-	-	-	-
Behenantha				X	X	-	X	-	X	-
Lychnis					-	-	-	-	-	X
Melandrium						-	-	-	X	X
Physolychnis							-	-	X	X
Psammophilae								-	-	-
Sclerocalicynae									-	-
Silene										-
Siphonomorpha										

DISCUSSION

In pollination syndromes the involved floral traits are expected to correlate with one another, but some traits may fit better to specific groups of pollinators than others, and some traits may be more variable or constrained than others (Fenster et al. 2004; Ollerton et al. 2009). In Sileneae, the synchronized flower opening during the evening or night (nyctinasty) and the emission of large amounts of scent at night (and low amounts during the day) should be robust flower traits for the definition of a nocturnal syndrome. However, they do not always correlate (e.g., *S. fuscata* and *S. giraldii*). Corolla color does not correlate with other flower traits either, as in the pink nyctinastic petals of *S. colorata*. Additionally, corolla color does not correlate with scent emission in *S. littorea* and *S. cambessedesii*. Both species emit large amounts of scent at night and have pink petals.

We found that many species showing high scent emissions during one part of the day also emit scent at the opposite part, sometimes with dramatic changes in composition. Intra-specific differences in scent production and composition between the day and night have previously been analyzed in only six *Silene* species. All of these species emitted more scent at the time of day expected for their syndrome (Castillo et al. 2014; Dötterl et al. 2012; Giménez-Benavides et al. 2007; Martinell et al. 2010; Waelti et al. 2008), with the exception of one species, *S. otites*, which emitted large amounts of scent at unexpected times (Dötterl et al. 2012).

Here on, we relate the flower odor changes found in Sileneae species between day and night with their function as an attractant that each compound might have based on the literature. Among the species that emitted more scent at night than during the day, some changed their scent composition considerably. Their night scents were dominated by aromatics (e.g., benzaldehyde and benzyl acetate), N-bearing compounds (e.g., 1-nitropentane, 2-methylbutyl aldoxime) or linalool and derivatives thereof (e.g., lilac aldehydes). These flower VOCs have the ability to attract settling moths or hawkmoth pollinators (Dobson 2006; Dötterl et al. 2006; Knudsen and Tollsten 1993; Meagher 2002; Raguso et al. 1996; Raguso and Light 1998). During the daytime, smaller amounts of other compounds, such as camphene or (*E*)-β-ocimene, were emitted, and these compounds could attract diurnal pollinators such as butterflies or bumblebees (Andersson 2003; Byers et al. 2014; Dobson 2006). In other species the main compound did not differ between night and day samples (e.g., (*E*)-β-ocimene). In such species, the same compounds might serve to attract diurnal and nocturnal Lepidoptera, but the greater emission of scent during the night seems to be an adaptive response to maximize nocturnal visits. Finally, some species emitted greater amounts of scent during the daytime (sabinene, β-pinene and camphene) that are typically associated with bee-pollinated plants (Dobson 2006), but may also attract moths at night (e.g., (*E*)-β-ocimene).

Our work is based on just a small time interval of day and night, and the studied species might have a more complex temporal variation, as shown for *S. otites* (Dötterl et al. 2012). However, the diel variations of scent composition and production detected do not fit with the diurnal or nocturnal syndromes as previously described for most of the Sileneae species. In fact, floral scent patterns of most of the studied species may point towards a more generalist pollination syndrome and a mixed pollination strategy (Dötterl et al. 2012). This may be the reason why most Sileneae species are visited both during the day and at night, even the “nocturnal” species that show inconspicuous white and closed flowers during the daytime.

The actual consent about the pollination syndrome concept is that syndromes reflect adaptation to primary pollinators, but some traits may not exclude the less efficient floral visitors, and these may also play a role in floral evolution (Rosas-Guerrero et al.

2014). Many case studies have demonstrated that there is not always a fit between visitors and real pollinators, as the pollination effectiveness may strongly differ among species (Brittain et al. 2013; Fishbein and Venable 1996; Javorek et al. 2002; Martén-Rodríguez et al. 2009; Motten et al. 1981; Waser and Price 1990). The study of pollination effectiveness by different flower visitors in a plant is difficult and costly, and sometimes context-dependent (Thompson, 1994, 1999). Unfortunately, there are not enough case studies to explore whether pollination syndromes correlate with the most effective pollinators in Sileneae. Only three species have both diurnal and nocturnal data on scent emission rates and pollinator effectiveness. In *S. sennenii* and *S. stellata* (Martinell et al. 2010; Reynolds et al. 2009), the dominance of nocturnal scents is in agreement with the higher pollinator effectiveness of moth pollinators. However, in *S. ciliata*, a pollination exclusion experiment showed that diurnal pollination provided greater fitness in spite of the prevalence of nocturnal scents (Giménez-Benavides et al. 2007).

There are other possible selective agents aside from pollinators that can model the flower scent in Sileneae, such as herbivores. For instance, several of the monoterpenoids we identified in samples collected during the day may have a defensive function against floral antagonists (Junker and Blüthgen 2010; Schiestl 2010) and not be under selection of diurnal pollinators. Flower scent may also be under conflicting selective pressures from both pollinators and herbivores in nursery pollination systems. Nursery pollination is widely distributed within Sileneae (Kephart et al. 2006). *S. latifolia* and *S. ciliata* form nursery pollination systems with *Hadena bicruris* and *H. consparcatoides*, respectively. Lilac aldehydes and benzaldehyde are the main compounds produced at night for *S. latifolia* and *S. ciliata*, respectively, and are (potential) attractants for each *Hadena* (Dötterl et al. 2006; Giménez-Benavides et al. 2007). If *Hadena* nursery pollinators act as parasite of its respective *Silene* (Giménez-Benavides et al. 2007; Petterson 1991; Reynolds et al. 2012), then these moths will create a negative selection pressure on the nocturnal scent. These plants could then make its floral scent more attractive for diurnal pollinators and thereby escape from this negative interaction. An exclusion of *Hadena* moths by still attracting other nocturnal

moths seems to be more difficult as other moths use similar compounds for host finding as *Hadena* (Dobson 2006; Plepys et al. 2002).

Finally, another cause that may explain the scent variation among and within Sileneae species is the phylogenetic constrains. Conservatism of the scent from ancestral species could lead to similarities between species within sections and dissimilarities with other sections. Congruence between fragrance and taxonomy at low taxonomic levels has been found before (Feulner et al. 2014; Levin et al. 2003). We have found some differences in scent composition in relation to taxonomy. The effect of *section* in taking part of the variance explained for *sampling time*, and decreased its effect. Therefore, the phylogeny better explains the differences in the scent composition than the sampling time. This result is only a preliminary attempt to the study phylogenetic signal, which will be further studied in a phylogenetic analysis, as it has been previously conducted for other flower traits such as pollen grain size and nectar sugar composition (Jürgens et al. 2012; Witt et al. 2013).

Most of the Sileneae species examined in this study emitted flower scents with attractive capacities for insects both during the day and at night. This example highlights that the assignment of a diurnal or nocturnal syndrome has not to be used as a clue to exclude the scent analysis of one part of the day, but because it may cause the loss of essential information to figure out the floral scent phenotype of a particular species. Detailed studies are needed to test if the different patterns of scents detected here are related with the most frequent and effective pollinators of each species. Questions addressed should be: (i) which compounds are involved in the attraction or repulsion of pollinators/florivores, (ii) which compounds are phylogenetically constrained, and (iii) which compounds are under conflicting or synergistic selection by different selective forces.

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SUPPLEMENTARY MARETIAL 1. Floral scent composition of the species analyzed in this study.

	S.colorata nigth	S.longicilia night	S.longicilia day	S.tridentata night	S.tridentata day	S.diclinis night	S.diclinis day	S.giraldii night	S.giraldii day	S.frivaldszkyana night	S.frivaldszkyana day
C5-branched chain compound											
Prenyl acetate*	-	-	-	-	-	-	-	-	-	0.04	-
Aromatics											
1, 4-Benzoquinone	0.03	-	-	1.82	0.26	-	-	-	-	-	-
Benzaldehyde	79.21	-	-	39.07	-	-	18.68	-	-	-	-
Benzylalcohol	1.54	0.21	-	-	-	-	-	-	-	-	-
Phenylacetaldehyde	10.90	-	-	-	-	-	62.51	-	-	-	-
2-Methoxyphenol	-	-	-	0.85	-	-	-	-	-	-	-
Methyl benzoate	1.27	-	-	-	-	-	-	0.45	13.10	-	-
2-Phenylethanol	1.36	-	-	-	-	100.00	18.22	-	-	1.05	-
p-methoxystyrene*	-	-	-	-	-	-	-	-	-	-	-
2-Phenylpropenal*	0.04	-	-	-	-	-	-	-	-	-	-
Benzyl acetate	-	46.30	22.72	-	-	-	-	-	-	2.06	-
Benzenepropanal*	0.12	-	-	-	-	-	-	-	-	-	-
1-Phenyl-1,2-propanedione	0.43	-	-	0.33	-	-	-	-	-	-	-
Methyl salicylate	1.44	-	-	-	-	-	-	-	-	-	-
Benzenepropanol	0.05	-	-	-	-	-	-	-	-	-	-
2-Phenylethyl acetate	-	-	-	-	-	-	0.27	-	-	0.06	-
p-methoxybenyaldehyde*	0.71	-	-	-	-	-	-	-	-	-	-
Hydroquinone*	-	-	-	0.45	-	-	-	-	-	-	-
Z-Cinnalmyl alcohol	-	-	-	-	-	-	-	-	-	-	-
E-Cinnamaldehyde	0.67	-	-	-	-	-	-	-	-	-	-
E-Cinnamyl alcohol	0.89	-	-	-	-	-	-	-	-	-	-
Methyl o-methoxybenzoate	-	-	-	-	-	-	-	-	-	-	-
Benzyl butyrate*	-	-	-	-	-	-	-	-	-	-	-
Eugenol*	-	-	-	-	-	-	-	-	-	-	-
3,4-Dimethoxystyrene	-	-	-	-	-	-	-	-	-	-	-
4-methoxyphenethyl alcohol*	-	-	-	-	-	-	-	-	-	-	-
Benzenepropyl acetate*	-	-	-	-	-	-	-	-	-	-	-
Methyleugenol*	-	-	-	-	-	-	-	-	-	-	-

	S.colorata nigth	S.longicilia night	S.longicilia day	S.tridentata night	S.tridentata day	S.diclinis night	S.diclinis day	S.giraldii night	S.giraldii day	S.frivaldszkyana night	S.frivaldszkyana day
Vanillin*	0.04	-	-	-	-	-	-	-	-	-	-
Isoamyl benzoate	-	-	-	-	-	-	-	-	-	-	-
E-Cinnamyl acetate*	-	-	-	-	-	-	-	-	-	-	-
Z-Methyl isoeugenol*	-	-	-	-	-	-	-	-	-	-	-
3,4-dimethoxybenzaldehyde*	-	-	-	-	-	-	-	-	-	-	-
E-Methyl isoeugenol*	-	-	-	-	-	-	-	-	-	-	-
Z-3-Hexenyl benzoate	-	-	-	-	-	-	-	-	-	-	-
3,4,5 trimethoxybenzaldehyde*	-	-	-	-	-	-	-	-	-	-	-
Isoelemecin*	-	-	-	-	-	-	-	-	-	-	-
Benzyl benzoate	-	-	-	0.69	-	-	-	-	-	-	-
Aromatic (m/z 105, 77, 51, 50, 137, 152)	0.07	-	-	-	-	-	0.13	-	-	-	-
N-bearing compounds											
2-methylbutylaldoxime	-	-	-	-	-	-	-	-	-	20.21	-
3-methylbutylaldoxime	-	-	-	-	-	-	-	-	-	0.40	-
1-Nitropentane*	-	-	-	-	-	-	-	-	-	72.14	-
Methyl nicotinate*	0.14	-	-	-	-	-	-	-	-	-	-
Phenylacetonitrile*	-	-	-	-	-	-	-	-	-	0.50	-
Octanenitrile*	-	-	-	-	-	-	-	-	-	-	-
2-Aminobenzaldehyde*	-	-	-	-	-	-	-	-	-	-	-
Aromatic aldoxime*	-	-	-	-	-	-	-	-	-	1.35	-
Decanenitrile*	-	-	-	-	-	-	-	-	-	-	-
Indole*	-	-	-	-	-	-	-	-	-	0.01	-
1-Nitro-2-Phenylethane*	-	-	-	-	-	-	-	-	-	0.22	0.55
2-(dimethylamino)benzaldehyde*	-	-	-	-	-	-	-	-	-	-	-
N-bearing (m/z 124, 151, 81, 95, 165, 53)	-	-	-	-	-	-	-	-	-	-	-
N-bearing (m/z 54, 55, 84, 39, 41, 64)	-	-	-	-	-	-	-	-	-	0.78	-
N-bearing (m/z 79,110, 43, 53, 92, 39)	-	-	-	-	-	-	-	-	-	-	-
N-bearing (m/z 83, 55, 43, 39, 98, 153)	-	-	-	-	-	-	-	-	-	-	-
Terpenoids											
Z-β-Ocimene	-	0.82	-	-	-	-	-	-	-	-	-
E-β-Ocimene	-	46.36	29.82	55.44	-	-	-	0.61	63.57	1.18	-

	S.colorata nigth	S.longicilia night	S.longicilia day	S.tridentata night	S.tridentata day	S.diclinis night	S.diclinis day	S.giraldii night	S.giraldii day	S.frivaldszkyana night	S.frivaldszkyana day
Camphene	-	0.59	-	-	17.43	-	-	14.09	-	-	56.95
Sabinene*	-	2.12	-	-	55.97	-	-	65.54	-	-	-
b-Pinene	-	0.67	-	-	26.34	-	-	18.58	-	-	42.50
α-Phellandrene	-	-	-	-	-	-	-	-	-	-	-
Z-Arbusculone	-	0.09	5.61	-	-	-	-	-	-	-	-
E-Arbusculone	-	-	-	-	-	-	-	-	-	-	-
Z or E-Ocimenone*	-	0.14	-	-	-	-	-	-	-	-	-
Epoxy ocimene*	-	-	-	-	-	-	-	-	-	-	-
Geraniol*	-	-	-	-	-	-	-	-	-	-	-
Geranyl acetate*	-	-	-	-	-	-	-	-	-	-	-
β-Bourbonene	-	-	-	-	-	-	-	-	2.20	-	-
Linalool	-	-	-	-	-	-	-	-	-	-	-
Allo or neoallo-ocimene*	-	-	-	-	-	-	-	-	-	-	-
E-Ocimene oxide	-	0.13	-	-	-	-	-	-	-	-	-
Z+E-Linalooloxide pyranoid	-	-	-	-	-	-	-	-	-	-	-
4-Oxoisophorone*	0.09	-	-	-	-	-	-	-	-	-	-
LilacAldA	-	0.24	17.19	-	-	-	-	-	-	-	-
LilacAldB+C	-	0.14	18.01	-	-	-	-	-	-	-	-
LilacAldD	-	<0.01	6.66	-	-	-	-	-	-	-	-
Lilac alcohol A	-	-	-	-	-	-	-	-	-	-	-
Lilac alcohol B+C	-	-	-	-	-	-	-	-	-	-	-
LilacAlc D	-	-	-	-	-	-	-	-	-	-	-
Lilac derivative (93, 43, 55, 81, 111, 67)	-	-	-	-	-	-	-	-	-	-	-
Lilac derivative (43, 55, 93, 111, 81, 39)	-	-	-	-	-	-	-	-	-	-	-
Lilac derivative (111, 43, 93, 77, 55, 136)	-	0.10	-	-	-	-	-	-	-	-	-
Lilac derivative (43, 71, 39, 55, 83, 107)	-	-	-	-	-	-	-	-	-	-	-
Lilac alcohol formate A	-	0.07	-	-	-	-	-	-	-	-	-
Lilac alcohol formate B+C	-	0.02	-	-	-	-	-	-	-	-	-
longifolene V4*	-	-	-	-	-	-	-	-	-	-	-
Germacrene D	-	-	-	-	-	-	-	-	2.13	-	-
E,E-a-Farnesene	-	-	-	-	-	-	-	-	-	-	-

	S.colorata nigth	S.longicilia night	S.longicilia day	S.tridentata night	S.tridentata day	S.diclinis night	S.diclinis day	S.giraldii night	S.giraldii day	S.frivaldszkyana night	S.frivaldszkyana day
β-Selinene*	-	-	-	-	-	-	-	0.73	19.01	-	-
E-nerolidol*	-	-	-	-	-	-	-	-	-	-	-
Farnesol/al isomer*	-	-	-	-	-	-	-	-	-	-	-
Cubenol*	-	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 43, 55, 93, 67, 111, 137)	-	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 43, 109, 79, 67, 134)	-	0.14	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 107, 91, 39, 79, 150, 122)	-	0.02	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 79, 105, 39, 135, 65)	-	0.14	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 93, 121, 43, 79, 67, 136)	-	0.01	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 119, 152, 39, 67, 79)	-	0.04	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 150, 39, 107, 79, 135)	-	0.14	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 119, 152, 39, 109, 79)	-	0.09	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 79, 108, 39, 91, 119, 43)	-	0.09	-	-	-	-	-	-	-	-	-
St (m/z 91, 105, 175, 189, 119, 204)	-	-	-	-	-	-	-	-	-	-	-
St (m/z 43, 95, 39, 79, 55, 59)	-	0.04	-	-	-	-	-	-	-	-	-
St (m/z 43, 39, 95, 55, 81, 67)	-	0.09	-	-	-	-	-	-	-	-	-
St (m/z 43, 95, 79, 119, 174, 189)	-	0.05	-	-	-	-	-	-	-	-	-
St (m/z 119, 161, 204, 105, 134, 91)	-	-	-	-	-	-	-	-	-	-	-
St (m/z 91, 39, 161, 204, 105, 79)	-	-	-	-	-	-	-	-	-	-	-
St (m/z 91, 133, 161, 189, 79, 150)	0.04	-	-	-	-	-	-	-	-	-	-
St-oxide (m/z 93, 91, 39, 43, 55, 67)	-	-	-	-	-	-	-	-	-	-	-
Spiroacetal											
2-ethyl-1,6 dioxaspiro[4.4]nonane*	-	-	-	-	-	-	-	-	-	-	-
Unknown compounds											
(m/z 94, 137, 39, 81, 43, 78)	-	-	-	-	-	-	-	-	-	-	-
(m/z 91, 135, 39, 107, 65, 79)	-	0.03	-	-	-	-	-	-	-	-	-
(m/z 103, 102, 77, 132, 51, 63)	0.84		-	-	-	-	-	-	-	-	-
(m/z 91, 65, 39, 105, 131, 146)	-	-	-	-	-	-	-	-	-	-	-
(m/z 121, 136 ,83, 69, 55, 42)	<0.01	-	-	-	-	-	-	-	-	-	-

	S.colorata nigth	S.longicilia night	S.longicilia day	S.tridentata night	S.tridentata day	S.diclinis night	S.diclinis day	S.giraldii night	S.giraldii day	S.frivaldszkyana night	S.frivaldszkyana day
(m/z 104, 91, 119, 65, 81 ,39)	0.01	-	-	-	-	-	0.19	-	-	-	-
(m/z 95, 39, 65, 50, 81, 124)	-	0.03	-	-	-	-	-	-	-	-	-
(m/z 109, 91, 67, 79, 43, 134)	-	0.85	-	-	-	-	-	-	-	-	-
(m/z 91, 43, 65, 162, 119, 145)	0.07	-	-	1.35	-	-	-	-	-	-	-
(m/z 81, 79, 43, 109, 53, 137)	-	-	-	-	-	-	-	-	-	-	-
(m/z 43, 67, 97, 59, 79, 113)	-	0.15	-	-	-	-	-	-	-	-	-
(m/z 43, 39, 67, 97, 79, 57)	-	-	-	-	-	-	-	-	-	-	-
(m/z 39, 43, 79, 107, 97, 55)	-	0.05	-	-	-	-	-	-	-	-	-
(m/z 43, 107, 79, 125, 137, 55)	-	0.04	-	-	-	-	-	-	-	-	-
(m/z 69, 41, 39, 81, 91, 133)	0.02	-	-	-	-	-	-	-	-	-	-
(m/z 43, 108, 58, 93, 123, 67)	-	-	-	-	-	-	-	-	-	-	-
(m/z 105, 57, 71, 43, 85, 177)	0.01	-	-	-	-	-	-	-	-	-	-
ng/mg of dry flower weight * min	2.35E+08	4.72E+08	1.32E+06	3.36E+07	5.81E+07	9.40E+05	1.12E+08	2.31E+08	9.67E+06	3.93E+07	1.48E+06
N	3	3	3	3	3	2	2	2	2	3	3

St= sesquiterpene. Mt=monoterpene. (*) are the new compunds for Sileneae.

	S.boryi night	S.boryi day	S.uniflora night	S.uniflora day	S.zawadzkii night	S.zawadzkii day	S.variegata night	S.variegata day	S.saxatilis night	S.saxatilis day	S. flavescens night
C5-branched chain compound											
Prenyl acetate*	0.05	-	-	-	-	-	-	-	-	-	-
Aromatics											
1, 4-Benzoquinone	-	-	-	-	-	-	-	-	-	-	-
Benzaldehyde	-	-	80.88	-	-	-	-	-	-	-	98.47
Benzylalcohol	7.81	-	-	-	-	-	-	-	-	-	-
Phenylacetaldehyde	-	-	-	-	-	-	-	-	-	-	-
2-Methoxyphenol	-	-	-	-	-	-	-	-	-	-	-
Methyl benzoate	-	-	-	-	-	-	-	-	1.11	-	-
2-Phenylethanol	-	-	0.22	-	-	-	1.21	-	-	-	-
p-methoxystyrene*	-	-	-	-	-	-	-	-	-	-	-
2-Phenylpropenal*	-	-	-	-	-	-	-	-	-	-	-
Benzyl acetate	5.95	-	-	-	-	-	-	-	-	0.29	-
Benzenepropanal*	-	-	-	-	-	-	-	-	-	-	-
1-Phenyl-1,2-propanedione	-	-	0.25	-	-	-	-	-	-	-	0.03
Methyl salicylate	-	-	4.27	-	-	-	-	-	1.99	-	-
Benzenepropanol	-	-	-	-	-	-	-	-	-	-	-
2-Phenylethyl acetate	-	-	-	-	-	-	-	-	-	-	-
p-methoxybenyaldehyde*	-	-	-	-	-	-	-	-	-	-	-
Hydroquinone*	-	-	-	-	-	-	-	-	-	-	-
Z-Cinnalmyl alcohol	-	-	-	-	-	-	-	-	-	-	-
E-Cinnamaldehyde	-	-	-	-	-	-	-	-	-	-	-
E-Cinnamyl alcohol	-	-	-	-	-	-	-	-	-	-	-
Methyl o-methoxybenzoate	-	-	0.03	-	-	-	-	-	-	-	-
Benzyl butyrate*	-	-	-	-	-	-	-	-	-	-	-
Eugenol*	-	-	-	-	-	-	-	-	-	-	-
3,4-Dimethoxystyrene	-	-	-	-	-	-	-	-	-	-	-
4-methoxyphenethyl alcohol*	-	-	-	-	-	-	0.05	-	-	-	-
Benzenepropyl acetate*	0.03	-	-	-	-	-	-	-	-	-	-
Methyleugenol*	-	-	-	-	-	-	-	-	-	-	-

	S.boryi night	S.boryi day	S.uniflora night	S.uniflora day	S.zawadzkii night	S.zawadzkii day	S.variegata night	S.variegata day	S.saxatilis night	S.saxatilis day	S. flavescens night
Vanillin*	-	-	-	-	-	-	-	-	-	-	-
Isoamyl benzoate	-	-	-	-	-	-	-	-	-	-	-
E-Cinnamyl acetate*	-	-	-	-	-	-	-	-	-	-	-
Z-Methyl isoeugenol*	-	-	-	-	-	-	-	-	-	-	-
3,4-dimethoxybenzaldehyde*	-	-	-	-	-	-	-	-	-	-	-
E-Methyl isoeugenol*	-	-	-	-	-	-	-	-	-	-	-
Z-3-Hexenyl benzoate	-	-	-	-	-	-	-	-	-	-	-
3,4,5 trimethoxybenzaldehyde*	-	-	-	-	-	-	-	-	-	-	-
Isoelemecin*	-	-	-	-	-	-	-	-	-	-	-
Benzyl benzoate	-	-	0.27	-	-	-	-	-	-	-	-
Aromatic (m/z 105, 77, 51, 50, 137, 152)	-	-	-	-	-	-	-	-	-	-	-
N-bearing compounds											
2-methylbutylaldoxime	-	-	-	-	-	-	-	-	-	-	-
3-methylbutylaldoxime	2.57	-	-	-	-	-	-	-	-	-	-
1-Nitropentane*	-	-	-	-	-	-	-	-	-	-	-
Methyl nicotinate*	-	-	-	-	-	-	-	-	-	-	-
Phenylacetonitrile*	-	-	-	-	-	-	-	-	-	-	-
Octanenitrile*	-	-	-	-	-	-	-	-	-	-	-
2-Aminobenzaldehyde*	-	-	-	-	-	-	-	-	-	-	-
Aromatic aldoxime*	-	-	-	-	-	-	-	-	-	-	-
Decanenitrile*	-	-	-	-	-	-	-	-	-	-	-
Indole*	0.74	-	-	-	-	-	-	-	-	-	-
1-Nitro-2-Phenylethane*	-	-	-	-	-	-	-	-	-	-	-
2-(dimethylamino)benzaldehyde*	-	-	-	-	-	-	-	-	-	-	-
N-bearing (m/z 124, 151, 81, 95, 165, 53)	-	-	-	-	-	-	-	-	-	-	-
N-bearing (m/z 54, 55, 84, 39, 41, 64)	-	-	-	-	-	-	-	-	-	-	-
N-bearing (m/z 79,110, 43, 53, 92, 39)	-	-	0.69	-	-	-	-	-	-	-	-
N-bearing (m/z 83, 55, 43, 39, 98, 153)	-	-	-	-	-	-	-	-	-	-	-
Terpenoids											
Z-β-ocimene	-	-	-	-	-	-	-	-	-	-	-
E-β-Ocimene	80.71	66.75	-	22.53	-	-	0.84	1.06	95.69	1.62	1.32
Camphene	-	-	-	-	37.57	-	8.01	18.62	-	10.22	-

	S.boryi night	S.boryi day	uniflora night	uniflora day	S.zawadzkii night	S.zawadzkii day	S.variegata night	S.variegata day	S.saxatilis night	S.saxatilis day	S. flavescens night
Sabinene*	-	-	-	-	-	-	-	54.09	-	69.55	-
b-Pinene	-	-	-	77.47	-	-	-	22.49	-	17.83	-
α-Phellandrene	-	-	-	-	-	-	-	-	-	-	-
Z-Arbusculone	-	-	5.54	-	5.04	16.84	8.14	0.59	-	-	-
E-Arbusculone	-	-	1.17	-	-	-	10.48	0.74	-	-	-
Z or E-Ocimenone*	0.19	-	-	-	-	-	-	-	-	-	-
Epoxy ocimene*	-	-	3.52	-	-	2.19	2.55	-	-	-	-
Geraniol*	-	-	0.10	-	-	-	0.79	-	-	-	-
Geranyl acetate*	0.28	-	-	-	-	-	-	-	-	-	-
β-Bourbonene	-	-	-	-	-	-	-	-	-	-	-
Linalool	-	-	-	-	1.01	-	-	-	-	-	-
Allo or neoallo-ocimene*	0.04	-	-	-	-	-	-	-	0.02	-	-
E-Ocimene oxide	0.24	-	-	-	-	-	-	-	0.47	0.34	0.02
Z+E-Linalooloxide pyranoid	-	-	-	-	-	-	-	-	-	-	-
4-Oxoisophorone*	-	-	-	-	-	-	-	-	-	-	-
LilacAldA	-	-	0.14	-	17.98	42.72	14.57	0.25	-	-	-
LilacAldB+C	-	-	1.41	-	21.97	3.17	31.25	0.81	-	-	-
LilacAldD	-	-	0.58	-	7.64	14.52	14.41	0.70	-	-	-
Lilac alcohol A	-	-	0.10	-	2.21	4.34	0.58	-	-	-	-
Lilac alcohol B+C	-	-	0.33	-	4.79	7.28	0.73	0.28	-	-	-
LilacAlc D	-	-	0.06	-	0.44	2.56	0.84	0.13	-	-	-
Lilac derivative (93, 43, 55, 81, 111, 67)	-	-	-	-	-	1.44	0.83	-	-	-	-
Lilac derivative (43, 55, 93, 111, 81, 39)	-	-	-	-	-	-	0.52	-	-	-	-
Lilac derivative (111, 43, 93, 77, 55, 136)	-	-	-	-	-	-	-	-	-	-	-
Lilac derivative (43, 71, 39, 55, 83, 107)	-	-	0.36	-	1.36	4.95	3.43	0.14	-	-	-
Lilac alcohol formate A	-	-	-	-	-	-	-	-	-	-	-
Lilac alcohol formate B+C	-	-	-	-	-	-	-	-	-	-	-
longifolene V4*	-	-	-	-	-	-	-	-	-	-	-
Germacrene D	-	-	-	-	-	-	-	-	-	-	-
E,E-a-Farnesene	0.03	-	-	-	-	-	-	-	-	-	-

	S.boryi night	S.boryi day	S.uniflora night	S.uniflora day	S.zawadzkii night	S.zawadzkii day	S.variegata night	S.variegata day	S.saxatilis night	S.saxatilis day	S. flavescens night
β-Selinene*	-	-	-	-	-	-	-	-	-	-	-
E-nerolidol*	0.06	-	-	-	-	-	-	-	-	-	-
Farnesol/al isomer*	0.21	-	-	-	-	-	-	-	-	-	-
Cubenol*	-	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 43, 55, 93, 67, 111, 137)	-	-	-	-	-	-	0.20	-	-	-	-
Mt-oxide (m/z 91, 43, 109, 79, 67, 134)	-	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 107, 91, 39, 79, 150, 122)	0.02	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 79, 105, 39, 135, 65)	0.11	-	-	-	-	-	-	-	0.18	-	-
Mt-oxide (m/z 93, 121, 43, 79, 67, 136)	-	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 119, 152, 39, 67, 79)	-	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 150, 39, 107, 79, 135)	0.11	3.71	-	-	-	-	-	-	0.30	0.14	<0.01
Mt-oxide (m/z 91, 119, 152, 39, 109, 79)	-	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 79, 108, 39, 91, 119, 43)	-	-	-	-	-	-	-	-	-	-	-
St (m/z 91, 105, 175, 189, 119, 204)	-	-	-	-	-	-	-	-	-	-	-
St (m/z 43, 95, 39, 79, 55, 59)	-	-	-	-	-	-	-	-	-	-	-
St (m/z 43, 39, 95, 55, 81, 67)	0.17	-	-	-	-	-	-	-	-	-	<0.01
St (m/z 43, 95, 79, 119, 174, 189)	0.13	-	-	-	-	-	-	-	-	-	-
St (m/z 119, 161, 204, 105, 134, 91)	-	-	-	-	-	-	-	-	-	-	-
St (m/z 91, 39, 161, 204, 105, 79)	-	-	-	-	-	-	-	-	-	-	-
St (m/z 91, 133, 161, 189, 79, 150)	-	-	-	-	-	-	-	-	-	-	<0.01
St-oxide (m/z 93, 91, 39, 43, 55, 67)	0.05	-	-	-	-	-	-	-	-	-	-
Spiroacetal											
2-ethyl-1,6 dioxaspiro[4.4]nonane*	-	-	-	-	-	-	-	-	-	-	-
Unknown compounds											
(m/z 94, 137, 39, 81, 43, 78)	-	-	-	-	-	-	-	-	-	-	-
(m/z 91, 135, 39, 107, 65, 79)	-	-	-	-	-	-	-	-	0.18	-	0.01
(m/z 103, 102, 77, 132, 51, 63)	-	-	-	-	-	-	-	-	-	-	-
(m/z 91, 65, 39, 105, 131, 146)	-	-	-	-	-	-	-	-	-	-	-
(m/z 121, 136 ,83, 69, 55, 42)	-	-	-	-	-	-	-	-	-	-	-
(m/z 104, 91, 119, 65, 81 ,39)	-	-	-	-	-	-	-	-	-	-	-

	S.boryi night	S.boryi day	S.uniflora night	S.uniflora day	S.zawadzkii night	S.zawadzkii day	S.variegata night	S.variegata day	S.saxatilis night	S.saxatilis day	S. flavescens night
(m/z 95, 39, 65, 50, 81, 124)	0.04	-	-	-	-	-	-	-	-	-	-
(m/z 109, 91, 67, 79, 43, 134)	0.20	-	-	-	-	-	-	-	-	-	-
(m/z 91, 43, 65, 162, 119, 145)	-	-	0.07	-	-	-	-	-	-	-	0.14
(m/z 81, 79, 43, 109, 53, 137)	-	-	-	-	-	-	0.56	0.10	-	-	-
(m/z 43, 67, 97, 59, 79, 113)	-	-	-	-	-	-	-	-	0.07	0.02	0.01
(m/z 43, 39, 67, 97, 79, 57)	0.27	-	-	-	-	-	-	-	-	-	-
(m/z 39, 43, 79, 107, 97, 55)	-	-	-	-	-	-	-	-	-	-	-
(m/z 43, 107, 79, 125, 137, 55)	-	-	-	-	-	-	-	-	-	-	-
(m/z 69, 41, 39, 81, 91, 133)	-	-	-	-	-	-	-	-	-	-	-
(m/z 43, 108, 58, 93, 123, 67)	-	29.54	-	-	-	-	-	-	-	-	-
(m/z 105, 57, 71, 43, 85, 177)	-	-	-	-	-	-	-	-	-	-	-
ng/mg of dry flower weight * min	1.45E+08	1.95E+05	1.70E+08	1.87E+07	6.65E+06	7.58E+06	1.21E+06	9.58E+06	7.69E+07	1.32E+08	1.05E+09
N	1	1	2	2	1	1	2	2	1	1	1

St= sesquiterpene. Mt=monoterpene. (*) are the new compunds for Sileneae.

	S.aegyptiaca night	S.aegyptiaca day	S.linicola night	S.linicola day	S.fraudatrix night	S.fraudatrix day	S.fuscata night	S.fuscata day	S.littorea night	S.littorea day
C5-branched chain compound										
Prenyl acetate*	-	-	-	-	-	-	-	-	-	-
Aromatics										
1, 4-Benzoquinone	-	-	-	-	-	-	-	0.19	-	-
Benzaldehyde	-	-	-	-	-	-	-	-	63.59	-
Benzylalcohol	-	-	-	-	-	-	-	-	0.58	-
Phenylacetaldehyde	-	-	-	-	-	-	-	-	-	-
2-Methoxyphenol	-	-	-	-	-	-	-	-	-	-
Methyl benzoate	-	-	-	-	-	-	-	-	0.02	-
2-Phenylethanol	-	-	-	-	-	1.96	-	-	0.01	-
p-methoxystyrene*	-	-	-	-	-	-	-	-	0.01	-
2-Phenylpropenal*	-	-	-	-	-	-	-	-	-	-
Benzyl acetate	-	-	-	0.63	-	-	-	-	35.72	-
Benzenepropanal*	-	-	-	-	-	-	-	-	-	-
1-Phenyl-1,2-propanedione	-	-	-	-	-	-	-	-	-	-
Methyl salicylate	-	-	-	-	-	-	-	-	-	-
Benzenepropanol	-	-	-	-	-	-	-	-	-	-
2-Phenylethyl acetate	-	-	-	-	-	-	-	-	<0.01	-
p-methoxybenyaldehyde*	-	-	-	-	-	-	-	-	-	-
Hydroquinone*	-	-	-	-	-	-	-	-	-	-
Z-Cinnalmyl alcohol	-	-	-	-	-	-	-	-	-	-
E-Cinnamaldehyde	-	-	-	-	-	-	-	-	-	-
E-Cinnamyl alcohol	-	-	-	-	-	-	-	-	-	-
Methyl o-methoxybenzoate	-	-	-	-	-	-	-	-	-	-
Benzyl butyrate*	-	-	-	-	-	-	-	-	-	-
Eugenol*	-	-	-	-	-	-	-	-	0.01	-
3,4-Dimethoxystyrene	-	-	-	-	-	-	-	-	0.01	-
4-methoxyphenethyl alcohol*	-	-	-	-	-	-	-	-	-	-
Benzenepropyl acetate*	-	-	-	-	-	-	-	-	-	-
Methyleugenol*	-	-	-	-	-	-	-	-	0.01	-

	S.aegyptiaca night	S.aegyptiaca day	S.linicola night	S.linicola day	S.fraudatrix night	S.fraudatrix day	S.fuscata night	S.fuscata day	S.littorea night	S.littorea day
Vanillin*	-	-	-	-	-	-	-	-	-	-
Isoamyl benzoate	-	-	-	-	-	-	-	-	-	-
E-Cinnamyl acetate*	-	-	-	-	-	-	-	-	-	-
Z-Methyl isoeugenol*	-	-	-	-	-	-	-	-	<0.01	-
3,4-dimethoxybenzaldehyde*	-	-	-	-	-	-	-	-	0.01	-
E-Methyl isoeugenol*	-	-	-	-	-	-	-	-	0.01	-
Z-3-Hexenyl benzoate	-	-	-	-	-	-	-	-	-	-
3,4,5 trimethoxybenzaldehyde*	-	-	-	-	-	-	-	-	-	-
Isoelemecin*	-	-	-	-	-	-	-	-	-	-
Benzyl benzoate	-	-	-	-	-	-	-	-	-	-
Aromatic (m/z 105, 77, 51, 50, 137, 152)	-	-	-	-	-	-	-	-	-	-
N-bearing compounds										
2-methylbutylaldoxime	-	-	-	-	-	-	-	-	-	-
3-methylbutylaldoxime	-	-	-	-	-	-	-	-	-	-
1-Nitropentane*	-	-	-	-	-	-	-	-	-	-
Methyl nicotinate*	-	-	-	-	-	-	-	-	-	-
Phenylacetonitrile*	-	-	-	-	-	-	-	-	-	-
Octanenitrile*	-	-	-	-	-	-	-	-	-	-
2-Aminobenzaldehyde*	-	-	-	-	-	-	-	-	-	-
Aromatic aldoxime*	-	-	-	-	-	-	-	-	-	-
Decanenitrile*	-	-	-	-	-	-	-	-	-	-
Indole*	-	-	-	-	-	-	-	-	-	-
1-Nitro-2-Phenylethane*	-	-	-	-	-	-	-	-	-	-
2-(dimethylamino)benzaldehyde*	-	-	-	-	-	-	-	-	-	-
N-bearing (m/z 124, 151, 81, 95, 165, 53)	-	-	-	-	-	-	0.29	1.48	-	-
N-bearing (m/z 54, 55, 84, 39, 41, 64)	-	-	-	-	-	-	-	-	-	-
N-bearing (m/z 79,110, 43, 53, 92, 39)	-	-	-	-	-	-	-	-	-	-
N-bearing (m/z 83, 55, 43, 39, 98, 153)	-	-	-	-	-	-	-	-	-	-
Terpenoids										
Z-β-ocimene	-	-	-	0.02	-	-	-	-	-	-
E-β-Ocimene	99.18	10.32	100.00	12.99	-	96.41	96.33	75.80	0.01	100.00

	S.aegyptiaca night	S.aegyptiaca day	S.linicola night	S.linicola day	S.fraudatrix night	S.fraudatrix day	S.fuscata night	S.fuscata day	S.littorea night	S.littorea day
Camphene	-	16.13	-	15.20	-	-	-	-	-	-
Sabinene*	-	53.38	-	47.90	-	-	-	-	-	-
b-Pinene	-	19.34	-	22.72	-	-	-	-	-	-
α-Phellandrene	-	-	-	-	-	-	-	-	-	-
Z-Arbusculone	-	-	-	-	-	-	-	-	-	-
E-Arbusculone	-	-	-	-	-	-	-	-	-	-
Z or E-Ocimenone*	-	-	-	-	-	-	-	-	-	-
Epoxy ocimene*	-	-	-	-	-	-	-	-	-	-
Geraniol*	-	-	-	-	-	-	-	-	-	-
Geranyl acetate*	-	-	-	-	-	-	-	-	-	-
β-Bourbonene	-	-	-	-	-	-	-	-	-	-
Linalool	-	-	-	-	-	-	-	-	-	-
Allo or neoallo-ocimene*	0.10	-	-	-	-	-	-	-	-	-
E-Ocimene oxide	-	-	-	0.08	100.00	1.64	-	-	-	-
Z+E-Linalooloxide pyranoid	-	-	-	-	-	-	-	-	-	-
4-Oxoisophorone*	-	-	-	-	-	-	-	-	-	-
LilacAldA	-	-	-	-	-	-	-	-	-	-
LilacAldB+C	-	-	-	-	-	-	-	-	-	-
LilacAldD	-	-	-	-	-	-	-	-	-	-
Lilac alcohol A	-	-	-	-	-	-	-	-	-	-
Lilac alcohol B+C	-	-	-	-	-	-	-	-	-	-
LilacAlc D	-	-	-	-	-	-	-	-	-	-
Lilac derivative (93, 43, 55, 81, 111, 67)	-	-	-	-	-	-	-	-	-	-
Lilac derivative (43, 55, 93, 111, 81, 39)	-	-	-	-	-	-	-	-	-	-
Lilac derivative (111, 43, 93, 77, 55, 136)	-	-	-	-	-	-	-	-	-	-
Lilac derivative (43, 71, 39, 55, 83, 107)	-	-	-	-	-	-	-	-	-	-
Lilac alcohol formate A	-	-	-	-	-	-	-	-	-	-
Lilac alcohol formate B+C	-	-	-	-	-	-	-	-	-	-
longifolene V4*	-	-	-	-	-	-	0.29	4.84	-	-
Germacrene D	-	-	-	-	-	-	-	-	-	-
E,E-a-Farnesene	-	-	-	-	-	-	-	-	-	-

	S.aegyptiaca night	S.aegyptiaca day	S.linicola night	S.linicola day	S.fraudatrix night	S.fraudatrix day	S.fuscata night	S.fuscata day	S.littorea night	S.littorea day
β-Selinene*	-	-	-	-	-	-	-	-	-	-
E-nerolidol*	-	-	-	-	-	-	-	-	-	-
Farnesol/al isomer*	-	-	-	-	-	-	-	-	-	-
Cubenol*	-	-	-	-	-	-	0.29	-	-	-
Mt-oxide (m/z 43, 55, 93, 67, 111, 137)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 43, 109, 79, 67, 134)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 107, 91, 39, 79, 150, 122)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 79, 105, 39, 135, 65)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 93, 121, 43, 79, 67, 136)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 119, 152, 39, 67, 79)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 150, 39, 107, 79, 135)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 119, 152, 39, 109, 79)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 79, 108, 39, 91, 119, 43)	-	-	-	-	-	-	-	-	-	-
St (m/z 91, 105, 175, 189, 119, 204)	-	-	-	-	-	-	-	-	-	-
St (m/z 43, 95, 39, 79, 55, 59)	-	-	-	-	-	-	-	-	-	-
St (m/z 43, 39, 95, 55, 81, 67)	-	-	-	0.16	-	-	-	-	-	-
St (m/z 43, 95, 79, 119, 174, 189)	-	-	-	-	-	-	-	-	-	-
St (m/z 119, 161, 204, 105, 134, 91)	-	-	-	-	-	-	0.61	-	-	-
St (m/z 91, 39, 161, 204, 105, 79)	-	-	-	-	-	-	2.19	17.68	-	-
St (m/z 91, 133, 161, 189, 79, 150)	-	-	-	-	-	-	-	-	-	-
St-oxide (m/z 93, 91, 39, 43, 55, 67)	-	-	-	-	-	-	-	-	-	-
Spiroacetal										
2-ethyl-1,6 dioxaspiro[4.4]nonane*	0.57	0.15	-	-	-	-	-	-	-	-
Unknown compounds										
(m/z 94, 137, 39, 81, 43, 78)	-	-	-	-	-	-	-	-	-	-
(m/z 91, 135, 39, 107, 65, 79)	0.09	-	-	0.06	-	-	-	-	-	-
(m/z 103, 102, 77, 132, 51, 63)	-	-	-	-	-	-	-	-	-	-
(m/z 91, 65, 39, 105, 131, 146)	-	-	-	-	-	-	-	-	-	-
(m/z 121, 136 ,83, 69, 55, 42)	-	-	-	-	-	-	-	-	-	-

	S.aegyptiaca night	S.aegyptiaca day	S.linicola night	S.linicola day	S.fraudatrix night	S.fraudatrix day	S.fuscata night	S.fuscata day	S.littorea night	S.littorea day
(m/z 104, 91, 119, 65, 81 ,39)	-	-	-	-	-	-	-	-	-	-
(m/z 95, 39, 65, 50, 81, 124)	-	-	-	-	-	-	-	-	-	-
(m/z 109, 91, 67, 79, 43, 134)	-	-	-	-	-	-	-	-	-	-
(m/z 91, 43, 65, 162, 119, 145)	0.05	0.69	-	-	-	-	-	-	-	-
(m/z 81, 79, 43, 109, 53, 137)	-	-	-	-	-	-	-	-	-	-
(m/z 43, 67, 97, 59, 79, 113)	-	-	-	0.25	-	-	-	-	-	-
(m/z 43, 39, 67, 97, 79, 57)	-	-	-	-	-	-	-	-	-	-
(m/z 39, 43, 79, 107, 97, 55)	-	-	-	-	-	-	-	-	-	-
(m/z 43, 107, 79, 125, 137, 55)	-	-	-	-	-	-	-	-	-	-
(m/z 69, 41, 39, 81, 91, 133)	-	-	-	-	-	-	-	-	-	-
(m/z 43, 108, 58, 93, 123, 67)	-	-	-	-	-	-	-	-	-	-
(m/z 105, 57, 71, 43, 85, 177)	-	-	-	-	-	-	-	-	-	-
ng/mg of dry flower weight * min	1.50E+07	2.98E+07	5.69E+05	1.29E+08	4.25E+05	5.93E+06	2.70E+07	2.09E+06	5.35E+09	2.95E+06
N	3	3	2	2	2	2	2	2	3	3

St= sesquiterpene. Mt=monoterpene. (*) are the new compunds for Sileneae.

	S.cambessedesii night	S.cambessedesii day	S.psammitis night	S.psammitis day	S.scabriflora night	S.scabriflora day	S.fabaria night	S.fabaria day	S.fabarioides night	S.fabarioides day
C5-branched chain compound										
Prenyl acetate*	-	-	-	-	-	-	-	-	-	-
Aromatics										
1, 4-Benzoquinone	-	-	-	-	-	-	4.22	9.24	-	-
Benzaldehyde	86.65	-	39.82	-	-	-	-	-	-	-
Benzylalcohol	3.32	-	2.38	-	-	-	-	-	-	-
Phenylacetaldehyde	-	-	-	-	-	-	-	-	-	-
2-Methoxyphenol	-	-	-	-	-	-	-	-	-	-
Methyl benzoate	-	-	-	-	-	-	-	-	-	-
2-Phenylethanol	0.38	-	1.50	-	-	-	-	-	-	-
p-methoxystyrene*	2.50	-	-	-	-	-	-	-	-	-
2-Phenylpropenal*	-	-	-	-	-	-	-	-	-	-
Benzyl acetate	-	-	-	0.40	-	-	-	-	-	-
Benzenepropanal*	0.87	-	-	-	-	-	-	-	-	-
1-Phenyl-1,2-propanedione	-	-	-	-	-	-	-	-	-	-
Methyl salicylate	-	-	-	-	-	-	-	-	-	-
Benzenepropanol	1.61	-	0.25	-	-	-	-	-	-	-
2-Phenylethyl acetate	-	-	-	-	-	-	-	-	-	-
p-methoxybenyaldehyde*	0.19	-	-	-	-	-	-	-	-	-
Hydroquinone*	-	-	-	-	-	-	2.16	2.64	-	-
Z-Cinnalmyl alcohol	0.11	-	0.32	-	-	-	-	-	-	-
E-Cinnamaldehyde	0.70	-	1.57	-	-	-	-	-	-	-
E-Cinnamyl alcohol	1.34	-	4.45	-	-	-	-	-	-	-
Methyl o-methoxybenzoate	-	-	-	-	-	-	-	-	-	-
Benzyl butyrate*	0.02	-	-	-	-	-	-	-	-	-
Eugenol*	-	-	-	-	-	-	-	-	-	-
3,4-Dimethoxystyrene	0.32	-	-	-	-	-	-	-	-	-
4-methoxyphenethyl alcohol*	-	-	-	-	-	-	-	-	-	-
Benzenepropyl acetate*	-	-	-	-	-	-	-	-	-	-
Methyleugenol*	-	-	-	-	-	-	-	-	-	-

	S.cambessedesii night	S.cambessedesii day	S.psammitis night	S.psammitis day	S.scabriflora night	S.scabriflora day	S.fabaria night	S.fabaria day	S.fabarioides night	S.fabarioides day
Vanillin*	-	-	-	-	-	-	-	-	-	-
Isoamyl benzoate	-	-	-	-	-	-	-	-	-	-
E-Cinnamyl acetate*	-	-	0.18	-	-	-	-	-	-	-
Z-Methyl isoeugenol*	-	-	-	-	-	-	-	-	-	-
3,4-dimethoxybenzaldehyde*	-	-	-	-	-	-	-	-	-	-
E-Methyl isoeugenol*	-	-	-	-	-	-	-	-	-	-
Z-3-Hexenyl benzoate	-	-	-	-	-	-	-	-	-	-
3,4,5 trimethoxybenzaldehyde*	-	-	0.04	-	-	-	-	-	-	-
Isoelemecin*	-	-	0.44	-	-	-	-	-	-	-
Benzyl benzoate	1.20	-	-	-	-	-	-	-	-	-
Aromatic (m/z 105, 77, 51, 50, 137, 152)	-	-	-	-	-	-	-	-	-	-
N-bearing compounds										
2-methylbutylaldoxime	-	-	38.93	-	-	-	-	-	-	-
3-methylbutylaldoxime	-	-	-	-	-	-	-	-	-	-
1-Nitropentane*	-	-	-	-	-	-	-	-	-	-
Methyl nicotinate*	-	-	-	-	-	-	-	-	-	-
Phenylacetonitrile*	-	-	-	-	3.46	-	-	-	-	-
Octanenitrile*	-	-	0.08	-	-	-	-	-	-	-
2-Aminobenzaldehyde*	-	-	-	0.07	-	-	-	-	-	-
Aromatic aldoxime*	-	-	-	-	2.77	-	-	-	-	-
Decanenitrile*	-	-	0.05	-	-	-	-	-	-	-
Indole*	-	-	-	2.40	-	-	-	-	-	-
1-Nitro-2-Phenylethane*	-	-	-	-	0.97	-	-	-	-	-
2-(dimethylamino)benzaldehyde*	-	-	-	0.04	-	-	-	-	-	-
N-bearing (m/z 124, 151, 81, 95, 165, 53)	-	-	-	-	-	-	-	-	-	-
N-bearing (m/z 54, 55, 84, 39, 41, 64)	-	-	8.29	-	-	-	-	-	-	-
N-bearing (m/z 79,110, 43, 53, 92, 39)	-	-	-	-	-	-	-	-	-	-
N-bearing (m/z 83, 55, 43, 39, 98, 153)	-	-	-	-	-	-	-	-	-	-
Terpenoids										
Z-β-ocimene	-	-	-	-	-	-	-	-	-	-
E-β-Ocimene	0.56	100.00	1.52	3.31	90.18	59.98	1.54	0.88	-	-

	S.cambessedesii night	S.cambessedesii day	S.psammitis night	S.psammitis day	S.scabriflora night	S.scabriflora day	S.fabaria night	S.fabaria day	S.fabarioides night	S.fabarioides day
Camphene	-	-	-	15.37	-	-	-	87.24	-	99.27
Sabinene*	-	-	-	78.30	-	-	-	-	-	-
b-Pinene	-	-	-	-	-	-	-	-	-	-
α-Phellandrene	-	-	-	-	-	-	-	-	-	-
Z-Arbusculone	-	-	-	-	-	-	-	-	-	-
E-Arbusculone	-	-	-	-	-	-	-	-	-	-
Z or E-Ocimenone*	-	-	-	-	-	-	-	-	-	-
Epoxy ocimene*	-	-	-	-	-	-	-	-	-	-
Geraniol*	-	-	-	-	-	-	-	-	-	-
Geranyl acetate*	-	-	-	-	-	-	-	-	-	-
β-Bourbonene	-	-	-	-	-	1.63	-	-	-	-
Linalool	-	-	-	-	-	9.76	91.12	-	97.82	-
Allo or neoallo-ocimene*	-	-	-	-	-	-	-	-	-	-
E-Ocimene oxide	-	-	-	-	-	-	-	-	-	-
Z+E-Linalooloxide pyranoid	-	-	-	-	1.71	18.91	-	-	-	-
4-Oxoisophorone*	-	-	-	-	-	-	-	-	-	-
LilacAldA	-	-	-	-	-	-	0.01	-	0.32	0.09
LilacAldB+C	-	-	-	-	-	-	0.37	-	1.27	0.46
LilacAldD	-	-	-	-	-	-	0.37	-	0.59	0.18
Lilac alcohol A	-	-	-	-	-	-	-	-	-	-
Lilac alcohol B+C	-	-	-	-	-	-	0.06	-	-	-
LilacAlc D	-	-	-	-	-	-	0.05	-	-	-
Lilac derivative (93, 43, 55, 81, 111, 67)	-	-	-	-	-	-	-	-	-	-
Lilac derivative (43, 55, 93, 111, 81, 39)	-	-	-	-	-	-	-	-	-	-
Lilac derivative (111, 43, 93, 77, 55, 136)	-	-	-	-	-	-	-	-	-	-
Lilac derivative (43, 71, 39, 55, 83, 107)	-	-	-	-	-	-	-	-	-	-
Lilac alcohol formate A	-	-	-	-	-	-	-	-	-	-
Lilac alcohol formate B+C	-	-	-	-	-	-	-	-	-	-
longifolene V4*	-	-	-	-	-	-	-	-	-	-
Germacrene D	-	-	-	-	-	-	-	-	-	-
E,E-a-Farnesene	0.22	-	0.16	-	-	-	-	-	-	-
β-Selinene*	-	-	-	-	-	8.50	-	-	-	-

	S.cambessedesii night	S.cambessedesii day	S.psammitis night	S.psammitis day	S.scabriflora night	S.scabriflora day	S.fabaria night	S.fabaria day	S.fabarioides night	S.fabarioides day
E-nerolidol*	-	-	-	-	-	-	0.10	-	-	-
Farnesol/al isomer*	-	-	-	-	-	-	-	-	-	-
Cubenol*	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 43, 55, 93, 67, 111, 137)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 43, 109, 79, 67, 134)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 107, 91, 39, 79, 150, 122)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 79, 105, 39, 135, 65)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 93, 121, 43, 79, 67, 136)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 119, 152, 39, 67, 79)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 150, 39, 107, 79, 135)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 91, 119, 152, 39, 109, 79)	-	-	-	-	-	-	-	-	-	-
Mt-oxide (m/z 79, 108, 39, 91, 119, 43)	-	-	-	-	-	-	-	-	-	-
St (m/z 91, 105, 175, 189, 119, 204)	-	-	-	-	-	0.66	-	-	-	-
St (m/z 43, 95, 39, 79, 55, 59)	-	-	-	-	-	-	-	-	-	-
St (m/z 43, 39, 95, 55, 81, 67)	-	-	-	-	-	-	-	-	-	-
St (m/z 43, 95, 79, 119, 174, 189)	-	-	-	-	-	-	-	-	-	-
St (m/z 119, 161, 204, 105, 134, 91)	-	-	-	-	-	-	-	-	-	-
St (m/z 91, 39, 161, 204, 105, 79)	-	-	-	-	-	-	-	-	-	-
St (m/z 91, 133, 161, 189, 79, 150)	-	-	-	-	-	-	-	-	-	-
St-oxide (m/z 93, 91, 39, 43, 55, 67)	-	-	-	-	-	-	-	-	-	-
Spiroacetal										
2-ethyl-1,6 dioxaspiro[4.4]nonane*	-	-	-	-	-	-	-	-	-	-
Unknown compounds										
(m/z 94, 137, 39, 81, 43, 78)	-	-	-	-	0.90	0.57	-	-	-	-
(m/z 91, 135, 39, 107, 65, 79)	-	-	-	-	-	-	-	-	-	-
(m/z 103, 102, 77, 132, 51, 63)	-	-	-	-	-	-	-	-	-	-
(m/z 91, 65, 39, 105, 131, 146)	-	-	-	0.11	-	-	-	-	-	-

	S.cambessedesii night	S.cambessedesii day	S.psammitis night	S.psammitis day	S.scabriflora night	S.scabriflora day	S.fabaria night	S.fabaria day	S.fabarioides night	S.fabarioides day
(m/z 121, 136 ,83, 69, 55, 42)	-	-	-	-	-	-	-	-	-	-
(m/z 104, 91, 119, 65, 81 ,39)	-	-	-	-	-	-	-	-	-	-
(m/z 95, 39, 65, 50, 81, 124)	-	-	-	-	-	-	-	-	-	-
(m/z 109, 91, 67, 79, 43, 134)	-	-	-	-	-	-	-	-	-	-
(m/z 91, 43, 65, 162, 119, 145)	-	-	-	-	-	-	-	-	-	-
(m/z 81, 79, 43, 109, 53, 137)	-	-	-	-	-	-	-	-	-	-
(m/z 43, 67, 97, 59, 79, 113)	-	-	-	-	-	-	-	-	-	-
(m/z 43, 39, 67, 97, 79, 57)	-	-	-	-	-	-	-	-	-	-
(m/z 39, 43, 79, 107, 97, 55)	-	-	-	-	-	-	-	-	-	-
(m/z 43, 107, 79, 125, 137, 55)	-	-	-	-	-	-	-	-	-	-
(m/z 69, 41, 39, 81, 91, 133)	-	-	-	-	-	-	-	-	-	-
(m/z 43, 108, 58, 93, 123, 67)	-	-	-	-	-	-	-	-	-	-
(m/z 105, 57, 71, 43, 85, 177)	-	-	-	-	-	-	-	-	-	-
ng/mg of dry flower weight * min	6.49E+08	3.66E+06	5.32E+07	3.24E+07	3.76E+06	1.59E+06	1.35E+07	4.14E+06	7.42E+06	1.28E+06
N	3	3	3	3	3	3	3	3	1	1

St= sesquiterpene. Mt=monoterpene. (*) are the new compunds for Sileneae.

	S.holzmannii night	L.coronaria night	L.coronaria day	S.tatarica night	S.tatarica day
C5-branched chain compound					
Prenyl acetate*	-	-	-	-	-
Aromatics					
1, 4-Benzoquinone	-	-	-	-	17.46
Benzaldehyde	-	-	-	-	-
Benzylalcohol	-	-	-	5.48	-
Phenylacetaldehyde	-	-	-	-	-
2-Methoxyphenol	-	-	-	-	-
Methyl benzoate	-	-	-	-	-
2-Phenylethanol	-	100.00	62.50	-	2.11
p-methoxystyrene*	-	-	-	-	-
2-Phenylpropenal*	-	-	-	-	-
Benzyl acetate	-	-	-	-	-
Benzenepropanal*	-	-	-	-	-
1-Phenyl-1,2-propanedione	-	-	-	-	-
Methyl salicylate	-	-	-	-	-
Benzenepropanol	-	-	-	-	-
2-Phenylethyl acetate	-	-	-	-	-
p-methoxybenyaldehyde*	-	-	-	-	-
Hydroquinone*	-	-	-	-	-
Z-Cinnalmyl alcohol	-	-	-	-	-
E-Cinnamaldehyde	-	-	-	-	-
E-Cinnamyl alcohol	-	-	-	0.46	-
Methyl o-methoxybenzoate	-	-	37.50	-	-
Benzyl butyrate*	-	-	-	-	-
Eugenol*	-	-	-	-	-
3,4-Dimethoxystyrene	-	-	-	-	-
4-methoxyphenethyl alcohol*	-	-	-	-	-
Benzenepropyl acetate*	-	-	-	-	-
Methyleugenol*	-	-	-	-	-

	S.holzmannii night	L.coronaria night	L.coronaria day	S.tatarica night	S.tatarica day
Vanillin*	-	-	-	-	-
Isoamyl benzoate	-	-	-	0.07	-
E-Cinnamyl acetate*	-	-	-	-	-
Z-Methyl isoeugenol*	-	-	-	-	-
3,4-dimethoxybenzaldehyde*	-	-	-	-	-
E-Methyl isoeugenol*	-	-	-	-	-
Z-3-Hexenyl benzoate	-	-	-	0.17	-
3,4,5 trimethoxybenzaldehyde*	-	-	-	-	-
Isoelemecin*	-	-	-	-	-
Benzyl benzoate	-	-	-	1.39	-
Aromatic (m/z 105, 77, 51, 50, 137, 152)	-	-	-	-	-
N-bearing compounds					
2-methylbutylaldoxime	-	-	-	42.10	-
3-methylbutylaldoxime	-	-	-	9.20	-
1-Nitropentane*	-	-	-	23.33	-
Methyl nicotinate*	-	-	-	-	-
Phenylacetonitrile*	-	-	-	-	-
Octanenitrile*	-	-	-	-	-
2-Aminobenzaldehyde*	-	-	-	-	-
Aromatic aldoxime*	-	-	-	-	-
Decanenitrile*	-	-	-	-	-
Indole*	-	-	-	-	-
1-Nitro-2-Phenylethane*	-	-	-	-	-
2-(dimethylamino)benzaldehyde*	-	-	-	-	-
N-bearing (m/z 124, 151, 81, 95, 165, 53)	-	-	-	-	-
N-bearing (m/z 54, 55, 84, 39, 41, 64)	-	-	-	5.88	-
N-bearing (m/z 79,110, 43, 53, 92, 39)	-	-	-	-	-
N-bearing (m/z 83, 55, 43, 39, 98, 153)	-	-	-	0.07	-
Terpenoids					
Z-β-ocimene	-	-	-	-	-
E-β-Ocimene	-	-	-	0.51	75.51
Camphene	-	-	-	-	-

	S.holzmannii night	L.coronaria night	L.coronaria day	S.tatarica night	S.tatarica day
Sabinene*	-	-	-	-	-
b-Pinene	-	-	-	-	-
α-Phellandrene	-	-	-	0.16	-
Z-Arbusculone	-	-	-	-	-
E-Arbusculone	-	-	-	-	-
Z or E-Ocimenone*	-	-	-	-	-
Epoxy ocimene*	-	-	-	-	-
Geraniol*	-	-	-	-	-
Geranyl acetate*	-	-	-	-	-
β-Bourbonene	-	-	-	-	-
Linalool	100.00	-	-	10.96	-
Allo or neoallo-ocimene*	-	-	-	-	-
E-Ocimene oxide	-	-	-	0.04	4.92
Z+E-Linalooloxide pyranoid	-	-	-	-	-
4-Oxoisophorone*	-	-	-	-	-
LilacAldA	-	-	-	-	-
LilacAldB+C	-	-	-	-	-
LilacAldD	-	-	-	-	-
Lilac alcohol A	-	-	-	-	-
Lilac alcohol B+C	-	-	-	-	-
LilacAlc D	-	-	-	-	-
Lilac derivative (93, 43, 55, 81, 111, 67)	-	-	-	-	-
Lilac derivative (43, 55, 93, 111, 81, 39)	-	-	-	-	-
Lilac derivative (111, 43, 93, 77, 55, 136)	-	-	-	-	-
Lilac derivative (43, 71, 39, 55, 83, 107)	-	-	-	-	-
Lilac alcohol formate A	-	-	-	-	-
Lilac alcohol formate B+C	-	-	-	-	-
longifolene V4*	-	-	-	-	-
Germacrene D	-	-	-	-	-
E,E-a-Farnesene	-	-	-	0.19	-
β-Selinene*	-	-	-	-	-
E-nerolidol*	-	-	-	-	-

	S.holzmannii night	L.coronaria night	L.coronaria day	S.tatarica night	S.tatarica day
Farnesol/al isomer*	-	-	-	-	-
Cubenol*	-	-	-	-	-
Mt-oxide (m/z 43, 55, 93, 67, 111, 137)	-	-	-	-	-
Mt-oxide (m/z 91, 43, 109, 79, 67, 134)	-	-	-	-	-
Mt-oxide (m/z 107, 91, 39, 79, 150, 122)	-	-	-	-	-
Mt-oxide (m/z 91, 79, 105, 39, 135, 65)	-	-	-	-	-
Mt-oxide (m/z 93, 121, 43, 79, 67, 136)	-	-	-	-	-
Mt-oxide (m/z 91, 119, 152, 39, 67, 79)	-	-	-	-	-
Mt-oxide (m/z 91, 150, 39, 107, 79, 135)	-	-	-	-	-
Mt-oxide (m/z 91, 119, 152, 39, 109, 79)	-	-	-	-	-
Mt-oxide (m/z 79, 108, 39, 91, 119, 43)	-	-	-	-	-
St (m/z 91, 105, 175, 189, 119, 204)	-	-	-	-	-
St (m/z 43, 95, 39, 79, 55, 59)	-	-	-	-	-
St (m/z 43, 39, 95, 55, 81, 67)	-	-	-	-	-
St (m/z 43, 95, 79, 119, 174, 189)	-	-	-	-	-
St (m/z 119, 161, 204, 105, 134, 91)	-	-	-	-	-
St (m/z 91, 39, 161, 204, 105, 79)	-	-	-	-	-
St (m/z 91, 133, 161, 189, 79, 150)	-	-	-	-	-
St-oxide (m/z 93, 91, 39, 43, 55, 67)	-	-	-	-	-
Spiroacetal					
2-ethyl-1,6 dioxaspiro[4.4]nonane*	-	-	-	-	-
Unknown compounds					
(m/z 94, 137, 39, 81, 43, 78)	-	-	-	-	-
(m/z 91, 135, 39, 107, 65, 79)	-	-	-	-	-
(m/z 103, 102, 77, 132, 51, 63)	-	-	-	-	-
(m/z 91, 65, 39, 105, 131, 146)	-	-	-	-	-
(m/z 121, 136 ,83, 69, 55, 42)	-	-	-	-	-
(m/z 104, 91, 119, 65, 81 ,39)	-	-	-	-	-
(m/z 95, 39, 65, 50, 81, 124)	-	-	-	-	-

	S.holzmannii night	L.coronaria night	L.coronaria day	S.tatarica night	S.tatarica day
(m/z 109, 91, 67, 79, 43, 134)	-	-	-	-	-
(m/z 91, 43, 65, 162, 119, 145)	-	-	-	-	-
(m/z 81, 79, 43, 109, 53, 137)	-	-	-	-	-
(m/z 43, 67, 97, 59, 79, 113)	-	-	-	-	-
(m/z 43, 39, 67, 97, 79, 57)	-	-	-	-	-
(m/z 39, 43, 79, 107, 97, 55)	-	-	-	-	-
(m/z 43, 107, 79, 125, 137, 55)	-	-	-	-	-
(m/z 69, 41, 39, 81, 91, 133)	-	-	-	-	-
(m/z 43, 108, 58, 93, 123, 67)	-	-	-	-	-
(m/z 105, 57, 71, 43, 85, 177)	-	-	-	-	-
ng/mg of dry flower weight * min	4.53E+03	1.72E+04	2.95E+04	4.25E+07	2.01E+06
N	1	1	2	3	3

St= sesquiterpene. Mt=monoterpene. (*) are the new compunds for Sileneae.

SUPPLEMENTARY MATERIAL 2. Green leaf volátiles (GLV) detected in eight Sileneae species (see Table 1).

	Compound		Compound
Aliphatics	Hexadiene	**Terpenoids**	Z-p-Mentha-2,8-dien-1-ol
	n-Heptanal		d-Limonene
	n-Octanal		Camphor
	n-Nonanal		Lavender lactone
	n-Decanal		Z-3-Hexen-1-ol butyrate
	n-Hexanal		Bornyl or isobornyl acetate
	n-Octane		Furan, 2, 4-dimethyl
	E-2-Decenal		β-Caryophyllene
	Z-3-Hexen-1-ol		cf Caryophylene oxide
	E-2-Hexen-1-ol		Longifolene
	Z-3-Hexen-1-ol acetate		α-Terpineol
	Hexylacetate		α-Caryophyllene
	Z-3-hexenylacetate		β-Cedrene
	E-2-hexenylacetate		α-Pinene
	Z-3-Hexen-1-ol butyrate		Unknown St 1 (m/z 105, 119, 161)
	(Z)-3-Hexenyl butyrate		Unknown St 2 (m/z 91, 105, 161)
	(E)-4,8 Dimethyl 1,3,7 nonatrienea		Unknown St 3 (m/z 91, 105, 204)
	Hexenol ester		Unknown St 4 (m/z 91, 161, 189)
Aromatics	Benzene, methoxy		Unknown St 5 (m/z 41, 77, 91)
	Benzyl alcohol, p-methyl		Unknown St 6 (m/z 39, 91, 131)
	Acetophenone		Unknown St-oxide 1 (m/z 39, 79, 136)
	Ethyltoluenea		Unknown St-oxide2 (m/z 39, 79, 91)
Miscellaneous	Alkene (m/z 41, 55, 69)		Unknown Mt-oxide (m/z 43, 55, 93)
Unknowns	Unknown 1 (m/z 43, 71, 108)		
	Unknown 2 (m/z 69, 97, 110)		
	Unknown 3 (m/z 78, 94, 105)		
	Unknown 4 (m/z 57, 79, 112)		

St= sesquiterpene. Mt=monoterpene.

CAPÍTULO 3/CHAPTER 3

Floral scent evolution in *Silene*: a multivariate phylogenetic analysis.

ABSTRACT

The composition and abundance of flower scent are key flower traits for chemical communication between plants and their pollinators. The qualitative and quantitative composition of floral scents vary within and among species, and are probably defined by balancing selection due to pollinators and herbivores but also by phylogenetic constraints. The congruence between flower fragrance and plant phylogeny has been studied previously but only focused on the qualitative dimension, and did not found much phylogenetic signal. We use phylogenetic principal component analysis (pPCA) and ancestral state reconstructions to analyze the evolution of 181 flower scent compounds in 38 *Silene* species. Our results show that the relative amount of some floral compounds correlates with the phylogeny of *Silene* at higher levels (genus and section) and produces a high phylogenetic signal. This signal was mainly characterized by the strong opposite effect between two monoterpenes, linalool and E-β-ocimene. Other compounds strongly differed among closely related species and are a likely consequence of evolutionary recent events. Our results show that pPCA combined with ancestral state reconstructions add a new dimension to the phylogenetic multivariate analyses used so far in evolutionary studies on floral scents.

INTRODUCTION

The composition of flower scent is an important flower trait for chemical communication between plants and their pollinators (Knudsen & Tollsen 1993, Knudsen *et al*., 2006). Therefore, like other flower traits such as size, shape and color, flower volatile compounds can be subjected to natural selection and adaptive evolution (Melendéz-Ackerman *et al*., 1997; Gómez *et al*., 2006; Parachnowitsch & Kessler 2010). The qualitative and quantitative compositions of floral scents vary within and among species (Knudsen & Gershenzon, 2006), and are probably defined by balancing selection due to pollinators and other selective agents (e.g., florivores), but also by phylogenetic constraints (Raguso, 2001). Phylogenetic effects on flower scents has been studied previously, but especially focused on the qualitative dimension (Azuma *et al*., 1999; Williams & Whitten, 1999; Barkman 2001; Levin *et al*., 2003; Steiner *et al*., 2011). By contrast, the quantitative information has been left aside because it has been suggested that the amount of scent compounds is too homoplastic to be used in phylogenetic studies (Barkman, 2001; Levin *et al*., 2003). However, floral scents can be specialized to attract a specific group of flower visitors not only through specific key compounds, but through particular ratios of more widespread compounds (Raguso, 2008). Recent works suggest that the variation in relative amounts of common volatile organic compounds (VOCs) is essential to differentially attract flower visitors. Therefore, the ratios of compounds may act as functional units of attraction and potentially be exposed to phenotypic selection (Andersson, 2003; Galen *et al*., 2011; Parachnowitsch *et al*., 2012). For instance, different relative amounts of the same VOCs lead to pollinator specificity, reproductive isolation and subsequently speciation in *Mitella*, *Silene* and several orchids species (Schiestl & Ayasse, 2002; Waelti *et al*., 2008; Ayasse *et al*., 2011; Okamoto *et al*., 2015; Sun *et al*., 2015). For these reasons the flower blend, not only the qualitative but also the quantitative dimension should be included in phylogenetic analyses on the evolution of floral scent.

Phylogeny reveals non-independence among trait values in taxa (Dobson, 1985; Felsenstein, 1985), and thus violates one of the basic assumptions required by most statistical tools (Harvey & Pagel, 1991). Phylogenetic comparative methods are designed to deal with this problem, but only few of them faces up with multivariate traits

(Desdevises *et al*., 2003; Giannini, 2003; Cadotte *et al*., 2013). The phylogenetic signal is the tendency of related species to resemble each other (Blomberg & Garland, 2002), and phylogenetic principal component analysis (pPCA) uncovers the phylogenetic signal in multivariate sets of traits, and also the variables that create dissimilarities among closely related species (Jombart *et al*., 2010). Besides pPCA has been used for other multivariate traits (Batalha *et al*., 2011; Logez *et al*., 2013), it has been never used before for flower VOCs analysis. Generally, flower compounds that are phylogenetically constrained were not yet discriminated from compounds that are less constrained and more variable among closely related species. In this work we use the pPCA approach to analyze flower VOCs, together with the analysis of reconstruction of ancestral states (qualitative data), to reveal the evolution of flower scent in *Silene* (Caryophyllaceae).

Silene is appropriate for this approach as most of the species are pollinated by insects and at least for nocturnal pollinators it was shown that floral scents are important cues/attractants (Dötterl *et al*., 2006; Giménez-Benavides *et al*., 2007; Jhumur *et al*., 2008). Further, scent data are available for a large number of species (Jürgens *et al*., 2002; Jürgens, 2004; Giménez-Benavides *et al*., 2007; Waelti *et al*., 2008; Jhumur *et al*., 2008; Martinell *et al*., 2010; Castillo *et al*., 2014; Chapter 2). For several species thereof are also molecular data available as they were included in molecular phylogenetic studies (Oxelman & Liden, 1995; Fior *et al*., 2006; Popp & Oxelman, 2007; Erixon & Oxelman, 2008; Rautenberg *et al*., 2012). Based on published scent and molecular data, and additional molecular data obtained in this study, we analyzed the evolution of flower scent in 38 species. We specifically studied: 1) the pattern of occurrence and the relative amount of which compounds are phylogenetically constrained and which compounds vary independent of phylogeny, 2) the evolution of switch on/off of biosynthetic routes, and 3) the possible relationship between the flower scent evolution and the function as pollinator attractant.

MATERIAL AND METHODS

Scent dataset

Silene is a diverse genus with >700 species (Oxelman *et al*., 2013). In *Silene*, nocturnal and diurnal pollination syndromes have been described and a recent work shows that the species typically emit, independent of the syndrome, some flower VOCs both at day

and night (Chapter 2). Our study is based on scent emitted at night (Table 1) for three reasons: (1) nocturnal species emit most volatiles at night (only seven of 155 volatiles were only emitted during day-time), (2) in diurnal species the volatile pattern emitted at night is the same as the pattern emitted at day-time (Chapter 2), and (3) there are more species with both genetic and scent information available for the night dataset. Anyway, the results based on scent emitted at day and compared with the result of the present text are in the Supplementary material 3.

Floral scents included were from 1 *Lychnis* and 37 *Silene* species (Table 1). Green leaf volatiles (GLVs, Visser *et al*., 1979; Light *et al*., 1993), which would not necessarily help pollinators to accurately locate the flowers (Honda *et al*., 1998), were omitted from the analyses (Chapter 2).

For each compound, the percentage of the total peak area in each sample was calculated as an estimate of relative amount (semiquantitative data) and used for subsequent analyses. As we have only one genetic dataset for each species available, the replicates available for scent composition per species were pooled (mean of replicates). For *S. otites* and *S. latifolia* scents were analyzed in different studies (Table 1; Jürgens *et al*., 2002; Dötterl *et al*., 2005; Waelti *et al*., 2007; Jhumur *et at*., 2008), which all obtained similar results, and their scents were also averaged (mean among studies).

Molecular phylogenetic dataset

We obtained an ultrametric tree that included 38 terminal taxa, 18 of which were sampled in this study and 20 of which were available in GenBank (Table 1). The ingroup comprised 37 terminals, all representatives of *Silene. Lychnis coronaria* was used as outgroup. The selected markers were the internal transcribed spacer complete repeat (ITS) of the nuclear ribosomal DNA and the maternally inherited chloroplast rps16 intron (rps16). We used maximum likelihood (ML) and Bayesian inference (BI) for the phylogenetic analyses using the combined dataset of ITS and rps16. The ultrametric tree used for the phylogenetic Principal Component Analyses (pPCA) was generated under a Bayesian approach. DNA extraction, amplification, sequencing, sequence alignment and phylogenetic analyses are described in detail in Supplementary material 1.

Table 1. List of the species used and the GenBank accession number. * New sequences of the current study (the accession numbers have to be confirmed by Genbank)

Taxa/Species	Scent reference	Accession GB	
		ITS	rps16
Lychnis			
L. coronaria	Chapter 2	X86891	FJ404912
Silene			
Subgen. Behenantha			
Sect. Atocion			
S. aegyptiaca	Chapter 2		EU314654
S. fraudatrix	Chapter 2	XXXXXX*	XXXXXX*
Sect. Behenantha			
S. fabaria	Chapter 2	X86851.1	
S. fabarioides	Chapter 2	XXXXXX*	XXXXXX*
S. holzmannii	Chapter 2	XXXXXX*	XXXXXX*
S. uniflora	Chapter 2	X86849.1	Z83173
S. variegate	Chapter 2	XXXXXX*	
S. vulgaris	Jürgens *et al*., 2002	FN821141	FN821317
Sect. Conoimorpha			
S. subconica	Jürgens *et al*., 2002	HQ334913.1	HQ334973
Sect.Dichotomae			
S. dichotoma	Jürgens *et al*., 2002	X86848.1	Z83174
Sect. Melandrium			
S. diclinis	Chapter 2	FN821103.1	FN821273
S. dioica	Waelti *et al*., 2008	FN821112	FN821280
S. latifolia 1 and 2	Jürgens *et al*., 2002 and Waelti *et al*., 2008	FN821128	FN821296
Sect. Viscosae			
S.viscosa	Jürgens *et al*., 2002	FN821148.1	FN821316
Sect. Psammophilae			
S. cambessedesii	Chapter 2	XXXXXX*	XXXXXX*
S. littorea	Chapter 2	FN821094	Z83185
S. psammitis	Chapter 2	XXXXXX*	XXXXXX*
Sect. Psysolychnis			
S. stellata	Castillo *et al*., 2014	DQ908667.1	DQ908847
S. zawadzkii	Chapter 2	X86883.1	Z83177
Subgen. Silene			
Sect. Auriculatae			
S. boryi	Chapter 2	XXXXXX*	XXXXXX*
S. linicola	Chapter 2	XXXXXX*	XXXXXX*
S. vallesia	Jürgens *et al*., 2002	X86870.1	
Subsect. Sclerocalycinae			
S. chloranta	Jürgens *et al*., 2002	XXXXXX*	XXXXXX*
S. frivaldszkyana	Chapter 2	XXXXXX*	XXXXXX*
S. saxatilis	Chapter 2	XXXXXX*	XXXXXX*

Taxa/Species	Scent reference	Accession GB	
		ITS	rps16
Sect. Silene			
S. ciliate	Giménez-Benavides *et al*., 2007	XXXXXX*	XXXXXX*
S. colorata	Chapter 2	X86842.1	JX560216
S. fuscata	Chapter 2	XXXXXX*	XXXXXX*
S. giraldii	Chapter 2	XXXXXX*	XXXXXX*
S. scabriflora	Chapter 2	XXXXXX*	XXXXXX*
Sect. Siphonomorpha			
S.flavescens	Chapter 2	XXXXXX*	XXXXXX*
S.italica	Jürgens *et al*., 2002	JX403422.1	
S.longicilia	Chapter 2	DQ059396.1	
S.nutans	Jürgens *et al*., 2002	AY936260.1	EF061361
S.otites1 and 2	Jürgens *et al*., 2002 and Jhumur *et al*., 2008	EF060233.1	EF061393
S.sennenii	Martinell *et al*., 2010	XXXXXX*	XXXXXX*
S.tatarica	Chapter 2	XXXXXX*	XXXXXX*

Phylogenetic Principal Component Analyses (pPCA) and reconstruction of ancestral states

pPCA. In the pPCA analysis, the phylogenetic resemblance in a complex set of continuous variables (flower VOCs in our case) is graphically summarized in two principal components (PC). The first PC denotes the global structure and reveals the VOCs that are more similar in related than distant species. The local structure is depicted in the second PC, which reveals the VOCs that create dissimilarities among closely related species. Phylogenetic principal component analysis was implemented in the *adephylo* package (Jombart & Dray, 2008) for R software (R Core Team, 2014). In the pPCA we used the measure of phylogenetic proximity underlying the test of Abouheif (1999) because of its goodness in detecting phylogenetic signal (Pavoine *et al*., 2008). From all the principal components from the pPCA output, the a*dephylo* package selects two, those with the largest (large variance and strong positive autocorrelation) and lowest (high variance and large negative autocorrelation) eigenvalues. These correspond with the highest and lowest phylogenetic signal respectively, namely global and local structures (Jombart *et al*., 2010).

Ancestral state reconstruction. Ancestral state reconstructions were performed for those compounds with the highest phylogenetic signal (species with $\geq 1e^{-2}$ or $\leq -1e^{-2}$ in the loading of PC1; see Supplementary material 2 and Fig. 1B) and most frequent (those

that appeared in more than 25% of the species: 2-phenylethanol, benzaldehyde, benzyl benzoate, benzyl alcohol, 2-methylbutyraldoxime, lilac aldehydes, linalool, and E-β-ocimene. To perform the reconstructions, VOCs were used as present/absent characters in a set of 5500 trees corresponding to the posterior tree sample from the MrBayes anaysis. We used a parsimony approach as implemented in MESQUITE software (Maddison & Maddison 2011). Only the nodes that reached BPP values ≥0.95 and ML bootstrap values ≥70% were taken into account.

RESULTS

Details about the complete scent composition of each species can be found in the Chapter 2.

Phylogenetic reconstructions

Results of the phylogenetic analyses and Bayesian trees, with BPP and ML bootstrap values, are described in Supplementary material 1. Our phylogenetic tree was quite consistent with the classification suggested by Oxelman *et al*., (2013). Exceptions were the two species of Sect. Atocion (*S. fraudatrix* and *S. aegyptiaca*), which did not belong to subgen. Behenantha, as already previously suggested (Erixon & Oxleman, 2008). Similarly, the species of Subsect. Sclerocalycinae studied here nested within Sect. Siphonomorpha, as in Slancarova *et al*., (2013) (Fig. 1).

Phylogenetic PCA

In our analysis of the 181 floral VOCs, the first PC denoted the global structures and the 36th PC denoted the local structures (Fig. 1). Global structures were mainly due to differential productions of linalool and E-β-ocimene (Fig. 1B). The species with the highest positive scores (PC1) were within subgen. Behenantha, and all of them produced high relative amounts of linalool (>90%). No other species produced similar high

amounts of this compound (Chapter 2), but linalool was also the most abundant

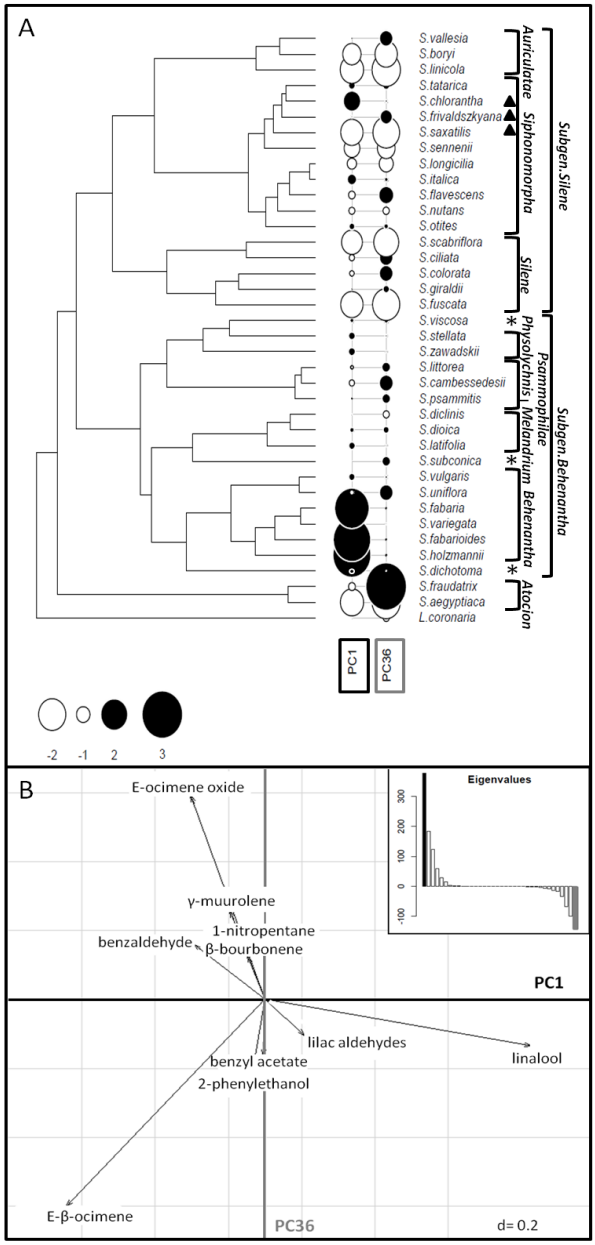

Figure 1. (A) Phylogeny of the species studied and results of the pPCA. Positive and negative scores on PC1 (global) and PC36 (local) are indicated by black and white circles, respectively. Symbol size is proportional to absolute values. (B) Loading of the main traits for the global and local principal components. Only the compounds with highest loadings on the PCs are depicted. The loadings of all compounds are shown in the Supplementary material 2. Inset barplot displays corresponding eigenvalues. d=0.2 is the mesh of the grid. *Silene* species are grouping in sections. * indicate Sect. *Viscosae* for *S. viscosa*, Sect. *Conoimorpha* for *S. subconica*, and Sect. *Dichotomae* for *S. dichotoma.* Black triangles denote species of Subsect. Sclerocalycinae.

compound in distantly related *S. chlorantha* (42%), which also had a relatively high positive score. The highest negative scores were found, with one exception (*S.*

aegyptiaca of section Atocion, affiliation to subgenus unclear), only in subgenus Silene (Fig. 1). E-β-ocimene, which was most responsible for this structure, was the most abundant compound in all these species (Chapter 2). All members of subgenus Behenantha had either positive scores or only small negative ones and none of the species in this subgenus emitted high relative amounts of E-β-ocimene. Besides the high phylogenetic signal of this monoterpene (global structure), E-β-ocimene had the strongest influence on the local structure (highest loading on PC 36, Fig. 1B), and species with high negative scores on PC1 often had high negative scores on PC36 as well. These high scores on PC 36 are especially due to the fact that in all sections of subgenus *Silene* only some of the species released high relative amounts of E-β-ocimene, whereas other species released only small amounts of this compound. 2-phenylethanol, benzyl acetate, linalool and lilac aldehydes also have negative loads, but much less than E-β-ocimene. Only one species (*S. fraudatrix*, affiliation to subgenus unclear) had high positive scores on PC36 and this was due to an extraordinary high relative amount of E-ocimene oxide. Besides E-ocimene oxide, the sesquiterpenes γ-muurolene and β-bourbonene, the nitrogen-containing compound 1-nitropentane, and the aromatic compound benzaldehyde were most responsible for the positive scores on the local PC (Fig. 1B). Therefore, species closely related often had quite different scores and emitted strongly different relative scent amounts of these compounds. To give an example, *S.scabriflora* (negative score) emitted high relative amounts of E-β-ocimene, compounds that occurred only in low amounts in *S. ciliata* (positive score), which itself released high relative amounts of benzaldehyde.

Ancestral state reconstructions

All aromatic compounds shared a similar pattern of appearance (Fig. 2). They were absent in the most basal nodes of the phylogeny, and almost all of them appeared at the section level (Melandrium, Psammophilae or Behenantha) or at the tips. Only benzaldeyde appeared already at a node ancestral to a subgenus (Behenantha, Fig. 2). The most frequent N-bearing compound, 2-methylbutyraldoxime, appeared in the common ancestor of Sect. Siphonomorpha (Fig. 2) and in most of the taxa of that section, whereas it only occurred in a few isolated tips (taxa) outside this section.

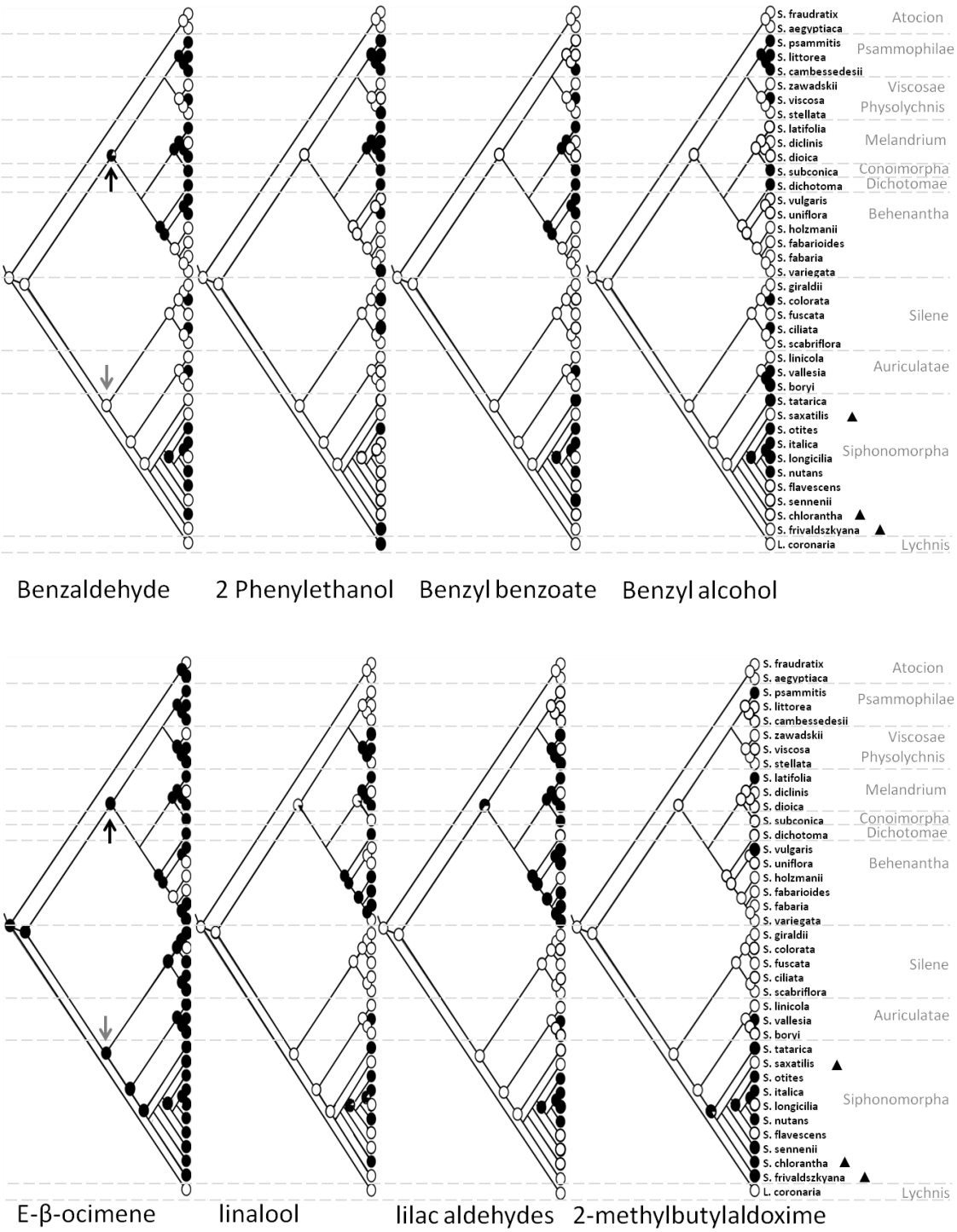

Figure 2. Reconstructions of the ancestral states. White color in the nodes and tips (circles) denotes the proportion of the trees in which the compound was absent, black when was present. The grey arrow marks the ancestral node to subgen. Silene and the black arrow marks the ancestral node to subgen. Behenatha. Black triangles denote species of Subsect. Sclerocalycinae. Name of sections are given in grey.

Linalool was absent in the most basal node, but may have appeared in the common ancestor of subgenus Behenantha (16% of reconstructions indicated presence at this node) and was present in all reconstructions in sections Melandrium, Behenantha,

Viscosae, and Physolychnis. The lilcac aldehydes were present in mostly the same common ancestors as linalool, but mainly varied in some of the tips. E-β-ocimene was present in the most basal node, in most of the internal nodes, and in most of the taxa.

DISCUSSION

Our results show that the percentage amount of some floral compounds correlates with the phylogeny of *Silene* at higher levels (genus and section) and produce a high phylogenetic signal. In other words, the semiquantitative pattern of floral scents can be explained to some extent by evolutionary conservatism. Previous studies have suggested that floral scents are too evolutionarily labile to be useful for phylogenetic inference (Williams & Whitten, 1999; Barkman, 2001), but others argued that there is some congruence between fragrance and phylogeny at low taxonomic levels (Levin *et al*., 2003). The lack of phylogenetic signal in other studies could be a consequence of using only the presence/absence of VOCs for analyses (Azuma *et al*., 1999; Williams & Whitten, 1999; Barkman, 2001; Levin *et al*., 2003; Steiner *et al*., 2011).

Flower scents are typically a combination of few to more than a hundred of volatile compounds in variable concentrations (Levin *et al*., 2001; Knudsen, 2002), from highly abundant compounds to minor ones. Thus, qualitative analyses only depict part of the variation of floral volatile profiles within a taxonomic group and masks information about taxonomic affiliation and evolution of floral signals available in relative scent patterns. First, two distant species may have a similar qualitative composition of VOCs but very divergent amounts of the different compounds. For example, *S. dichotoma* (Sect. Behenantha) and *S. chlorantha* (Sect. Siphonomorpha) have a similar qualitative scent composition (share 12 of 22 and 18 compounds respectively), but release their compounds in very different relative amounts. Scent of *S. dichotama* is dominated by benzaldehyde and *S. chlorantha* emits high amounts of linalool. Second, two sister species may emit some different compounds but very similar ratios of the common ones. For example, *S. sennenii* and *S. saxatilis* (section Siphonomorpha) produce 16 and 9 compound respectively, sharing only 2, but both species produce mainly benzaldehyde. Consequently these two taxa have a similar chemical trait (Fig. 1), which would not have been detected by quantitative analyses alone.

Our results prove that pPCA, combined with ancestral state reconstruction of particular VOCs, is a powerful tool to explore the phylogenetic signal of floral volatiles in complex multivariate datasets. Furthermore, local structures of the pPCA may uncover the scent compounds that are consequence of the most recent evolutionary processes (Jombart *et al*., 2010), like speciation caused by diversifying selection (i.e., different pollinator) or niche partition. Therefore, pPCA helps us to understand semiquantitative multivariate dependence among species' flower scents due to their phylogenetic relationships, whereas ancestral state reconstructions give insight into the presence/absence of each floral volatile (i.e. switch on/off of biosynthetic routes) along the evolutionary history of *Silene*.

The phylogenetic signal of flower scents in *Silene* species as shown by pPCA was characterized by a strong opposition between two monoterpenes, linalool and E-β-ocimene (PC1 in Fig. 1). This denotes that the higher the emission of linalool in a species or clade, the lower the emission of E-β-ocimene, and reversely. Interestingly, the finding that these two compounds have an opposite behavior is not obvious in the ancestral state reconstructions. More than this, the presence of E-β-ocimene in the flower scents turned out to be a plesiomorphic state, suggesting evolutionary conservatism across *Silene* in the production and emission (qualitatively) of this compound. Contrarily, the emission of linalool was homoplasic, suggesting convergence in a derived VOC that emerged independently in different moments in the evolution of *Silene*. It is well known that pollinator and herbivore-mediated selection may be exerted by single species or functional groups of pollinators, and this may generate convergent evolution of floral traits (Van der Pijl, 1960; Faegri & Van der Pijl, 1979; Fenster *et al*., 2004). Overall, while there was only small qualitative variation in E-β-ocimene, a compound that varied strongly in its relative amounts, linalool varied both qualitatively and quantitatively along the phylogenetic tree.

Monoterpenes, such as linalool and E-β-ocimene, are synthesized by the plasmidial methylerythritol phosphate (MEP) pathway from geranyl pyrophosphate (GPP) (Tholl, 2006; Dudareva *et al*., 2013) and our results suggest that E-β-ocimene synthase (Bohlmann *et al*., 2000; Dudavera *et al*., 2003) and linalool synthase (Pichersky *et al*., 1995; Nagegowda *et al*., 2008) compete for the same precursor (GPP), or that only one

of the enzymes but not both together are upregulated at the transcriptional level (Shimada *et al*., 2005). The latter scenario could occur, when both compounds are effective in attracting pollinators and the production of both compounds together would be functionally redundant. Indeed, both, E-β-ocimene (together with Z-β-ocimene) and linalool are related with nocturnal or crepuscular pollination by moths and hawkmoths (Fraser *et al*., 2003; Dobson, 2006; Dötterl *et al*., 2006; Martinell *et al*., 2010; Thöming & Knudsen 2014, Riffel *et al*., 2013), the main nocturnal pollinator groups in *Silene* (Jürgens *et al*., 2002; Giménez-Benavides *et al*., 2007; Martinell *et al*., 2010). This suggests that E-β-ocimene is not only phylogenetically constrained but is also involved in pollinator attraction, whereas linalool specifically evolved for pollinator attraction in nocturnal *Silene* species.

Lilac aldehydes showed a similar phylogenetic pattern to linalool in the ancestral state reconstructions (Fig. 2) and also had positive scores on PC1 in the pPCA analysis (Fig. 1). This finding is not surprising when considering that linalool is a precursor in the biosynthesis of lilac compounds (Burkhardt & Mosadl, 2003; Kreck *et al*., 2003). Lilac aldehydes are highly attractive for moths (Plepys *et al*., 2002; Dötterl *et al*., 2006).

The compounds responsible for local structure in the pPCA analysis strongly differed in relative amount in closely related species (Fig. 1, Supplementary material 2), and these changes are a likely consequence of evolutionary recent events. Among these compounds were aromatics (2-phenylethanol, benzaldehyde and benzyl acetate) and terpenoids (E-β-ocimene, E-ocimene oxide, and lilac aldehydes). These aromatic compounds are, similarly to several terpenoids (see above), associated with moth pollinated flowers (Heath *et al*., 1992; Knudsen & Tollsten 1993; Meagher, 2002; Dobson, 2006; Giménez-Benavides *et al*., 2007). The presence of the enzymatic route that produces aromatic compounds (the shikimate/phenylalanine pathway, Dudareva *et al*., 2013) arised independently in two clades (Sect. Siphonomorpha and the complex from Sect. Behenantha to Sect. Psammophilae in Fig. 2). Also E-ocimene-oxide, γ-muurolene , β-bourbonene and 1-nitropentane are responsible for local structures. These compounds are not common within *Silene* species (Jürgens *et al*., 2002; Jürgens, 2004; Chapter 2) and their function as moth-attractor is unknown. Moreover, 1-nitropentane acts as a deterrent of the hawkmoth *Manduca sexta* (Shields & Hildebrand 2001). It remains unclear why these compounds (with high score on PC 36) differ in their

relative emission rates among closely related species. A possible explanation is that closely related species attract moths with different olfactory preferences (that could contribute to reproductive isolation of sympatric taxa). These evolutionary recent events may be due to the attraction of pollinator but also to the attraction of both pollinators and herbivores as in nursery pollination. Lilac aldehydes and benzaldehyde are olfactory cues of two nursery pollination associations between *S. latifolia and Hadena bicruris,* and between *S. ciliata* and *H. consparcatoides* (Dötterl *et al*., 2006; Giménez-Benavides *et al*., 2007).

In conclusion, we have found that phylogenetically controlled multivariate analyses (phylogenetic PCA) add a new dimension to the phylogenetic analyses used so far in evolutionary studies on floral scents. Based on the relative amount of floral scents, we identified compounds that were phylogenetically constrained. On the other hand our analyses revealed that other compounds strongly differed among closely related *Silene* species, possibly due to interaction with environmental factors, such as pollinators. Together with qualitative investigations such as ancestral state reconstructions, which were used in previous phylogenetic studies on floral scents and focus on the presence and absence of single compounds, such analyses help to better understand how floral scents have diverged.

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SUPPLEMENTARY MATERIAL 1. Phylogenetic reconstructions.

MATERIALS AND METHODS

Taxon sampling

We obtained molecular data for 38 taxa (Table 1). The ingroup comprised 37 terminals, all representatives of *Silene. Lychnis coronaria* was used as outgroup.

DNA extraction, amplification and sequencing

DNA was extracted from 18 dried herbarium specimens (Table 1). Total DNA was extracted using the Qiagen DNeasy Plant MiniKit, according to the manufacturer's instructions. The selected markers for this study were the internal transcribed spacer complete repeat (ITS) of the nuclear ribosomal DNA, and the maternally inherited chloroplast rps16 intron (rps16).

PCR amplifications were performed using a reaction mixture containing 2.5 µl 10X DNA polymerase Biotoosl® buffer (containing MgCl2 2 mM, 10 mM Tris-HCl, pH 8·0, 50 mM KCl, 1 mM EDTA, 0·1% Triton X-100), 1 µL of dinucleotide triphosphate (dNTPs), containing 10mM of each base, 2 µL of each primer (10 µM), 0,75 µL of Biotools ®DNA polymerase (1U μL^{-1}) and 11,75 µL dH_2O. Finally, 20 µl of this mixture was added to 5 µl of DNA from each specimen. To amplify the intron in the rps16 gene we used the primers rpsF and rpsR2R (Oxelman et al., 1997). To amplify the ITS1 the 5.8S and the ITS2 we used the primers P17 and 26S-82R (Popp and Oxelman, 2001). The PCR settings for all primer combinations were as follows: initial denaturing at 95˚C for 5 min and 45 cycles (95˚C for 30 s, 56-58˚C for 1 min. and 72˚C for 2 min.) followed by a final extension at 72˚C for 7 min.

Before sequencing, the PCR products were purified using the PCR-M® Clean-up System of Viogene or the enzymatic method Exo-sap-IT© provided by USB Corporation. For ITS, P16b (Popp et al., 2005) and ITS4R (White et al., 1990) and for rps16, rpsF2a and rpsR3R (Popp et al., 2005) were used as internal sequencing primers.

Sequence alignment and phylogenetic analyses

Sequences were aligned using the multiple sequence alignment software MAFFT version 7.110 (Katoh et al., 2002, Katoh and Toh 2008a). The G-INS-i algorithm was used for the rps16 sequences and the Q-INS-i for the ITS sequences (Katoh and Toh 2008b). Major insertions and ambiguous regions in the alignments were identified and eliminated with Gblocks version 0.91b (Castresana, 2000) using the relaxed parameter values suggested by Talavera and Castresana (2007).

We assessed congruence analysing the datasets separately by ML bootstrapping, to detect possible conflicts among genetic markers. Conflict was understood as bootstrap support (≥ 70%; Hillis and Bull, 1993) for one marker, contradicted with significant support by another. No incongruence was found and the data were concatenated into a single dataset.

We used maximum likelihood (ML), and Bayesian inference (BI) for the phylogenetic analyses using the combined dataset. We achieved ML analyses in RAxMLGUI 1.3, a graphical front-end for RAxML (Randomized Accelerated Maximum Likelihood for High Performance Computing; Stamatakis, 2006), using the GTRCAT model of nucleotide substitution (a GTRGAMMA approximation with optimization of individual per-site substitution rates). We partitioned the dataset by gene. The same model was applied to all partitions because of constraints of the software RAxML. We performed a total of 10 runs and assessed node support via 10000 bootstrap replicates (ML + thorough bootstrap; n. threads 2).

Bayesian analysis (Huelsenbeck et al., 2001) was achieved with the software MrBayes 3.2.1 (Ronquist and Huelsenbeck, 2003). We selected the model of nucleotide substitution that fitted best for every particular partition, according to the Akaike Information Criterion (AIC) in jModeltest (Posada, 2008). We used full likelihood optimization and searched only among the 24 models implemented in MrBayes. A GTR+I+Γ model was selected for the ITS, and a GTR+Γ models were selected for the rps16 gene, in both datasets respectively. Selection of priors and convergence assessment was achieved following Millanes et al. (2014).

The ultrametric trees used in the pPCA analyses were generated under a Bayesian approach using BEAST version 1.7.5 (Drummond and Rambaut 2007). Ultrametric trees were constructed employing the same substitution models as in the analyses performed in MrBayes. BEAST input files were generated using BEAUti (Drummont and Rambaut 2007). In all cases we used a relaxed lognormal clock (Drummond et al. 2006) using a yule speciation prior and assuming constant population size to estimate branch lengths. The substitution models, the rate heterogeneity and the base frequencies were unlinked across partitions in the combined analysis. For each individual dataset and for the combined dataset, three independent MCMC analyses were run for 100 million generations, sampling trees every 10000 generations. We assessed convergence by examining the likelihood plots through time using Tracer version 1.5 (Rambaut and Drummond 2007), after removing 10% of the sample as burn-in, and observed that the effective sample sizes (ESS) of all parameters of interest were > 200. Tree files from the independent runs were combined using LogCombiner (Drummont and Rambaut 2007). The posterior tree sample was summarized using TreeAnnotator (Rambaut and Drummond 2007) after discarding the first 5000 trees of each run as burn-in.

RESULTS

DNA sequences and phylogenetic analyses

We generated 35 new sequences, which were aligned together with 53 sequences already available in GenBank (Table 1). Of the 38 terminals, 1 lack ITS, and 5 rps16. The combined matrix included a total of 1350 characters of aligned DNA sequences from the nuclear and chloroplastic genes: ITS rDNA (608 bp), and rps16 (742 bp)

The Bayesian analyses of the combined dataset halted after 1100000 generations, at which time the average standard deviation of splits across runs in the last half of the analysis was 0.009 (<0.01). Potential Scale Reduction Factor (PSRF) values for all model parameters as well as all branch lengths were close to 1. (the highest PSRF value of the model parameters was 1.024 only in one case). We considered the three runs to have converged and that our samples were valid estimates of the posterior

distribution. A majority rule consensus tree was constructed from the 5500 trees of the stationary tree sample.

Phylogenetic reconstructions obtained by the two inference methods and the three software packages, that is, ML (RAxML) and BI (MrBayes and BEAST) were congruent so, only the topology corresponding to the Bayesian analyses from BEAST is shown in Figs. 1 & 2. Supplementary Fig. 1 shows the best ML trees with Bayesian posterior probabilities (as inferred by MrBayes) and ML bootstrap values added.

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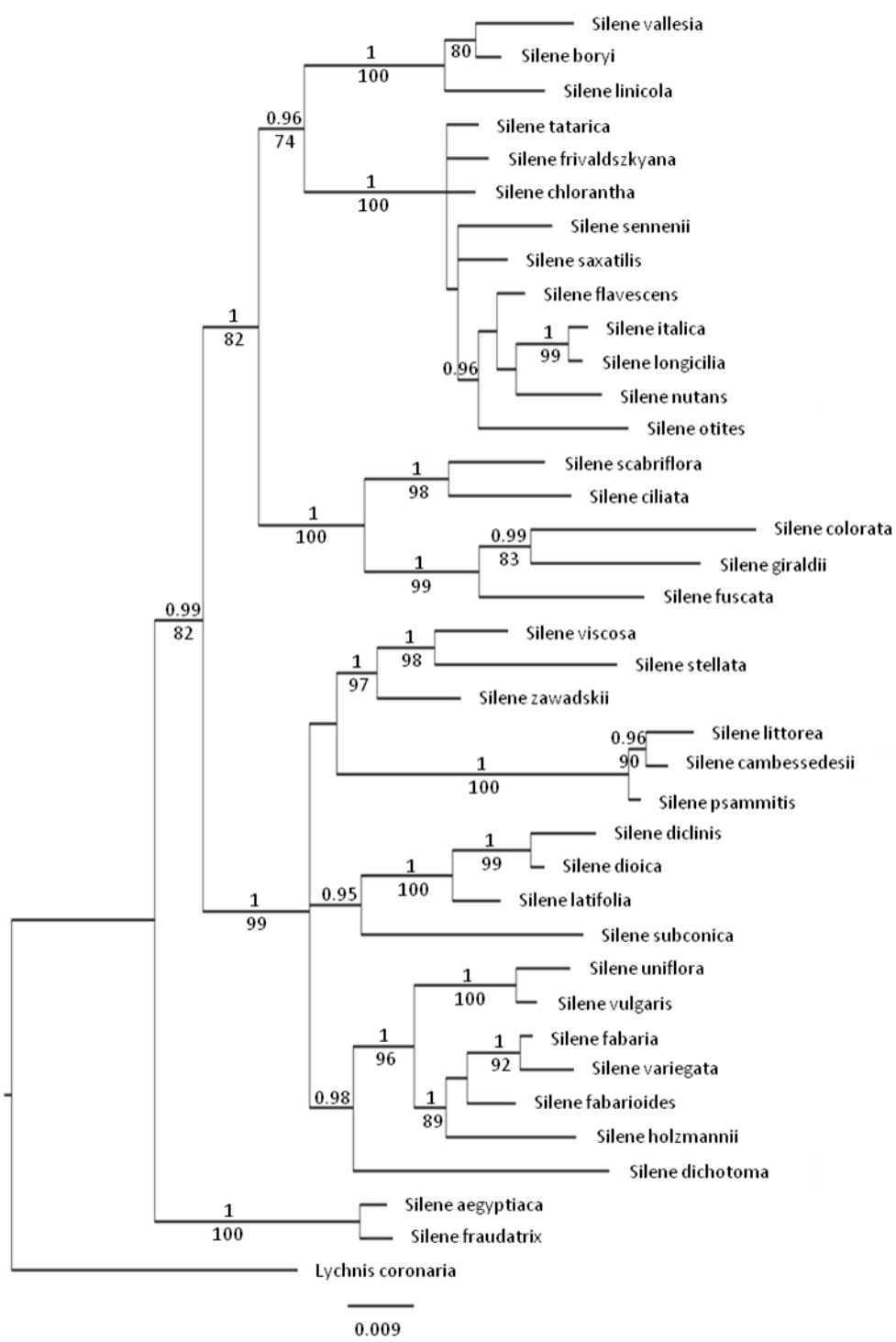

Supplementary Fig. 1. ML phylogenetic reconstructions using ITS and rps16. Bayesian posterior probabilities (BPP) ≥0.95, and ML bootstrap values ≥70%, are indicated above and below branches, respectively. Branch lengths are scaled to the expected number of nucleotide substitutions per site.

SUPPLEMENTARY MATERIAL 2. Loading of each compound in the PCs. The compounds are organized in each PC from highest values to lowest.

Compounds	PC1	Compounds	PC36
Linalool	7,30E-01	E-Ocimene oxide	5,95E-01
Lilac aldehyde B+C	1,95E-01	γ-Muurolene	2,59E-01
Lilac aldehyde A	1,09E-01	1-Nitropentane	1,69E-01
Lilac aldehyde D	7,69E-02	Benzaldehyde	1,62E-01
Z-Arbusculone	5,55E-02	ß-Bourbonene	1,29E-01
E-Arbusculone	5,34E-02	Phenylacetaldehyde	6,58E-02
p-Cresol	3,84E-02	unknown 2	5,02E-02
Benzyl benzoate	2,63E-02	unknown 5	3,29E-02
Camphene	2,55E-02	γ-Cadinene	3,22E-02
Epoxyocimene	1,78E-02	ST 9	2,81E-02
unknown lilac derivative 8	1,67E-02	δ-Cadinene	2,41E-02
Benzyl alcohol	1,61E-02	α-Copaene	2,11E-02
2-Methylbutyraldoxime	1,20E-02	Sabinene	2,07E-02
1,2-Dimethoxybenzene	1,16E-02	ST 8	1,81E-02
1,4-Benzoquinone	1,10E-02	unknown 16	1,56E-02
Lilac alcohol B+C	9,17E-03	Z-ß-ocimene	1,53E-02
Lilac alcohol D	6,04E-03	unknown 3	1,47E-02
Hydroquinone	5,63E-03	unknown benzenoid 1	1,38E-02
Lilac alcohol A	4,92E-03	1,2-Dimethylbenzene	1,00E-02
unknown lilac derivative 7	4,73E-03	3-Methylbutyraldoxime	9,55E-03
Geraniol	3,80E-03	(E,E)-α-Farnesene	9,04E-03
unknown lilac derivative 5	3,79E-03	Methyl benzoate	8,62E-03
Methyl salicylate	3,44E-03	unknownEster	7,02E-03
Carvone	3,20E-03	ß-Pinene	6,91E-03
unknown N bearing2	3,00E-03	unknown 19	6,32E-03
3-Pyridinecarboxaldehyde	2,91E-03	Z.E.Linalooloxidepyranoid	5,68E-03
unknown 27	2,64E-03	Methyl salicylate	5,57E-03
Z-Carveole	2,56E-03	unknown 18	5,39E-03
unknown 13	2,54E-03	unknown 20	4,71E-03
unknown lilac derivative 6	2,39E-03	unknown 21	3,34E-03
α-Pinene	2,19E-03	unknown benzenoid 4	2,95E-03
unknown N bearing3	1,19E-03	E-Linalool oxide furanoid	2,24E-03
6-methyl-5-hepten-2-one	1,03E-03	Phenylacetate	1,89E-03
unknown 4	1,01E-03	unknown 17	1,56E-03
MT oxide 1	9,05E-04	unknown 6	1,34E-03
3-Methylbutyraldoxime	7,22E-04	unknown 27	1,33E-03
unknown lilac derivative 3	6,63E-04	Hotrienol	1,26E-03
unknown lilac derivative 1	6,03E-04	1-Phenyl-1,2-propanedione	6,94E-04
unknown 30	5,55E-04	p.Methoxybenzaldehyde	6,94E-04
2-Methoxyphenol	5,43E-04	α-Gurjunene	6,55E-04
ß-farnesene	5,31E-04	Ethylbenzoate	5,61E-04
unknown 29	4,59E-04	2,2,6-trimethyl-2-vinyl-5-ketotetrahydropyran	5,02E-04
unknown 26	4,33E-04	1-Hydroxylinalool	4,94E-04

α-Phellandrene	3,17E-04
4-Methoxyphenethylalcohol	2,47E-04
E-Nerolidol	1,97E-04
1,8-Cineole	1,52E-04
unknown lilac derivative 2	1,37E-04
Z-3-Hexenyl benzoate	7,61E-05
Methyl o-methoxybenzoate	5,89E-05
unknownN.bearing4	5,53E-05
Isoamyl benzoate	4,92E-05
unknown 28	3,93E-05
MT oxide 2	3,88E-05
MT 1	2,86E-05
MT oxide 9	2,58E-05
MT oxide 8	2,49E-05
unknown 8	2,48E-05
unknown lilac derivative 4	2,03E-05
unknown lilac derivative 9	1,92E-05
unknown 9	1,83E-05
unknown 11	1,30E-05
ST 1	1,27E-05
MT oxide 6	1,16E-05
unknown 12	1,05E-05
unknown lilac derivative 10	5,38E-06
MT oxide 5	3,68E-06
Z-Methyl isoeugenol	-7,03E-07
unknown benzenoid 3	-2,22E-06
Z-Jasmone	-2,22E-06
ST 7	-2,22E-06
Methyl eugenol	-3,16E-06
3,4-Dimethoxybenzaldehyde	-3,16E-06
Eugenol	-3,51E-06
Benzyl butyrate	-3,59E-06
E-Methyl isoeugenol	-4,57E-06
unknown 14	-4,83E-06
3,4,5-Trimethoxybenzaldehyde	-6,45E-06
Decanenitrile	-6,89E-06
unknown 25	-8,45E-06
MT oxide 10	-9,54E-06
octanenitrile	-1,16E-05
MT oxide 3	-1,38E-05
unknown 22	-1,81E-05
2-Phenylethylacetate	-1,99E-05
unknown 24	-2,42E-05
E-Cinnamyl acetate	-2,68E-05
unknown 7	-3,11E-05
MT 2	-3,46E-05
Benzenepropylacetate	-3,63E-05

Butyl benzoate	4,18E-04
Indole	3,67E-04
Z-Linalool oxide	2,81E-04
ß-Selinene	2,64E-04
Pentyl benzoate	2,54E-04
Methyl nicotinate	2,24E-04
Hexanol	2,22E-04
unknown lilac derivative 5	1,66E-04
unknown benzenoid 2	1,64E-04
4-Oxoisophorone	1,50E-04
Geranyl acetate	1,31E-04
Prenylacetate	1,29E-04
unknown 10	1,25E-04
2-Phenylethylacetate	1,25E-04
Z-3-Hexenyl benzoate	1,24E-04
unknown 13	1,11E-04
unknown aromatic compound	1,10E-04
α-Pinene	1,05E-04
unknown lilac derivative 6	1,05E-04
Geranyl Isovalerate	1,04E-04
Farnesol/al isomer	9,62E-05
Geraniol	9,57E-05
ST 4	6,57E-05
Vanillin	6,23E-05
2-Phenylpropenal	5,91E-05
MT 2	5,90E-05
MT oxide 1	3,96E-05
unknown 24	3,20E-05
unknown 22	2,40E-05
ST oxide	2,19E-05
MT oxide 10	1,63E-05
Benzenepropylacetate	1,54E-05
unknown 25	1,12E-05
4-Methoxyphenethylalcohol	1,08E-05
unknown 14	6,39E-06
unknown benzenoid 3	5,28E-06
Z-Jasmone	5,28E-06
ST 7	5,28E-06
unknownN.bearing4	4,69E-06
Isoamyl benzoate	4,17E-06
Z-Methyl isoeugenol	-6,39E-06
E-Nerolidol	-8,55E-06
6-methyl-5-hepten-2-one	-1,64E-05
ST 3	-1,85E-05
MT oxide 5	-1,94E-05
Methyl o-methoxybenzoate	-2,09E-05
unknown 7	-2,25E-05

unknown 23	-4,44E-05
2-Phenylpropenal	-4,47E-05
Vanillin	-4,71E-05
ST 4	-4,77E-05
ST oxide	-5,16E-05
Geranyl Isovalerate	-6,08E-05
3,4-Dimethoxystyrene	-6,43E-05
Isoelemicin	-6,51E-05
unknown benzenoid 2	-6,87E-05
Z-Cinnamylalcohol	-7,09E-05
Prenylacetate	-7,23E-05
unknown aromatic compound	-8,33E-05
unknown 1	-9,98E-05
Pentyl benzoate	-1,13E-04
4-Oxoisophorone	-1,14E-04
Allo or neoallo ocimene	-1,22E-04
MT oxide 4	-1,26E-04
ST 3	-1,28E-04
Fenchyl acetate	-1,28E-04
Hexanol	-1,30E-04
1-Phenyl-1,2-propanedione	-1,48E-04
ST 2	-1,56E-04
MT oxide 7	-1,60E-04
Z or E-Ocimenone	-1,65E-04
Z-Linalool oxide	-1,66E-04
Methyl nicotinate	-1,69E-04
Butyl benzoate	-1,86E-04
Farnesol/al isomer	-2,26E-04
Ethylbenzoate	-2,50E-04
unknown N bearing1	-2,60E-04
Longifolene V4	-2,60E-04
Cubenol	-2,60E-04
α-Gurjunene	-2,75E-04
1-Hydroxylinalool	-2,90E-04
unknown 10	-2,93E-04
2,2,6-trimethyl-2-vinyl-5-ketotetrahydropyran	-2,94E-04
Geranyl acetate	-3,08E-04
Benzenepropanal	-3,11E-04
2-ethyl-1,6-dioxaspiro 4.4nonane	-3,96E-04
Benzenepropanol	-4,01E-04
p-methoxystyrene	-4,75E-04
ST 6	-5,51E-04
unknown 17	-6,97E-04
Hotrienol	-7,38E-04
Indole	-8,16E-04
p.Methoxybenzaldehyde	-8,99E-04
unknown 15	-9,70E-04

MT oxide 3	-2,30E-05
unknown 28	-2,39E-05
unknown lilac derivative 10	-2,84E-05
3,4-Dimethoxybenzaldehyde	-2,87E-05
Methyl eugenol	-2,87E-05
MT oxide 7	-3,05E-05
Eugenol	-3,19E-05
E-Methyl isoeugenol	-4,15E-05
Benzyl butyrate	-4,40E-05
unknown lilac derivative 4	-5,14E-05
unknown 12	-5,53E-05
ST 2	-5,68E-05
unknown 23	-5,73E-05
MT oxide 6	-6,13E-05
ST 1	-6,73E-05
unknown 11	-6,88E-05
3,4,5-Trimethoxybenzaldehyde	-9,48E-05
MT oxide 4	-9,49E-05
Decanenitrile	-1,01E-04
unknown lilac derivative 9	-1,02E-04
Z or E-Ocimenone	-1,17E-04
MT oxide 8	-1,32E-04
MT oxide 9	-1,36E-04
unknown 26	-1,50E-04
α-Phellandrene	-1,65E-04
octanenitrile	-1,70E-04
MT 1	-1,72E-04
unknown 9	-1,78E-04
MT oxide 2	-2,05E-04
aromatic aldoxime	-2,30E-04
unknown N bearing1	-2,58E-04
Longifolene V4	-2,58E-04
Cubenol	-2,58E-04
unknown lilac derivative 2	-3,41E-04
ß-farnesene	-3,51E-04
1,8-Cineole	-3,79E-04
E-Cinnamyl acetate	-3,94E-04
unknown N bearing3	-4,22E-04
ST 6	-5,48E-04
unknown 1	-6,29E-04
1-Nitro-2-phenylethane	-6,65E-04
unknown 30	-6,86E-04
Allo or neoallo ocimene	-6,92E-04
3,4-Dimethoxystyrene	-7,78E-04
Hydroquinone	-8,03E-04
unknown 29	-8,77E-04
Z-Cinnamylalcohol	-9,53E-04

unknown 6	-1,01E-03	Isoelemicin	-9,57E-04
ß-Selinene	-1,10E-03	2-Methoxyphenol	-1,00E-03
1-Nitro-2-phenylethane	-1,11E-03	unknown 15	-1,09E-03
E-Linalool oxide furanoid	-1,31E-03	unknown 8	-1,18E-03
Phenylacetate	-1,38E-03	unknown lilac derivative 1	-1,50E-03
E-Cinnamaldehyde	-1,40E-03	1,4-Benzoquinone	-1,52E-03
unknown 21	-1,49E-03	unknown lilac derivative 8	-1,52E-03
E-Cinnamylalcohol	-1,62E-03	Epoxyocimene	-1,65E-03
ST 5	-1,97E-03	unknown lilac derivative 3	-1,65E-03
unknown benzenoid 4	-2,08E-03	Benzenepropanal	-1,82E-03
unknown 20	-2,10E-03	ST 5	-1,96E-03
unknown 18	-2,41E-03	Z-Carveole	-2,38E-03
unknown 19	-2,82E-03	α-Farnesene	-2,68E-03
α-Farnesene	-3,26E-03	phenylacetonitrile	-3,05E-03
(E,E)-α-Farnesene	-3,38E-03	unknown 4	-3,23E-03
aromatic aldoxime	-3,42E-03	Carvone	-3,33E-03
Myrcene	-3,64E-03	E-Cinnamaldehyde	-3,41E-03
phenylacetonitrile	-3,90E-03	2-ethyl-1,6-dioxaspiro 4.4nonane	-4,09E-03
Benzyl acetate	-4,11E-03	Benzenepropanol	-4,21E-03
1-Nitropentane	-4,91E-03	Myrcene	-4,23E-03
Methyl benzoate	-5,11E-03	Lilac alcohol D	-4,54E-03
Phenylacetaldehyde	-5,75E-03	p-methoxystyrene	-5,82E-03
unknown benzenoid 1	-5,80E-03	Lilac alcohol A	-6,75E-03
Z.E.Linalooloxidepyranoid	-6,41E-03	Fenchyl acetate	-7,82E-03
ST 8	-6,76E-03	3-Pyridinecarboxaldehyde	-8,35E-03
unknown 16	-6,95E-03	E-Arbusculone	-8,60E-03
Z-ß-ocimene	-7,13E-03	E-Cinnamylalcohol	-1,12E-02
1,2-Dimethylbenzene	-7,57E-03	unknown lilac derivative 7	-1,37E-02
α-Copaene	-7,88E-03	unknown N bearing2	-1,57E-02
unknown 3	-8,48E-03	Lilac alcohol B+C	-1,61E-02
δ-Cadinene	-9,01E-03	1,2-Dimethoxybenzene	-2,13E-02
ST 9	-1,05E-02	Lilac aldehyde D	-2,38E-02
γ-Cadinene	-1,20E-02	2-Methylbutyraldoxime	-2,44E-02
unknownEster	-1,31E-02	Z-Arbusculone	-3,06E-02
unknown 5	-2,10E-02	Benzyl benzoate	-3,09E-02
unknown 2	-2,35E-02	p-Cresol	-3,56E-02
ß-Pinene	-2,74E-02	Benzyl alcohol	-4,05E-02
2-Phenylethanol	-3,48E-02	Camphene	-4,95E-02
ß-Bourbonene	-4,81E-02	Lilac aldehyde A	-1,06E-01
γ-Muurolene	-9,70E-02	Lilac aldehyde B+C	-1,27E-01
Sabinene	-9,86E-02	Linalool	-1,36E-01
Benzaldehyde	-1,97E-01	Benzyl acetate	-1,66E-01
E-Ocimene oxide	-2,04E-01	2-Phenylethanol	-2,19E-01
E-ß-Ocimene	-5,44E-01	E-ß-Ocimene	-6,02E-01

SUPPLEMENTARY MATERIAL 3

In this supplementary material we evaluated the congruence between the phylogeny of Sileneae and the flower scent emitted during day. Unfortunately day and night flower scent information is not available for the same species to compare and analyze the day and night VOC evolution in a unique phylogenetic tree. Therefore, we constructed another phylogenetic tree with slight differences in taxon sampling, and the results are not directly comparable to the tree with the scent produced at night.

RESULTS

The 100 floral VOCs emitted at day were summarized in the global PC (PC1) and the local PC (PC33) (Figure 1A). PC1 was mainly defined by the opposite loads of E-β-ocimene to linalool, phenylacetaldehyde and 2-phenylethanol (Figure 1B). *Atocion*, *Viscaria* and *Eudianthe*, outside the clade formed by *Lychnis* and *Silene*, shared few or none E-β-ocimene production (negative scores). The three species of *Lychnis* and the two species of setc. Atocion were the sister group of the rest of the *Silene* species but had opposite scores. *Lychnis* species did not emit E-β-ocimene and produced some aromatics like phenylacetaldehyde and 2-phenylethanol. Sect. Atocion species produced mainly E-β-ocimene and sabinene. In the subgen. Behenantha, the highest negative scores were found in sect. Psammophilae, with *S. littorea* and *S. cambessedesii* producing 100% of E-β-ocimene. In the subgen. Behenantha there were also species with high positive scores, in the sect. Physolychnis and sect. Melandrium, because they produced high mounts of 2 phenylethanol, phenylacetaldehyde or lilac aldehydes. Species of subgen. Silene had scores of different magnitude and sign within each section. PC33 showed the compounds which variation were close-to-tips, like benzyl benzoate, benzaldehyde, β-pinene, camphene and sabinene. For example, *S. psammitis* differs from the other two species of sect. Psammophilae because *S. psammitis* produced high amounts of sabinene.

In the day reconstructions (Figure 2), benzaldehyde was present in the basal node, suggesting that this compound was lost during the evolution of *Silene*, to reappear secondarily in sects. Melandrium and Silene, and in *Lychnis*. By contrast, Linalool was absent in the basal node but then appeared in the common ancestor of *Atocion* and

Viscaria and in some tips within *Silene*. E-β-ocimene was present in the basal node and in most of the internal nodes. However, E-β-ocimene was absent in the common ancestors of sections Physolychnis and Atocion, and *Lychnis.*

DISCUSSION

Floral scents produce phylogenetic signal in Sileneae when semiquantitative data are used. Despite day and night trees were slightly different, they share some patterns. Both at night and day, the phylogenetic signal of flower scentwas dominated by a strong opposition between two monoterpenes, linalool and E-β-ocimene, but during day there were also 2-phenylethanol and phenylacetaldehyde. Also at night and day there are compounds of recent evolution that produce differences between species. This is the case of benzaldehyde that appears several times in the phylogeny and could be due to shared selective pressures exerted by pollinators, hervibores and nursery pollinators. The species of sect. Behenantha have an evolutionary conservative scent at night, with high production of linalool and derivates as lilac aldehydes, but during day they produce different amounts of sabinene, camphene or β-pinene that are result of more recent evolutionary events. If sabinene, camphene or β-pinene attract pollinators with different olfactory preferences exerting different selective pressures, therefore these compounds could contribute to reproductive isolation and speciation. Different key compounds, which attract different pollinators, can produce reproductive isolation between closely related plant species (Waelti et al. 2008) .The study of the diurnal and nocturnal scent evolution in Sileneae may reflect the recent and past evolutionary pressures that have modeled the present scent composition.

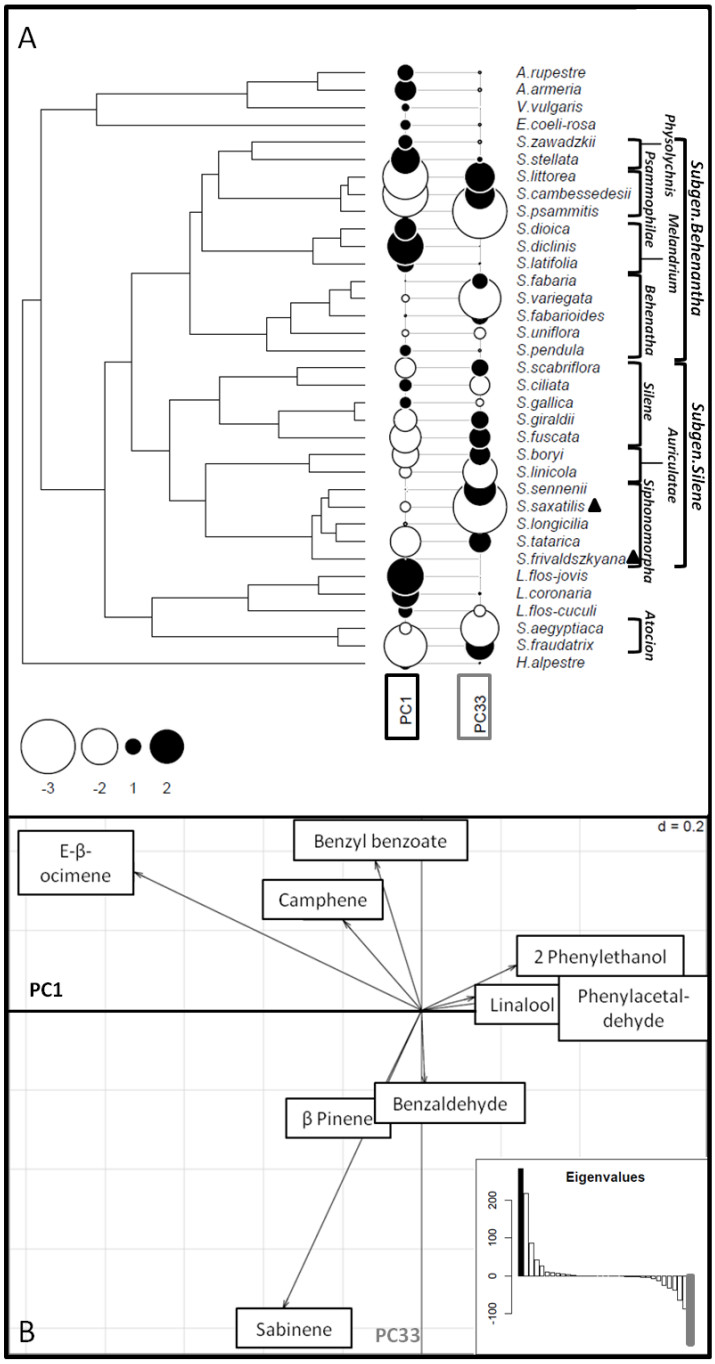

Figure 1. pPCA diurnal scent. (A) PC1 (global) and PC33 (local) principal component. Positive and negative scores are indicated by black and white circles, respectively. Symbol size is proportional to absolute values. (B) Loading of the main traits (see text) for the first global and local principal component. Inset barplot display corresponding eigenvalues. d=0.2 is the mesh of the grid. Black indicates the global and grey the local principal component. *Silene* species are grouping in sections. Black triangles denote species of sub.sect.Sclerocalycinae.

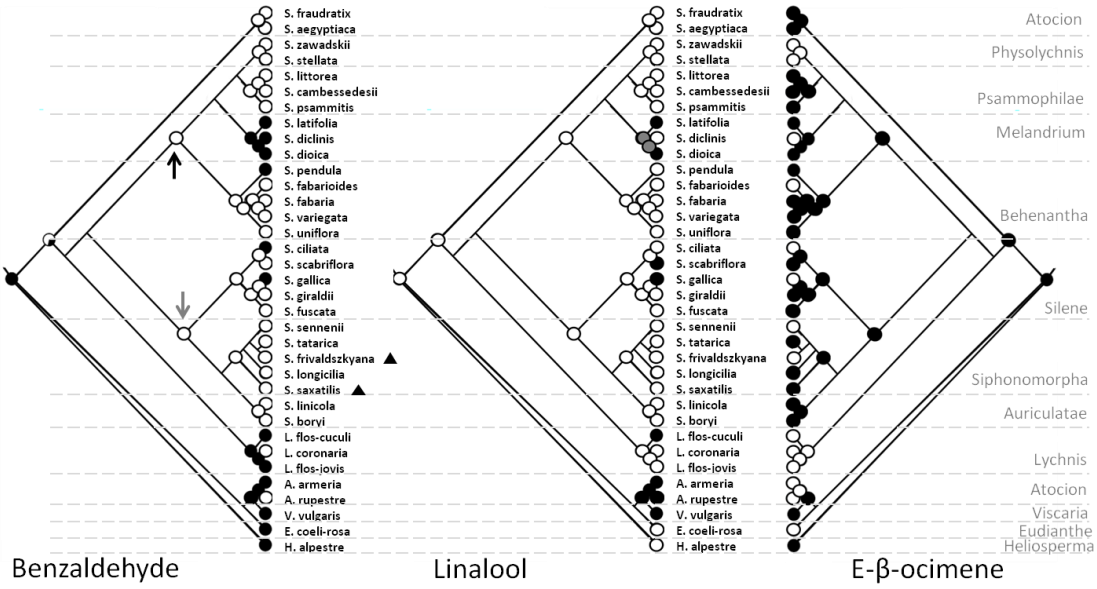

Figure 2. Day reconstructions of the ancestral estates for benzandeyde, linalool and E-β-ocimene. White color in the nodes and tips (circles) denotes the proportion of the trees in which the compound was absent, black when was present and grey when the reconstruction of the character was equivocal. Grey arrow marks the ancestral node to subgen. *Silene* and black arrow marks the ancestral to subgen. Behenatha. Section names are given in grey. Black triangles denote species of sub.sect.Sclerocalycinae.

CAPÍTULO 4/CHAPTER 4

Flower circadian rhythm restricts/constraints pollination generalization and prevents the escape from a pollinator-seed predating specialist in *Silene*.

ABSTRACT

1. Flower traits as scent, nectar and color are important for the attraction of pollinators but also predators. The circadian coordination of some of these traits is important for the attraction of different pollinators at different times in a day, and defines the diurnal and nocturnal pollination syndromes.

2. In this study we test how some flower traits correlate in a circadian rhythm to attract different pollinator guilds. For this aim we chose *Silene colorata*, a species with mixed floral features between the diurnal and nocturnal syndromes of pollination which can be attractive for both diurnal and nocturnal pollinators. Moreover, among the nocturnal visitors *S. colorata* have a nursery pollinator, *Hadena sancta,* whose larvae predate on reproductive structures of the plant. The effect of day vs. night pollination and predation exerted by *Hadena* on the plant reproductive success, were tested in three populations.

3. Our result showed that the repeated petal opening at night was correlated with high nectar and scent production. However, high flower attraction can be maintained in the early morning depending on the environmental conditions. Hand pollinations showed that pollen deposition were similarly effective at night and morning, but higher than in the afternoon. These results are consistent with field studies because both diurnal and nocturnal insects visit the flowers of *S. colorata*. Diurnal and nocturnal visit rates varied among populations, and fruit predation by larvae of *H. sancta* was proportional to nocturnal pollination service in two of the three populations studied.

4. The analyzed flower traits suggest that *S. colorata* is predominantly nocturnal, but favorable environmental conditions may length the optimal period of flower attraction and pollination towards early morning. The net outcome of the *S. colorata-H. sancta* interaction shifts between mutualism to parasitism when other pollinators are present, but the plant is very limited to avoid this nursery pollinator as consequence of its nocturnal pollination syndrome.

INTRODUCTION

Plant circadian clocks coordinate the physiology of individuals to their surrounding environment, and have evolved to enhance fitness in response to predictable changes such as light/dark cycles (McClung 2006; Sanchez et al. 2011). The biological rhythms that affect plant reproduction can be divided in the seasonal cycles of flowering phenology, mediated mainly by day length, and the daily rhythms of flower advertisement and reward, like opening-and-closure movements (van Doorn & van Meeteren 2003; McClung 2006), nectar production (Cruden et al. 1983) or diel variation of flower scent (Dobson 2006; Knudsen et al. 2006). The rhythmic movement of plant organs in response to the onset of darkness is called nyctinasty (Darwin 1880; Palmer & Asprey 1958; Satter & Galston 1981). It has long been presumed that flower nyctinasty and dynamics of nectar secretion and scent emission have evolved to match the flower attractiveness to the time of activity of the most efficient pollinators (Dudareva et al. 2000; van Doorn & van Meeteren 2003; Pacini & Nepi 2007), as a part of the pollination syndromes. Pollination syndromes are defined as the suite of floral traits associated with the attraction and utilization of a specific group of animals as pollinators (Faegri & van der Pijl 1979; Fenster et al. 2004). Specific combinations of floral traits should reflect selection mediated by the pollinators that visit the flowers most frequently and/or effectively (Stebbins 1970). Although the pollination syndrome concept has been controversial in past decades (Waser et al. 1996; Ollerton 2007) recent works suggest that, in most of the plants studied, pollination syndromes predict accurately the most effective pollinators (Rosas-Guerrero et al. 2014). However, it is assumed that adaptation to a particular type of pollinator by floral specialization may incur in fitness trade-offs, reducing the effectiveness of generalist pollinators (Galen & Newport 1987; Hurlbert et al. 1996; Aigner 2004) and preventing the escape from specialized herbivores and pathogens (Strauss & Whittall 2006). These reasons, together with the spatio-temporal variation in the abundance and identity of the most effective pollinators, explain from an evolutionary perspective the prevalence of generalized over specialized pollination systems (Herrera 1996; Waser et al. 1996; Gómez & Zamora 2006; Johnson & Steiner 2000; Fenster et al. 2004; Waser & Ollerton 2006).

In *Silene* (Caryophyllaceae), two pollination syndromes have been traditionally described, nocturnal and diurnal (Lindman 1897; Greuter 1995). Diurnal species usually

have pink or red corollas, and flowers are usually open during day and night. These species do not show obvious changes of scent intensity between day and night as perceived by the human nose (Greuter 1995; Jürgens 2004; Jürgens 2006). Nocturnal species have white or pale flowers that show repeated petal opening and intense scent emission in the evening/night (Greuter 1995; Jürgens et al. 2002; Jürgens 2006). Flower nyctinasty has been barely studied in *Silene*, and is likely caused by the different rate of water loss from the cells of the upper and lower epidermis of petals, due to a combination of changes in light intensity and soil moisture (Halket 1931). Also, in *S. latifolia* the petals are responsible of the emission of specific nocturnal VOCs mediating plant-insect communication (Dötterl & Jürgens 2005). Although diurnal and nocturnal pollination syndromes are apparently well defined in *Silene* (but see Chapter 2), some questions remain unclear. First, few case studies have addressed whether pollination syndromes of *Silene* species predict accurately the most effective pollinators (Giménez-Benavides et al. 2007; Reynolds et al. 2009; Martinell et al. 2010). Second, some key flower traits have received less attention despite they may be also regulated by circadian rhythms, such as the dynamic of nectar secretion, anther dehiscence, pollen viability and stigmatic receptivity. One previous study showed that nectar volume increases from afternoon until midnight, and then decreases, in four nocturnal *Silene* species (Witt et al 1999), but the observed pattern was not contrasted with pollinator data. Pollen germination depends on time after dehiscence in *S. dioica* and *S. acutifolia* (Bassani et al. 1994; Buide & Guitan 2002), but stigmatic age does not affect stigmatic receptivity in *S. latifolia* (Young & Gravitz 2002). And third, it is not clear whether the daily variation in these traits may affect the interaction between *Silene* species and their specialist nursery pollinators. The moths of the genus *Hadena* (Noctuidae) pollinate many *Silene* species, but also use the flowers and fruits as food resource for their larval offspring. The outcome of this interaction may shift between mutualistic to antagonistic depending on the presence and importance of other pollinators (Giménez-Benavides et al. 2007; Reynolds et al. 2012). For these reasons, the Silene-Hadena system has recently emerged as a nice model system to understand the evolution of mutualisms (Kephart et al. 2006). To understand the relative contribution of *Hadena* and copollinators to the reproductive output of *Silene*, we need to know which flower traits are important for the attraction and efficiency of each group of flower visitors.

In this study we evaluate the functional coherence of the combination of flower traits exhibited by *S. colorata*, a species with mixed floral features between the diurnal and nocturnal syndromes. *S. colorata* has flowers with pink corolla but shows a marked nyctinasty and emit flower scent during night, but not at midday (Chapter 2). Our specific objectives were: 1) to characterize the diel variation of flower traits related with the attraction of pollinators (petal opening, emission of scent and secretion of nectar); 2) to investigate the dependence on pollinators analyzing the breeding system of the species; 3) to assess whether anther dehiscence and pollination success are synchronized with flower nyctinasty; 4) to determine whether the floral phenotype of *S. colorata* can predict its pollinators; and 5) to explore the influence of these floral traits in the net outcome of the interaction with its *Hadena* nursery pollinator.

We expect that the combination of flower traits of *S. colorata* may attract a generalist suite of flower visitors, both diurnal and nocturnal. The flower nyctinasty and high scent emission at night suggest that this species is more specialized on nocturnal visitors, but these traits have not been characterized in detail. We expect that flower opening, scent emission, nectar secretion and anther dehiscence will follow the same circadian rhythm. The net outcome of the *Silene-Hadena* interaction will depend on the importance of the complete suite of flower visitors as pollinators.

MATERIAL AND METHODS

Plant material

S. colorata Poiret (Caryophyllaceae) is an annual plant with a height of 15-60 cm. Calyx is 10-15mm in length and petal limbs are 5-12 mm, bipartite and pink. Fruit capsules open at the top when ripe and hold 45-85 seeds of 1-1,5 mm in diameter (Talavera 1991). Flowers are protandrous and the anthesis (first opening of the flower from the bud stage) is at sunset. The petal limbs remain open during all night and close (rolling themselves) early in the morning. Nonetheless, the sexual parts of flowers remain accessible when petals are completely rolled (personal observation). This species inhabits croplands and sideways of the Mediterranean region, north of Iran, Arabia and the Canary Islands (Talavera 1990). In our area of study (Madrid, Spain) the flowering period usually spans from April to June.

Plants used in this study grew from seeds in the greenhouse of the Universidad Rey Juan Carlos (Móstoles, Madrid 40º20'02"N, 3º52'57"W, altitude 651 m). Seeds were obtained directly from natural populations in summer 2011 and 2012 (Supplementary material 1) and stored in silica gel at ambient temperature until next spring, when they were sown in 5 cm seedling trays. After three months, plantlets were transferred to 2 l pots until flowering. Plants grew outdoors in an insect exclusion structure from June to July 2012 and 2013. Pollinator observations were done in the populations of origin.

Effect of light intensity and soil moisture on timing and duration of flower opening

Since petal nyctinasty is related with water content in limb cells (Halket 1931), we expected that plants exposed to high light intensity and/or dry soil close their petals earlier in the morning, and open them later in the evening, than those exposed to low light intensity and/or wetter soil. To explore this, we subjected 29 plants to a factorial experiment with two levels of light intensity and two levels of soil moisture: "Shadow-Wet" (N=8), "Shadow-Dry" (N=4), "Sun-Wet" (N=8) and "Sun-Dry" (N=9). "Wet" plants were supplied with 60 min of drip irrigation every day, and "Dry" plants every two days. "Sun" plants were exposed to direct solar radiation, whereas "Shadow" plants were placed under shading net. The light intensity was 191.25 and 42.25 µmol photon/ m^2*s in the "Sun" and "Shadow" treatments, respectively (mean of two days at 8:00, 16:30 and 20:30 h, with a Field Scout Quantum Light Meter, Spectrum Technologies, Plainfield, USA). In the "Sun" treatment the temperature varied between 21.8-39.3 ºC in the morning (7:30 to 11:00 h) and between 31.8-23.1 ºC during evening-night (20:30-00:00 h). In the "Shadow" treatment the temperature varied between 21.9- 32.6 ºC in the morning and between 31.9- 24 ºC during evening-night.

The dynamics of petal opening and closure at dusk and dawn were calculated by measuring the corolla diameter every 30 minutes, from 20:30 to 00:00 h and from 7:30 to 11:00 h, respectively. Measurements were made with a digital caliper on a total of 259 flowers (mean ±SE) of flowers per plant in each treatment: "Shadow-Wet" 9.0±2.7, "Shadow-Dry"6.8±2.8, "Sun-Wet" 9.2±1.0 and "Sun-Dry" 9.6±2.6), from 11th to 18th July 2013. In each flower, the maximum diameter achieved was considered as 100% of the corolla opening, and was used to calculate the percentage of flower opening at each time interval.

Dynamic of flower scent emission

Previous analysis reported that *S. colorata* did not emit scent at midday, unlike the typical *Silene* species with diurnal pollination syndrome (Chapter 2). However, we wanted to assess whether *S. colorata* emits flower scent at the beginning of the day, and in that case, to compare the emission rate and composition with nocturnal samples. From 11th to 18th July 2013, we sampled flower VOCs from 11 plants using a dynamic head-space method. Inflorescences were enclosed in polyethylene oven bags for 5 min, and the emitted volatiles were then trapped for another 5 min in adsorbent tubes (Dötterl et al. 2005; Dötterl & Jürgens 2005) with a 9 V battery-operated pump (Giménez-Benavides et al. 2007). The number of flowers per inflorescence ranged between 2 and 10, and age of flowers was 1-4 days. Nine samples were taken during night (between 21:30 to 23:15) and 11 samples during day (between 7:50 to 9:30), when most of the flowers were at least partially open. Surrounding air samples were taken as negative controls to distinguish between floral compounds and ambient contaminants. Since we also wanted to assess whether flower VOCs are emitted from the petal limbs or from other parts of the flower, we sampled three plants of the "Shadow-wet" treatment after removing all the petal limbs (hereafter "no petals limbs" samples). After scent sampling, the flowers in each bag were counted and clipped, and they were dried in an oven at 60ºC for 24 hours to obtain their dry weight. To control for the emission of green leaf volatiles (GLVs, Visser et al. 1979, Light et al. 1993) we took one sample from vegetative parts (leaves and stems) and the volatiles detected were deleted from the matrix of flower scent compounds.

The volatiles trapped were analyzed by GC/MS using an automatic thermal desorption (TD) system (TD-20, Shimadzu, Japan) coupled to a Shimadzu GCMS-QP2010 Ultra equipped with a ZB-5 fused silica column (5 % phenyl polysiloxane; 60 m, i.d. 0.25 mm, film thickness 0.25 µm, Phenomenex). The samples were run with a split ratio of 1:1 and a constant helium carrier gas flow of 1.5 ml/min. The GC oven temperature started at 40°C, then increased by 6°C/min to 250°C and held for 1 min. The MS interface worked at 250°C. Mass spectra were taken at 70 eV (EI mode) from m/z 30 to 350. GC/MS data were processed using the GCMSolution package, Version 2.72 (Shimadzu Corporation 2012).Identification of the compounds was carried out using the NIST 11, Wiley 9,

FFNSC 2, and Adams (Adams 2007) databases as well as the database available in MassFinder 3. Some of the compounds were confirmed by comparing mass spectra and retention times with those of synthetic reference compounds. Total scent emission was estimated by injecting known amounts of monoterpenoids, aromatics, and aliphatics (added to adsorbent tubes). The mean response of these compounds (mean peak area) was used to determine the total amount of each compound extracted from the adsorbent tubes (Dötterl et al. 2005). For each sample and compound we calculated the absolute amount emitted (ng) by flower (number), dry weight (gr), and time (min).

Nectar secretion dynamic

To characterize the temporal variation of nectar production, we took samples from 464 flowers of 23 plants (20±3.1 flowers per plant), from 12 June to 16 July 2013. Before the initiation of anthesis, several cohorts of flowers were randomly marked with color codes to control for flower age and sexual stage. Flowers were sampled until three days after begin of anthesis. Nectar was sampled in three time intervals, morning (10-13 h), evening (17-19 h) and night (21-23 h), with 0.25 µl calibrated microcapillaries (Drummond Scientific Co.). The length of the nectar column was measured with a digital caliper to calculate the extracted volume (µl). The calyx tube of *S. colorata* is deep and narrow, so the nectar extraction implied destruction of the flowers. Therefore, we quantified the nectar accumulated from the anthesis until each time of measure (Witt et al. 1999). Sample size was high (n=30-70 on each time interval) to cope with destructive sampling, with the intrinsic variation of nectar measurement, and with the high frequency of nectarless flowers (Witt et al. 1999).

Anther dehiscence, breeding system and pollination success throughout day

To assess whether nyctinastic flower opening is coupled with the release of pollen grains and the elongation of the style, we carried out direct observations from initial flower opening until the fourth day of each flower. We observed 2-3 flowers from five plants in June 2012, and we captured a long series of photographs every 15 minutes with a 90 mm macro lens to make a time-lapse sequence (Bieleski et al. 2000).

To analyze whether nyctinastic flower movements are correlated with variation in pollination success throughout day, we performed a hand pollination experiment on June-July 2012. We randomly assigned 263 flowers from 117 plants (2±0.2 flowers per plant) to one of the following time intervals, “Morning” (9:00-11:00 h) “Afternoon” (15:30-19:00 h) and “Night” (21:00-23:00 h). Pollinated flowers were in the second or third day of the female state. Pollen was collected immediately before pollination. All hand-pollinations were performed with pollen from another plant of the same population (intrapopulation xenogamy), assigned randomly. Additionally, to investigate the breeding system of *S. colorata* (i.e. its dependence on pollinators), we randomly assigned 296 flowers from 137 plants (3.0±0.1 flowers per plant) to one of the following treatments: (1) spontaneous autogamy (flowers were individually bagged but not hand pollinated), (2) geitonogamy (hand-pollination with pollen from other flower of the same plant), (3) intrapopulation xenogamy, and (4) interpopulation xenogamy. Pollen donors were randomly assigned within treatment. For the breeding system study, pollinations were made only at night (21:00-23:00 h) because it was the time interval that achieved the highest pollination success in the previous experiment (see results).

In both experiments, pollen was supplied by taking one anther of the donor flower with forceps and brushed it on the stigmas of the recipient one. Flowers were bagged with organza bags (4x3 cm) before and after hand pollinations to avoid pollen contamination, until fruit ripening. After 2-4 weeks we sampled all fruits to calculate fruit set (proportion of flowers setting fruit) and the number of seeds per fruit. To describe the breeding system we calculated a modification of the self-incompatibility index (ISI, Zapata & Arroyo 1978). ISI was calculated both with fruit set and number of seeds, dividing the success of geitonogamy by the success of intrapopulation or interpopulation xenogamy. Values ≤ 0.25 indicate self-incompatibility (Sobrevilla & Arroyo 1982; Faria et al. 2012). Finally, we tried to explore the variation in pollen viability throughout day by germination tests. Pollen collected at the same time intervals was placed onto Petri dishes containing agar with 30% of sucrose (Buide & Guitan 2002). However, the culture medium used to test the pollen viability did not work in this species, despite it was the best medium for a relative species, *S. acutiflora* (Buide & Guitan 2002).

Flower visitation rates and reproductive success in natural populations

To assess whether floral phenotype of *S. colorata* is adapted to a particular type or functional group of pollinators, we conducted a field test from 5th to 22nd May 2012. Censuses of flower visitors were made in three natural populations of *S. colorata,* for a total of 15-20 hours of observation per population. To collect visitation data, we established five 1 x 1 m sampling plots in each population. Each plot contained several plants, ranging the number of flowers per plot between 25 and 320 in the flowering peak. Diurnal observations were made on sunny days without wind, at different time intervals from 10:00 to 19:00 h. At each time interval, we made observations of 5-10 minutes in each plot and noted the identity and number of contacts of insect species with the reproductive structures of the flowers. Nocturnal observations were conducted with customized digital video cameras equipped with near-infrared light (Giménez-Benavides & Prieto-Benítez, unpublished). Video recordings from 21:00 h-01:00 h were visualized afterwards to note identity and frequency of flower visitors. We choose this time interval because it usually accounts for most of the nocturnal moth visits in other *Silene* species (Petterson 1991; Martinell et al. 2010). We could not accurately distinguish among moths species in the night video records. All visiting insects touching sexual organs were considered pollinators regardless of the efficacy of the visit. Insects were grouped into functional groups to calculate visitation rates (visits flower^{-1} hour^{-1}) per *time* (morning: 9:00-15:00 h), afternoon: 15:00-19:00 h, and night: 21:00-1:00 h). The minutes of observation per population range between 150-200 in the morning, 50-100 in the afternoon and 672-1078 in the night.

2-3 weeks after pollination census (25 of May-4 of June 2012), we randomly sampled 10 plants that have completed the full life cycle in each 1 x 1 m plot. We counted the total number of flowers (dried or aborted) and fruits produced per plant to calculate the fruit set. The rate of fruit predation (number of predated fruits/total number of fruits) by the *Hadena* nursery pollinators was also estimated because the larvae of these moths leave a characteristic hole in the capsules (pers. obs). Non-predated fruits were dissected to count the number of seeds. Outside the plots, we also collected green fruits that were carried to the laboratory. After some days the *Hadena* larvae that emerged from the parasitized fruits thereof were reared until adult stage to identify the species.

Statistical analysis

To explore the effect of light intensity and soil moisture on the dynamic of flower opening, a LMM (linear mixed model) was carried out with the following explanatory variables: *light* (Shadow and Sun), *moisture* (Wet and Dry), *time* (every 30 minutes), *plant* and the interaction *light*moisture*time*. These factors were computed as fixed effects and *plant* as random effect. The percentage of petal opening was arcsin (square root(X)) transformed before analysis to achieve normality.

To analyze the variation of flower scent depending on the *treatment* (morning, night and flowers without petals), a set of GLM analyses were made for total scent production and the production of each compound independently. To depict variation in floral scent composition among samples we used non-metric multidimensional scaling (NMDS) with the Bray-Curtis pairwise matrix of similarities (Clarke & Gorley 2006). To avoid that NMDS were largely influenced by the most abundant compounds the data were forth root transformed (Clark & Warwick 2001).

To explore the nectar dynamic, a GLM was applied with *day* (1st, 2nd and 3rd), *time of sampling* (morning, evening and night) and the interaction *day* * *time of sampling* as the explanatory variables. To evaluate the breeding system, and the differences in pollination success throughout the day, we use linear models (LM) for fruitset and GLMs for number of seeds. *Treatment* (geitonogamy, intrapopulation xenogamy and interpopulation xenogamy) and *pollination time* (morning, afternoon and night) were used as explanatory factors for breeding system and pollination success, respectively. Autogamic hand crossings were excluded from the analyses because they did not produce any fruit (see results).

To explore differences in flower visitation rate, we carried out a GLM with *population*, *time* and *population*time*. Fruit set, fruit predation (LM) and seed number (GLM) comparisons between populations were performed with *population* as explanatory factor. To test the effect of the visitation rate on the pollination success we performed four (total, morning, afternoon and night rates) LM and four GLM regression for fruit set and seed number respectively. For these GLM regressions we used Poisson error structure because the seed number was positive integer values. For the rest of GLM models we used a tweedie error structure because of the zero-inflated distribution of the data (Dunn & Smyth 2005; Tascheri et al. 2010). Post-hoc analyses were performed

with the Tukey HDS test. Analysis were implemented with the *tweedie, nlme, and argricolae* packages (Pinheiro et al. 2013; Dunn 2014; Mendiburu 2014) for R software (R Core Team 2014), except the NMDS that was implemented in PRIMER 6.1.11 (Clarke and Gorley 2006).

RESULTS

Effect of light intensity and soil moisture on flower nictinasty

There were significant effects of *light intensity* ($F_{1,1651}$=8.57, *P*=0.007) and *time of measure* ($F_{13,1651}$=498.8, *P*<0.001) on the percentage of petal opening. *Soil moisture* had a marginal effect ($F_{1,1651}$=4.01, *P*=0.057) but the interaction *moisture*light*time* was highly significant ($F_{13,1651}$=3.93, *P*<0.001). In the evening, petals of the Shadow/Wet treatment were most unrolled at 21-21:30 h (Figure 1). All treatments reach the 100% opening at 22-22:30 h and remained open until dawn. In the morning, petals of Shadow/Dry treatment were the first to close at 7:30-8 h, followed by plants under the Sun/Dry conditions at 8:30-9 h. Plants under the Shadow/Wet treatment maintained the petals more open than Dry treatments until 10-10:30 h. The closest petals at the end of the morning were those under Sun treatments, although differences were not significant.

Dynamic of flower scent emission

The GC-MS analyses showed that two of the night samples did not emit any scent. These plants had flowers with the petals not completely open when volatiles were trapped (21:10 h). These two samples were not taken into account in the GLM analysis. Another night sample with the petals excised did not emit scent either. These three samples were not taken into account in the NMDS analysis. Morning and night samples formed different groups in the NMDS analysis (Figure 2). Samples without petals were placed between the morning and night groups. The standardized total scent production (ng flower $gr^{-1}min^{-1}$) was higher in night samples ($F_{2,18}$=48.62 *P*<0.001) (Figure 3). There were significant differences in *treatment* in some compounds (benzaldehyde $F_{1,8}$=9.57 *P*=0.015; benzyl alcohol $F_{2,18}$=12.04 *P*<0,001; p-benzoquinone $F_{2,18}$=6.23 *P*=0,009; E-caryophyllene $F_{1,9}$=0.85 *P*=0.38) but not in 2-phenylethanol ($F_{2,18}$=0.74 *P*=0,49) and benzyl acetate ($F_{2,18}$=0.67 *P*=0,52). Benzylaldehyde and E-caryophyllene were emitted in

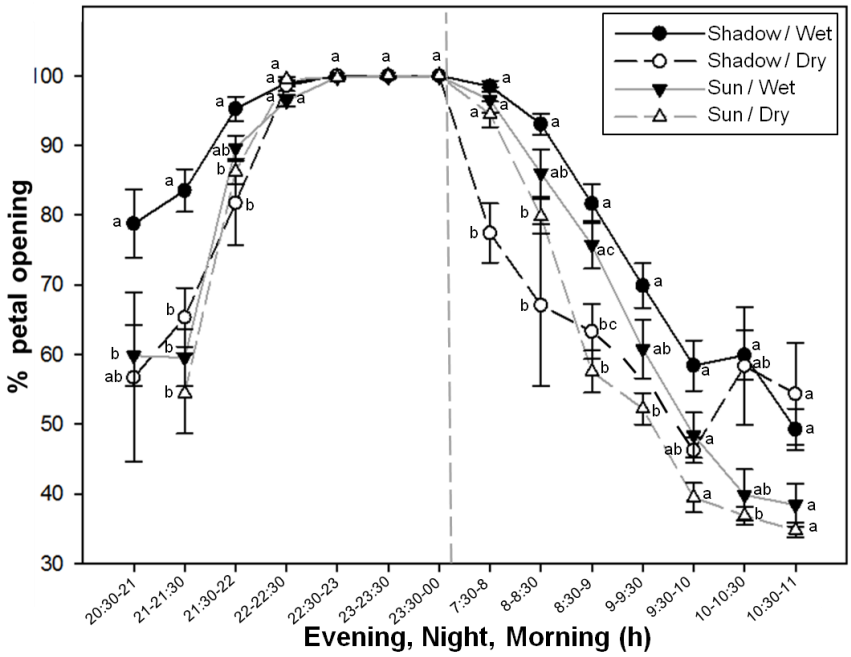

Figure 1. Dynamic of flower opening (mean and SE of % petal opening) on plants subjected to four treatments of light intensity/soil moisture. Different letters indicate significant differences among treatments at the same time period. Vertical dashed line denotes the pass from night to morning. At 9-9:30 and 20:30-21 time intervals there were no measures in the Shadow/Dry and Sun/Dry treatments, respectively.

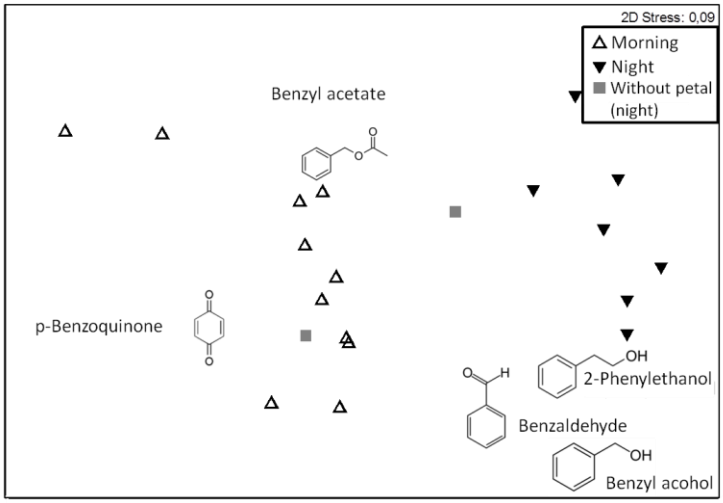

Figure 2. Non-metric multidimensional scaling (NMDS) of flower scent samples of *S. colorata* at morning, night, and night with petals excised. Main volatile compounds are depicted.

high amounts at night but not at day. The emission of benzyl alcohol was higher at night than at day. Conversely, three unknown compounds were only emitted in the morning and the emission of p-benzoquinone was higher at day than at night. The excision of the

petal limbs reduced the emission of benzaldehyde and wiped out the emission of benzyl alcohol, but increased the amount of p-benzoquinone, and did not affect to E-caryophyllene, 2-phenylethanol and benzyl acetate (Figure 3).

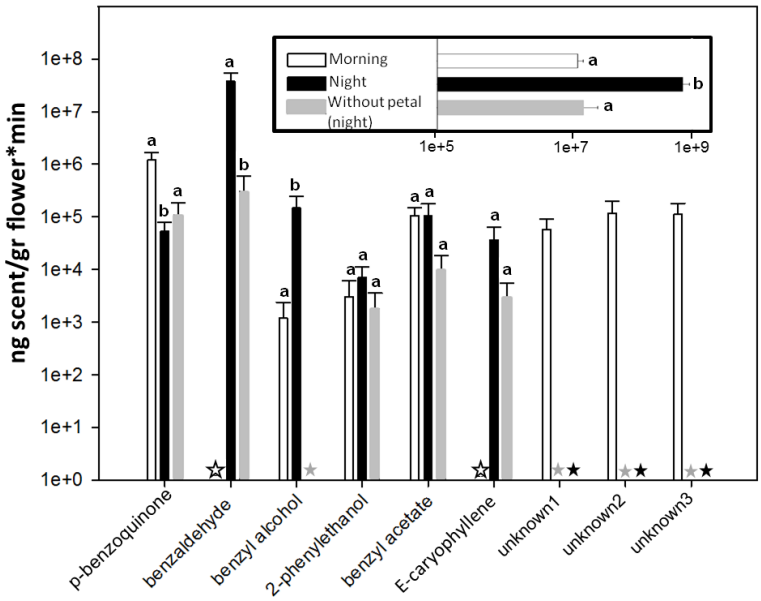

Figure 3 Mean and SE of emission of each floral volatile compound (vertical bars). Inserted plot (horizontal bars) denotes the total scent emission. Stars denote no production of a compound. Different letters indicate significant differences among treatments. Both graphs are in Log scale. N was 11, 7 and 3 in "Morning", "Night" and "Without petal (night)", respectively.

Anther dehiscence, breeding system and pollination success throughout day

Anther dehiscence of *S. colorata* took place immediately after the flower bud burst. The two whorls of five stamens dehisced sequentially on first and second night, and they withered at dawn. The style elongation began at dusk of the third day. Both anther dehiscence and style elongation are perfectly synchronous with nyctinastic flower opening. The pollen was of fresh and dusty appearance from anther dehiscence until mid morning, but then it turned dry and clumpy (personal observation).

In the breeding system experiment, the spontaneous autogamy treatment did not produce any fruit. There were no differences between geitonogamy, intrapopulation xenogamy and interpopulation xenogamy in fruitset ($F_{2,17}$=2.2 P=0.14) and in the number of seeds ($F_{2,271}$=0.33 P=0.71). The ISI values indicated that *S. colorata* self-compatible (fruit set ISI=0.86 and seed number ISI= 0.91 for interpopulation xenogamy;

fruit set ISI=1.01 and seed set ISI=0.95 for intrapopulation xenogamy). Both fruitset and number of seeds were lower in the afternoon than in the morning and at night pollinations ($F_{2,17}$=13.12, *P*<0.001 and $F_{2,262}$=25.71, *P*<0.001 respectively, Figure 4).

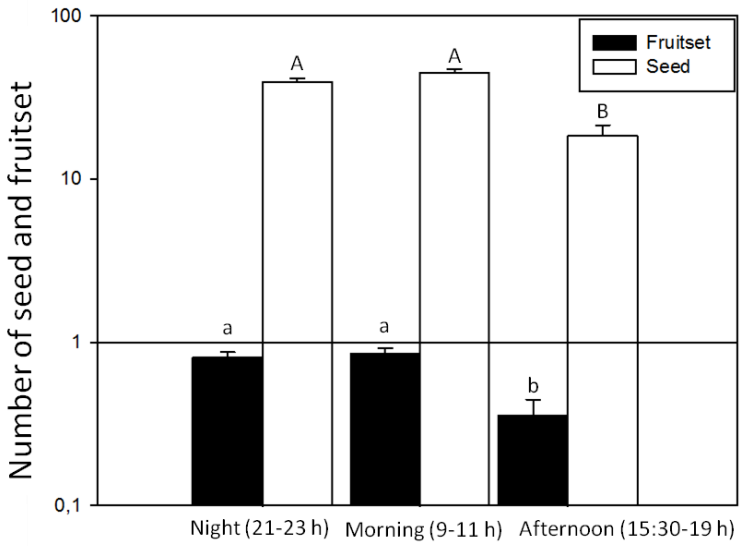

Figure 4. Pollination success throughout day. Mean and SE of number of seed and fruitset derived from hand pollinated flowers of *S. colorata*. Different capital and lower case letters indicate significant differences among treatments. The figure is in Log scale. For both fruit set and seed number N was 90, 127 and 46 in Morning, Night and Afternoon, respectively.

Nectar dynamic

Nectar volume was very low at all time intervals (range=0 - 0.21 µl). There was a high proportion of flowers that did not produce nectar (Figure 5). Nonetheless, there were significant differences in nectar volumes between time intervals ($F_{2, 455}$=9.03, *P*<0.001) and in the interaction *day* * *time of sampling* ($F_{6, 455}$=6.54, *P*<0.001) but not between days ($F_{2, 455}$=1.71, *P*<0.18). The first morning after flower bud burst, nectar volume was high, and then decreased in the course of the day (Figure 5). In the second day, flowers showed the same pattern but there were no significant differences among daytimes. In the third day (first in female stage) there were no significant differences either (Figure 5).

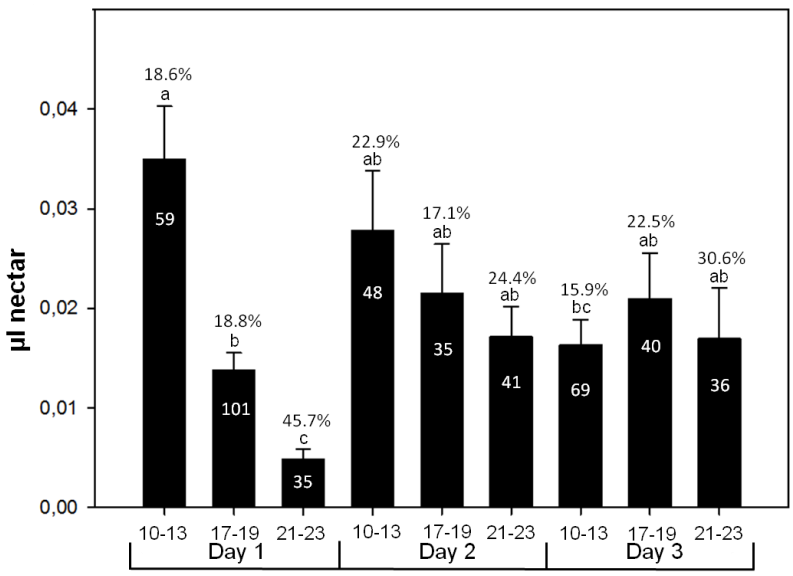

Figure 5. Mean nectar volume (µl) accumulated from the anthesis until the time of measure (h) in flowers of *S. colorata*. Note that first measure corresponds to the morning after the flower bud opening at dusk. On day 1 and 2 flowers are in male stage, on day 3 flowers reach the female stage. Error bars denote SE. Different letters indicate significant differences among time intervals. Numbers above each bar denote the precentage of nectarless flowers. Number within each bar denote the N.

Flower visitation rates, reproductive success and fruit predation by the nursery pollinator

Field results showed that *S. colorata* was visited by both diurnal and nocturnal insects (Figure 6). At day time (morning and afternoon), small bees were the most frequents visitors (range from 100% to 81% of diurnal visits), followed by bombilid flies (0-18%) and hoverflies (0-1%). At night all visits were by moths (Noctuidae and Geometridae). All larvae emerged from fruits of *S. colorata* were from *Hadena sancta*. The visitation rate was lower in the afternoon than in the morning and night ($F_{2, 43}$=5.50, *P*=0.008). Also, there were differences between populations ($F_{2, 43}$=7.85, *P*=0.002) (Figure 6) and a significant effect of the interaction between population and time interval ($F_{4, 43}$=5.26, *P*=0.002) (Figure 6). Fruit set and number of seeds were not different among populations ($F_{2, 12}$=0.41, *P*=0.67 and $F_{2,12}$=0.59, *P*=0.57, respectively). None of the visitation rates correlated with fruitset ($F_{1, 13}$=0.67, *P*=0.43; $F_{1, 13}$=0.02, *P*=0.89; $F_{1, 13}$=1.59, *P*=0.23; $F_{1, 12}$=2.45, *P*=0.14; for total, morning, afternoon and night visitation rate respectively). There was a positive influence of the total ($Z_{1, 12}$=4.04, *P*=0.04), night ($Z_{1, 12}$=2.16, *P*=0.031), and afternoon ($Z_{1, 11}$=2.03, *P*=0.042) visitation rate on the seeds number, but

not of the morning visitation rate ($Z_{1, 11}$=-1.84, *P*=0.14). There were differences among populations of the fruit predation rate by *Hadena sancta* ($F_{2, 11}$=4.51, *P*=0.037) (Figure 6).

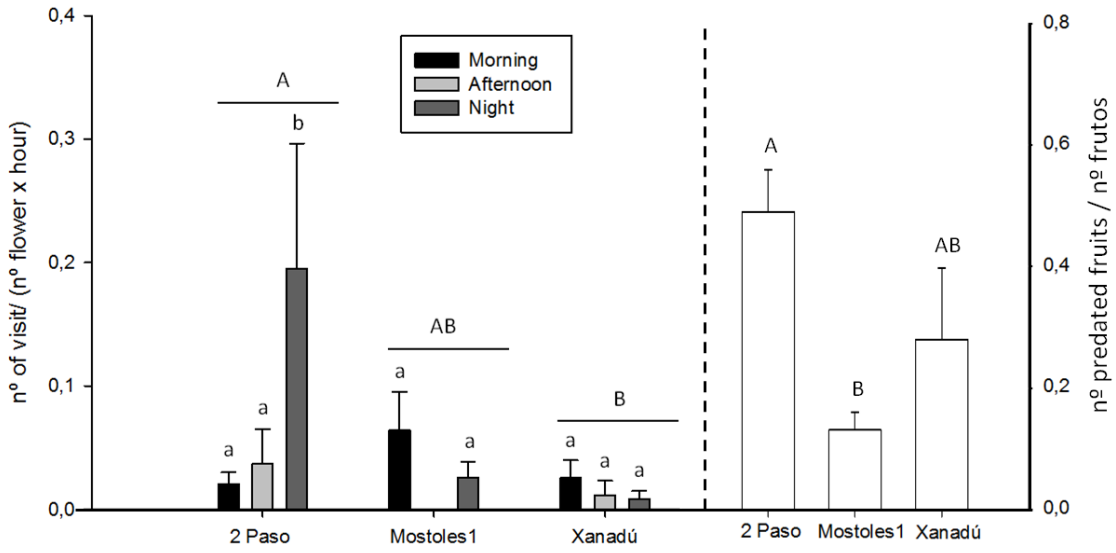

Figure 6. Left: mean and SE of flower visitation rate (visits flower^{-1} hour^{-1}) per population and time (morning, afternoon and night). Right: mean and SE of fruit predation rate per population. Different capital letters indicate significant differences among populations. Different lower case letters denote differences in time period within each population. N=5 per time and/or population.

DISCUSSION

Flower traits and nyctinasty

The first observation of the floral biology of *S. colorata* is an interesting contradiction, it has bright pink petals pointing towards a diurnal pollination system, but flowers are fully-opened only at night. Flower color has been one of the classical phenotypic traits for definition of pollination syndromes (Faegri & van der Pijl 1979; Proctor et al. 1996), but it must be taken with caution as key trait (Waser et al. 1996). Night blooming species often have white or pale corollas presumably to reflect the moonlight and to attract animal pollinators, whereas bright-colored corollas may be more attractive during day, when visual cues may be more important (Menzel & Shmida 1993; White et al. 1994). However, it is known that petal color transitions are very frequent in plant evolution (Weiss 1991; Weiss 1995; Rausher 2008), and are consequence of few genetic changes (Hoballah et al. 2007; Wu et al. 2013). Color polymorphisms are also frequent at intraspecific level, sometimes without shifts in pollinators (Schemske & Bierzychudek 2001; Reynolds et al. 2009; Wang et al. 2013). In Sileneae the flower color is not very

reliable as predictor trait for diurnal or nocturnal pollination, as shown in a recent study (Chapter 2).

Our results suggest that the nyctinastic petal rolling in *S. colorata* is triggered by changes in light intensity and can be accelerated or delayed by soil water content. *S. colorata* flowers start to open at dusk, when the light intensity decreases. In shadow microhabitats (e.g. beneath trees or shrubs) the light intensity decreases earlier at dusk and increases later at dawn, and the soil retain more water. In consequence the flowers open earlier and close later, extending the period of flower display resulting in an increase in the visibility for evening and early morning flower visitors. In sunny and dry microhabitats, petal closure is accelerated at dawn and the petals remain rolled during all day, reducing the temporal range of floral display. The closing and opening of petals was also influenced by light intensity and air humidity in *Silene saxifraga* (Halket 1931). Halket showed that the closing of petals is due to the loss of cell water content by transpiration, and the opening is due to cell refilling likely in response to a combination of sugar uptake and degradation of polysaccharides (van Door & van Meeteren 2003). The maintenance of turgor in the corollas requires a constant input of water from vegetative parts (Ram & Rao 1984), and this demand may involve a high cost of reproduction especially in dry environments (Nobel 1977; Galen et al. 1999). Since light is the most important input signal to the circadian clock (McClung 2006; Sanchez et al. 2011) an increase in light intensity may regulate the flower closure to anticipate the plant to the high evapotranspirational demand in the central hours of the day. This strategy could be very advantageous in the Mediterranean-type climate that dominates the distribution range of *S. colorata*. Therefore the maintenance of flower nyctinasty in *S. colorata* may be constrained by a tradeoff between water economy and visibility for diurnal pollinators.

Nectar secretion, flower scent and sexual phases

We have also found that nyctinastic petal opening is synchronized with nectar secretion dynamics. In *S. colorata* there is a higher nectar production and accumulation at night, at least the first day and probably also the second day after anthesis. At the third day (first in female phase) this pattern was lost. It remains to be tested the nectar dynamics during the rest of the female stage. Nectar production increases at night also in other

Silene species with a nocturnal pollination syndrome (Witt et al. 1999) and similar diel patterns have been previously described in other species pollinated by nocturnally active animals (Cruden et al. 1983; Tschapka & von Helversen 2007; Amorim et al. 2013). Since insect visits were excluded in our experiment, the reduction in nectar volume from night to afternoon could be due to evaporation or resorption. Evaporation of nectar may be limited in this species due to the high osmolality provided by the high hexose (glucose and frutose) content (Witt et al. 2013) and the availability of nectar within a long floral tube. However, nectar resorption is a widespread strategy in unvisited flowers, presumably to recover the resources invested in nectar production (Burquez & Corbet 1991; Pacini & Nepi 2007). The high number of nectarless flowers in this species is in accordance with previous findings in several plant species and also in other *Silene* (Brink 1982; May 1988; Gilbert et al. 1991; Witt et al. 1999).

Nyctinastic flower opening and nectar secretion are also synchronized with the emission of high amounts of scent at night. The corolla usually produces most of the volatile organic compounds (VOCs) of the flower scent (Dobson 1990; Bergström et al. 1995). When petal limbs were abscised, the total scent emission rate at night decreased significantly, and was similar to emission rate of intact flowers at morning. This suggests that the emission of the most abundant flower VOCs at night, benzaldehyde and benzyl alcohol, takes place mainly in the expanded petals, whereas other compounds (i.e., p-benzoquinone, E-caryiphyllene, 2-phenylethanol and benzyl acetate) are released from other floral organs (Dötterl & Jürgens 2005). Petals are also responsible of the benzenoid emission in *S. latifolia* (Dötterl & Jürgens 2005) and are also the main scent producer in *Petunia* and *Antirrhinum* flowers (Dudareva et al. 2000; Verdonk et al. 2003). When petals are rolling in the morning, the scent production cease, and in the afternoon *S. colorata* do not produce any flower scent (Chapter 2) until the petals are completely open at night.

Anther dehiscence and style elongation were also synchronized with petal opening. Anther dehiscence at dusk may provide a fresh atmosphere for pollen during night, but in the morning, temperature increases and the pollen grains get dry and clump together (personal observation), decreasing its viability (Nepi et al. 2001). In the nocturnal *S. latifolia*, invitro pollen germinability reaches the maximum at midnight and then decreases (Aonuma et al. 2013). Contrarily, in the diurnal *S. acutifolia*, pollen

germinability declined progressively after dehiscence at daytime (Buide & Guitan 2002). Unfortunately the culture medium used by these authors did not work in *S. colorata,* so we cannot prove the loss of viability at daytime. In any case, our experiment showed that hand pollinations yielded higher reproductive success (both fruitset and number of seeds) at night and morning than in the afternoon. These differences may indeed be due to the reduction of pollen viability, but may also be due to a decrease in stigma receptivity, since it is known that high temperatures at midday can reduce the stigmatic receptivity (Hedhly et al. 2005).

Breeding system, pollinators and predators

S. colorata is self-compatible as many other *Silene* species (Bocquet 1968). Spontaneous autogamy is unviable due to protandry, so this species depends on flower visitors even for geitonogamous pollination. It is supposed that the selection pressures exerted by pollinators on flower traits determine the pollination syndromes (van der Pijl 1960; Faegri 1979; Fenster et al. 2004), with flowers adapted to the most effective pollinators (Stebbins 1970; Wilson & Thomson 1996). As we have seen, the floral traits of *S. colorata* point mainly towards nocturnal pollination by moths. The opening of petals at dusk may attract moths visually and additionally provide a landing platform for settling moths. At the same time, the production of nectar reward increases in parallel with the emission of high amounts of benzaldehyde, benzyl alcohol, 2-phenylethanol and benzyl acetate, all of them related with the attraction of moths (Heath et al. 1992; Meagher 2002; Giménez-Benavides et al. 2007; Dobson 2006). These flower VOCs are also present in the nocturnal scent of other *Silene* species with moth pollinators (*S. subconica*, *S. viscosa*, *S. latifolia* and *S. ciliata)* (Jürgens et al 2002; Dotter et al. 2005; Giménez-Benavides et al. 2007)). This combination of traits may lead the moths to remove the fresh pollen just after dehiscence and to deposit it on the young receptive styles, yielding a high reproductive success.

In the morning other insects such as bees and bombilids also visit *S. colorata* and may pollinate the flowers with the remaining pollen. Although we previously found that *S. colorata* did not emit flower VOCs at midday (Chapter 2), the flowers still emit a small amount of scent before the complete petal closure, which differs in composition to the nocturnal one. p-Benzoquinone and 2-phenylethanol have the potential to attract the

bees found on the flowers (Knudsen & Mori 1996; Burger et al. 2012; Dötterl & Vereecken 2010), whereas benzyl acetate and benzyl alcohol, are associated with butterfly and bee pollination (Honda et al. 1998; Dobson 2006; Dötterl & Vereecken 2010). The interplay of color and scent is also essential for host-plant finding and recognition for diurnal insects like bees (Burger et al. 2010). Pink corollas are attractive to bees (Menzel & Shmida 1993), so they can be a visual cue of *S. colorata* early in the morning, when they are still open, and even when they are completely rolled in the afternoon (personal observation). Little bees visited the flowers of *S. colorata* to collect pollen, and bombilids to drink the nectar. Both rewards are less abundant but still available at day, especially in the early morning if they have not been consumed in the previous night. It is also interesting to note that three unknown compounds are emitted in significant amounts only in the morning. These compounds may act as olfactory cues for diurnal attraction of pollinators or deterrent of herbivores.

The pollination syndrome concept predicts that the flower traits mentioned above make *S. colorata* more susceptible to be effectively pollinated by nocturnal pollinators (moths in this case). The combination of traits aimed to maximize flower attraction (petal opening, scent and nectar reward) favor flower visits at night, and secondarily at early morning. The correlated traits aimed at maximize pollen removal, deposition and fertilization (anther dehiscence, style elongation and, supposedly, pollen viability and/or stigmatic receptivity) should also favor the pollination success at night, and secondarily at early morning. Unfortunately, we do not have data on the pollination effectiveness (pollen removed and deposited per single visit and hour) of each flower visitor. These data are the best way to assess the pollinator importance (effectiveness together with visitation rate), and to understand whether the pollination syndrome of *S. colorata* is effectively fitted to a particular group of pollinators (Ollerton 1996; Waser et al. 1996; Fenster et al. 2004). Anyway, our hand-pollination experiment clearly showed that one pollen grain has higher probability to develop a mature seed when it is effectively deposited in the stigmatic surface of a flower at night and early morning than in the afternoon.

Our pollinator censuses showed that the abundance of day and night visitors varies between the three populations (Figure 6), and moths were the most frequent visitors only in one population. Moth visits were positively correlated with number of seeds per

fruit (but did not with fruit set). Also, the number of seeds was correlated with afternoon and total visit rates. This result suggests that the combined effect of diurnal and nocturnal pollination may compensate the reproductive success when nocturnal pollinators are scarce. Complimentarity of diurnal and nocturnal pollination has been described before (Miyake & Yahara 1999; Wolf et al. 2003; Amorim et al. 2013), and diurnal pollinators are frequent and have a substantial role in the plant reproductive success in other *Silene* species with nocturnal syndrome (Giménez-Benavides et al. 2007; van Putten et al. 2007; Reynolds et al. 2009; Martinell et al. 2010). The presence of the nursery pollinator *Hadena sancta* in the nocturnal pollinator guild of *S. colorata* (which is for the first time described as host in the present work), may explain why there was no positive effect of the visitation rate on the fruit set. *H. sancta* pollinate the plant but also rear its offspring in the flowers and developing fruits. Although the pollinator service provided by *Hadena* species may be prominent, the net effect of the interaction may be negative when the cost of fruit predation is taken into account (Kephart et al. 2006). Unfortunately, we could not accurately distinguish *Hadena* from other moths in the night video records to estimate its visitation frequency. However, overall nocturnal visitation rate was positively related with fruit predation by Hadena larvae in two out of three populations (2 Paso and Mostoles) (Figure 6) suggesting that loss by fruit predation is proportional to pollination service in this two populations. The outcome of nursery pollination has been studied in other *Silene-Hadena* systems (Petersson 1991; Reynolds et al. 2012). These works have shown that the frequency of the nursery pollinators and copollinators contributes to shifts between mutualism and parasitism with the host plant, and this outcome also varies in space and time. If the net outcome of the *S. colorata-H. sancta* interaction shifts towards parasitism, the plant would be very limited to avoid this partner as consequence of the nocturnal pollination syndrome.

In summary, we have found that *S. colorata* has a suite of pollination traits aimed to maximize pollination at night. However, its bright petal color and the diurnal scent may help to increase its reproductive success by additional pollination in the morning. The extension of the optimal period of flower attraction and pollination towards early morning will depend on environmental conditions. Pollination and predation differ between populations, thus selective pressures exerted over flower traits may also vary (Thompson 1994, 1999). Studies that involve several population and various years are

needed to clarify the constancy or lability of selective pressures acting over floral traits of *S. colorata*.

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SUPPLEMENTARY MATERIAL 1. Localities where came from the seed and the data of flower visitor census.

Locality	Seed/Census	Coordinates	Height (m)	Experiment
Navas del Rey	Seed	40º24N, 4º15W	920	Flower opening
Navas del Rey	Seed	40º24N, 4º15W	920	Scent
Navas del Rey	Seed	40º24N, 4º15W	920	Nectar secretion
Hoyo de Manzanares	Seed	40º37N, 3º54W	982	Breeding system and pollination timing
Embalse de Valmayor	Seed	40º34N, 4º02W	867	Breeding system and pollination timing
Valdemorillo	Seed	40º29N, 4º04W	818	Breeding system and pollination timing
Xanadu	Seed/Census	40º16N, 3º55W	617	Breeding system and pollination timing Flower visitation
Mostoles	Seed/Census	40º20N, 3º52W	650	Breeding system and pollination timing Flower visitation
Poveda	Seed	40º19N, 3º28W	551	Breeding system and pollination timing
Sotillo*	Seed	40º22N, 3º56W	598	Breeding system and pollination timing
2Paso	Census	40º23N, 4º10W	579	Flower visitation

*Seed from Sotillo were used in 2012 and 2013 and taken in the previous year respectively.

CAPÍTULO 5/CHAPTER 5

Spatio-temporal variation in the interaction outcome between a nursery pollinator and its host plant when other pollinators, fruit predators and nectar robbers are present.

ABSTRACT

Nursery pollination can be very specialist or facultative with the presence of co-pollinators. Facultative nursery pollinator systems can shift from mutualism to commensalism or antagonism depending on different community contexts. The facultative nursery pollination is commonly observed between *Silene* and *Hadena* species. *S. colorata* has a mixed floral phenotype between diurnal and nocturnal syndromes, is visited by diurnal and nocturnal insects, and it hosts the nursery pollinator *Hadena sancta*. To understand the context in which the nursery pollination of *S. colorata* and *H. sancta* develops, we studied not only the interactions of both partners in this system and the possible effects of the copollinators, but also the relationships of other organisms that can influence in this system. We used a geographic mosaic theory of coevolution framework to find out how the temporal and spatial variations of all these interactions can affect the outcome of the *S. colorata* and *H. sancta* interaction. There were differences in the nocturnal and diurnal visitation rate among populations. Also the frequency of robbed flowers, predated fruits and fruitset varied among populations and years. There no influence of the visitation rate on the fruitset but there was effect on the female fecuntidy and this effect varied between years. We found a high negative effect on the fruitset of the *Hadena* larvae predation and nectar robbers. The lack of influence in the fruit pollination of the flower visit rate of *H. sancta* and the negative effects produced by its larvae point to a clear parasitism as in other *Silene-Hadena* systems. However other organisms affect both nursery pollination partners, and therefore influence in this outcome of this system.

INTRODUCTION

Nursery pollinator insects use their host plant tissues to rear their larvae. This system can be very specialist as the obligate mutualism between yuccas and yucca moths (Pellmyr 2003) or facultative with the presence of co-pollinators (Thompson & Pellmyr 1992). The sign of the interaction in the facultative nursery pollinator systems can shift from mutualism to commensalism or antagonism depending on different community contexts (Dufay & Anstett 2003; Thompson & Fernandez 2006). The transitions between interactions outcomes depend on the fluctuations in density of the nursery pollination partners (Holland & DeAngelis 2009), but also copollinators can influence pairwise interactions and cause shifts between mutualism and parasitism (Bronstein et al. 2003). The facultative nursery pollination is commonly observed between some *Silene* (Caryophyllaceae) and *Hadena* (Noctuidae) species (Kephart et al. 2006). The outcome of the nursery pollination interactions has been studied in some *Silene-Hadena* pairs of species (Petersson 1991; Reynolds et al. 2012; Kula et al. 2014). These examples have shown that density of the nursery pollinators, copollinators and plant host contributes to shifts between mutualism and parasitism, and this outcome also varies in time and space in accordance to the geographic mosaic theory of coevolution (Thompson 2005).

Most of the *Silene* species that host *Hadena* have a nocturnal syndrome of pollination (Jürgens et al 2002a; Kephart et al. 2006). Pollination syndromes are defined as the suite of floral traits associated with the attraction and utilization of the major pollinators, i.e., important mediators of selection of the floral characters (Stebbins 1951; Vogel 1954; Faegri & van der Pijl 1979; Fenster et al., 2004). In *Silene,* two pollination syndromes have been traditionally described, nocturnal and diurnal (Lindman 1897, Greuter 1995).

S. colorata was initially described as diurnal because of the pink-colored petals (Jürgens et al. 2002, Witt et al. 2013). However, recent work pointed out that this species has a mixed floral phenotype between diurnal and nocturnal syndromes (Chapter 4). In fact, pollinator census in three populations of central Spain have shown that *S. colorata* is visited by diurnal (mainly bees and bee flies) and nocturnal (mainly Noctuid moths) insects, and it hosts the nursery pollinator *Hadena sancta* (Chapter 4).

The diurnal pollination is possible but limited by the lost of visibility of the closed petals, the decrease of sent and nectar production and the loss of pollination ability with respect to night (Chapter 4).

In the present study, to understand better the context in which the nursery pollination of *S. colorata* and *H. sancta* develops, we study not only the interactions of both partners in this system and the possible effects of the copollinators, but also the relationships of other organisms that can influence in this system. We use a geographic mosaic theory of coevolution (Thompson 2005) framework to find out how the temporal and spatial variations of all these interactions can affect the outcome of the *S. colorata* and *H. sancta* interaction. Our main objectives are, 1) to figure out how the variation of the diurnal and nocturnal density of pollinators among years or populations affects to *S. colorata* fitness and 2) to understand how the different organism interact in this nursery pollination system. This is also the first detailed study of the *Hadena-Silene* interaction in a typical zone of the Mediterranean region, which is a center of diversity of both *Hadena* and *Silene* (Talavera 1990; Greuter et al. 2015; Hacker 1992, 1996, 1999). Our specific objectives were: 1) to assess how diurnal and nocturnal pollinators affect to *S. colorata* fitness; 2) to figure out the negative effects on *S. colorata* of the different seed and flower predators; 3) to investigate the spatio-temporal variation of the interaction that affect this nursery pollination system; 4) to insight the outcome of the *H. sancta-S. colorata* in this context.

MATERIALS AND METHODS

Species and study sites

S. colorata Poiret (Caryophyllaceae) is an annual plant of 15-60 cm in high. Flowers are protandrous and petal limbs are bipartite and pink. The anthesis (first opening of the flower from the bud stage) is at sunset. The petal limbs remain open during all night and close (rolling themselves) every morning. Nonetheless, the sexual parts of flowers remain uncovered when petals are completely rolled. Nectar production is higher at night than during day, the scent flower emission star in the evening and cease in the morning, and the pollination success in a hand pollination experiment is lower in the afternoon than at night and morning (Chapter 4). Previous works in three natural populations showed that *S. colorata* has a mixed pollinator guild composed by diurnal and nocturnal insects, and it hosts the pollinator seed-predating moth *Hadena sancta* (Chapter 4). *S. colorata* is a widespread species in the Mediterranean region, and inhabits natural and seminatural pastures, roadsides, croplands and open areas in shrublands and woodlands.

Hadena sancta is a night active moth with a wingspan of 26-28 mm, with a Mediterranean distribution (Jose Luis Yela, unpublished results), where it has other eight *Silene* species as hosts (Chapter 1). After drink nectar and pollinate the flower of *S. colorata*, the female moth ovoposit on the ovary or the interior of the calyx walls. The larva of the first instar enters in the young fruit (still green) and feed on the ovules. When the larva has consumed all the seeds leave the fruit to look for more fruits or flower to eat until complete its development. Then pupae and hibernate in the ground until next spring.

We selected ten population of *S. colorata* in 2012 and eight in 2013, located in Madrid province (Table 1). These populations encompassed an altitudinal range of ca. 400 m and diverse plant communities from natural and roadside pastures to shrub formations and open oak woods (Table 1). Vegetation was composed by siliceous annual pastures interspersed with chamaephytes (e.g. *Thymus vulgaris, T. mastichina*,), shrubs (e.g., *Lavandula pedunculata*, *Rosmarinus officinalis, Retama sphaerocarpa, Cytisus scoparius, Daphne gnidium*) and trees (e.g., *Quercus ilex subsp. Ballota, Olea europaea*,

Pinus pinea). In each population we established five sampling plots (1 x 1 m) in areas of moderate to high density of the focal species. Individual census was difficult because the species grows in dense clumps, so plant density was estimated as number of flowers per sampling plot.

Table 1. Description of the studied populations. Accu. Prec. is the volume of rainfall accumulated until spring (from 1th January to 31th May). Plant density means (SE) are given.

Population	Latitude, longitude	Elevation	Year	Accu. Prec. mm	plant density	Surrounding habitat
Móstoles 1	40º20'2.12''N, 3º53'3.79''O	645 m	2012 2013	100-120 200-250	26.83 (6.9) 22.61 (4.1)	olive grove
Móstoles C	40°18'18.49"N, 3°53'55.89"O	662 m	2012 2013	100-120 200-250	14.69 (2.4) 6.71(1.1)	pasture with sparse shrubs
Hoyo	40°37'13.15"N, 3°54'2.61"O	987 m	2012 2013	200-250 300-400	40.29 (9) 18.92 (2.9)	open oak wood
2 Paso	40°23'4.86"N, 4°10'44.67"O	576 m	2012 2013	100-120 200-250	13.66 (2.3) 13.46 (3.9)	roadside pasture with shrubs
Piedras	40°24'3.82"N, 4°15'8.78"O	744 m	2012 2013	100-120 200-250	24.89 (6.2) 13.96 (3.3)	roadside pasture with trees and shurbs
Valdemorillo	40°29'27.14"N, 4°2'59.27"O	816 m	2012 2013	200-250 200-250	7.28 (1.2) 19.29 (2.6)	pasture with shurbs
Xanadú	40°16'56.23"N, 3°56'8.03"O	621 m	2012 2013	100-120 200-250	22.45 (5.9) 7.10 (0.9)	pasture with shurbs
Torrelodones	40°34'53.97"N, 3°56'8.99"O	880 m	2012	200-250	10.05 (2.6)	pasture with trees and shurbs
Sotillo	40°22'16.60"N, 3°56'37.89"O	593 m	2012	100-120	5.35 (0.6)	Pine wood
Embalse	40°34'45.66"N, 4° 2'32.18"O	836 m	2012	200-250	10.17 (1.9)	oak wood

Estimation of flower visitation rates

During the flowering season (from April to June 2012 and 2013), we made diurnal and nocturnal observations of flower visitors within each population and plot. Diurnal observations were made on sunny days without wind. We made 5 min censuses (N= 879 censuses, corresponding to 73 observation hours evenly distributed among experimental plots). The censuses were made about 1 m from the plots to avoid the disturbance of foraging behavior. Every census day, we counted the number of open flowers per plot as estimate of flower display size. Nocturnal observations were conducted with customized weatherproof digital video cameras equipped with near-infrared light (Giménez-Benavides & Prieto-Benítez, unpublished). Each census date, we placed one camera 15-30 cm in front of each sampling plot and counted the

number of open flowers the camera was framing. We recorded each plot continuously from dusk until dawn (about 21:00 to 6:30 h). Video records were visualized afterwards to note the identity and frequency of flower visitors, and were supplemented with direct census and captures sporadically. We visualized 477 hours evenly distributed among experimental plots. All diurnal and nocturnal insects touching sexual organs were considered pollinators regardless of the efficacy of the visits. Insects were grouped into functional groups to calculate visitation rates (visits flower^{-1} hour^{-1}) per time period (early night: 21-01 h, late night: 01-06 h, morning: 09-12 h and midday: 12-15 h). We also calculate the total day and night visitation rates and the proportion of visits, as the day visitation rate/ (day + night visitation rates). This proportion summarize the prevalence of day versus night visits; populations with values > 0.5 had more diurnal than nocturnal visits and reversely.

Estimation of reproductive success and predispersal fruit predation

Two-three weeks after pollination censuses, we randomly sampled 10 plants that have completed the full life cycle in each 1 x 1 m plot. With a careful examination of the reproductive structures, were able to distinguish between wilted flowers (non-fecunded), predated flowers (wilted and with external and internal signs of herbivory), flowers attacked by nectar robbers (wilted and with robbery holes in the calyx), predated fruits (initiated seed capsules that were predated before ripening) and final fruits (intact ripe capsules). In each plant, we counted the total number of flowers and fruits of each state to calculate the following variables: rate of predated flowers (number of predated flowers/total number of flowers), rate of nectar robbery (number of robbed flowers/total number of flowers), rate of predated fruits (number of predated fruits/total number of fruits), initial fruitset (total number of fruits/ total number of flowers) and final fruitset (non-predated fruits/ total number of flowers). To estimate the number of seeds per fruit we collected and dissected one fruit per sampled plant before dehiscence and seed dispersal. When all fruits in the plant were dehisced or predated, we took another fruit from a neighboring plant. The dissected fruits that had larvae developing into them were discarded of the seed counting..

Female fecundity (total number of seeds produced per plant) was calculated by multiplying the number of final fruits per plant by the number of seeds per fruit.

Additionally, to explore the identity and abundance of seed eating larvae, we collected 100-400 immature fruits every census date in each population and year. We harvested these fruits several meters from the plots to avoid interference. Batches of 50 fruits were stored in Petri dishes under laboratory conditions. Every 2-3 days, the larvae emerging from each Petri dish were counted and classified when possible, and they were reared until pupation to get adult moths for accurate identifications. We placed the larvae in Petri dishes individually to avoid intraspecific predation (Brantjes, 1976; Peschken and Derby, 1990; Elzinga et al 2002; Reynols et al 2012). Every 3-4 days they were fed with green fruits of *S. colorata* that were collected in the vicinity of the same populations. Petri dishes were filled with a thin layer of vermiculite to provide a ground-like substrate for pupating. Previous trials showed that most *Hadena* are univoltine and chrysalides need long diapauses to emerge (pers. obs.), thus we placed the dishes in an incubation chamber (Selecta Hotcold GL, Barcelona, Spain) under 12-h light/12 h-dark photoperiod and 15/10 ºC temperature. Next spring, dishes were taken out and adult moths were identified to the species level with the help of an expert. This approach also provided us an estimate of the mean number of fruits eaten by a single larva to reach the pupae stage.

Data analysis

Before statistical analysis, all dependent variables were checked and transformed to fulfill the assumptions of parametric analyses if possible. Fruitset was log transformed and visitation rate and rate of predated flowers were square root transformed. Mean values per plot and population were used for subsequent analysis. To explore differences in the number of flowers per plant, rate of nectar robbery, rate of predated flowers, initial fruitset, rate of predated fruits and final fruitset we run a set of ANOVAs with *year, population* and the interaction *year*population.* To explore variation in the rate of visited flowers among populations and periods of day, we carried out an ANOVA for each year of study, with the following explanatory variables: *population*,

time (early night, late night, morning and midday) and the interaction *population*time*. To analyze whether visitation rate differed between years, we run a LMM (linear mixed model) with *year*, *time* and the interaction *year*time* as fixed factors and *population* as random factor. Post-hoc pairwise analyses were performed with the Tukey HDS test. Another LMM was made to test the influence of plant density on the initial fruitset. At level population (mean values per population) we made regressions (both years separately) to figure out the effect of number of flower on the proportion of visits and the effect of this proportion on female fecundity. We used a LM for the first regression. The female fecundity regression was done using GLM with Poisson distribution because of positive integer values. Effects were tested using simple regressions at population scale, both linear and quadratic. We retained the regression providing the best fit (highest explained variance and lowest P-values). ANOVAs, LMMs and, Tukey HDS tests were done at plot scale (five plots per population). All these analysis were implemented in R (R Core Team 2014), with the nlme and agricolae packages (Pinheiro et al 2013, Mendiburu 2014).

D-sep analysis

We performed a d-sep test of casual graphs (Shipley 2009; Laliberté & Tylianakis 2012) to explore the relationships among biotic variables that explain the reproductive success of *S. colorata*. This methodology allows the analysis of complex multivariate causal schemes represented in a path diagram, and relaxes some of the limitations of standard structural equation models, including non-normal data distribution, small sample sizes and allowing the inclusion of random variables (Grace 2006; Shipley 2009). The d-sep approach is based on an acyclic graph that depicts the hypothetical relationships and independence claims between variables, and these are tested using the C statistic. The Figure 1 shows our multivariate causal hypothesis. The direct relationships and the d-sep claim were tested using the data per plot of all population in both years. We first hypothesized that the number of flowers per plant determined diurnal and nocturnal rates of pollinator visits, proportion of robbed flowers, and abundance of larvae developing into the fruits. Our rearing assay showed that *Hadena*

sancta was not the only seed-eating insect. We found a substantial proportion (see results) of fruits parasitized by larvae of a leaf weevil (*Hypera* sp.), so we use the two estimates. We also expected the frequency of nectar robbery and parasitized fruits to affect rate of pollinator visits, and thereby pollination (i.e. initial fruitset). It is common to find the larvae of *Hadena* eating flowers (Reynold et al. 2012), so high abundance of caterpillars and high rate of night visits (including *Hadena*) might have a direct effect on the frequency of flower predation. Flower predation might also determine visit rates to remaining flowers and pollination success. Finally, our model proposes that reproductive success of *S. colorata* (expressed as final fruitset per plant) depends ultimately on pollination success, frequency of fruit predation and number of flowers per plant. All the analyses in the d-sep were LMM, with *population* and *year* as random factors. To reduce the causal hypothesis, we selected the predictors that have significant effect.

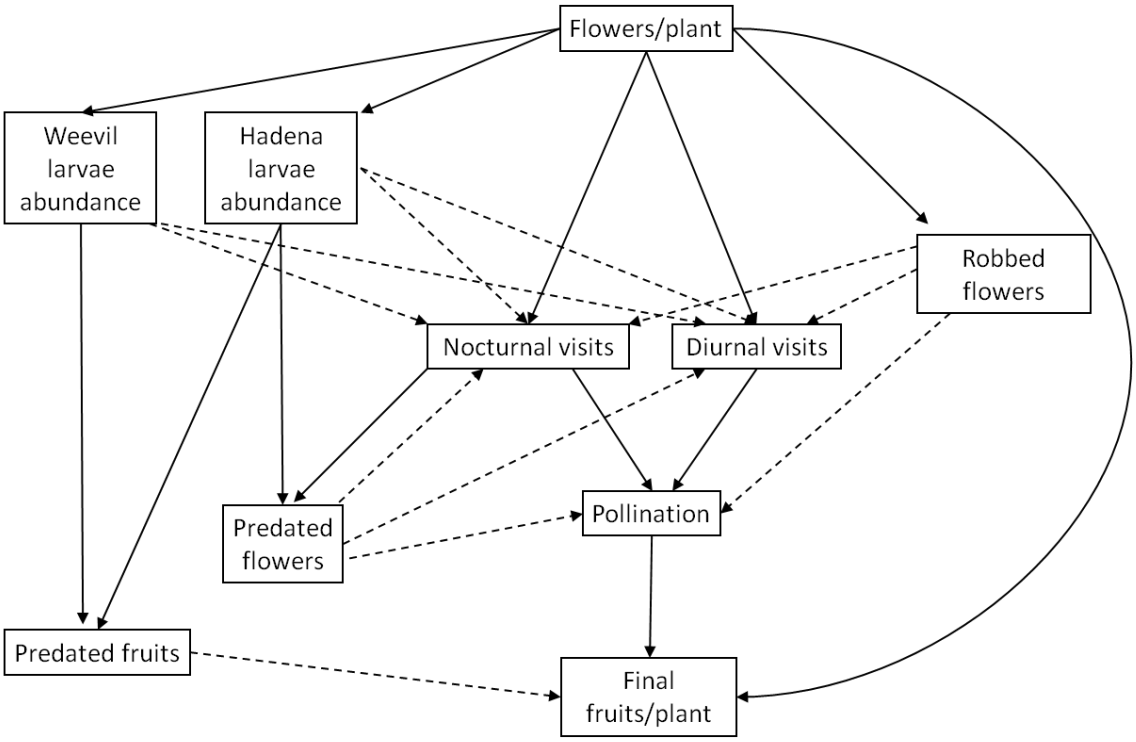

Figure 1. Path diagram showing the hypothetical relationships among biotic variables that may have a direct or indirect effect in the final fruit set of *S. colorata*. Biotic variables are flower/plant (florwer per plant), *Hadena* larvae density (*Hadena* larvae per fruit), weevil larvae density (weevil larvae per fruit), nectar robbers (flower attacts by nectar robbers), nocturnal visit (nocturnal visit rate), diurnal visit (diurnal visit rate), predated fruits, predated flowers and pollination (total fruit set). Full and dashed lines mean possible positive and negative relationships respectively.

RESULTS

We found diurnal and nocturnal flower visitors in all populations of *S. colorata* studied. In 2012 we found five functional groups of insects (bees, hoverflies, bee flies, flies and moths). In 2013 another functional group (bumblebees) was added (Table 2). The most abundant diurnal visitors were bees (averaging 25.68% of visits in 2012) and *Bombus terrestris* (averaging 12.86% of visits in 2013). Nocturnal video records did not provide sufficient quality to discern among species, so night visit rate was computed for all moths together. However, direct censuses and captures allowed the identification of some species (Noctuidae: *H. sancta*, *Autographa gamma* and *Noctua sp.*; Geometridae: one morphospecies). Visitation rate was different among periods of day and populations both years (Figure 2). In both years post-hoc pairwise analyses showed that visitation rate at early night was significantly higher than at late night, morning and midday. The interaction term was significant (Table 3). When both years were analyzed together, there were also differences among periods of day, but neither year nor the interaction terms were significant (Table 3). There were differences among population in the number of flower and the rate of nectar robbery (Figure 3), and also in the initial fruitset, rate of predated fruits and final fruitset (Figure 4), but not in the rate of predated flowers and number of seeds per fruit (Table 4). There were differences between years in the 3 variables related with the flowers (Figure 3), the 3 related with the fruits (Figure 4) and also in the number of seeds (Table 4).

We could not estimate the number of seeds per fruit in Torrelodones in 2012 because in the final sampling date most fruits were dispersed, and only one of the green fruits collected was not parasitized by *Hadena* or weevil larvae. The day-night visit proportion was not affected by the number of flowers per plant in 2012 and 2013 ($F_{1,7}$=0.35, P=0.76; $F_{1,5}$=0,94 P=0.37, respectively). However, day-night visit proportion had different significant relationships with the female fecundity between years (Figure 5). In 2012 we found a linear relation with upward tendency, whereas in 2013 we found a pure quadratic concave shape (Figure 5).

Table 2. Relative visit rates of the functional groups of *S. colotara* flower visitors.

Population	Year	Bees %	*Bombus* %	Hoverflies %	Bee flies %	Flies %	Other diurnal %	Moths %
Móstoles C	2012	63.2	0	0.7	10.6	0	0	25.5
	2013	1.8	4.2	0	0	0	0.6	93.4
Móstoles 1	2012	56.4	0	0	0	3.9	0	39.8
	2013	0	0	61.6	24.6	0	0	13.8
Hoyo	2012	30.1	0	0	24	0	0	45.9
	2013	3.2	0	0	9.5	20.6	0	66.7
2º Paso	2012	21.9	0	0	2.7	0	0	75.4
	2013	0	0	12.5	0	0	3.7	83.8
Piedras	2012	10.8	0	0	0	10.8	25.3	53
	2013	0.6	58.1	0	0	0	0	41.2
Valdemorillo	2012	0	0	0	0	23.3	0	76.7
	2013	0	0	11.8	0	27.6	7.9	52.6
Xanadú	2012	74.4	0	0	0	0	0	25.6
	2013	0	0	0	5.2	20.9	0	73.9
Torrelodones	2012	0	0	0	0	0	0	100
El Sotillo	2012	0	0	0	5.1	0	35.9	58.9
Embalse	2012	0	0	0	0	0	64.7	35.3

Bombus (*B. terrestris*), Hoverflies (*Scaeva pyrastri, Sphaerophoria scripta, Eristalis tenax*), Bee flies (Bombylius major).

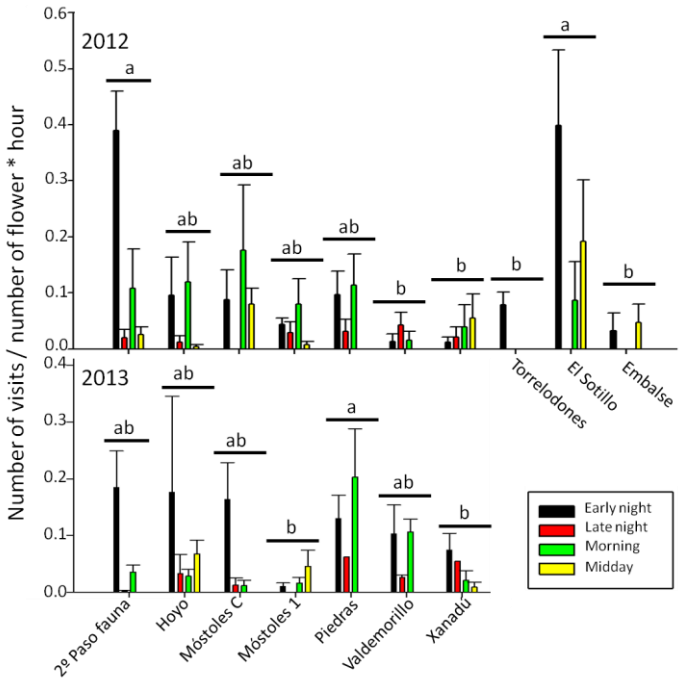

Figure 2. Flower visitation rate in the studied populations on both years. Vertical lines denote standard errors. Different letters denote differences among populations within years.

Table 3. Results of lineal models for visitation rate. * denotes significant differences.

	Visitation rate		
	df	F	*P*
2012			
periods of day	3, 141	10.05	< 0.001*
population	9, 141	4.04	< 0.001*
periods of day x population	27, 141	2.21	0.022*
2013			
periods of day	3, 77	6.76	< 0.001*
population	6, 77	2.98	0.011*
periods of day x population	15, 77	1.87	0.04*
2012 & 2013			
periods of day	3, 266	13.84	< 0.001*
year	1, 266	0.58	0.45
periods of day x year population	3, 266	0.06	0.98

Table 4. Results of lineal models for variables related with *S. colorata* fitness. * denotes significant differences

	Nº of flower per plant				**Initial fruit set**		
	df	F	*P*		df	F	*P*
year	1, 68	36.1	< 0.001*	year	1, 68	20.22	< 0.001*
population	9, 68	10.89	< 0.001*	population	9, 68	2.31	0.025*
year x population	6, 68	3.16	0.001*	year x population	6, 68	0.5	0.81
	Rate of nectar robbery				**Rate of predated fruits**		
year	1, 68	70.82	< 0.001*	year	1, 68	20.63	< 0.001*
population	9, 68	10.84	<0.001*	population	9, 68	3.48	< 0.001*
year x population	6, 68	3.35	0.006*	year x population	6, 68	1.46	0.2
	Rate of predated flowers				**Final fruti set**		
year	1, 68	6.76	0.011*	year	1, 68	40.36	< 0.001*
population	9, 68	1.82	0.08	population	9, 68	2.45	0.018*
year x population	6, 68	2.61	0.025*	year x population	6, 68	0.98	0.44
	Nº of seed per fruit						
year	1, 62	39.26	< 0.001*				
population	8, 62	1.65	0.129				
year x population	6, 62	0.98	0.142				

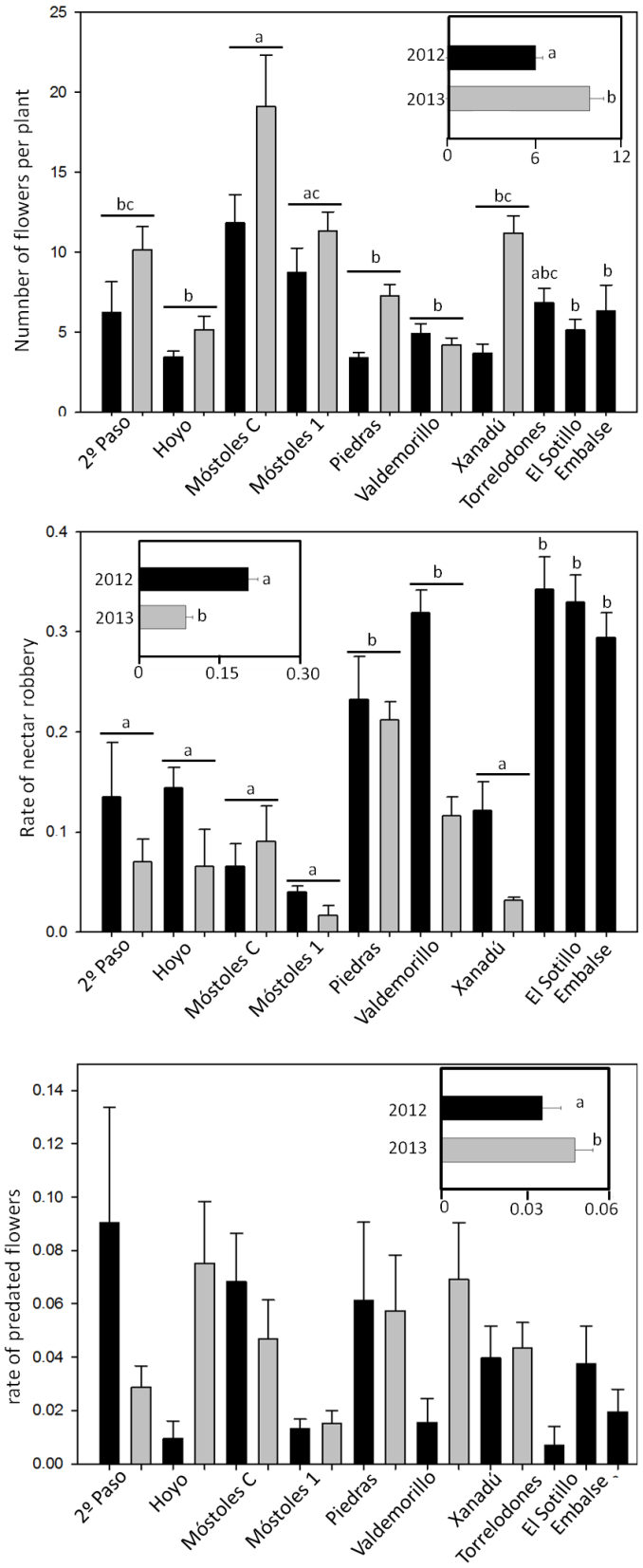

Figure 3. Number of flowers per plant, rate of nectar roberry and rate of predated flowers in the studied populations on both years. Vertical lines denote estandar errors. Different letters denote differences among populations.

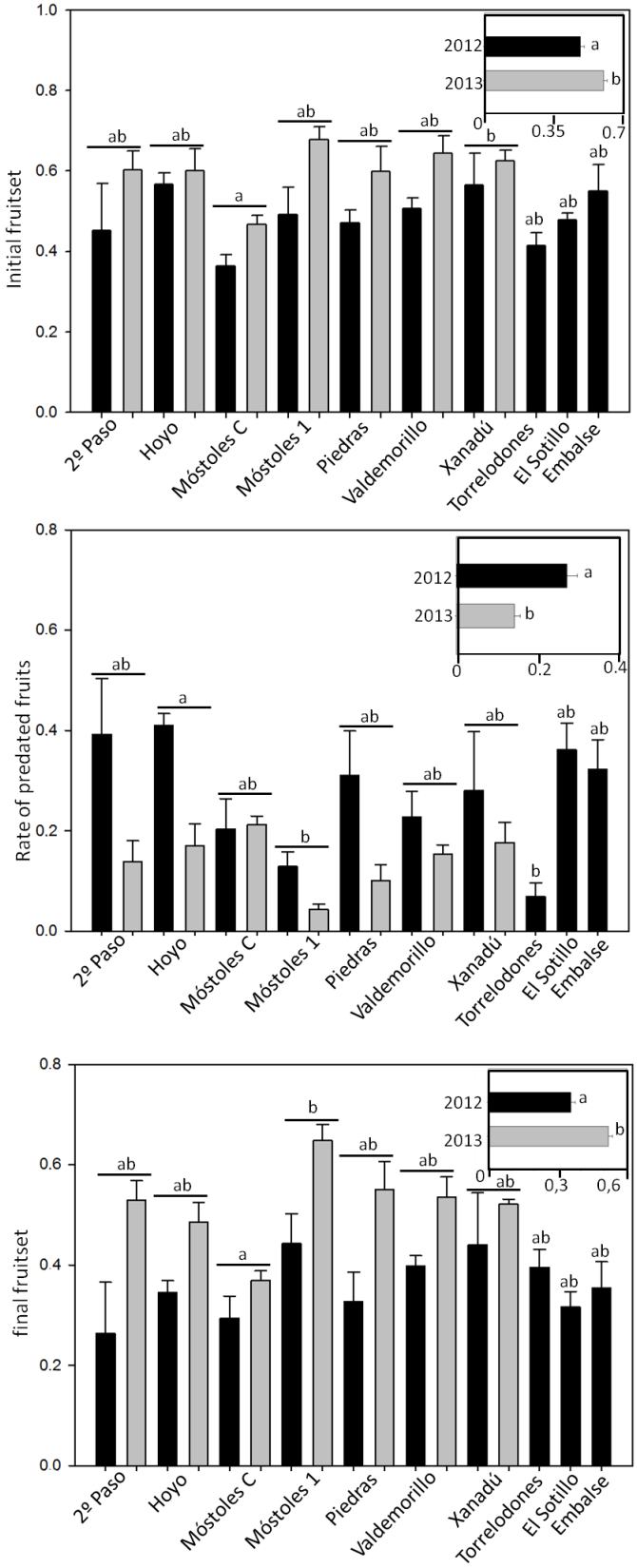

Figure 4. Initial fruitset, rate of predated fruits and final fruitset in the studied populations on both years. Vertical lines denote estandar errors. Different letters denote differences among populations.

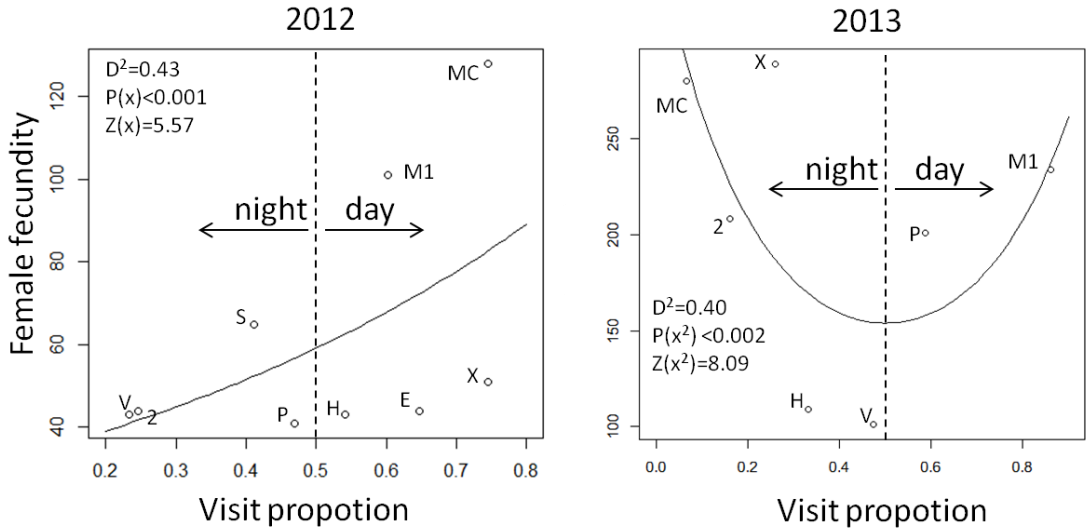

Figure 5. Relationships between the proportion of pollinator visits (diurnal rate of visit/(diurnal + nocturnal rate of visit)) and the female fecundity, each year separately.

In 2012 and 2013, 27.03 % and 16.62 % of the fruits hosted one *Hadena* larva, whereas 6.14 % and 3 % hosted more than one, respectively. During this work, we have not detected any other Hadena species hosted by *S. colorata*. Under laboratory conditions, we assessed that each *H. sancta* larva needs a mean (±SD) of 32±7 green fruits to reach the pupae state (n=38, range=15-48). We also found two species of konobiont parasitoids within larvae of *H. sancta*, one tachinid fly (Diptera: Tachinidae) only in Hoyo population (in 12.5 % of *Hadena* larvae), and one parasitoid wasp (Hymenoptera: Braconidae/Ichneumonidae) in seven populations. We found this endoparasitoid in the 5.6 % and 0.6 % of the fruits in 2012 an 2013, respectively. In 2012 and 2013, 13.51 % and 8.16 % of the fruits were parasitized by one *Hyperia* weevil, whereas 4.9 % and 0.3 % had more than one. In most of the cases the adult phase of weevils emerged directly from collected fruits, so they only need the primary fruit to reach the pupal stage.

There was no effect of the plant density on the fruit set ($F_{1,67}$=3.14, *P*=0.08). In the d-sep analysis, the model presented in the figure 6 was not rejected by the data (Supplementary material 1). The model highlighted the lack of direct or indirect effects of night and day visitation rates on the final fruit set. The number of weevils per fruits affected negatively to the night visitation rate ($F_{1,58}$=13.43, *P*>0.001), but it had no effect in the final fruit set and therefore was left out of the model. The numbers of *Hadena* larvae per fruit had an indirect negative effect on the final fruit set through the

rate of predated flowers, which ultimately affected the pollination success (Figure 6). The numbers of *Hadena* larvae per fruit had another indirect negative effect on the final fruit set through the rate of predated fruits. It is very interesting to note the strong negative effect of the proportion of flowers visited by nectar robbers in the pollination success, and therefore a negative indirect effect on the final fruit set.

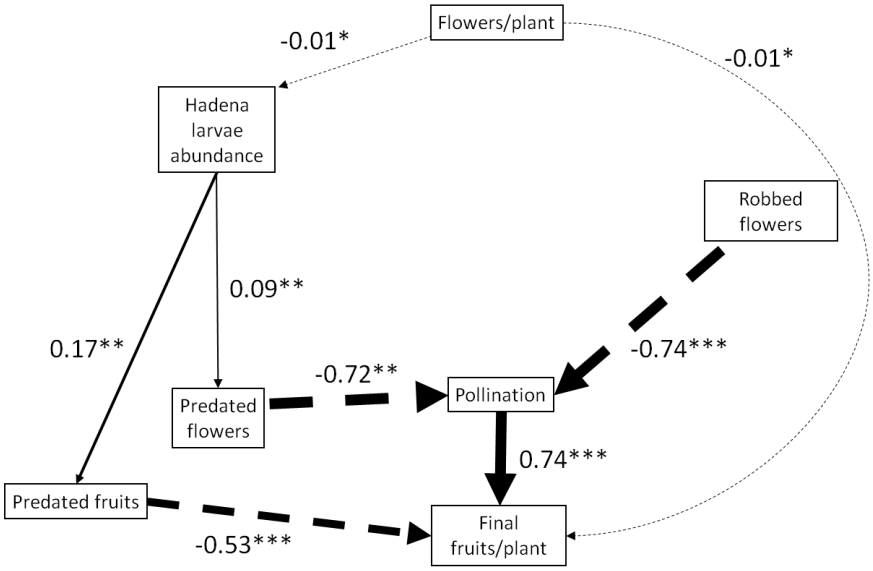

Figure 6. Biotic variables affecting, direct or indirectly, on the final fruit set per plant (Final fruit/plant). Result of the conceptual d-sep graph showing only the significant causal paths. Full and dashed lines mean positive and negative relationships, respectively. The slope of regression of each independent variable is given with p-values (***) <0.001, (**) ≥ 0.001 < 0.01 and (*) ≥ 0.01 < 0.05.

DISCUSSION

In the present study, we show how the spatiotemporal variation of several mutualistic and parasitic interactions affects the reproductive performance of a plant. Initially, we centered our study in the interaction outcome of the plant, *S. colorata,* and its nursery pollinator *H. sancta*, because some previous studies showed that *Silene-Hadena* interaction systems may shift between mutualism and parasitism depending on the pollination context of populations (Petersson 1991; Kepthar et al. 2006; Giménez Benavides et al. 2007; Reynolds et al. 2012). In contrast to obligate nursery pollination interactions, the facultative *Silene-Hadena* system is enriched by the presence of other pollinators and shows some degree of unspecificity between partners (Kephart et al., 2006; Bernasconi et al. 2009). This makes the system very interesting to assess in the

context of the geographic mosaic theory of coevolution (Thompson 2005). However, as fieldwork made progress we found that the initial system got more complex, with presence of nectar robbers, parasitoids of *Hadena* larvae and other fruit predators. All these organisms also may have a significant role in the interaction outcome of the focal system, so an effort is needed to integrate them in this context.

The ten *S. colorata* populations differed in many abiotic and biotic aspects. Site selection was done deliberately to cover the wide environmental range of this plant species in Central Spain, and we also had contrasting weather conditions between years. There were more flowers per plant in 2013 than in 2012 in all populations, probably because of higher accumulated rainfall in 2013 compared to 2012 (AEMET 2013, Table 1). However, we are not so interested in the environmental causes of plant variation across populations and years, but on the consequences of variation of biotic interactions on the *Silene-Hadena* system. In spite of variation in flower display between years, the overall flower visitation rate was very similar. However, flower visitation rate varied significantly among populations, and among hourly intervals within populations. The interpopulation variation seems to be not related with surrounding habitats, because some similar populations (e.g. those within oak woods) had contrasting visit rates. The variation of visit rates among hourly intervals is also clear between years within population, with only two out of seven populations showing a similar interannual pattern (2ºPaso and Piedras). The variation in frequency of pollinator assemblages between years and populations had been described previously in different plant species, and also in *Silene* (Bertin 1982; Herrera 1988; Fishbein &Venable 1996; Fenster & Dudash 2001; Reynolds et al. 2009; Martinell et al. 2010). On the other hand, the studied populations also showed high spatiotemporal variation in density of seed-eating larvae, both *H. sancta* and Hyperia sp., and in the frequency of nectar robbery. Therefore, we have a geographic mosaic of *S. colorata* populations that differ in frequency and composition of pollinators, nursery pollinators, nectar robbers and flower-fruit predators. Also ours populations varied in the fruit pollination (initial fruit set), but this variation was not related with plant density as in *S. stellata* (Kula et al. 2014).

S. colorata have its floral traits mainly focus to attract nocturnal pollinators. This pollination syndrome predicts the main pollinator should be nocturnal moths (Rosas-Guerrero et al. 2014). Therefore we expected that *S. colorata* plants in the populations with more nocturnal visits will have a higher pollination success (initial fruit set). But the overall of the populations and years show that there was a lack of influence of the nocturnal and diurnal visitors on the potential fruit set. Exclusion pollinators experiment in several *Silene* species have found a differential fruit set between day and night pollination (Giménez-Benavides et al. 2007; Reynolds et al. 2009; Martinell et al. 2010). Previous hand pollination experiment in *S. colorata* revealed that passive autogamy does not produce any fruit (Chapter 4), therefore *S. colorata* is completely dependent of pollen vectors. There have several reasons why our expectation about nocturnal pollination is not met. First, our pollination censuses may not have quantified the real pollinator's spectra. However we found that diurnal and nocturnal pollination in *S. colorata* affect to the female fecundity. Second, in those populations with more visits in the early morning respect nocturnal visits had more reproductive success (female fecundity), as in 2012 (Móstoles 1 and C), because the flowers are still open and probably the remaining pollen is fresh and the styles are receptive (Chapter 4). Third, the no relationship of the visitation rate with the initial fruit set may depend on the effectiveness of the pollinators. We have not quantified the effectiveness, however visitation rates may play a more important role in pollination importance than effectiveness (Vazquez et al. 2005; Salhi & Cornner 2006). The differences between years in the relation of female fecundity with the day night visits proportion may be related with the effectiveness of certain pollinators. For example in 2012, populations with high rate of nocturnal visits have low female fecundity, but in 2013 populations with high rate of nocturnal visits have the highest female fecundity. This could be owed to a change within the nocturnal pollinators with different effectiveness (*Autographa gamma* was more abundant in 2013 than 2012 and Geometridae reversely, personal observations).

Although there was not relationship between diurnal or nocturnal pollination rates with *S. colorata* fruit pollination success (d-sep), we found a clear relationship with the flower and fruit predation mediated by *H. sancta*. Before pollination, flowers of *S. colorata* are exposed to florivory (i.e. herbivory focused on floral tissues). During our

day and night census, we only found larvae of *Hadena sancta* predating the flowers of *S. colorata*. When *Hadena* larvae feed on flowers, they usually eat the calyx and the ovary, so it reduces the total number of available flowers to pollination. After pollination, *H. sancta* larva should move between plants because the mean number of flowers (potential fruits) per plant (15) is not enough to reach the pupae state. Therefore, the lack of influence in the fruit pollination of the flower visit rate of *H. sancta* (indirectly measured in the nocturnal visit rate) and the negative effects produced by its larvae point to a clear parasitism as in other *Silene-Hadena* systems (Petterson 1991; Giménez-Benavides et at. 2007; Reynolds et al. 2012). Moreover, we detected other organisms that affect this nursery pollination, as the nectar robbers affecting the fruit set and therefore both partners. Nectar robbers harm the flower calyx to extract the nectar and do not pollinate, but the flower reduces its nectar reward to attract pollinators (McCall & Irwin 2006). The weevils seem to produce few fruit set lose. Also the parasitoids, that been described previously in *S. latifolia* (Elzinga et al. 2007), may reduce the seed predation and the number of *H. sancta*. Anyway, only in populations or years with a low or null abundance of co-pollinators *H. sancta* would have a mutualistic relationship with *S. colorata*. But in our extensive study with 10 populations we did find only few situations that could fulfill this requisite. In 2013, in the populations with high female fecundity and low visit proportion (Figure 5), this system could be mutalistic if the density of moths co-pollinator are lower than *H. sancta*. This requisite is also difficult to find in other systems when most *Silene* species seem to be generalists (Chapter 2). We think that this study, together with the previous realized in *Silene-Hadena* nursery-pollinations (Petterson 1991; Giménez-Benavides et at., 2007; Reynolds et al., 2012), close the debate of the "parasitism-mutualism" in this system.

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SUPPLEMENTARY MATERIAL 1. Conditional independence tests applied in the hypothesis of the d-sep model implied by the hypothesized path models.

D-sep clain of independence	Model formula	Variable whose partial regression slope should be zero	*P*
(FP, PFR)|{HA}	PF~HA+FPR	FP	0.41
(FP, PFL)|{HA}	PF~HA+FPL	FP	0.075
(FP, RF)|Ø	RF~FP	FP	0.59
(FP, FF)|{PFR,IF}	FF~PFR+IF+FP	FP	0.7
(HA, RF)|{FP}	RF~FP+HA	HA	0.027
(HA, IF)|{FP, PFL, RF}	IF~FP+PFL+RF+HA	HA	0.72
(HA, FF)|{PFR, IF, FP}	FF~FP+PFR+IF+HA	HA	0.46
(PFL, PFR)|{HA}	PFL~HA+PFR	PFR	0.84
(RF, PFR)|{HA}	PFR~HA+RF	RF	0.35
(PFR, IF)|{HA, PFL, RF, FP}	IF~HA+RF+PFL+FP+PFR	PFR	0.87
(PFL, RF)|{HA}	RF~HA+PFL	PFL	0.57
(PFL, FF)|{PFR, IF, HA}	FF~PFR+IF+HA+PFL	PFL	0.98
(RF,FF)|{IF, PFR}	FF~IF+PFR+RF	RF	0.88

C statitic	*P* of C	df
26.93	0.41	26

HA, Hadena laveae abundance; FF; final fruitset; FP, flower per plant; IF; initial fruitset; PFL, predated flowers; PFR, predated fruits; RF, robbed flowers.

The probability of observing this value of C by chance if the data were actually generated by the causal process is 0.41, thus correctly preventing us from rejecting the model at a significance level of 0.05 (Shipley 2009).