ECOLOGÍA EVOLUTIVA DEL POLIMORFISMO ESTILAR EN *Narcissus papyraceus* KER-GAWL. (AMARYLLIDACEAE).

EVOLUTIONARY ECOLOGY OF STYLAR POLYMORPHISM IN *Narcissus papyraceus* KER-GAWL. (AMARYLLIDACEAE).

Tesis Doctoral

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Mayo de 2013

Directores: Dr. Juan Arroyo y Dr. F. Xavier Picó

Ecología evolutiva del polimorfismo estilar en *Narcissus papyraceus* Ker-Gawl. (Amaryllidaceae).

Memoria que Violeta Simón Porcar, Licenciada en Biología, presenta para optar al grado de Doctora por la Universidad de Sevilla a 15 de mayo de 2013.

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INFORMAN:

Que esta memoria fue realizada bajo su dirección en el Departamento de Biología Vegetal y Ecología de la Universidad de Sevilla y en el Departamento de Biología Integrativa de la Estación Biológica de Doñana (CSIC). Ante lo cual, considerando que tiene la suficiente entidad para constituir un trabajo de Tesis Doctoral, autorizan su presentación ante el Consejo de Departamento y la Comisión de Doctorado.

Y para que así conste, firman el presente documento en Sevilla a 15 de mayo de 2013.

Fdo.: Juan Arroyo Marín **Fdo.: F. Xavier Picó Mercader**

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A mi madre

A mi padre

ÍNDICE

"A first-hand study is always instructive, and often... full of surprises."

R.A. Fisher 1965

RESUMEN GENERAL

Narcissus papyraceus es un geófito del oeste del Mediterráneo que posee dimorfismo estilar, un polimorfismo similar a la heterostilia pero que carece tanto de reciprocidad sexual perfecta entre morfos como de sistema de incompatibilidad heteromórfico. Sin embargo, como en la heterostilia, la estabilidad evolutiva del dimorfismo estilar depende de las tasas de cruzamiento entre morfos. Las poblaciones de *N. papyraceus* son dimórficas (con morfos longistilo y brevistilo) en el centro y sur de su distribución, pero monomórficas (con morfo longistilo) en su límite norte. Con el fin de encontrar las causas de este patrón espacial, en esta Tesis Doctoral se estudian en detalle (i) el sistema de incompatibilidad de la especie y (ii) los patrones de polinización y de cruzamientos efectivos de los morfos florales, y se infieren (iii) los procesos demográficos poblacionales. Se muestra que, dado su sistema de autoincompatibilidad totalmente homomórfico, los patrones de polinización son altamente determinantes del éxito reproductor de los morfos florales. La mayor reciprocidad sexual dentro del morfo longistilo que dentro del morfo brevistilo favorece mayores tasas de cruzamientos entre los individuos del morfo longistilo. Además, el morfo brevistilo sufre una merma de su éxito reproductor femenino cuando actúan los polinizadores de probóscide corta. Las desventajas del morfo brevistilo se ven acentuadas por posibles eventos demográficos históricos en las poblaciones y por la selección dependiente de la frecuencia, lo cual puede haber abocado a este morfo a la extinción en las poblaciones del norte de la distribución de la especie.

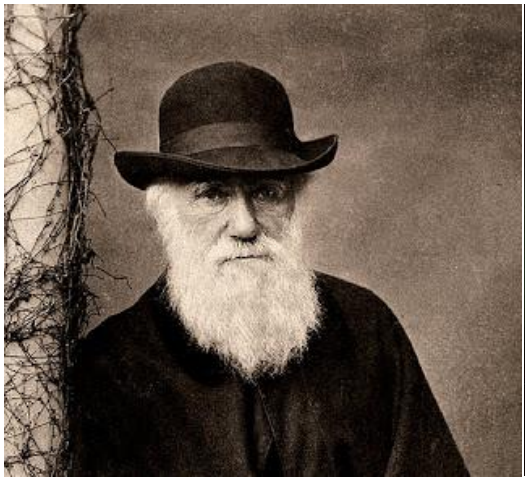
GENERAL ABSTRACT

Narcissus papyraceus is a western Mediterranean geophyte having stylar dimorphism, a polymorphism similar to heterostyly but lacking both perfect sexual reciprocity between morphs and heteromorphic incompatibility system. However, as in heterostyly, evolutionary maintenance of stylar dimorphism depends on disassortative mating rates. Populations of *N. papyraceus* are dimorphic (with long- and short-styled morphs) in the center and south of its range, but monomorphic (with the long-styled morph) in its northern boundary. In order to find the causes of this geographical pattern, in this PhD thesis they are studied in detail (i) the incompatibility system of the species and (ii) the patterns of pollination and effective mating of floral morphs, and they are inferred (iii) the population demographic processes. It is find out that, given their fully homomorphic self-incompatibility system, pollination patterns are highly determinant of reproductive success of floral morphs. Higher sexual reciprocity within L-morph than within S-morph favors higher rates of assortative mating in the L-morph. In addition, the S-morph suffers depletion of female reproductive success under the action of short-tongued pollinators. The disadvantages of the S-morph are accentuated by possible historical demographic events in populations and the frequency-dependent selection, which may have doomed this morph to extinction in northern populations of the species' distribution.

CAPÍTULO 1

INTRODUCCIÓN GENERAL

GENERAL INTRODUCTION



La ecología evolutiva en el campo de la biología floral

La ecología evolutiva integra la biología evolutiva con la ecología, considerando los aspectos históricos y contemporáneos que influyen en los patrones de variación y el valor adaptativo de estos últimos (Fox *et al.* 2001, Mayhew 2006). Esta rama de la ciencia se fundamenta en el pilar central de la teoría darwinista de la evolución (Darwin 1859): los organismos se adaptan generación tras generación a su ambiente por medio de la selección natural. Así, tanto las interacciones intraespecíficas como las interespecíficas, y las de los organismos con las condiciones geográficas, climáticas o fisiográficas de su hábitat, pueden ser fuerzas selectivas directas o indirectas que determinen evolución fenotípica en los organismos implicados (Moore *et al.* 1997, Hairston *et al.* 2005, Svensson *et al.* 2005, Estes *et al.* 2013).

Uno de los principales objetos de estudio de la ecología evolutiva en plantas son sus estrategias de reproducción. Desde la autogamia obligada de las flores cleistógamas hasta la alogamia necesaria de las plantas dioicas, las angiospermas presentan los más variados mecanismos para separar las funciones sexuales al nivel espacial y/o temporal. Así, la monoecia, la poligamia, la ginodioecia, la androdioecia, la heterodicogamia o la heterostilia, entre otros, permiten promover la fecundación cruzada y/o evitar la autointerferencia (Barrett 1998, 2002, Renner 2001). Aunque algunos de estos sistemas de reproducción habían sido descritos desde antiguo (Clusius 1583, Linnaeus 1735), no fue hasta la segunda mitad del siglo XIX cuando se inquirió sobre su significado adaptativo. Fue entonces cuando Charles Darwin (1809–1882) sentó las bases de la ecología evolutiva en el campo de la biología floral a través de sus obras *Fertilisation of Orchids* (1862a; título abreviado), *The Effects of Cross*

and Self-Fertilisation in the Vegetable Kingdom (1876) y *The Different Forms of Flowers on Plants of the Same Species* (1877).

Posiblemente, *Forms of Flowers* ha sido la obra más influyente en el campo de la biología floral evolutiva hasta nuestros días (Pannell 2009), acaso porque coronando su propio trabajo experimental (1862b) y el de botánicos como Gray (1842), Hildebrand (1863, 1865, 1867), Delpino (1867) o Müller (1869), Darwin trató con especial interés y extensión la heterostilia. Desde entonces, este polimorfismo estilar, codificado supuestamente en algunos grupos de plantas por un supergen que segrega de forma mendeliana (Lewis & Jones 1992), ha recibido una gran atención como sistema modelo en estudios evolutivos (véanse las revisiones de Vuilleumier 1967, Ganders 1979, Barrett 1992 y Barrett & Shore 2008).

La heterostilia

La heterostilia consiste en la presencia de dos (distilia) o tres (tristilia) morfos florales dentro de una misma población que difieren de forma recíproca en la posición de sus órganos sexuales. Así, en el caso de la distilia, las anteras del morfo de estilo largo o longistilo (L de aquí en adelante) están a la misma altura que el estigma del morfo de estilo corto o brevistilo (S, del inglés *short*, de aquí en adelante), y viceversa (Figura 1). En el caso de la tristilia ocurre lo mismo con tres niveles y morfos florales (L, S y M, este último de longitud de estilo mediana). Comúnmente, el polimorfismo morfológico, llamado *per se* hercogamia recíproca (Webb & Lloyd 1986), se asocia con un sistema de incompatibilidad, llamado heteromórfico, que impide la fecundación propia y entre individuos del mismo morfo. Además, se asocian también

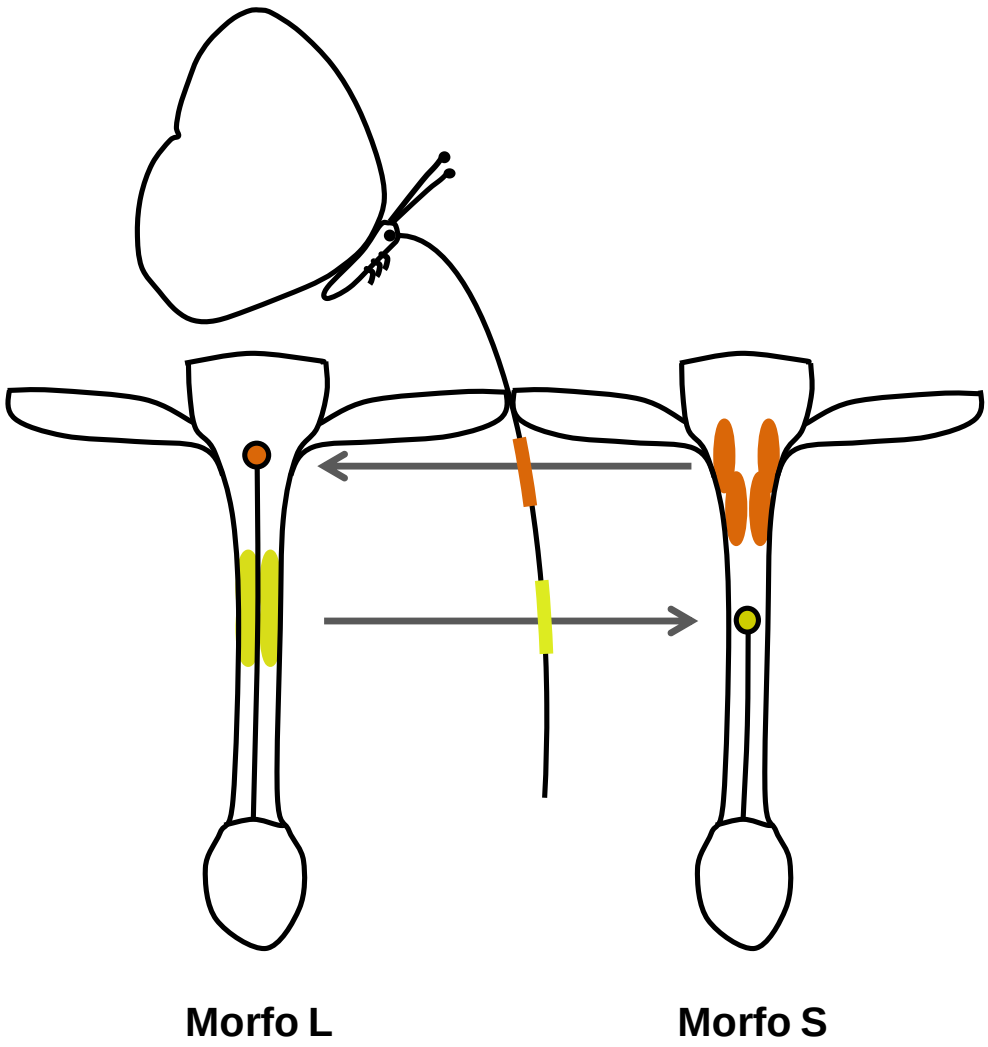


Figura 1. Morfos florales de una planta con distilia común. Morfo de estilo largo (L) y morfo de estilo corto (S) con el patrón teórico de transferencia de polen mediado por un polinizador entre los dos niveles recíprocos de órganos sexuales marcado con flechas. El mismo color en el estigma y las anteras indica compatibilidad fisiológica.

polimorfismos en otros caracteres secundarios, principalmente en la forma del estigma y de los granos de polen, llamados caracteres ancilares. El significado evolutivo de cada uno de estos componentes, su orden de aparición y su ligamiento han sido objeto de intenso estudio desde mediados del siglo XX, sin haberse llegado aún a una solución definitiva (véase revisión en Ornduff 1992).

Darwin (1877; pág. 245) interpretó la heterostilia como un mecanismo de promoción de la fecundación cruzada, en el que los distintos morfos, aunque todos hermafroditas, se relacionan entre ellos "*como los machos y hembras de las plantas dioicas o de los animales superiores*". El mecanismo debía ser, sin lugar a dudas, su adaptación a un transporte eficaz del polen por parte de los polinizadores entre los distintos niveles de órganos sexuales (Figura 1). Dicho esto, es importante notar que todas las especies heterostilas son polinizadas por animales. Además, la mayoría posee flores actinomorfas con un tubo floral bien desarrollado y un número limitado de estilos y estambres (Lloyd & Webb 1992a), cualidades que podrían favorecer la precisión en la transferencia del polen y por ende el buen funcionamiento de este polimorfismo y su estabilidad evolutiva.

Darwin consideró que en la evolución de la heterostilia la hercogamia recíproca tenía que haber aparecido en primer lugar, seguida de los caracteres ancilares y la incompatibilidad heteromórfica. La incompatibilidad heteromórfica no era adaptativa en su opinión, puesto que debe conllevar la esterilidad de cada individuo con la mitad de la población, la de su mismo morfo. Con términos actuales, podríamos decir que dividiría el tamaño efectivo poblacional por la mitad sin comportar otras posibles ventajas, como la liberación del coste de una función sexual que ocurre en plantas dioicas (Bawa 1980). Por tanto, Darwin propuso que la incompatibilidad heteromórfica había aparecido como un subproducto de la

hercogamia recíproca y que los polimorfismos en el estigma y el polen debían jugar un papel importante en el reconocimiento y admisión del polen del morfo opuesto.

Tras Darwin, otros investigadores han propuesto secuencias evolutivas para la aparición de los distintos caracteres de la heterostilia. Algunos autores han apoyado su secuencia de pasos, hercogamia recíproca seguida de autoincompatibilidad: Ernst (1936), Anderson (1973), Lloyd y Webb (1992b) y Richards (1998). Por el contrario, otros consideraron que el primer paso en la evolución de la heterostilia debía de haber sido la autoincompatibilidad heteromórfica: Crowe (1964), Baker (1966), Yeo (1975) y Charlesworth y Charlesworth (1979). Por su parte, Mather y de Winton (1941) propusieron que ambas características se habían adquirido simultáneamente.

La promoción de la fecundación cruzada

Tras el debate sobre el orden de aparición de las dos características principales de la heterostilia se esconde una pregunta de ecología funcional clave: ¿puede la hercogamia recíproca por sí sola facilitar la fecundación cruzada entre distintos morfos? Si la respuesta es afirmativa, el polimorfismo estilar podría ser estable sin necesidad de incompatibilidad heteromórfica, como Darwin propuso. En caso contrario, el polimorfismo habría de perderse recurrentemente y, por tanto, la aparición de la heterostilia podría haber resultado del camino inverso y ser dependiente de la incompatibilidad heteromórfica.

La prevalencia de la fecundación cruzada entre distintos morfos sobre la fecundación cruzada dentro de un mismo morfo es requisito indispensable para la estabilidad de cualquier polimorfismo intrapoblacional. Los cruzamientos entre morfos son un mecanismo de selección negativamente dependiente de la frecuencia

(Fisher 1930), en el que el morfo menos común disfruta de la ventaja de tener un mayor número posible de parejas (Eckert *et al.* 1996, Gleiser *et al.* 2008, Shang *et al.* 2012). Así, los cruces entre morfos garantizan un equilibrio dinámico en la proporción de morfos en la población (Heuch 1979). Por el contrario, si los cruzamientos dentro de un morfo cualquiera prevalecen, este aumentaría su frecuencia en la población pudiendo llegar a dominarla y haciendo al otro desaparecer. Por tanto, los patrones de cruzamiento de los morfos determinan la frecuencia de los mismos en una población. Un caso extremo de prevalencia de los cruzamientos dentro de cada morfo llevaría a su aislamiento reproductor. Lloyd y Webb (1992b) modelaron la necesidad de prevalencia de los cruces entre morfos para el equilibrio del polimorfismo estilar en una población con las siguientes ecuaciones:

$$\frac{1}{2}\,(q_{LS} + q_{SL}) > q_{LL} \qquad \text{Ec. 1}$$

$$\frac{1}{2}\,(q_{LS} + q_{SL}) > q_{SS} \qquad \text{Ec. 2}$$

$$q_{LS} > q_{LL} \qquad \text{Ec. 3}$$

$$q_{SL} > q_{SS} \qquad \text{Ec. 4}$$

Las ecuaciones 1 y 2 corresponden a condiciones en las que la fertilidad femenina está limitada por la cantidad o calidad de polen recibido, y las ecuaciones 3 y 4 a condiciones sin limitación por polen. En ellas, *q* representa el éxito reproductor, y los subíndices L y S representan los morfos de estilo largo y corto respectivamente, con el morfo materno en primer lugar. Para que se mantenga el polimorfismo, deben

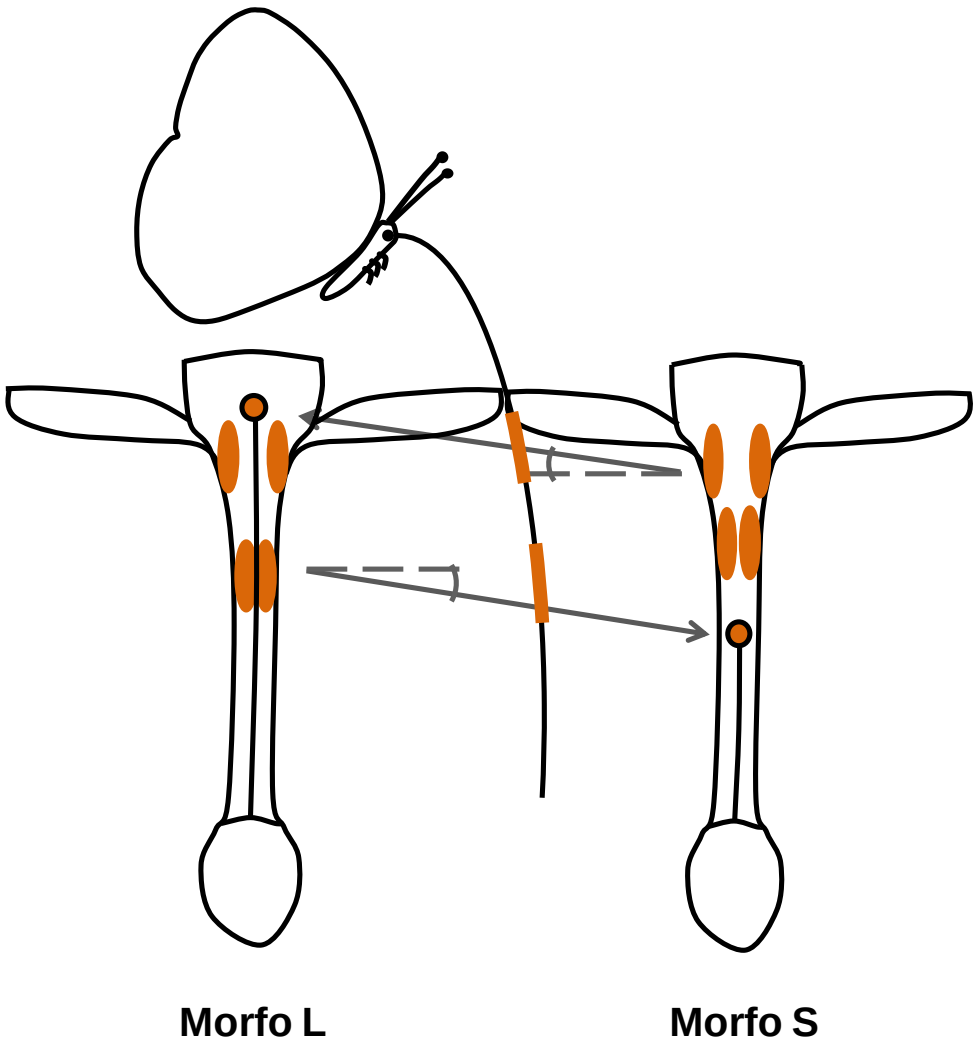


Figura 2. Morfos florales de una planta con dimorfismo estilar. Morfo de estilo largo (L) y morfo de estilo corto (S) con el patrón teórico de transferencia de polen entre sus dos niveles de órganos sexuales marcado con flechas, que muestran una correspondencia imperfecta de alturas. El mismo color en el estigma y las anteras indica compatibilidad fisiológica entre individuos. Nótese que el verticilo inferior de anteras se sitúa ligeramente más arriba en el morfo S; este es el caso de *Narcissus papyraceus*.

cumplirse las ecuaciones 1 y 2 o bien las ecuaciones 3 y 4, dependiendo de las condiciones de limitación por polen.

Aunque Darwin definió la heterostilia como el conjunto de hercogamia recíproca, incompatibilidad heteromórfica y polimorfismos ancilares (pues la mayoría de casos por él conocidos portaban esta combinación de rasgos), algunos autores actuales consideran que el único carácter indispensable para definir una especie (o más apropiadamente una población) como heterostila es la presencia de hercogamia recíproca (Barrett & Shore 2008). De hecho, la existencia de plantas heterostilas sin sistema de incompatibilidad heteromórfico es un apoyo para la hipótesis darwinista de la evolución de la heterostilia. Mientras que las ecuaciones anteriores son de obligado cumplimiento en las plantas con sistema de autoincompatibilidad heteromórfico, probar si los cruzamientos entre morfos dominan en plantas heterostilas sin sistema de autoincompatibilidad heteromórfico es un punto clave para probar si la hercogamia recíproca por sí sola puede mantenerse en la naturaleza y en qué condiciones.

El dimorfismo estilar

Existe un tipo de polimorfismo estilar parecido a la heterostilia pero que carece de su perfecta reciprocidad morfológica: el dimorfismo estilar (Barrett *et al.* 2000). El dimorfismo estilar consiste en la presencia en una población de dos morfos florales que difieren en la posición de su estigma pero no en la de sus anteras. Así, existe un morfo de estilo largo (L) y un morfo de estilo corto (S), cuyos estigmas se colocan respectivamente por encima o por debajo de las anteras, las cuales permanecen a una

altura constante (Figura 2). Tanto el modelo de Lloyd y Webb (1992b) como el de Charlesworth y Charlesworth (1979), los únicos modelos cuantitativos disponibles sobre la evolución de la heterostilia, consideraron que el dimorfismo estilar es un paso morfológico intermedio en la transición evolutiva desde el monomorfismo estilar hacia la heterostilia. Dado que los dos morfos estilares carecen de la reciprocidad sexual de los morfos heterostilos (Figura 2), se consideró que serían incapaces de promover eficazmente los cruzamientos entre morfos en ausencia de una incompatibilidad heteromórfica. Esta característica debería proporcionar al dimorfismo estilar una gran inestabilidad evolutiva, que habría de desembocar o bien en su pérdida o bien en una rápida transición a la heterostilia. Se conocen especies heterostilas repartidas por todo el mundo y en un total de 28 familias de angiospermas, es decir, en aproximadamente el 7,5% de las mismas (Barrett & Shore 2008). Por su parte, el dimorfismo estilar se ha descrito dentro de algunas de estas mismas familias (Amaryllidaceae, Boraginaceae, Linaceae, Primulaceae), y también en otras familias dentro de las cuales no se conocen taxones heterostilos (Epacridaceae, Ericaceae, Liliaceae; Barrett *et al.* 2000). La mayoría de las especies con dimorfismo estilar que han sido estudiadas carecen de incompatibilidad heteromórfica. Por tanto, cabe preguntarse si, siendo mucho menos frecuente que la heterostilia en la naturaleza, el dimorfismo estilar es estable en estos grupos.

El polimorfismo estilar en Narcissus

Uno de los pocos ejemplos de plantas en los que el polimorfismo estilar no se asocia con la incompatibilidad heteromórfica ni con polimorfismos ancilares es el género *Narcissus* (Barrett *et al.* 1996, Barrett & Harder 2005). Este género engloba una gran

variación en la posición de los órganos sexuales. Muchas de las especies de *Narcissus* presentan polimorfismo estilar. De las 41 especies estudiadas, 13 son dimórficas estilares (Santos-Gally *et al.* 2013a), una es distila (*N. albimarginatus*; Arroyo & Barrett 2000) y otra es tristila (*N. triandrus*; Barrett *et al.* 1997). El polimorfismo aparece en distintas secciones del género, en algunas de las cuales parece haber surgido de forma independiente (Pérez *et al.* 2004, Santos-Gally *et al.* 2013a). El resto de las especies presentan hercogamia de aproximación, con el estigma situado por encima del verticilo inferior de anteras (incluyendo el fenotipo no hercógamo respecto al verticilo superior de anteras; Santos-Gally *et al.* 2013a), carácter que parece ser derivado en la filogenia (Pérez-Barrales *et al.* 2006). Hasta ahora, no se conoce ningún otro género de angiospermas en el que aparezca tal variedad de polimorfismos estilares. La mayoría de las especies de *Narcissus*, incluyendo aquellas con polimorfismo estilar, tienen un sistema de autoincompatibilidad homomórfico, por lo que los individuos son autoincompatibles pero tanto las polinizaciones dentro de cada morfo como las polinizaciones entre distintos morfos producen la misma cantidad de semillas (Barrett *et al.* 1997, Baker *et al.* 2000, Pérez-Barrales *et al.* 2006, Santos-Gally *et al.* 2013b). Unas cuantas especies son plenamente autocompatibles (Baker *et al.* 2000, Pérez-Barrales *et al.* 2006, Santos-Gally *et al.* 2013a). Estas características han hecho del género *Narcissus* un interesante sistema de estudio de la evolución de la heterostilia, que ha generado un gran número de estudios tanto a escala macroevolutiva (p. ej. Graham & Barrett 2004, Pérez *et al.* 2004, Pérez-Barrales *et al.* 2006, Santos-Gally *et al.* 2013a) como microevolutiva (p. ej. Thompson *et al.* 2003, Cesaro & Thompson 2004, Cesaro *et al.* 2004, Hodgins & Barrett 2006, 2008, Pérez-Barrales *et al.* 2008, Pérez-Barrales & Arroyo 2010, Thompson *et al.* 2012, Santos-Gally *et al.* 2013b).

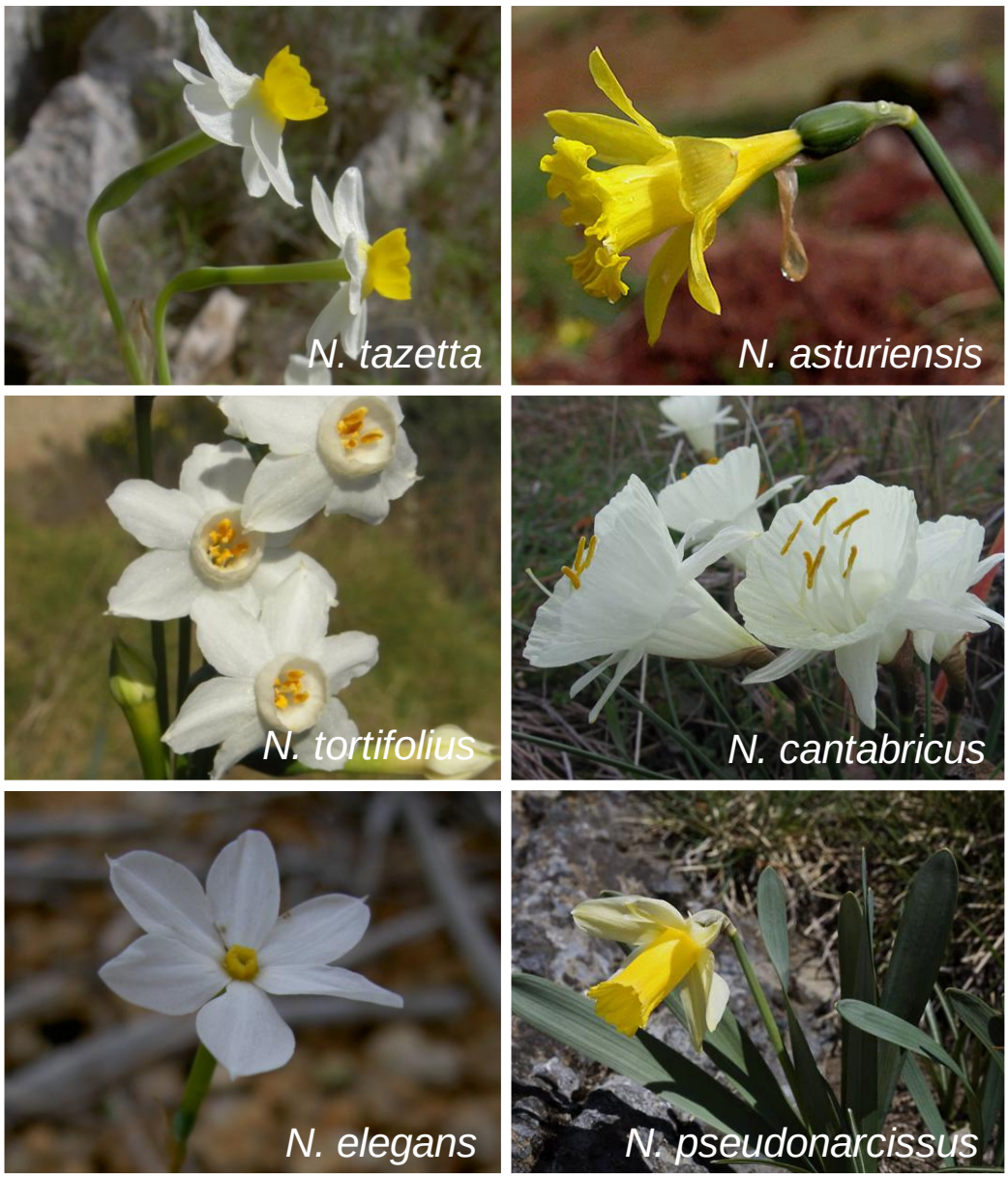


Figura 3. Algunos representantes del género *Narcissus* en la Península Ibérica. A la izquierda, tres especies que ejemplifican la morfología de tubo floral estrecho y corona poco desarrollada. A la derecha, el segundo grupo morfológico con tubo floral ancho y corona muy desarrollada.

El género Narcissus *L.*

Narcissus L. es un género de plantas perteneciente a la familia Amaryllidaceae (orden Asparagales, clase Monocotyledoneae). Las Amaryllidaceae cuentan con unos 59 géneros y 850 especies que se distribuyen por todo el mundo (Aedo 2013). Una de las regiones de máxima diversidad de la familia es la Cuenca Mediterránea, de donde *Narcissus* es endémico (Santos-Gally *et al.* 2012). En esta región se encuentran también los géneros más estrechamente emparentados con *Narcissus*: *Sternbergia* (de su misma tribu Narcisseae), *Pancratium* y *Vagaria* (tribu Pancratieae), y *Acis*, *Galanthus* y *Leucojum* (tribu Galantheae) (Meerow & Snijman 1998, Lledó *et al.* 2004, Meerow *et al.* 2006).

El género *Narcissus* es el más diverso de su clado. Con entre 16 y 160 especies reconocidas por diversos autores, la taxonomía del género ha sido y sigue siendo controvertida (Fernandes 1975, Dorda & Fernández-Casas 1989, Blanchard 1990, Mathew 2002). La hibridación entre especies y la poliploidización son comunes (Fernandes 1951, 1967, 1968, Marques *et al.* 2007, 2010), factores que no han facilitado la labor nomenclatural, como tampoco lo ha hecho el profuso cultivo de variedades ornamentales desde el Siglo XV (Hanks 2002, van Dijk 2003) y el posterior asilvestramiento de algunas de ellas. Más de la mitad de las especies descritas se encuentran en la Península Ibérica, donde muchas son endémicas y varias se encuentran amenazadas (Barra *et al.* 2011).

Los narcisos son plantas herbáceas bulbosas y perennes. Poseen hojas basales lineares y de uno a varios escapos que terminan en inflorescencias en forma de umbela, con entre una y 15 flores según la especie (Worley *et al.* 2000, Aedo 2013). Las flores son hermafroditas, actinomorfas y trímeras. El perianto está formado por seis tépalos, normalmente de colores blanco y/o amarillo (aunque *N. viridiflorus* es

completamente verde), que forman un tubo floral. En la parte superior del tubo nacen las seis partes libres de los tépalos y, normalmente, una corona. Existe gran variación en la forma del perianto en función de la arquitectura del tubo floral y de la corona. Hay dos grupos morfológicos principales: uno con tubo floral ancho y corona ancha y muy desarrollada, y otro con tubo floral estrecho y corona estrecha y reducida (Santos-Gally *et al.* 2013a; Figura 3). Los estambres están adnatos al interior del tubo floral y se disponen en dos verticilos trímeros. El gineceo se compone de un ovario ínfero y triloculado, un estilo alargado y un estigma capitado. El fruto es una cápsula dehiscente sin ningún mecanismo especial de dispersión de las semillas.

Los narcisos silvestres florecen entre otoño y principios de primavera, con un buen número de especies florecientes en pleno invierno. Las flores son visitadas por polillas, mariposas diurnas, abejas solitarias, abejas sociales y sírfidos (Pérez-Barrales *et al.* 2006, Navarro *et al.* 2012, Santos-Gally *et al.* 2013a).

El caso de Narcissus papyraceus *Ker-Gawler.*

Narcissus papyraceus es una de las especies de *Narcissus* en las que se conoce el dimorfismo estilar (Arroyo *et al.* 2002). Así, la especie posee dos morfos florales que difieren en la longitud de su estilo: un morfo de estilo largo (L), cuyo estigma se sitúa por encima del verticilo inferior de anteras, y un morfo de estilo corto (S), cuyo estigma se coloca por debajo de dicho verticilo[1] (Figura 4). Las anteras se colocan a

[1] Un examen de las células epidérmicas estilares ha revelado que la diferencia en la longitud estilar no resulta de un cambio en el número de células (media ± SD= 65.8 ± 10.6 células; $F_{1,29} = 0.10$; $P = 0.75$; GLM), sino de un cambio en la elongación de las mismas (media ± SD: morfo L=185.4 ± 28.7 μm; morfo S=115.7 ± 13.9 μm; $F_{1,74} = 159.6$; $P < 0.001$; GLM).

alturas equivalentes en ambos morfos, aunque el verticilo inferior se sitúa ligeramente más elevado en el morfo S (Pérez-Barrales & Arroyo 2010). No existen diferencias en ningún otro rasgo floral ni vegetativo entre ambos morfos (pero véase Pérez-Barrales *et al.* 2007).

Las poblaciones de *N. papyraceus* presentan una proporción de morfos florales que varía desde aproximadamente 1:1 hasta 1:0 para L:S (Arroyo *et al.* 2002). Es decir, virtualmente no existen poblaciones en las que domine el morfo S ni se ha encontrado una sola población monomórfica S. Por el contrario, el morfo L se encuentra presente en todas las poblaciones, las domina y llega a formar poblaciones monomórficas L. La proporción de morfos de las poblaciones no se distribuye al azar, sino que sigue un patrón geográfico. Las poblaciones más próximas al Estrecho de Gibraltar tienen proporciones iguales de ambos morfos (1L:1S). La frecuencia del morfo S se reduce conforme las poblaciones se alejan del Estrecho, llegando a desaparecer en las poblaciones del límite norte del área de distribución, en el valle del Guadalquivir, que son monomórficas L. Hacia el centro de Marruecos, el morfo S se mantiene en todas las poblaciones en pequeña proporción (Figura 5).

El patrón geográfico de la proporción de morfos en *N. papyraceus* ha sido estudiado desde diversas perspectivas. En el primer trabajo en que el patrón fue exhaustivamente descrito (Arroyo *et al.* 2002) la proporción de morfo S se correlacionó positivamente con el tamaño poblacional. Estudios posteriores analizaron el papel de los polinizadores, el éxito reproductor femenino y los patrones de cruzamiento en la proporción de morfos en las poblaciones (Pérez-Barrales *et al.* 2007, Pérez-Barrales & Arroyo 2010, Santos-Gally *et al.* 2013b). Estos trabajos apoyaron la hipótesis del papel principal de los visitantes florales sobre la frecuencia de aparición del morfo S. En concreto, Pérez-Barrales *et al.* (2007) y Santos-Gally *et al.* (2013b) mostraron una correlación negativa entre la frecuencia del morfo S en las

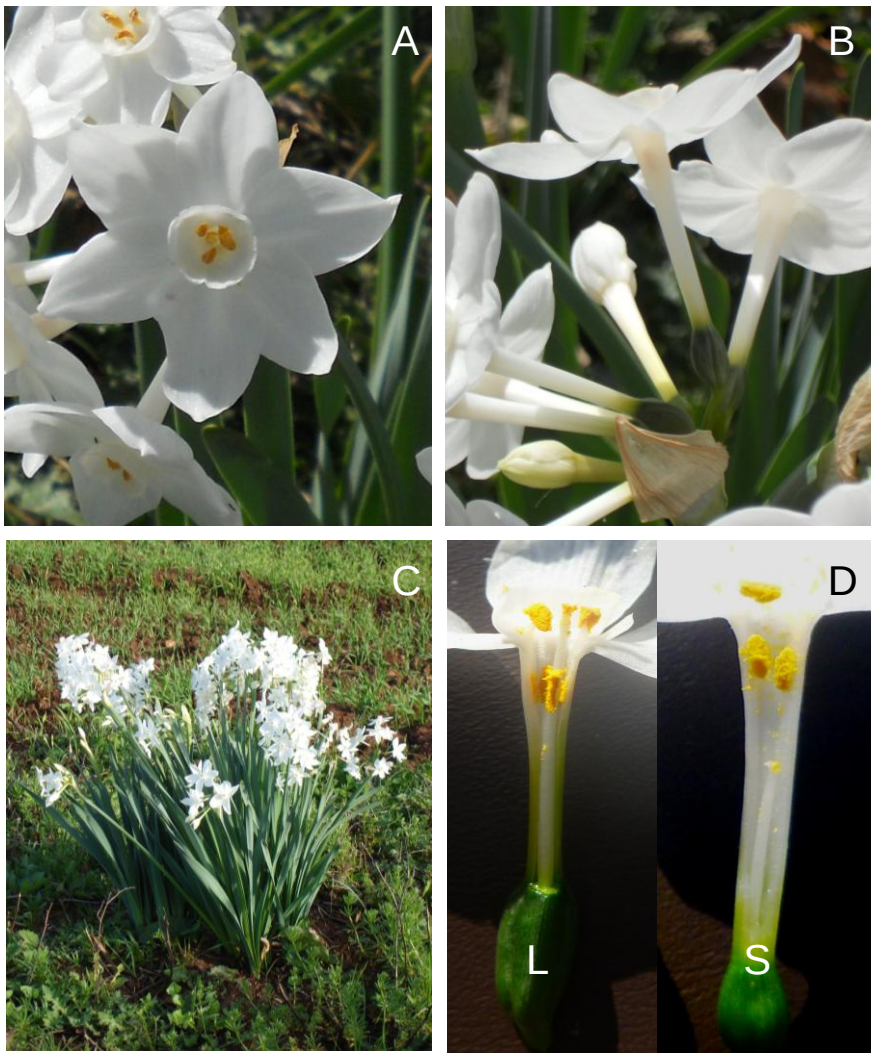


Figura 4. Morfología de *Narcissus papyraceus*. (A) Detalles de la corona y los tépalos y (B) del tubo floral. (C) Agrupación típica de escapos de un mismo genotipo en el campo debido a la reproducción vegetativa por fragmentación de bulbos. (D) Morfos florales de estilo largo (L) y de estilo corto (S); tras la disección, faltan 1–2 anteras por verticilo.

poblaciones y la tasa de visitas de polinizadores de probóscide corta (sírfidos y abejas). Por su parte, Pérez-Barrales y Arroyo (2010) mostraron un menor éxito reproductor femenino del morfo S y mayores tasas de cruzamientos entre individuos del morfo L en el área natural monomórfica de la especie. La hipótesis propuesta por estos investigadores es que los polinizadores de probóscide corta reducen el éxito reproductor femenino del morfo S porque no alcanzan a polinizar su estigma a la vez que incrementan las tasas de cruzamiento entre individuos del morfo L, que tiene por tanto mayor éxito masculino y femenino. Así, los polinizadores de probóscide corta podrían determinar la menor frecuencia o la pérdida del morfo S en las poblaciones.

Características de Narcissus papyraceus *como modelo de estudio*

Narcissus papyraceus es una especie situada hacia la base de la filogenia del género (Graham & Barrett 2004, Santos-Gally *et al.* 2013a). Es una especie longeva; aunque se desconoce su tiempo de vida máximo o promedio, la primera reproducción sexual ocurre a partir del tercer o cuarto año (observaciones personales). Los individuos poseen varios escapos florales y hasta quince flores por inflorescencia. La reproducción vegetativa, por medio de la fragmentación de los bulbos, determina en ocasiones que los genotipos consten de grandes agrupaciones de escapos florales. Las flores son blancas y tienen el tubo floral estrecho (Figura 4). Su olor dulzón, determinado por una alta concentración de indol (Dobson *et al.* 1997), es característico y causa de los nombres vernáculos que la especie tiene en el sur de España.

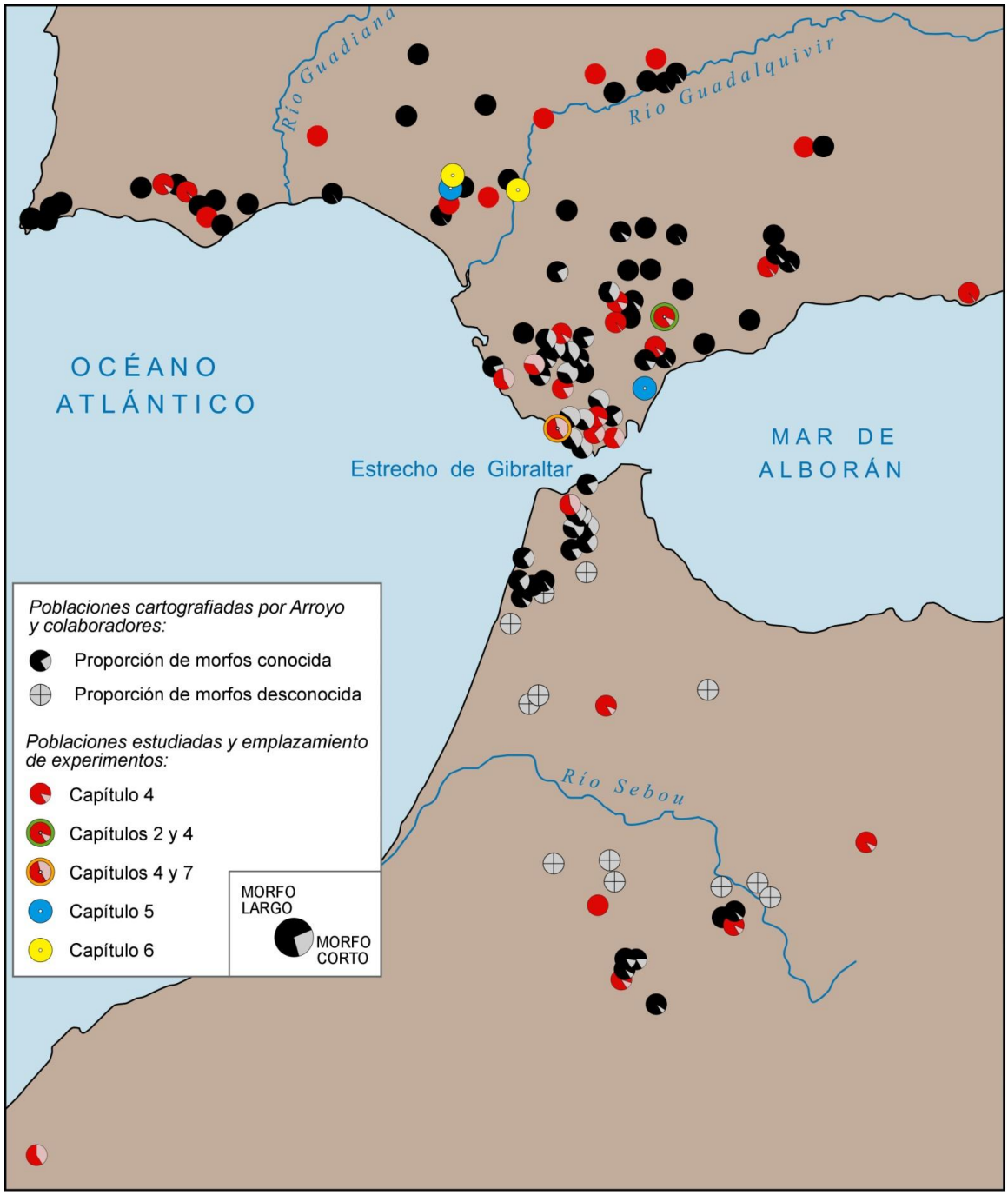


Figura 5. Poblaciones de *Narcissus papyraceus* en la principal área de distribución de la especie, el margen occidental de la Cuenca Mediterránea, y sitios de estudio de esta Tesis Doctoral.

El área de distribución de *N. papyraceus* se extiende por toda la Cuenca Mediterránea. No obstante, aunque se conocen poblaciones en Argelia, Francia, Italia, Grecia y la antigua Yugoslavia, algunas de dudoso origen nativo (Blanchard 1990), la gran mayoría de las poblaciones de la especie se reparte entre el sur de España y el norte de Marruecos. Así, *N. papyraceus* tiene su principal área de distribución en la zona del Estrecho de Gibraltar, en la cual se centra la presente Tesis Doctoral (Figura 5). En esta zona, las poblaciones presentan un gradiente de densidad y tamaño poblacional decreciente con la distancia al Estrecho. En las provincias de Cádiz, en España, y de Sidi-Kacem, en Marruecos, se encuentran las mayores densidades poblacionales, con poblaciones de hasta varios miles de individuos distribuidas frecuentemente a menos de cinco kilómetros unas de otras. La mayoría de las poblaciones están a baja altitud, aunque algunas llegan a situarse hasta a 1200 m sobre el nivel del mar. Casi todas se localizan en hábitats muy perturbados, como pastos, cultivos y márgenes de caminos, ocupando suelos preferentemente arcillosos o limosos sobre sustratos rocosos de composición muy variada (Tabla S1).

En el entorno del Estrecho de Gibraltar las poblaciones de *N. papyraceus* florecen entre diciembre y marzo en un gradiente latitudinal de sur a norte. La intensidad de floración de las poblaciones es muy dependiente de una alta precipitación otoñal e invernal. Las condiciones invernales durante su floración determinan un ambiente de polinización inestable, al que los individuos hacen frente con flores que duran hasta quince días en espera de la visita de un polinizador y una floración individual total que se prolonga hasta un mes, una estrategia típica de las plantas de floración invernal (Primack 1985). Al nivel de población, la floración puede durar hasta dos meses. Los visitantes florales de *N. papyraceus* son insectos generalistas que incluyen lepidópteros diurnos (géneros *Pieris*, *Vanessa* y *Gonopteryx*),

polillas diurnas o nocturnas (*Macroglossum stellatarum* y *Autographa gamma* principalmente), abejas solitarias (*Anthophora*, *Andrena* y *Eucera*), abejorros (*Bombus*), abejas sociales (*Apis mellifera* principalmente) y sírfidos (*Eristalis*, *Merodon*, *Eupeodes* y *Syritta*) (Pérez-Barrales *et al.* 2007, Santos-Gally *et al.* 2013b). *Narcissus papyraceus* es autoincompatible, pero tanto las polinizaciones entre plantas del mismo morfo como las polinizaciones entre plantas de morfos diferentes son igualmente viables (Arroyo *et al.* 2002, Santos-Gally *et al.* 2013b).

Objetivos de la Tesis Doctoral

A pesar de las varias aproximaciones con las que se había estudiado el patrón geográfico de la proporción de morfos en *Narcissus papyraceus*, la mayoría de las cuestiones implicadas permanecían sin una evidencia empírica directa y/o detallada. La presente Tesis Doctoral profundiza en el estudio de las causas de dicho patrón, gracias al empleo de métodos exhaustivos. En concreto, se estudian en detalle (i) el sistema de autoincompatibilidad de la especie; (ii) los procesos históricos demográficos de formación y cambio de las poblaciones; (iii) los patrones de transferencia de polen dentro de cada morfo y entre distintos morfos determinados por los polinizadores de probóscide larga y de probóscide corta en la especie; y (iv) los patrones de éxito reproductor de los morfos florales y de cruzamientos dentro de cada morfo y entre distintos morfos. Así, el objetivo de la Tesis Doctoral es dar respuesta a las siguientes preguntas:

- ¿Existe una incompatibilidad heteromórfica críptica que pudiese afectar diferencialmente a los patrones de cruzamiento de los morfos en las poblaciones?
- ¿Pueden la similitud genética de las poblaciones o procesos estocásticos, como la deriva genética, haber influido sobre las proporciones de morfos y la reversión al monomorfismo?
- ¿Cuál es el papel efectivo de los distintos tipos de polinizadores en los patrones de cruzamiento y en el éxito reproductor de los morfos?
- ¿Explican los patrones de cruzamiento en las poblaciones la estabilidad y/o pérdida del polimorfismo?

De forma global, esta Tesis Doctoral persigue descubrir si el dimorfismo estilar es capaz de promover los cruzamientos entre individuos de distintos morfos y es por tanto una condición estable en *N. papyraceus*, y si es así, bajo qué circunstancias.

Estructura de la Tesis Doctoral

Para acometer el estudio de distintos elementos involucrados en el funcionamiento y la pérdida del dimorfismo estilar en las poblaciones de *Narcissus papyraceus*, la presente Tesis Doctoral posee cinco capítulos centrales con datos empíricos propios.

En el segundo capítulo, *La autoincompatibilidad homomórfica y ovárica en* Narcissus papyraceus *(Amaryllidaceae) se debe a una respuesta precigótica*, se estudia la naturaleza de la reacción de autoincompatibilidad de la especie con técnicas de microscopía de luz blanca y fluorescencia. Los objetivos de este capítulo son detectar posibles diferencias crípticas en el proceso de fecundación entre polinizaciones dentro de cada

morfo y entre distintos morfos, y describir el sitio exacto y el mecanismo de la autoincompatibilidad de la especie.

En el tercer capítulo, *Nuevos marcadores microsatélites para* Narcissus papyraceus *(Amaryllidaceae) y amplificación en otras especies congéneres*, se desarrollan marcadores moleculares microsatélites nucleares para su empleo en estudios ecológicos y poblacionales en *N. papyraceus*, y se investiga su posible aplicación en otras especies de *Narcissus*.

En el cuarto capítulo, *Genética de poblaciones y variación en la proporción de morfos florales en toda el área de distribución de la especie dimórfica estilar* Narcissus papyraceus *(Amaryllidaceae)*, se estudian los patrones de variación y estructuración genética en poblaciones de *N. papyraceus* usando los microsatélites desarrollados en el capítulo tercero. El objetivo de este trabajo es conocer la posible influencia de procesos estocásticos como los cuellos de botella o los efectos fundadores en la proporción de morfos florales en las poblaciones. Además, se investiga la relación de estas características poblacionales con la variación climática territorial.

En el quinto capítulo, *Los insectos de probóscide larga promueven la transferencia de polen entre los morfos estilares de* Narcissus papyraceus *(Amaryllidaceae)*, se investigan en detalle los patrones de polinización dentro de cada morfo y entre morfos que promueven los dos tipos distintos de polinizadores de *N. papyraceus*: de probóscide larga y de probóscide corta. Además, se estudian los patrones de remoción de polen y de autopolinización para tener un esquema completo de la interacción de los morfos florales con sus visitantes, e inquirir cuál puede ser el papel de los últimos en el mantenimiento del dimorfismo estilar en la naturaleza.

En el sexto capítulo, *Los cruzamientos entre morfos prevalecen en la especie dimórfica estilar sin reciprocidad sexual* Narcissus papyraceus *(Amaryllidaceae)*, se investigan la fertilidad y el éxito de padreamiento de los morfos florales y los patrones de

cruzamiento dentro de cada morfo y entre morfos distintos para prever la estabilidad del dimorfismo estilar en *N. papyraceus* bajo diversas condiciones. Los datos se obtuvieron a partir de poblaciones experimentales fabricadas con individuos genotipados con los microsatélites desarrollados en el tercer capítulo, con distintas proporciones de morfos florales y localizadas en las áreas naturales dimórfica y monomórfica de la especie.

Los cinco capítulos se encuentran estructurados en formato de artículo científico. Así, pueden leerse independientemente y en el orden deseado, aunque considero más adecuado el orden que siguen en la Tesis. Los capítulos están escritos en inglés por ser este el idioma estándar de comunicación en ciencia, aunque incluyen una traducción del Resumen al español. La Tesis termina con un séptimo capítulo de Discusión General en el que se integran todos los resultados y se discuten sus implicaciones en la ecología evolutiva del dimorfismo estilar, para terminar con las Conclusiones Generales de la Tesis Doctoral.

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Tabla S1. Coordenadas y sustrato geológico de 31 poblaciones de *Narcissus papyraceus* muestreadas en el Capítulo 4.

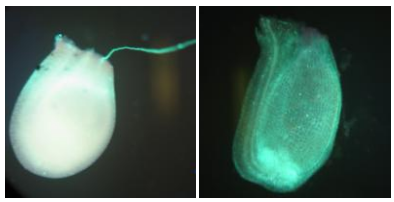
Pob.	Lat. (ºN)	Long. (ºW)	Altit. (msnm)	Edad (*)	Litología (*)
1	34º12,6'	4º08,4'	401	Cuaternario	Arenas y limos
2	33º51,0'	4º52,2'	905	Jurásico basal	Dolomías
3	34º49,8'	5º32,4'	224	Oligoceno-Mioceno	Arcillas y limos
4	35º43,2'	5º44,4'	55	Cuaternario	Arenas, arcillas y carbonatos
5	33º57,0'	5º34,2'	299	Mioceno superior	Arcillas
6	33º37,8'	5º25,9'	914	Jurásico basal	Limos y arcillas con cantos
7	32º39,6'	8º39,6'	166	Cuaternario	Limos arenosos
8	37º50,4'	5º18,6'	152	Cámbrico inferior	Pizarras y calizas
9	37º28,2'	4º21,0'	783	Jurásico medio	Calizas
10	37º45,6'	5º34,8'	331	Mioceno superior	Calizas karstificadas y arcilla
11	36º45,0'	3º25,8'	495	Triásico inferior	Cuarcitas y filitas
12	37º29,4'	7º15,0'	197	Carbonífero superior	Pizarras y areniscas
13	37º13,2'	6º26,4'	34	Plioceno	Arenas arcósicas
14	36º37,8'	5º27,0'	507	Eoceno-Oligoceno	Calizas y margas
15	36º45,0'	5º30,6'	300	Triásico	Arcillas con yesos
16	36º12,6'	5º34,8'	102	Mioceno inferior	Arcillas (localmente sulfatos)
17	36º08,4'	5º36,0'	252	Cuaternario	Arcillas (loc. sulfatos) y arenas
18	36º04,2'	5º33,0'	420	Cuaternario	Arcillas, arenas y carbonatos
19	36º19,2'	5º46,2'	31	Mioceno inferior	Areniscas silíceas
20	36º22,2'	6º06,6'	24	Mioceno superior	Margas (loc. arenosas)
21	36º24,6'	5º54,6'	44	Mioceno inferior	Arcillas (loc. sulfatos)
22	36º28,8'	5º43,8'	74	Cuaternario	Arenas (loc. calcáreas) y arcillas
23	36º06,0'	5º43,8'	164	Cretácico superior	Calizas y margas
24	36º40,2'	5º13,2'	962	Cretácico superior	Calizas
25	36º28,8'	5º17,4'	366	Cretácico inferior	Calizas
26	36º54,0'	4º34,2'	523	Mioceno inferior	Areniscas y margas
27	37º14,4'	8º02,4'	209	Triásico sup.-Jurásico inf.	Arcillas, sulfatos y carbonatos
28	37º15,0'	8º06,0'	314	Triásico sup.-Jurásico inf.	Arcillas, sulfatos y carbonatos
29	37º06,0'	7º51,0'	199	Jurásico superior	Calizas
30	37º14,4'	6º14,4'	12	Cuaternario	Limos y arcillas
31	37º33,0'	5º56,4'	27	Cuaternario	Gravas cuarcíticas

(*) Información tomada de: cartografía geológica MAGNA (1:50.000) del Instituto Geológico y Minero de España, hojas 921, 941, 962, 980, 989, 1001, 1038, 1050, 1056, 1064, 1069, 1070, 1071, 1074 y 1077 (1975–1991); Carte Géologique du Maroc 1:1.000.000, Service Géologique du Maroc (1985); Carta Geológica de Portugal 1:500.000, Serviços Geológicos de Portugal (1992).

CAPÍTULO 2

LA AUTOINCOMPATIBILIDAD HOMOMÓRFICA Y OVÁRICA EN *Narcissus papyraceus* (AMARYLLIDACEAE) SE DEBE A UNA RESPUESTA PRECIGÓTICA

HOMOMORPHIC OVARIAN SELF-INCOMPATIBILITY IN *Narcissus papyraceus* (AMARYLLIDACEAE) IS DUE TO A PRE-ZYGOTIC RESPONSE



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Unpublished.

Resumen

La autoincompatibilidad ovárica, que incluye reacciones pre- y postcigóticas, es un mecanismo complejo del que todavía desconocemos muchos detalles sobre su función y significado. La presencia conjunta de autoincompatibilidad ovárica homomórfica (no dialélica) y polimorfismo estilar es una rara combinación que el género *Narcissus* presenta. Por lo general, las especies polimórficas estilares presentan incompatibilidad heteromórfica (dialélica), la cual impide la fecundación entre individuos del mismo morfo y así ayuda a mantener la misma proporción de morfos florales en las poblaciones. Por lo tanto, estudiar la autoincompatibilidad de *Narcissus* es interesante tanto para intentar desentrañar la naturaleza de la reacción de rechazo como para evaluar posibles diferencias crípticas en el proceso de fertilización en polinizaciones entre individuos del mismo y de distinto morfo. Examinamos el sistema de reproducción de *Narcissus papyraceus*, una especie con dimorfismo estilar que tiene proporciones de morfos sesgadas en la mayoría de sus poblaciones. Para ello, se estudió el crecimiento del tubo polínico en el pistilo y la respuesta de los óvulos después de polinizaciones manuales controladas. El crecimiento de los tubos polínicos en los pistilos autopolinizados y en los pistilos polinizados por su mismo morfo o por el morfo opuesto fue en ambos morfos similar hasta la penetración del micropilo ovular, pero posteriormente un fallo precigótico pareció afectar a los gametofitos masculino y femenino en los pistilos autopolinizados. Una proporción de óvulos en estos pistilos mostró signos de colapso y los tubos polínicos se bloquearon o comportaron anormalmente antes de entrar en el saco embrionario. Una parte de los óvulos no penetrados también mostró signos de degeneración posiblemente debido a la falta de estimulación ovárica. No encontramos indicios concluyentes de funcionamiento diferencial entre las polinizaciones entre individuos

del mismo y de distinto morfo en ninguno de los dos morfos, lo que nos permite descartar posibles efectos del sistema de reproducción en *Narcissus papyraceus* como causa del sesgo en las proporciones de morfos en las poblaciones y confirmar el carácter excepcional del mecanismo de autoincompatibilidad en esta especie polimórfica.

Palabras clave: Amaryllidaceae, crecimiento del tubo polínico, degeneración ovular, dimorfismo estilar, *Narcissus*, sistema de incompatibilidad.

Abstract

Ovarian self-incompatibility, which includes pre- and post-zygotic reactions, is a complex mechanism for which we still lack many details of its function and significance. The joint presence of homomorphic (i.e. non diallelic) ovarian self-incompatibility with stylar polymorphism is a rare combination that is found in the genus *Narcissus*. Usually, style-polymorphic species have heteromorphic (i.e. diallelic) incompatibility, which prevents fertilization between individuals of the same morph, thereby helping to maintain equal proportions of floral morphs in populations. Hence, self-incompatibility in *Narcissus* is of particular interest when attempting to unravel the nature of the rejection reaction and assess possible cryptic differences in the fertilization process in intra- and inter-morph crosses. We examined the breeding system of *Narcissus papyraceus*, a style-dimorphic species that has biased morph ratios in most of its populations. We studied pollen tube growth in the pistil and ovule fate after experimentally controlled hand pollinations. The growth of pollen tubes in selfs and intra- and inter-morph crosses was similar up to the point of micropyle

penetration in both morphs, but subsequently a pre-zygotic failure appeared to affect male and female gametophytes in selfed pistils. A proportion of ovules from self-pollinated flowers showed signs of collapse and self-pollen tubes were blocked or behaved abnormally before entering the embryo sac. Non-penetrated ovules also showed signs of degeneration possibly due to the failure of ovary stimulation. We did not find any conclusive signs of differential functioning between intra- and inter-morph pollinations in any morph. These results enable us to rule out the possible effects of the breeding system in *Narcissus papyraceus* as a cause of morph-ratio biases and confirm the exceptional nature of the self-incompatibility mechanism in this polymorphic species.

Keywords: Amaryllidaceae, incompatibility system, *Narcissus*, ovule degeneration, pollen tube growth, stylar dimorphism.

Introduction

Self-incompatibility has evolved repeatedly in hermaphroditic flowering plants as a means of avoiding self-fertilization and promoting cross-fertilization, and thereby increasing the genetic pool of the progeny (Charlesworth & Charlesworth 1987, Johnston 1992, Uyenoyama *et al.* 1993, Igic *et al.* 2008, Goldberg *et al.* 2010). Three major mechanisms of self-incompatibility that differ in the site of the rejection reaction and their genetic control have been described in flowering plants: homomorphic gametophytic, homomorphic sporophytic and heteromorphic sporophytic incompatibility (de Nettancourt 1977, Gibbs 1986, Dickinson *et al.* 1992,

Hinata *et al.* 1993, Barrett & Cruzan 1994, Kao & McCubbin 1996). In these incompatibility systems, pollen tube growth is blocked at the stigma or in the style as a result of a rapid response to the pollen-pistil interaction. A delay in the rejection response may be the cause of a less well known and, possibly, less efficient self-incompatibility mechanism, namely ovarian or late-acting self-incompatibility (LSI; Seavey & Bawa 1986, Gibbs & Bianchi 1993, Sage *et al.* 1994).

The difficulty of observing post-pollination events within the ovules has hindered our understanding of the possible mechanisms operating in cases of late-acting self-incompatibility in certain species (Gibbs & Bianchi 1999, Lipow & Wyatt 1999, Aguilar & Bernardello 2001, LaDoux & Friar 2006). In the few detailed studies that are available, both pre- and post-zygotic reactions have been proposed (Cope 1962, Kenrick *et al.* 1986, Gibbs *et al.* 1999, Sage & Sampson 2003, Valtueña *et al.* 2010, Finatto *et al.* 2011, Ford & Wilkinson 2012). However, other studies have stimulated a debate regarding the possibility that selfing failure in some species is due to early acting inbreeding depression rather than any novel LSI mechanism (Klekowski 1988, Manicacci & Barrett 1996, Nic Lughadha 1998, Lipow & Wyatt 2000, Bittencourt *et al.* 2003, Koehl *et al.* 2004, Hao *et al.* 2012). Late-acting self-incompatibility has been viewed as a maladaptive system because it reduces considerably the number of ovules available for subsequent cross-pollinations (ovule-discounting; Dulberger 1964, Waser & Price 1991, Broyles & Wyatt 1993, Vaughton 1993, Vaughton *et al.* 2010), although it may be advantageous by permitting some selfing after mixed self/cross pollinations (Bertin & Sullivan 1988, Sage *et al.* 1999).

Self-incompatibility and ovule discounting are widespread in the genus *Narcissus*, where they are associated with stylar polymorphism (Barrett *et al.* 1997, Baker *et al.* 2000, Arroyo *et al.* 2002, Pérez-Barrales *et al.* 2006, Santos-Gally *et al.* 2013a). Commonly, species with stylar polymorphism have a sporophytic

heteromorphic (i.e. di- or tri-allelic) incompatibility system that prevents both self-fertilization and crosses between individuals of the same floral morph. By contrast, the incompatibility mechanism in polymorphic *Narcissus* seems to be homomorphic (i.e. to have a multiallelic basis) since floral morphs are compatible and only self-fertilization is precluded (Bateman 1952, Dulberger 1964). The lack of heteromorphic incompatibility has important implications for the evolution of polymorphism in populations of *Narcissus* species. Equal proportions of floral morphs are obligate in populations of heterostylous species with heteromorphic incompatibility systems (Fisher 1930, Heuch 1979, Barrett 1993) given the putative genetic codification of one morph as a recessive homozygote and the other as a heterozygote (i.e. crosses *SS* × *Ss* are an obligatory consequence across generations; Lewis & Jones 1992). The floral morph ratios in populations of species with homomorphic incompatibility systems will depend on pollination patterns among floral morphs that are influenced by their floral architecture (Cesaro & Thompson 2004, Pérez-Barrales & Arroyo 2010, Chapters 5 and 6). However, cryptic differences in the incompatibility systems of floral morphs (Eckert & Allen 1997, Wu *et al.* 2010) could cause deviations between the effective mating patterns and the pollen flow (Cruzan & Barrett 1996). Consequently, a detailed examination of the SI mechanism is warranted in *Narcissus* species that present morph ratio bias in some populations.

Detailed studies in style-dimorphic *Narcissus tazetta* and tristylous *N. triandrus* have identified an ovarian self-incompatibility reaction (Dulberger 1964, Sage *et al.* 1999) and have discarded the possibility of heteromorphic differences in the fertilization process, although they have disagreed on whether the rejection occurred pre- or post-zygotically. Given the apparent taxonomic clustering of ovarian self-incompatibility (Gibbs & Bianchi 1999), it is to be expected that other *Narcissus* species will show similar ovarian SI mechanisms. However, self-incompatibility

systems are labile, often break down (Johnston & Schoen 1996, Pailler & Thompson 1997, Pérez-Barrales *et al.* 2006, Bramow *et al.* 2013, Santos-Gally *et al.* 2013a) and can vary among related species or floral morphs (Bawa & Beach 1983, Manicacci & Barrett 1996, Pailler & Thompson 1997, Brys *et al.* 2008, Ferrero *et al.* 2012).

In the present study, we investigated the breeding system of *Narcissus papyraceus*, a style-dimorphic species that has biased morph ratios in most populations. Our aim was to (i) assess the occurrence of late-acting self-incompatibility in this species, (ii) investigate in detail the nature of the rejection reaction and (iii) explore possible cryptic differences in the fertilization process between intra- and inter-morph pollinations that might account for the observed bias in morph ratios. We looked at the behaviour of pollen tubes and the response of ovules after self-, intra- and inter-morph pollinations, and then addressed the following questions: i) do pollen tubes behave in a similar way in the style after each pollination type?; ii) are there any differences in the rates of ovule penetration or fertilization?; and iii) do the ovules respond in a similar way to self-, same-morph and inter-morph pollen?

Material and Methods

Study species and experimental crosses

Narcissus papyraceus Ker-Gawler is a winter-flowering species endemic to the Mediterranean Basin that is very common at the south-western edge of its range. It is a style-dimorphic bulbous geophyte and individuals bear either long-styled (L-) or

short-styled (S-) flowers (Arroyo *et al.* 2002). Flowers are trimerous and so have a tricarpellar pistil with a trilobate stigma, a stylar canal and an inferior ovary with three locules; the ovules have axial placentation. The androecium consists of six stamens in two unequal whorls with three anthers at each level. The pollen to ovule ratio is around 4,400 (J. Arroyo, unpublished data). Previous hand-pollinations in *N. papyraceus* have shown that this species is self-incompatible but that crosses either between or within morphs are fertile, and, in addition, that prior self-pollination reduces seed set in chase cross-pollinations (i.e. ovule-discounting takes place; Arroyo *et al.* 2002, Santos-Gally *et al.* 2013b). The flowers are visited by butterflies, moths, hoverflies and bees (Pérez-Barrales *et al.* 2007, Santos-Gally *et al.* 2013b). Long- and short-styled morphs are present in populations of *N. papyraceus* on both sides of the Strait of Gibraltar. However, the short-styled morph gradually disappears towards the northern and the southern limits of its distribution, and populations in the northern part of its range (southwest Spain) are monomorphic for the long-styled morph (Arroyo *et al.* 2002, Santos-Gally *et al.* 2013b).

Experimental pollinations were carried out from 22 February to 2 March 2011 in a single natural dimorphic population near Ronda (Málaga province, S Spain; 36º40' N, 5º13' W, 962 m a.s.l.). Each day during that period, 10 young individuals of each morph were selected and three flowers per individual were emasculated prior to anther dehiscence. The following day, we pollinated these flowers with self- or intra-morph or inter-morph pollen. For inter- and intra-morph pollinations, a single pollen donor was selected at random, located at least 10 m from the maternal parent to minimize biparental inbreeding. After the hand pollination treatment, plants were bagged to prevent further pollination by insects. Pistils were harvested at 20 and 30 h and thereafter daily for 2–7 days post-pollination; pistils were fixed in a solution of 2.5% glutaraldehyde in a phosphate buffer. One day after fixing, samples were

washed with a 0.03 M phosphate buffer and dehydrated in a 30–50–70% ethanol series and stored in the latter until further use.

Histochemical and microscope preparation

We used three kinds of preparations to study the kinetics and performance of pollen tubes under each pollination treatment. First, we monitored pollen performance in squash preparations of pistils for each harvesting time, pollination treatment and floral morph. Pistils were softened in 5% sodium sulphite for 20 hours and styles separated from the ovaries. Then, the styles were boiled in 5% sodium sulphite for 20 sec (Martin 1959, Jefferies & Belcher 1974) and the ovules were removed from the ovary. Styles and ovules were mounted separately on microscope slides, stained with 0.1% aniline blue in 0.1 N K_3PO_4 (Martin 1959) and lightly squashed by applying a cover-slip. Preparations were examined under a Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany) equipped with UV epifluorescence with a band pass 340–380 exciter filter and an LP 425 barrier filter for the detection of callose. We observed a total of 122 pistils under fluorescent microscopy (Table S1). However, we ruled out for further study a number of samples that had immature stigmas (see Results section). Also, although it was impossible to quantify the total number of pollen grains on the stigma, samples with less than fifty germinated pollen grains were discarded from further screening, being considered as inadequately hand-pollinated. Thus, in all we studied 78 pistils as squash preparations (Table S1).

In the style, we measured the pollen tube length as the percentage of the style length reached by the longest pollen tube, and also recorded the number of pollen

tubes at the base of the style. With the extracted ovules, we counted: (i) total number of ovules, (ii) penetrated ovules, (iii) total number of degenerated ovules as shown by callose accumulations, callosed cell walls or visibly plasmolized contents, and (iv) penetrated-degenerated ovules.

In a second series of observations we embedded ovaries in paraffin for histological examination, a technique that permitted a more detailed view of the pollen tube growth inside the ovules and the occurrence of fertilization. Since pollen tubes of both floral morphs had exhibited the same behaviour up to this stage (see Results section), we only examined S-morph ovaries in the paraffin sections. As well, given that pollen tubes were observed to penetrate the ovules from the third day onwards (see Results section), we examined with paraffin sections samples harvested 3–7 days after hand pollination for each of the three different pollination treatments. We sequentially dehydrated ovaries with Tertiary Butyl Alcohol (TBA 85%, 95%, 100% and absolute) and then gradually included them in pure paraffin over 10 days. Ovaries were cut longitudinally in 10 μm sections with a rotary microtome Leica Multicut 2045 (Leica Microsystems, Wetzlar, Germany) and sections were mounted on microscope slides with Histomount (CellPath, Hemel, UK). Preparations were deparaffined and hydrated with three sequential baths in Histoclear II (CellPath, Hemel, UK), followed by ethanol:histoclear II (1:1), ethanol 100%, 70%, 40% and water, and then stained with 0.1% aniline blue in 0.1 N K_3PO_4. The preparations were observed in an epifluorescence microscope with the light settings described above. Each ovule penetrated by a pollen tube through the exostome was scored for pollen tube penetration as far as (i) the micropyle (the whole aperture through the integuments), (ii) the nucellus and (iii) the embryo sac. We also scored signs of degeneration with plasmolysis and/or callosic accumulations in the embryo sac (iv) and abnormal pollen tube penetration in the ovule (see Results section; v). We

examined a total of 205 ovules penetrated through the exostome out of a total of over 1000 ovules scrutinized in 18 pistils in paraffin preparations (Table S1).

To evaluate whether fertilization had occurred or not, the same paraffin preparations were subjected to a third treatment following the observation of the pollen tube growth. The aniline blue stain was washed out and the microscope slides were stained with Gerlach staining (Safranin, Crystal-Violet and Light-Green; Gerlach 1969) to detect visible synergid cells, whose presence we interpreted as indicating a lack of syngamy, in ovules that had been previously recorded as penetrated by a pollen tube into the embryo sac. We explored a total of 56 ovules from 7 pistils of the three pollination treatments, which were harvested on the sixth and seventh days after pollination to ensure that the developmental status was as advanced as possible (Table S1).

Statistical analysis

We used Generalized Linear Models (GLMs) with a Gaussian distribution of errors to analyze the variation in the data taken in the squash preparations. The total number of ovules was analyzed as a function of floral morph and log transformed to adjust better to the normal distribution. The pollen tube length was not analyzed since the lack of variation and the small sample size in each combination of floral morph, pollination treatment and harvesting time rendered this process untenable. To examine possible differences in the growth rate of pollen tubes among pollination treatments, we analyzed separately for each harvesting day the variation in the number of pollen tubes at the base of the style as a function of pollination treatment by pooling data from both floral morphs. Pollen tubes entered massively into the

ovary from the third day after pollination onwards (see Results section) and so the computed variables (number of pollen tubes at the style base, penetrated ovules, degenerated ovules and penetrated-degenerated ovules) were analyzed as a function of the pollination treatment and floral morph, with data pooled from the third to the seventh day. We used the cosine of the variables number of penetrated ovules and total number of degenerated ovules to adjust better to the normal distribution. Bonferroni *post hoc* analyses were employed to assess differences among the three pollination treatments when this factor had a significant effect on the response variable.

Data from paraffin preparations, i.e. micropyle penetration, nucellus penetration, embryo sac penetration, embryo sac degeneration, ovules with equivocal pollen tube penetration and presence of synergid cells, were analyzed with GLMs with binomial distribution of errors and with the pollination treatment as a single factor. Subsequently, we employed Bonferroni *post hoc* analyses as described above. All the analyses were performed with the package *stats* of the software R (R Core Team 2009).

Results

Immature stigmas had well-defined stigmatic papillae with neither secretion nor pollen adhesion (Figure 1a). S-morph stigmas matured massively by the third day after anthesis, by which time a secretion had covered the shapeless papillae, thereby allowing pollen adhesion (Figure 1b). This process occurred one day later in L-

morph stigmas (Figure 1c). Exceptionally cold weather on the last days of the experimental pollinations may have provoked the delay in stigma maturation. This may also have caused some pistils to exhibit a somewhat slower sequence of post-pollination events. However, the results for these samples are highly unlikely to affect the observed overall sequence of post-pollination events, particularly since the time sequences for our species were similar to those reported for *Narcissus triandrus* by Sage *et al.* (1999).

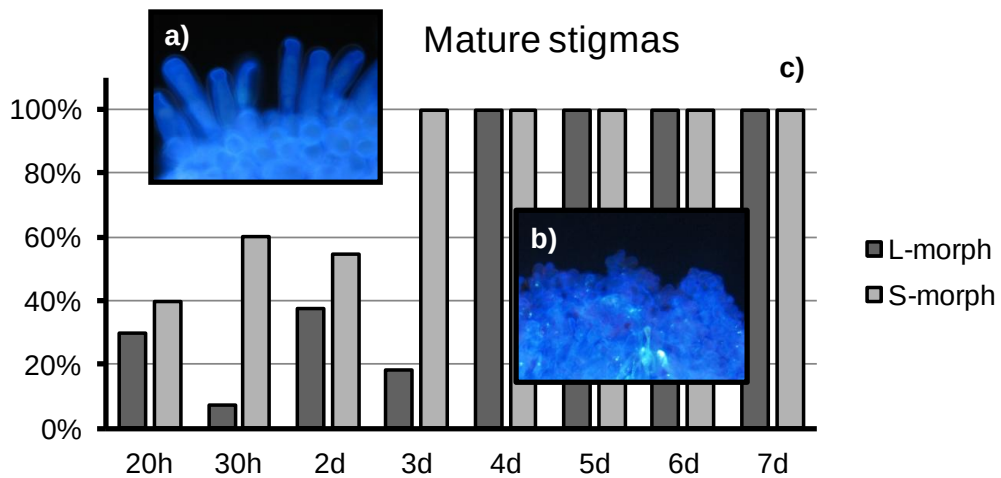

Figure 1. Stigma maturation in the long- (L-) and the short- (S-) styled morphs of *Narcissus papyraceus*. (a) Immature stigmatic papillae. (b) Mature stigmatic papillae. (c) Percentage of examined samples with mature stigmas harvested at different times after anthers dehiscence.

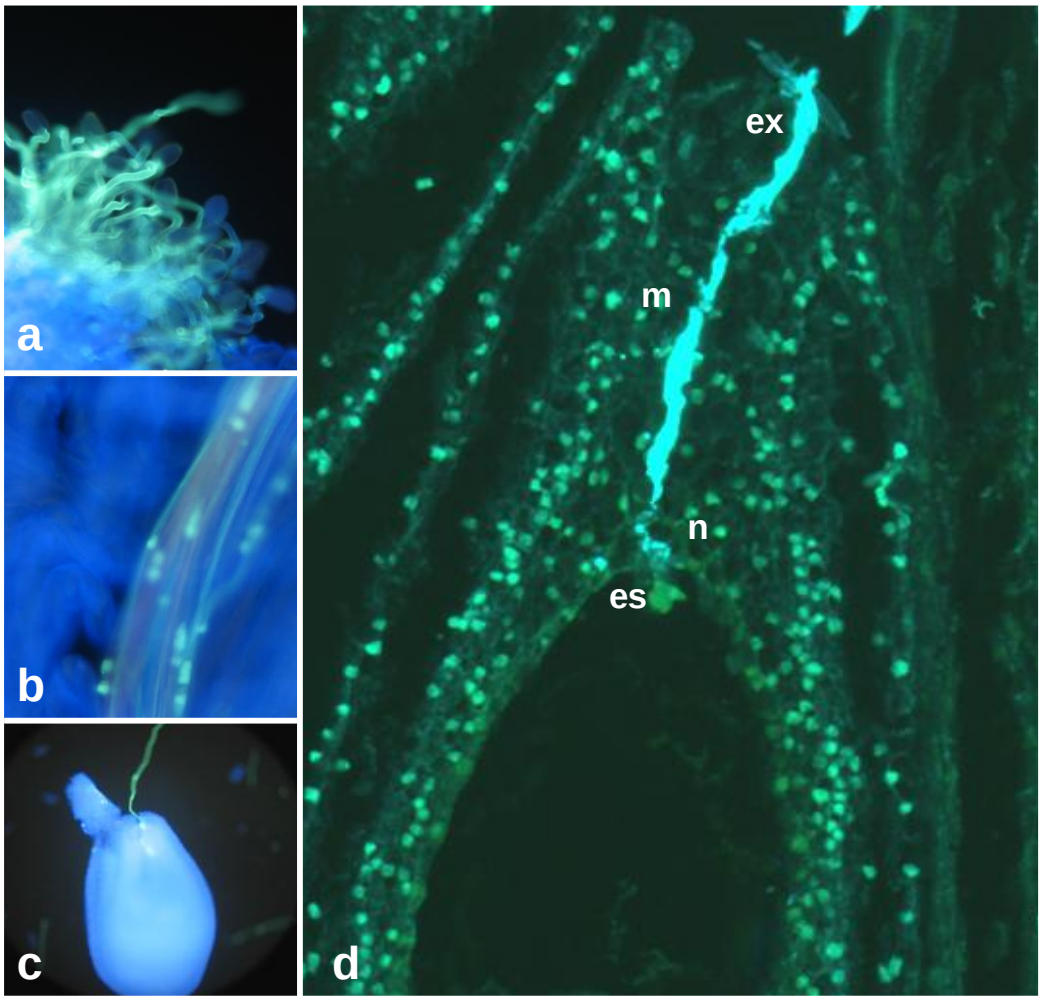

Figure 2. Pollination process in *Narcissus papyraceus*. (a) Pollen grains germinating at the stigma surface. (b) Pollen tubes traversing the stylar canal. (c) Pollen tube entering the ovule through the exostome. (d) Cross pollen tube traversing the micropyle and reaching the embryo sac of a compatible individual. es, embryo sac; ex, exostome; m, micropyle; n, nucellus.

Table 1. Results of the Generalized Linear Models used to test the effect of pollination treatment, floral morph and their interaction over four variables scanned in squash preparations. (i, ii) Variables for the advance of the pollen tube. (iii, iv) Variables for the status of the ovules. Degrees of freedom (d.f.) and *F*-values for each variable and factor are given. Significance: **, $P < 0.01$; *, $P < 0.05$; *ns*, non significant.

	d.f.	(i) Pollen tubes at the style base	(ii) Penetrated ovules	(iii) Degenerated ovules	(iv) Penetrated-degenerated ovules
Floral morph (M)	1	1.42 *ns*	4.12 *	0.21 *ns*	1.5 *ns*
Pollination treatment (P)	2	1.51 *ns*	0.38 *ns*	1.14 *ns*	6.54 **
M x P	2	2.21 *ns*	1.32 *ns*	4.96 *	0.04 *ns*
Error	59				

Pollen performance in the style and ovule penetration

Pollen grains germinated massively in mature stigmas 30 hours after pollination without any observable difference among pollination treatments or floral morphs (Figure 2a). Pollen tubes started to reach the base of the S-style earlier (30–48 h) than in the L-style (72 h; Figures 2b and 3). There were no significant differences from the third to the seventh day in the number of pollen tubes at the style base among floral morphs or pollination treatments (overall average ± SD: 21.6 ± 18.7; Table 1). Analysis of the differences in the number of pollen tubes at the style base among pollination treatments separatedly at each harvesting time only gave significantly

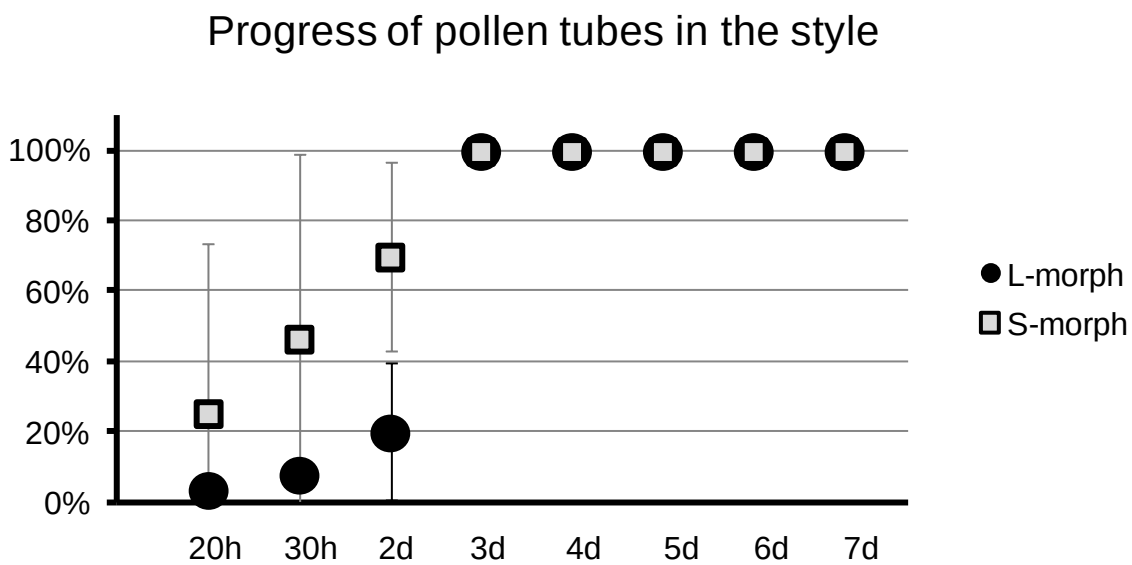

Figure 3. Pollen tube growth in the style of the long- (L-) and the short- (S-) styled morphs of *Narcissus papyraceus*. Pollen tube length is expressed as the percentage of the style length.

different results for the seventh day, when the number of pollen tubes for intra-morph pollinations was significantly higher than for inter-morph pollinations (N = 17, d.f. = 2, $F = 6.3$, $P = 0.01$; Bonferroni *post hoc*). There were no differences in samples for other harvesting times (N = 4–13, d.f. = 1–2, $F > 3.1$, $P > 0.11$; GLM). Pollen tubes penetrated ovules from the third day onwards (Figure 2c) without differences among pollination treatments (Table 1). A marginally significant difference was found for the effect of floral morph on the number of penetrated ovules (Table 1) and in this case ovule penetration was lower in the L-morph than in the S-morph (averages ± SD: L-morph, 10.7 ± 10.3; S-morph, 19.6 ± 16.1).

In the paraffin preparations (Figure 2d), 83% of ovules penetrated through the exostome were penetrated as far as the micropyle without differences among any of the three pollination treatments (Table 2a). However, the pollination treatment did affect nucellus and embryo sac penetrations (Figure 4a and Table 2a). Self-pollen tubes penetrated the nucellus in 39% of ovules and the embryo sac in 24% of ovules. For inter-morph pollinations, we observed pollen tubes within the nucellus and embryo sac in 66% and 61% of ovules, respectively, while in intra-morph crosses pollen tubes entered the nucellus and the embryo sac in 87% and 79% of ovules, respectively. The results of the Bonferroni *post hoc* analysis gave significant differences between the self-pollination treatment and both cross-pollination treatments for both variables ($P < 0.01$), but not between inter- and intra-morph pollinations ($P > 0.07$). The pollination treatment also had a significant effect on the proportion of ovules with abnormal pollen tube penetration (Table 2a), that is, the pollen tube penetrated the ovule via an anomalous path rather than through the micropyle (Figures 4b and 4c). In our samples, 11% of self-pollen tubes, 0% of intra-morph and 6% of inter-

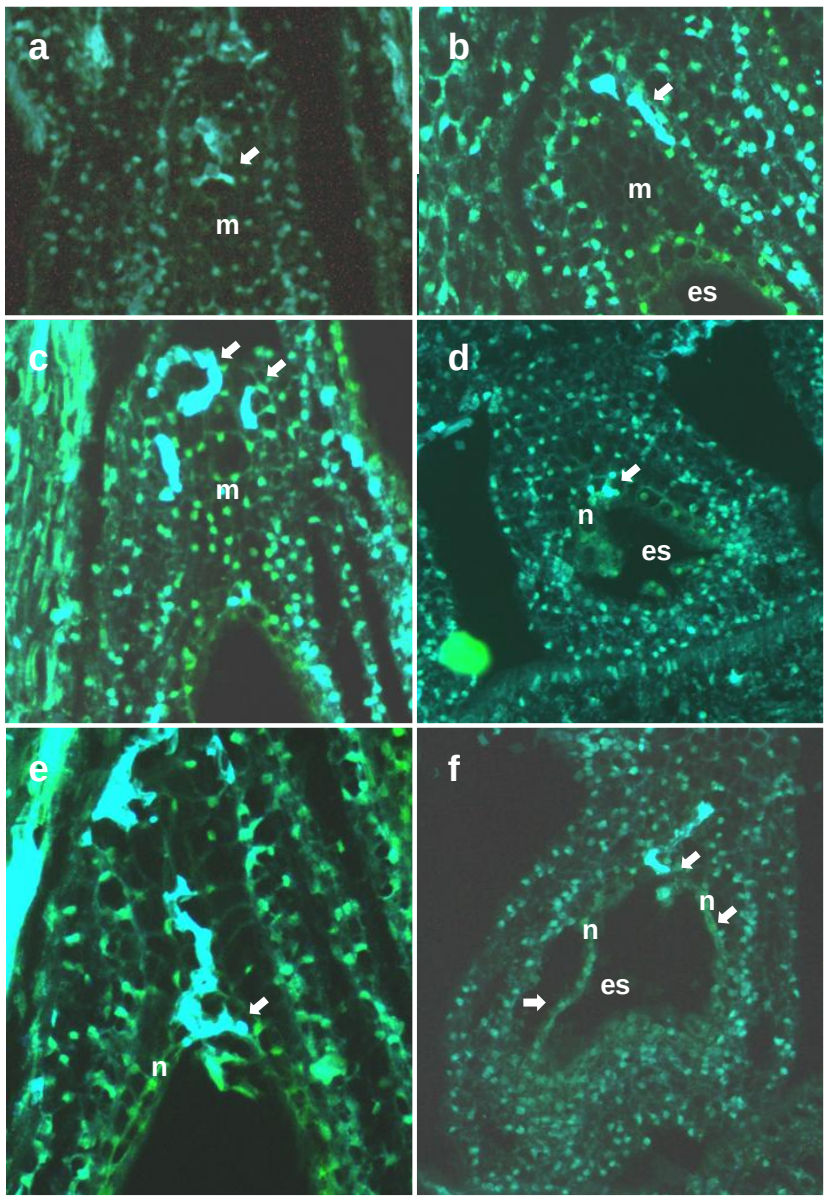

Figure 4. Selfed ovules in *Narcissus papyraceus*. (a) Collapsed pollen tube in the micropyle. (b,c) Aberrant pollen tubes penetrating the ovule through the integuments. (d) Callose accumulation in synergid cells. (e,f) Pollen tube reaching a degenerated embryo sac with callosic or plasmolyzed walls. es, embryo sac; m, micropyle; n, nucellus.

morph pollen tubes had anomalous ovule penetrations. However, the results of the Bonferroni *post hoc* did not reveal any significant difference among the three treatments ($P > 0.06$).

Table 2. Results of the Generalized Linear Models used to test the effect of pollination treatment over seven variables scanned in paraffin preparations. a) Variables scanned with aniline blue staining of ovules penetrated by pollen tubes through the exostome. b) Variable scanned with Gerlach staining of ovules penetrated by pollen tubes as far as the embryo sac. Degrees of freedom (d.f.) and *F*-values for each variable are given. Significance: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; *ns*, non significant.

	d.f./ d.f. error	Variable	*F*-value
a)	2 / 204	Micropyle penetration	1.47 *ns*
		Nucellus penetration	15.29 ***
		Embryo sac penetration	22.32 ***
		Abnormal pollen tube penetration	4.03 *
		Embryo sac degeneration	25.35 ***
b)	2 / 55	Synergid cells	5.67 **

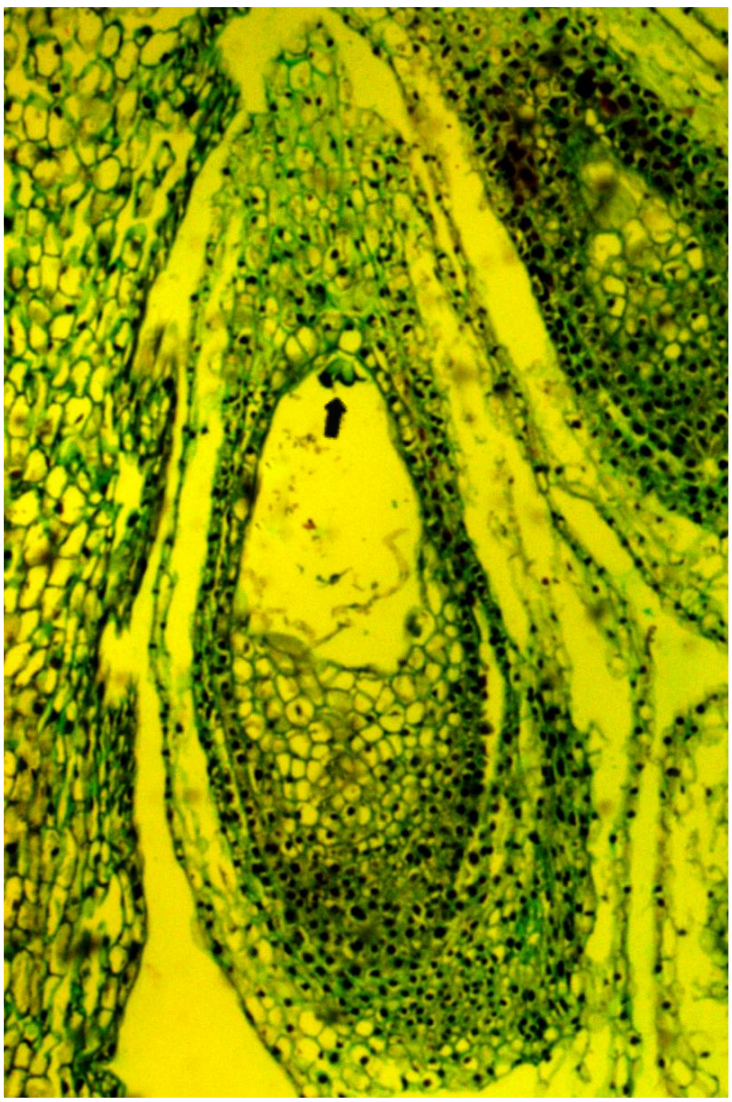

Figure 5. Self-pollinated ovule of *Narcissus papyraceus* penetrated by a pollen tube until the embryo sac that has not undergone syngamy and hence has visible synergid cells, seven days after pollination.

Ovule response

The ovaries in *Narcissus papyraceus* had an average (± SD) of 61 ± 11 ovules in both floral morphs (N = 78, d.f. = 1, $F = 0.34$, $P = 0.56$). The interaction of the factors floral morph and pollination treatment had a significant effect on the number of

degenerated ovules observed in the squash preparations (Table 1). Overall, the number (average ± SD) of degenerated ovules was higher in the L-morph (19.7 ± 15.6) than in the S-morph (11.8 ± 8.7); it was greatest after self-pollinations (20.1 ± 12.8), had an intermediate value for intra-morph pollinations (15.3 ± 15.1) and reached its lowest values after inter-morph pollinations (10.7 ± 10.6). The results of the Bonferroni test showed significant differences between self- and inter-morph pollination treatments ($P = 0.04$). The pollination treatment had a significant effect on degeneration in penetrated ovules (Table 1). The number (average ± SD) of penetrated ovules with visible signs of degeneration in squash preparations was higher in self-pollinated pistils (5.52 ± 6.21) than in intra-morph (1.62 ± 3.04) or inter-morph pollinations (1.11 ± 1.73). In this case, the Bonferroni test found significant differences between self-pollination and both cross-pollination treatments ($P < 0.04$). In the paraffin samples, the effect of pollination treatment on the degeneration of penetrated ovules was also highly significant (Table 2a): 31% of self-pollinated ovules but no cross-pollinated ovules showed signs of degeneration (Figures 4d, 4e and 4f). Hence, the results of the Bonferroni *post hoc* test showed significant differences between self-pollination and both intra- and inter-morph pollination treatments ($P < 0.01$).

The pollination treatment affected significantly the number of penetrated ovules that had distinct synergid cells (Table 2b). Up to 77% of selfed ovules had visible synergid cells, which reflects the lack of penetration into the female gametophyte and hence a lack of fertilization (Figure 5). By contrast, both synergid cells were only visibly intact in 53% and 23% of the ovules penetrated by intra- and inter-morph pollen tubes, respectively. The Bonferroni test results revealed a significant difference between self- and inter-morph pollination treatments ($P < 0.01$).

Discussion

In this study we demonstrated that the breeding system of *Narcissus papyraceus* involves a mechanism of homomorphic ovarian self-incompatibility that resembles that reported in two other *Narcissus* species, *N. tazetta* (Dulberger 1964) and *N. triandrus* (Sage *et al.* 1999). Ovarian self-incompatibility in *N. papyraceus* appears to be due to a pre-zygotic rejection reaction caused by the presence of self-pollen tubes in the pistil, as reported by Sage *et al.* (1999) in the most detailed study to date of this genus. In addition, we found evidence of greater protandry in the L-morph than in the S-morph, as occurs in *N. assoanus* (Cesaro *et al.* 2004). Given the close positioning of the L-stigma to the anthers, marked protandry would help prevent high self-pollination rates.

Pollen-pistil interaction is mediated through a series of signals from the stigma to the ovary (McCubbin & Kao 1996, Gaude & McCormick 1999, Herrero 2000, 2001, Qin *et al.* 2009); furthermore, it is known that the growth of pollen tubes in the style can stimulate changes within the ovary (Tupy 1961, Deurenberg 1976, Herrero & Dickinson 1979, Hormaza & Herrero 1992, O'Neill 1997). Given that we only examined ovules in ovaries that had been penetrated by pollen tubes, we were unable to determine whether the rejection response was induced by long-distance signalling of self-pollen tubes in the style (i.e. Sage *et al.* 1999) or in the ovary. Nevertheless, it is obvious that a generalized collapse involving a high proportion of ovules and pollen tubes occurs in the ovary of selfed pistils. Ovules degenerated and pollen tubes stopped prior to nucellus penetration in a greater proportion of selfed than outcrossed pistils. The degeneration of non-penetrated ovules after self-pollination may be a reflection of the strength of the chemical signalling and so

histochemical studies may provide further valuable insights into the mechanisms of ovarian self-incompatibility in this genus (Sage *et al.* 2006). At this point, we have shown that callose accumulations in the ovule integuments and embryo sacs appear as a response to incompatible pollination, as reported by Knox (1984) and Bell (1995). Thus, we report here a further example of what has been considered by some authors as an inefficient self-incompatibility system with a misleading evolutionary meaning (Lewis 1979, Wyatt & Broyles 1994), but that may in fact be surprisingly common in nature (Seavey & Bawa 1986, Gibbs & Bianchi 1999).

Previous studies on polymorphic *Narcissus* species have shown that intra-morph pollinations are compatible (Dulberger 1964, Barrett *et al.* 1997, Baker *et al.* 2000, Arroyo *et al.* 2002, Pérez-Barrales *et al.* 2006, Santos-Gally *et al.* 2013b). In the present study, intra- and inter-morph pollination treatments in *N. papyraceus* did not differ overall in the observed growth rates of pollen tubes in the style or in the rates of ovule penetration. Mixed pollinations and paternity analyses would be necessary to rule out definitely the existence of cryptic heteromorphic incompatibility or interaction effects between pollen tubes from different sources (Jones 1994, Snow 1994, Eckert & Allen 1997, Wu *et al.* 2010). However, the greater rate of embryo sac penetration after intra- than after inter-morph pollinations but the more frequent occurrence of syngamy after inter- than after intra-morph pollinations found in our study point to a total equivalence of floral morphs in breeding. The general higher incidence of ovule penetrations in the S- morph than in the L-morph samples found in our squash preparations are not transformed into different fecundities according to other studies of this species (Arroyo *et al.* 2002, Pérez-Barrales & Arroyo 2010, Chapter 6).

The frequency of occurrence of the floral morphs in any population will depend on the prevailing mating patterns. Two factors contribute to mating success: pollination patterns and physiological compatibility. Differences in the incompatibility system between floral morphs could seriously influence morph ratios in some style-polymorphic species (Pailler & Thompson 1997, Hodgins & Barrett 2006, Brys *et al.* 2008). However, we show here that no differences exist in the strength of inter-compatibility within and between morphs, and that all cross pollinations are equally fertile in *N. papyraceus*. This situation has two important implications for the floral morph ratio in this species. Firstly, we can discard the possibility that a breeding disadvantage in the S-morph explains its lower frequency or its loss in populations. Secondly, we can assume that pollination patterns in populations of *N. papyraceus* translate directly into mating success and hence are the main determinants of floral morph ratios in populations in the absence of any stochastic processes in population formation and dynamics (Chapter 4).

The occurrence of homomorphic late-acting self-incompatibility in style-polymorphic species has only been recognized to date in the genus *Narcissus* (Dulberger 1964, Sage *et al.* 1999) and in *Anchusa officinalis* (Philipp & Schou 1981, Schou & Philipp 1983), although it may also occur in other Boraginaceae (see Ferrero *et al.* 2012). An ovarian rejection site has also been reported in some heterostylous species with heteromorphic incompatibility (Bawa & Beach 1983, Anderson & Barrett 1986, Scribailo & Barrett 1991). However, two differences exist between heteromorphic and homomorphic incompatibility. Firstly, while in heterostylous species with heteromorphic self-incompatibility ovarian rejection occurs only in one of the morphs (Bawa & Beach 1983), in species with homomorphic incompatibility (*N. tazetta*, *N. triandrus* and *Anchusa officinalis*) the rejection response always occurs in the ovary, as we found in the present study. Secondly, although much more study is

still needed of the genetics of ovarian self-incompatibility, heteromorphic incompatibility seems to be determined by a diallelic gene expressed in sporophytic tissues (Ganders 1979, Barrett & Cruzan 1994), while a multiallelic gene expressed in gametophytic tissues or a polygenic control might act in homomorphic ovarian self-incompatibility (Seavey & Bawa 1986).

The marked differences between heteromorphic and homomorphic late-acting self-incompatibility enhance the uniqueness of the breeding system of style-polymorphic species of *Narcissus*. In addition, the absence of ancillary polymorphic traits in these species (Barrett *et al.* 1997, Arroyo *et al.* 2002) reinforces the rarity of this combination. It is accepted that stylar polymorphism and heteromorphic incompatibility have evolved repeatedly and independently in different heterostylous clades (Lloyd & Webb 1992). Hence, the different breeding system in polymorphic *Narcissus* may be a sign of the variety in the convergent evolutionary mechanisms that are involved in the appearance of stylar polymorphism. Nevertheless, the drivers for these patterns still need to be unravelled.

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Table S1. Number of samples of each morph, pollination treatment and harvesting time examined in the present study. (a) Number of mature and immature (in parenthesis) pistils examined in squash preparations. (b) Number of penetrated ovules examined in paraffin preparations stained with aniline blue and Gerlach (in parenthesis).

a)

	L-morph			S-morph		
	inter	intra	self	inter	intra	self
20h	0 (2)	0 (1)	3 (4)	2 (1)	0 (1)	0 (1)
30h	0 (4)	0 (4)	1 (5)	2 (1)	0 (1)	1
2d	0 (1)	0 (1)	3 (3)	1 (1)	2 (2)	3 (2)
3d	0 (2)	1 (3)	1 (4)	2	2	2
4d	3	2	3	1	1	2
5d	2	3	3	2	1	2
6d	1	2	2	2	1	2
7d	4	4	2	3	1	3

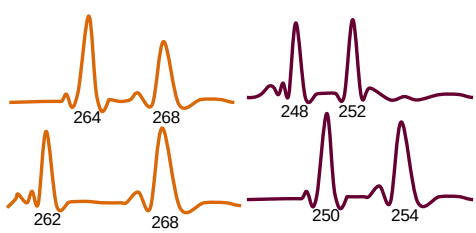
b)

	S-morph		
	inter	intra	self
3d	11	0	0
4d	2	11	24
5d	11	5	9
6d	44 (25)	7 (4)	30 (5)
7d	2 (1)	15 (13)	34 (8)

CAPÍTULO 3

NUEVOS MARCADORES MICROSATÉLITES PARA *Narcissus papyraceus* (AMARYLLIDACEAE) Y AMPLIFICACIÓN EN OTRAS ESPECIES CONGÉNERES

NEW MICROSATELLITE LOCI FOR *Narcissus papyraceus* (AMARYLLIDACEAE) AND CROSS-AMPLIFICATION IN OTHER CONGENERIC SPECIES



V.I. Simón, F.X. Picó & J. Arroyo (2010)
American Journal of Botany 97: e10–e13.

Resumen

Se optimizaron y caracterizaron loci microsatélites a partir de una librería genómica de la especie polimórfica *Narcissus papyraceus* para estudios de genética poblaciones. Once marcadores que amplificaron exitosamente mostraron polimorfismo al probarlos en 50 individuos de dos poblaciones del sur de España y norte de Marruecos. El número total de alelos por locus varió entre cuatro y 15. Entre siete y 10 loci amplificaron exitosamente en otras ocho especies de *Narcissus*. Estos marcadores posibilitarán estudios de diversidad genética en *N. papyraceus* a lo largo de su área de distribución y la realización de análisis de paternidad entre individuos de distinta morfología floral.

Palabras clave: Amaryllidaceae, amplificación cruzada, microsatélite, *Narcissus papyraceus*.

Abstract

Microsatellite loci from a genomic library of polymorphic species *Narcissus papyraceus* were optimized and characterized for studies of population genetics. Eleven markers that were successfully amplified showed polymorphism when tested on 50 individuals from two populations in South Spain and North Morocco. Overall the number of alleles per locus ranged between four and 15. Between seven and 10 loci successfully amplified in other eight *Narcissus* species. These markers will enable for

genetic diversity studies of *N. papyraceus* across its distribution range and conduct paternity analyses among individuals differing in flower morphology.

Keywords: Amaryllidaceae, cross-amplification, microsatellite, *Narcissus papyraceus*.

Introduction

The genus *Narcissus* (Amaryllidaceae) is well known for its morphological diversity due to the vast amount of within- and among-species floral variation affecting both perianth and sex organs. Both polymorphisms make the genus a good model system to study floral evolution and stylar polymorphism (Barrett *et al.* 1996, Pérez-Barrales *et al.* 2006). The genus is circunmediterranean and the highest diversity is found in the Iberian Peninsula and Northwestern Africa. One of the most common species occurring in the core of the center of diversity of the genus (South Spain and North Morocco) is *Narcissus papyraceus* Ker-Gawler. The species is self-incompatible and exhibits stylar dimorphism and a differential geographic distribution of style-length morphs (Arroyo *et al.* 2002) and perianth traits (Pérez-Barrales *et al.* 2007, 2009). Here we characterize 11 new polymorphic microsatellite loci for *N. papyraceus* and their cross-amplification in other eight *Narcissus* species collected in the field. These markers will be used to assess the genetic differentiation and structure across the species' distribution range, and conduct paternity analyses to investigate the ecological and genetic mechanisms underlying variation in stylar polymorphism.

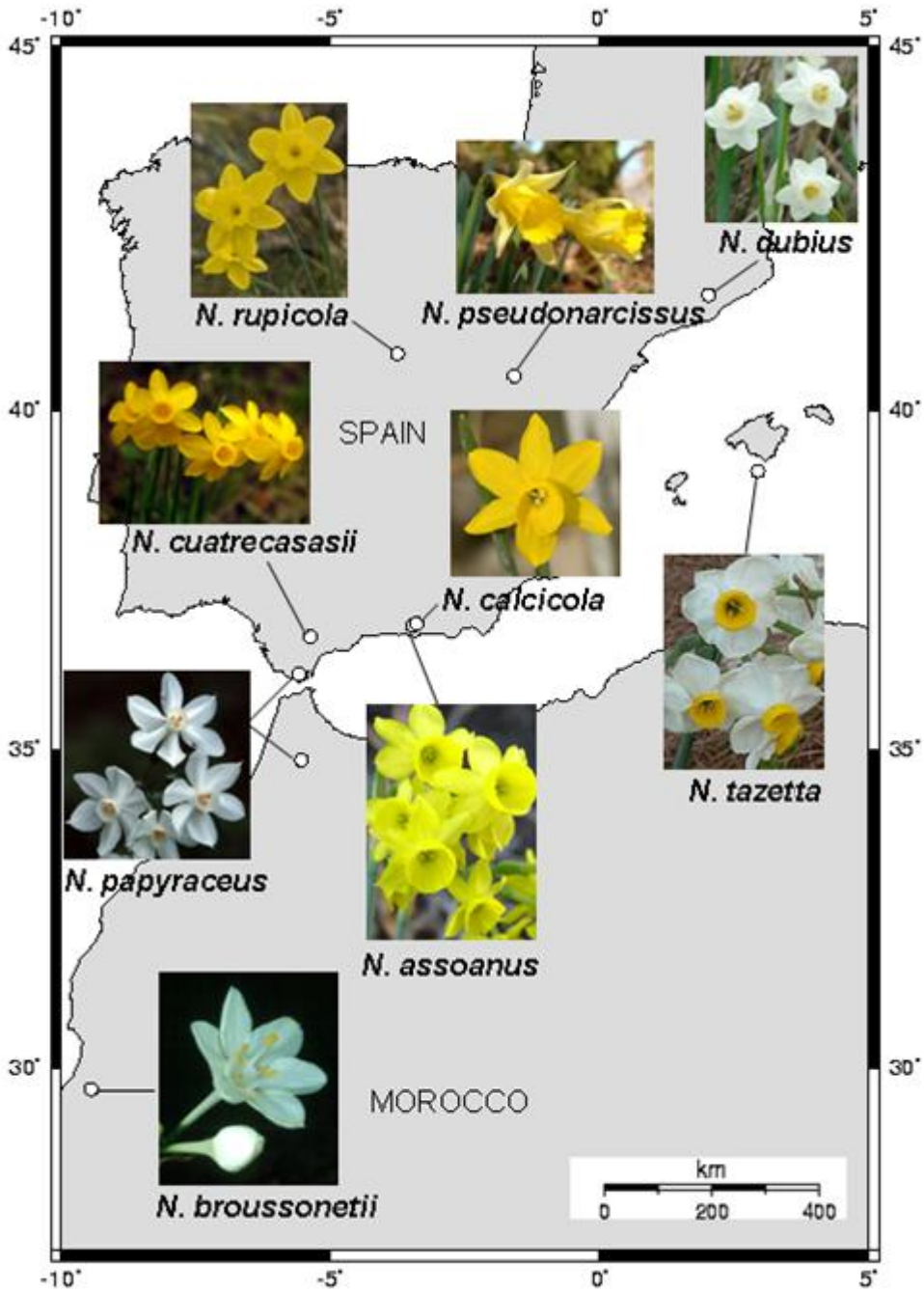


Figure 1. Geographic location of sampling populations for *Narcissus papyraceus* and the other congeneric species of study in Spain and Morocco.

Table 1. Characteristics of 11 microsatellite loci of *Narcissus papyraceus*. GenBank accession numbers (below loci names), repeat motifs, forward (F) and reverse (R) primer sequences, allele size ranges and optimal annealing temperatures (T_a) are given.

Locus (GenBank accession)	Repeat motif	Primer sequence (5' to 3')	Product size (bp)	T_a[1]
A5 (GU271106)	AC_{23}	F-CCACGATTCCAATATGAATTTG R-TATGCACACCTGGTATGTCAAG	238–298	58
A109 (GU271107)	$TA_{10}CA_{14}$	F-GATTGTCAACAAGCATGATATG R-ATGTCGAGTGGATATGGTTATG	100–132	57
A116 (GU271108)	CA_{26}	F-GCCATGTTTTATGCCTGA R-ATCCTCACCGGAATCAAC	262–316	58
A121 (GU271109)	GT_{27}	F-GGGAGGACCCTAAATCAAGTA R-GCCTAATAAAGCTGCTATCCC	156–202	58
A131 (GU271110)	GT_{11}	F-AGCTCTCTGTGTGTGTTTCAC R-GGTGACCGTGTCAATTACAC	119–129	58
A134 (GU271111)	GT_{22}	F-ACCTCGCTTATGGGTGAG R-ATTTGATACTCGTGGATGGATA	276–306	58
B7 (GU271112)	GA_{15}	F-AACCGTTGTCTCCTCTCTATG R-TTCTCCCTCTCTCTTCATTTC	136–184	57
B104 (GU271113)	GA_{16}	F-CTGCTACACCATTAGAGACACC R-ACATCCACTGGTAACAAATCTG	156–176	59
B109 (GU271114)	TC_{10}	F-TTCCAACAAGATAACGAACCT R-AAACCGAACCTACACTAAGAGG	179–191	58
B112 (GU271115)	TC_{18}	F-CCATTCGACCACACCTACCT R-CCAAGCTCCAAATCTTCGTC	286–332	59
B131 (GU271116)	GA_{24}	F-AAACCCACCTTCAAACGA R-TGGAAACTTGTGCCCATAC	162–186	59

Notes: [1] Temperature of annealing (T_a) is given for non-labeled primers.

Methods and Results

Microsatellite libraries were developed following Jones *et al.* (2002) by Genetic Identification Services (www.genetic-id-services.com). Extracted DNA (approximately 100 μg) was digested with different standard restriction enzymes. Fragments were ligated and cloned into an *E. coli* strain. After incubation, a total of 100 randomly-chosen recombinant clones were selected, purified and sequenced. Finally, primer pairs were designed for all 100 clones. After visual inspection of the sequences, we selected a total of 29 primer pairs. Overall fifty individuals from two populations were employed to test the amplification and polymorphism of primer pairs. Populations were located in South Spain (Cádiz; 36°8' N, 5°35' W; N = 35) and North Morocco (Ouezzane; 34°49' N, 5°32' W; N = 15; Figure 1).

For primer testing, DNA was isolated from silica-dried leaf samples using a previously described protocol (Bernartzky & Tanksley 1986) without mercaptoethanol. Polymerase chain reactions were performed in 25 μl of reaction mixture containing 50 ng/μl of template genomic DNA, 1x PCR buffer, 1.5 mM MgCl, 1 mg/ml BSA, 0.034 μM forward primer, 0.25 μM reverse primer, 0.4 μM forward dye-labelled M13 primer, 0.05 mM each dNTP and 1.25 U Taq polymerase. Samples were incubated in a Touch-Down PCR in a Biometra TGradient Thermal Cycler, with an initial 5 min of denaturation at 94 ºC, 27 cycles at 94 ºC for 30 s, annealing at 67−43 ºC for 30 s (1 ºC decrease in each cycle), and extension at 72 ºC for 30 s, 17 cycles at 94 ºC for 30 s, 53 ºC for 30 s, 72 ºC for 30 s, and a final extension at 72 ºC for 10 min. Polymerase chain reaction products were analyzed on an ABI 3130×1 Genetic Analyzer and sized using GeneMapper v.4.0 (Applied Biosystems) and LIZ 500 size standard. Cross-amplification was conducted on two

field-collected samples of eight *Narcissus* species (Figure 1) following the same protocol described above.

Table 2. Results of initial primer screening in two populations of *Narcissus papyraceus*. Sample size (N), number of alleles (n_a), observed heterozygosity (H_o), genetic diversity (H_s) and *P*-values for the Hardy-Weinberg equilibrium (HWE) test are given for each marker and population.

Locus	Cádiz (Spain)					Ouezzane (Morocco)				
	N	n_a	*Ho*	*Hs*	HWE	N	n_a	*Ho*	*Hs*	HWE
A5	30	15	0.400	0.878	0.000†	15	14	0.933	0.882	0.378
A109	10	4	0.200	0.575	0.034†	14	12	0.214	0.883	0.000†
A116	26	15	0.385	0.885	0.000†	11	7	0.091	0.806	0.000†
A121	30	10	0.833	0.839	0.752	15	9	0.667	0.716	0.016†
A131	31	4	0.161	0.506	0.003†	15	5	0.067	0.598	0.000†
A134	34	13	0.912	0.731	0.466	15	7	0.733	0.591	0.031†
B7	21	12	0.190	0.885	0.000†	11	7	0.273	0.570	0.002†
B104	27	10	0.667	0.869	0.226	15	8	0.333	0.824	0.002†
B109	15	4	0.400	0.436	0.013†	12	5	0.333	0.646	0.004†
B112	26	10	0.308	0.482	0.000†	12	9	0.250	0.854	0.002†
B131	31	8	0.710	0.724	0.000†	15	8	0.600	0.704	0.182

Notes: †Significant departure from HWE ($P < 0.05$).

After excluding those that did not amplify or were monomorphic, we selected 11 primer pairs that showed polymorphism (Table 1). The number of observed alleles per locus (n_a), observed heterozygosity (H_o), genetic diversity (H_s) and tests for Hardy-Weinberg equilibrium (HWE) were calculated using GENALEX

v.6.3 software (Peakall & Smouse 2006). Tests for linkage disequilibrium were performed using FSTAT v.2.9.3 software (Goudet 1995).

The mean number of alleles per locus was 9.545 (range: 4–15) and 8.273 (range: 5–14) for the Spanish and Moroccan population, respectively (Table 2). On average, observed heterozygosity was 0.470 (range: 0.161–0.912) and 0.409 (range: 0.067–0.933) for the Spanish and Moroccan population, respectively (Table 2). Mean genetic diversity was 0.710 (range: 0.436–0.885) and 0.734 (range: 0.570–0.883) for the Spanish and Moroccan population, respectively (Table 2). A total of eight and nine microsatellite loci significantly departed from Hardy-Weinberg equilibrium for the Spanish and Moroccan population, respectively (Table 2). The species' self-incompatible breeding system accounts for this result. None of the 11 microsatellite loci exhibited significant linkage disequilibrium ($P > 0.002$ in all cases; nominal level = 0.000909). Between seven and 10 microsatellite loci also amplified in the other *Narcissus* species (Table 3).

Table 3. Amplification of 12 microsatellite loci in eight *Narcissus* species. Plus and minus signs mean successful and unsuccessful amplifications, respectively.

Locus	Species[1]							
	N. pseudonarcissus ssp. *eugeniae*	*N. tazetta*	*N. assoanus*	*N. rupicola*	*N. cuatrecasasii*	*N. dubius*	*N. broussonetii*	*N. calcicola*[3]
A5	+	+	+	+	+	+	+	+
A109	+	-	-	-	-	+	+	-
A116	-	+	-	-	-	+	+	-
A121	+	+	+	+	+	+	+	+
A131	+	+	+	+	+	+	+	+
A134	+	+	+	+	+	+	+	+
A136[2]	+	-	+	+	+	+	+	+
B7	+	+	+	+	+	+	+	+
B104	+	+	+	+	+	+	+	+
B109	+	-	-	-	+	-	-	+
B112	+	+	+	-	+	+	+	+
B131	-	+	-	+	+	+	+	-

Notes: [1]Geographical coordinates of populations: *N. pseudonarcissus* ssp. *eugeniae*: 40º29′ N, 1º35′ W; *N. tazetta*: 39º8′ N, 2º56′ E; *N. assoanus*: 36º51′ N, 3º29′ W; *N. rupicola*: 40º48′ N, 3º46′ W; *N. cuatrecasasii*: 36°41′ N, 5°22′ W; *N. dubius*: 41º37′ N, 2º1′ E; *N. broussonetii*: 29º39′ N, 9º26′ W; *N. calcicola*: 36º53′ N, 3º24′ W. [2]This marker was monomorphic in *N. papyraceus*. Primer pairs: F-ACTTTGAGTTCCGCTTCAG and R-ACACCCTTTATGTTGAGTGC; motif: (CA_{13}) GATATA (CA_9); product size: 182 bp; temperature of annealing: 58 ºC. [3]The population sampled of *N. calcicola* is highly isolated from the core range of the species in central Portugal.

Conclusions

These microsatellites will be adequate for genetic diversity studies across the species' distribution range in the Iberian Peninsula and Northwestern Africa, separated by the Strait of Gibraltar. This region has proved to harbor a high biodiversity and biogeographical significance due to its complex history (Rodríguez-Sánchez *et al.* 2008). This will provide insights into the geographic structure of genetic diversity that reflects the evolutionary history of the species and the palaeogeographical setting of the region. Furthermore, the characteristics of these markers make them suitable to conduct paternity analysis among *N. papyraceus* individuals, which will permit to understand the dynamics of flower morphology evolution. Finally, the successful cross-amplification of these markers can allow study of similar questions on other *Narcissus* species.

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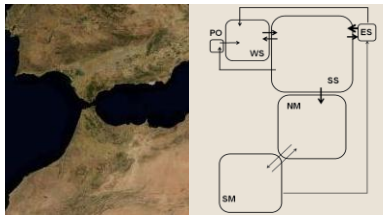
CAPÍTULO 4

GENÉTICA DE POBLACIONES Y VARIACIÓN EN LA PROPORCIÓN DE MORFOS FLORALES EN TODA EL ÁREA DE DISTRIBUCIÓN DE LA ESPECIE DIMÓRFICA ESTILAR *Narcissus papyraceus* (AMARYLLIDACEAE)

RANGE-WIDE POPULATION GENETICS AND VARIATION IN FLORAL MORPH RATIO IN STYLE-DIMORPHIC *Narcissus papyraceus* (AMARYLLIDACEAE)



V.I. Simón-Porcar, F.X. Picó & J. Arroyo.

Unpublished.

Resumen

Los modelos teóricos establecen que la selección natural y los patrones de cruzamiento determinan la proporción de morfos florales en poblaciones de plantas con polimorfismo estilar. No obstante, se ha propuesto que la historia demográfica inferida a partir de datos moleculares también puede explicar la variación en la proporción de morfos florales. En este estudio, empleamos microsatélites nucleares para valorar los patrones de variación genética en poblaciones de *Narcissus papyraceus*, una planta dimórfica estilar cuyas proporciones de morfos florales varían a lo largo de su área de distribución en el SW de la Cuenca Mediterránea. Basándonos en el concepto centro-margen, planteamos la hipótesis de que las poblaciones con mayor variación fenotípica en el centro de la distribución de la especie tendrán también una mayor diversidad genética. Las diversidades fenotípica y genética podrían decrecer conjuntamente hacia los bordes de la distribución de la especie, constreñidas por el efecto de eventos fundadores o cuellos de botella en condiciones ambientales subóptimas. De acuerdo con observaciones previas de las proporciones de morfos florales en la especie, encontramos la mayor frecuencia del morfo de estilo corto en las poblaciones centrales con un patrón decreciente hacia la periferia. El patrón geográfico en la proporción de morfos florales de las poblaciones no estuvo relacionado con su estructura genética. Las tasas de migración fueron relativamente altas, lo cual explicó la débil estructura genética geográfica de las poblaciones de *N. papyraceus* estudiadas. La diversidad genética y el tamaño efectivo poblacional fueron mayores en las poblaciones centrales isopléticas que en las poblaciones monomórficas del norte del área. Los menores tamaños efectivos poblacionales en el límite norte del área de distribución de la especie pueden atribuirse a una mayor

deriva genética como consecuencia de efectos fundadores o cuellos de botella, y podrían ser responsables de la pérdida de polimorfismo en estas poblaciones septentrionales. La combinación de datos moleculares y ecológicos, como correlaciones climáticas y el conocimiento sobre la biología de la polinización de la especie, proporciona la clave para una mejor comprensión de la ecología evolutiva de las plantas dimórficas estilares.

Palabras clave: diversidad genética, estructura genética, heterostilia, patrón centro-margen, tamaño efectivo poblacional, tasas de migración.

Abstract

Theoretical models state that natural selection and mating patterns account for floral morph ratio in style-polymorphic plant populations. However, it has been proposed that demographic history inferred from molecular data can also explain variation in floral morph ratio. In this study, we used nuclear microsatellites to assess genetic patterns of variation in populations of *Narcissus papyraceus*, a style-dimorphic plant whose floral morph ratios vary throughout its distribution range in the SW Mediterranean Basin. Based on the central-marginal concept we hypothesized that populations exhibiting higher phenotypic variation in the species' core distribution should also have greater genetic diversity. Phenotypic and genetic diversity should both decline towards the edges of the species' range, constrained by the effect of

founder events or bottlenecks in sub-optimal environmental conditions. According to previous observations of floral morph ratios in this species, we found greater short-styled morph frequency in central populations with a declining pattern towards periphery. The geographic pattern in floral morph ratios in populations was not related to their genetic structure. Migration rates were relatively high, accounting for the overall weak geographical genetic structure of the studied *N. papyraceus* populations. Genetic diversity and effective population size were greater in central isoplethic than in northern monomorphic populations. The reduced effective population sizes at the northern edge of the plant's range can be attributed to greater genetic drift as a consequence of either founder events or bottlenecks, and might be responsible for the loss of polymorphism in these northern populations. Furthermore, the combination of molecular and ecological data, such as climatic correlates and knowledge of the species' pollination biology provides the key to a better understanding of the evolutionary ecology of style-dimorphic plants.

Keywords: central-marginal pattern, effective population size, genetic diversity, genetic structure, heterostyly, migration rates.

Introduction

Heterostylous and style-length morphs are phenotypic variants that differ in the position of their sex organs within the flower (Lewis & Jones 1992, Barrett 2002) and whose functional genetic basis has only just begun to be studied in detail (Labonne *et al.* 2008, Yoshida *et al.* 2011, Ushijima *et al.* 2012). Evolutionary models state that natural selection maintains heterostyly and stylar dimorphism in populations through enhanced disassortative mating (Lloyd & Webb 1992a, 1992b), a mechanism by which in flowers inter-morph mating is more frequent than intra-morph mating (Baker *et al.* 2000a, Barrett *et al.* 2000, Thompson *et al.* 2003, Cesaro & Thompson 2004). Disassortative mating is driven by pollen transfer in differentiated parts of the pollinator's bodies, as well as by a heteromorphic incompatibility system (Barrett & Shore 2008) that only allows for cross-fecundation between floral morphs. Theoretically, populations in equilibrium are expected to have an even proportion of floral morphs (i.e. isoplethy) when mating is completely disassortative. However, the heteromorphic incompatibility system is not always present and crosses between individuals with the same floral morph are thus permitted (Dulberger 1964, Philipp & Schou 1981, Barrett *et al.* 1997, Baker *et al.* 2000b, Arroyo *et al.* 2002, Pérez-Barrales *et al.* 2006, Brys *et al.* 2008, Ferrero *et al.* 2012). In this case, an increase in assortative mating in one of the floral morphs can lead to biased floral morph ratios and, eventually, to the loss of polymorphism in the population (Cesaro & Thompson 2004, Pérez-Barrales & Arroyo 2010).

Field studies have revealed that variation in floral morph ratios exists in many populations of style-polymorphic plants (Ganders 1979, Arroyo & Dafni 1995, Baker

et al. 2000b, van Rossum & Triest 2006). Beyond among-population differences in mating patterns, it has been shown that observed patterns of variation in floral morph ratios in populations can be accounted for by historical and contemporary demographic processes (Heuch 1980, Morgan & Barrett 1988, Husband & Barrett 1992a, 1992b, Eckert & Barrett 1995, Eckert *et al.* 1996, Kéry *et al.* 2003, Barrett & Shore 2008, Barrett *et al.* 2009, 2010, Zhou *et al.* 2012). For example, it is widely accepted that founder events or past bottlenecks can lead to the random loss of genetic variants (Nei *et al.* 1975). As a result, populations may undergo a considerable loss of neutral genetic diversity and a probable reduction in the allelic diversity of the loci responsible for floral morph variation (Barrett *et al.* 2009), which could seriously affect floral morph ratios in plant populations. In this sense, it has been suggested that the effects of frequency-dependent selection through disassortative mating on the maintenance of floral polymorphisms can strongly be weakened by demographic factors (Barrett *et al.* 2010). Hence, assessments inferred from neutral molecular markers of the role of population history and demographic processes in floral morph ratios variation will form an important part of studies dealing with among-population variation in floral polymorphism (Hodgins & Barrett 2007, Barrett *et al.* 2010, Zhou *et al.* 2012).

The central-marginal concept forms part of one of the ecological scenarios that may considerably enhance the assessment of the effects of demographic factors on floral polymorphism. Under this hypothesis, ecological, demographic and genetic features of populations are expected to differ depending on their location (i.e. core *vs.* periphery) within species' distribution ranges (Eckert *et al.* 2008 and references therein). In particular, peripheral populations are likely to be more isolated and have smaller populations due to generally sub-optimal environmental (e.g. climatic)

conditions at range margins (Chase & Leibold 2003). These population features are usually associated with reduced genetic diversity (Soulé 1973, Lammi *et al.* 1999, Gapare & Aitken 2005, Mandák *et al.* 2005, Eckstein *et al.* 2006, Cornman & Arnold 2007, Pfeifer *et al.* 2009, Leonardi *et al.* 2012), mainly because of increasing genetic drift and decreasing gene flow between populations that occur at range edges. Furthermore, marginal plant populations have also been found to have less phenotypic variation (Agnew 1968, Grant & Antonovics 1978, Cronberg *et al.* 1997, Eckert *et al.* 2008, Paiaro *et al.* 2012), probably due to a lack of the genetic variation needed for trait variation (Paul *et al.* 2011). That being said, we hypothesize that if historical and demographic processes have played an important role in shaping centre-margin patterns of variation in floral morph ratios across a style-polymorphic species' range, genetic diversity and gene flow will be greater at the core of the species' range and will decline towards its range margins.

The main goal of this study was to examine the genetic features of the populations of the style-dimorphic plant *Narcissus papyraceus* as a means of disentangling possible demographic causes of variation in floral morph ratios. Previous work on this species has detected a centre-margin pattern of variation in floral morph ratios (Barrett *et al.* 1996, Arroyo *et al.* 2002, Santos-Gally *et al.* 2013), which has been associated with variability in assortative *vs.* disassortative mating rates caused by among-population variation in the visiting pollinator fauna (Pérez-Barrales *et al.* 2007, Pérez-Barrales & Arroyo 2010, Santos-Gally *et al.* 2013). Alternatively, founder events, bottlenecks and their consequent genetic drift might explain the decline of polymorphism in the perimeter areas of this plant's range (Arroyo *et al.* 2002), a scenario that is still to be properly explored. Thus, the main hypothesis that we test here is that stochastic population processes determine the pattern of

population morph ratios across the range of the species. Nuclear microsatellite genotyping was used to analyze populations with different floral morph ratios throughout a substantial part of the *N. papyraceus*' distribution range in the SW Mediterranean Basin. Our specific objectives were (i) to estimate genetic diversity and genetic structure of *N. papyraceus* populations, (ii) to infer effective population size and migration rates among populations, (iii) to determine the relationship between genetic attributes of populations and variation in floral morph ratios, and (iv) to analyze the association between climatic variation across core and peripheral distribution areas and variation in floral morph ratios and genetic diversity.

Materials and Methods

Study species and source populations

Narcissus papyraceus Ker-Gawler (Amaryllidaceae) is a long-lived geophyte common in SW Mediterranean Basin whose range is centred upon the Strait of Gibraltar (Figure 1). The flowering period ranges from December to March. Inflorescences last up to one month and sequentially display up to 15 flowers. The species is self-incompatible but completely within-morph compatible (Arroyo *et al.* 2002, Chapter 2). It is visited by a wide array of generalist pollinators such as hoverflies, solitary bees, butterflies and moths (Arroyo *et al.* 2002, Pérez-Barrales *et al.* 2007, Santos-Gally *et al.* 2013).

Narcissus papyraceus is style-dimorphic with individuals exhibiting either long- (L-morph) or short-styled (S-morph) flowers. The two morphs differ in the position

of the style, which is either above or below the stamens whose position in the floral tube is mostly invariable (Arroyo *et al.* 2002). Populations of *N. papyraceus* vary in the proportion of L- and S-morphs. Around the Strait of Gibraltar, populations tend to be isoplethic (1:1 ratio of L- to S-morph individuals). In the Iberian Peninsula, the S-morph frequency decreases northwards and in the northern part of its range populations are monomorphic for the L-morph (Arroyo *et al.* 2002). In Morocco, on the other hand, the S-morph is maintained in L-biased populations (Santos-Gally *et al.* 2013).

In winter 2008–2009, we selected a total of 31 *N. papyraceus* populations throughout the species' range (Figure 1 and Table 1). In agreement with geographical criteria, populations were classified into six regions defined by rivers (e.g. Guadiana, Guadalquivir and Sebou) and mountain ranges (e.g. Baetic Mountains) on both sides of the Strait of Gibraltar (Figure 1). The names and codes of the six geographical regions (Figure 1) were: Central Morocco (CM), Northern Morocco (NM), Gibraltar Area (GI), Guadalquivir Basin (GB), Baetic Mountains (BM) and Algarve (AL). The number of populations sampled per region (N = 2–12) was determined by the abundance of the species within each region (Figure 1).

We estimated population size by counting floral clumps. In large populations, floral clumps were counted across defined areas and extrapolated for the complete population. For each population, we estimated the morph ratio by visual determination of the floral morph of 25–213 randomly chosen flowers from different individuals, totaling 3,514 flowers. Individuals were separated from each

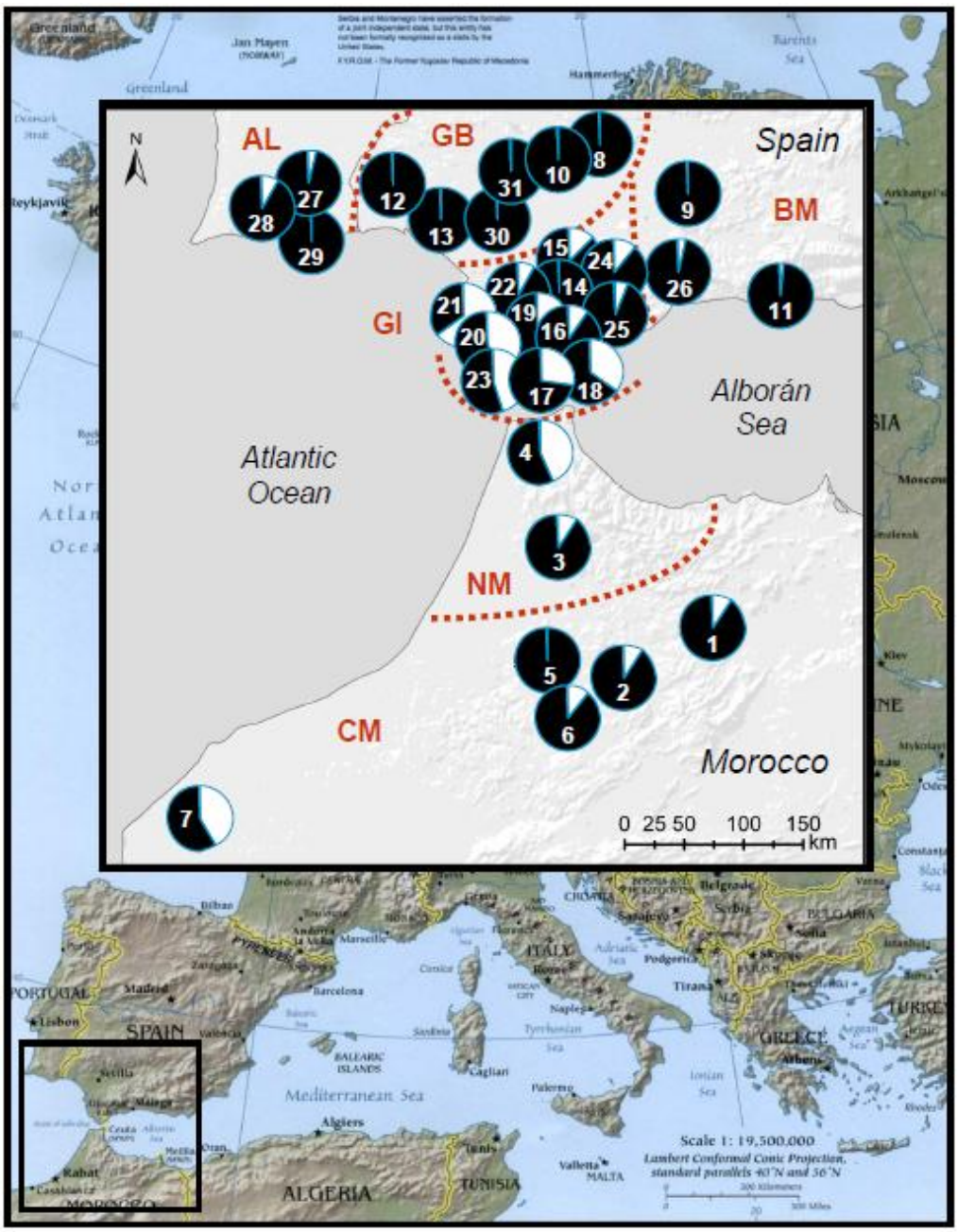


Figure 1. Map with the location of the 31 *Narcissus papyraceus* populations studied in six geographic regions. The study area is located within the square on the map. The proportion of L- (black) and S-morph (white) within each population is indicated. Region codes: CM, Central Morocco; NM, Northern Morocco; GI, Gibraltar Area; GB, Guadalquivir Basin; BM, Baetic Mountains; AL, Algarve.

other by at least one meter to sample different genets given the low dispersal ability of *N. papyraceus* clones (Arroyo *et al.* 2002). Populations were classified into three groups according to the S-morph frequency (Table 1): monomorphic (i.e. all individuals were L-morph), anisoplethic (i.e. 1–30% of individuals were S-morph), and isoplethic (i.e. 31–64% of individuals were S-morph). Strongly S-biased and S-monomorphic populations have never been reported for the species (Arroyo *et al.* 2002) and none were found in our survey.

Marker genotyping

For each population, we collected leaf tissue from 13–24 randomly chosen individuals, totaling 512 *N. papyraceus* plants (Table 1). Sampled plants were separated from each other by at least one meter. We collected samples separately from each floral morph in six isoplethic and two anisoplethic populations (Table 1). In the other 23 populations, we collected samples from the dominant L-morph (Table 1). Leaf tissue was dried out in silica gel and later frozen at -80 ºC until DNA extraction.

DNA was isolated following Bernartzky & Tanksley's (1986) protocol without mercaptoethanol. We genotyped all samples with eight nuclear microsatellite markers previously tested for polymorphism (A5, A109, A116, A121, B7, B104, B109 and B112; Simón *et al.* 2010). We performed polymerase chain reactions (PCR) in 25 μL of reaction mixture containing 50 ng of template DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.1 μM fluorescently labeled (6-FAM™, VIC®, NED™ and PET® dyes) forward primer, 0.1 μM reverse primer, 0.05 mM each dNTP and 1.25 U Taq polymerase. PCRs were performed in a Biometra Gradient Thermal Cycler (Biometra, Göttingen, Germany), with an initial 5 min of denaturation at 94°C, 45 cycles at 94°C for 30 s, annealing at different temperatures depending on the marker

(57 ºC for A109 and B7; 58 ºC for A116, A121 and B109; 59 ºC for B104 and B112) for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Polymerase chain reaction products were analyzed on an ABI 3130 × 1 Genetic Analyzer and sized using GeneMapper v.4.0 (Applied Biosystems, Foster City, USA) and GeneScan™ 500 LIZ size standard. We genotyped 9–52 individuals twice for each locus to calculate the mean genotyping error rate per locus that was 1.0 ± 1.5%.

Genetic data analysis

For each *N. papyraceus* population, we estimated the mean number of alleles per locus (n_a), the mean genetic diversity (H_S), the fixation index (F_{IS}) and the proportion of polymorphic loci (PL) using GENALEX v.6 (Peakall & Smouse 2006). We estimated allelic richness (R_S) and private allelic richness (R_P) with HP-RARE v.1 (Kalinowski 2005). Differences in genetic diversity parameters among geographical regions, floral morph ratio groups (monomorphic, anisoplethic or isoplethic) and genetic clusters estimated with STRUCTURE (see the Results section) were tested with General Linear Models and Student-Newman-Keuls *post hoc* tests in SPSS v.17 (SPSS Inc., Chicago, IL, USA). For the six isoplethic and two additional anisoplethic populations (Table 1), we computed the same genetic parameters for each group of L- and S-morph individuals and analyzed differences between floral morphs using Student's *t*-tests.

The genetic structure of *N. papyraceus* was estimated by means of Bayesian analyses with STRUCTURE v.2.3.1 (Pritchard *et al.* 2000, Falush *et al.* 2003) using non-redundant multilocus genotypes (N_G = 510). We performed different analyses under admixture and non-admixture models with and without prior information on

individual assignment to pre-defined populations. We established a burn-in time of 50,000 iterations and 20,000 MCMC (Markov Chain Monte Carlo) iterations assuming a correlated frequencies model. We repeated simulations 10 times for each number of clusters (*K*) from 1 to 10. We used STRUCTURE HARVESTER v.0.6.1 (Earl & von Holdt 2011), which implements the method described by Evanno *et al.* (2005), to find the most accurate *K* value. We averaged the matrix from the different replicates of STRUCTURE on the real number of genetic clusters with CLUMPP v.1.1.2 (Jakobsson & Rosenberg 2007). The genetic diversity of the resulting genetic clusters was also characterized as described above, assigning each population categorically to the predominant genetic cluster.

Genetic differentiation was computed by means of nested analyses of molecular variance (AMOVA; Excoffier *et al.* 1992). All *F*-statistics (Wright 1951, Weir & Cockerham 1984) and their significance, based on 1,000 permutations, were estimated with GENALEX for the three following population groupings: AMOVAs decomposed the genetic variance (i) among geographical regions and populations within regions, (ii) among floral morph ratio groups (monomorphic, anisoplethic and isoplethic) and populations within each floral morph ratio group, and (iii) between genetic clusters estimated with STRUCTURE (see the Results section) and populations within genetic clusters. We also assigned each population categorically to the predominant genetic cluster for AMOVA and MIGRATE analyses (see below).

Genetic relationships among populations were also analyzed with Factorial Analysis of Correspondences (FCA) using GENETIX v.4.05.2 (Belkhir *et al.* 1996). We computed isolation-by-distance (IBD) with Mantel tests (Mantel 1967) performed with matrices of pairwise genetic and log-transformed geographical distances among populations. *P*-values were estimated from 1,000 permutations. We

also conducted IBD based on pairwise genetic and log-transformed geographical distances among regions using the geographical centroids from all populations within each region. All genetic distances for IBD tests were based on *D*, estimated as F_{ST} / [1 – F_{ST}] (Rousset 1997).

We used the software MIGRATE v.2.3.2 (Beerli & Felsenstein 2001) to compute by means of maximum likelihood analyses the parameter $\Theta = 4N_e\mu$, where N_e is the population effective size and μ is the mutation rate, and the migration rate as $M = m/\mu$, where m is the immigration rate. We estimated Θ and M values with their 95% confidence intervals (CI) for the six geographical regions, the three floral morph ratio groups (monomorphic, anisoplethic and isoplethic), and the genetic clusters given by STRUCTURE (see the Results section). We performed maximum likelihood analysis over 10 short MCMC, sampling 500 out of 50,000 visited genealogies, three long MCMC, sampling 5,000 out of 500,000 visited genealogies, and burning periods of 10,000 discarded genealogies. The starter genealogy was random and the maximum likelihood estimates started with the F_{ST} value. For the sake of accuracy, we repeated all analyses five times, establishing the estimates from the previous run as starter parameters. Convergence was met at the second run of the simulation.

Spatial analyses

We assessed the spatial autocorrelation of S-morph frequency and all genetic diversity parameters (PL, n_a, H_S, R_S, and F_{IS}) of *N. papyraceus* populations using PASSaGE v.2 (Rosenberg & Anderson 2011). We calculated Moran's *I* (Moran 1950) for each parameter. All *P*-values and standard deviations for each distance class were

estimated from 1,000 permutations and 1,000 bootstrap replicates, respectively. The relationship between S-morph frequency, population size and genetic diversity parameters of *N. papyraceus* populations were analyzed with Simultaneous Autoregressive Models (SAR) using the software SAM v.4.0 (Rangel *et al.* 2010). SAM uses Generalized Least Squares (GLS) to estimate regression parameters including an additional term in the model for the autocorrelation matrix of the errors (Beale *et al.* 2010). We conducted SAR to correlate population size with S-morph frequency, population size with population genetic diversity parameters, S-morph frequency with population genetic diversity parameters, and S-morph frequency with the proportional membership to the main genetic cluster given by STRUCTURE (see the Results section). Genetic diversity parameters were log-transformed to improve data adjustment to the model. Finally, we estimated the correlation between S-morph frequency, given as Euclidean distances among populations, and genetic differentiation, given by *D*, with a partial Mantel test controlling for the log-transformed geographical position of populations given by the geographical distance matrix.

Climatic records

For the 31 *N. papyraceus* populations, we obtained climatic data from the WorldClim v.1.4 global climate database (Hijmans *et al.* 2005). WorldClim bioclimatic database encompasses 19 variables representing annual trends and seasonality in temperature and precipitation (see http://www.worldclim.org/; Table S1). Given the inherent spatial autocorrelation of weather data, we also explored the relationship between the 19 climatic variables and S-morph frequency in populations with SAR models.

Table 1. Geographic location, population size, S-morph frequency (%) and sample size (*N*) of each floral L- and S-morph of 31 *Narcissus papyraceus* populations (*N* = 512 individuals).

Pop. Number	Region Code	Lat. (ºN)	Long. (ºW)	Altitude (m a.s.l.)	Pop. Size	S-morph (%-type)	L-morph (N)	S-morph (N)
1	CM	34º12,6'	4º08,4'	401	200	10 (A)	15	–
2	CM	33º51,0'	4º52,2'	905	150	10 (A)	15	9
3	NM	34º49,8'	5º32,4'	224	500	10 (A)	15	–
4	NM	35º43,2'	5º44,4'	55	2000	43 (I)	10	10
5	CM	33º57,0'	5º34,2'	299	100	0 (M)	16	–
6	CM	33º37,8'	5º25,9'	914	300	11 (A)	15	–
7	CM	32º39,6'	8º39,6'	166	400	41 (I)	10	10
8	GB	37º50,4'	5º18,6'	152	200	0 (M)	15	–
9	BM	37º28,2'	4º21,0'	783	300	0 (M)	15	–
10	GB	37º45,6'	5º34,8'	331	700	0 (M)	15	–
11	BM	36º45,0'	3º25,8'	495	200	2 (A)	14	–
12	GB	37º29,4'	7º15,0'	197	300	0 (M)	16	–
13	GB	37º13,2'	6º26,4'	34	80	0 (M)	19	–
14	GI	36º37,8'	5º27,0'	507	300	2 (A)	15	–
15	GI	36º45,0'	5º30,6'	300	300	13 (A)	15	–
16	GI	36º12,6'	5º34,8'	102	2000	11 (A)	15	–
17	GI	36º08,4'	5º36,0'	252	700	28 (A)	10	10
18	GI	36º04,2'	5º33,0'	420	150	35 (I)	10	10
19	GI	36º19,2'	5º46,2'	31	150	18 (A)	13	–
20	GI	36º22,2'	6º06,6'	24	100	43 (I)	10	10
21	GI	36º24,6'	5º54,6'	44	80	64 (I)	10	10
22	GI	36º28,8'	5º43,8'	74	1500	8 (A)	15	–
23	GI	36º06,0'	5º43,8'	164	2000	45 (I)	9	9
24	GI	36º40,2'	5º13,2'	962	3000	11 (A)	15	–
25	GI	36º28,8'	5º17,4'	366	1500	6 (A)	15	–
26	BM	36º54,0'	4º34,2'	523	300	4 (A)	16	–
27	AL	37º14,4'	8º02,4'	209	25	4 (A)	16	–
28	AL	37º15,0'	8º06,0'	314	250	9 (A)	15	–
29	AL	37º06,0'	7º51,0'	199	400	0 (M)	16	–
30	GB	37º14,4'	6º14,4'	12	250	0 (M)	15	–
31	GB	37º33,0'	5º56,4'	27	50	0 (M)	14	–

Region codes: CM, Central Morocco; NM, Northern Morocco; GI, Gibraltar Area; GB, Guadalquivir Basin; BM, Baetic Mountains; AL, Algarve. Floral morph ratio type: M, Monomorphic; A, Anisoplethic; I, Isoplethic.

Finally, we conducted SAR models to correlate climatic variables with each of the genetic diversity parameters of *N. papyraceus* populations.

Results

Population characteristics

The 31 sampled *N. papyraceus* populations were located between 32º39,6' N – 37º50,4' N and 3º25,8' W – 8º39,6' W (Figure 1 and Table 1). Altitudes ranged between 12 and 962 m a.s.l. (Table 1). Weather records indicated that the annual mean temperatures and total annual precipitation were in the range 14.2–18.6 ºC and 339–862 mm, respectively (Table S1). Estimates of population size varied from a low of 25 to a high of 3,000 individuals (Table 1). S-morph frequency varied between 0 and 64% (Table 1). The highest proportions of S-morph individuals were found in populations from Morocco and the Gibraltar Area (Figure 1 and Table 1).

Genetic diversity, differentiation and structure

The percentage of polymorphic loci varied between 62.5 and 100% among *N. papyraceus* populations (among-population mean PL ± SD = 95.2 ± 8.3; Table S2). The mean number of alleles per locus (n_a) ranged between 2.9 and 11.8 among populations (mean n_a ± SD = 8.0 ± 1.9), genetic diversity (H_S) between 0.33 and 0.81 (mean H_S ± SD = 0.69 ± 0.08) and allelic richness (R_S) between 1.7 and 3.2 (mean R_S ± SD = 2.8 ± 0.3; Table S2). Private allelic richness (R_P) was low in all populations,

varying between 0.03 and 0.27 (mean $R_P \pm$ SD = 0.16 ± 0.06). Fixation indexes (F_{IS}) were positive and ranged from 0.24 to 0.55 among populations with the exception of population 31, which had a coefficient of -0.33 (mean $F_{IS} \pm$ SD = 0.37 ± 0.15; Table S2).

The six geographical regions differed significantly for n_a and R_S ($F_{5,25} > 2.97$, $P < 0.031$ in both cases). PL, H_S and F_{IS} did not vary significantly among geographical regions ($P > 0.14$ in all cases). For n_a, Moroccan and Gibraltar Area populations exhibited higher values than in the other regions (Table 2A). By contrast, differences in R_S were less pronounced among geographical regions. For n_a and R_S, the populations from Morocco and Algarve ranked as the most and least diverse, respectively (Table 2A).

The three groups of floral morph ratio (monomorphic, anisoplethic and isoplethic) did not differ significantly for PL or F_{IS} ($P > 0.56$ in both cases) but were significantly different for n_a, H_S and R_S ($F_{2,30} > 3.82$, $P < 0.034$ in all cases). The isoplethic and monomorphic groups of populations had the highest and lowest values for H_S and R_S, respectively (Table 2B). For n_a, the anisoplethic group of populations exhibited the highest value, while the isoplethic and monomorphic groups had the lowest values (Table 2B). Within the dimorphic populations in which L- and S-morph individuals were sampled, none of the genetic diversity parameters differed significantly between floral morphs ($P > 0.11$ in all comparisons).

The Bayesian structuring method detected two genetic clusters. One group included all the Moroccan populations and the Iberian coastal populations 11 and 18 (the Moroccan cluster; Figure 2), while the second group consisted of the remaining Iberian populations (the Iberian cluster; Figure 2). This result was obtained with a non-admixture model when we supplied information of the prior individual

assignment to pre-defined populations. In all other cases, the analysis did not detect structured groups in the genetic data set. The two genetic clusters differed significantly for n_a, H_S and R_S ($F_{1,30} > 4.41$, $P < 0.045$ in all cases), the Moroccan cluster being more diverse than the Iberian cluster, but not for PL or F_{IS} ($P > 0.49$ in both cases; Table 2C).

AMOVAs showed that in all hierarchical models approximately 90% of the genetic variation occurred among individuals within populations (Table 3). Genetic differentiation among geographical regions ($F_{RT} = 0.025$) and floral morph ratio groups ($F_{RT} = 0.001$) was low but significant (Table 3A and 3B). Genetic differentiation among populations within geographical regions ($F_{ST} = 0.105$) and among populations within groups of floral morph ratio ($F_{ST} = 0.100$) was also significant (Table 3A and 3B). Albeit low, AMOVA also detected significant differences between genetic clusters ($F_{RT} = 0.019$) and among populations within genetic clusters ($F_{ST} = 0.109$; Table 3C).

Factorial Analysis of Correspondences (FCA) yielded three axes explaining 8.9%, 7.3% and 6.6% of the variance, respectively. The first axis clearly separated populations from Morocco and Algarve (Figure 2). In general, the consistency of the results obtained with FCA and Bayesian methods supported the idea that the geographical genetic structure of *N. papyraceus* is weak (Figure 2). The Mantel test did not detect isolation-by-distance with population pairwise genetic differences ($P = 0.13$), but found isolation-by-distance when using pairwise genetic differences among the six geographical regions ($r = 0.47$, $P = 0.004$).

Table 2. Mean (± SD) genetic diversity parameters of 31 *Narcissus papyraceus* populations grouped by (A) geographic region, (B) floral morph ratio group, and (C) genetic cluster estimated with STRUCTURE.

(A)						
Geographic regions	N	PL (%)	n_a	H_S	R_S	F_{IS}
Central Morocco	5	92.50 ± 6.85	9.72 ± 0.62 a	0.734 ± 0.035	3.00 ± 0.10 ab	0.33 ± 0.07
Northern Morocco	2	100.00 ± 0.00	10.35 ± 0.97 a	0.770 ± 0.056	3.10 ± 0.17 a	0.37 ± 0.11
Gibraltar Area	12	95.83 ± 6.15	8.25 ± 0.40 ab	0.707 ± 0.023	2.89 ± 0.07 ab	0.40 ± 0.05
Guadalquivir Basin	6	93.75 ± 15.31	6.92 ± 0.56 bc	0.642 ± 0.032	2.65 ± 0.10 ab	0.31 ± 0.06
Baetic Mountains	3	95.83 ± 7.22	7.03 ± 0.80 bc	0.677 ± 0.046	2.77 ± 0.14 ab	0.35 ± 0.09
Algarve	3	95.83 ± 7.22	5.23 ± 0.80 c	0.613 ± 0.046	2.53 ± 0.14 b	0.48 ± 0.09
(B)						
Floral morph ratio groups	N	PL (%)	n_a	H_S	R_S	F_{IS}
Monomorphic	9	93.06 ± 2.82	6.89 ± 0.55 b	0.638 ± 0.026 b	2.66 ± 0.08 b	0.33 ± 0.05
Anisoplethic	16	95.31 ± 2.11	9.75 ± 0.68 a	0.698 ± 0.019 ab	2.86 ± 0.06 ab	0.39 ± 0.04
Isoplethic	6	97.92 ± 3.45	7.88 ± 0.42 b	0.750 ± 0.031 a	3.00 ± 0.10 a	0.37 ± 0.06
(C)						
Genetic clusters	N	PL (%)	n_a	H_S	R_S	F_{IS}
Moroccan cluster	9	95.83 ± 2.82	9.57 ± 0.53 a	0.738 ± 0.027 a	2.99 ± 0.08 a	0.34 ± 0.05
Iberian cluster	22	94.89 ± 1.81	7.30 ± 0.34 b	0.671 ± 0.017 b	2.76 ± 0.05 b	0.38 ± 0.03

Number of populations (N), percentage of polymorphic loci (PL), number of alleles per locus (n_a), genetic diversity (H_S), allelic richness (R_S) and the fixation index (F_{IS}). Means with different letters differ significantly from one another ($P < 0.05$; Student-Newman-Keuls *post hoc* test).

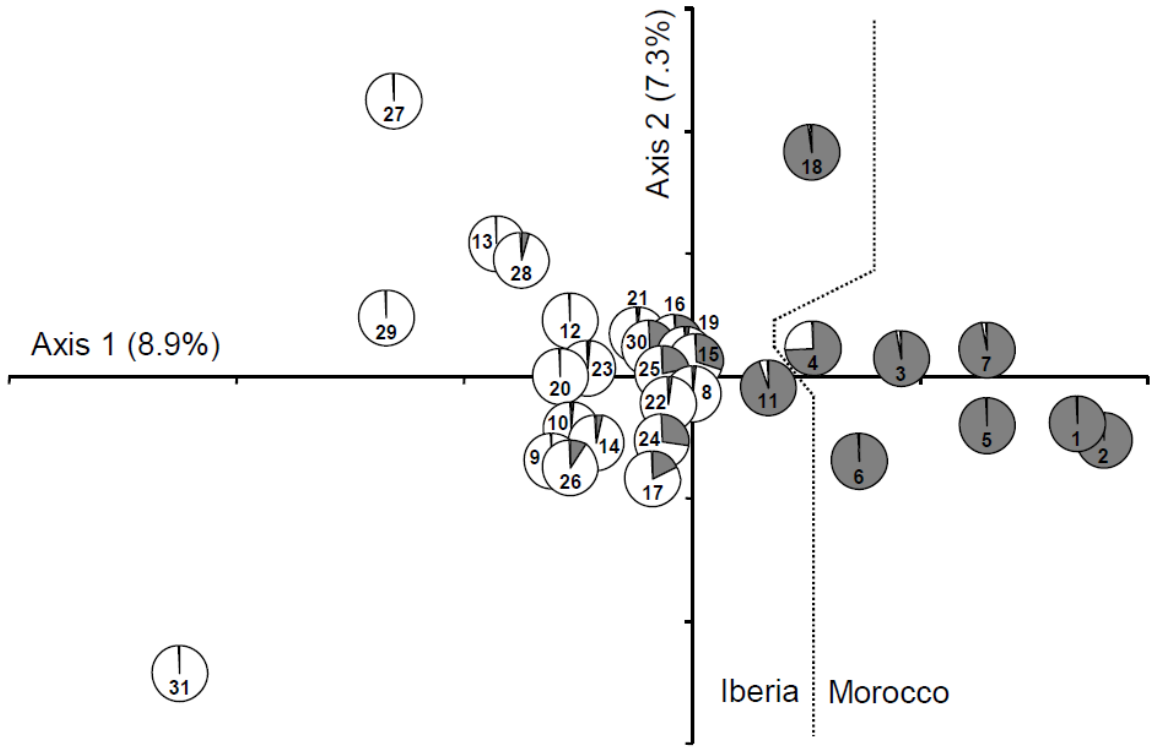


Figure 2. Genetic structure of the *Narcissus papyraceus* populations given by STRUCTURE and Factorial Analysis of Correspondences (FCA). Populations are represented along the first two axes of FCA with the percentage of explained variance in parenthesis. The dotted line separates Moroccan from Iberian populations independently of the genetic cluster. The Moroccan (grey) and Iberian (white) genetic clusters are indicated as the proportional population membership to each genetic cluster.

Table 3. Analyses of Molecular Variance (AMOVA) for 31 *Narcissus papyraceus* populations grouped by (A) geographic region, (B) floral morph ratio group and (C) genetic cluster estimated with STRUCTURE.

(A)			
Geographic regions	d.f.	Variance components	Fixation indices
Among geographic regions	5	2.47	0.025***
Among populations within regions	25	8.02	0.105***
Among individuals within populations	481	43.00	0.480***
Within individuals	512	46.51	0.535***
(B)			
Floral morph ratio groups	d.f.	Variance components	Fixation indices
Among floral morph ratio groups	2	0.07	0.001*
Among populations within groups	28	9.97	0.100***
Among individuals within populations	481	43.22	0.480***
Within individuals	512	46.74	0.533***
(C)			
Genetic clusters	d.f.	Variance components	Fixation indices
Among genetic clusters	1	1.91	0.019***
Among populations within clusters	29	8.94	0.109***
Among individuals within populations	481	42.88	0.481***
Within individuals	510	46.26	0.537***

Degrees of freedom (d.f.), variance components (%) and fixation indices for each level of hierarchical variation are given. Significance: ***, $P < 0.001$; *, $P < 0.05$.

Θ values and migration rates

All Θ estimates obtained with MIGRATE differed significantly, based on the overlap of 95% CI between Θ values, among geographical regions, floral morph ratio groups and genetic clusters (Table 4). The only exception was the Θ values for Central and Northern Morocco. Populations from the Gibraltar Area and Algarve exhibited the highest and lowest Θ values, respectively (Table 4A). The Θ value from the Gibraltar Area was six-times higher than that from Algarve. The Θ value of isoplethic populations doubled that of the monomorphic populations (Table 4B). The Moroccan genetic cluster showed a higher Θ value than that of the Iberian cluster, although the difference was not as marked (Table 4C).

Populations from the central Gibraltar Area received more migrants from other regions (Table 4A). On the other hand, the peripheral regions of Algarve and Central Morocco received few migrants from other regions (Table 4A). Populations from the Gibraltar Area and Baetic Mountains donated by far the most migrants to the rest of the regions (Table 4A). In general, there was a trend towards greater migration rates between adjacent geographical regions (Table S3). Among floral morph ratio groups, we also detected differential patterns of migration. Anisoplethic populations almost doubled the number of immigrants with respect to monomorphic and isoplethic populations (Table 4B). Differences in emigration rates were less pronounced among floral morph ratio groups, although monomorphic and isoplethic populations had the highest and the lowest emigration rates, respectively (Table 4B). The Moroccan and Iberian genetic clusters exchanged a similar number of migrants (Table 4C).

Table 4. Results from MIGRATE analyses of 31 *Narcissus papyraceus* populations grouped by (A) geographic region, (B) floral morph ratio group and (C) genetic cluster estimated using STRUCTURE.

(A)			
Geographic regions	Θ value	Immigrants	Emigrants
Central Morocco	5.54 (5.23 – 5.87) b	5.02 (4.32 – 5.80) c	4.72 (4.04 – 5.51) c
Northern Morocco	6.02 (5.43 – 6.70) b	8.75 (7.53 – 10.11) b	4.75 (4.00 – 5.58) c
Gibraltar Area	7.21 (6.92 – 7.52) a	11.91 (10.95 – 12.95) a	13.68 (12.24 – 15.24) a
Guadalquivir Basin	3.87 (3.66 – 4.09) c	8.76 (7.75 – 9.87) b	7.28 (6.34 – 8.31) b
Baetic Mountains	1.59 (1.51 – 1.67) d	8.05 (7.06 – 9.13) b	11.84 (10.66 – 13.13) a
Algarve	1.20 (1.13 – 1.28) e	5.67 (4.74 – 6.75) c	5.90 (5.05 – 6.85) bc
(B)			
Morph ratio groups	Θ value	Immigrants	Emigrants
Monomorphic	4.69 (4.50 – 4.92) c	7.92 (7.48 – 8.39) c	13.58 (12.83 – 14.40) a
Anisoplethic	9.29 (8.92 – 9.68) b	15.29 (14.51 – 16.20) a	10.05 (9.53 – 10.62) b
Isoplethic	10.33 (9.74 – 10.98) a	8.94 (8.42 – 9.48) b	8.52 (8.06 – 9.06) c
(C)			
Genetic clusters	Θ value	Immigrants	Emigrants
Moroccan cluster	10.29 (9.86 – 10.75) a	5.16 (4.92 – 5.41) a	4.34 (4.13 – 4.55) b
Iberian cluster	8.45 (8.18 – 8.75) b	4.34 (4.14 – 4.55) b	5.16 (4.92 – 5.41) a

Θ values and net number of immigrants and emigrants from the fifth final consecutive simulation are given for each group of populations. The 95% CI are indicated in parenthesis. For each variable and group of populations, values with different letters differ significantly from one another given the overlap of their 95% CI.

Spatial patterns

Analysis of spatial patterns revealed significant spatial autocorrelation for the S-morph frequency. The S-morph frequency was significantly spatially autocorrelated within a range of 50 km (Moran's $I = 0.41$, $P < 0.05$). By contrast, none of the genetic diversity parameters exhibited significant spatial autocorrelation ($P > 0.05$ in all cases).

The Simultaneous Autoregressive Models (SAR) found significant positive relationships between S-morph frequency and genetic diversity parameters such as n_a and R_S (Figure 3). H_S was marginally positively correlated with S-morph frequency (Figure 3). The SAR model was not significant for the correlation between S-morph frequency and proportional membership to the Moroccan genetic cluster ($P = 0.51$). Partial Mantel tests did not find significant associations between S-morph frequency and genetic differences among populations ($P = 0.15$). Population size did not significantly correlate with S-morph frequency or with population genetic diversity parameters ($P > 0.27$ in all cases).

Climatic effects

SAR models found significant negative relationships between S-morph frequency and temperature range-related variables ($P < 0.003$ in all cases; Table 5) such as the mean diurnal range (i.e. the difference between maximum and minimum monthly temperatures), temperature seasonality (i.e. standard deviation of temperature records), the maximum temperature of the warmest month and the annual

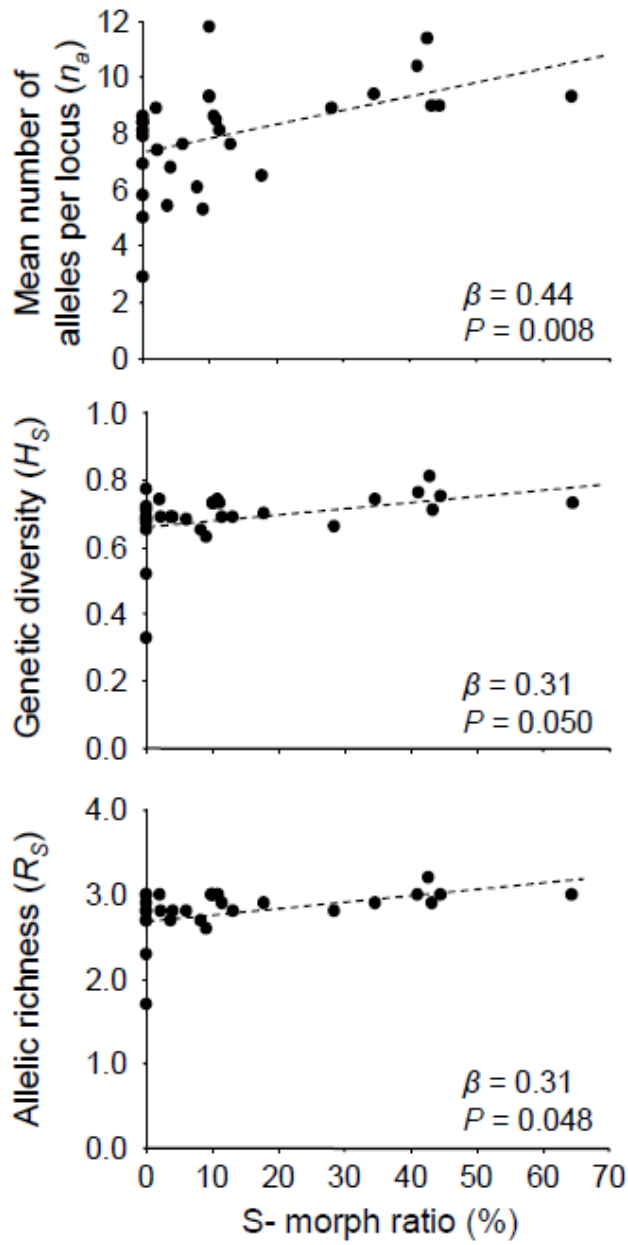


Figure 3. Linear relationships between S-morph frequency and mean number of alleles per locus (n_a), genetic diversity (H_S) and allelic richness (R_S). Correlation coefficients and *P*-values are given.

temperature range (i.e. the difference between maximum temperature of the warmest month and minimum temperature of the coldest month). Relationships between S-morph frequency and precipitation-related variables such as precipitation in the wettest month and precipitation seasonality (i.e. the coefficient of variation of precipitation records) were significantly positive ($P < 0.003$ in all cases; Table 5). We found no significant relationships between climatic records and genetic diversity parameters ($P > 0.28$ in all cases).

Table 5. Results of Simultaneous Autoregressive Models (SAR) for six climatic records obtained from the WorldClim global climate database that were significantly correlated with the S-morph frequency in 31 *Narcissus papyraceus* populations.

Climatic record	R^2	β	F-value
Mean diurnal range (bio2)	23.4	-0.490	10.35**
Temperature seasonality (bio4)	28.8	-0.550	15.10***
Maximum temperature of the warmest month (bio5)	26.7	-0.544	12.76***
Annual temperature range (bio7)	26.9	-0.534	13.27***
Precipitation in the wettest month (bio13)	20.4	0.418	10.93**
Precipitation seasonality (bio15)	30.3	0.525	18.81***

Bioclimatic variable codes are given in parenthesis. R^2, correlation coefficients (β), and F-values are given. Significance: ***, $P < 0.001$; **, $P < 0.01$.

Discussion

Genetic diversity and structure of Narcissus papyraceus *populations*

The inference of demographic history from genetic data provides insights into the processes shaping floral polymorphism in populations. In this study, we employed molecular markers to depict patterns of genetic variation and to infer demographic processes in populations of the style-dimorphic plant *Narcissus papyraceus* throughout its distribution range in the Western Mediterranean Basin. Our results showed that *N. papyraceus* populations maintain high levels of genetic diversity across its distribution range (Table 2) with a trend of increasing genetic diversity in central populations. This result suggests that central populations with higher S-morph frequency perform better demographically, thereby boosting genetic diversity, as the central-marginal hypothesis predicts (Eckert *et al.* 2008). In plants, genetic diversity and population demographic performance have been found to be positively correlated (Ellstrand & Elam 1993, Vergeer *et al.* 2003, Montesinos *et al.* 2009) because variability in the main demographic events (e.g. recruitment and mortality) strongly affects the genetic composition of populations.

Our analyses revealed that genetic diversity in *N. papyraceus* was poorly structured, as shown consistently by ordination, Bayesian and genetic variance partitioning methods. Only the Strait of Gibraltar acted as a geographical barrier for *N. papyraceus,* differentiating genetically Moroccan populations from most of the Iberian populations (Figure 2), as has been shown for other plant species occurring in this area (Ortiz *et al.* 2007, Arroyo *et al.* 2008, Rodríguez-Sánchez *et al.* 2008). It has long been accepted that the occurrence of gene flow prevents genetic differentiation

and weakens the geographic genetic structure of natural populations (Slatkin 1987). This could be the case of *N. papyraceus* given that we found relatively high levels of gene flow among populations, with greater gene flow between neighbouring locations (Tables 4 and S3) and some indication of dispersal between Morocco and the Iberian Peninsula. In fact, dispersal across the Strait of Gibraltar was probably involved in the formation of this species' range, which is subsequent to the opening of the Strait (Santos-Gally *et al.* 2012).

Genetic patterns and floral morph ratios in populations of Narcissus papyraceus

The lack of correspondence between the marked geographical distribution of stylar polymorphism and the weak geographical structure of genetic variation in *N. papyraceus* is the key result of our study. Gene flow among populations could lead to the homogenization of genetic pools across the whole of the species' distribution. As a result, *N. papyraceus* will have few limitations when moving alleles of the loci responsible for style-morph variation among populations and so the short-styled morph could potentially be found everywhere in the plant's range. Therefore, the geographical distribution of stylar polymorphism in *N. papyraceus*, reflected in the occurrence of isoplethic populations in the centre of the distribution and the gradual loss of the short-styled morph in peripheral populations, requires alternative explanations.

An explanation for the geographical distribution of floral polymorphism in *N. papyraceus* can be found in the comparison of estimated effective population size among populations given by the parameter Θ. Our results indicated that northern

peripheral monomorphic populations exhibited significant lower Θ values than those from central isoplethic populations (Table 4). Such a difference can be interpreted in terms of greater genetic drift in the former as a result of past bottlenecks, or as a consequence of founder events in the formation of marginal populations, as suggested by Arroyo *et al.* (2002). In addition, these authors' extensive survey included a total of 66 *N. papyraceus* populations and demonstrated that population sizes in northern peripheral populations dominated by long-styled individuals were lower than those of central isoplethic populations (Arroyo *et al.* 2002). A restricted flowering in our sampling year may have prevented us from finding any such correlation between population size and S-morph frequency. Hence, northern peripheral and central areas of the plant's distribution might have different histories in terms of population bottlenecks or founder events and consequent genetic drift. More intense genetic drift in northern peripheral populations might have eliminated the genetic variation that is required for floral polymorphism, which the gene flow, slightly restricted to periphery, would as yet have been unable to restore (Husband & Barrett 1992a, 1992b, Barrett *et al.* 2009).

Selective factors accounting for the fixation of the L-morph

Although bottlenecks and founder events may explain the reduction of floral polymorphism in marginal populations, they are unable to clarify the consistent bias and fixation of the L-morph (Eckert & Barrett 1992). Such a recurrent presence of L-monomorphic populations could respond to selective environmental forces acting against the S-morph in the northern range. In this regard, it has been recently argued that the loss of the short-styled morph individuals in peripheral populations of *N.*

papyraceus was correlated with massive visitation rates by short-tongued pollinators (Santos-Gally *et al.* 2013). This supported the hypothesis that the replacement of legitimate long-tongued pollinators (e.g. mostly moths) by short-tongued pollinators (e.g. mostly hoverflies) – incapable of pollinating short-styled morph flowers efficiently – represents an additional factor accounting for the scarcity or loss of this floral morph at the limits of its range (Pérez-Barrales *et al.* 2007, Pérez-Barrales & Arroyo 2010).

In addition, we have shown that the occurrence of isoplethic morph ratios in central populations was significantly associated with environmentally benign conditions, with gentler temperature fluctuations and greater precipitation that contrast with the harsher seasonality in L-dominated inland populations. In *N. triandrus* it was proposed that climatic gradients could affect floral morph ratios via the allometric effects of perianth variation (Barrett *et al.* 2004, Hodgins & Barrett 2008). Despite not directly exploring this possibility here, the separation of climate-shaped vegetative traits from perianth traits and morph ratio variation in *N. papyraceus* (Pérez-Barrales *et al.* 2009) contradicts this suggestion. Nevertheless, there are two possibilities that seem to include the role of climate in the central-marginal concept. First, although no case of morph-specific differences in the climatic niche of style-polymorphic species has ever been documented (Kéry *et al.* 2003), propitious climatic conditions may enhance population performance and have led to large population sizes in the central part of the plant's range (Brown 1984), which could be a prerequisite for the maintenance of stylar polymorphism (Baker *et al.* 2000a, Arroyo *et al.* 2002, Brys *et al.* 2008). Second, given that activity by different groups of insect pollinators varies according to weather conditions (Morgan & Heinrich 1987, Yela & Holyoak 1997, Wilson *et al.* 2009, Mutshinda *et al.* 2011, Robinson *et al.* 2012), above

all in critical periods such as mid-winter, the climate gradient may affect floral morph ratio indirectly through the shift of the pollinator arrays operating in the dimorphic and the monomorphic areas, as previous studies have reported (Pérez-Barrales *et al.* 2007, Santos-Gally *et al.* 2013).

Conclusions

It is clear that a thorough understanding of stylar dimorphism in plants does not depend on the knowledge of any single factor and, indeed, the duality of ecological and genetic factors in the shaping of floral morph variation in *N. papyraceus* has been long known to be crucial (Arroyo *et al.* 2002). Alongside recent studies on other plant species (Hodgins & Barrett 2007, Barrett *et al.* 2010, Zhou *et al.* 2012, see also Meeus *et al.* 2012), our study has stressed the role of molecular markers in understanding the demographic history of style-dimorphic plants and their geographical patterns of variation in floral morph ratios. Here we showed that historical processes that affect effective population size and genetic diversity seem to have influenced the morph ratios in populations together with environmental gradients. The combination of molecular data and complementary ecological data (e.g. the assessment of pollinator efficiency, fitness of floral morphs, variation in mating patterns; Chapters 5 and 6) is still needed if we are to fully understand the evolutionary ecology of style-dimorphic plants.

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Table S1. Coordinates and 19 climatic variables from the WorldClim database analyzed in the 31 populations of study (continues in the next page).

Pop.	Lat. (ºN)	Long. (ºW)	bio1	bio2	bio3	bio4	bio5	bio6	bio7	bio8
1	34º12,6'	4º08,4'	181	123	39	6397	357	43	314	104
2	33º51,0'	4º52,2'	156	138	42	6163	342	15	327	111
3	34º49,8'	5º32,4'	185	120	41	5563	344	55	289	115
4	35º43,2'	5º44,4'	179	89	42	4250	294	84	210	138
5	33º57,0'	5º34,2'	186	132	44	5547	356	58	298	128
6	33º37,8'	5º25,9'	152	142	43	6055	337	14	323	90
7	32º39,6'	8º39,6'	182	100	41	4688	310	70	240	134
8	37º50,4'	5º18,6'	175	124	39	6517	360	43	317	103
9	37º28,2'	4º21,0'	146	116	37	6521	328	19	309	77
10	37º45,6'	5º34,8'	168	125	39	6463	354	38	316	98
11	36º45,0'	3º25,8'	165	101	38	5433	310	50	260	109
12	37º29,4'	7º15,0'	167	94	39	5098	302	62	240	113
13	37º13,2'	6º26,4'	179	95	38	5401	317	67	250	120
14	36º37,8'	5º27,0'	156	96	39	5090	294	49	245	104
15	36º45,0'	5º30,6'	165	96	39	5140	303	59	244	112
16	36º12,6'	5º34,8'	171	79	40	4048	274	78	196	129
17	36º08,4'	5º36,0'	164	85	40	4316	278	67	211	122
18	36º04,2'	5º33,0'	159	91	40	4582	284	57	227	104
19	36º19,2'	5º46,2'	172	75	38	4217	275	79	196	127
20	36º22,2'	6º06,6'	176	71	36	4408	277	84	193	131
21	36º24,6'	5º54,6'	173	73	36	4482	278	79	199	126
22	36º28,8'	5º43,8'	171	78	37	4591	283	75	208	123
23	36º06,0'	5º43,8'	168	79	39	4130	274	75	199	126
24	36º40,2'	5º13,2'	142	105	39	5440	293	28	265	87
25	36º28,8'	5º17,4'	164	91	40	4653	289	63	226	117
26	36º54,0'	4º34,2'	159	103	39	5487	306	44	262	102
27	37º14,4'	8º02,4'	162	96	42	4445	290	65	225	118
28	37º15,0'	8º06,0'	149	97	42	4492	281	53	228	105
29	37º06,0'	7º51,0'	164	93	42	4382	287	67	220	121
30	37º14,4'	6º14,4'	182	104	38	5667	333	63	270	120
31	37º33,0'	5º56,4'	184	122	39	6163	362	55	307	117

bio1 = Annual Mean Temperature; bio2 = Mean Diurnal Range (Mean of monthly (max temp - min temp)); bio3 = Isothermality (bio2/bio7) (* 100); bio4 = Temperature Seasonality (standard deviation *100); bio5 = Max Temperature of Warmest Month; bio6 = Min Temperature of Coldest Month; bio7 = Temperature Annual Range (bio5-bio6); bio8 = Mean Temperature of Wettest Quarter. Temperatures are given in ºC × 10.

Table S1. Coordinates and 19 climatic variables from the WorldClim database analyzed in the 31 populations of study (begins in the previous page).

Pop.	bio9	bio10	bio11	bio12	bio13	bio14	bio15	bio16	bio17	bio18	bio19
1	267	268	104	570	95	2	67	250	16	20	250
2	238	239	81	527	69	4	56	195	26	28	188
3	257	258	115	821	148	0	78	411	12	12	411
4	232	236	127	774	145	0	77	377	11	18	373
5	258	260	117	531	88	1	66	231	14	15	223
6	232	235	80	690	103	4	61	278	27	30	270
7	241	244	125	339	59	0	73	163	5	7	148
8	261	261	95	612	88	2	60	245	19	19	237
9	234	234	68	650	87	6	56	251	30	30	248
10	254	254	89	634	90	2	61	257	22	27	247
11	238	238	100	358	50	3	56	146	16	22	139
12	231	234	104	528	78	2	61	227	19	26	210
13	247	250	112	528	81	1	63	226	15	23	207
14	222	225	95	788	126	1	70	360	18	26	346
15	232	234	103	732	119	1	69	335	17	24	316
16	222	225	121	839	146	1	76	402	15	22	389
17	219	222	111	850	145	1	76	404	14	23	399
18	218	222	104	846	142	1	76	403	14	24	403
19	225	228	120	786	134	0	73	361	15	22	348
20	231	235	121	659	115	1	70	290	14	23	276
21	229	233	117	723	123	1	70	324	15	23	309
22	229	233	115	751	126	1	71	343	16	23	326
23	220	224	117	862	149	0	76	404	14	22	398
24	213	215	77	809	121	3	67	358	24	32	348
25	223	227	108	750	126	1	72	358	16	24	340
26	231	232	93	621	95	3	65	276	21	27	261
27	219	223	109	553	91	1	67	260	18	21	247
28	207	211	96	629	100	2	65	287	24	25	277
29	219	224	112	544	90	1	68	258	17	21	242
30	254	256	111	550	84	1	64	235	17	23	217
31	265	266	108	563	83	1	63	237	18	22	224

bio9 = Mean Temperature of Driest Quarter; bio10 = Mean Temperature of Warmest Quarter; bio11 = Mean Temperature of Coldest Quarter; bio12 = Annual Precipitation; bio13 = Precipitation of Wettest Month; bio14 = Precipitation of Driest Month; bio15 = Precipitation Seasonality (Coefficient of Variation); bio16 = Precipitation of Wettest Quarter; bio17 = Precipitation of Driest Quarter; bio18 = Precipitation of Warmest Quarter; bio19 = Precipitation of Coldest Quarter. Temperatures are given in $^{o}C \times 10$; precipitation values are given in mm.

Table S2. Genetic diversity (± SD) parameters for each of the 31 *Narcissus papyraceus* populations of study.

Population	PL	n_a	H_S	R_S	F_{IS}
1	87.5	9.3 (1.5)	0.73 (0.11)	3.0 (0.3)	0.37 (0.07)
2	87.5	11.8 (1.8)	0.73 (0.11)	3.0 (0.3)	0.24 (0.05)
3	100.0	9.3 (1.3)	0.73 (0.08)	3.0 (0.2)	0.33 (0.10)
4	100.0	11.4 (1.2)	0.81 (0.06)	3.2 (0.2)	0.40 (0.07)
5	100.0	8.6 (1.1)	0.72 (0.10)	3.0 (0.3)	0.36 (0.09)
6	87.5	8.5 (1.4)	0.73 (0.11)	3.0 (0.3)	0.43 (0.11)
7	100.0	10.4 (1.1)	0.76 (0.07)	3.0 (0.2)	0.25 (0.08)
8	100.0	8.1 (1.5)	0.67 (0.09)	2.7 (0.3)	0.26 (0.10)
9	87.5	6.9 (1.5)	0.65 (0.10)	2.7 (0.3)	0.27 (0.10)
10	100.0	7.9 (1.1)	0.68 (0.07)	2.8 (0.2)	0.46 (0.12)
11	100.0	7.4 (0.9)	0.69 (0.09)	2.8 (0.3)	0.32 (0.08)
12	100.0	5.8 (0.7)	0.71 (0.06)	2.8 (0.2)	0.47 (0.12)
13	100.0	8.4 (1.0)	0.77 (0.05)	3.0 (0.2)	0.55 (0.08)
14	100.0	8.9 (1.3)	0.74 (0.10)	3.0 (0.3)	0.31 (0.09)
15	100.0	7.6 (1.0)	0.69 (0.10)	2.8 (0.3)	0.42 (0.10)
16	100.0	8.6 (0.9)	0.74 (0.06)	3.0 (0.2)	0.37 (0.09)
17	100.0	8.9 (1.3)	0.66 (0.11)	2.8 (0.3)	0.35 (0.09)
18	100.0	9.4 (1.3)	0.74 (0.05)	2.9 (0.2)	0.37 (0.14)
19	100.0	6.5 (0.9)	0.70 (0.06)	2.9 (0.2)	0.41 (0.12)
20	100.0	9.0 (1.3)	0.71 (0.09)	2.9 (0.3)	0.44 (0.12)
21	87.5	9.3 (1.4)	0.73 (0.11)	3.0 (0.3)	0.39 (0.09)
22	87.5	6.1 (1.0)	0.65 (0.10)	2.7 (0.3)	0.41 (0.13)
23	100.0	9.0 (1.1)	0.75 (0.06)	3.0 (0.2)	0.38 (0.10)
24	87.5	8.1 (1.4)	0.69 (0.11)	2.9 (0.3)	0.46 (0.07)
25	87.5	7.6 (1.5)	0.68 (0.10)	2.8 (0.3)	0.45 (0.08)
26	100.0	6.8 (0.8)	0.69 (0.09)	2.8 (0.3)	0.45 (0.12)
27	100.0	5.4 (0.9)	0.69 (0.05)	2.7 (0.2)	0.53 (0.10)
28	100.0	5.3 (0.8)	0.63 (0.07)	2.6 (0.2)	0.46 (0.11)
29	87.5	5.0 (1.0)	0.52 (0.10)	2.3 (0.3)	0.46 (0.13)
30	100.0	8.4 (1.3)	0.69 (0.09)	2.9 (0.3)	0.46 (0.08)
31	62.5	2.9 (0.7)	0.33 (0.12)	1.7 (0.3)	-0.33 (0.20)

Percentage of polymorphic loci (PL), mean number of alleles (n_a), genetic diversity (H_S), allelic richness (R_S), and the fixation index (F_{IS}) per locus.

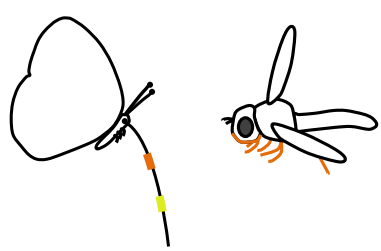
Table S3. Migration rates among geographic regions estimated with MIGRATE. The 95% CI are given in parenthesis. Columns and rows represent exporting and receiving migrants, respectively.

Geographic Region	Central Morocco	Northern Morocco	Gibraltar Area	Guadalquivir Basin	Baetic Mountains	Algarve
Central Morocco	–	1.77 (1.58 – 1.99)	1.09 (0.94 – 1.26)	0.29 (0.21 – 0.38)	1.40 (1.23 – 1.59)	0.47 (0.37 – 0.58)
Northern Morocco	1.59 (1.35 – 1.86)	–	3.43 (3.08 – 3.82)	1.37 (1.15 – 1.62)	1.15 (0.95 – 1.38)	1.21 (1.00 – 1.44)
Gibraltar Area	1.40 (1.24 – 1.58)	0.35 (0.28 – 0.44)	–	3.30 (3.05 – 3.56)	5.50 (5.17 – 5.83)	1.37 (1.21 – 1.54)
Guadalquivir Basin	0.12 (0.07 – 0.19)	1.46 (1.26 – 1.68)	2.82 (2.54 – 3.12)	–	2.57 (2.30 – 2.85)	1.80 (1.58 – 2.04)
Baetic Mountains	1.58 (1.37 – 1.82)	0.43 (0.33 – 0.55)	3.87 (3.53 – 4.22)	1.10 (0.93 – 1.29)	–	1.06 (0.90 – 1.25)
Algarve	0.02 (0.01 – 0.07)	0.73 (0.57 – 0.92)	2.47 (2.16 – 2.81)	1.22 (1.00 – 1.46)	1.23 (1.01 – 1.48)	–

CAPÍTULO 5

LOS INSECTOS DE PROBÓSCIDE LARGA PROMUEVEN LA TRANSFERENCIA DE POLEN ENTRE LOS MORFOS ESTILARES DE *Narcissus papyraceus* (AMARYLLIDACEAE)

LONG-TONGUED INSECTS PROMOTE DISASSORTATIVE POLLEN TRANSFER BETWEEN STYLE-LENGTH MORPHS OF *Narcissus papyraceus* (AMARYLLIDACEAE)



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Under review.

Resumen

En las flores hermafroditas, la hercogamia recíproca (p.ej. la heterostilia) mejora la transferencia de polen entre morfos florales evitando la autointerferencia de los órganos sexuales. Por el contrario, la polinización entre morfos podría verse comprometida en flores con dimorfismo estilar, que carecen de reciprocidad sexual perfecta entre ellos. Se ha considerado que este funcionamiento subóptimo explica por qué el dimorfismo estilar es raro en la naturaleza.

Algunas especies con polimorfismo estilar reciben una amplia gama de visitantes florales, incluyendo insectos de probóscide larga que se alimentan de néctar e insectos de probóscide corta que se alimentan de polen. Las diferencias en la morfología y el comportamiento de estos dos tipos de insectos podrían manifestarse en distintos patrones de polinización en cada morfo floral.

En un experimento de campo usando flores emasculadas e intactas, se estudiaron los patrones de polinización mediada por diferentes tipos de insectos (de probóscide larga y de probóscide corta) en los dos morfos florales (de estilo largo y de estilo corto) de la planta dimórfica estilar *Narcissus papyraceus*. Se investigaron los patrones de transferencia de polen entre y dentro de los morfos estilares en polinizaciones cruzadas, así como las tasas de autopolinización y de remoción de polen, para cada morfo floral y mediados por cada tipo de insecto.

Los insectos de probóscide larga fueron polinizadores eficientes de ambos morfos florales, puesto que tomaron poco polen de las anteras pero depositaron cantidades relativamente grandes en los estigmas. Aunque la transferencia de polen al morfo longistilo fue igualmente alta desde los morfos longistilo y brevistilo, la

transferencia de polen al morfo brevistilo fue mayor desde el morfo longistilo que desde el brevistilo. Los insectos de probóscide corta se llevaron grandes cantidades de polen de las anteras, pero depositaron sólo unos pocos granos de polen en los estigmas de las flores longistilas y un número insignificante de granos en los estigmas de las flores brevistilas, independientemente del morfo de la planta donadora.

En este estudio proporcionamos apoyo empírico para la hipótesis de que, bajo la acción de los polinizadores de probóscide larga, los patrones de transferencia de polen en la planta dimórfica estilar *N. papyraceus* se parecen a los de las especies heterostilas. Además, encontramos que los insectos de probóscide corta actúan principalmente como ladrones de polen, y por tanto limitan el éxito reproductor masculino de ambos morfos estilares y el éxito reproductor femenino de las plantas de morfo brevistilo. En vista de estos resultados, proponemos que las diferentes eficacias de polinización de los visitantes florales son clave para determinar la proporción de morfos de las poblaciones de esta especie de *Narcissus*.

Palabras clave: autopolinización, dimorfismo estilar, eficacia de los polinizadores, heterostilia, *Narcissus*, patrones de cruzamiento, polinización, polinizadores de probóscide corta, remoción de polen.

Abstract

In hermaphroditic flowers, reciprocal herkogamy, e.g. heterostyly, enhances pollen transfer between floral morphs (disassortative) while avoiding self-interference

between sexual organs. By contrast, disassortative pollination might be compromised in style-dimorphic flowers, which lack perfect reciprocity between different floral morphs. This sub-optimal functioning has been considered to explain why stylar dimorphism is rare in nature.

Some style-polymorphic species receive a wide array of floral visitors, including long-tongued insects that feed on nectar and short-tongued insects that feed on pollen. Differences in the morphology and behaviour of these two insect types could be manifested as different pollination patterns in each floral morph.

In a field-based experiment using emasculated and intact flowers, we studied pollination patterns mediated by different insect types (long- and short-tongued) in the two floral morphs (long- and short-styled) of the style-dimorphic *Narcissus papyraceus*. We investigated patterns of pollen transfer between and within style-length morphs in cross pollinations, as well as self-pollination and pollen removal rates, for each floral morph mediated by each insect type.

Long-tongued insects were efficient pollinators of both floral morphs as they removed little pollen from the anthers but deposited comparatively large amounts on the stigmas. Although disassortative and assortative pollen transfer were equally high to the long-styled morph, the former prevailed in the short-styled morph. Short-tongued insects removed large amounts of pollen from the anthers but deposited only a few pollen grains on the long-styled stigmas and a negligible number of grains on short-styled stigmas regardless of the morph of the donor.

In this study we provide empirical support for the hypothesis that, under the action of long-tongued pollinators, pollen transfer patterns in the style-dimorphic *N. papyraceus* resemble those of heterostylous species. In addition, we found that short-

tongued insects act mostly as pollen thieves, thereby limiting the male fitness of both style morphs on top of depleting the female fitness of S-morph plants. In view of these results, we propose that the differing pollination efficiencies of floral visitors are key in determining the morph ratio of populations in this *Narcissus*.

Keywords: heterostyly, mating patterns, *Narcissus*, pollen removal, pollination, pollinator efficiency, self-pollination, short-tongued pollinators, stylar dimorphism.

Introduction

The pollination efficiency of floral visitors functions in terms of how their morphology and behaviour oblige them to interact with the sexual organs of flowers. Floral visitors are only concerned about obtaining rewards, usually in the form of nectar and/or pollen. Thus, the challenge in floral evolution is the development of architectures – especially in the disposition of the sexual organs – that will optimize the pollination process. In hermaphroditic flowers this optimization should not only encourage cross-pollination but also establish a compromise between gains of fitness through male and female functions and the avoidance of self-interference (Barrett 2002). To this end, many hermaphroditic flowers display herkogamy, that is, the spatial separation of the female and male organs in the flower (Webb & Lloyd 1986).

Approach, reverse and reciprocal herkogamy are the commonest types of herkogamy and refer, respectively, to the positioning of the stigma above or below

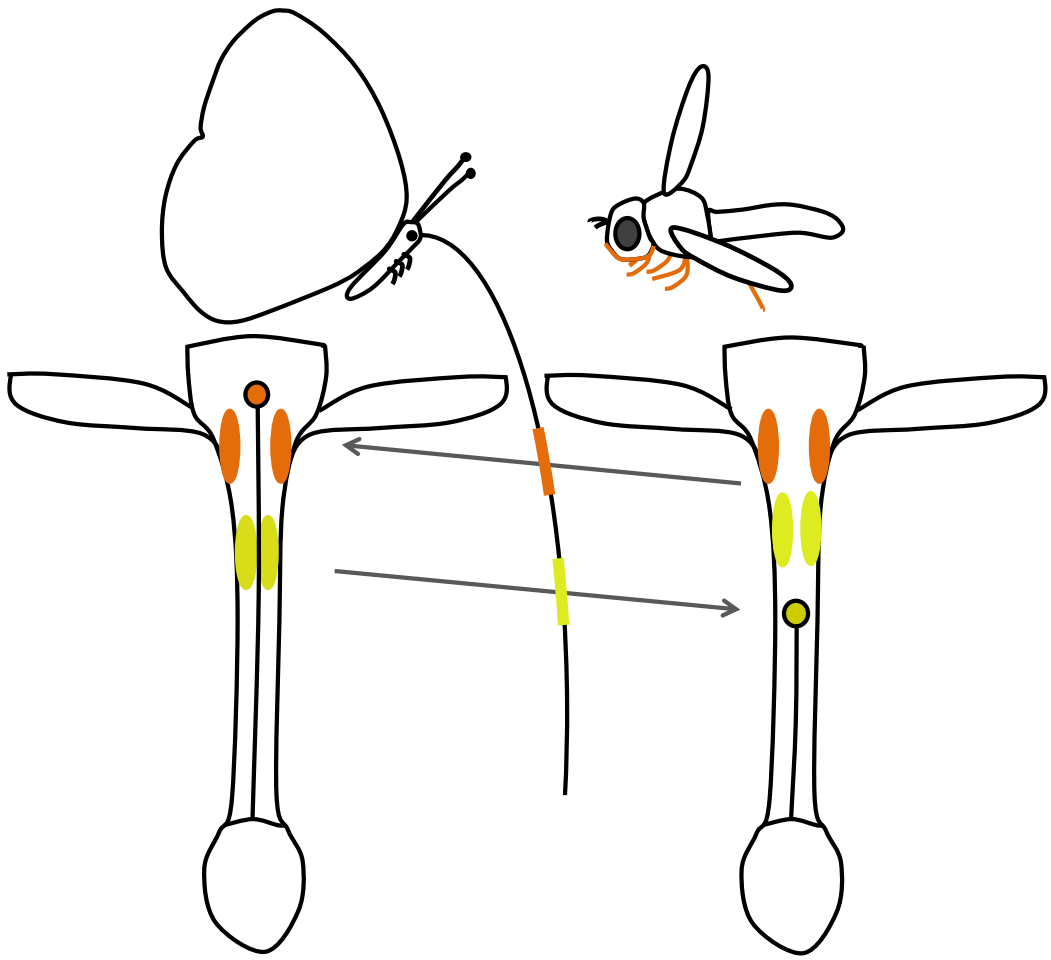

Figure 1. Possible pollen transfer patterns between style-length morphs mediated by long- and short-tongued pollinators. Note that the lower anther whorl in the short-styled morph (right) is slightly higher than in the long-styled morph (left). Colours indicate sexual organ level.

the anthers (Webb & Lloyd 1986) and to the existence of two or three floral morphs within populations that differ reciprocally in the position of the stigmas and anthers in the flower. The most typical form of reciprocal herkogamy is heterostyly (Darwin 1877, Vuilleumier 1967, Ganders 1979). In herkogamous flowers, each sexual organ comes into contact with a different part of the pollinator's body, a process that could potentially compromise the pollination efficiency between flowers whose sexual organs have the same disposition (Cresswell 2000). In contrast, the pollen transfer between heterostylous floral morphs (i.e. disassortative) is enhanced, a key fact for explaining the evolution of heterostyly according to Darwin's (1877) hypothesis (Lloyd & Webb 1992). Suboptimal efficiency in disassortative pollen transfer is expected to occur in style-dimorphic species, which have two floral morphs with approach and reverse herkogamy but low reciprocity due to the equal positioning of anthers in different floral morphs (Figure 1). Stylar dimorphism is uncommon and in evolutionary models appears as an unstable transitional stage towards heterostyly (Charlesworth & Charlesworth 1979, Lloyd & Webb 1992).

In recent decades, several studies have addressed pollen transfer patterns between floral morphs of heterostylous plants. Most such studies examine stigma loads in natural populations taking advantage of the dimorphism in pollen size or sculpture that is usually associated with floral morphs (Ganders 1974, 1976, Ornduff 1975a, b, Barrett & Glover 1985, Nicholls 1986, Björkman 1995, Ree 1997, Brys *et al.* 2008, Sánchez *et al.* 2010, see also Pailler *et al.* 2002, Lau & Bosque 2003). By contrast, to date only a single study (Stone & Thomson 1994) has ever dealt with pollen transfer patterns between approach and reverse herkogamous morphs; this study concluded that these morphs are able to promote disassortative pollination themselves without any need for reciprocal stigma-anther positioning. Less attention

has been paid to the pollinators responsible for such patterns, notwithstanding the fact that the pollination efficiency of floral visitors may vary in terms of the floral morph (Ornduff 1975c, Ornelas *et al.* 2004, Adler & Irwin 2006, Schlindwein & Medeiros 2006, Taki *et al.* 2009).

Long-tongued pollinators feeding on nectar at the bottom of the floral tube and carrying pollen on their proboscis are regarded as the genuine pollinators that are responsible for the legitimate and precise pollination between style morphs in typical long- and narrow-tubed flowers of heterostylous species (Lloyd & Webb 1992; Figure 1). However, many heterostylous and style-dimorphic species are not specialized to receive long-tongued pollinators and, instead, host a varied array of floral visitors including, commonly, flies and bees either in sympatry (Domínguez *et al.* 1997, Chen & Zhang 2010, Ferrero *et al.* 2011) or allopatry (Arroyo & Dafni 1995). Short-tongued insects feeding on the pollen of stamens in the upper part of the floral tube might be barely capable of reaching low-level sexual organs (Beach & Bawa 1980, Baker *et al.* 2000a, Dos Santos 2002), a fact that should alter legitimate pollen transfer patterns in populations (Dos Santos & Wittmann 2000; Figure 1). However, to date the difference in pollination patterns between long- and short-tongued floral visitors in style-length morphs has received little attention and remains empirically untested.

We studied the pollination efficiency of the long- and short-tongued insects that visit the two floral morphs (approach- and reverse-herkogamous) of the style-dimorphic *Narcissus papyraceus*. This species exhibits dimorphic populations in the centre and south of its distribution, but monomorphic populations of the approach-herkogamous morph in the northern part of its range (Arroyo *et al.* 2002, Santos-Gally *et al.* 2013). It has been argued that the short-tongued insects prevailing in the

flower's northern range lead to the disappearance of reverse-herkogamous individuals that cannot be pollinated efficiently, while long-tongued pollinators mediating sufficient levels of disassortative mating maintain dimorphism in the plant's populations in the central and southern parts of its range (Arroyo *et al.* 2002, Pérez-Barrales & Arroyo 2010). To test this hypothesis, we studied the pollination efficiency of long- and short-tongued insects in each floral morph from the female function perspective (i.e. pollen deposition on stigmas), and complemented it with the male function perspective (i.e. pollen removal from anthers), as well as the case of self-interference (i.e. self-deposition of pollen on stigmas). Finally, we examined whether pollen transfer patterns satisfy the conditions of the evolutionary model of Lloyd and Webb (1992) regarding the stability of stylar dimorphism in *N. papyraceus*.

Materials and Methods

Study Species

Narcissus papyraceus Ker-Gawler is a style-dimorphic species with individuals with approach or reverse herkogamy (Arroyo *et al.* 2002; Figure 1). Both floral morphs, long- (L) and short- (S) styled hereafter, have two whorls of stamens whose only significant difference is that the lower anther whorl is placed slightly higher in the S-morph (Pérez-Barrales & Arroyo 2010). *N. papyraceus* possesses a late-acting self-incompatibility system equivalent in both morphs (Chapter 2) that causes ovule discounting to occur after self-pollination, although within- and between-morph cross pollinations are equally fertile (Arroyo *et al.* 2002). The herkogamy (i.e. the

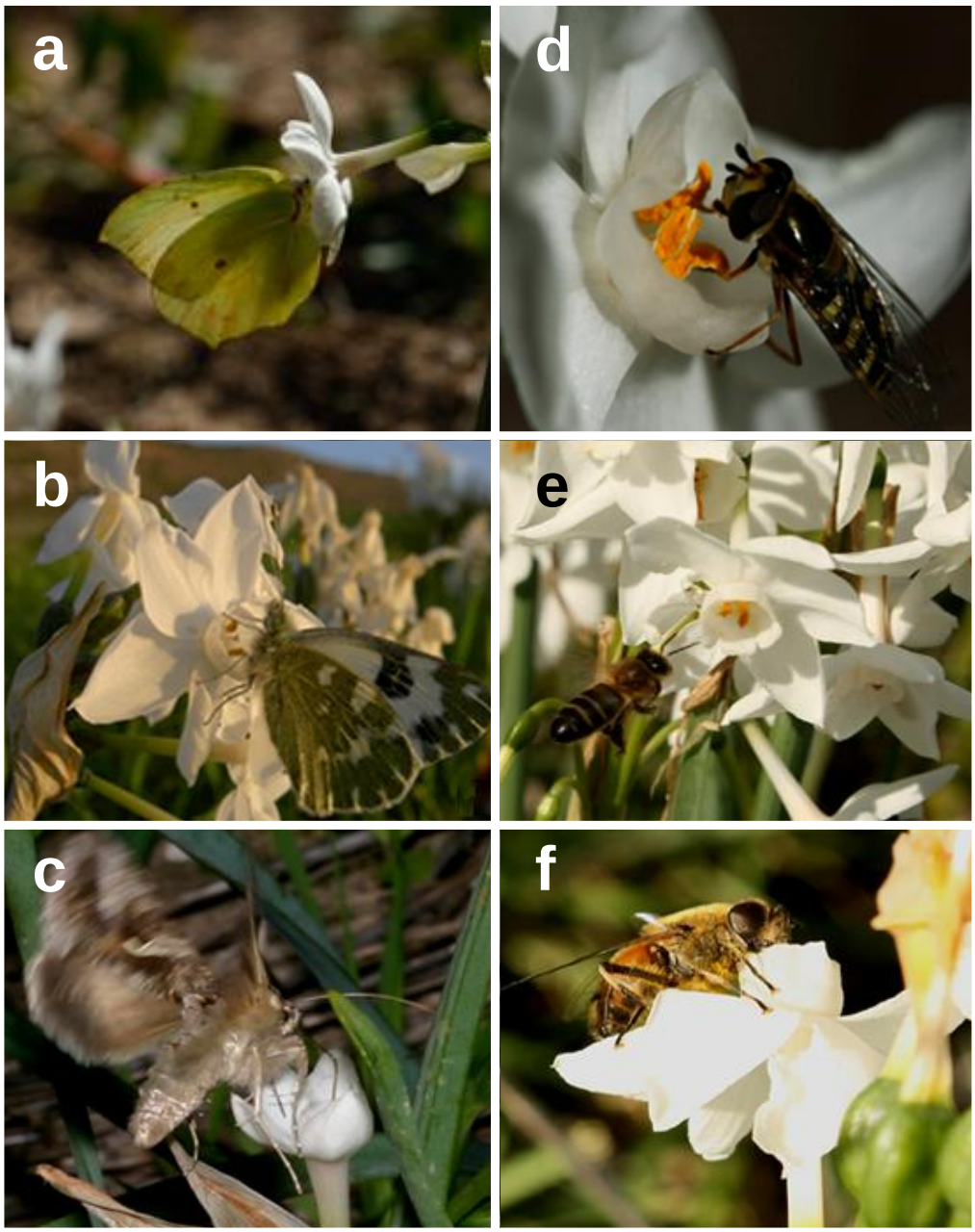

Figure 2. *Narcissus papyraceus* and some of its natural floral visitors. (a) *Gonepteryx cleopatra*; (b) *Pontia daplidice*; (c) *Autographa gamma*; (d) *Eupeodes* sp.; (e) *Apis mellifera*; (f) *Eristalis tenax*.

stigma-anther separation) is greater in the S- than the L-morph (Pérez-Barrales & Arroyo 2010, Santos-Gally *et al.* 2013). There is no other variation in perianth traits between these two morphs (Pérez-Barrales *et al.* 2007), including floral scent (R. Santos-Gally, unpublished).

As rewards for pollinators, the flowers of both morphs produce equal amounts of nectar (R. Pérez-Barrales & J. Arroyo, unpublished data) and large amounts of pollen, which are located at the bottom and the top of the floral tube, respectively. Despite its apparent adaptation to pollination by long-tongued nectar-foraging insects, the floral visitors of *N. papyraceus* include both nectar- and pollen-feeders. The nectar-feeders consist of insects with a long proboscis that can reach the base of the floral tube and are mainly butterflies and moths (e.g. genera *Pieris*, *Vanessa*, *Autographa* or *Macroglossum*), while the pollen-feeders are above all short-tongued insects such as syrphids (genera *Eristalis* or *Eupeodes)*, other flies and social bees such as *Apis* (Santos-Gally *et al.* 2013; Figure 2).

Plant material

Narcissus papyraceus plants of both morphs were grown in winter 2011–2012 in a greenhouse in the University of Seville from 250 bulbs collected in winter 2009 from two natural populations close to Tarifa (Cádiz province, S Spain; 36°6' N, 5°44' W; 200 individuals) and Ronda (Málaga province, S Spain; 36º40' N, 5º13' W; 50 individuals). The different phenology of individuals in each population provided us with long-lasting material. Prior to flowering, each new floral stem was marked and trimmed to leave just four flowers in order to avoid important differences in phenology and developmental stages. Floral buds were emasculated before anthesis

in half of the floral stems of each morph. Finally, all the floral stems were bagged to avoid insect visits and were left untouched until use in the field experiment, which took place 5–7 days after the first flower opened (within the lifespan of a non-pollinated flower).

Field experiment

We used the virgin (unvisited) floral stems of *N. papyraceus* to carry out a field experiment on 10 January–10 March 2012. The experiment was performed in two natural sites in the Guadalquivir Valley (SW Spain; coordinates 37º17' N, 6º25' W; 37º13' N, 6º01' W), within the natural range of *N. papyraceus* but far from any wild population. Although both sites had similar sub-shrub Mediterranean vegetation, during the experiment different pollinator communities were present. On each day with suitable weather conditions for insect activity, the experiment was performed in one or the other site, either at midday (beginning at 12.00 h) or at night (beginning at 20.00 h).

Ensuring that all the non-emasculated floral stems were of the same morph, every day we cut 6–10 virgin floral stems of *N. papyraceus* in the greenhouse and transported them to the experimental site in water pots. In the field, the floral stems were unbagged and planted separately in the ground and then observed for a period of two hours. During nocturnal sessions, we used a headlamp with a red light source to visualize the flowers without disturbing the insects. Eventually, the natural pollinators at each site were attracted by the floral stems and visited the flowers. Emasculated flowers attracted fewer visitors, which, nevertheless, behaved in the same fashion as when visiting non-emasculated flowers. When an insect was noted at

the floral stems, it was prevented from visiting any flower more than once. Each visited flower was collected separately in a vial and it was awarded a score for its morph, its position in the insect visit sequence and the identity of the insect visitor. We identified the insect visitor to species, genus or, in some cases, family level, and classified them as long-tongued (LT) or short-tongued (ST) type according to the distance (more or less than 5 mm) between the tip of the proboscis and bottom of the floral tube (Santos-Gally *et al.* 2013).

We collected three types of samples: i) cross-pollination, ii) self-pollination and iii) remaining-pollen on anthers. Cross-pollination samples were emasculated receptive flowers visited by an insect that had previously visited a non-emasculated donor flower. Taking into account the floral morph of the receptive and the donor flowers, four types of crosses were possible: assortative cross-pollinations L×L and S×S, and disassortative cross-pollinations L×S and S×L (hereafter, the receptive morph is always mentioned first). Self-pollination samples consisted of the styles of donor flowers that had been visited first in an insect's visit sequence, while the remaining-pollen samples were entire donor flowers collected after a single insect visit. To collect self-pollination samples we dissected the non-emasculated flowers very carefully with a scalpel to separate the style from the anthers without provoking additional self-pollen deposition. We also collected non-visited flowers as control groups for remaining-pollen and self-pollination samples in order to compare them, respectively, with the total pollen production by anthers and autonomous self-pollination of flowers. For cross-pollination, the control group consisted of the receptive emasculated flowers visited first by an insect in its visit sequence, to guarantee that there was no *N. papyraceus* pollen flow from outside the experiment.

Sample processing

We processed cross-pollination and self-pollination samples in the laboratory the same day after the diurnal sessions or on the next morning in the case of the nocturnal sessions. We used a compound microscope to count the number of pollen grains of *N. papyraceus* deposited on the stigmas. We carefully removed the stigmas from the styles and stained them with a melted fuchsine jelly (Beattie 1972) on a clean microscope slide. Due to their small size and different morphology, the pollen grains of a few other co-flowering species carried and deposited by pollinators were easily detected and discarded.

The remaining-pollen samples were immediately frozen at -80 ºC and processed later on. In these samples, we counted the total number of pollen grains that remained on the anthers in each stamen whorl. Using fine tweezers, we carefully removed the upper and lower anthers from the floral tube before defrosting and placed them separately into vials with 0.5 ml of electrolyte solution (Isoton II, Beckman Coulter Inc., Fullerton, CA). The mixture was then sonicated with an UltraSONIC8891 (Cole-Parmer, Illinois, USA) for five minutes to ensure that all pollen grains were released from the anthers. Each sample was brought to a total volume of 51 ml with Isoton II and the pollen grains were counted using a particle counter (Multisizer 3, Beckman Coulter Inc.). We ran each sample three times and calculated the average value.

Data analyses

For cross-pollination samples, we first explored the effect of the number of prior-visited flowers on the pollen transfer to receptive stigmas. To this end, we set up two

binomial variables, 'multiple donors' (i.e. one *vs.* several donors) and 'multiple emasculated' (i.e. zero *vs.* one or more emasculated flowers). We analyzed the number of cross-pollen grains deposited on receptive stigmas as a function of both explanatory variables with Generalized Linear Models (GLM) with negative binomial-distributed errors given that the data appeared over-dispersed relative to a Poisson model. Neither multiple donors nor multiple emasculated had a significant effect on the number of pollen grains deposited on receptive stigmas ($P > 0.077$). These variables were excluded in further analysis for the sake of simplicity. Then, we analyzed the number of pollen grains deposited on the receptive stigma in terms of the insect type (LT and ST), the donor floral morph (L and S) and the recipient floral morph (L and S) with a second GLM with negative binomial-distributed errors. We explored the differences among groups based on Bonferroni *post hoc* test. The control samples of cross-pollination were excluded from the analysis as they had no *N. papyraceus* pollen grains.

We employed the mean number of cross pollen grains deposited on stigmas by each insect type in the four types of crosses to examine the fulfillment of the model equations of Lloyd and Webb (1992) for the stability of stylar polymorphism in populations, which for pollen-limited conditions are:

$$\frac{1}{2}(q_{LS} + q_{SL}) > q_{LL} \quad \text{Eq. 1}$$

$$\frac{1}{2}(q_{LS} + q_{SL}) > q_{SS} \quad \text{Eq. 2}$$

and for non pollen-limited conditions:

$$q_{LS} > q_{LL} \quad \text{Eq. 3}$$

$$q_{SL} > q_{SS} \quad \text{Eq. 4}$$

where q is the number of pollen grains deposited on stigmas and the receptive morph is mentioned before the donor morph.

In self-pollination samples, we compared the number of self-pollen grains deposited on the stigmas by the two insect types in each floral morph with a GLM with a negative binomial distribution of errors, and then determined differences among groups based on Bonferroni *post hoc* test. In this case, the control group had few pollen grains, probably due to the difficulties encountered during dissection. However, the amount of self-pollen grains deposited on control samples was much lower than on visited samples (see Results section) and so we excluded the control group from the analysis.

The remaining-pollen data had a normal distribution and so we were able to use GLMs with Gaussian-distributed errors for the analysis. First, we tested whether (i) the anthers pollen load in control samples varied significantly between floral morphs and floral stems from different individuals, pooling both anthers whorls and for each whorl level separately. Since the anthers' pollen load differed only between floral morphs (see Results section) we analyzed separately for each floral morph (ii) the effect of insect type on the total remaining pollen, excluding from the analysis the factor 'individual'. The control group had similar anthers pollen loads to the flowers

Table 1. Number of samples of each type collected for all of the insect genera that contributed to the experiment. Receptive × Donor morphs (L, S) are given for each of the four types of cross pollinations. LT: long-tongued; ST: short-tongued.

		Cross-pollination				Self-pollination		Remaining-pollen	
		L-morph		S-morph		L-morph	S-morph	L-morph	S-morph
	Control	5		5		3	3	16	30
		L×L	L×S	S×L	S×S				
LT	*Pieris*	22	14	24	13	7	9	11	15
	Eucera	1	4	0	3	0	2	0	2
	Autographa	7	0	3	0	10	0	0	0
	Total	30	18	27	16	17	11	11	17
ST	*Apis*	14	1	0	0	0	3	0	3
	Andrena	4	0	1	1	0	0	0	0
	Eristalis	6	1	10	2	5	0	10	0
	Eupeodes	5	9	2	8	0	3	2	1
	Syritta	7	2	3	2	0	2	7	0
	Small flies	3	2	1	8	0	0	0	0
	Total	39	15	17	21	5	8	19	4

visited by long-tongued insects and so could be included in the analyses. Likewise, we performed Bonferroni *post hoc* analyses to assess differences among control group and groups visited by each insect type. In addition, we analyzed (iii) the effect of anther level on the remaining pollen after the visit of each insect type, pooling data from both floral morphs to increase the robustness of the analysis. Given that short-tongued insects removed more pollen from the upper anther whorl (see Results section), we repeated the (ii) analysis for each floral morph separately for the remaining pollen in the upper (iv) and lower (v) whorl levels, including the control group. All the analyses were performed with *R* 2.13, using *MASS* and *stats* libraries (R Core Team 2009).

Results

During the experiment, we collected a total of 183 cross-pollination samples with 10 controls, 41 self-pollination samples with six controls, and 51 remaining-pollen samples with 46 controls (Table 1). Short-tongued visitors mainly included bees of the genera *Apis* (*A. mellifera*) and *Andrena*, and hoverflies such as *Eristalis* (*E. tenax*), *Syritta* (*S. pipiens*) and *Eupeodes*. Long-tongued visitors included butterflies of the genus *Pieris* (*P. brassicae*), moths of the genus *Autographa* (*A. gamma*, the only nocturnal visitor), and a few visits by nectar-feeding bees of the genus *Eucera*. Due to the stochasticity and scarcity of insect visits during the experiment, most insect species did not provide samples of each type (Table 1). However, differences in morphology

and behaviour at flowers between insect types should be much greater than differences among insect species within each insect type.

Assortative and disassortative cross-pollination

The number of pollen grains deposited on stigmas after cross-pollination was significantly dependent on the insect type, on both parental morphs (donor and receptive), on the interaction of the receptive morph with the insect type, and on the three-factors interaction (Table 2). Overall, long-tongued insects (N = 91) deposited a higher number of pollen grains than short-tongued insects (N = 92; total means ± SD: 141 ± 157 *vs.* 14 ± 39 pollen grains, respectively; Figure 3). The *post hoc* comparison revealed significant differences between the number of pollen grains deposited by short-tongued insects in all types of crosses and the number of pollen grains deposited by long-tongued insects in all types of crosses ($P < 0.049$) except assortative cross-pollinations from S-morph donor flowers to S-morph receptive flowers (S×S crosses; $P = 1$; Figure 3). Long-tongued insects deposited fewer pollen grains in assortative crosses S×S than in the other three types (means ± SD: 35 ± 50 *vs.* 162 ± 163 overall L×L, L×S and S×L, respectively; $P < 0.006$), although the difference was not significant with disassortative L×S crosses ($P = 0.309$; Figure 3). Short-tongued insects deposited a negligible amount of pollen grains on the stigmas of the short-styled flowers (2 ± 5). Regardless of the insect visitor type, more pollen grains were deposited on long-styled stigmas (N = 102; 88 ± 138) than on short-styled stigmas (N = 81; 65 ± 121). Similarly, more pollen grains were deposited when the donor flower was long-styled (N = 113; 97 ± 147) than when it was short-styled (N = 70; 46 ± 91).

The terms for the model equations of Lloyd and Webb (1992) estimated for long-tongued pollinators, qLL_{LT} = 177; qSS_{LT} = 35; qLS_{LT} = 131; qSL_{LT} = 167; $1/2(qLS_{LT} + qSL_{LT})$ = 149, satisfied the conditions of equations 2 and 4 for both pollen-limited and non-pollen-limited circumstances. By contrast, the estimates for short-tongued pollinators, qLL_{ST} = 28; qSS_{ST} = 2; qLS_{ST} = 10; qSL_{ST} = 2; $1/2(qLS_{ST} + qSL_{ST})$ = 6, only met the condition of equation 2 for pollen-limited circumstances.

Table 2. Results of the GLM model for the effects of parental floral morphs and visitor type on the number of pollen grains deposited in cross-pollinations. Degrees of freedom (d.f.) and *F*-values are given. Significance: ***, $P < 0.001$; **, $P < 0.01$; *ns*, non significant.

	d.f.	*F*-value	*P*-value
Error	182		
Visitor type (V)	1	100.479	<0.001***
Receptive floral morph (R)	1	24.538	<0.001***
Donor floral morph (D)	1	18.503	<0.001***
V x R	1	10.591	0.001**
V x D	1	0.242	0.623 *ns*
R x D	1	0.290	0.590 *ns*
V x R x D	1	6.706	0.009**

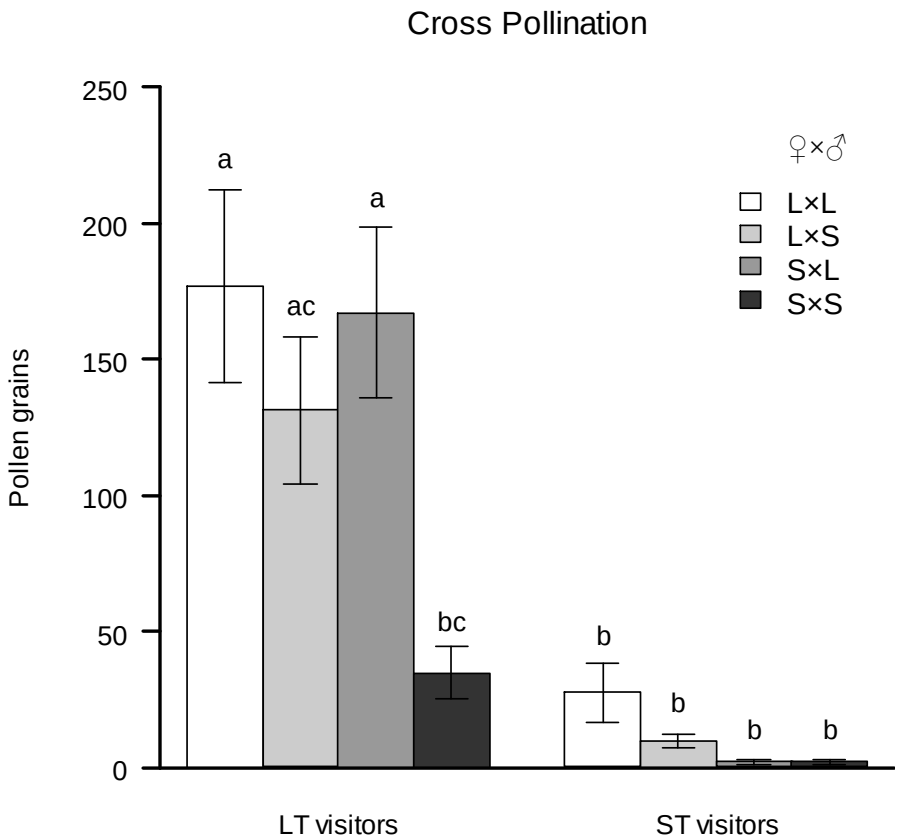

Figure 3. Pollen deposited on the stigmas of emasculated *N. papyraceus* flowers in four types of crosses between parental morphs following the action of two different insect types. Equal letters above bars indicate non-significant differences among groups based on *post hoc* analyses. Receptive × Donor morphs (L, S) are given for the four types of cross-pollinations, two assortative (L×L, S×S) and two disassortative (L×S, S×L). LT = Long-tongued insects; ST = Short-tongued insects. Average numbers of pollen grains with 95% confidence intervals are given.

Self-pollination

Control samples for self-pollination had 16 ± 18 pollen grains in the L-morph and 6 ± 10 pollen grains in the S-morph (N = 3 in both cases). In visited flowers, the floral morph and its interaction with the insect visitor type had a significant effect on the number of self-pollen grains deposited on the stigma (Table 3). Self-pollination was greatest in long-styled flowers after a visit by a long-tongued pollinator (328 ± 280 pollen grains) and in short-styled flowers after a visit by a short-tongued pollinator (201 ± 165; Figure 4). However, *post hoc* analyses only found significant differences between self-pollination conducted by long-tongued pollinators in L- and S-morphs ($P = 0.022$; Figure 4). It is worth noting that the mean (± SD) number of ovules in *N. papyraceus* is 61 ± 11 ovules (Chapter 2), thus a high proportion of them could be affected by self-pollination.

Table 3. Results of the GLM model for the effects of floral morph and visitor type on the number of pollen grains deposited by self-pollination. Degrees of freedom (d.f.) and *F*-values are given. Significance: **, $P < 0.01$; *, $P < 0.05$; *ns*, non significant.

	d.f.	*F*-value	*P*-value
Error	40		
Visitor type (V)	1	1.270	0.260 *ns*
Floral morph (M)	1	3.904	0.048*
V x M	1	9.053	0.003**

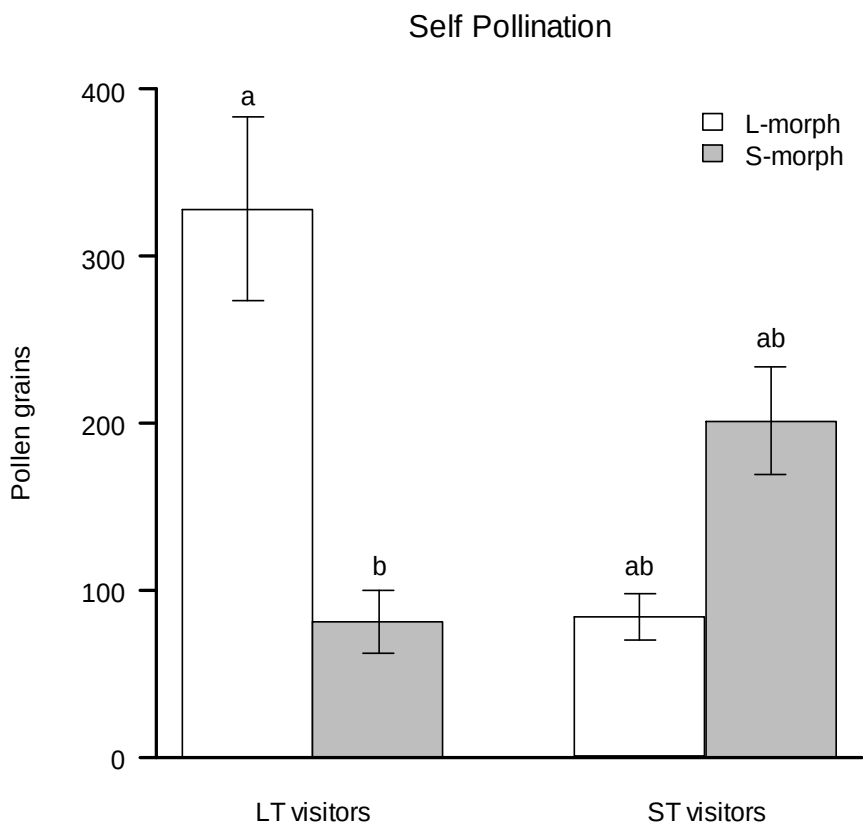

Figure 4. Self-pollen deposited on stigmas of non-emasculated *N. papyraceus* flowers of both morphs after a single visit by either a long- (LT) or a short-tongued (ST) insect. Equal letters above bars indicate non-significant differences among groups based on *post hoc* analyses. Average numbers of pollen grains with 95% confidence intervals are given.

Table 4. Results of the GLM models for the effects of (A) visitor type and (B) whorl level on remaining pollen. Degrees of freedom (d.f.) and *F*-values are given. Significance: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; *ns*, non significant. LT: long-tongued; ST: short-tongued.

A	L-morph			S-morph		
	d.f.	*F*-value	*P*-value	d.f.	*F*-value	*P*-value
Effect of visitor type						
Error	45			50		
i) Total remaining pollen	2	7.226	0.002**	2	11.951	<0.001***
ii) Upper anther whorl remaining pollen	2	11.604	<0.001***	2	10.425	<0.001***
iii) Lower anther whorl remaining pollen	2	2.581	0.087 *ns*	2	4.234	0.02*
B	LT-insects			ST-insects		
	d.f.	*F*-value	*P*-value	d.f.	*F*-value	*P*-value
Error	55			45		
Effect of whorl level on remaining pollen	1	1.842	0.18 *ns*	1	14.062	<0.001***

Remaining pollen

Preliminary analyses of the control samples of remaining-pollen detected a significant effect of the floral morph on the total anther pollen load ($F_{45,1} = 5.462$; $P = 0.024$). L-morph flowers had a lower load of pollen grains on anthers than the S-morph flowers (averages 152,320 ± 31,279 and 171,787 ± 23,874, respectively). This total difference was due to a significantly smaller load on the lower anther whorl (73,717 ± 17,547 *vs.* 86,213 ± 16,299; $F_{45,1} = 5.778$; $P = 0.021$), the upper anther whorl having comparable amounts of pollen (78,604 ± 18,106 and 85,575 ± 16,226; $F_{45,1} = 1.703$; $P = 0.199$). The number of pollen grains on each anther whorl did not differ among individuals ($F_{45,1} < 0.575$; $P > 0.452$).

We found a significant effect for the insect visitor type on the total remaining pollen for both floral morphs (Table 4A). In each case, short-tongued insects collected more pollen than long-tongued insects (total averages remaining pollen after a single visit: ST-visited flowers, 112,760 ± 39,794; LT-visited flowers, 165,342 ± 31,606). There was no significant difference between the control and LT-visited groups in the total remaining pollen for any floral morph ($P = 1$), although there were differences between ST-visited flowers and both control and LT-visited groups ($P < 0.007$; Figure 5). Short-tongued insects collected significantly more pollen from upper anthers than from lower anthers (remaining pollen after a single visit: upper whorl, 43,952 ± 24,158; lower whorl, 68,809 ± 20,664; Table 4B); long-tongued insects collected pollen equally from both anther levels (remaining pollen: upper whorl, 79,014 ± 24,363; lower whorl, 86,328 ± 14,828; Table 4B). Consequently, the separate analyses for remaining pollen on each whorl for each floral morph showed an increased effect of insect type for remaining pollen on the upper whorl in comparison with the lower anther whorl (Table 4A). There was no significant difference between the control and the LT-visited groups in any case ($P > 0.293$).

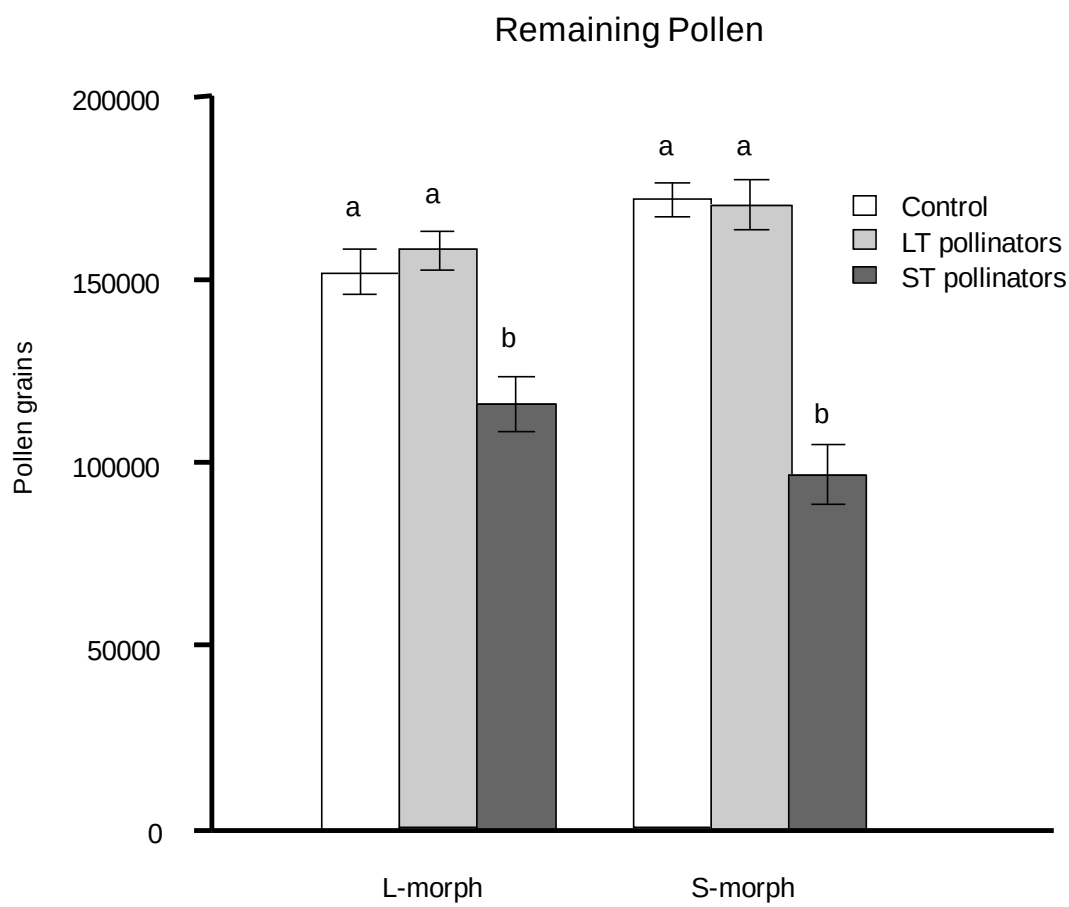

Figure 5. Total number of pollen grains remaining in flowers of *N. papyraceus* after visits of each pollinator type (LT: long-tongued insect; ST: short-tongued insect) with anther whorls pooled. Equal letters above bars indicate non-significant differences among groups based on *post hoc* analyses performed independently in each floral morph. Average numbers of pollen grains with 95% confidence intervals are given.

Discussion

Pollen transfer patterns between floral morphs has been a recurrent topic in studying heterostylous species in which pollen dimorphism facilitates the assessment of naturally occurring stigma loads. By contrast, here we studied different pollen transfer patterns mediated by differing pollinator types in style morphs of *Narcissus papyraceus* using an experimental approach that circumvents the lack of pollen dimorphism in this plant.

Assortative and disassortative cross-pollen transfer by long-tongued insects

In agreement with theoretical predictions for heterostylous species (Lloyd & Webb 1992), we found that disassortative pollination can occur between different style-length morphs of *Narcissus papyraceus* when the most effective long-tongued pollinators are involved. Long-tongued pollinators transferred similar amounts of intra- and inter-morph cross-pollen to the long-styled morph but, interestingly, favored inter-morph pollen transfer to the short-styled morph. This pattern may reflect the greater reciprocity of heights between the lower anthers of the L-morph and the stigma of the S-morph but the equal reciprocity of the L-stigma with the upper anthers of both morphs (Baker et al 2000a, Cesaro & Thompson 2004). Due to the presence of two stamen whorls, the pattern resembles the situation in heterostylous species (Ganders 1976, Olesen 1979, Schou 1983, Wolfe & Barrett 1989, Washitani *et al.* 1994, Ornelas *et al.* 2004, Hernández & Ornelas 2007, Wolfe *et al.* 2009), in which the short-styled morph usually receives more legitimate pollen than the long-styled morph, and confirms the assertion of Stone and Thomson

(1994) that disassortative pollination can occur between non-reciprocal herkogamous floral morphs.

Our data on assortative and disassortative pollen transfer by long-tongued pollinators satisfied the conditions defined by Lloyd and Webb (1992) for the appearance of the S-morph in populations and hence partially support the occurrence of stylar dimorphism in *N. papyraceus* despite the lack of any heteromorphic incompatibility system. The fulfillment of the equations for both pollen-limited and non pollen-limited conditions enhances the significance of this pattern given the variable pollination conditions of natural populations in winter-flowering *Narcissus* (Baker *et al.* 2000b). However, similar assortative and disassortative pollen transfer to the long-styled morph would explain the dominance of such morph in most dimorphic populations of *N. papyraceus* and would facilitate the reversion to L-monomorphism in some of them (Arroyo *et al.* 2002). Cesaro and Thomspon (2004) examined seed production in experimental arrays and reported a similar pattern in style-dimorphic *N. assoanus*, though in their study only the equation 3 of the Lloyd and Webb's model (1992) was not met. Thus, it is not surprising that monomorphism has never been reported in their species (Thompson *et al.* 2012). Hence, the maintenance of stylar dimorphism through appropriate levels of disassortative mating might in fact be commoner than expected, thereby explaining the prevalence of this mating system in low reciprocal *Narcissus* species (Barrett *et al.* 1996, Barrett & Harder 2005, Chapter 6). In other unrelated style-dimorphic species, reciprocity is apparently a key factor in determining possibilities of disassortative mating (Ferrero *et al.* 2011), although this possibility still needs to be tested directly. In *N. papyraceus* the disposition of sexual organs in floral morphs implies that other

factors than cross-pollination patterns should account for the maintenance of stylar dimorphism.

One such additional factor could be the observed patterns of self-interference. The advantage of the L-morph given the cross-pollen transfer patterns under the action of long-tongued visitors in our study may be offset by the fact that long-tongued pollinators induced higher levels of self-pollination in the L-morph, probably due to direct body contact with the upper anthers and L-stigma. Although herkogamy cannot completely prevent self-interference of sexual organs in heterostylous species either (Ornduff 1975a, 1979, Thomson & Thomson 1989, Schlindwein & Medeiros 2006), the negative effect of self-interference through self-pollination may be increased in *N. papyraceus* given its late-acting incompatibility system. This allows self-pollen to enter the micropyle and render ovules ineffective, a mechanism for ovule discounting (Chapter 2) that has been shown to exist in other *Narcissus* species (Dulberger 1964, Sage et al. 1999). Hence, long-tongued insects may establish an equilibrium in the mating success of both floral morphs.

Pollination efficiency of short-tongued floral visitors

Unlike long-tongued insects, short-tongued insects were ineffective pollinators in the long-styled morph but, notably, were also incapable of pollinating the S-morph stigmas, hence compromising the female function of these individuals. In addition, short-tongued insects induced higher levels of self-pollination in the S-morph, probably due to flower shaking (as they manipulate flowers to collect pollen) and short-level pollen drop, thus depleting the fecundity of the S-morph doubly.

Moreover, while nectar foraging caused long-tongued pollinators to remove pollen unintentionally and thus in small amounts, the feeding requirements of short-tongued insects implied the deliberate removal of pollen grains. Thus, short-tongued insects exhibited a role as pollen thieves (Wilson & Thomson 1991, Hargreaves *et al.* 2009) and could also affect significantly the male function of individuals of both morphs (Lau & Galloway 2004, Santos-Gally *et al.* 2013). In fact, on a number of occasions during the experiment, we observed how hoverflies foraged the entire pollen load of the upper anther whorl and then left the flower immediately after cleaning their bodies (Holloway 1976). It is worth noting that in flowers visited by short-tongued insects the function of the low-level sexual organs is compromised (Beach & Bawa 1980, Baker *et al.* 2000a). The feeding of short-tongued insects on the upper anther whorl may suppose a disadvantage for the long-styled morph due to the reduction of the same-level pollen available for its pollination. However, such disadvantage may be compensated by their restricted pollination ability. As a consequence, in some L-monomorphic populations of *N. papyraceus*, the low efficiency of the short-tongued insects that are the most frequent visitors could still be sufficient to guarantee the reproductive success of plants. More work on the role of both pollinator types in wild populations and actual pollinator frequencies is required to ascertain the net mutualism-antagonism balance.

Conclusions

The pollination efficiency of long- and short-tongued insects in differing style-length morphs has often been invoked as the selective force determining the morph ratio in populations of *Narcissus papyraceus* (Arroyo *et al.* 2002, Pérez-Barrales *et al.* 2007, Santos-Gally *et al.* 2013). It has been proposed that, while long-tongued pollinators

maintain dimorphism in populations through increased rates of disassortative pollen transfer, a greater proportion of short-tongued insects leads to the loss of the S-morph by reducing its female fitness. In this study, we have highlighted that the mechanism of pollinator-mediated selection of morph frequency is somewhat more complex than previously hypothesized.

Most of pollination studies in style-polymorphic species stress the fact that the mutualism is quite generalist and that functioning is mostly depending on sex organ reciprocity, based on overall pollen deposition or seed set patterns (e.g. Ganders 1974, Ferrero *et al.* 2011). However, when a diversity of pollinator types does exist in a given system, we believe that there is still a need to investigate in greater depth the function of different floral visitors as a complement to pollinator censuses and overall pollen deposition. Our approach has demonstrated the contrasting roles of different insect types in the pollination of the different style-length morphs of *Narcissus papyraceus*: while long-tongued insects promote disassortative pollination to the S-morph, even in the absence of perfect reciprocity, and meanwhile they increase self-interference in the L-morph, short-tongued pollinators could suppress the female fitness of the S-morph by depositing more self- than cross- pollen. Hence, the relative proportion of each pollinator type in a given population should strongly determine the frequency of the S-morph. We thus provide clear empirical support for the Darwinian hypothesis of the evolution of stylar polymorphism based on the effect of pollinators in each morph.

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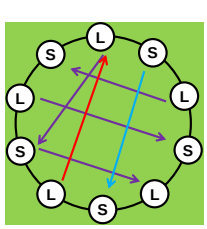
CAPÍTULO 6

LOS CRUZAMIENTOS ENTRE MORFOS PREVALECEN EN LA ESPECIE DIMÓRFICA ESTILAR SIN RECIPROCIDAD SEXUAL *Narcissus papyraceus* (AMARYLLIDACEAE)

DISASSORTATIVE MATING PREVAILS IN NON-RECIPROCAL STYLE-DIMORPHIC *Narcissus papyraceus* (AMARYLLIDACEAE)



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Resumen

La presencia de polimorfismos fenotípicos dentro de las poblaciones ha desafiado a los ecólogos evolutivos desde hace mucho tiempo. En las flores hermafroditas, uno de dichos polimorfismos es la aparición de morfos sexuales cuya presencia depende de los cruces entre individuos de distinto *vs.* del mismo morfo. En las especies heterostilas los morfos florales promueven la polinización entre ellos mediante la colocación de sus estigmas y anteras en posiciones recíprocas. Algunas especies presentan dimorfismo estilar sin una colocación recíproca de las anteras y se consideran un paso intermedio en la evolución del monomorfismo a la heterostilia. No obstante, se ha planteado que el dimorfismo estilar también se mantiene gracias a los cruzamientos entre los individuos de distintos morfos. Además, se ha planteado que la variación en la tasa de cruzamientos entre morfos y diferencias en la fecundidad o el éxito de padreamiento entre los morfos florales son responsables de la variación en la proporción de los mismos. Para evaluar estas hipótesis, investigamos los patrones de cruzamiento, fecundidad y éxito de padreamiento de los morfos estilares en *Narcissus papyraceus*, una especie con poblaciones dimórficas (morfos longistilo y brevistilo) y monomórficas (morfo longistilo) en distintas regiones dentro de su área de distribución. Establecimos poblaciones experimentales con diferentes proporciones de morfos en dos sitios distintos dentro de las regiones naturales dimórfica y monomórfica, exponiéndolas a los polinizadores naturales. Usamos análisis de paternidad basados en microsatélites para medir el éxito de padreamiento de los morfos y la tasa de cruzamientos entre morfos. Se obtuvo un éxito de padreamiento similar en ambos morfos florales y altas tasas de cruzamientos entre morfos en la mayoría de las poblaciones. Estimamos la fecundidad y exploramos el rendimiento de la progenie de ambos morfos. Los morfos florales

exhibieron una fecundidad similar en todas las poblaciones. Las plántulas de las madres brevistilas y de los cruzamientos entre morfos rindieron mejor que las de las madres longistilas y de los cruzamientos entre individuos del mismo morfo, respectivamente. Aunque los resultados no pudieron explicar por completo la pérdida de dimorfismo en el norte del área de distribución de la especie, proporcionaron evidencia experimental directa de la estabilidad evolutiva del dimorfismo estilar en *N. papyraceus*. Estos resultados refuerzan la idea darwiniana de que la prevalencia de los cruzamientos entre morfos es clave para el mantenimiento de dimorfismo estilar.

Palabras clave: análisis de paternidad, cruzamientos entre morfos, dimorfismo estilar, éxito de padreamiento, fecundidad femenina, *Narcissus*, proporción de morfos.

Abstract

The presence of phenotypic polymorphisms within populations has long challenged evolutionary ecologists. In hermaphroditic flowers, one such polymorphism is the occurrence of mating types that depend on disassortative (inter-morph) *vs.* assortative (intra-morph) mating. In heterostylous species different morphs enhance disassortative pollination through reciprocal placement of stigmas and anthers. Some species display stylar dimorphism without reciprocal anther placement and are considered an intermediate step in the evolution from monomorphism to heterostyly. However, it has been hypothesized that stylar dimorphism is also maintained due to disassortative mating. In addition, it has been hypothesized that variable rates of disassortative mating along with differential fecundity or siring

success among floral morphs are responsible for variation in morph ratio. In order to evaluate these hypotheses, we investigated mating patterns, fecundity and siring success of style-length morphs in *Narcissus papyraceus*, a species with dimorphic (long-styled and short-styled) and monomorphic (long-styled) populations in contrasting regions of its distribution area. We established experimental populations with different morph ratios in two different sites within naturally occurring dimorphic and monomorphic regions and exposed them to ambient pollinators. We used microsatellite-based paternity analysis to measure the siring success of morphs and the extent of disassortative mating. Similar siring success of floral morphs and increased rates of disassortative mating occurred in most populations. We estimated fecundity and explored the performance of progeny from different parental morphs. Floral morphs exhibited similar fecundity in all populations. Seedlings from S-maternal parents and from disassortative mating events performed better than those from L-maternal parents and assortative mates, respectively. Although these results could not completely explain the loss of dimorphism in the species' northern range, they provided direct experimental evidence for the evolutionary stability of stylar dimorphism in *N. papyraceus*. These results reinforce the Darwinian idea that prevailing inter-morph mating is key for the maintenance of stylar dimorphism.

Keywords: disassortative mating, female fecundity, morph ratio, *Narcissus*, paternity analysis, siring success, stylar dimorphism.

Introduction

Sexual polymorphisms have arisen multiple times along the evolution of flowering plants in response to selection favouring outcrossing among other factors (Darwin 1877, Barrett 2002). Dioecy is an extreme sexual polymorphism for enforcing outcrossing, but hermaphrodites can also manifest outcrossing through physiological incompatibility (Castric & Vekemans 2004), contrasting phenology (Renner 2001) and variable positioning of sex organs in different floral morphs (Barrett *et al.* 2000). The outcome of such processes is the presence of mating morphs within populations, whose maintenance depends on the prevalence of mating between morphs, i.e. disassortative, over mating within morphs, i.e. assortative (Eckert *et al.* 1996, Pannell *et al.* 2005). Disassortative mating is a major mechanism of negative frequency dependent selection that increases the mating opportunities of the less frequent morph in a population, leading to the equilibrium ratio of floral morphs (Fisher 1930, Heuch 1979, Barrett *et al.* 2004). On the other hand, many sexual polymorphisms in hermaphroditic plants do not avoid assortative mating completely and this may lead their populations to vary in morph ratio. It is expected that an increase in assortative mating in one morph would raise its frequency in a population, which in turn would eventually drive that population towards monomorphism (Baker *et al.* 2000a). It has also been suggested that transitory differences in fecundity or siring success among floral morphs could be the factors contributing to variation in morph ratio (Eckert & Barrett 1995, Baker *et al.* 2000a, Hodgins & Barrett 2008, Pérez-Barrales & Arroyo 2010).

Heterostyly is a sexual polymorphism in which two or three morphs differ in the position of sexual organs, which are placed reciprocally in the different floral

morphs. In this way, morphs have enhanced pollen transfer between them through precise pollen delivery and deposition by pollinators (Darwin 1877, Barrett & Shore 2008). Typically, this morphological polymorphism is accompanied by a physiological heteromorphic incompatibility system which impedes self- and within-morph mating success (Ganders 1979), although exceptions occur (Casper 1985, Eckert & Barrett 1994, Ferrero *et al.* 2012). Stylar dimorphism, a condition similar to heterostyly, consists of the occurrence of two floral morphs that differ in the position of the stigma (above or below the anthers), while the anthers maintain a constant position across floral morphs. Thus, style-dimorphic plants lack the reciprocity between sex organs displayed by typical heterostylous species. Most style-dimorphic species also lack a heteromorphic incompatibility system (Barrett 1992). The evolutionary models of Charlesworth and Charlesworth (1979) and later Lloyd and Webb (1992) propose that stylar dimorphism represents an intermediate step in the evolution from monomorphism to heterostyly. In particular, Lloyd and Webb (1992) suggested that floral morphs are maintained in populations if disassortative mating results in greater reproductive success than assortative mating, i.e.

$$\frac{1}{2}\left(q_{LS} + q_{SL}\right) > q_{LL} \qquad \text{Eq. 1}$$

$$\frac{1}{2}\left(q_{LS} + q_{SL}\right) > q_{SS} \qquad \text{Eq. 2}$$

under pollen-limited conditions, or

$$q_{LS} > q_{LL} \qquad \text{Eq. 3}$$

$$q_{SL} > q_{SS} \qquad \text{Eq. 4}$$

under non pollen-limited conditions. In these equations, *q* represents the reproductive success, and the subscripts *L* and *S* represent long-styled and short-styled floral morphs respectively, the maternal morph being in first position. Due to the lack of reciprocity in spatial placement of sexual organs and absence of heteromorphic incompatibility, stylar dimorphism could be unlikely to meet the requirements above, and the polymorphism would be unstable. In fact, stylar dimorphism, which has been reported in six families of angiosperms, is much less common than heterostyly, which has been reported in 28 families (Barrett & Shore 2008).

In spite of its overall rarity, stylar dimorphism is widespread in the Mediterranean genus *Narcissus* (Barrett & Harder 2005). This observation seems to contradict the model proposed by Lloyd and Webb (1992) and might result from the effectiveness of stylar dimorphism in promoting disassortative mating in the genus in the absence of heteromorphic incompatibility (Cesaro & Thompson 2004). On the other hand, population morph ratios show considerable variation in style-dimorphic *Narcissus* species (Arroyo & Dafni 1995, Barrett *et al.* 1996, Baker *et al.* 2000b, Arroyo *et al.* 2002, Thompson *et al.* 2012, Santos-Gally *et al.* 2013). In order to explain morph ratio variation in *Narcissus*, some studies have investigated the association between morph ratio and differential maternal fitness between floral morphs (Baker *et al.* 2000a, Pérez-Barrales & Arroyo 2010). However, total fitness also includes a paternal component and a full understanding of selection driving morph ratios requires that both maternal and paternal fitness are taken into account. To date, the assessment of the relationship between morph ratio and mating patterns in *Narcissus* is mostly based on indirect procedures (Thompson *et al.* 2003, Cesaro & Thompson 2004, Pérez-Barrales & Arroyo 2010). However, the increasing use of paternity analysis to investigate gender-based patterns of reproductive success in natural populations (e.g.

Meagher 1986, Smouse *et al.* 1999, Wright & Meagher 2004, Kitamoto *et al.* 2006, Rosas & Domínguez 2009, see also Kulbaba & Worley 2012) yields a more complete picture of the relationship between reproductive success and morph ratio variation (Hodgins & Barrett 2008).

Due to the widespread occurrence of stylar dimorphism in *Narcissus*, this genus represents a good system for investigating the relationship between stylar dimorphism and reproductive success. However, no study to date has dealt with each involved factor: female fitness, male fitness, mating patterns and their relationship with population morph ratio in a style-dimorphic plant. To undertake this aim, the present study focusses on *Narcissus papyraceus* Ker-Gawler, a winter-flowering, style-dimorphic geophyte whose floral morphs present very low reciprocity between stigma and anthers placement. The flowers present two stamen whorls (upper and lower) and stigmas in different morphs occur at different heights, above or below the lower stamen whorl. In long-styled (L-) flowers, the lower stamen whorls are slightly shorter than those of short-styled (S-) flowers (Pérez-Barrales & Arroyo 2010). Both morphs co-occur in populations around the Strait of Gibraltar, but in the northern limit of the species range (Guadalquivir Basin, in SW Spain) populations are monomorphic for the L-morph (Arroyo *et al.* 2002). The species has a late acting self-incompatibility system (Chapter 2) that permits either between- and within-morph fecundation (Arroyo *et al.* 2002), and a long and narrow floral tube with nectaries at the bottom that suggests an adaptation to pollination by long-tongued insects (Pérez-Barrales *et al.* 2007). Nevertheless, a variety of pollinators visit flowers of *N. papyraceus*, including long-tongued butterflies and moths and short-tongued hoverflies and bees (Pérez-Barrales *et al.* 2007, Santos-Gally *et al.* 2013). There is some evidence that short-tongued pollinators are more common in the monomorphic region in southwest Spain (Santos-Gally *et al.* 2013), which might have led to the disappearance

of the S-morph by two processes. First, short-tongued pollinators might be unable to reach the stigma of S-flowers, restricting their female fecundity. Second, these pollinators might change pollen transfer patterns between morphs, increasing assortative mating in the L-morph (Pérez-Barrales & Arroyo 2010). The maintenance of the stylar dimorphism in populations around the Strait of Gibraltar could be due to higher rates of disassortative mating in that region owing to the prevalence of long-tongued pollinators.

We report on an experiment to assess the maternal and paternal fitness of each floral morph and mating success within and between style-length morphs in *N. papyraceus* after open pollination in the field in contrasting regions of its distribution area, i.e. dimorphic and monomorphic natural regions. We compared the maternal and paternal fitness of floral morphs in terms of fecundity and siring success and estimated levels of assortative and disassortative mating to evaluate Lloyd and Webb's model for the maintenance of stylar polymorphism. We also compared progeny performance at early stages of development as an additional component of fitness of floral morphs (e.g. post-dispersal mating success). Our experimental approach included experimental populations with genotyped plants, which increased the probability of paternity assignment while allowing us to test the effect of different morph ratios on mating patterns. Ultimately, we aimed to determine whether stylar dimorphism, despite limited reciprocity of sex organs, can lead to disassortative mating, and whether variation in the extent of disassortative mating accounts for the absence or presence of stylar dimorphism in the monomorphic and dimorphic regions of *N. papyraceus* distribution area.

Material and Methods

Source material and parental genotyping

In December 2008, we collected 150 *Narcissus papyraceus* individuals from a single population close to Tarifa (Cádiz province, S Spain; 36°6' N, 5°44' W). This population is located in the center of the distribution range of the species and is isoplethic (equal proportions of L- and S-morph), large (thousands of plants) and harbours a high genetic diversity (H_s = 0.75; Chapter 4). We collected individuals separated by at least two meters from each other to ensure collecting different genets, given the intense vegetative reproduction but low bulb dispersal of the species. Individuals were collected after the end of the flowering period to diminish plant stress. Thus, we determined the morph from the wilted flowers to collect equal numbers of the two style-length morphs. Plants were labeled, potted and moved to a glasshouse at the University of Seville. Plants were watered at levels consistent with natural conditions until the end of winter and kept in darkness during summer. This procedure was repeated for two years until massive flowering occurred in winter 2010–2011. In November 2010, we collected leaf samples from each individual, which were immediately frozen at -80 ºC. DNA was isolated following Bernartzky and Tanksley's (1986) protocol without mercaptoethanol, and each individual was genotyped for four specific microsatellite markers which had high genetic variability and good amplification rates (A116, A121, B104 and B112; Simón *et al.* 2010). Polymerase Chain Reactions (PCR) were performed in 25 μL of reaction mixture containing 50 ng of template DNA, 1 × PCR buffer, 1.5 mM MgCl2, 0.1 μM fluorescently labeled (6-FAM™, VIC®, NED™ and PET® dyes) forward primer, 0.1 μM reverse primer, 0.05 mM each dNTP and 1.25 U Taq polymerase. PCRs were

performed in a Biometra Gradient Thermal Cycler (Biometra, Göttingen, Germany), with an initial 5 min of denaturation at 94 °C, 45 cycles at 94°C for 30 s, annealing at 58 ºC (markers A116 and A121) or 59 ºC (markers B104 and B112) for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Polymerase chain reaction products were analyzed on an ABI 3130 × 1 Genetic Analyzer and sized using GeneMapper v.4.0 (Applied Biosystems, Foster City, USA) and GeneScanTM 500 LIZ size standard.

Field experiment

The field experiment was performed during the flowering season 2010–2011. We set up a total of 15 artificial populations in two sites within the dimorphic and the monomorphic natural distribution regions of the species (Figure 1a). In the dimorphic region, experimental plots were located at Finca de la Alcaidesa (36º18' N, 5º22' W; Alcaidesa hereafter), 16 km from the closest known naturally occurring population. In the monomorphic region, experimental plots were located at Pinares de Hinojos (37º17' N, 6º25' W; Hinojos hereafter), 8 km from the closest known naturally occurring population.

Three types of populations were established at each site: Isoplethic, L-biased and S-biased populations, with proportions of 1:1, 3:1 and 1:3 of L- to S-styled plants, respectively (Figure 1b). Each population was composed of 8–12 individuals, with morphs alternating in a circular pattern in order to ensure regular distances between individuals (Figure 1b). Plants were selected to ensure synchronic flowering and high multilocus microsatellite diversity for marker-based paternity assignment within populations. For each experimental population, the number of alleles with the

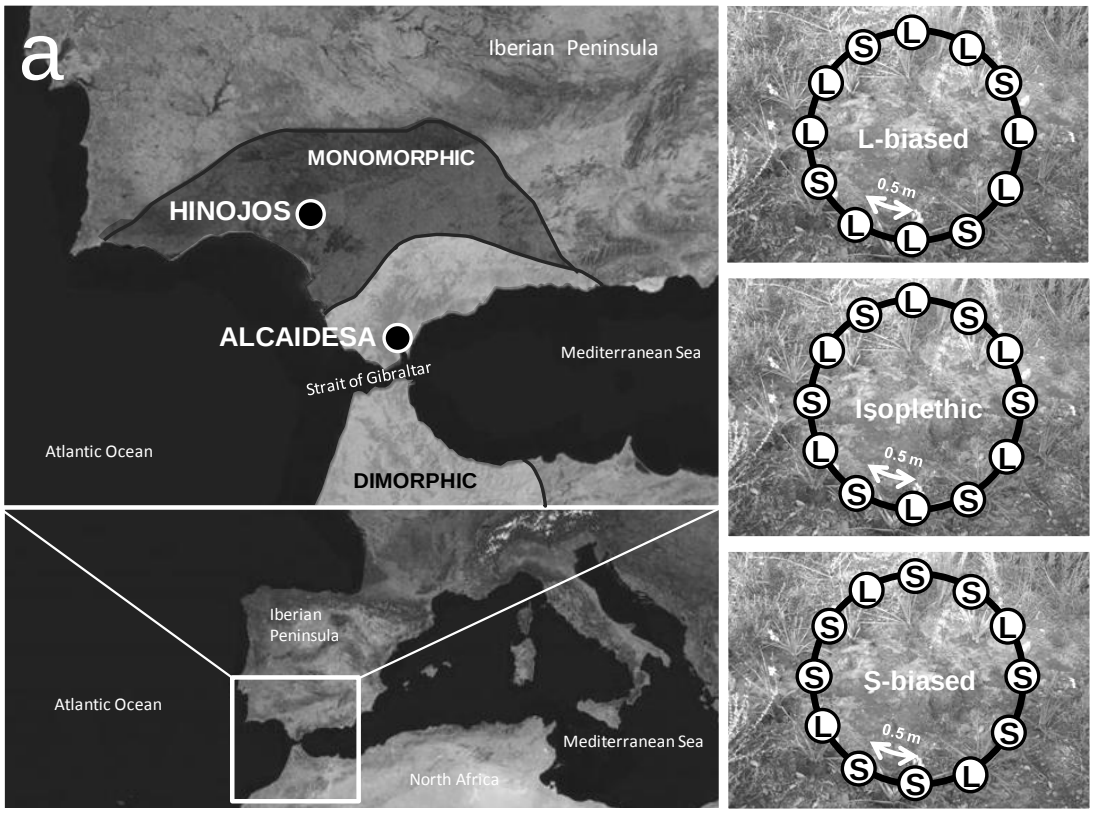

Figure 1. (a) Main distribution range of *Narcissus papyraceus* and experimental sites in the monomorphic and dimorphic regions of the species distribution range. (b) Arrangement of experimental population types with different morph ratios. L, L-morph; S, S-morph.

four markers ranged from 24 (Population 5) to 47 (Population 11). The populations were set up to be synchronous with the natural flowering period of natural populations at each region to ensure similar pollinators to them. At Alcaidesa, three replicates of each experimental population type were maintained from 23 December 2010 until 21 January 2011. The means of maximum and minimum daily

temperatures during this period were 16.9 ºC and 9.7 ºC, respectively, and the mean daily rainfall was 35.8 mm (Spanish National Meteorological Agency 2012). At Hinojos, two replicates of each experimental population type were maintained from 11 until 31 January 2011 with mean maximum and minimum temperatures of 14.9 ºC and 6.3 ºC, respectively, and mean daily rainfall of 2.7 mm (Spanish National Meteorological Agency 2012). These values were within the normal ranges of climatic conditions in the flowering period of each region in a period of 50 years excepting significantly higher rain rates at Alcaidesa during the experiment (mean daily max.temp/min.temp/rainfall; Alcaidesa: 15.9 ºC / 8.2 ºC / 4.2 mm; Hinojos: 14.3 ºC / 6.4 ºC / 2.2 mm; Climatic Data from WorldClim database, Hijmans *et al.* 2005). Populations within each site were located at least 300 meters apart from each other to prevent pollen flow between them (Barthelmess *et al.* 2006). We counted the number of flowers per individual 2–4 times in each population to estimate the total number of flowers during the experiment. Populations were exposed to natural pollinators at each site. Plants were removed from the field after flower withering and kept in the greenhouse until fruit maturation.

Three populations were damaged by wild boars: one isoplethic and one L-dominant replicate at Alcaidesa, and one L-dominant replicate at Hinojos. These populations were removed from the study leaving 12 populations in our final analysis. Some flowers and fruits were also grazed by insects or snails (0.04% of total number of flowers) and were discarded for the female fecundity analysis. Individuals affected by herbivory in half or more of the flowers (0.05% of total number of individuals) were not included in the female fecundity analysis, but all individuals were included in the paternity analysis.

Female fecundity

In March 2011, we collected all matured fruits. For each maternal individual we counted fruits and seeds per fruit and estimated fruit set, the average number of seeds per fruit and total seed production per plant as female fecundity measures. Fruit and seed production were strongly dependent on flower position. Seed production of the first flower was statistically indistinguishable from that of the second, third and fourth flowers ($F_{7, 948} = 8.01$; $P > 0.27$; ANOVA and Tukey HSD), but was significantly different from that of the fifth to eighth flowers ($P < 0.04$). Thus, we only used the four first flowers per inflorescence to calculate female fecundity. We performed Generalized Linear Models (GLM) for the fixed effects of floral morph (L-morph and S-morph), morph ratio (isoplethic, L-biased and S-biased) and region (Alcaidesa and Hinojos) on fruit set, the average number of seeds per fruit and total seed production. We employed a binomial distribution of errors to analyze fruit set and Gaussian distribution of errors to analyze the average number of seeds per fruit and total seed production. Analyses were conducted with the package *stats* of the software R 2.13.1 (R Core Team 2011).

Progeny experiment and genotyping

Seeds were stored at 4 ºC in darkness until the next season. In November 2011, we sowed a total of 2,566 seeds from 315 fruits in trays (30 × 50 cm) with a mixture of peat and vermiculite grade 2 (3:1; PROJAR, Málaga, Spain), which were watered to field capacity twice a week for 18 weeks until germination and growing was stopped in March 2012. Fruits from each individual were distributed haphazardly in trays, whose position was changed weekly to homogenize growing conditions. Seed germination, seedling height, anomalous growth (i.e. distorted or discolored leaves)

and survival were monitored weekly during the entire experiment. To ensure enough DNA material, we selected the tallest seedling of each fruit (N = 306). Since germination percentage and maximum height did not differ between maternal style morphs or mating combinations (see Results section), sampling bias was regarded as negligible. The aboveground fractions of seedlings were dried in silicagel, and DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Chatsworth, CA, USA). Seedling genotyping for the four markers (A116, A121, B104 and B112) was performed under the same conditions as for parents but with an annealing temperature of 50 ºC. Including parental individuals, we genotyped 40 individuals twice to estimate genotyping error per locus, which was 1.4 ± 0.2%.

Paternity analysis and deviations from random mating

As all experimental populations were dimorphic, four types of crosses were possible: L×L, L×S, S×S and S×L (hereafter, we refer to the maternal morph in first position). We analyzed the deviation of the observed number of crosses of each type in each population from the expected number under the null model of random mating. The observed number of crosses of each mating combination in each population was determined with paternity exclusion analyses. In order to maximize precision and percentage of assignment, we combined direct and probabilistic assignments of seedlings. First, progeny and parental genotypes were compared to categorically assign a single paternal parent to all possible seedlings in each population. Autogamy was considered as some flowers set seeds by selfing (Arroyo *et al.* 2002). From the 306 genotyped seedlings, we could assign directly a total of 217 seedlings (71%) to a single paternal parent. Seedlings that remained unassigned were subjected to exclusion analysis with the software CERVUS 3.0 (Marshall *et al.* 1998), which

assigned categorically the most probable paternal parent to each seedling with 80% of confidence based on a log-likelihood ratio (Meagher 1986). We ran separate analyses for each population, simulating 10,000 offspring with 100% candidate paternal parents sampled and allowing for selfing. The mistyping error rate was set as the estimated genotyping error (0.014), and a minimum of two loci typed was required. CERVUS assigned probabilistically 44 seedlings (14%) to a single father. Hence, in this study we assigned a total of 261 seedlings (85%) with an overall assignment probability of 96.8%. A total of 18 seedlings (7.4 %) from 11 maternal parents were self-fertilized, being 15 seedlings from S-morph mothers. Self-fertilized seedlings were discarded for the analyses of mating patterns between floral morphs, which were performed on 7–39 assigned seedlings per population (N = 243 in total). Final contingency tables with the observed number of crosses of each mating combination in each population were obtained including the seedlings assigned directly and probabilistically to a single paternal parent.

The expected number of crosses between i and j morphs in the population p (E_{ijP}) under random mating was estimated as:

$$E_{ijP} = S_P \times \left(\frac{F_{iP} \times F_{jP}}{F_P}\right) \qquad \text{Eq. 5}$$

were S is the number of seeds assigned to a paternal parent and F is the number of flowers, which in the case of random mating determines the frequency of crosses for each combination. In each population, we performed a X^2 test with 10,000 permutations to determine the goodness-of-fit between the observed and expected distributions and its significance. Analyses were performed with the package *stats* of the software R 2.13.1 (R Core Team 2011). Finally, to evaluate the model equations

of Lloyd and Webb (1992) in each experimental population, we employed the observed number of seedlings from each cross combination as *q* values in equations 1, 2, 3 and 4.

Siring success modelling

We used the software PatQuest 4.0 (Meagher 2002) to directly estimate the effect of the parental morphs on mating success in each population. The software employs maximum likelihood to estimate the relative siring success of each individual male (λ_i, i=1, …, number of males), and the effect of phenotypic characters (β_k, k=1, …, number of phenotypic characters evaluated) and maternal-paternal interactions (γ) such as distance between mating pairs, using univariate or multivariate log-linear models (Smouse & Meagher 1994, Smouse *et al.* 1999, Wright & Meagher 2004). We conducted four analyses. Univariate models were employed to investigate (1) the variation in λ_i among individuals and (2) the effect of the spatial distance between mating pairs (γ_s). Multivariate models were applied to assess (3) the joint effect of the number of flowers per individual (β) and the interaction between mating-pair morphs (γ_m) in the mating success, and (4) the joint effect of the λ distribution and the interaction between mating-pair morphs (γ_m).

The interaction between mating-pair morphs (γ_m) was evaluated using a morph distance between individuals, with a distance of 0 for individuals of the same morph and a distance of 1 for individuals of different morph. In all cases but (4) (Meagher 2002), we assessed the significance of the estimated parameters by feature-based permutations (Smouse *et al.* 1999) and by pedigree-based bootstrap (Morgan & Conner 2001) with 1,000 iterations. As we did with female fecundity measures, we performed a GLM for the fixed effects of floral morph (L-morph and S-morph), morph ratio (isoplethic, L-biased and S-biased) and region (Alcaidesa and Hinojos)

on siring success of individuals (λ), using Gaussian distribution of errors with the package *stats* of the software R 2.13.1 (R Core Team 2011).

Progeny performance

We analyzed the variation in the performance of seedlings from different maternal morphs and mating combinations. Some seedlings exhibited anomalous growth (2.4% overall) or died (1% overall) so these seedlings were excluded from the following analyses. From the remaining number of seeds sown (N = 2,492) we estimated sibship performance for each of 89 maternal individuals (range 1–104 seedlings per maternal sibship). We estimated percentage of germination and average germination time of seedlings of each maternal sibship. We adjusted a logistic model with a spline to the growth curves of the seedlings as implemented in the R package *grofit* (Kahm *et al.* 2010). We extracted the parameters Λ (length of lag phase), μ (maximum growth rate), A (maximum growth) and I (the integral) from the models for each seedling. Seedlings germinated from the second to the sixteenth week, hence the growth curve parameters were strongly dependent on germination time. Growth curve parameters of seedlings germinated in the first month of the experiment differed significantly from those of seedlings germinated in the second, third and fourth month ($F_{3,255} > 43.71$; $P < 0.001$; ANOVA and Tukey HSD). Hence, we estimated the average parameters for each maternal sibship with the seedlings germinated in the first month (weeks 1–4). We performed GLMs with Gaussian-distributed errors for the fixed effect of the maternal morph (L-morph and S-morph) on each parameter of maternal sibship performance: percentage of germination, average germination time, average length of lag phase, average maximum growth rate, average maximum growth and average integral.

With the subset of the seedlings that were assigned to a paternal parent (N = 243) we also analyzed the effect of both maternal and paternal morphs on the four growth curve parameters (Λ, μ, A, I). Since the tallest seedlings were selected for genotyping, there were no differences among the growth curves of seedlings germinated at different times along the experiment (germination range 2–10 weeks; $F_{2,109} < 0.49$; $P > 0.61$; ANOVA and Tukey HSD), thus we pooled data from all seedlings. We estimated the average parameters for each combination of maternal individual and paternal morph (range 1–8 seedlings per combination). We performed GLMs with Gaussian-distributed errors for the fixed effects of the maternal and paternal morphs on the four growth curve parameters (Λ, μ, A, and I). Bonferroni *post hoc* test were conducted to assess the difference between mating combinations when the interaction of maternal and paternal morph had a significant effect on the response variable.

Results

Female fecundity

Floral morph, morph ratio and region did not have a significant effect on fruit set, average number of seeds per fruit and total seed production (Table 1). Only the interaction among floral morph, morph ratio and region had a significant effect on fruit production (Table 1). The mean (± SD) number of fruits produced per individual was 1.6 ± 1.3, the average number of seeds per fruit was 9.0 ± 6.2 and total seed production was 14.5 ± 14.9 (Table S1).

Table 1. Results of the Generalized Linear Models for the fixed effects of floral morph, morph ratio and region on three female fecundity measures: fruit set, average number of seeds per fruit and total seed production per plant. Degrees of freedom (d.f.) and *F*-values are given. Significance: *, $P < 0.05$; *ns*, non significant.

Factor	Fruit set		Average number of seeds per fruit		Total seed production	
	d.f.	*F*-value	d.f.	*F*-value	d.f.	*F*-value
Floral Morph (FM)	1	1.480 *ns*	1	0.002 *ns*	1	1.628 *ns*
Morph Ratio (MR)	2	1.761 *ns*	2	0.803 *ns*	2	0.964 *ns*
Region (RE)	1	0.141 *ns*	1	0.039 *ns*	1	0.001 *ns*
FM × MR	2	0.989 *ns*	2	0.039 *ns*	2	1.266 *ns*
FM × RE	1	3.122 *ns*	1	2.339 *ns*	1	0.077 *ns*
MR × FM	2	0.236 *ns*	2	0.624 *ns*	2	0.099 *ns*
FM × MR × RE	2	3.873 *	2	0.754 *ns*	2	1.058 *ns*
error	113		85		85	

Paternity analysis and deviations from random mating

Populations 5 (Alcaidesa, S-biased), 8 (Hinojos, Isoplethic), 11 and 12 (Hinojos, S-biased) showed significant departures from random mating (Table 2). In all these cases, the observed number of dissasortative mates was higher than expected by chance for both morphs (except L-morph in population 11 where the observed value was similar to the expected one), while the observed number of assortative mates was lower than expected for both morphs. This result was consistent with the trend shown by the remaining populations, albeit with non-significant departures from random mating. When comparing morphs, disassortative mating was higher than expected by chance in six and 10 populations for the L- and the S-morph, respectively, while assortative mating was higher only in three and two populations for the L- and the S-morph, respectively (Table 2). Based on the observed number of seedlings from each mating combination, the Lloyd and Webb conditions meaning prevalence of disassortative mating to the L-morph both in pollen-limited and non pollen-limited conditions (Equations 1 and 3), were satisfied in seven populations, none of them L-biased (Table 2). The Lloyd and Webb conditions representing prevalence of disassortative mating to the S-morph in pollen-limited and non pollen-limited conditions (Equations 2 and 4), were satisfied in eight populations, none of them S-biased (Table 2). Three populations in Hinojos and none in Alcaidesa met all of the Lloyd and Webb conditions (Equations 1–4) for maintenance of stylar dimorphism.

Table 2. Observed / expected number of seedlings from each possible mating combination in each experimental population (maternal morph in first position). Results of the goodness-of-fit test between both distributions are given. The last column indicates the equations of the Lloyd and Webb (1992) model that were fulfilled in each population (Eqs.1 and 3: high disassortative mating to the L-morph in populations with pollen-limited and non pollen-limited conditions, respectively; Eqs. 2 and 4: high disassortative mating to the S-morph in populations with pollen-limited and non pollen-limited conditions). Significance: **, $P < 0.01$; *, $P < 0.05$; *ns*, non significant.

Region	Morph Ratio	Pop	N	L × L	L × S	S × S	S × L	X^2-value	L-W Eqs.
Alcaidesa	Isoplethic	1	35	15 / 16.1	11 / 7.6	0 / 3.6	9 / 7.6	5.21 *ns*	2,4
	Isoplethic	2	7	2 / 1.9	1 / 1.7	1 / 1.6	3 / 1.7	1.45 *ns*	2,4
	L-biased	3	17	9 / 8	3 / 3.7	0 / 1.7	5 / 3.7	2.34 *ns*	2,4
	L-biased	4	15	7 / 6.2	6 / 3.5	0 / 1.9	2 / 3.5	4.56 *ns*	2,4
	S-biased	5	39	0 / 0.8	9 / 4.7	19 / 28.7	11 / 4.7	16.67 **	1,3
	S-biased	6	16	0 / 1.8	4 / 3.6	11 / 7	1 / 3.6	6.02 *ns*	1,3
	S-biased	7	19	0 / 1.1	3 / 3.5	11 / 11	5 / 3.5	1.92 *ns*	1,3
Hinojos	Isoplethic	8	30	3 / 7.2	12 / 7.5	4 / 7.8	11 / 7.5	8.63 *	1,2,3,4
	Isoplethic	9	11	1 / 2.2	2 / 2.7	3 / 3.3	5 / 2.7	2.73 *ns*	1,2,3,4
	L-biased	10	17	6 / 7.6	3 / 3.8	3 / 1.9	5 / 3.8	1.61 *ns*	2,4
	S-biased	11	24	0 / 0.2	2 / 2.1	14 / 19.5	8 / 2.1	17.56 **	1,3
	S-biased	12	13	0 / 1	6 / 2.6	3 / 6.8	4 / 2.6	8.33 *	1,2,3,4

Table 3. Results of univariate and multivariate models performed with PatQuest for the factors affecting siring success of individuals in each experimental population. γ_s: effect of the spatial distance between mating pairs (univariate model); $\beta_{(\gamma m)}$: effect of the number of flowers per individual estimated in a multivariate model jointly with the interaction between mating-pair morphs, $\gamma_{m(\beta)}$. $\gamma_{m(\lambda)}$: interaction between mating-pair morphs estimated jointly with the λ distribution. Significance based in feature-based permutations (Smouse *et al.* 1999; left) and based in pedigree-based bootstrap (Morgan & Conner 2001; right) are given. **, $P < 0.01$; *, $P < 0.05$; *ns*, non significant.

Region	Morph Ratio	Pop	N	γ_s	X^2	$\beta_{(\gamma m)}$	X^2	$\gamma_{m(\beta)}$	X^2	$\gamma_{m(\lambda)}$
Alcaidesa	Isoplethic	1	37	0.238	0.616 *ns*/*ns*	0.022	2.779 *ns*/*ns*	0.588	0.334 *ns*/*ns*	1.484
	Isoplethic	2	12	-0.850	1.574 *ns*/*ns*	0.111	0.887 *ns*/*ns*	0.200	0.006 *ns*/*ns*	0.250
	L-biased	3	19	-0.019	0.003 *ns*/*ns*	0.059	2.841 *ns*/*ns*	0.600	0.148 *ns*/*ns*	0.956
	L-biased	4	21	-0.400	0.688 *ns*/*ns*	-0.012	0.075 *ns*/*ns*	3.800	4.569 */*	1.525
	S-biased	5	43	-0.159	0.452 *ns*/*ns*	0.027	0.608 *ns*/*ns*	2.713	5.201 ns/*	1.444
	S-biased	6	20	0.150	0.131 *ns*/*ns*	0.043	0.997 *ns*/*ns*	-1.625	0.846 *ns*/*ns*	1.088
	S-biased	7	22	0.063	0.018 *ns*/*ns*	-0.229	4.100 *ns*/*ns*	1.700	1.160 *ns*/*ns*	1.269
Hinojos	Isoplethic	8	36	-0.619	4.544 *ns*/*	0.002	0.010 *ns*/*ns*	3.750	8.929 */**	1.175
	Isoplethic	9	12	-0.313	0.528 *ns*/*ns*	0.574	7.420 *ns*/**	2.025	0.563 *ns*/*ns*	0.600
	L-biased	10	28	-0.450	1.619 *ns*/*ns*	0.170	5.729 ns/*	-1.775	1.588 *ns*/*ns*	-0.444
	S-biased	11	41	-1.556	25.1417 */**	0.063	3.484 *ns*/*ns*	0.250	0.031 *ns*/*ns*	0.356
	S-biased	12	15	0.100	0.0569 *ns*/*ns*	-0.066	0.147 *ns*/*ns*	3.975	4.741 **/**	2.094

Siring success modelling

The univariate log-linear regression for the γ_s parameter showed that only two populations (Populations 8 and 11; Hinojos) had a significant negative effect of spatial distance between mates based on pedigree bootstrapping (Table 3), indicating that the position of individuals within experimental population was mostly irrelevant to mating combinations. The multivariate equation for the β and γ_m parameters revealed a significant positive effect of the number of flowers per individual (β) on siring success in populations 9 and 10 (Hinojos), based on pedigree bootstrapping ($P < 0.02$; Table 3). The interaction between parental morphs (γ_m) was positive and significant in populations 4, 5, 8, and 12 (two from each region, Alcaidesa and Hinojos), indicating increased mating success between individuals of different morph ($P < 0.04$; Table 3). Only populations 6 (Alcaidesa, S-biased) and 10 (Hinojos, L-biased) showed a negative γ_m parameter, corresponding to the populations with higher observed than expected assortative mating in the S-morph noted above (Tables 2 and 3). For the joint estimate of γ_m and λ, every population except 10 had a positive value for γ_m. The siring success was highly variable among individuals, with λ values ranging from 0 to 0.42 (Table S1). Floral morph, morph ratio and region did not have a significant effect on siring success ($F_{2,119} < 2.3$; $P > 0.1$).

Table 4. Results of the progeny growth experiment. (A) Results of the Generalized Linear Models testing the effect of maternal morph on four growth curve parameters measuring the average performance of maternal siblings germinated at the first month of the experiment (i), and the effect of both parental morphs and their interaction on the performance of maternal individuals × paternal morph sibling combinations estimated from genotyped seedlings assigned to a paternal parent (ii). Degrees of freedom (d.f.) and *F*-values are given. Significance: *, $P < 0.05$; *ns*, non significant. (B) Mean (± SD) of four parameters that define the growth curves of the progeny from different parental morphs. N, number of maternal families (i) or maternal individuals × paternal morph combinations (ii). μ, maximum growth rate (mm/week); Λ, length of lag phase (weeks); A, maximum growth (mm); I, integral (mm*week). L, L-morph; S, S-morph. Different letters in the Λ column indicate significant differences among mating types based on Bonferroni *post hoc* correction.

A	Fixed effect	d.f. / d.f. error	μ	Λ	A	I
i	Maternal morph (M)	1 / 47	0.076 *ns*	1.626 *ns*	2.102 *ns*	4.718 *
ii	Maternal morph (M)	1 / 109	0.14 *ns*	4.95 *	2.38 *ns*	4.01 *
	Paternal morph (P)		0.62 *ns*	0.08 *ns*	0.17 *ns*	0.05 *ns*
	M × P		0.64 *ns*	5.39 *	0.10 *ns*	2.19 *ns*

B	Parental morphs	N				
i	L maternal parent	15	58.6 ± 10.4	3.9 ± 1.7	235.1 ± 39.2	2169.5 ± 369.1
	S maternal parent	33	59.5 ± 10.3	3.5 ± 0.7	251.3 ± 34.4	2402.2 ± 332.4
ii	L × L cross type	36	54.2 ± 12.9	6.0 ± 2.0 a	229.4 ± 45.4	1711.0 ± 521.2
	S × S cross type	69	57.2 ± 11.1	5.5 ± 2.2 ab	236.0 ± 47.4	1875.7 ± 645.5
	L × S cross type	54	58.0 ± 10.9	5.4 ± 1.8 ab	238.0 ± 56.9	1909.7 ± 656.5
	S × L cross type	65	57.3 ± 11.9	5.2 ± 1.8 b	243.6 ± 44.9	1981.4 ± 615.4

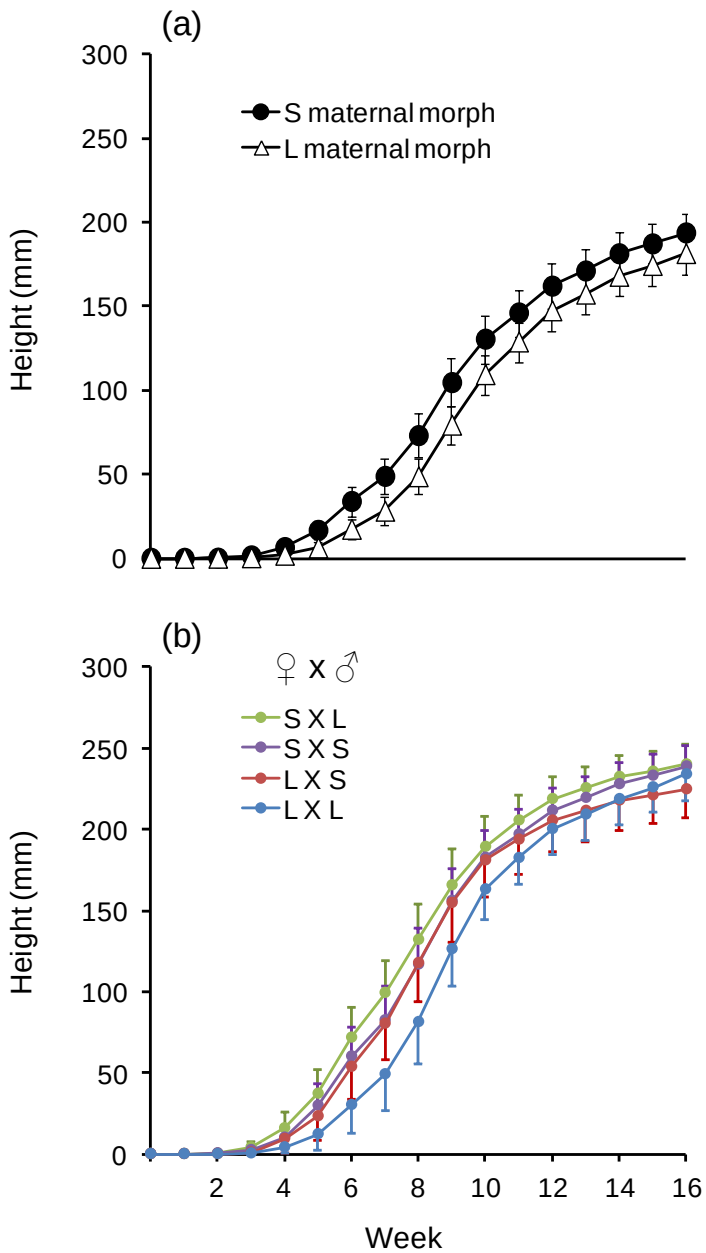

Figure 2. Growth of the progeny of each maternal morph (a) and mating combination (b) throughout 16 weeks in a greenhouse experiment. Each data point is the average height of sibling combinations with 95% confidence intervals.

Progeny performance

There were no significant differences between maternal morphs in the percentage of germination ($F_{1,88}$ = 0.293, P = 0.59). The overall percentage of germination was 84.4%. The progeny from S-morph maternal parents germinated significantly earlier than those from L-morph maternal parents (mean ± SD: S-morph progeny: 7.5 ± 1.6 weeks; L-morph progeny: 8.3 ± 1.5 weeks; $F_{1,86}$ = 6.422, P = 0.013). The logistic models yielded average (± SD) parameters μ = 49.0 ± 9.3 (mm / week), Λ = 7.3 ± 1.4 (week), A = 189.1 ± 40.6 (mm), and I = 1267.6 ± 436.6 (mm × week). The growth curve of the progeny from S-morph maternal parents had significantly higher Integer than progeny from L-morph maternal parents (Table 4 and Figure 2a). The Λ parameter differed significantly among progeny from different parental morph combinations and maternal morphs in the subset analysis (Table 4). The mean values and the Bonferroni *post hoc* test indicated a better performance for the offspring from disassortative matings, the progeny from S × L crosses performing significantly better than the progeny from L × L crosses (Table 4 and Figure 2b).

Discussion

In this study we found evidence of frequent disassortative mating in the style-dimorphic *Narcissus papyraceus*, thus one of the key conditions for the evolutionary stability of stylar dimorphism in this species was met. We validated results from previous studies in this and other species of *Narcissus*, which were based on indirect approaches to incorporating paternal success in style-dimorphic plants (Thompson *et*

al. 2003, Cesaro & Thompson 2004, Pérez-Barrales & Arroyo 2010) as well as on direct paternity analysis of heterostylous *N. triandrus* (Hodgins & Barrett 2008). Specifically, our results showed increased rates of dissasortative mating in the S-morph. This trend may well reflect the more accurate correspondence between the L-morph lower stamens and the S-morph stigma, which could lead the S-morph to receive more pollen from the opposite morph than the L-morph does (Cesaro & Thompson 2004, Chapter 5).

Effects of morph ratio variation

Disassortative mating prevailed in most populations at both sites, Alcaidesa (isoplethic dimorphic natural region) and Hinojos (L-monomorphic natural region). Hence, our results on mating patterns of *N. papyraceus* cannot explain the disappearance of the S-morph in the northern limit of the species range. The only advantage reported for the S-morph in the dimorphic region was its higher assortative mating in one population at Alcaidesa, a result in concordance with experimental S-monomorphic populations in that region (Pérez-Barrales & Arroyo 2010). In contrast, we found similar fecundity of both floral morphs in both regions, which cannot account for the disappearance of the S-morph in the northern range either. In contrast to our study, the previous work by Pérez-Barrales and Arroyo included monomorphic populations of L- and S-morphs and isoplethic populations but not anisoplethic populations, which may explain the different results. Inter-annual variability in pollinator activity might also bring about variable patterns of mating success of floral morphs. Even though our experiment has not been able to detect differences between the monomorphic and dimorphic regions, the assessment

of mating patterns among populations of different morph ratios sheds light into the maintenance of biased populations and the reversion to monomorphism.

According to the model of Lloyd and Webb (1992), populations should meet the conditions for increased disassortative mating in both morphs (i.e. satisfy equation 1 and/or 3 for the L-morph, and equations 2 and/or 4 for the S-morph) to reach an equilibrium morph ratio. In our study, this occurred in three populations, two isoplethic and one S-biased. In the rest of populations, only two conditions were met (either equations 1 and 3, or equations 2 and 4). In such cases the model equations 1 and 3 for disassortative mating prevailing to the L-morph were never satisfied in L-biased populations, while equations 2 and 4 for disassortative mating prevailing to the S-morph were never satisfied in S-biased populations (Table 2). According to the theory on frequency dependent selection, this result indicates that assortative mating prevailed in the dominant morph in each population. Given the lack of heteromorphic incompatibility in *N. papyraceus*, this should lead to maintenance of biased morph ratios. This could account for the stability of biased populations and the reversion to monomorphism in the northern range of *N. papyraceus*, though the consistent loss of the S-morph would require additional explanations. One such explanation may come from the putative inheritance system in *Narcissus* (Dulberger 1964), according to which assortative mating in the S-morph (heterozygote for the diallelic gene of stylar polymorphism) would lead to a percentage of L-morph individuals (homozygote recessive). This could account for the absence of S-biased populations in the wild, though the genetic base of stylar dimorphism needs to be explored. Mating disadvantages of the S-morph under the action of short-tongued pollinators could also account for the observed pattern (Santos-Gally et al 2013, Chapter 5).

Effects of parental morphs on progeny performance

In our study, we found some distinct features in the progeny from different parental morphs that could potentially have implications for the occurrence of floral morphs in the wild. First, we attributed a higher number of seedlings from S-maternal parents to self-fertilisation, as shown from previous hand pollinations (Arroyo *et al.* 2002). Two factors acting in concert may account for the higher probability of selfing in the S-morph: the late-acting self-incompatibility system of imperfect action of *N. papyraceus* (Chapter 2), similar to those described in *N. tazetta* (Dulberger 1964) and *N. tri*and*rus* (Sage *et al.* 1999), and the reduced dichogamy of the S-morph (Chapter 2), as previously reported for *N. assoanus* (Cesaro *et al.* 2004).

The second distinct feature of the S-morph was the better performance of the progeny of S-morph maternal parents, which germinated earlier than progeny of L-morph maternal parents. This result contradicts expected effects of pollen competition in long styles (Mulcahy & Mulcahy 1975, Mulcahy *et al.* 1983, McKenna 1986, Armbruster 1996), and may derive from genetic differences between floral morphs. Quantitative genetic approaches would be an effective means by which to explore the mechanisms underlying this difference, such as pleiotropic effects of the alleles codifying style-length morphs (Eckert & Barrett 1995). Alternatively, the concealing of the S-stigmas could permit their longer receptivity due to lesser pollination events, which would allow to the S-morph to mate with a higher number of males increasing progeny fitness due to sexual selection (Simmons 2005) or resource partitioning (Barton & Post 1986). Intra-fruit paternity analyses would be valuable to test these hypotheses.

Our results also showed a trend of progeny from disassortative mating events to perform better than progeny from assortative mating events, which suggests that

in addition to being more frequent disassortative mating has an extra advantage that is expressed in the next generation. Unpublished work (V.I. Simón-Porcar) has shown a slight difference in the genetic pool of floral morphs in the natural population where the parental individuals were collected. Hence, increased heterozygosity may also be a reason for the better growth of the seedlings from disassortative mates (Szulkin *et al.* 2010 and references therein), as recently shown for different ecological conditions in the region (González-Varo *et al.* 2012).

Disassortative mating in Narcissus

Theory on the evolution of sexual polymorphism emphasizes the role of disassortative mating in the evolutionary maintenance of different floral morphs in populations. In the evolution of heterostyly, enhanced inter-morph mating should be important for the establishment of intermediate stylar dimorphism (Lloyd & Webb 1992). In this study, we have directly estimated mating patterns for the first time in a style-dimorphic plant. We have provided direct experimental evidence for the potential of stylar dimorphism in promoting disassortative mating in *N. papyraceus*, supported by congruent results from goodness-of-fit tests, siring success modelling and the fulfillment of at least two Lloyd and Webb's (1992) model conditions in each experimental population. Taken together, our results help explain the commonness of stylar dimorphism in *Narcissus*, in agreement with previous studies on the genus (Thompson *et al.* 2003, Cesaro & Thompson 2004, Hodgins & Barrett 2008, Pérez-Barrales & Arroyo 2010).

Disassortative mating is obligate in perfect heterostylous species due to their heteromorphic incompatibility system. The finding of disassortative mating in *Narcissus* polymorphic species, where both inter- and intra-morph crosses can

succeed, reveals the central role of morphological reciprocity of sexual organs between floral morphs in promoting disassortative pollination, even when such reciprocity is imperfect. Though no differences have been found in the rates of disassortative mating between regions with long- and short-tongued pollinators, disassortative pollination is probably conducted by long-tongued pollinators (Santos-Gally *et al.* 2013, Chapter 5). The incompatibility system in *Narcissus* is probably shared with ancestors of the genus and is unrelated with the promotion of disassortative mating. Hence, our results could provide the basis for the maintenance of stylar polymorphisms in other taxa which also lack the heteromorphic incompatibility system, such as some Boraginaceae (Barrett & Cruzan 1994, Ferrero *et al.* 2012), and supports the independence of sexual polymorphism and physiological heteromorphic incompatibility.

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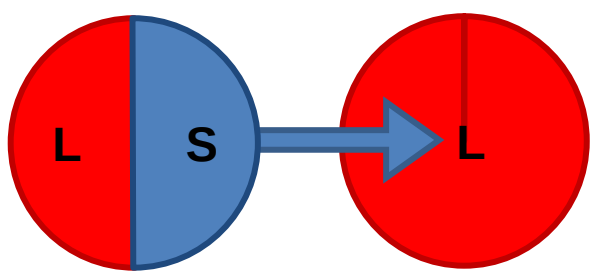
Table S1. Mean (± SD) for fruit set, average seed set per fruit, total seed production and siring success (λ parameter) of floral morphs in each experimental region and morph ratio.

Floral Morph	Region	Morph Ratio	N individuals	N fruits	Fruit set	Average number of seeds per fruit	Total seed production	N individuals	Siring success (λ)
L-morph	Alcaidesa	Isoplethic	10	15	1.5 ± 1.3	7.3 ± 3.4	10.9 ± 11.6	11	0.128 ± 0.123
		L-biased	14	23	1.6 ± 1.4	6.3 ± 4.6	10.7 ± 15.9	15	0.089 ± 0.078
		S-biased	7	9	1.3 ± 1.6	11.7 ± 8.3	12.1 ± 13.2	7	0.100 ± 0.105
	Hinojos	Isoplethic	9	15	1.7 ± 1.5	9.9 ± 6.2	17.9 ± 18.9	9	0.110 ± 0.114
		L-biased	9	9	1.0 ± 0.7	11.9 ± 10.7	10.7 ± 10.7	9	0.058 ± 0.077
		S-biased	5	8	1.6 ± 0.5	8.8 ± 8.6	12.4 ± 9.4	5	0.090 ± 0.070
S-morph	Alcaidesa	Isoplethic	9	13	1.4 ± 1.1	9.2 ± 5.1	14.3 ± 16.1	11	0.054 ± 0.063
		L-biased	4	5	1.3 ± 1.0	8.0 ± 2.5	10.0 ± 8.7	5	0.132 ± 0.068
		S-biased	20	38	1.9 ± 1.1	10.4 ± 7.3	19.8 ± 18.2	21	0.110 ± 0.097
	Hinojos	Isoplethic	9	11	1.2 ± 1.1	6.2 ± 4.1	8.7 ± 11.8	9	0.112 ± 0.140
		L-biased	3	9	3.0 ± 1.0	7.1 ± 2.3	22.7 ± 13.0	3	0.160 ± 0.155
		S-biased	15	33	2.2 ± 1.7	9.2 ± 4.8	18.5 ± 15.7	15	0.102 ± 0.069

CAPÍTULO 7

DISCUSIÓN GENERAL

GENERAL DISCUSSION



Factores implicados en el patrón de variación de la proporción de morfos en las poblaciones de Narcissus papyraceus

La proporción de morfos en las poblaciones de las plantas heterostilas que poseen el típico sistema de incompatibilidad heteromórfico varía ocasionalmente, fruto de procesos estocásticos (Eckert & Barrett 1995, Ågren & Ericson 1996, Kéry *et al.* 2003, Zhou *et al.* 2012) o de diferencias en el éxito reproductor de los morfos tras la quiebra o el debilitamiento del propio sistema de incompatibilidad (Pailler & Thompson 1997, Brys *et al.* 2008, Consolaro *et al.* 2011). En las especies polimórficas estilares sin sistema de incompatibilidad heteromórfico, a estas dos causas se suma la variación en los patrones de polinización, que determinan las tasas de cruzamientos entre individuos del mismo y de distintos morfos. El nivel de reciprocidad de los órganos sexuales en estas plantas y la interacción de estos con los distintos polinizadores podrían ser los principales determinantes de dichos patrones.

El escenario poblacional para la expresión y desempeño de los morfos estilares

La correlación positiva entre el tamaño poblacional y la frecuencia del morfo S en las poblaciones de *Narcissus papyraceus* llevó a Arroyo *et al.* (2002) a plantear la hipótesis de que la pérdida de polimorfismo en las poblaciones del norte del área de distribución de la especie fuese debida a eventos fundadores. En el Capítulo 4 se ha mostrado que procesos estocásticos poblacionales y la deriva genética resultante podrían haber reducido el tamaño efectivo de las poblaciones en los márgenes del área de distribución de la especie en torno al Estrecho de Gibraltar (especialmente en

el margen norte). La correspondencia del patrón del tamaño efectivo poblacional con la proporción de morfos indica que los factores demográfico-históricos pueden tener un efecto sobre la proporción de morfos y la reversión al monomorfismo en las poblaciones de *N. papyraceus*, al reducir su acervo genético. No obstante, a falta de un estudio biogeográfico con marcadores moleculares de herencia materna (cf. Hodgins & Barrett 2007) que permita determinar el origen de las poblaciones, el estudio con marcadores microsatélites nucleares muestra la estrecha relación de las poblaciones monomórficas y dimórficas. Así, en conjunto, los procesos de formación y dinámica poblacionales no pueden haber determinado por sí solos la presencia o ausencia del morfo S en las poblaciones periféricas de *N. papyraceus*, aunque pueden haberlas favorecido.

Curiosamente, otros estudios en plantas heterostilas han sugerido una relación causa-efecto opuesta entre el tamaño efectivo poblacional y la proporción de morfos. Mientras que aquí se considera que los procesos demográficos pueden reducir el tamaño efectivo poblacional y la diversidad genética, lo cual podría alterar la proporción de morfos en las poblaciones, otros autores han propuesto que son los patrones de cruzamiento y proporción de morfos en la población los que determinan el tamaño efectivo poblacional. En concreto, un aumento en la tasa de cruzamientos entre individuos de un morfo podría reducir el tamaño efectivo poblacional en términos del número de emparejamientos disponibles para el otro morfo y, en consecuencia, se reduciría también la diversidad genética en las poblaciones (Asmussen & Basnayake 1990, Husband & Barrett 1992, Meeus *et al.* 2012). Esta última interpretación amplía las consecuencias evolutivas de los patrones de cruzamiento de los morfos florales. Aunque no carente de lógica para especies con incompatibilidad heteromórfica total o parcial, la total falta de limitación para los

cruzamientos entre individuos del mismo morfo en *N. papyraceus* hacen menos factible la hipótesis planteada por estos autores.

Un último factor estocástico poblacional que no se ha estudiado hasta la fecha y que podría tener implicaciones en la proporción de morfos, por su influencia sobre los patrones de cruzamiento y el éxito de los morfos, es la estructura espacial intrapoblacional (Ishihama *et al.* 2003, 2006). En *N. papyraceus* no hay un mecanismo especial de dispersión de semillas, por lo que es esperable que los genotipos emparentados se agreguen en el espacio, alrededor del genotipo materno. De hecho, en las poblaciones naturales dimórficas puede observarse que los morfos no se distribuyen al azar, sino que frecuentemente se agregan en parches[2]. Dado que este hecho no pudo explorarse con la aproximación experimental del Capítulo 6, sería interesante investigar los patrones de cruzamiento de los morfos en poblaciones naturales con un modelo espacialmente explícito. La cierta agregación espacial de los morfos podría aumentar la tasa de polinizaciones entre individuos del mismo morfo, debido a la tendencia de los insectos a visitar plantas próximas entre sí (Waser 1982, Gómez & Zamora 1999).

Los patrones de cruzamiento de los morfos estilares

Tras examinar la colocación de los órganos sexuales en *Narcissus assoanus* y *N. dubius*, que es similar a la de *N. papyraceus*, Baker *et al.* (2000a) propusieron que la misma correspondencia en altura del estigma del morfo L con el verticilo superior de anteras de ambos morfos podría hacer que el morfo L tuviese la misma probabilidad de

[2] El índice de agregación de Pielou (1961), que va desde -1 (total mezcla de morfos) a 1 (total agregación), se calculó en seis poblaciones isopléticas, resultando en un rango de 0.023–0.328, que se desvía significativamente del cero ($P = 0.004$; *t*-test).

recibir polen de individuos L y S. Por el contrario, la colocación ligeramente más arriba del verticilo inferior de anteras en el morfo S hace que el estigma de este morfo se corresponda mejor con el verticilo inferior de anteras del morfo L (véase la Figura 2 en la Introducción General), lo cual facilitaría las polinizaciones de S por parte de L. Baker *et al.* (2000a) demostraron con un modelo cuantitativo que en tal escenario el morfo L pasa a dominar y finalmente fijarse en las poblaciones. Este hecho podría explicar la recurrente dominancia y fijación del morfo L en las poblaciones de *N. papyraceus*, *N. assoanus* y *N. dubius* y otras especies de *Narcissus* con polimorfismo estilar (Arroyo & Dafni 1995, Baker *et al.* 2000b, Barrett *et al.* 2004).

En el Capítulo 5 queda manifiesto que los patrones de transferencia de polen mediados por insectos de probóscide larga se ajustan a la predicción de Baker *et al.* (2000a) a partir de la colocación de los órganos sexuales en la flor. Así, la extrapolación de estos resultados a un largo plazo implicaría que la transferencia de polen por parte de estos polinizadores de probóscide larga no mantendría el polimorfismo en las poblaciones por sí sola, como comúnmente se ha pensado. Sin embargo, un elemento podría compensar la ligera ventaja del morfo L dado el patrón de transferencia de polen por los polinizadores de probóscide larga: la alta deposición de polen propio (no compatible) que podría reducir su fecundidad por medio del descuento de óvulos debido al tipo de incompatibilidad de esta especie (Capítulo 2). Por tanto, la polinización por parte de los insectos de probóscide larga parece establecer un equilibrio en el éxito reproductor de ambos morfos estilares. Por el contrario, la perdida del morfo S parece ser inevitable bajo la acción de los polinizadores de probóscide corta, tanto en base a su patrón de transferencia de polen como a la deposición de polen propio en los estigmas. Así, los resultados del experimento de polinización son acordes con el patrón descubierto por Santos-Gally *et al.* (2013a), en el que la frecuencia de polinizadores de probóscide larga en las

poblaciones no se correlaciona con la frecuencia de morfo S, pero sí lo hace, negativamente, la frecuencia de polinizadores de probóscide corta.

Habiendo descartado diferencias crípticas en el proceso de fecundación tras las polinizaciones entre individuos del mismo y de distintos morfos (Capítulo 2), los patrones de polinización (transferencia y deposición de polen propio) deberían ser los únicos determinantes de los patrones de cruzamiento en las poblaciones de *N. papyraceus*. La similitud del patrón de transferencia de polen por parte de los polinizadores de probóscide larga (Capítulo 5) con los resultados obtenidos independientemente en el experimento con análisis de paternidad (Capítulo 6) refleja esta correspondencia; en ambos estudios se obtuvieron altas tasas de cruzamientos entre distintos morfos y mayores tasas de cruzamientos entre individuos L que entre individuos S. A su vez, este paralelismo indica que, contrariamente a lo esperado, los cruzamientos ocurridos en el experimento de análisis de paternidad fueron mediados por polinizadores de probóscide larga tanto en el área dimórfica como en el área monomórfica de la especie. La alta fecundidad femenina del morfo S en el área monomórfica del experimento también denuncia la actuación de los polinizadores de probóscide larga en este área, lo cual contradice los patrones generales encontrados a partir de censos de polinizadores en la especie (Pérez-Barrales *et al.* 2007, Santos-Gally *et al.* 2013a). Poco se sabe acerca de cómo la variación climática interanual u otros elementos determinan la variabilidad demográfica en las comunidades de estos polinizadores. Para estudiarlo, sería necesaria la repetición continuada de censos de polinizadores con un registro exhaustivo de las condiciones ambientales.

A pesar del paralelismo, las tasas de cruzamientos entre individuos L fueron aparentemente más altas en el experimento de transferencia de polen que en el de análisis de paternidad. El efecto de la autopolinización antes nombrado podría marcar esta diferencia o, independientemente, podría resultar de una competencia

polínica entre los distintos morfos dentro del pistilo. Para explorar esta última opción, sería interesante realizar polinizaciones mixtas controladas con polen de ambos morfos, imitando a las polinizaciones naturales, y llevando a cabo un análisis de paternidad posterior (cf. Sage *et al.* 1999). Finalmente, el desarrollo de la progenie procedente de distintos morfos maternos y tipos de cruzamientos (Capítulo 6) también podría afectar a la desviación de los patrones de éxito reproductor de los morfos de sus patrones de polinización, aparentemente en beneficio del morfo S y de los cruces entre individuos de distintos morfos. Así, los eventos postdispersivos también podrían operar a favor del mantenimiento del dimorfismo en las poblaciones.

Obviando los vectores y los procesos de polinización implicados, en el experimento sobre los patrones de cruzamiento (Capítulo 6) es notable que en todas las poblaciones experimentales se cumplieron al menos las ecuaciones de Lloyd y Webb (1992a) para uno de los dos morfos. Además, en tres poblaciones (dos con la misma proporción de ambos morfos y otra con mayor proporción de S) se cumplieron las condiciones para los dos morfos. Tomados en su conjunto, los resultados de este capítulo indican la prevalencia de los cruzamientos entre morfos distintos y por tanto la posible estabilidad general del dimorfismo estilar en la especie.

Sin embargo, dicha estabilidad podría encontrarse en un delicado equilibrio. La selección dependiente de la frecuencia puede jugar en contra de la persistencia del dimorfismo en las poblaciones de esta especie sin incompatibilidad heteromórfica. En tanto que en las especies con incompatibilidad heteromórfica existe una selección negativamente dependiente de la frecuencia que equilibra la proporción de morfos en la población, en las especies sin incompatibilidad heteromórfica puede ocurrir lo

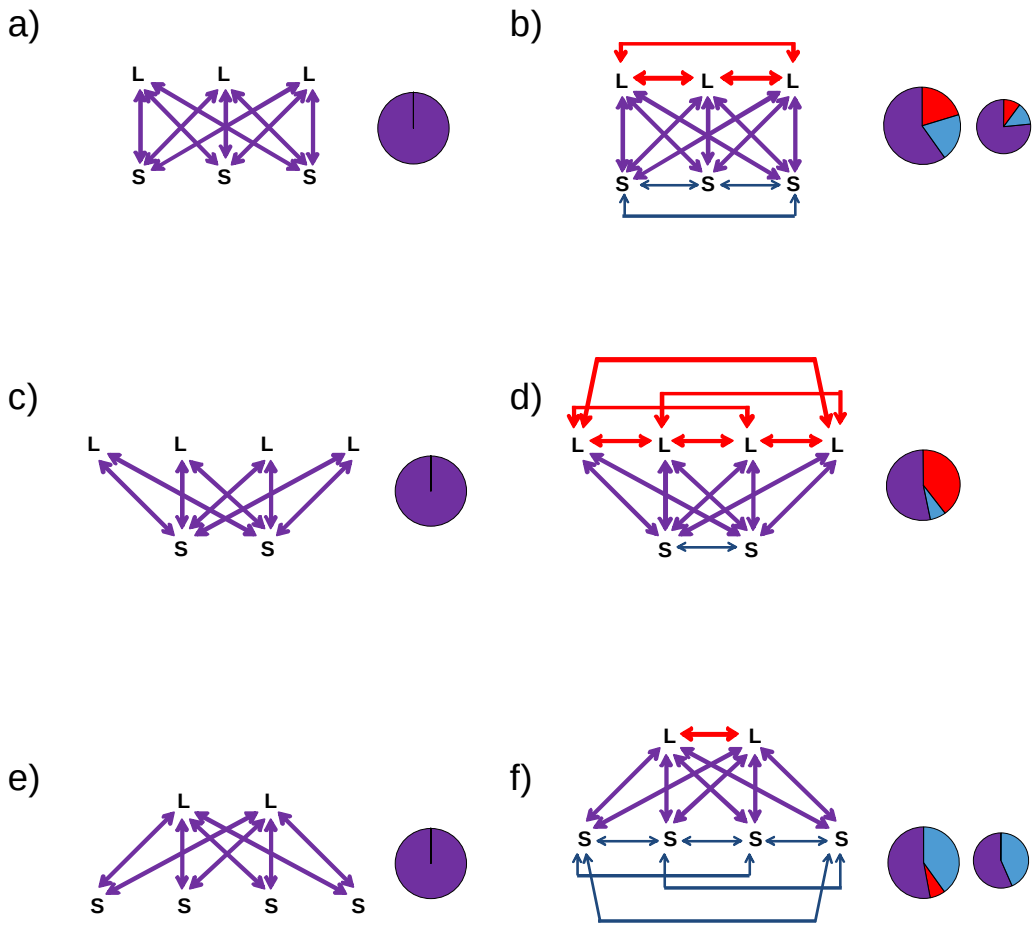


Figura 1. Selección dependiente de la frecuencia de los morfos. Poblaciones con proporciones L:S de 1:1 (a, b), 2:1 (c, d), y 1:2 (e, f) en especies con incompatibilidad heteromórfica (a, c, e) y en *N. papyraceus* (b, d, f). Las flechas indican todos los cruzamientos recíprocos posibles al azar. Los colores indican progenie del morfo L (rojo), de ambos morfos (morado), o de morfo S, al menos predominantemente (azul). El grosor de las flechas indica la probabilidad teórica del cruzamiento de acuerdo a la reciprocidad sexual de los morfos implicados. Los gráficos circulares indican la frecuencia teórica al azar de cada tipo de cruce (en grande) y la extraída como promedio en las poblaciones experimentales con desviaciones significativas de los cruzamientos al azar del Capítulo 6 (en pequeño; no hubo poblaciones L-dominantes con desviaciones del azar).

contrario. Mientras que los individuos del morfo poco común se cruzan con los del morfo más frecuente, estos últimos pueden cruzarse entre sí y aumentar por tanto su frecuencia hasta fijarse en la población, por lo que acontecería una selección positivamente dependiente de la frecuencia (Figura 1). Aunque en el Capítulo 6 se observó esta selección positiva tanto en las poblaciones experimentales con morfo L dominante como en las poblaciones experimentales con morfo S dominante, la menor reciprocidad sexual entre los individuos del morfo S podría reducir en cierta medida la selección positiva en este morfo. En tal contexto, es fácil descifrar la importancia del papel de los procesos estocásticos poblacionales (Capítulo 4) en la reversión al monomorfismo, pues estos procesos podrían determinar tanto ligeras desviaciones de la proporción 1:1 de morfos florales como la pérdida total de un genotipo poco frecuente.

La pérdida del morfo S

En síntesis, los resultados de la Tesis Doctoral indican que varias causas no excluyentes han podido determinar la menor frecuencia o pérdida recurrente del morfo S en muchas de las poblaciones de *Narcissus papyraceus* (Figura 2). En primer lugar, aunque el dimorfismo parece estar garantizado bajo la acción de polinizadores de probóscide larga (Figura 2a), sin el efecto de la deposición de polen propio en los estigmas L por parte de estos polinizadores el morfo L podría tener una ventaja reproductora (Figura 2b). Por su parte, el efecto de los polinizadores de probóscide corta debe suponer la pérdida directa del morfo S (Figura 2c). La presencia de polinizadores de probóscide corta en una población ha de reducir forzosamente el éxito reproductor femenino del morfo S. No obstante, la reversión al

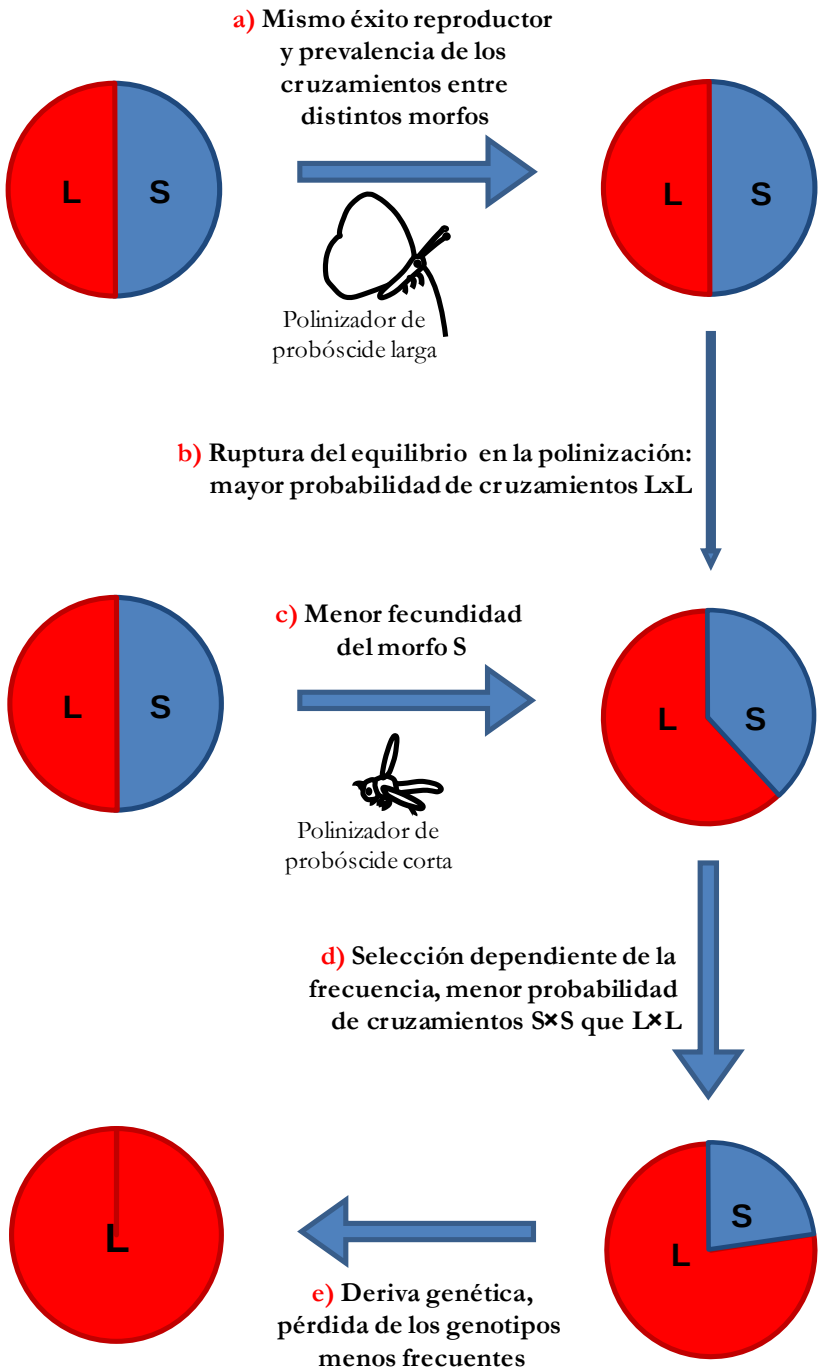


Figura 2. Escenario de pérdida del morfo S a partir de una proporción inicial 1:1 de morfos L:S. El orden de los mecanismos implicados puede variar.

monomorfismo L en las poblaciones podría no requerir que estos visitantes florales de probóscide corta fuesen dominantes en exceso, pues se ha visto que una ligera desventaja para un morfo puede terminar comportando su pérdida en la población por el efecto de la selección dependiente de la frecuencia (Figura 2d). Esta selección actuaría especialmente en contra del morfo S por su menor reciprocidad sexual, que dificulta los cruces S×S en comparación con los cruces L×L. Además, factores demográficos desestabilizadores en la formación de las poblaciones (e.g. efectos fundadores, cuellos de botella) y la deriva genética pueden también favorecer la reversión al monomorfismo (Figura 2e).

Se ha sugerido que la base genética de los morfos florales podría favorecer la pérdida del morfo S en las poblaciones (Arroyo & Dafni 1995). El modelo de herencia de un locus dialélico propuesto por Dulberger (1964) para *Narcissus tazetta*, según el cual el morfo L es homocigoto recesivo (*ss*) y el morfo S tiene el alelo dominante (*Ss*; con inexistencia virtual del genotipo *SS*), es el mismo que el sistema mendeliano propuesto para algunas plantas distilas (véase la revisión de Lewis & Jones 1992). En estas, los cruces entre morfos distintos (*ss* × *Ss*) obligados por el sistema de incompatibilidad heteromórfico dan lugar a una progenie 1:1 de genotipos *ss* : *Ss*; manteniendo el equilibrio de la proporción de morfos en la población generación tras generación. En ausencia de la incompatibilidad heteromórfica, los cruces *ss* × *ss* (entre individuos de morfo L) dan lugar a progenie *ss* (morfo L), mientras que los cruces *Ss* × *Ss* (entre individuos de morfo S) dan lugar a progenie 1*SS* : 2*Ss* : 1*ss* (morfos S y L). Esta idea sugiere que el morfo S tiene una desventaja, puesto que no podría establecer poblaciones monomórficas. No obstante, es notable que una pequeña tasa de cruzamientos entre individuos del morfo S llevaría al genotipo *SS* a aumentar en la población con el paso de las generaciones, reduciendo la desventaja del morfo S. Además, el carácter dominante del alelo que

supuestamente determina este morfo también debería reducir dicha desventaja en cuanto aconteciesen cruces *SS* × *ss*. Richards (1997, 1998) sugirió que el genotipo *SS* era letal, lo cual impediría las ventajas explicadas, pero su afirmación no posee evidencia empírica (Shore & Barrett 1985, Eckert & Barrett 1993). Así pues, el papel de la base genética de los morfos florales en su frecuencia de aparición debe ser muy secundario, por la falta de incompatibilidad ligada al morfo. No obstante, sería necesario estudiar a fondo esta base genética y los posibles efectos directos (longitud estilar) e indirectos (desarrollo de la progenie, Capítulo 6) que puedan afectar al éxito de los morfos.

La aplicación del modelo evolutivo de la heterostilia de Lloyd y Webb al género Narcissus

Esta Tesis Doctoral se enmarca en un programa de investigación encaminado a probar el modelo de Lloyd y Webb (1992a, b; Figura 3) de la evolución de la heterostilia en el género *Narcissus*. Los resultados han demostrado que el dimorfismo estilar en *Narcissus papyraceus* puede, en la medida en que su limitada reciprocidad le permite, promover cruzamientos entre distintos morfos. La independencia de este hecho de la existencia de un sistema de incompatibilidad heteromórfico apoya el modelo de Lloyd y Webb. No obstante, la aplicación de todos los puntos de dicho modelo a *Narcissus* debería matizarse.

Debe tenerse en cuenta que el modelo evolutivo de Lloyd y Webb fue desarrollado, en lo que se refiere a la incompatibilidad, para sistemas heteromórficos. El modelo expresa, como lo hiciese Darwin en 1877, que la incompatibilidad heteromórfica no puede seleccionarse antes que la hercogamia recíproca porque por sí sola implica que la mitad de las polinizaciones cruzadas son incompatibles, y por tanto reduce el éxito reproductor. Por consiguiente, su propuesta de sucesión de eventos (hercogamia recíproca seguida por incompatibilidad) no sería obligada en las especies polimórficas con un sistema de incompatibilidad homomórfico (circunscritas a los géneros *Narcissus, Anchusa* y a algunas otras Boraginaceae), donde todas las polinizaciones cruzadas son compatibles independientemente de los morfos implicados, supuestamente debido a la existencia de muchos alelos que gobiernan el sistema de incompatibilidad (Lloyd & Webb 1992a; pág. 197).

Lloyd y Webb (1992a; pág. 202) aceptaron que la fuerza de la selección a favor de la polinización cruzada dependería de la secuencia en la que habían aparecido los principales rasgos de las especies heterostilas, y que si los antepasados hubiesen sido autoincompatibles, la selección contra la autopolinización probablemente habría tenido un papel mucho más importante en la aparición de la heterostilia que el que ellos le habían asignado. La autopolinización (o en general, la interferencia sexual) limita el éxito masculino y femenino reduciendo la liberación de polen propio y la recepción de polen ajeno, por lo cual se considera una fuerza selectiva importante en la evolución floral (Barrett 2002) que conduce a la selección gradual de fenotipos hercógamos (Karron *et al.* 1997, Stone & Motten 2002, Herlihy & Eckert 2007, Larrinaga *et al.* 2009, Navarro *et al.* 2012, Luo & Widmer 2013, pero véase Medrano *et al.* 2005). Suele también asumirse que la evitación de la autofecundación es la principal fuerza selectiva promotora de la autoincompatibilidad (Charlesworth & Charlesworth 1987).

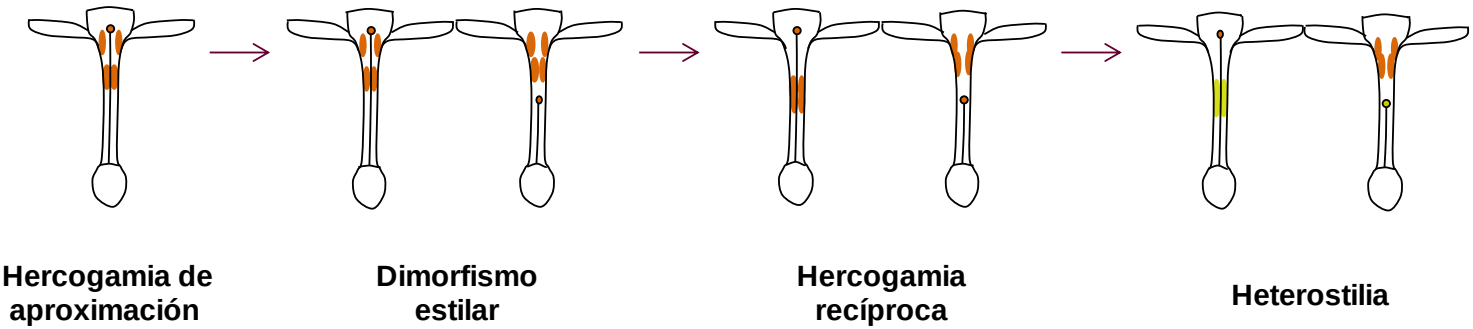


Figura 3. Modelo evolutivo de Lloyd y Webb (1992 a, b) de la evolución de la heterostilia. Las dos primeras transiciones responden a mutaciones morfológicas sencillas y discretas seleccionadas para la promoción de la fecundación cruzada. La tercera transición (aparición de la incompatibilidad heteromórfica) responde a un cambio gradual en la reacción polen-pistilo seleccionado para evitar la autopolinización y potenciar así el efecto de la hercogamia recíproca.

Aunque son necesarios más estudios comparados con grupos emparentados, es probable que, dada su ocurrencia en el género *Narcissus* (Dulberger 1964, Sage *et al.* 1999, Santos-Gally *et al.* 2013b, Capítulo 2) y en otras Amaryllidaceae (*Sternbergia*, *Galanthus*, *Pancratium, Cyrtanthus*; Eisikowitch & Galil 1971, Dafni & Werker 1982, Chudzik *et al.* 2002, Vaughton *et al.* 2010, véase también Gibbs & Bianchi 1999, Pérez-Barrales *et al.* 2006), la autoincompatibilidad de acción tardía y homomórfica sea ancestral en *Narcissus* (Lloyd & Webb 1992a; pág. 165). Por tanto, la evitación de la autointerferencia podría haber tenido un papel importante como fuerza selectiva para la aparición del dimorfismo estilar en *Narcissus*. Esta fuerza podría ser intensa, ya que la autoincompatibilidad de acción tardía conduce a importantes niveles de descuento de óvulos tras la autopolinización, disminuyendo la producción de semillas tras polinizaciones cruzadas posteriores (Dulberger 1964, Waser & Price 1991, Vaughton 1993, Barrett *et al.* 1997, Sage *et al.* 1999, Arroyo *et al.* 2002, Vaughton *et al.* 2010, Capítulo 2).

El papel de la autointerferencia como fuerza selectiva en la evolución del polimorfismo estilar en *Narcissus* fue considerada por Yeo (1975) y Barrett *et al.* (1996). Estos últimos autores modificaron las ecuaciones del modelo de Lloyd y Webb (1992a) para el mantenimiento del dimorfismo estilar en las poblaciones incluyendo un término para el descuento de óvulos en los morfos florales de *Narcissus*, y concluyeron que las diferencias entre morfos en el descuento de óvulos podrían promover la evolución del dimorfismo estilar. Estudios posteriores en las especies polimórficas del género también han considerado este punto de vista (Barrett *et al.* 1997, Cesaro *et al.* 2004).

La aparición del polimorfismo estilar en *Narcissus* se asocia con tubos florales estrechos (Graham & Barrett 2004, Pérez-Barrales *et al.* 2006, Santos-Gally *et al.* 2013b), donde se incluye el verticilo inferior de anteras. Esta asociación se ha

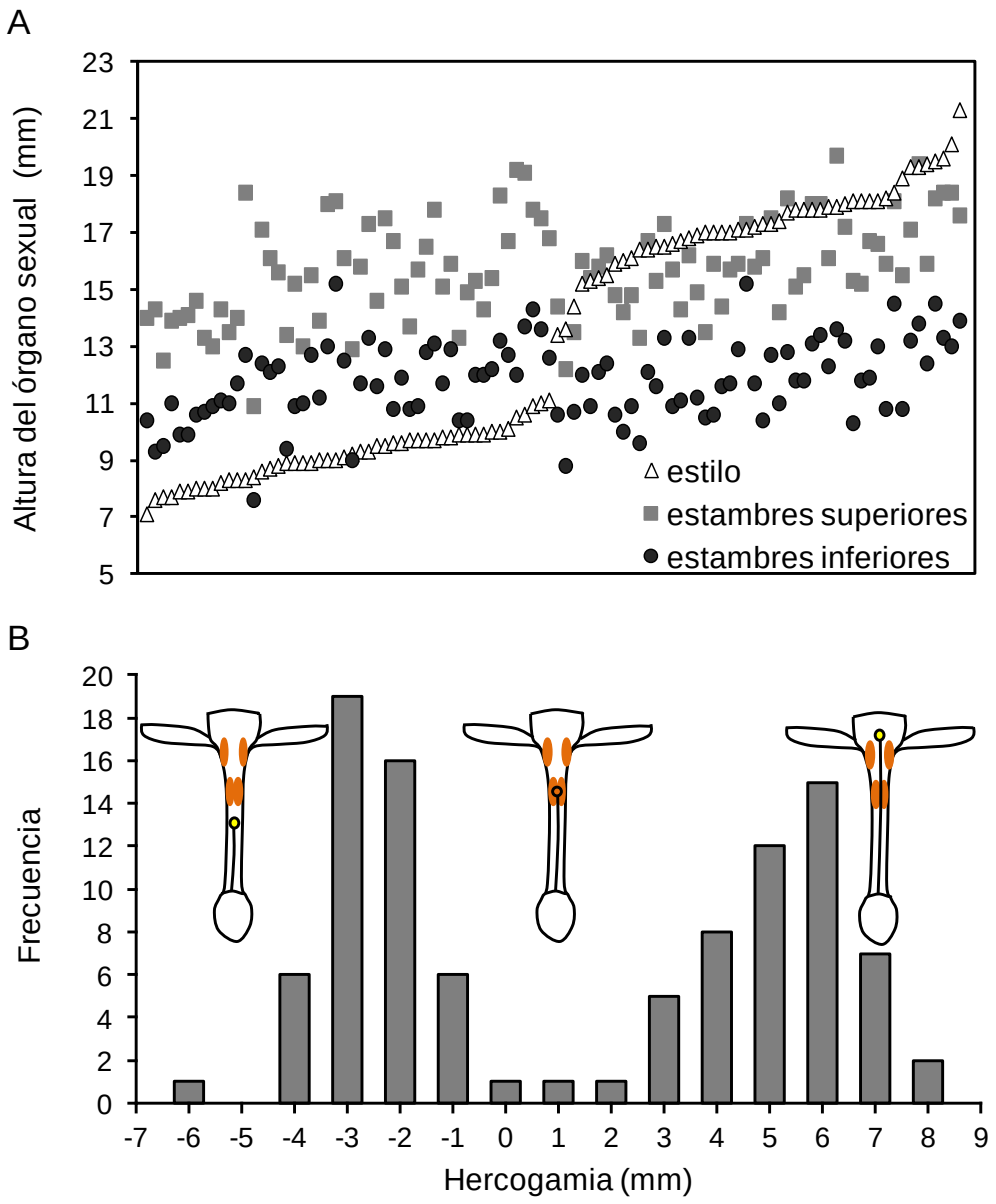


Figura 4. Colocación de los órganos sexuales en una muestra de 100 individuos seleccionados al azar en la población dimórfica de *Narcissus papyraceus* en Bolonia (Provincia de Cádiz). (A) Altura del estigma y de las anteras de flores de distintos individuos ordenados por la longitud de su estilo. (B) Distribución de frecuencias de hercogamia respecto al verticilo inferior de anteras. Datos de Arroyo *et al.* (2002).

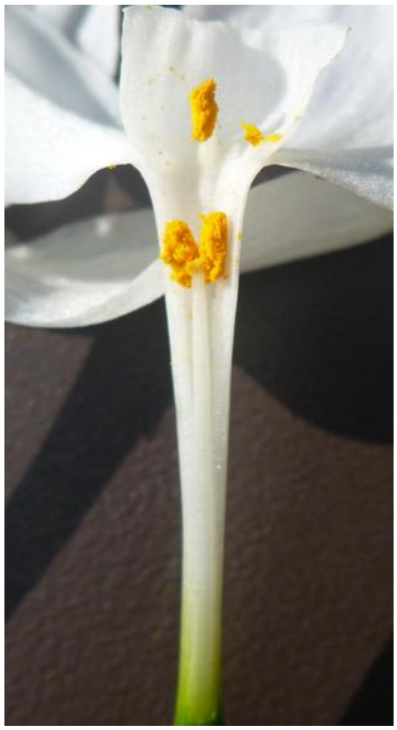


Figura 5. Estigma virgen de un individuo de *Narcissus papyraceus* con fenotipo estilar intermedio en el que se aprecia un alto nivel de autopolinización.

atribuido a la restricción de movimientos que el tubo floral estrecho impone al polinizador cuando este introduce su probóscide en la flor, favoreciendo la precisión en la transferencia del polen. Independientemente, la asociación entre dimorfismo estilar y tubo floral estrecho podría deberse también a un aumento de la autointerferencia entre los órganos sexuales en un espacio reducido. En las especies dimórficas estilares como *N. papyraceus*, la longitud del estilo muestra una distribución bimodal solapada, con el mínimo de dicha distribución situado exactamente al nivel del verticilo inferior de anteras (Figura 4). Una exploración morfológica deja patente que un estigma situado al mismo nivel que el verticilo inferior de estambres en una especie de *Narcissus* de tubo floral estrecho está "preso" entre sus propias anteras (Figura 5). Por tanto, una hipótesis plausible es que una masiva autopolinización incompatible y su consiguiente descuento de óvulos y de polen en individuos con estigmas al mismo nivel que el verticilo inferior de estambres podrían seleccionar

negativamente este fenotipo[3]. Más aun cuando se ha demostrado que en algunas especies dimórficas de *Narcissus* el morfo S carece de dicogamia (Cesaro *et al.* 2004, Capítulo 2). Así, podría existir en mitad del tubo floral un espacio mal adaptativo para la posición del estigma (correspondiente al mínimo de la distribución bimodal de la altura de los estigmas) que podría haber actuado como "catalizador" en la aparición del dimorfismo estilar.

Estado ancestral y selección del polimorfismo estilar

A pesar de retomar la idea darwinista de la promoción de la fecundación cruzada como fuerza selectiva del polimorfismo estilar, Lloyd y Webb contradijeron las ideas de Darwin sobre la forma ancestral del polimorfismo (gran variación en la posición de los órganos sexuales) y el proceso de aparición de los morfos estilares (selección gradual). La distribución ancestral continua en la longitud de los estilos y la selección gradual propuestas por Darwin (1877), aunque no son condición necesaria, acentuarían el papel selectivo de la autointerferencia en la aparición del dimorfismo estilar en *Narcissus*, al poder actuar sobre una gama amplia de fenotipos. Así, podría conjeturarse que en un ancestro con gran variación en la longitud estilar, tubo floral estrecho y autoincompatibilidad homomórfica y de acción tardía, la evitación de la

[3] En *N. papyraceus*, el solapamiento de la distribución bimodal de las longitudes de estilo es más notable en las poblaciones centrales, grandes y fenotípicamente más variables de la especie, alrededor del Estrecho de Gibraltar (Figura 4). Un examen de los patrones de polinización y de fecundidad femenina de individuos con el estigma al mismo nivel que el verticilo inferior a partir de un estudio piloto sobre un total de 104 individuos en la población de *N. papyraceus* de Bolonia (Cádiz, España, 36°6'N, 5°44'W) mostró que este fenotipo recibe mayor carga de polen pero tiene menor fecundidad que los individuos de estilo largo o corto, lo cual se explica fácilmente si el exceso de polen es propio (autoincompatible y provocador de descuento de óvulos; Tabla S1).

autointerferencia y la autopolinización podrían haber sido fuerzas que seleccionaran gradualmente el dimorfismo estilar en sus inicios. La hercogamia recíproca podría haberse seleccionado posteriormente (ahora sí, tal y como Lloyd y Webb propusieron) por la promoción de la polinización cruzada. Esta hipótesis podría probarse con un modelo teórico, obteniendo parámetros a partir de experimentos de selección fenotípica como el estudio piloto mostrado.

A día de hoy no existe evidencia empírica sólida a favor de la hipótesis de Lloyd y Webb ni de la de Darwin en cuanto al estado ancestral de la posición de los órganos sexuales en *Narcissus*. Graham y Barrett (2004) apoyaron con un estudio filogenético la condición ancestral de Lloyd y Webb (hercogamia de aproximación). La conclusión de Graham y Barrett se basó en considerar los géneros hermanos *Sternbergia* y *Galanthus* y a *N. broussonetii* y *N. elegans* como especies monomórficas con hercogamia de aproximación. No obstante, los géneros externos no se han estudiado en profundidad y se carece de estudios poblacionales extensivos sobre la distribución de sus longitudes de estilo. Además, la asunción de la condición monomórfica de *N. broussonetii* y *N. elegans* sobre la base de una población limitada y del examen de ejemplares herborizados era incorrecta, pues Santos-Gally *et al.* (2013b) han demostrado su dimorfismo. Por tanto, son necesarios muchos más estudios morfométricos detallados en grupos emparentados con *Narcissus* para determinar fielmente la condición ancestral en cuanto a la variabilidad fenotípica en la longitud del estilo. El sujeto de estudio de estos posibles análisis comparados debe ser tanto la longitud media del estilo (y su posición respecto de las anteras) como su variabilidad intrapoblacional.

Si la diferenciación genética de los estilos en largos y cortos en *Narcissus* se debe a una única mutación sencilla y de efecto discreto (Lloyd & Webb 1992a) tampoco es sabido. El modelo genético del locus dialélico de Dulberger (1964) se

basó en la segregación de morfos obtenida tras la realización de polinizaciones manuales, una metodología que no puede por sí sola desentrañar la arquitectura genética del dimorfismo estilar. Para ello, sería necesario realizar un análisis de loci de carácter cuantitativo (QTLs) sobre variables fenotipos estilares, incluyendo el fenotipo intermedio, con los que podría distinguirse si la variación en la longitud estilar se debe mayoritariamente a un locus, como sugieren el trabajo de Dulberger y el modelo de Lloyd y Webb (véase Yoshida *et al.* 2011), o a muchos, como cabría esperar en el caso de la adaptación fenotípica gradual propuesta por Darwin (véanse Shore & Barrett 1990, Fishman *et al.* 2002, Chen & Tanksley 2004, Luo & Widmer 2013 para esta visión alternativa). El gran tamaño del genoma de las monocotiledóneas como *Narcissus* (Zonneveld 2008), y el notable esfuerzo que debería invertirse en el desarrollo de marcadores moleculares para fabricar el mapa de ligamiento, supondrían sin duda dificultades en la ejecución de un estudio de QTLs en el género.

El dimorfismo estilar más allá de Narcissus

Aparte de en *Narcissus*, la asociación de polimorfismo estilar e incompatibilidad homomórfica sólo ocurre en *Anchusa* (donde es de acción tardía; Dulberger 1970, Philipp & Schou 1981, Schou & Philipp 1983), y en algunas otras especies de Boraginaceae de los géneros *Lithodora* y *Glandora* (Ferrero *et al.* 2012). En estas últimas, aunque el mecanismo no se ha estudiado en detalle, también podría tratarse de incompatibilidad de acción tardía (véase Gibbs & Bianchi 1999). Por lo tanto, la hipótesis aquí expuesta podría aplicarse también a algunos de estos géneros. Es notable que estos taxones también se caracterizan por una baja reciprocidad de los morfos florales, con un patrón de variación de la longitud del estilo similar a *N.*

papyraceus (véanse la figura 3 en Philipp & Schou 1981 y la figura 2 en Ferrero *et al.* 2009 y 2011), y que en algunas especies de *Lithodora* y *Glandora* hay poblaciones tanto con dimorfismo estilar como con heterostilia, fruto de una variación gradual (Ferrero *et al.* 2011; véanse también Thompson *et al.* 2012, Haddadchi 2013). Además, es interesante observar que una variación continua en la longitud del estilo, desde la hercogamia reversa (estigma situado bajo las anteras) hasta la de aproximación, ocurre en otra especie de la misma subfamilia Boraginoideae, *Mertensia fusiformis* (Forrest *et al.* 2011).

Consideración final

Para terminar, es importante reseñar de nuevo la validez de algunos de los principales puntos del modelo evolutivo de Lloyd y Webb (1992a, b) en el género *Narcissus*. Aunque la incompatibilidad homomórfica podría haber sido previa a la aparición del dimorfismo estilar y la selección en contra de la autointerferencia podría haber tenido un papel en su aparición, la promoción de la polinización cruzada podría haber seleccionado de forma importante la posición de los órganos sexuales a continuación. Por ejemplo, el movimiento hacia arriba del verticilo inferior de anteras en el morfo S de *N. papyraceus* y de otros *Narcissus* dimórficos estilares y la selección de fenotipos heterostilos y tristilos en las especies derivadas *N. albimarginatus* y *N. triandrus*, mejoran claramente la reciprocidad entre morfos florales de las especies dimórficas (Pérez-Barrales *et al.* 2006). Además, como Lloyd y Webb modelaron siguiendo las ideas de Darwin, la prevalencia de los cruzamientos entre individuos de distintos

morfos es necesaria para el mantenimiento del polimorfismo en las poblaciones. El papel selectivo de los polinizadores es también importante, pues además de mediar precisamente dicha polinización cruzada entre morfos florales, pueden determinar fuertemente la pérdida de polimorfismo, como esta Tesis Doctoral ha demostrado.

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Tabla S1. Resultados de un estudio de campo sobre la carga de polen en los estigmas y la fecundidad femenina de individuos con estilo largo (L; *N* = 31), corto (S; *N* = 29) o intermedio (I; *N* = 44). a) Valores medios (± DE) para la carga de polen y tres medidas de fecundidad femenina tomadas sobre las dos primeras flores de cada inflorescencia para cada morfo estilar y el fenotipo intermedio. b) Resultados de GLMs con distribución binomial negativa probando el efecto del morfo estilar sobre cada variable. Letras iguales junto a los valores medios indican falta de diferenciación significativa entre morfos estilares de acuerdo con un análisis *post hoc* de Bonferroni. g.l.= grados de libertad. Significancia: ***, *P* < 0.001; **, *P* < 0.01; *ns*, no significativo.

	Morfo estilar	Carga de polen	Producción de frutos	Semillas por fruto	Producción total de semillas
a	L	256.30 ± 444.36 a	0.89 ± 0.25	17.15 ± 9.28 a	31.44 ± 17.25 a
	S	327.62 ± 362.83 a	0.86 ± 0.3	15.82 ± 7.87 a	29.34 ± 14.95 a
	I	579.72 ± 723.77 b	0.81 ± 0.34	9.63 ± 6.75 b	18.05 ± 14.05 b
b	g.l. / g.l. error	2 / 179	2 / 103	2 / 90	2 / 90
	F-valor	8.58	1.03	8.79	3.138
	P-valor	<0.001***	0.357 *ns*	<0.001***	0.001**

CONCLUSIONES GENERALES

1- *Narcissus papyraceus* posee un sistema de incompatibilidad homomórfico que no impide ni obstaculiza los cruzamientos entre individuos del mismo morfo estilar. Así, los patrones de polinización deben ser determinantes por sí mismos del éxito reproductor de los morfos y su frecuencia en las poblaciones.

2- El rechazo a los tubos polínicos propios ocurre en el ovario y de manera precigótica, comportando la degeneración de óvulos penetrados y no penetrados. Así, la autopolinización en *Narcissus papyraceus* implica un visible descuento de óvulos, como ya se había sugerido en base a experimentos de polinización manual.

3- Los microsatélites nucleares desarrollados para *Narcissus papyraceus* son altamente variables y por tanto útiles para estudios de genética de poblaciones y análisis de paternidad. Además, son transferibles a otras especies de *Narcissus*, por lo que podrían emplearse para resolver nuevas cuestiones ecológicas y evolutivas en el género, incluso más allá del polimorfismo estilar (e.g. estructura genética espacial intrapoblacional, distancias de polinización y dispersión).

4- El patrón geográfico en la proporción de morfos estilares en las poblaciones de *Narcissus papyraceus* no se correlaciona con el parentesco genético de las poblaciones, aunque sí con su diversidad genética y tamaño efectivo poblacional. Eventos fundadores o cuellos de botella, y la deriva genética resultante de ellos, pueden haber influido en la pérdida del polimorfismo en el límite norte del área de distribución de la especie.

5- De acuerdo con la diferente reciprocidad de los órganos sexuales en los morfos de *Narcissus papyraceus*, el patrón de transferencia de polen mediado por polinizadores de probóscide larga conlleva altas tasas de deposición de polen en cruces L×L, L×S y S×L, y bajas tasas de deposición de polen en cruces S×S. La ventaja reproductora del morfo L según este patrón puede verse compensada por la alta deposición de polen propio, no compatible. Así, la interacción de los polinizadores de probóscide larga con las flores de esta especie implica un equilibrio en el éxito reproductor de ambos morfos.

6- Los polinizadores de probóscide corta son poco eficaces, pues se llevan grandes cantidades de polen de las anteras pero depositan un número limitado de granos en los estigmas del morfo L y virtualmente ningún grano en los estigmas del morfo S. El éxito reproductor femenino del morfo S debe ser nulo bajo la acción de estos visitantes florales, dada además la cantidad de polen propio, no compatible, que depositan en sus estigmas.

7- El patrón de cruzamientos efectivos entre los morfos florales, inferido a partir de análisis de paternidad con microsatélites en poblaciones experimentales en las áreas dimórfica y monomórfica de la distribución de la especie, reflejó la actuación de polinizadores de probóscide larga en ambas áreas. Dicho patrón indicó la prevalencia de los cruzamientos entre individuos de distintos morfos en la mayoría de las poblaciones.

8- De acuerdo con la teoría de la selección dependiente de la frecuencia, los individuos del morfo menos frecuente en las poblaciones experimentales se cruzaron más frecuentemente con el morfo dominante, mientras los individuos de este último se cruzaron más entre ellos. Este resultado indica que pequeñas desviaciones de la proporción 1:1 de morfos pueden verse acentuadas con el tiempo y por tanto el dimorfismo estilar podría permanecer en un frágil equilibrio en las poblaciones.

9- La pérdida del morfo S en las poblaciones del margen norte del área de distribución de *Narcissus papyraceus* debe haber sido determinada por su desventaja reproductora bajo la acción de los polinizadores de probóscide corta, potenciada por efectos demográficos desestabilizadores en la formación de las poblaciones y los patrones de cruzamiento determinados por la reciprocidad sexual y la frecuencia de los individuos en ausencia de incompatibilidad heteromórfica.

10- Los resultados en los patrones de polinización indican que el dimorfismo estilar puede promover cruzamientos entre morfos opuestos, lo cual apoya la hipótesis de Darwin (1877) y de Lloyd y Webb (1992). No obstante, la excepcionalidad del sistema de autoincompatibilidad ovárico de las especies polimórficas de *Narcissus* no fue considerada por estos modelos evolutivos de la heterostilia, y podría haber tenido implicaciones en la evolución del dimorfismo estilar en el género.

GENERAL CONCLUSIONS

1- *Narcissus papyraceus* has a homomorphic incompatibility system that does not prevent or hinder crosses between individuals of the same style morph. Thus, pollination patterns by themselves must be decisive for the reproductive success of morphs and their frequency in populations.

2- The rejection of self-pollen tubes occurs in the ovary prezygotically, and involves the degeneration of penetrated and non-penetrated ovules. Thus, self-pollination in *Narcissus papyraceus* results in visible ovule discounting, as suggested on the basis of prior hand-pollination experiments.

3- The nuclear microsatellites developed for *Narcissus papyraceus* are highly variable and therefore useful for studies of population genetics and paternity analysis. They are transferable to other species of *Narcissus*, so they could be employed to address new ecological evolutionary questions in the genus, also beyond stylar polymorphism (e.g. intrapopulation genetic structure, pollination and dispersal distances).

4- The geographic pattern of floral morph ratios in *Narcissus papyraceus* populations is not associated with the genetic relatedness of populations, although it is with their genetic diversity and effective population size. Founder events or bottlenecks and genetic drift derived from them may have influenced the loss of polymorphism.

5- Due to the different reciprocity of sexual organs of morphs in *Narcissus papyraceus*, the pattern of pollen transfer mediated by long-tongued pollinators rise to high rates of pollen deposition in crosses L×L, L×S and S×L, and low rates of pollen deposition in crosses S×S. The L-morph reproductive advantage given this pattern may be offset by high non-compatible self-pollen deposition. Thus, the interaction of long-tongued pollinators with flowers of this species implies a balance in the reproductive success of both morphs.

6- The short-tongued pollinators are ineffective because they remove large amounts of pollen from the anthers but deposit a limited number of grains on the stigmas of the L-morph and virtually no grains on the stigmas of S-morph. The female reproductive success of S-morph must be zero under the action of these floral visitors, given also the amount of non-compatible self-pollen that they deposit on their stigmas.

7- The pattern of effective fertilisation between floral morphs, inferred from microsatellite-based paternity analysis in experimental populations in the dimorphic and monomorphic areas of the species' distribution range, reflected the performance of long-tongued pollinators in both areas. This pattern indicated the prevalence of disassortative mating in most populations.

8- In accordance with the theory of frequency-dependent selection, individuals of the less common morph on experimental populations were crossed over more frequently to the dominant morph, whereas the latter individuals were crossed over each other. This result indicates that small deviations from a 1:1 ratio of morphs may be accentuated with time and therefore stylar dimorphism could be maintained in populations in a fragile equilibrium.

9- The loss of the S-morph in populations in the northern margin range of *Narcissus papyraceus* must have been determined by its reproductive disadvantage under the action of short-tongued pollinators, powered by destabilizing demographic events in the formation of populations and crossing patterns determined by sexual reciprocity and the frequency of morphs in the absence of heteromorphic incompatibility.

10- Results of pollen transfer patterns indicate that stylar dimorphism can promote disassortative mating, which supports Darwin's (1877) and Lloyd and Webb's (1992) hypothesis. However, the uniqueness of ovarian self-incompatibility system in stylar-polymorphic *Narcissus* was not considered by these evolutionary models of heterostyly, and could have implications for the evolution of stylar dimorphism in the genus.

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