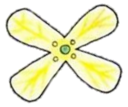
I was born in 1997 in Almería, Spain. I obtained my Biology degree in 2019 at University of Granada. Where I also cursed a master in Genetics and Evolution. Since my access to the University, I have been collaborating with the Department of Genetics helping with ongoing projects until I started working in my PhD in 2020. During these years, I have been mentoring undergraduate students and I obtained my first project for researches in early stages of the investigator career by the Spanish Association of Terrestrial Ecology (AEET).

Departamento de Genética

Speciation associated to evolutionary transitions
Mechanisms driving the ploidy effects on the diversification in a selfing species complex



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Ana García Muñoz

Tesis doctoral
Granada
2023

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UNIVERSITAS · GRANATENSIS · 1531

Facultad de Ciencias

Departamento de Genética

Biología Fundamental y de Sistemas

Ana García Muñoz

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Speciation associated to evolutionary transitions

Mechanisms driving the ploidy effects on the diversification in a selfing species complex

Memoria presentada por Ana García Muñoz para optar al Grado de Doctora en Biología por la Universidad de Granada. Esta memoria ha sido realizada bajo la dirección del Dr. Mohamed Abdelaziz Mohamed y el Dr. Antonio Jesús Muñoz Pajares, Doctores en Biología por la Universidad de Granada.

Fdo.: **Ana García Muñoz**

La doctoranda

Fdo.: **Mohamed Abdelaziz**

Director de tesis

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Director de tesis

Mohamed Abdelaziz, Profesor Titular de Universidad, y Antonio Jesús Muñoz Pajares, Profesor Contratado Doctor, ambos doctores y profesores del Departamento de Genética de la Universidad de Granada,

CERTIFICAN:

Que los trabajos de investigación desarrollados en la Memória de Tesis Doctoral: ***Speciation associated to evolutionary transitions. Mechanisms driving the ploidy effects on the diversification in a selfing species complex***, son aptos para ser presentados por Ana García Muñoz ante el Tribunal que en su día se designe, para aspirar al Grado de Doctor en Biología por la Universidad de Granada.

Y para que así conste, en cumplimiento de las disposiciones vigentes, extiendo el presente certificado a mayo del 2023.

Fdo.: **Mohamed Abdelaziz**

Director de tesis

Fdo.: **A. Jesús Muñoz Pajares**

Director de tesis

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La doctoranda, Ana García Muñoz, y los directores de la tesis, Mohamed Abdelaziz y Antonio Jesús Muñoz Pajares, garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por la doctoranda bajo la dirección de los directores de tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada, 23 de mayo de 2023

Fdo.: **Ana García Muñoz**

La doctoranda

Fdo.: **Mohamed Abdelaziz**

Director de tesis

Fdo.: **A. Jesús Muñoz Pajares**

Director de tesis

El presente trabajo se ha desarrollado en el grupo de ***Genética Evolutiva*** usando las instalaciones con las que cuenta el **Departamento de Genética** de la Universidad de Granada (España) y mediante colaboraciones establecidas con el grupo ***FLOWer Lab***, del Centre for Functional Ecology, Associate Laboratory Terra, Departament of Life Sciences de la Universidad de Coímbra (Portugal) y con el grupo ***MESSY Lab*** del Departamento de Biología, Instituto Universitario de Investigación Marina (INMAR) de la Universidad de Cádiz (España). Los trabajos de campo se han llevado a cabo en la Península Ibérica y Marruecos. El desarrollo de la presente tesis ha sido financiado gracias al proyecto *Entendiendo las transiciones evolutivas en los sistemas reproductivos de plantas: el paso "prohibido" de especies autógamas a alógamas (**OUTevolution**)*, proyecto I+D+i del **Programa Estatal de Investigación** del **Ministerio de Ciencia e Innovación**, que además de financiar la investigación, procuró a la estudiante un contrato de técnico asociada al proyecto.

A mi madre, por su apoyo incondicional

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Premios

Segundo premio a la mejor comunicación oral:

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Modesto Berbel, **Ana García -Muñoz**, José Carlos del Valle, Eduardo Narbona, Mohamed Abdelaziz, A. Jesús Muñoz-Pajares. 2021. ¿Es el polimorfismo de color floral una señal honesta para polinizadores las zonas híbridas? **II Simposio Anual de Botánica Española**. Madrid.

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RESUMEN

En la presente tesis doctoral se aborda el estudio de los mecanismos involucrados en fenómenos de diversificación dentro un clado multiploide y autógamo en elque parece que se están dando procesos de transición evolutiva en el sistema reproductivo hacia la alogamia. En especial se ha explorado el efecto de los diferentes niveles de ploidía encontrados en este complejo de especies como promotor de potenciales transiciones evolutivas. Entre los factores involucrados en estas transiciones ecológicas en plantas, hemos evaluado el sistema reproductivo predominante de las especies *Erysimum wilczekianum* y *Erysimum incanum* así como las características más prominentes en cuanto a fenotipo floral e inversión reproductiva tanto masculina como femenina. En el ***capítulo 1*** identificamos el nivel hexaploide en poblaciones de *E. incanum* que presentan una distribución aislada en las montañas del Alto Atlas y del Antiatlas marroquí. Esta ploidía mostró una serie de características que podrían promover el éxito de eventos de polinización cruzada, aun cuando la especie está ampliamente descrita como autocompatible y con mecanismos que facilitan la auto-polinización. La presencia de barreras reproductivas asimétricas es común entre especies que presentan distintos sistemas reproductivos, tal como ocurre entre las dos especies con sistemas reproductivos más divergentes en el ***capítulo 2***. Además, la alta intensidad de las barreras previas a la polinización sugiere que ambas especies se encuentran en un estado temprano de divergencia. El efecto de la ploidía sobre la interrelación establecida entre los caracteres fenotípicos medidos y su efecto sobre la eficacia biológica es explorada en varias poblaciones de ***E. incanum***. Así, en el ***capítulo 3*** se demuestra el efecto de las presiones selectivas sobre caracteres estrechamente relacionados con estrategias reproductivas más asociadas a la alogamia, y cómo esta selección es más intensa en ploidías superiores. Sin embargo, no encontramos un efecto de la ploidía sobre la plasticidad fenotípica exhibida por los caracteres en el ***capítulo 4***, aunque sí demostramos el potencial evolutivo de ésta explorando los beneficios y costes para la eficacia biológica asociados a caracteres cuya plasticidad es heredable. La ploidía tampoco afectó a la relación inversa que encontramos entre la plasticidad y la integración fenotípica en *E. incanum* en el ***capítulo 5***. En este capítulo, usando herramientas de genética cuantitativa, proponemos a la selección natural como mecanismo responsable del papel limitante que la integración fenotípica de los caracteres ejerce sobre sus plasticidades, al presentar la primera una respuesta a la selección más intensa que la que pueda presentar la plasticidad. Por último, exploramos el papel que tiene la ploidía y niveles opuestos de heterocigosidad, conseguidos mediante cruces controlados en plantas de *E. incanum*, sobre los escenarios resultantes de la interacción entre estos. En este último ***capítulo 6*** demostramos que los distintos niveles de ploidía y heterocigosidad no son competitivamente excluyentes entre ellos y que, por el contrario, incurren en escenarios de coexistencia y de efectos prioritarios. La combinación de los resultados obtenidos en esta tesis llaman la atención sobre la posibilidad de eventos de diversificación asociados a transiciones en sistemas reproductivos desde la autogamia dentro de un complejo de especies autógamo, algo inesperado según el marco teórico vigente.

INTRODUCCIÓN GENERAL

Y OBJETIVOS

La diversidad biológica observada en la naturaleza es una de las consecuencias más evidentes de la actuación de la selección natural como mecanismo clave en los eventos de especiación y diversificación (Darwin's, 1859; Endler, 1986). En este contexto, la especiación ecológica resulta de especial interés al promover la aparición y evolución de barreras al flujo genético entre poblaciones debido a la interacción con factores ambientales tanto bióticos como abióticos (Nosil, 2012). Estos factores actúan como agentes de la selección divergente, responsable a su vez de la acumulación de diferencias fenotípicas y genéticas que contribuyen al aislamiento reproductivo entre las especies incipientes (Mayr, 1947; Rundle & Nosil, 2005). La divergencia entre especies mediada por selección natural se cimenta tanto en la selección directa sobre rasgos fenotípicos que influyen en la aparición y el mantenimiento de barreras reproductivas, como en la selección indirecta sobre rasgos genéticamente correlacionados que favorece el funcionamiento modular de los organismos.

Los rasgos conocidos como *caracteres mágicos* (*magic traits* en inglés; Gavrilets, 2004) contribuyen de forma simultánea a la divergencia ecológica y al aislamiento reproductivo (Servedio et al., 2011; Servedio & Kopp, 2012). Por tanto, juegan un papel fundamental no solo en la diversificación de los organismos, sino en la velocidad con la que ésta se da. En angiospermas las flores son estos caracteres mágicos que combinan la capacidad de dirigir la divergencia entre diferentes formas existentes en una población y su función principal reproductora. Como resultado podemos observar maravillados la diversidad de patrones florales que presentan las angiospermas desde la aparición de las primeras estructuras florales (One Thousand Plant Transcriptomes Initiative, 2019), fruto de la evolución del amplio abanico de estrategias reproductivas que presentan las plantas. Desde mecanismos que aseguran la reproducción mediante la polinización autógama, a específicas interacciones con polinizadores que aseguran una eficiente transferencia de polen entre individuos. Los requerimientos fenotípicos de cada sistema reproductivo son el producto de la selección sobre determinados caracteres que aseguran una óptima eficacia biológica. Un cambio en el sistema reproductivo dentro de una especie puede conllevar la divergencia en los rasgos fenotípicos asociados a este.

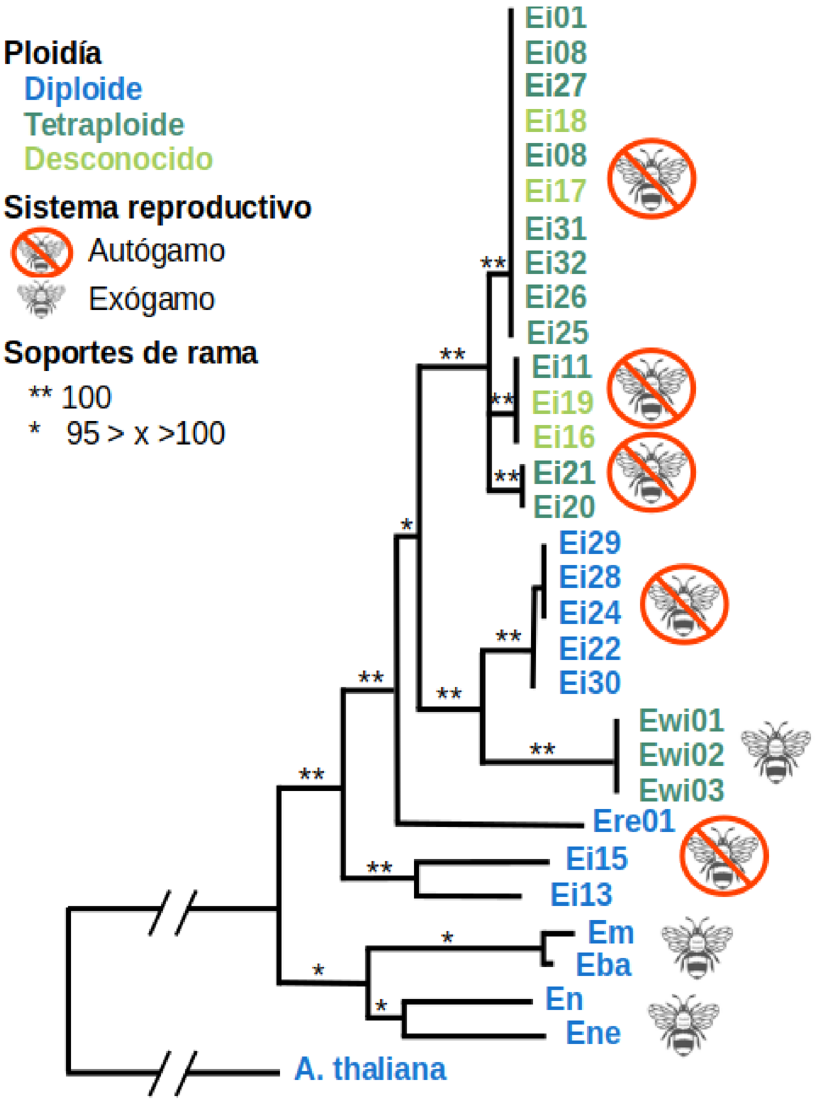

Figura I.1: Representación esquemática de las relaciones evolutivas de las especies del complejo incanum (Ei = *E. incanum,* Ewi = *E. wilczekianum*, Ere = *E. repandum*) y de otras especies de Erysimum de la Península Ibérica (Em = *E. mediohispanicum*, Eba = *E. baeticum,* En = *E. nevadense*) y Marruecos (Ene = E. nervosum). Se representan además los niveles de ploidía de cada población y el sistema reproductivo de los diferentes clados (A. thaliana = *Arabidopsis thaliana)*.

La transición hacia la autogamia es uno de los ejemplos de convergencia evolutiva más comunes y mejor descritos en planta (Wright et al., 2013; Slotte et al., 2013). Este cambio a nivel de estrategia reproductiva lleva asociado un cambio en rasgos florales y reproductivos que son recogidos bajo el término *síndrome de autogamia* (del inglés *selfing syndrome*; Darwin, 1876; Ornduff, 1969; Sicard & Lenhard, 2011). Este se manifiesta como una reducción del tamaño floral y una inversión de recursos menor en la función masculina, tanto en producción de polen, como en producción de otras recompensas y atrayentes que potencian la polinización cruzada, la cual pasa a segundo plano en contraposición a las ventajas derivadas de la autogamia, también ampliamente descritas (Darwin, 1876; Fisher, 1949; Lloyd, 1979, 1992; Aarssen, 2000; Kennedy & Elle, 2008). En este marco teórico, las transiciones evolutivas y sus consecuencias son un motor de diversificación en las plantas. De hecho, la divergencia en los ratios de auto-polinización así como los cambios en el fenotipo floral, especialmente en los rasgos con gran influencia en el comportamiento de los polinizadores como son el tamaño y la forma floral, han sido identificados como mecanismos precursores de aislamiento reproductivo asociado a una transición en el sistema reproductivo (Gómez et al., 2008; 2009; Parachnowitsch & Kessler, 2010). Sin embargo, la reducida diversidad genética provocada tras varios eventos de auto-polinización ha llevado a considerar a la autogamia un callejón sin salida en términos evolutivos (Takebayashi & Morrell, 2001; Igic & Busch, 2013) generando la conocida hipótesis de la *autogamia como callejón evolutivo sin salida* (*selfing as evolutionary dead end*, SEDE; Takebayashi & Morrell, 2001, y las citas en el contenidas), marco conceptual en el que se desarrollan la mayoría de las investigaciones sobre evolución de los sistemas reproductivos en plantas.

Por otro lado, el fenotipo, independientemente de una mayor asociación al sistema reproductivo o al aislamiento reproductivo, se ve influenciado por mecanismos genéticos. Un ejemplo muy extendido en la historia evolutiva de las plantas con flores es el evento de poliploidización (Soltis & Soltis, 1999; Otto and Whitton, 2000; Leebens-Mack et al., 2006; Soltis et al., 2009; Wood et al., 2009). Se han identificado los efectos sobre el fenotipo y la maquinaria metabólica de los incrementos de copias génicas, pero el alcance que los niveles superiores de ploidía pueden tener en el potencial evolutivo de un grupo no es del todo claro. La poliploidización puede darse en especies autógamas, pudiendo generar un extra de diversidad a nivel genético y a nivel fenotípico entre individuos que reproductivamente tienen su diversidad genética mermada. Mediante su efecto en estos caracteres involucrados con el sistema reproductivo y en los ya mencionados *magic traits*, la ploidía podría jugar un papel esencial sobre los cambios evolutivos que, en última instancia, actuarían como motor de divergencia.

Dentro de los mecanismos que actúan potencialmente como promotores de diversidad y evolución, la plasticidad fenotípica ha constituido un tema de debate central en el ámbito de la biología evolutiva en las últimas décadas (Price et al., 2003; Schlichting, 2004; Fitzpatrick, 2012). La plasticidad fenotípica se ha definido como la habilidad de un genotipo individual para expresar variación en su fenotipo asociado como respuesta al ambiente (Pigliucci et al., 2006). El potencial adaptativo de la plasticidad fenotípica, por tanto, está siendo un sujeto de estudio actual en muchos trabajos cuyas conclusiones muestran un amplio contraste (Relyea, 2002; Nussey et al., 2005; Murren et al., 2015; Acasuso-Rivero et al., 2019; Svensson et al., 2020). Sin embargo, a pesar de la capacidad adaptativa de la plasticidad fenotípica demostrada en determinados contextos y organismos, la inherente correlación genética entre rasgos, conocida como integración fenotípica (Pigliucci, 2003), tiene un efecto importante sobre su potencial evolutivo al limitar los cambios en rasgos fenotípicos interrelacionados en pos de una mejor funcionalidad de los órganos y organismos (Gianoli & Palacio-López, 2009; Damián et al., 2020; Matesanz et al., 2021). Los estudios sobre las bases genéticas de la capacidad de plasticidad fenotípica tampoco han arrojado resultados claros sobre el efecto de ciertos mecanismos como la poliploidía (Sánchez Vilas and Pannell, 2017; Shimizu-Inatsugi et al., 2017; Van de Peer et al., 2017; Wei et al., 2019). Sin embargo, el incremento de copias cromosómicas aportado por los eventos de poliploidización podrían tener efectos no solo sobre la plasticidad fenotípica, sino también sobre la interacción de esta con la integración fenotípica.

Los procesos de especiación y diversificación de especies son también el resultado de la interacción entre organismos en la naturaleza. De hecho, la diversidad de especies que observamos en la naturaleza sigue un patrón de distribución determinado por estas dinámicas poblacionales que a su vez construye las comunidades ecológicas. Como predice la teoría moderna de la coexistencia, el destino de una especie en la comunidad viene moldeado por el balance de la habilidad competidora de cada organismo que puede resultar en escenarios de coexistencia o de exclusión de especies (Chesson, 2000). Dentro del contexto ecológico en el que se desarrollan estas teorías, la influencia de factores genéticos como la ploidía y la diversidad genética sobre los escenarios resultantes de las interacciones entre organismos ha pasado desapercibida. Sin embargo, el conocimiento de estas interacciones entre mecanismos genéticos y dinámicas ecológicas ayudarían a entender el alcance de los cambios evolutivos que se plantean en esta tesis, usando como sistema de estudio el complejo de especies *Erysimum incanum*.

El género *Eryismum s.l.* es uno de los más diversos de la familia Brassicaceae. Presenta un amplio rango de distribución en el hemisferio norte (Polatschek, 1986), habitando el norte y el centro de América y África, aunque la mayoría de especies se encuentran en Eurasia (Al-Shehbaz et al., 2006), siendo la cuenca del Meditarráneo un punto caliente de diversificación que alberga cientos de especies (Blanca & Morales, 1990; López et al., 1991; Ollero and Saiz, 2002). Se han identificado mecanismos de adaptación local y eventos de hibridación entre especies dentro de este género (Gómez et al., 2009; Abdelaziz, 2013; Osuna-

Mascaró et al., 2023), así como procesos de poliploidización (Clot, 1991). Dentro del género nosotros llamamos complejo de especies *Erysimum incanum* al compuesto por poblaciones anuales, monocárpicas y con caracteristicas muy similares distribuidas en la cuenca occidental mediterránea (Nieto-Feliner & Clot, 1993; Abdelaziz et al., 2014). Dentro del complejo, la especie *Erysimum incanum* presenta poblaciones diploides ($2n = 2x = 16$ cromosomas) y tetraploides ($2n = 4x = 32$; Nieto-Feliner & Clot, 1993). Las poblaciones diploides muestran una distribución vicariante entre las montañas del Rif y de los Pirineos mientras que las poblaciones tetraploides presentan una distribución similar en el sudeste de la Península Ibérica y las montañas del Medio Atlas, Alto Altlas y Antiatlas (Nieto-Feliner & Clot, 1993; Fennane & Ibn-Tattou, 1999). La especie *E. incanum* ha sido descrita como autógama, mostrando rasgos típicos del síndrome selfing como es la reducción en el tamaño floral y la producción de polen, así como el mecanismo del *Anther rubbing,* recientemente descrito y que favorece la auto-polinizción *a priori* (Abdelaziz et al., 2019). Dentro del complejo *E. incanum*, la especie *Erysimum wilczekianum* ha sido descrita como una especie de limitada distribución en las montañas del Medio Atlas. La reproducción de esta especie es mayormente mediada por polinizadores y presenta un fenotipo más relacionado con la polinización cruzada en cuanto a tamaño floral, indicando su entomofilia. Recientes análisis filogenéticos basados en el cloroplasto completo apuntan a que *E. wilczekianum* constituye, junto a *E. incanum* y *E. repandum*, un clado monofilético dentro del género *Erysimum* (Figura I.1). Este clado en el que se encuentran todos los *taxa* que conforman el complejo de especies *E. incanum* parece ocupar una posición filogenética basal en reconstrucciones que incluyen un rango más amplio de especies del género *Erysimum* (Gómez et al., 2014; Moazzeni et al., 2014; Züst et al., 2020). Estos resultados sugieren una transición hacia la exogamia dentro de un grupo cuyo potencial evolutivo se suponía mermado tras una larga historia evolutiva de autogamia, lo cual pone en entredicho al paradigma actual y presenta un nuevo patrón en el origen de la biodiversidad.

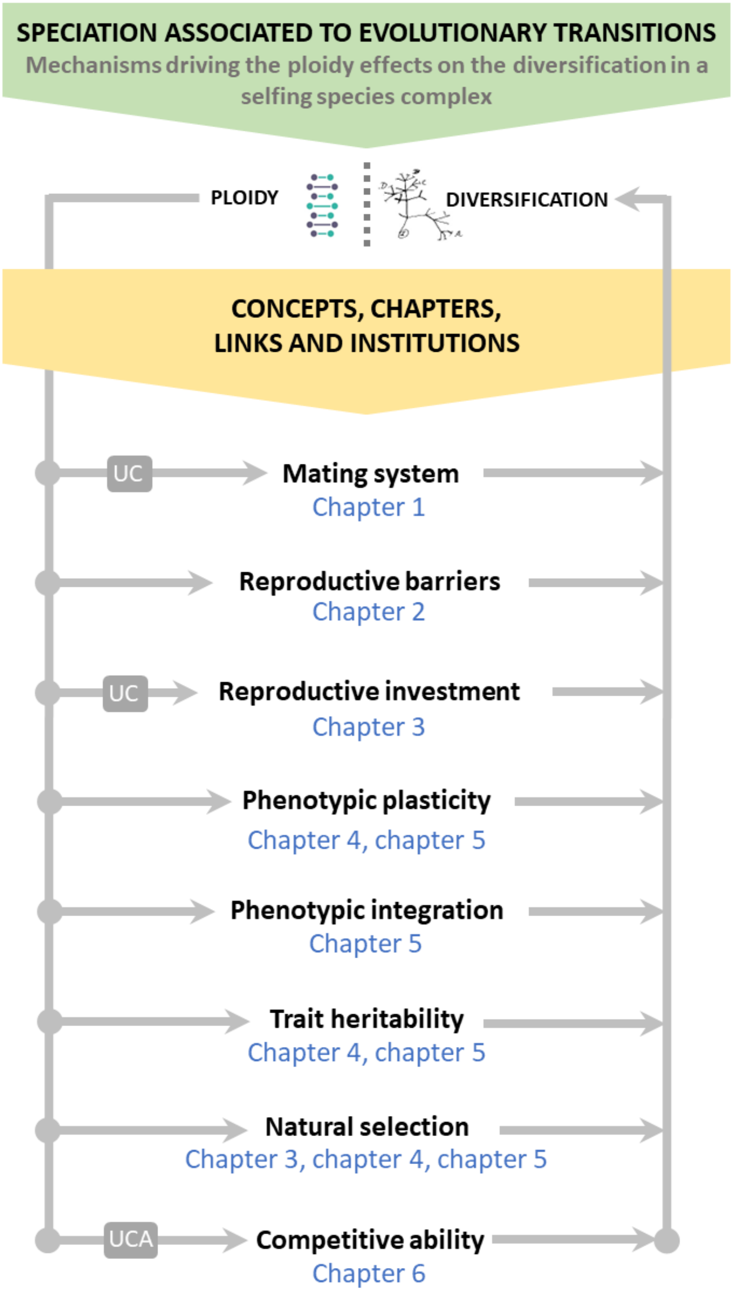

Figura I.2: Representación esquemática de los conceptos, capítulos y conexiones establecidas entre ellos en la presente tesis. También se indican las instituciones con las que se ha colaborado para el desarrollo de alguno de los capítulos: Universidade de Coimbra (UC) y Universidad de Cádiz (UCA).

OBJETIVOS

El objetivo principal de la presente Tesis Doctoral es explorar el papel de la ploidía sobre los mecanismos involucrados en la diversificación dentro un clado autógamo en el que están ocurriendo procesos de divergencia en el sistema reproductivo y, como consecuencia, en el potencial evolutivo exhibido por caracteres primarios y secundarios relacionados con las nuevas estrategias reproductivas. Este objetivo general se compone de una serie de objetivos específicos (Figura I.2):

Objetivo 1. *Explorar el efecto de la ploidía en el sistema reproductivo dentro del complejo de especies Erysimum incanum.*

Para esta tarea, llevaremos a cabo cruces controlados a nivel inter e intra individual en plantas de *E. incanum* y de *E. wilczekianum*, además de medir caracteres florales y de inversión reproductiva tanto masculina como femenina. Previamente, exploraremos los diferentes niveles de ploidía. Estimaremos el coeficiente de depresión por endogamia a partir de la producción de semillas obtenidas de cruces intra e inter individuales. Este coeficiente, junto a los rasgos florales y a ratio pollen/óvulos, nos aportará información sobre la estrategia reproductiva dominante de cada especie y nivel de ploidía.

Objetivo 2. *Analizar el aislamiento reproductivo entre E. incanum y la especie con rasgos entomófilos de este clado, E. wilczekianum.*

Para este objetivo realizaremos también una serie de cruces controlados entre individuos de diferente especie en ambos sentidos para evaluar las barreras reproductivas entre las especies y posibles asimetrías en ellas. El aislamiento reproductivo entre ambas especies se estimará a través de diferentes barreras reproductivas que actúan secuencialmente a lo largo de la vida de la planta, obteniendo componentes que limitan el flujo génico tanto antes como después de la polinización. Nuestro experimento nos permitirá estudiar tanto la primera como la segunda generación de híbridos, cuyas eficacia biológica será comparada con las especies parentales. La magnitud de cada una de las barreras reproductivas estimadas nos aportará información sobre la fase en la que estas dos especies se encuentran dentro del proceso de especiación.

Objetivo 3*. Analizar el efecto de la ploidía sobre los caracteres sexuales primarios y secundarios en E. incanum.*

Para este objetivo, mediremos una serie de rasgos relacionados con la reproducción como son el tamaño floral, la posición relativa de los órganos sexuales dentro de la flor y la inversión relativa entre polen y óvulos (ratio P/O), así como la eficacia biológica medida y la producción de semillas, para cada una de las ploidías. Mediante la relación global de estos caracteres entre ellos mismos y con la eficacia biológica, cuantificamos las presiones selectivas que actúan sobre ellos en cada ploidía. Dada la importancia del ratio P/O en estudios de reproducción floral, evaluaremos su efecto sobre los patrones selectivos que se dan en el resto de caracteres.

Objetivo 4. *Explorar el efecto de la ploidía sobre los patrones de plasticidad fenotípica en E. incanum.*

Para este objetivo, calcularemos un índice de plasticidad fenotípica (*RDPI*) para un total de trece rasgos medidos en plantas sometidas a dos tratamientos: condiciones controladas dentro de invernadero y condiciones estocásticas fuera del invernadero en un jardín común. Se comparará la plasticidad fenotípica de cada rasgo entre los tres niveles de ploidía identificados en *E. incanum*. De esta forma se evaluarán costes, efectos neutrales o beneficios sobre la eficacia biológica asociada a la habilidad de expresar plasticidad fenotípica.

Objetivo 5. *Explorar la relación establecida entre plasticidad e integración fenotípica a lo largo de las diferentes ploidías de E. incanum.*

Evaluaremos el efecto de la plasticidad fenotípica sobre la integración fenotípica presentada por los caracteres bajo los dos tratamientos utilizados para calcular los índices de plasticidad en el objetivo anterior. Además se explorará el efecto de la ploidía sobre esta relación. Caracterizaremos los componentes de la plasticidad y la integración fenotípica para un posterior análisis de mecanismos de cambio evolutivo que pudieran explicar su relación causal.

Objetivo 6. *Analizar los efectos de la variación en ploidía sobre las estimas de heredabilidad de caracteres y la heredabilidad de las plasticidades de los mismos.*

Llevaremos a cabo estimaciones de la heredabilidad para cada uno de los caracteres, además de sus respectivas plasticidades fenotípicas. Esta heredabilidad será usada para analizar los patrones adaptativos que exploraremos asociados a la plasticidad y la integración fenotípica.

Objetivo 7. *Efecto de la selección natural sobre caracteres, plasticidad fenotípica e interacción entre caracteres.*

Para este objetivo, calcularemos medidas de selección natural sobre los valores de los caracteres estudiados en sujetos experimentales bajo diferentes condiciones ambientales y sobre las plasticidades de dichos caracteres, usando varios componentes de eficacia biológica. Analizaremos la intensidad relativa de la selección indirecta sobre los caracteres correlacionados y la selección directa sobre la plasticidad fenotípica para evaluar el alcance de la integración limitando la capacidad evolutiva de la plasticidad. Todas estas relaciones se compararán entre los diferentes niveles de ploidía de *E. incanum* y entre los módulos funcionales en los que los caracteres medidos han sido agrupados.

Objetivo 8. *Explorar el efecto de mecanismos de diversidad genética sobre los escenarios resultantes de las interacciones ecológicas.*

Para este último objetivo, aplicaremos modelos de competencia ecológica para evaluar el resultado de la interacción entre las distintas ploidías y entre reducidos y elevados niveles de heterocigosidad en *E. incanum.* De esta forma, se evaluarán estos dos componentes de diversidad genética sobre la habilidad competitiva y los posibles escenarios de exclusión por competencia o coexistencia que derivarían de ésta a nivel intraespecífica.

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Chapter 1

Mechanisms driving mating system transitions in a multiploidy selfing species complex

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ABSTRACT

The evolution of mating systems in plants has been central to understand the rise of their diversity on Earth. Different mechanisms have been identified driving this evolution, and the transition to self-fertilisation is proposed as a paradigmatic example of convergent evolution. However, exploring these mechanisms in a multiploidy selfing species complex is scarcely possible. In the present work, we used 1,123 plants from the different taxa composing the *Erysimum incanum* species complex to carry out over 19,000 controlled crosses to evaluate pollen-stigma compatibility. These results on breeding preferences and reproductive investment, along with estimates on phenotypic traits, ploidy levels and genomic diversity per taxa, allowed us to characterise mating systems in this clade. We found three different ploidy levels, one more than the previously described in this group. Furthermore, significant differences were found between the species and ploidy levels analysed for the breeding preferences and traits related to the reproductive investment, floral size and relative position of reproductive organs. We also described significant differences in the genomic diversity associated with the diverging mating system exhibited by the taxa. Overall, our findings suggest that diploid individuals are mainly selfers, while polyploids (tetraploids and hexaploids) are less tolerant to self-fertilisation. Into the species complex, *E. wilczekianum* showed the most evident traits of an evolutionary transition to outcrossing mating, exhibiting the highest differences from the diploid plants. This transition in the mating system to outcrossing in a selfing clade associated with speciation and polyploidisation events is highly unexpected and has not been described in nature before.

Key words *Erysimum*, self-fertilisation, inbreeding depression, outbreeding depression, pollen-ovule ratio, flower size, herkogamy, genome size, pollen-stigma interaction

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INTRODUCTION

Patterns of genetic transmission between generations shape the mating system in every organism. The vast majority of flowering plants are hermaphrodites, developing pollen and ovules in the same flower. However, they display an impressive variation in mating strategies, including the development of organs and functions to optimise mating. The evolution of mating systems in plants and the mechanisms driving their diversity in nature have called the attention of scientists since Darwin (Darwin 1877; Jain 1976). Although increases in ploidy level have been widely associated with self-fertilisation for the stability of new karyotypes (Stebbins 1950; Levin 1975), the study of the different mechanisms interacting to control mating systems in multiploidy species complex is not common because to have variability at these two traits is not so frequent in nature. Here we characterise the mating systems of the taxa conforming the multiploidy and selfing *Erysimum incanum* species complex (Feliner 1991) and the drivers shaping the mating system transition associated with changes in ploidy and diversification.

Plants are sessile organisms, but mating is not random since individuals have developed mechanisms to improve reproduction success and increase the effectiveness of the transmission of genes to the progeny by a wide variety of reproductive strategies. Reproductive strategies in plants range from self-compatible systems promoted by autonomous or facilitated self-pollination, to plants showing self-incompatibility mechanisms, whose reproduction relies entirely on pollination vectors. Intermediate states between both extreme strategies are common in plants and are known as mixed mating systems (Goodwillie et al., 2005). However, even though mixed mating is common (Aide 1986; Barrett and Eckert 1990), a transition towards selfing is expected to occur in the majority of plant groups as it has been described in the evolutionary history of many taxa as a good example of convergent evolution (Levin and Kerster 1974; Zhang et al. 2022). The adaptive consequences of selfing are known since classical works from Darwin and Fisher, who hypothesised a high spreading ability of self-fertilisation alleles because of the transmission of the whole genetic material and the independence of mates and pollination vectors, conferring a reproductive assurance that grants such individuals a unique colonising ability (Darwin 1877; Fisher 1949; Lloyd 1979, 1992; Holsinger 1996).

Differences among mating systems involve relevant consequences on the genetic structure and the evolutionary dynamics of populations (Allard 1975; Charlesworth and Wright 2001), contributing to plant population variability and, ultimately, being the substrate for speciation and diversification observed in the wild. Speciation is expected to be reduced in selfing lineages, and thus, selfing is usually regarded as an evolutionary dead-end (Takebayashi and Morrell 2001; Igic and Busch 2013). This hypothesis has been supported and well documented by the many examples of convergent evolution to selfing, leading to a wide knowledge of the evolutionary consequences of this evolutionary transition (Stebbins 1950; Grant 1981; Charlesworth and Charlesworth 1987; Charlesworth 1992; Takebayashi and Morrell 2001). However, the reverse transition (from selfing to outcrossing) has been considered unexpected in nature and directly overlooked (Takebayashi and Morrell 2001; Igic and Busch 2013). Even the maintenance of mixed mating systems has become controversial regarding this theoretical framework (Flaxman, 2000; Plaistow et al. 2004). Because transitions to selfing are expected to be completed, mixed mating systems are considered an intermediate step in the transition from outcrossing to selfing (Goodwillie et al., 2005).

Selfing advantages are counteracted by fitness reductions experienced by inbred offspring, known as inbreeding depression (Darwin 1877; Charlesworth and Charlesworth 1987; Charlesworth and Willis 2009). This reduction in reproduction success occurs when recessive deleterious alleles become unmasked when the frequency of homozygotes increases (Charlesworth and Charlesworth 1987). In this scenario, inbreeding depression should evolve to reduce its effect in populations where self-pollination is frequent because deleterious alleles are purged by natural selection (Lande and Schemske 1985; Schemske and Lande 1985; Charlesworth and Charlesworth 1987; Husband and Schemske 1996). The limitation imposed by the low genetic diversity exhibited by selfing plants to cope with unpredictability in changing environments could be too constraining for the evolutionary outcomes of selfing populations. In contrast, cross-pollination in outcrossing species increases heterozygosity, which can be translated to more allelic combinations and even heterosis events. Predominantly outcrossing plants show inbreeding depression when self-pollination occurs, because the recessive deleterious alleles have not been purged. For this reason, the inbreeding depression index, which ranges from -1 to 1, is an accurate coefficient for characterising mating systems (Lohr and Haag 2015).

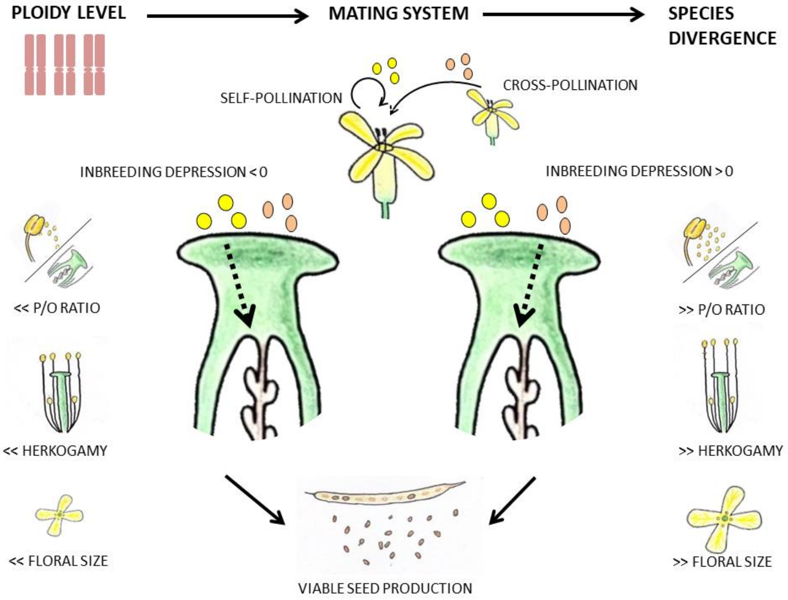

Figure 1.1. Mating system is shaped by several traits at different levels: tolerance to own or foreign pollen, relative reproductive investment to male and female function (P/O ratio), relative position between sexual organs (herkogamy) and floral attractiveness (for example, the floral size). Whole genome duplications could be affecting these components used as predictors of the mating system and, thus, on the evolutionary dynamics driving the speciation and species diversification.

Polyploidisation is the result of whole genomic duplications, having the potential to increase genetic diversity through new allelic combinations and interactions. Even though it is well-known how ploidy affects the plant phenotype (Jürgens et al., 2002; Te Beest et al., 2012; Moghe and Shiu 2014), its influence on mating system evolution is a more arguing topic despite the high incidence of polyploidisation events across the evolutionary history of flowering plants (Grant 1981; Soltis and Soltis 1999; Otto and Whitton 2000; Leebens-Mack et al. 2006; Soltis et al. 2009; Van de Peer et al. 2021). The effect of ploidy on mating system variation has been traditionally discussed from a theoretical framework which suggests that inbreeding depression is reduced in polyploid lineages, explaining the colonisation ability of polyploids in selfing species (Lande and Schemske 1985; Barrett and Eckert 1990; Husband and Schemske 1996; Ronfort 1999; Soltis and Soltis 1999; Tate and Simpson 2004; Barringer 2007). However, only a few experimental works have compared populations differing in both ploidy and mating systems (Johnston and Schoen 1996; Husband and Schemske 1997; Miller and Venable 2000; Rosquist 2001; Mable 2004), while studies addressing this question in populations sharing the same mating system are even scarcer (Barringer and Geber 2008). Since there is variation in reproductive strategies and their associated mating traits at intra-specific levels, the consideration of several populations for an accurate mating systems characterisation has gained importance in the last years (Whitehead et al. 2018). Indeed, Barringer and Geber (2008) discussed the influence of adding or removing a single population in the inbreeding depression estimation, and this can even be more important among populations differing in the genome copies.

Different attributes are related to pollination and plant reproduction and, hence, shape organisms' mating systems. Some of them involve primary sexual traits, such as reproductive investment or pollen-stigma recognition and rejection; while others are related to secondary sexual traits, such as flower attractiveness (Ornduff 1969; Barrett and Harder 1996). The corolla size might be one of the most representative traits related to plant attractiveness, enhancing pollinator visitation and pollen exportation (Herrera 1996; Gómez et al. 2008; Caruso et al. 2019). In hermaphrodite plants, the relative performance of sexual organs plays an important role in pollination. While hermaphroditism can facilitate autonomous self-pollination in selfing plants, the co-occurrence of both sexual organs is problematic in plants exhibiting any significant level of inbreeding depression. Then, traits such as the anthers-stigma separation, known as herkogamy, are essential to avoid sexual conflict and the problems derived from inbreeding depression (Charlesworth and Willis 2009; Armbruster et al. 2014) The evaluation of traits directly or indirectly related to reproduction is informative about the predominant reproductive strategy followed by plants. Genetic factors can influence the variation in these traits, which mediate the mating system evolution, as most of them have been demonstrated to have a genetic basis (Kruszewski and Galloway 2006; Eckert et al. 2009; Karron and Mitchell 2012; Cruzan and Barrett 2016).

Regarding primary sexual traits and the sex allocation theory (Charnov 1987, 1996), the relative relationships between pollen production and ovule amount by flower (i.e., P/O ratio) has been used as a conservative indicator in the mating systems characterisation (Cruden, 1977). This index is expected to show high values in outcrossing species since they must produce great amounts of pollen to be exported. Conversely, P/O ratio is lower in selfing species because the arrival of pollen grains to the stigma of the same flower is assured. The values of the P/O ratio are often correlated with other floral traits mentioned above, such as floral size and herkogamy (Galloni et al. 2007), suggesting that all these traits are closely related between them (Stebbins et al., 1971; Barrett and Eckert 1990; Lloyd et al., 1990) and with plant mating strategy. This index is important to recognise the mating system shown by a species, but it can vary among populations and even among individuals (Cruden, 2000). This is explained by the influence of environmental aspects on outcrossing and selfing rates, which are responsible for the mating system variation among locally adapted populations and even among years within the same population (Moeller and Gebre 2005; Cheptou and Avendaño 2006). Variation in the mating system involves relevant macro and microevolutionary consequences due to changes in population genetics and the interaction with other species (Charlesworth and Wright, 2001).

We studied the mechanisms driving the mating system evolution within a multiploidy species complex using several components for its characterisation (Figure 1.1). These components are the inbreeding depression, the reproductive investment (and P/O ratio), the floral traits related to pollination and pollen-stigma interaction. We compared these parameters between the taxa in the *Erysimum incanum* species complex, including different species and subspecies, and variation in ploidy level, floral size and geographic distribution. Moreover, the species complex has been described as a selfing clade, as most of the species composing it are selfing (Nieto-Feliner and Clot, 1993; Fennane and Ibn-Tattou, 1999). The main aims of this work were to: (i) evaluate the ploidy levels of the different populations included in this study; (ii) characterise the mating system of each taxa and the mechanisms shaping them; (iii) explore the variation of secondary reproductive traits influencing the evolution of mating system in the species complex; (iv) and explore the consequences of this divergence in mating systems on the genomic variability and diversification, into this group, of closely related species.

MATERIALS AND METHODS

Study system

In the Brassicaceae family, the genus *Erysimum* L. is one of the most diverse, and its species have been described as native plants in Eurasia, North and Central America and North Africa

(Al-Shehbaz et al., 2006). Ploidy variations are frequent in the genus, across but also within species (Clot, 1991). Diversification in the genus was also promoted by patterns of local adaptation and hybridisation between lineages (Abdelaziz. 2013). *Erysimum incanum* can be considered a species complex, including annual and monocarpic species and subspecies inhabiting the Eastern part of the Iberian Peninsula, Southeast of France and the four main mountain ranges in Morocco (Nieto-Feliner and Clot 1993; Fennane and Ibn-Tattou 1999; Abdelaziz et al. 2014). The species complex harbors diploids ($2n = 2x = 16$ chromosomes) and tetraploids ($2n = 4x = 32$ chromosomes; (Nieto-Feliner and Clot 1993; Fennane and Ibn-Tattou 1999). Diploids of *E. incanum* present a vicariant distribution in the Rif and the Pyrenees mountains, where species *E. incanum mairei* and *E. aurigeratum* were described, respectively. *Erysimum repandum* is also a diploid species, previously considered *E. incanum* (Nieto-Feliner and Clot, 1993; Fennane and Ibn-Tattou, 1999), inhabiting the Eastern half of the Iberian Peninsula (Nieto-Feliner and Clot, 1993). In the tetraploid level, we find *E. incanum incanum* that also presents a vicariant distribution in the Iberian Peninsula and Morocco (Nieto-Feliner and Clot 1993; Fennane and Ibn-Tattou 1999). *Erysimum incanum incanum* in Morocco was described as inhabiting Middle Atlas, High Atlas and Antiatlas (Nieto-Feliner and Clot 1993; Fennane and Ibn-Tattou 1999). Finally, *E. wilczekianum* is a tetraploid and entomophilous species, endemic to the Middle Atlas where they can be found growing close to *E. incanum* but never in sympatry (Nieto-Feliner and Clot 1993; Fennane and Ibn-

Tattou 1999; Abdelaziz et al. in prep.). All these taxa in the *Erysimum incanum* species complex form a monophyletic clade when we use genomic data for phylogenomic reconstruction (Abdelaziz et al., in prep.).

We can state that the *E. incanum* species complex exhibits selfing syndrome, as the predominant phenotypes include small, hermaphroditic and self-compatible flowers (Feliner 1991) and a recently described mechanism promoting prior selfing (*anther rubbing*; Abdelaziz et al. 2019). However, within the species complex, *E. wilczekianum*, a species with only three known populations, develops hermaphroditic but bigger flowers, visited by a generalist assemblage of pollinators in natural populations (***Chapter 2***).

In the present work we used plants from 11 populations from *E. incanum* species complex: two diploid populations, five tetraploid populations, including *E. incanum* (3) and *E. wilczekianum* populations (4), and four hexaploid populations. A total of 19039 hand crosses were carried out on 1123 plants of these populations. The population origin, the total number of plants and crosses made per population can be found in Table 1.1.

Genome size measurements

We estimated the genome size from young leaves using flow cytometry (FCM) and inferred the ploidy level following our previous knowledge of genome size variation in the *Erysimum* genus (Nieto-Feliner et al., 1993; Fennane et al., 1999; Muñoz-Pajares et al., 2018). We carried out the genome size analyses as described by Muñoz-Pajares et al. (2018).

Between 10 and 20 individuals were analysed per population from every range where these species occur (Table 1.1). Populations showing homogeneous values for ploidy level were considered as composed mainly of a single cytotype.

Experimental design

Controlled crosses experiment

Flowers from each species were subjected to two different treatments: (1) Selfing, in which pollen grains from the own flower were deposited in the style, and (2) Intra-Population Outcrossing, in which flowers were pollinated with pollen from another individual from the same population. For outcrossing treatment, flowers were carefully emasculated just before opening to avoid self-pollination, especially in the *E. incanum* populations because of the anther-rubbing mechanisms. Each manipulated flower was tagged to identify the type of treatment once the plant life cycle was finished. At the end of the experiment, 6980 selfing and 380 outcrossing hand-made crosses were done on 759 *E. incanum* plants. On 364 *E. wilczekianum* plants, we carried out 10398 selfing and 1655 outcrossing crosses. The number of individuals and total crosses in each population are detailed in Table 1.1.

Evaluation of fitness

When the plants were dry and the fruits ripened, these were recollected to count the number of viable and aborted seeds, and non-fertilised ovules for artificially crossed fruits. We compared four pre-dispersal fitness components among *E. wilczekianum* and the three ploidies of *E. incanum*. These components are the result of pollen-stigma-ovule interaction. This interaction can fail at two different stages: (1) before fertilisation because of the inhibition of pollen tubes, which are not able to reach the ovary and the ovules are not fertilised, and (2) after fertilisation, where pollen tubes reach the ovules but the fertilisation produce aborted seeds. The fitness component, ***seedset***, refers to the proportion of viable seeds produced per fruit compared to the total of ovules, while ***fertility***, is the proportion of fertilised ovules (viable seeds and aborts) compared to the total of ovules per fruit. A third fitness component, ***fertility success***, is the proportion of viable seeds compared to the number of fertilised ovules. When the fruit was not developed, probably because pollen tube growth failed on stigma, the seedset for this treatment was considered zero.

Inbreeding depression estimate

The relative reproductive success of inbred line crosses compared to the outcrossing line is called ***inbreeding depression***. The inbreeding depression coefficient (ID) was calculated using only the seedset from selfing and outcrossing treatments by individual plant, population, ploidy and species, following the Ågren and Schemske (1993) approach:

$$ID = 1 - \frac{Ws}{Wo} \text{ if } w_s < w_o \qquad \textbf{(1.1)}$$

and $$ID = \frac{Wo}{Ws} - 1 \text{ if } w_s < w_o \text{ ;} \qquad \textbf{(1.2)}$$

where $\boldsymbol{w_s}$ is the fitness components, the seedset in this case, from selfing treatment, and $\boldsymbol{w_o}$, the seedset from outcrossing treatment. Inbreeding depression ranges from -1 to 1. When negative and significantly different from zero, ***outbreeding depression*** occurs, meaning that selfing offspring show higher fitness than outcrossing ones (Frankham et al. 2011).

Significant positive values correspond to inbreeding depression, so fitness from outcrossing treatment is superior to selfing treatment. The significant values of these variables were calculated by computing the 99% confidence intervals using bootstrapping with 10 000 permutations, using the package *boot* v. 1.3-28 in R (Canty and Ripley 2017). Values not significantly different from zero mean no fitness differences between selfing and outcrossing offspring.

Plant phenotype measurement

For one flower in anthesis in a subset of 475 individuals of both species, we measured the following phenotypic traits: corolla diameter (i.e., the distance between the edge of a petal and the edge of the opposite one), the length of the long stamens (i.e., the distance between the basis of the long filament and the anther) and the height of the style (i.e., the distance between the insertion point of the style in the basis of the corolla tube and the stigma surface). We calculated herkogamy as the difference between the stamen length and the style height, showing positive values when the stigma surface is above the stamens while a negative herkogamy occurs when the stamens are higher than the style, facilitating the drop of own pollen on the stigma surface. From these flowers, we collected half of the stamens (two long stamens and one short stamen) and preserved them in alcohol 70° for counting pollen grains. Male and female reproductive investments were estimated at the end of the plant life cycle as pollen and ovule production per flower, respectively. Pollen grains and the number of ovules produced per flower were also used to calculate the P/O ratio described by

(Cruden 1977). By multiplying the number of pollen grains and ovules by the number of flowers, we estimated every plant individual's male and female reproductive investment.

Pollen growth and germination analysis

In a subset of 174 flowers, these were harvested 72 hours after a self-pollinating treatment and preserved in alcohol 90° to follow the aniline blue staining modified protocol proposed by (Xie et al. 2017). We obtained a qualitative estimation of the performance of the pollen-pistil interaction by the visualisation under UV microscopy. This approach gave us an estimation of each species' tolerance to its own pollen, which is the process underlying self-compatibility. To explore the performance of the pollen germination independently of the plant receptor, we did an experiment of pollen germination in vitro following a modified protocol by (Nogueira et al. 2016) in another subset of 170 pollen samples from both species.

Genomic diversity estimations

We have obtained whole genome sequencing data from one individual per studied population. For that, we collected leaf material conserved in silica gel until DNA extraction, performed using the GenElute™ Plant Genomic DNA Kit (SIGMA) following manufacturer instructions. The purity, integrity and concentration of the resulting DNA were evaluated by means of agarose gel visualisations and spectrophotometric methods (Nanodrop and Qubit). Individual Illumina libraries were performed using the Collibri ES DNA Library Prep Kit (ThermoFisher) and sequenced by Novogene using a NovaSeq sequencing system. The resulting fastq files were aligned using bwa (Burrows-Wheeler

Alignment Tool; H. Li and Durbin 2009) and the *E. cheiranthoides* genome as a reference (http://erysimum.org/). We used samtools (Li et al. 2009; Li et al. 2012) to convert sam files into bam files and bcftools to perform variant calls. Finally, the proportion of heterozygotic sites was estimated using the VCFTools (Danecek et al. 2011).

Statistical analyses The autonomous selfing success and inbreeding depression were compared between the two species and among ploidies and populations within *E. incanum* using ANOVA analyses and the Tukey test implemented in the R stat package. In addition, the relationships among the corolla diameter, herkogamy, P/O ratio and inbreeding depression for different species and ploidies were performed using the package stat in R. We performed generalised linear mixed model analyses to explore the effect of treatments and ploidies (fixed factors and their interaction) on the fitness components of *E. incanum* populations. These models were performed separately for seedset, fertility and fertility success. The individual plants nested in the population were considered random factors. The different models were compared using package lme4 (Bates et al. 2009) and Akaike information criterion (AIC), Bayesian information criterion (BIC), log-likelihood (LogLik), chi-squared test (X^2). All analyses were performed using R Statistical Software (v4.2.1; Team 2021).

RESULTS

Genome size We found three different genome size ranges in the populations included in this study. According to our previous knowledge of genome size variation in the *Erysimum* genus (Nieto-Feliner and Clot 1993; Fennane and Ibn-Tattou 1999), it was possible to assign each individual and population to a specific ploidy level, corresponding to diploids, tetraploids and hexaploids (Table 1.1). Diploid and tetraploid levels have previously been described in this species complex (Nieto-Feliner and Clot 1993; Fennane and Ibn-Tattou 1999), but the hexaploid cytotype is new. *Erysimum wilczekianum* showed values similar to the tetraploid populations of *E. incanum* (Table 1.1). As the genome size was homogeneous in every individual of each population studied, we assumed that each population was mainly composed of a single cytotype.

Species	**Pop**	**Origin**	**N plants**	**N crosses**	**Genome size**	**Ploidy**	**ID**	**99% C.I.**
E. incanum	Ei12	Rif	67	196	0.35 ± 0.01	2x	-0.310	**[-0.317, -0.302]**
E. incanum	Ei13	Pyrenees	19	34	0.34 ± 0.01	2x	-0.399	**[-0.469, -0.331]**
E. incanum	Ei08	Middle Atlas	302	4013	0.78 ± 0.10	4x	-0.009	**[-0.014, -0.005]**
E. incanum	Ei09	Baetic	110	572	0.78 ± 0.03	4x	-0.125	[-0.162, 0.088]
E. incanum	Ei11	Baetic	46	203	0.84 ± 0.07	4x	-0.111	**[-0.121, -0.101]**
E. incanum	Ei16	High Atlas	90	527	1.10 ± 0.03	6x	0.043	**[0.017 ,0.070]**
E. incanum	Ei17	High Atlas	25	641	1.12 ± 0.04	6x	0.562	**[0.553, 0.571]**
E. incanum	Ei18	Antiatlas	74	543	1.13 ± 0.03	6x	0.108	**[0.098, 0.118]**
E. incanum	Ei19	Antiatlas	26	246	1.11 ± 0.02	6x	-0.390	**[-0.397, -0.384]**
E. wilckzekianum	Ewi01	Middle Atlas	69	2584	0.71 ± 0.04	4x	0.542	**[0.539, 0.540]**
E. wilckzekianum	Ewi02	Middle Atlas	295	9480	0.73 ± 0.03	4x	0.588	**[0.586, 0.589]**

Table 1.1. Summary of the species and populations (Pop) accompanied by the geographical origin and the number of plants and crosses used in this study. Genetic information of each population is given by the genome size (mean ± SD) and the ploidy level. Values of inbreeding depression (ID) and its 99% confidence interval (99% C.I.) calculated by bootstrapping for populations are shown. In bold are shown the significant values for confidence intervals.

Treatment performance

The species *E. incanum* produced a significantly higher seedset than *E. wilczekianum* when the fruits analysed resulted from selfing (F = 4178, p-value < 0.0001). However, the data showed an important degree of dispersion (Figure 1.2A). We explored the mean differences in seedset from the inbred fruits. We found significant differences among all the populations (Figure 1.2B). In general, seedset after selfing was higher in diploid populations and decreased with the ploidy level, but the highest value was observed in a hexaploid population. The lowest value for selfing seedset was found in *E. wilczekianum* populations (Figure 1.2B). In *E. incanum*, variation in selfing seedset values across populations increases with ploidy (Figure 1.2B).

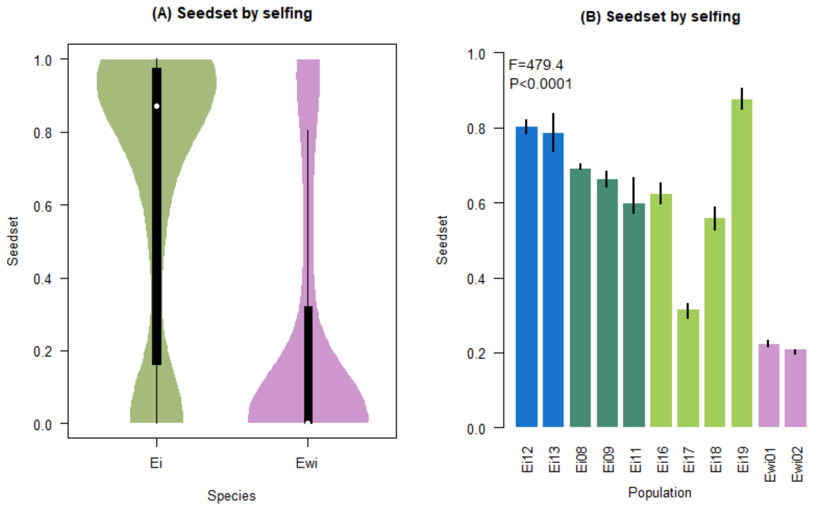

Figure 1.2. (A) Seedset produced by each species from selfing fruits and (B) mean values and SE of seedset from selfing fruits in each *E. incanum* and *E. wilczekianum* population. Blue bars indicate *E. incanum* diploid populations, turquoise bars indicate *E. incanum* tetraploid populations, green bars indicate *E. incanum* hexaploid populations and purple bars indicate *E. wilczekianum* tetraploid populations.

When comparing ploidies within *E. incanum*, mean values of the three fitness components were significantly different in the selfing treatment, with diploids showing the highest values (Figure 1.3A). However, no significant differences were found when we explored the same fitness components for the fruits resulting from outcrossing treatment (Figure 1.3B). The GLMM analyses revealed that the type of treatment (selfing or outcrossing) and the ploidy level had an effect on seedset but the best explanatory model included the interaction between these two factors (Table 1.2). These factors gained importance independently for the rest of the fitness components as fertility and fertility success. (Table S1.1 and S1.2).

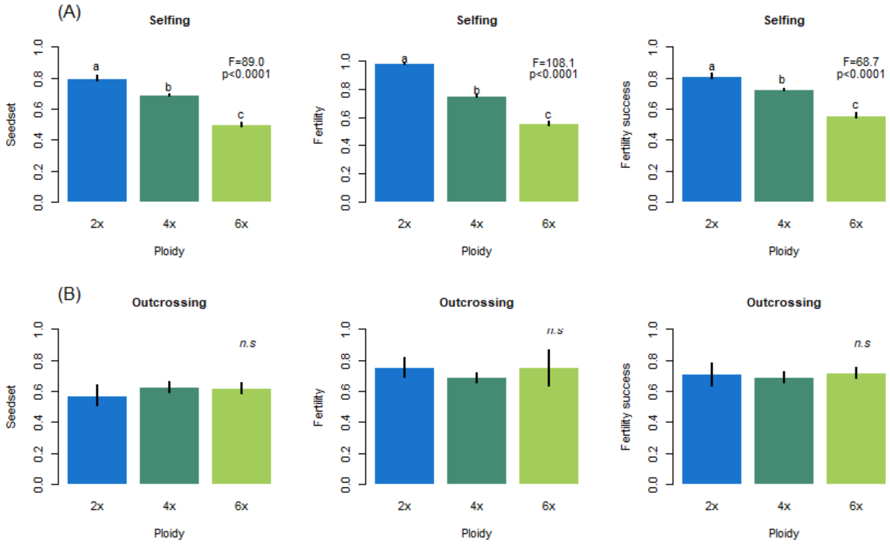

Figure 1.3. Mean values of seedset, fertility and fertility success among ploidies from fruits subjected to (A) selfing treatment and (B) outcrossing treatment.

Inbreeding depression

Average inbreeding depression values per population were significant for every population but one of the tetraploid populations (Ei09). *E. wilczekianum* populations exhibited the highest values of inbreeding depression. Diploid and tetraploid populations of *E. incanum* showed mostly significant negative values of inbreeding depression, indicating a higher success for selfing treatment in these ploidies, known as ***outbreeding depression***. The magnitude of the outbreeding depression was higher in diploids. Tetraploids showed lower but significant values of outbreeding depression in populations Ei08 and Ei11. The population Ei09 was the only one showing no significant values of inbreeding or outbreeding depression. In contrast, significant and positive values of inbreeding depression were found in most hexaploid populations. Only the Ei19 population showed significant values of outbreeding depression, similar to those exhibited by diploid populations.

	Seedset					
Model	AIC	BIC	logLik	χ^2	df	*P-value*
Intercept	2973.4	2999.3	-1482.7			
Model 1	2970.8	3003.2	-1480.4	4.539	1	**<0.05***
Model 2	2967.1	3070.8	-1467.5	25.733	11	**<0.01****
Model 3	2946.3	3609.4	-1453.2	26.804	3	**<0.0001******

Table 1.2. Outcome of the GLMM testing the effect of the treatment and the ploidy as fixed factors together with their interaction on *E. incanum* seedset. The individual plant and the population appear as random factors nested within the ploidy level. Significance p-values are indicated in bold (*p-value<0.05; **p-value<0.01; ***p-value<0.001; ****p-value<0.0001).
Model 1: Seedset ~ Treatment + (1 |Population/Plant)
Model 2: Seedset ~ Ploidy + (Ploidy |Population/Plant)
Model 3: Seedset ~ Treatment * Ploidy + (Ploidy |Population/Plant)

On average, *E. wilczekianum* showed a higher inbreeding depression value, while *E. incanum* tended to show an outbreeding depression behaviour (Figure 1.4A). However, when we compared the different ploidies within *E.*

incanum, they showed significant differences in inbreeding depression patterns. Thus, diploids showed a clear outbreeding depression pattern, while hexaploids showed the highest inbreeding depression values. Tetraploids showed values close to the non-difference between selfing and outcrossing performance (Figure 1.4B).

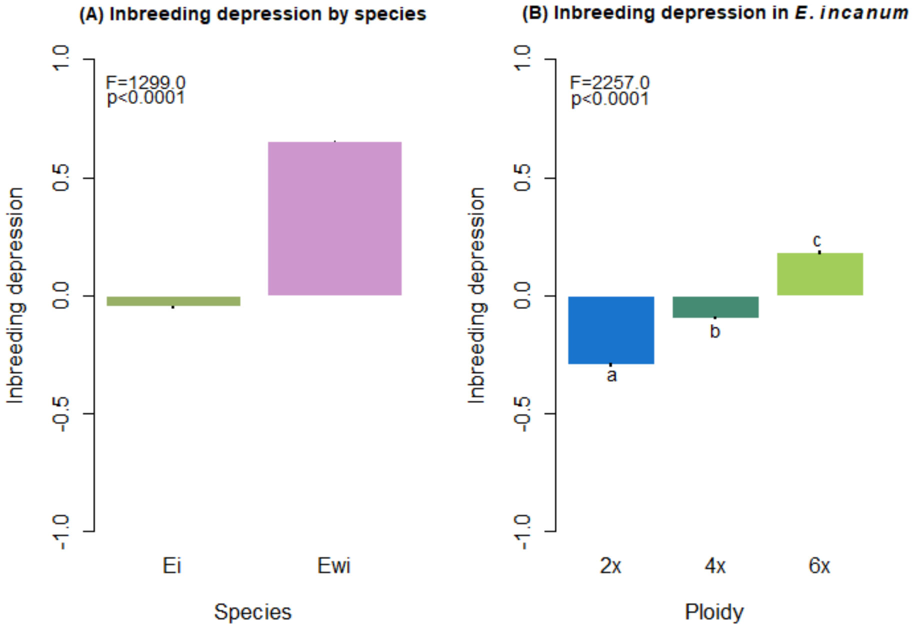

Figure 1.4. Mean values of inbreeding depression at seedset stage (A) between the species *E. wilczekianum* and *E. incanum and* (B) among the three ploidy levels exhibited by the species *E. incanum*.

Pollen tube germination and growth

No differences were found in the proportion of pollen grains germinated between the two species in the *in vitro* experiments (*F* = 0.33, *P-value* = 0.566; Figure 1.5A). However, we did find a different performance of the pollen tube growth for the selfing treatment between the two species (X^2=4.32, *df*=1, *P-value* < 0.03), with self-pollen tubes failing to grow in *E. wilczekianum*. In contrast, they successfully grew into the ovary of *E. incanum* (Figure 1.5B).

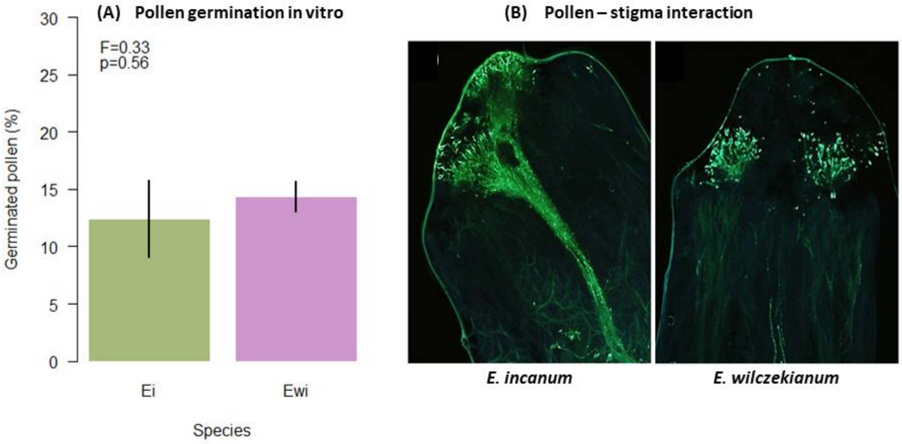

Figure 1.5. Performance of pollen tubes growth (A) in vitro, as the proportion of germinated pollen grains from both species in the same time interval and (B) the visualisation by UV luminescence of pollen tubes growing on the own stigma surface in a self-pollinated flower *E. wilczekianum* (left) and *E. incanum* (right), where pollen tubes growing until the ovary can be observed.

Reproductive investment

Regarding reproductive investment as an important indicator of the mating system, we found that flowers of the species *E. wilczekianum* produced substantially more pollen than *E. incanum* (Figure 1.6A), while the latter allocated more resources to the ovule production (Figure 1.6B). This relative resource allocation to male and female function was translated to a higher P/O ratio exhibited by *E. wilczekianum* (Figure 1.6C). Comparison of reproductive investment among ploidies within *E. incanum* showed an increase in pollen production in hexaploids. In contrast, tetraploids allocated more resources to ovule production (Figure S1.1).

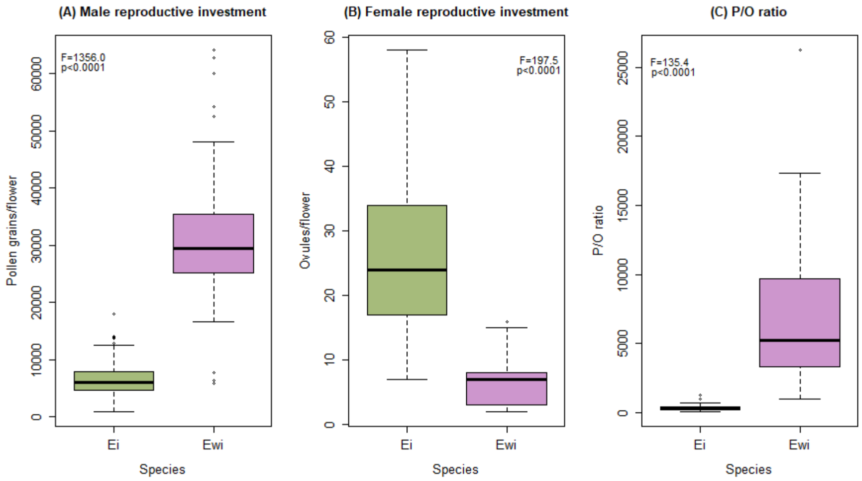

Figure 1.6. Differences in reproductive investment between *E. incanum* and *E. wilczekianum*: (A) male reproductive investment as the number of pollen grains per flower (B) female reproductive investment as the ovules amount per flower and (C) the relative amount between pollen and ovules as the P/O ratio.

Phenotypic traits and mating system relationships

When we explored the data set as a whole (Figure 1.7), we found a significant and positive relationship between P/O ratio and inbreeding depression per plant (Pearson's product-moment correlation; t = 5.78; df= 82, p-value < 0.0001). Plants allocating more resources to male function had larger flowers (t = 9.76; df=118; p-value < 0.0001) and a greater separation between stamens and stigma (t = 3.93; df=118; p-value < 0.001). Likewise, larger flowers (t = 10.62; df=81 p-value < 0.0001) and higher values of herkogamy (t = 5.03; df=81, p-value < 0.0001) were found in plants showing higher values of inbreeding depression. Finally, larger flowers were significantly correlated with positive values of herkogamy, while smaller flowers showed negative herkogamy (t = 10.05; df = 431; p-value < 0.0001). Individuals from each species appeared, forming two well-defined clusters in the correlations (Figure 1.7).

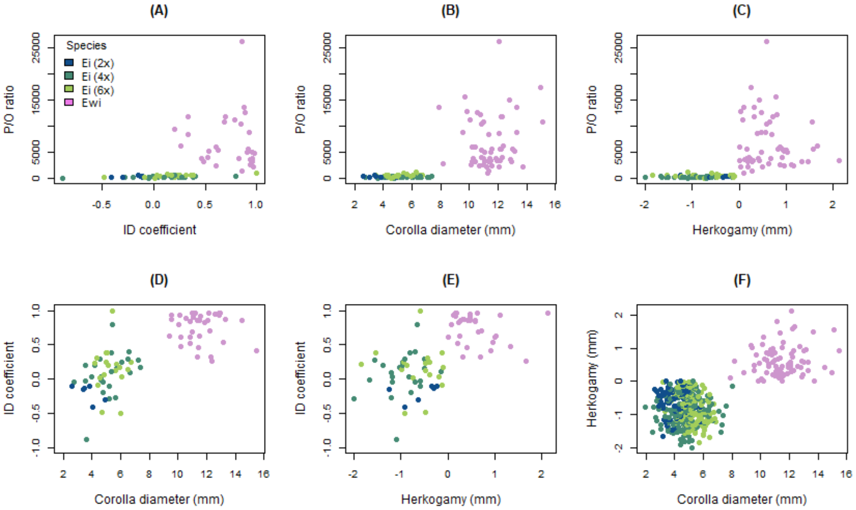

Figure 1.7. Correlations among mating traits related to inbreeding depression (ID coefficient), reproductive investment and floral phenotype shown by *E. incanum* and *E. wilczekianum* individuals.

However, less significant correlations were found when we explored these same relationships separating by species and ploidies in *E. incanum* (Figure 1.8). In diploids, only a significant correlation between herkogamy and inbreeding depression was found (t = 10.63, df = 3, p-value < 0.01). For tetraploid plants, there was only a significant positive correlation between inbreeding depression and P/O ratio (t = 2.44, df=26, p-value < 0.05), but plants with higher inbreeding depression marginally showed larger flowers (t = 1.96, df=22, p-value < 0.1). However, hexaploid plants exhibited a strong correlation between inbreeding depression and P/O ratio (t = 4.67, df =17, p-value < 0.001), but also a significant relationship between bigger flowers and higher values of P/O ratio (t = 2.16, df=21, p-value < 0.05). A strong significant correlation between larger flowers and higher herkogamy was found in hexaploids (t= -3.56, df = 132, p-value < 0.001). For *E. wilczekianum*, none of these relationships showed significant values (Figure 1.8).

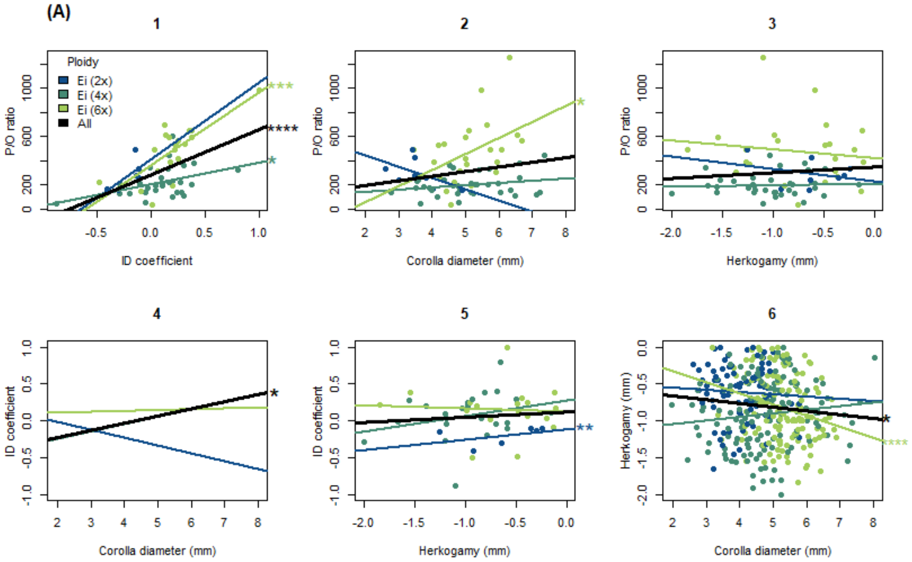

Figure 1.8. Continues to the next page with the legend.

Patterns across ploidies

The traits examined in the previous section showed significant differences among the three ploidy levels within *E. incanum* (Figure 1.9). Diploid and hexaploid plants showed significantly different values of inbreeding depression. Diploids exhibited significant values of outbreeding depression, while hexaploids showed significant values of inbreeding depression. Tetraploids exhibited intermediate values between the other two ploidies and were not significantly different from zero (Figure 1.9). However, it is important to consider that the inbreeding depression can vary enormously within a ploidy level, being only the populations of *E. wilczekianum* and diploids of *E. incanum* which showed coherent values of inbreeding and outbreeding depression, respectively (Table 1.1). The mean value of the P/O ratio was significantly higher in hexaploids than in lower ploidies. Corolla size increased significantly with the ploidy level (Figure 1.9). The separation between anthers and stigma (herkogamy) increased with the ploidy.

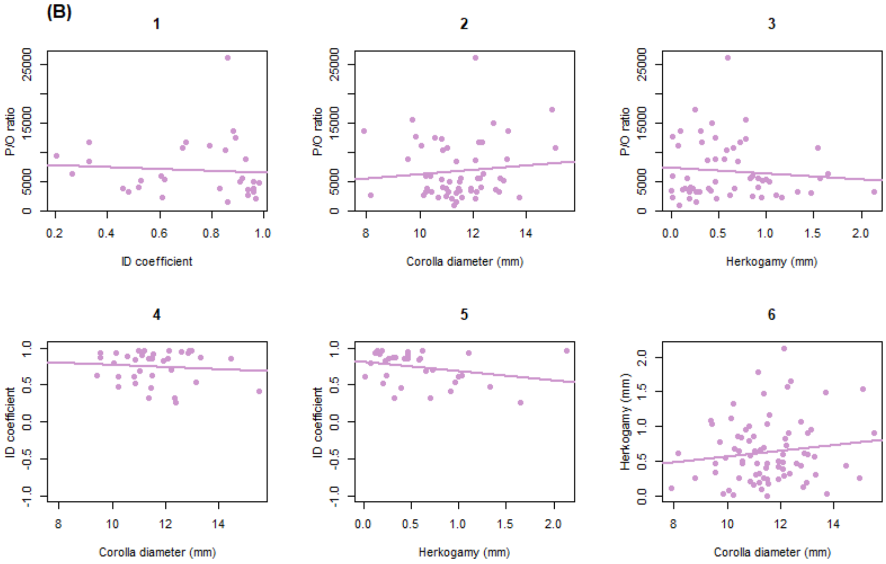

Figure 1.8. Correlations among mating traits in individuals from (A) *E. incanum* individuals and (B) *E. wilczekianum* individuals. *p-value<0.05; **p-value<0.01; ***p-value<0.001; ****p-value<0.0001.

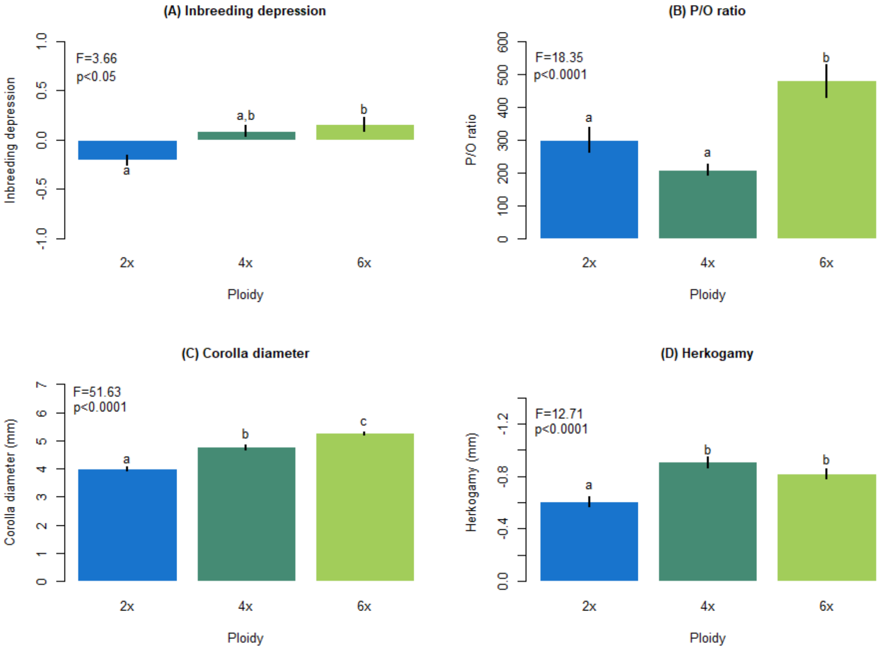

Figure 1.9. Mean values and SE values of mating traits measured in a subset of *E. incanum* plants (N=475) showing the three different levels of ploidy. Letters refer to these groups that are significantly different (P-value < 0.05).

Genomic diversity

In addition to the differences in mating traits among ploidies, we found an important inter-ploidy variation in the level of heterozygosity exhibited by wild genotypes (Figure 1.10). Genetic analyses revealed that hexaploid plants were more heterozygotic. The level of heterozygosity was lower in diploids, while *E. incanum* polyploids and *E. wilczekianum* shared a higher heterozygosity. Interestingly, the heterozygosity level of the outcrossing *E. wilczekianum* is not significantly different from those observed in polyploid individuals of the (so far considered) selfing *E. incanum*.

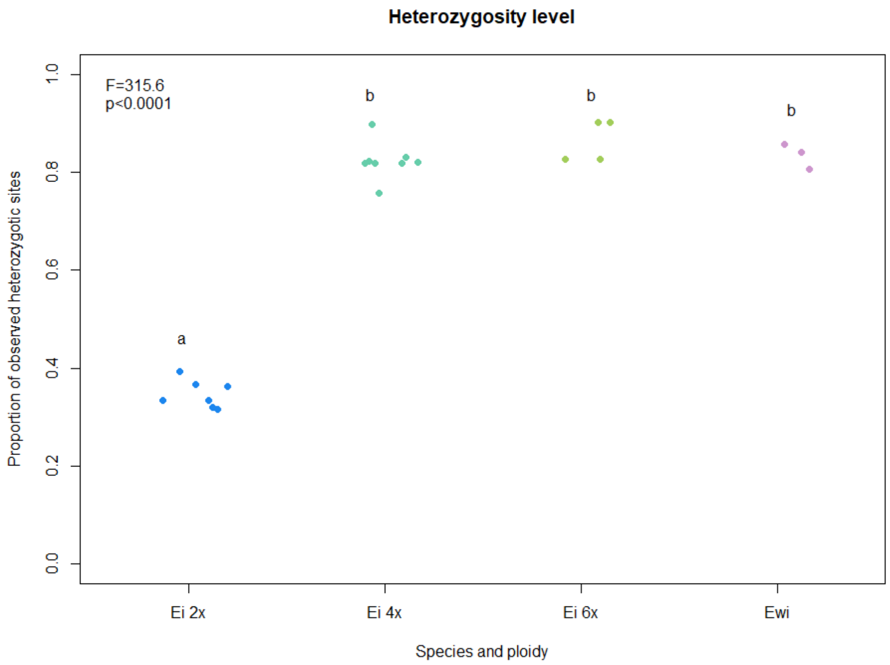

Figure 1.10. Heterozygosity level shown by *E. wilczekianum* and the three ploidies within E. incanum. We included the heterozygosity of a related diploid species as reference. Letters refer to these groups that are significantly different (P-value < 0.05).

DISCUSSION

In this study, we describe the mating systems of closely related taxa in the *E. incanum* species complex based on estimates of inbreeding depression, male and female relative reproductive investment, floral traits related to pollination, and pollen-stigma interaction. On the one hand, *E. wilczekianum* showed a strong inbreeding depression and low seed production in self-pollinated flowers. These results, together with a high P/O ratio, large flowers and a positive herkogamy that avoids self-pollination, suggest that *E. wilczekianum* exhibits a reproductive strategy based on allogamy (Table 1.3). On the other hand, the *E. incanum* species generally produced a higher seedset in selfing fruits and experienced lower inbreeding depression compared to their relative, suggesting a reproductive system based on autogamy. This selfing behaviour is supported by the negative values of herkogamy, lower values of P/o ratio and corolla size found in this species, which facilitate self-pollination. However, we found a gradient of selfing tolerance associated with the changes in ploidy. Diploid plants showed the most tolerant pattern to self-reproduction (Table 1.3).

Inbreeding depression is the main force opposing inbreed reproduction by optimising the reproductive success of cross-reproduction. However, combining successive generations of closely related reproduction and inbreeding depression facilitates the shift to inbreed reproduction through deleterious allele purge. The existence of a certain level of inbreeding depression in selfing species is challenging due to the complete purge of recessive deleterious alleles that are expected after many selfing events (Lande and Schemske, 1985). The outcrossing events in a selfing population would influence the population's genetic structure, providing genetic diversity by the allele combinations in the offspring. Nevertheless, changes to outcrossing reproduction in selfing populations are not expected (Takebayashi and Morrell, 2001; Igic and Busch, 2013). In our case, diploid populations, probably the ancestors of the rest of the ploidies, showed high values of selfing tolerance. Contrary to theory expectations about high selfing rates in polyploids, we found higher values of inbreeding depression as ploidy increases (Lande and Schemske 1985). In this sense, doubling gene copies after polyploidisation may have increased the mutation rate just by chance (bigger genomes) and may have a central role in masking recessive deleterious alleles, exhibiting less tolerance to selfing when this happens.

Population	**Mating traits**						**Predicted mating**
	Selfing	**ID coefficient**	**P/O ratio**	**Corolla size**	**Herkogamy**	**Heterozygosity**	
***E. incanum* (2x)**	++	--	-	--	-	-	S
***E. incanum* (4x)**	+	-	-	-	-	+	S-Out
***E. incanum* (6x)**	-	+	+	+	-	+	S-Out
E. wilczekianum	--	++	+	++	+	+	Out
	Fig. 3	**Fig. 4**	**Fig. 6**	**Fig. 6**	**Fig. 7**	**Fig. 10**	

Table 1.3. Summary of the comparisons of measured mating traits among *E. wilczekianum* and ploidies within *E: incanum* and interpretation of these values according to the expected mating system described by the literature. High and low values are indicated by “+” and “-“, respectively, for selfing, P/O ratio, corolla size, herkogamy and heterozygozity. Negative and positive values of the ID coefficient were indicated by “-“ and “+”, respectively. S= selfing, Out= outcrossing.

One of the main mechanisms modulating inbreeding depression and the mating system of the studied population is the pollen-stigma interaction. This is the ability of pollen tubes to grow in the stigma surface and into the pistil. Here, we demonstrated that *E. wilczekianum* rejects its own pollen grains because they could not develop their pollen tubes. Indeed, we found more fruits completely aborted than aborted seeds within fruits in selfing treatments. In contrast, *E. incanum* allows not only the growth of self-pollen tubes but also the growth of pollen from other individuals (including *E. wilczekianum* pollen, see ***Chapter 2****)*. *Erysimum incanum* is not visited by pollinators, so rarely foreign pollen arrives, and a mechanism of recognition and rejection of pollen is not expected to develop in this species (Lipow and Wyatt 2000). So, apparently, *E. incanum s*hows no filters when allowing the growth of pollen tubes into the pistil. Strong mechanisms, however, seem to exist in *E. wilczekianum* to avoid non-optimum pollen tube growth, modulating a reproduction strategy mainly driven by the interaction with a generalist pollinator assemblage (***Chapter 2***). The absence of pollen rejection in *E. incanum* and similar pollen tube growth rate in vitro open the door to the fact that *E. incanum* could occasionally suffer outcrossing events. Higher ploidies, producing bigger flowers, would be more prone to these events. A more exhaustive study would be necessary to explore the frequency of these events in nature and their consequences on the population genetic structure and reproductive strategies of these plants.

We have studied inbreeding depression and mating traits at the population level, as recent studies suggest for a proper mating system characterisation (Whitehead et al. 2018). Variations for inbreeding depression, tolerance to selfing and outcrossing rates were described previously in the wild among populations and different years (Schoen 1982; Lyons and Antonovics 1991; Goodwillie and Ness 2005; Vallejo-Marín et al. 2014). Our result highlights the importance of attending to among-population variation in characterising species mating systems. The fact that mating systems can vary in space and time within a single species invites thinking about how mechanisms modifying self-fertilisation respond rapidly to natural selection (Jain 1976).

In addition, inbreeding depression can be estimated using different fitness components across offspring life and the magnitude exhibited by each one of them can be different (Jain 1976). For example, classical works (Husband and Schemske 1997) stated that inbreeding depression is more substantial in the earlier phases of offspring development, while other studies suggest a different magnitude of inbreeding depression across post-dispersal stages of fitness (Grueber et al. 2010). We have tested the differences in our system study using seedset, as it is the first consequence of the pollen-stigma interaction. However, other fitness aspects, such as offspring germination and survival, could be interesting for inferring the consequences on genetic diversity and structure of the populations (Barrett and Harder 2017), especially among different ploidies.

Ecological factors affecting outcrossing rates among populations are well described (Moeller and Gebre 2005; Cheptou and Avendaño 2006), but genetic factors seem less explored. Here, some traits potentially related to pollination could be affected by the ploidy level in *E. incanum.* Indeed, our results revealed a clear pattern of trait values increasing with the ploidy. Together with inbreeding depression, the magnitude of P/O ratio and the higher pollen production in hexaploids strongly indicate that a larger amount of pollen grains could be exported instead of being used only for self-pollination. The use of P/O ratio to predict the reproductive strategy has been demonstrated by several experimental works beyond the classical theory (Johnston and Schoen

1996; Sato and Yahara 1999; Fishman and Stratton 2004; Goodwillie and Ness 2005; Lozada-Gobilard et al. 2019). Some examples include the works in *Clarkia*, which demonstrated that flower size and herkogamy are reliable indicators of its mating system. In this species, small flowers showed reduced herkogamy and were mainly self-pollinated, while larger flowers showed a higher degree of herkogamy and were mainly outcrossing (Barringer and Geber 2008; Delesalle et al., 2008). Interestingly, Mazer et al. (2009) found that P/O ratio was a highly canalised trait in *Clarkia* selfing species compared to its outcrossing relatives. This trait showed a significant variation within *E. incanum* among ploidies. In addition, the larger corolla and herkogamy exhibited by hexaploid plants indicate that these traits can function as pollinator attractive, enhancing the probability of outcrossing. The heterozygosity positions quantified in the higher levels of ploidy support this fact.

Population patterns observed can be thought to result from processes taking place at the intra-population level. The correlation between corolla size and herkogamy across all *E. incanum* ploidies suggests that indirect selection would play a significant role in the evolution of these traits and their genetic correlation. Similar patterns were found in *Clarkia* previously (Barringer and Geber 2008). More negative herkogamy values in larger flowers might allow for greater exposure of the anthers for pollen export by pollinators, who are expected to visit these flowers more frequently. In addition, avoiding sexual interference by herkogamy is particularly important in these populations showing a strong inbreeding depression. Genetic correlations between morphological and reproductive traits are found in previous studies (Stanton and Young 1994; Young et al. 1994; Ashman 2003), while the lack of such correlations in similar studies suggests that this covariance does not always exist (Mazer et al., 2007).

The inbreeding depression was related to a higher P/O ratio and larger flowers in *E. incanum*, whereas corolla size was related to the P/O ratio. The lack of correlations with inbreeding depression in hexaploids could be because of the high variation we found between populations with individuals with this ploidy. In all cases, the correlation between inbreeding depression and P/O ratio is maintained, suggesting that P/O ratio is an accurate index for predicting the primary reproductive strategy (Cruden, 1977). The correlation between P/O ratio and corolla size can be due to a greater resource allocation to primary and secondary reproductive traits. However, the increase in P/O ratio seems to be driven by the inbreeding depression magnitude. In tetraploids, the correlation of inbreeding depression with corolla size might suggest that larger flowers are subjected to cross-pollination more than expected. A similar pattern was found in other *Erysimum* species (Abdelaziz et al., 2014).

An increase in flower size has a demonstrated effect on the frequency of pollinator visits (Kennedy and Elle, 2008) and their role as selective pressures (Gómez et al., 2009), being proposed as an indicator of outcrossing rate

(Goodwillie et al., 2010). In our study system, we observe a drastic change in corolla size between *E. incanum* and *E. wilczekianum* and no such drastic change between ploidies. The changes in corolla size would be the main force modifying the pollinator attractiveness by the species in the complex (***Chapter 2***). We also found larger corollas with more herkogamy as ploidy increases. Similar patterns were described in previous studies (Webb and Lloyd 1986; Barrett and Eckert 1990), but also the opposite patterns (Tate and Simpson, 2004). So, the ploidy changes can be associated with changes in the flower size but not necessarily with changes in selfing rate (Husband and Schemske, 1997).

Traits related to pollination are helpful in determining the mating system. Plants depending on pollinators for reproduction will show traits enhancing insect attraction with showy corollas, scents, reward (nectar or pollen), among other traits. Rewards are expected to be scarce in *E. incanum*, where the flower size is improper for an outcrossing plant. However, *E. wilczekianum* exhibits an important production of pollen grains. In addition, as correlations showed, there is a clear separation in the range of values of corolla diameter and herkogamy between *E. incanum* and *E. wilczekianum*. The marked differences between them in morphological and reproductive traits suggest that the mating system is strongly associated with traits involved in pollination. The differences in floral morphology are translated to the mating system evolution and the diversity of pollination syndromes observed in the wild (Barrett and Harder 2017). In our case, the surprising point is that this happened in a selfing clade.

CONCLUSIONS

Our study accurately described *E. wilckzekianum* as a predominantly self-incompatible species, with important cross-pollination in nature (see ***Chapter 2***) supported by the resource allocation to enhance the pollination visit probability. Our results were consistent for the populations included in this study, exhibiting high levels of heterozygosity, P/O ratio, herkogamy and corolla diameter. While the mating system exhibited by *E. incanum* is predominantly selfing when we consider the entire species, we found a large variation among ploidies and even among populations within ploidies. Diploids exhibited a proper selfing mating with significant outcrossing depression patterns, the smallest flower sizes, and reduced herkogamy. However, hexaploid plants seem to experience significant inbreeding depression and have bigger flowers, herkogamy and P/O ratio within the *E. incanum* species. Taking into account the phylogenetically selfing background of this clade and the strong selfing mating system of the diploid taxa, the results shown in this study highlight the role of polyploidy changes in shaping different evolutionary trajectories and promoting the rise of outcrossing mating systems in a selfing clade. Changes in population genetic structure, genome architecture and ecological interaction could culminate in speciation events associated with this kind of infrequent transition in plant mating systems.

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AUTHOR CONTRIBUTIONS

AGM, AJMP and MA thought and designed the experiments. AGM, CF and MNMG conducted the greenhouse experiments. JL and SC conducted the ploidy analysis. AGM, AJMP and MA made the statistical analyses and designed the tables and figures. AJMP and AGM made the genomic analyses. AGM wrote the first draft of this manuscript and all the rest of authors made significant contributions to the draft. AJMP, SC and MA got the funds to develop this study. AJMP and MA supervised all the study.

DATA AVAILABILITY STATEMENT

Data is stored in XXX for review purposes, and will make it available in a public repository upon acceptance.

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SUPPORTING INFORMATION

Additional Supporting Information can be found in the following pages.

Appendix S1.1:

Table S1.1. Outcome of the GLMM testing the effect of the treatment and the ploidy as fixed factors together with their interaction on *E. incanum* fertility. The individual plant and the population appear as random factors nested within the ploidy level. Significance p-values are indicated in bold (*p-value<0.05; **p-value<0.01; ***p-value<0.001; ****p-value<0.0001).

	Fertility success					
Model	AIC	BIC	logLik	χ^2	df	*P-value*
Intercept	3411.4	3437.7	-1701.7			
Seedset ~ Treatment + (1 |Population/Plant)	3399.4	3431.8	-1694.7	13.977	1	**<0.001*** **
Seedset ~ Ploidy + (Ploidy |Population/Plant)	3399.2	3502.9	-1683.6	22.186	11	**<0.05***
Seedset ~ Treatment * Ploidy + (Ploidy |Population/Plant)	3377.9	3501.0	-1670.0	27.326	3	**<0.0001********

Table S1.2. Outcome of the GLMM testing the effect of the treatment and the ploidy as fixed factors together with their interaction on *E. incanum* fertility success. The individual plant and the population appear as random factors nested within the ploidy level. Significance p-values are indicated in bold (*p-value<0.05; **p-value<0.01; ***p-value<0.001; ****p-value<0.0001).

	Fertility success					
Model	AIC	BIC	logLik	χ^2	df	*P-value*
Intercept	3307.5	3326.8	-1650.8			
Seedset ~ Treatment + (1 |Population/Plant)	3297.9	3323.8	-1645.0	11.555	1	**<0.0001**** **
Seedset ~ Ploidy + (Ploidy |Population/Plant)	3276.8	3341.4	-1628.4	33.116	6	**<0.0001**** **
Seedset ~ Treatment * Ploidy + (Ploidy |Population/Plant)	3257.6	3341.5	-1615.8	25.261	3	**<0.0001**** **

Appendix S1.2:

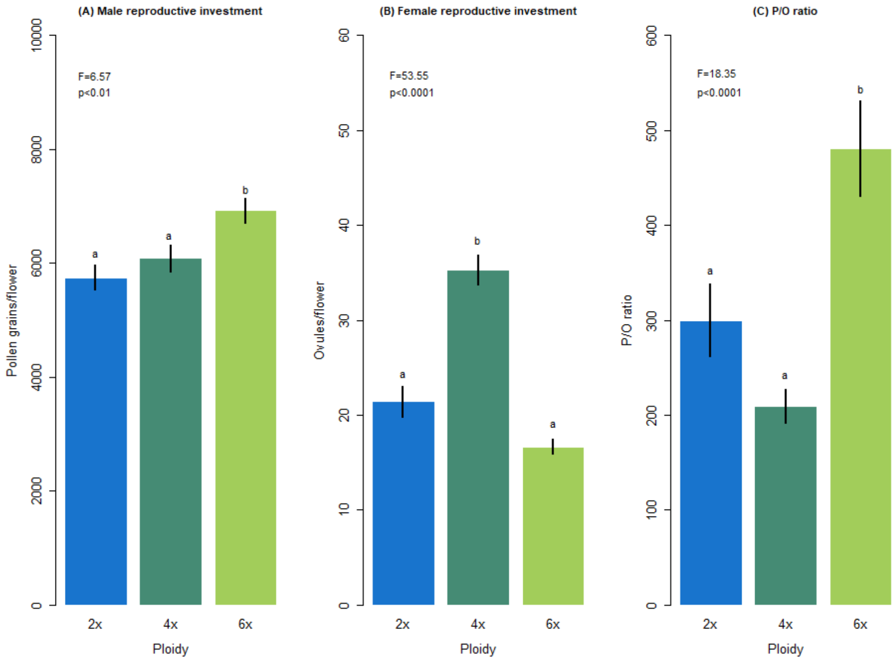

Figure S1.1. Differences in (A) male reproductive investment estimated by pollen production, (B) female reproductive investment estimated by the ovule amount and (C) P/O ratio values among ploidies within *E. incanum*. Letters indicate significant differences among ploidies.

Chapter 2

Mechanisms driving reproductive isolation in close related species with diverging mating systems

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ABSTRACT

Speciation requires the evolution of mechanisms driving reproductive isolation between divergent species. Different reproductive isolation components act subsequently, having their effects prior or posterior to pollination and contributing in different degrees to the total gene flow limitation. We estimated the magnitude of the total reproductive isolation and multiple reproductive barriers between the selfing species *Erysimum incanum* and the outcrossing species *Erysimum wilczekianum.* We found a stronger contribution to reproductive isolation in pre-pollination barriers which included ecogeography, phenology, and pollinator preferences. Post-pollination barriers, including hybrid seed formation and overall performance exhibited by the first and second hybrid generation, showed a strong asymmetry, with the outcrossing species rejecting interspecific pollen. The resulting hybrids exhibited lower germination rates than the selfing parental species but similar rates than the outcrossing parental species and those patterns were consistent in the second hybrid generation. Regarding phenotype and fertility, hybrid individuals were similar to the selfing parental species but outperformed both parental species in growth. The summary of our findings suggests that reproductive isolation is not complete between these two species, which are found in an early stage of the speciation process. In addition, the mating system and the traits related to mating seem to be the drivers of the observed reproductive isolation within this selfing *Erysimum* clade.

Key words Asymmetric barriers, ecological divergence*, Erysimum*, hybridization, pollinator preferences

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INTRODUCTION

The speciation process requires the existence of genetic differences among populations which are accumulated by the rise of reproductive barriers that reduce or even avoid the gene flow between them (Mayr, 1947). Ecological speciation occurs when, in addition to reproductive isolation, the formation of new species is promoted by divergent natural selection driven by ecological factors (Coyne and Orr, 1998; Nosil, 2012). In most cases, this speciation process involves multiple traits, some of them promoting reproductive isolation and some other promoting divergent selection. It may occur, however, that a given trait affecting reproductive isolation is also subjected to divergent selection. These traits that simultaneously influence reproductive barriers and are closely related to an ecological factor are known as magic traits (Servedio and Kopp, 2012). Although magic traits are commonly related to assortative mating in animals, floral traits such as size, color or shape can be considered a magic trait in pollinated plants (Servedio et al., 2011; Haller et al., 2012).

Flower attractiveness has a broadly described influence on frequency and diversity of pollinator visits (Stanton et al., 1986; Erickson and Pessoa, 2022), which are the principal vectors moving genetic material between individuals (Handel, 1983; Deynze et al., 2005; Hoyle and Cresswell, 2007; Wang et al., 2021). Thus, differences in floral shape may lead to a shift in pollinator assemblages promoting the emergence of reproductive barriers due to assortative mating. Nevertheless, a certain degree of gene flow often remains between incipient species. Nearly 25% of plant species are forming hybrids at the present (Mallet, 2007), meaning that they are not completely isolated and making the species term difficult to apply in plants when it is based on reproductively isolated groups. Quantifying the actual gene flow between incipient species is useful for understanding speciation processes. In addition, the phenotypic characterization of populations and the interplay with the environment is important to identify patterns of divergent selection that might, ultimately, originate isolation between groups potentially leading to the formation of new species.

The level of reproductive isolation observed between two species is the result of the interaction of multiple components (also called reproductive barriers), which contribute differently to the total gene flow reduction (Ramsey et al., 2003; Nosil, 2007; Lowry et al., 2008a; Matsubayashi and Katakura, 2009). Reproductive barriers are commonly classified according to their occurrence prior or posterior to the pollination event, so the terms pre- and post-pollination barriers are often employed. (Ramsey et al., 2003; Lowry et al., 2008a; Baack et al., 2015). Pre-pollination barriers involve all stages preceding the deposition of pollen into stigma and that may influence the success of mating, including the existence of differences in ecogeographic distribution, phenology and pollinator preferences.

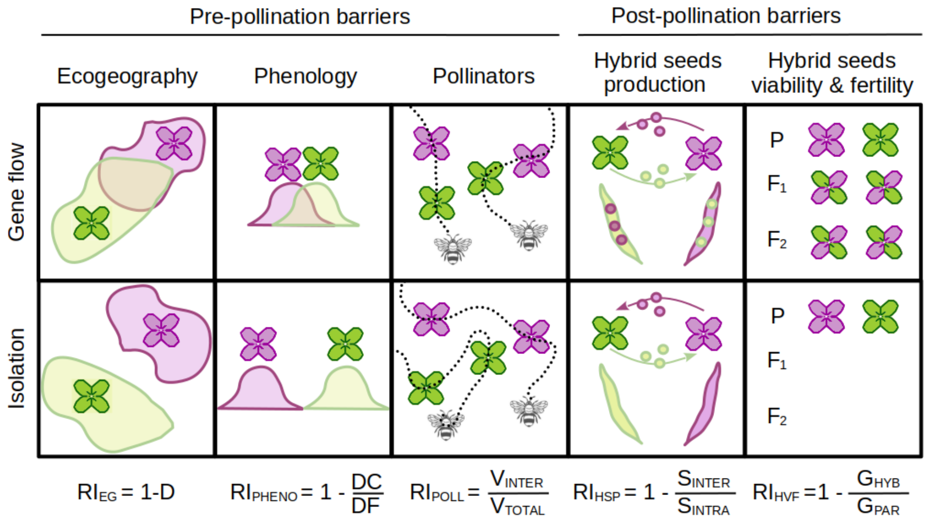

Figure 2.1. Summary of the expected outcomes for every reproductive barrier under the scenarios of gene flow and reproductive isolation. Equations used to quantify individual reproductive isolation barriers (RI) are also shown (D: Schoener's D index; DC: days co-flowering; DF: days flowering; V_{INTER}: Interspecific visits; V_{TOTAL}: total visits; S_{INTER}: Interspecific seedset; S_{INTRA}: Intraspecific seedset; G_{HYB}: Germinated hybrids; G_{PAR}: Germinated parentals).

Similarly, post-pollination barriers refers to the rest of steps influencing mating success after the arrival of pollen to the stigma, involving from the seed formation and germination to the establishment of the offspring in following generations (Figure 2.1). The relative magnitude of every barrier provides valuable information: on the one hand, it is an indicator of the stage in which two incipient species are in the speciation process. For example, pre-pollination barriers are higher in recently diverged species while post-pollination barriers are strong when the speciation process is almost completed (Hendry et al. 2007; Nosil 2012). On the other hand, the relative magnitude of barriers sheds light on the main mechanisms promoting isolation. For example, soft pre-pollination barriers while strong post-pollination barriers may denote a greater accumulation of molecular and genetic differences between species that avoid the inter-cross. In the following lines we will briefly describe the mentioned pre- and post-pollination barriers, also summarized in Figure 2.1.

Ecogeographical isolation involves geographical and ecological factors that explain the observed distribution of the studied species. Hybrid formation can potentially be influenced by the overlapping degree and by the seed and pollen dispersion ability of the parental species (Honnay et al., 2002). Furthermore, species distribution is sensitive to changes in ecological variables and the adaptive ability of the parental species (Duffy and Jacquemyn, 2019). Although the ecogeographical barrier can be considered the first barrier to gene flow, few studies have addressed this issue (Baack et al., 2015). The probability for encountering potential mates is reduced due to large differences in habitat preferences, which can be controlled by genetic factors. (Schemske, 2000; Ramsey et al., 2003; Sobel et al., 2010). It is possible to test differences in habitat preference between taxa using species distribution modeling (SDM; Peterson, 2003; Kozak et al., 2008). This approach is considered as the best alternative to calculate the isolation degree based on differences or similarities in potential habitat occupation when transplant studies are not possible (Cunningham et al., 2009; Sobel and Chen, 2014).

Phenological isolation occurs when the mating likelihood is reduced as a result of an asynchrony in flowering between individuals or by a timing difference between male pollen production and female sexual organs receptivity, avoiding the existence of gene flow even when synchronic flowering plants coexist (Primack, 1985; Husband and Schemske, 1996). Reproductive barriers due to phenology would be reduced when flowering for both species overlaps in a high degree, increasing the probability of mating, while species are phenologically isolated when the overlapping degree is null (Elzinga et al., 2007).

Pollinator preferences influence gene flow between individuals in several ways. On one hand, pollinators can maintain well isolated two species even in sympatry when they show strong fidelity to their host plant. On the other hand, generalist pollinators move pollen among different species and allow for the gene exchange among populations (Grant, 1949). The frequency of pollinator visits are determined by floral attractiveness and rewards (Møller et al., 1995; Waelti et al., 2009), so cross-pollination mediated by insects can be expected according to the traits shaping the floral attractiveness shown by each species.

Hybrid seeds production results from interspecific crosses. It is determined by the interaction pollen-stigma, which is the first and one of the strongest post-pollination barriers (Lowry et al., 2008a; Sambatti et al., 2012). Since some of the proteins involved in pollen recognition are often related to self-incompatibility mechanisms, the mating system of the parental species can play an important role contributing to the strength of this barrier (Koelling and

Mauricio, 2010; Tovar-Méndez et al., 2014) resulting in asymmetric reproductive barriers. In fact, the "weak inbred-strong outbreeder (WISO)" hypothesis (Brandvain and Haig, 2005) predicts an unbalanced hybrid seed production because the parental conflict shows opposite forces between species with different mating systems, being these forces more intense in outcrossing species and weaker in selfing species (Eberhard and Cordero, 1995; Haig, 2000). So, pollen from outcrossing species would be successful fertilizing ovules of selfing species but the inverse process would fail.

Viability and fertility of the first and second hybrid generations compose the post-pollination barriers after hybrid seed dispersion. Hybrids often outperform the parental species in terms of germination or growth which is known as hybrid vigor (East, 1936) although the opposite, hybrid weakness, also can occur (Koinange and Gepts, 1992). However, hybrids can suffer an abrupt decrease in fitness in following generations referred to as hybrid breakdown (Templeton et al., 1986). So the ultimate success of gene flow between different species would be determined by the performance of hybrid generations.

Due to their greater magnitude and their importance in the first stages of species divergence, the vast majority of experimental works are focused in pre-pollinations barriers (Ferriol et al., 2009; Nosil, 2012; Matsumoto et al., 2019; Cardona et al., 2020). As a consequence, post-pollination barriers, which are less intense in early stages of speciation, are underestimated. Other limitations in experimental studies on reproductive isolation is the overrepresentation of only a few genera (Baack et al., 2015) and the lack of a whole estimation of all the components shaping the total reproductive isolation, but see Ramsey et al. (2003) and Lowry et al. (2008b). However, a whole estimation of the reproductive isolation is necessary due to the interaction among reproductive barriers and how they influence themselves. Indeed, simulation models of Nosil and Yukilevich (2008) have demonstrated that different forms of reproductive barriers can favor themselves under different degrees of selection. The rapid evolution of one of the barriers which is under strong selection would avoid the rise of the following reproductive barriers because selection cannot act against them. The maintenance and increase of a reproductive barrier require the reinforcing effect on selection for this barrier as it would be a trait itself (Nosil and Yukilevich, 2008) while the evolution of a barrier also can constrain and even, avoid, the evolution of other barriers because removes all the previous gene flow (Agrawal et al., 2011). Unfortunately, it is hard to assess all the existing barriers in a single system study due to the efforts in field and lab work and the necessity of monitoring the individual across its life history, which is challenging especially in species with long life cycles.

Here, we studied the extent of reproductive isolation between two species from the *Erysimum incamum* species complex which exhibit a contrasting mating system.

We quantify the total reproductive isolation for each species and the relative contribution of all the reproductive barriers. So, we estimated pre-pollination barriers including ecogeographical separation, flowering phenology and pollinator preferences and post-pollination barriers from the pollen-stigma interaction until the survival of the second generation of hybrids, testing the occurrence of the hybrid breakdown. The estimation of the relative contribution to total reproductive isolation of each barrier allows us to accurately identify the degree of ecological divergence between the two species.

MATERIALS AND METHODS

Study system

We focus our study in two species from the genus *Erysimum L.*, which is one of the most diverse in the Brassicaceae family, with species inhabiting Eurasia, North Africa and North and Central America (Al-Shehbaz et al., 2006). These two species are well differentiated in the mating system and in traits shaping the flower (***Chapter 1***). *Erysimum incanum* is a selfing species showing the main characteristics of the selfing syndrome (Sicard and Lenhard, 2011) such as small flowers, reduced nectar and pollen production and reduced pollen-ovule (P/O) ratio. Three different ploidy levels have been identified in this species: diploids, tetraploids and hexaploids (Nieto Feliner, 2014***; Chapter 1***). Diploid populations are found in the Rif and the Pyrenees mountains, tetraploids populations are found in southwest of the Iberian Peninsula and the Middle Atlas mountains (Fennane and Ibn-Tattou, 1999) while hexaploids have been found only in the High Atlas and Middle Atlas mountains ***(Chapter 1)***. The other species, *Erysimum wilczekianum*, exhibits a cross-pollination reproductive system mediated by insects and shows larger flowers and a strong inbreeding depression (***Chapter 1***). This species is found only in Morocco, with a narrow distribution near to tetraploid populations of *E. incanum* (Fennane and Ibn-Tattou, 1999).

Phylogenetic studies revealed that *E. wilczekianum* forms a clade with allogamous reproduction within a selfing clade (**Introduction**; Abdelaziz et al. in prep.). This is an unexpected result according to the traditional premise that selfing is an evolutionary dead end. These characteristics make this species complex an interesting study system for speciation and ecological divergence driven by magic traits related to floral phenotype and mating systems.

Reproductive isolation estimation

We quantified individual components of reproductive isolation throughout the taxa life cycle by a series of field studies and greenhouse experiments. Estimates of the total reproductive isolation and the contribution of individual components are detailed below following the methods proposed by Ramsey et al. (2003).

Pre-pollination reproductive barriers

Ecogeographical isolation

We found a minimal distance of 500 m between *E. wilczekianum* and the closest *E. incanum* population, which are both tetraploids (Fennane and Ibn-Tattou, 1999). This distance could be based on differences in habitat preferences exhibited by each species. For this reason and to cope with possible mapping distribution dismissal, we decided to test the differences in habitat preferences and the potential overlapping degree by SDM. We mapped the spatial distribution of four tetraploid *E. incanum* populations and two *E. wilczekianum* populations inhabiting Morocco.

Based on our own records, we constructed the distribution model of both species in Maxent v.3.4.4 (Phillips and Others, 2005) using the principle of maximum entropy (Guisan and Thuiller, 2005). We used bioclimatic variables from Worldclim v.2 dataset (www.wordclim.org; Fick and Hijmans, 2017) at 1 km^2 resolution. After discarding variables with high collinearity (Guisan et al., 2017), we focused on the average precipitation (VIF = 1.84), average temperature (VIF = 3.03) and two other bioclimatic variables related to precipitation seasonality and the mean diurnal range of temperature (VIF = 3.26 and VIF = 1.87, respectively) which have been demonstrated to be key for determining plant distribution by other works (Dixon and Busch, 2017; Duffy and Jacquemyn, 2019). We also considered elevation and geological variables from ISRIC-WISE v3.1 database (Batjes, 2012). Distribution model was built on 50 Maxent replicates selecting the cloglog format output. We maintained standard default settings (converge threshold = 0.00001, iterations = 500, default prevalence = 0.5) and used the equal training specificity and sensitivity (ETSS) approach in order to classify accurately suitable or unsuitable sites. This threshold represents the umbral at which predictions about suitability or unsuitability habitats have an equal probability of being correct (Freeman and Moisen, 2008; Chen et al., 2010). We converted the predicted distribution models from ASCII to raster format in order to visualize as maps in ArcGis v.10.8.1 (Esri, 1995).

To assess the differences in niche preferences we compared the species distribution models using the Schoener's D index (Schoener, 1970), which is an indicator of habitat overlapping between the species. Specifically, we used the niche identity test implemented in the R package ENMTools v.1.0.6 (Warren et al., 2021a; b) in R v3.1.2 (Core Team, 2014). Niche identity test uses the Schoener's D index to evaluate the probability of two species niches to be identical. This metric ranges from 0 when niches are not overlapping, to 1 when niches of both species are completely overlapped (Schoener and Gorman, 1968; Püts et al., 2020). This index has been used in studies of reproductive isolation in plants as the ecogeographical barrier (Glennon et al., 2012; Willis and Donohue, 2017) as:

$$RI\ ecogeograhical = 1 - Schoener's\ D\ index \qquad \textbf{(2.1)}$$

We compared the empirical value of the D index from our data with the null distribution generated by 100 randomly permuted species distribution models in order to test the existence of significant differences in habitat preferences between the two species.

Phenological isolation

According to our personal observations, the flowering for both species overlaps in the wild, with *E. wilczekianum* starting to flower earlier. To remove environmental effects, we tested the phenological barrier in common greenhouse conditions. We sowed seeds from two populations of *E. wilczekianum* and from the closest population of *E. incanum*. We checked germinated plants daily and, once the first flower opened in one of the individuals, we monitored plants every two days during a month. Specifically, we recorded the number of plants showing at least one open flower and the number of open flowers per plant. We also noted the date of flowering termination for each species and estimated the phenological barrier by the degree of overlapped flowering following the method proposal by (Husband and Sabara, 2004):

$$RI\ phenology\ = 1 - \frac{number\ of\ days\ co{-}flowering\ species\ 1\ and\ species\ 2}{total\ number\ of\ days\ flowering\ species\ 1};\ \text{for the species 1}$$

(2.2.1)

$$RI\ phenology\ = 1 - \frac{number\ of\ days\ co{-}flowering\ species\ 1\ and\ species\ 2}{total\ number\ of\ days\ flowering\ species\ 2};\ \text{for the species 2}$$

(2.2.2)

Pollinator preference isolation

We tested the reproductive barrier driven by pollinator preferences by setting an experimental array with 30 potted plants (15 per species) separated from each other by 0.5 m and randomly arranged in a rectangle (5x6 pots). Before starting the observations, we measured floral traits (petal length, corolla diameter and corolla tube length) from one flower of each individual of the array and counted the number of flowers in anthesis at the moment of the observations in order to test the effect of specific trait values on visit frequency. The experiment was performed in two sites, corresponding to the habitat of *E. incanum* and the habitat of *E. wilczekianum.* We repeated the observations twice in each site, moving the pots randomly every day. Two observers noted flights that insects performed between plants from the same or different species during observation periods of 50 minutes between 10.30 am and 17.30 pm, local time. We only noted the flights where pollinators touched the reproductive part of the flower, considering the pollen transfer as effective. We obtained a symmetric measurement of isolation between both species:

$$RI\ pollinator\ = \frac{Inter-specific\ flights}{Inter\ and\ intra-specific\ \ flights} \qquad \textbf{(2.3)}$$

Finally, we compared the amount of visits between the two sample sites by ANOVA analyses in order to detect whether the frequency of visits is influenced by the local pollinator assemblage. We assessed if pollinators showed preferences by the traits exhibited in each species by Pearson's product-moment correlations.

Post-pollination reproductive barriers

Hybrid seeds production

We estimated the first post-pollination barrier to gene flow comparing the seed production from intraspecific and interspecific crosses. These crosses were performed in both directions, that is, *E. incanum* and *E. wilczekianum* acting both as pollen donor and receptor. To perform hybrid crosses in *E. incanum*, we carefully emasculated flowers before opening in the plant receptor to avoid the anther-rubbing mechanisms described in this species (Abdelaziz et al., 2019). Then, we added pollen from *E. wilczekianum* plants. Since *E. incanum* is predominantly selfing according to previous studies (***Chapter***

1) auto-pollinated crosses served as control in *E. incanum.* Following the same rationale, to perform hybrid crosses in *E. wilczekianum* we emasculated flowers before opening and added pollen from *E. incanum* plants. Because *E. wilczekianum* exhibits high values of inbreeding depression (***Chapter 1***), we performed cross-pollinated crosses between different individuals to obtain the seed production from intraspecific crosses which served as control. In all cases, each crossed flower was labeled to identify the type of treatment once the plant life cycle was finished. When it occurred, fruits were recollected to count the number of viable seeds, aborted seeds and non-fertilized ovules. The sum of these three parameters resulted in the amount of produced ovules per flower. The seedset was the number of viable seeds divided by the total amount of ovules. In total, we conducted 162 hybrid crosses in *E. incanum* and 131 hybrid crosses in *E. wilczekianum*. We used 1204 and 954 control crosses in *E. incanum* and *E. wilczekianum*, respectively. All these crosses involved 121 *E. incanum* and 255 *E. wilczekianum* plants. We estimated reproductive barrier due to hybrid seed formation applying the method proposed by (Lowry et al., 2008b)) to each species:

$$RI\ hybrid = 1 - \frac{Seedset\ from\ inter{-}specific\ crosses}{Seedset\ from\ intra{-}specific\ crosses} \qquad \textbf{(2.4)}$$

Viability and fertility of the hybrids

Finally, we sowed a subset of 300 hybrid seeds resulting from *E. incanum* and 300 seeds from each parental species. We only selected viable seeds which are easily differentiated from aborted seeds by the color and consistency. We estimated the hybrid viability by comparing its germination with the germination of the parental seeds. Since we did not sow seeds of hybrids from *E. wilczekianum*, we compared the hybrid germination of *E. incanum* hybrids with both parental species. Germination was measured as the number of germinated seeds that reach the rosette size by the total of seeds that were sowed in each pot. Here, the germination was an approximation of survival because mortality in these plants is not common once the plants become rosettes. The reproductive barrier due to the viability of first hybrid generation (F1) was estimated as:

$$RI\ viability\ of\ F1 = 1 - \frac{Proportion\ of\ germinated\ \ hybrid\ plants}{Proportion\ of\ germinated\ Ei\ plants}; \quad \mathbf{(2.5.1)}$$

$$RI\ viability\ of\ F1 = 1 - \frac{Proportion\ of\ germinated\ \ hybrid\ plants}{Proportion\ of\ germinated\ Ewi\ plants} \quad \mathbf{(2.5.2)}$$

Additionally, we estimated the growth ratio counting the leaves. When plants started to bloom, we measured floral size traits (petal length, corolla diameter and corolla tube length, described above). In addition, we measured nectar production in order to obtain a more accurate picture of differences in floral phenotype between hybrid individuals and parental species. Nectar was measured in a flower per plant measuring nectar amount using glass Drummond® capillary 0.5 µL.

Hybrid fertility was estimated as the seedset produced by these plants. For that, we counted the number of seeds, aborts and unfertilized ovules in four fruits per plant. We did not manipulate flowers, allowing for autonomous self-pollination because the phenotype was similar to *E. incanum* regarding flower size so we considered that the mating system would be similar. The barrier due to hybrid fertility was calculated only for the hybrids from *E. incanum* due to the unbalanced production of hybrids from each species as pollen receptors. The barrier due to the fertility exhibited by the first hybrid generation was calculated as:

$$RI\ fertility\ of\ F1 = 1 - \frac{Seedset\ of\ hybrids}{Seedset\ of\ Ei} \qquad \textbf{(2.6)}$$

We obtained a second generation of hybrids sowing a total of 560 seeds produced by self-pollinated fruits from the first generation of hybrids. We followed the methodology to compare the viability in this second hybrid generation (F2) comparing again with *E. incanum* individuals by:

$$RI\ viability\ of\ F2 = 1 - \frac{Proportion\ of\ germinated\ hybrid\ plants}{Proportion\ of\ germinated\ Ei\ plants} \qquad \textbf{(2.7)}$$

Absolute and relative contribution of reproductive barriers

Following the methods proposed by Coyne and Orr (1998) and Ramsey et al. (2003), we also calculated the absolute and relative contribution of each barrier and the total reproductive isolation. The absolute contribution (AC) of a barrier to the reproductive isolation is the gene flow removed that was not eliminated by previous barriers and can be estimated as:

$$ACn\ =\ RIn\ (1 - \sum ACi); \qquad \textbf{(2.8)}$$

In this notation *n* takes values from 1 to 5 to represent barriers due to ecogeography, phenology, pollinators, hybrid seeds production and hybrids viability and fertility, respectively. Thus the absolute contribution of the ecogeography is equal to the estimated reproductive isolation (RI ecogeograhical) whereas for pollinators the absolute contributions of ecogeography and phenology must be subtracted. When the absolute contribution of every barrier have been estimated, it is possible to quantify the total reproductive isolation (T) by summing these values as follows:

$$T = \sum ACi \, ; \qquad \textbf{(2.9)}$$

This total reproductive isolation T varies from 0 (complete lack of isolation) to 1 (complete isolation between species). Finally, we can estimate the relative contribution of each barrier (RCn) to the total reproductive isolation (T) as:

$$RCn = \frac{ACn}{T}; \qquad \textbf{(2.10)}$$

with *n* taking values from 1 to 5 to represent the different barriers as detailed for ACn. All these parameters were estimated for both *E. incanum* and *E. wilczekianum* in order to consider the asymmetries in the barriers to gene flow.

RESULTS

Total reproductive isolation

The strength of the total reproductive isolation and the absolute and relative component contributions of the different barriers are summarized in Table 1. The species *E. incanum* and *E. wilczekianum* experimented a similar and high total reproductive isolation, with values of T = 0.9997 and 0.9998, respectively. These values were mainly due to pre-pollination barriers such as ecogeographical and pollinator isolation. Even though pre-pollination barriers are high and symmetric, we found a strong asymmetry in post-pollination barriers involving the hybrid seed formation.

	COMPONENTS OF REPRODUCTIVE ISOLATION (RI)		ABSOLUTE CONTRIBUTION TO TOTAL ISOLATION (AC)		RELATIVE CONTRIBUTION TO TOTAL ISOLATION (RC)	
	E. incanum	*E. wilczekianum*	*E. incanum*	*E. wilczekianum*	*E. incanum*	*E. wilczekianum*
Ecogeographical isolation	0.892	0.892	0.89200	0.89200	89.222%	89.210%
Floral phenology	0.271	0.075	0.02926	0.00810	2.927%	0.810%
Pollinator preference	0.985	0.985	0.07755	0.09840	7.757%	9.841 %
Hybrid seed formation	0.143	0.926	0.00016	0.00138	0.016%	0.138 %
H_1 viability	0.478	0.041	0.00048	<0.00001	0.048%	<0.001%
Hybrid performance	0.173	-	0.00009	-	0.009%	-
H_2 viability	0.501	-	0.00021	-	0.021%	-
		Total isolation (T)	**0.99975**	**0.99989**		

Table 1. Summary of the individual reproductive barriers, shown by each species and the absolute (AC) and relative (RC) contribution of each barrier to total reproductive isolation (T).

Pre-pollination reproductive barriers

Ecogeographical isolation

We used the Maxent approach based on the ETSS threshold that resulted in constructing reliable distribution models for both species according to the obtained parameters. One of these indicators of model quality is the area under receiver operator curve (AUC) which assesses the model fit by plotting the proportion of predicted presences and the proportion of predicted absences (Peterson, 2003; Phillips and Others, 2005). It ranges from 1 (the model is optimum predicting the habitat suitability) to 0.5 (the model is inaccurate in estimating the habitat suitability). The AUC that we obtained from our species distribution models were

0.938 for *E. incanum* and AUC = 0.999 for *E. wilczekianum* species. Error estimates are produced by the 95% confidence intervals on the ETSS threshold values. Distribution range is quite different among species because *E. incanum* shows a broader niche compared to *E. wilczekianum* (Figure 2.2). We calculated the overlapping degree with the Schoener's D index and performed a niche identity test that showed that D index values for the empirical distribution of this species pairwise were lower than values from randomly generated species distributions (empirical D index = 0.108; permuted D index = 0.306) suggesting that habitats are significantly different between the two species. Based on the empirical D index, the reproductive barrier due to ecogeographical differences was **0.892** (1-0.108).

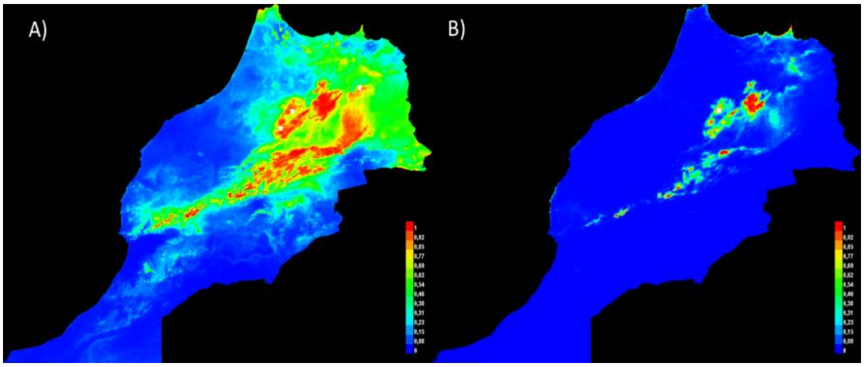

Figure 2.2. Potential distribution of (A) *E. incanum* and (B) *E. wilczekianum* calculated by Maxent. Colors represent the likelihood of the species occupation. Red zones predict the presence of the species with complete likelihood and blue zones indicate the no existence of the species. White points indicate the real presence of the species.

Phenological isolation

Plants of *E. wilczekianum* started flowering in early February (7th February was the date of the first flower opening) and first flowers of *E. incanum* opened just a week later (14th

February). The two species shared a part of the flowering period but *E. wilczekianum* finished blooming earlier than *E. incanum* (12th May), which kept producing new flowers until 8th June. Although the flowering period was shorter, *E. wilczekianum* showed a greater amount of simultaneously open flowers per plant (Figure 2.3). In total, *E. incanum* maintained its flowers open during 118 days while *E. wilczekianum* showed open flowers during 93 days. Flowering overlap between the two species occurred during a total of 86 days, so the reproductive barriers caused by the phenology was **0.2711** for *E. incanum* and it was **0.0752** for *E. wilczekianum.*

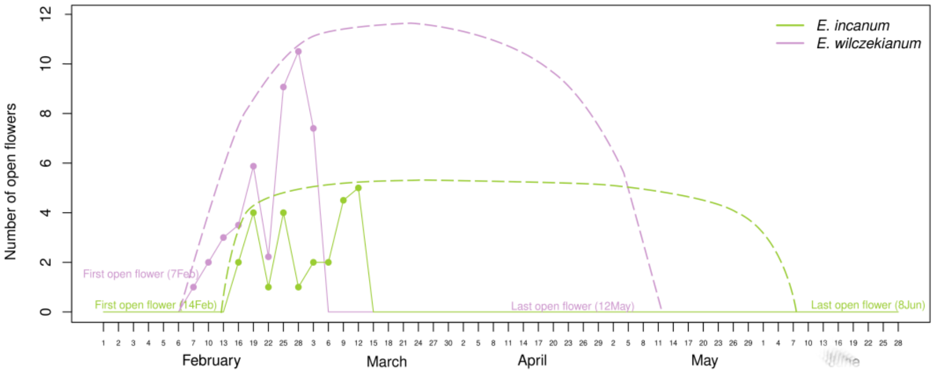

Figure 2.3. Amount of open flowers per plant shown by *E. incanum* and *E. wilczekianum* along their flowering period. Solid lines represent monitored plants during 30 days after the first open flower in *E. wilczekianum.* Dashed lines represent the inferred flowering until the last day with open flowers in each species.

Pollinator preference isolation

A single insect was able to successively visit several flowers in the same plant of our experimental display. In fact, we noted a maximum of 20 flowers touched by a single pollinator in a plant individual. We found that *E. wilczekianum* was much more visited than *E. incanum* (254 and 4 visits, respectively, Figure 2.4). We also followed the pollinator flights among plants to account for intra- and inter-specific visits, being the majority of flights exclusively between *E. wilczekianum* plants. Specifically, we noted a total of 141 inter-specific flights occurred and only 2 movements were between *E. wilczekianum* and *E. incanum*. The remaining 139 flights involved flowers from *E. wilczekianum* only. Therefore, the reproductive barrier driven by pollinator preferences was **0.9858** for both species. In addition, we found that the frequency of visits to each species was not affected by the site where the observations were made (ANOVA; *F* = 0.146, *P*-value = 0.704 for the visits in *E. incanum* and *F* = 2.108, *P*-value = 0.150 for *E. wilczekianum* visits).

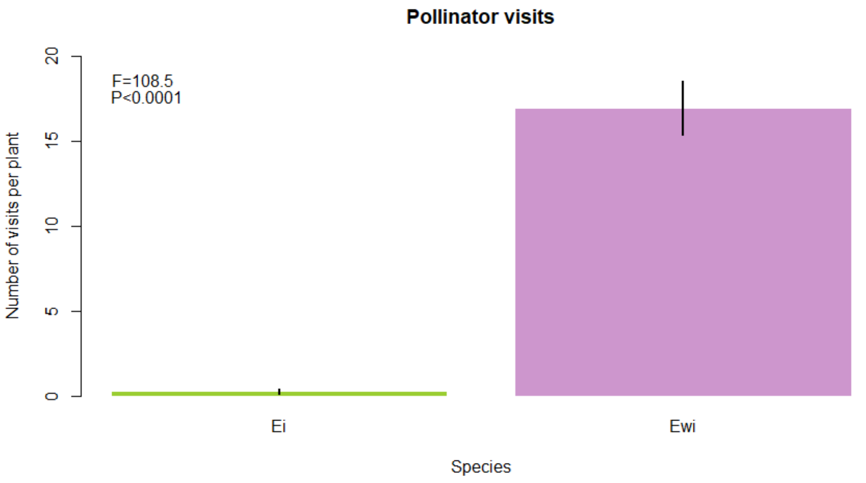

Figure 2.4. Total amount of visits received by plant individuals from *E. incanum* and *E. wilczekianum* in the two study sites.

Regarding the phenotypic traits of our experimental plants, *E. wilczekianum* had larger flowers with longer corolla tubes (Figure 2.5A and 2.5B) whereas both species showed the same amount of open flowers at the moment of the observation (Figure 2.5C). Thus, insects exhibited a higher preference for visiting plants with larger corollas and longer corolla tubes (Figure 2.5D and 2.5E) but they did not show a preference for plants showing more open flowers at that moment (Figure 2.5F).

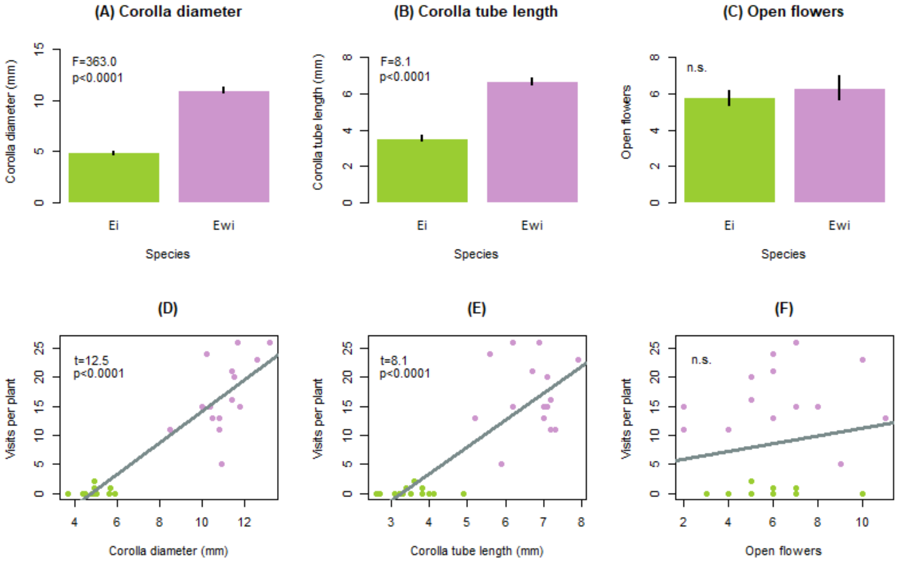

Figure 2.5. Differences in (A,B) floral size and (C) the number of open flowers prior to pollinator observations and (D,E,F) correlations between these traits and the number of visits by pollinators.

Post-pollination reproductive barriers

Hybrid seed formation

We found that the hybrid crosses when *E. incanum* was the pollen acceptor produced a high seedset, although it was lower than the seedset produced by selfing (Figure 2.6A; *F* = 110.6, *P-value* < 0.0001). In contrast, the hybrid crosses mainly failed when *E. wilczekianum* acted as the plant receptor, so we found a strong difference between the seedset from outcrossing and hybrid treatments in this species (Figure 2.6B; $F = 637.3$, P-value < 0.0001).

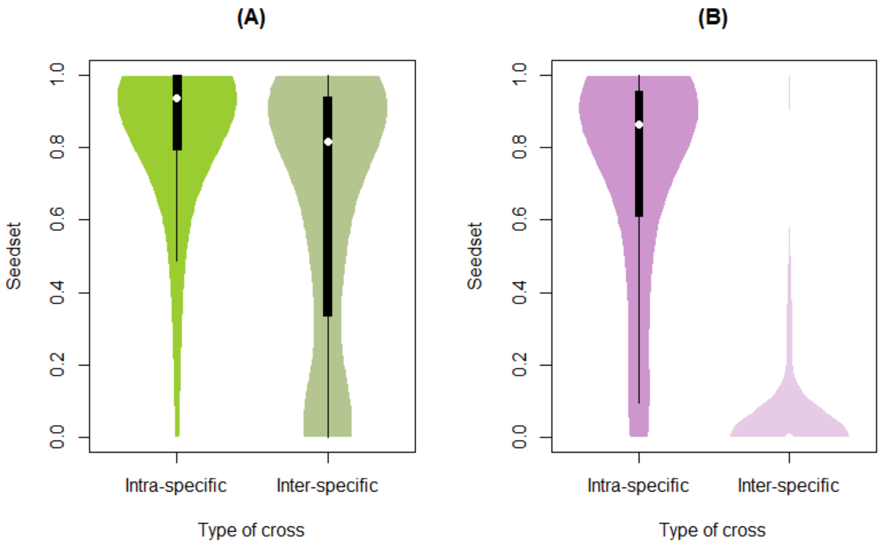

Figure 2.6. Differences in the seedset produced for (A) *E. incanum* plants through intraspecific crosses (self-pollination) and its hybrid and for (B) *E. wilczekianum* plants through interspecific crosses (cross-pollination) its hybrid.

According to these differences, we obtained a strong asymmetry in the reproductive isolation due to hybrid seed formation between both species. This reproductive barrier was **0.143** when *E. incanum* received interspecific pollen while *E. wilczekianum* frequently rejected the foreign pollen, resulting in a reproductive barrier of **0.926** in this outcrossing species.

Viability of the first and second hybrid generation

We found a strong decrease in the germination of hybrids compared to their parental species *E. incanum* although the germination of these hybrids was not significantly different from the germination exhibited by *E. wilczekianum* which was also lower than the germination of *E. incanum* (Figure

2.7A). We calculated a reproductive barrier due to differences in viability of **0.4781** between *E. incanum* and its hybrids while the reproductive barrier between these hybrids and *E. wilczekianum* would be **0.04080** due to the almost null difference in the total number of germinated plants (Figure 2.7A). The germination of the seeds produced by the hybrid plants was quite similar to the germination of their parents and, thus, the same pattern of germination compared to the parental species (Figure 2.7A). Hybrids from the second hybrid generation exhibit a germination significantly different from *E. incanum* but similar to both the hybrids from the first generation and *E. wilczekianum* (Figure 2.7A). Therefore, reproductive barriers in this ultimate stage were similar, with the reproductive barrier due to the viability of the second hybrid generation showing a value of **0.501**.

We found that hybrids produced more leaves than both parental species at the same stage of the life cycle, indicating that hybrids grew faster (Figure 2.7B). Likewise, although hybrids did not differentiate significantly from *E. wilczekianum* in germination, the leaves production was much higher. As we previously knew, *E. wilczekianum* flowers were larger (Figure 2.8) and produced nectar while the nectar amount in *E. incanum* was practically null (Figure 2.8D). We found an intermediate size in hybrid flowers compared with the parental species, although the difference between the hybrid and *E. incanum* was not significant (Figure 2.8). Even though the nectar amount in the first generation of hybrids was not significantly different from *E. incanum*, it tended to be increased (Figure 2.8D).

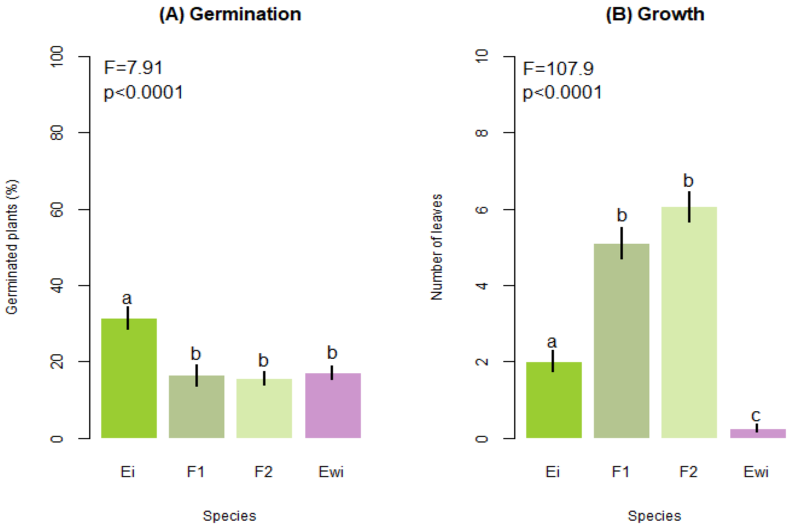

Figure 2.7. Differences in the germination proportion and the growth at first life stages among the parental species and their hybrids. Germination was considered as a viability measurement to calculate the reproductive barriers. Letters indicate significant differences. F1 and F2 refer to first and second hybrid generations, respectively.

Although the seed production of hybrid plants tended to be lower than the parental species, this difference was not significant, at least when we allowed for autonomous self-pollination (Figure 2.9). We compared the hybrid seedset with the seedset from *E. incanum* species to obtain a reproductive barrier due to the hybrid fertility of **0.1735.**

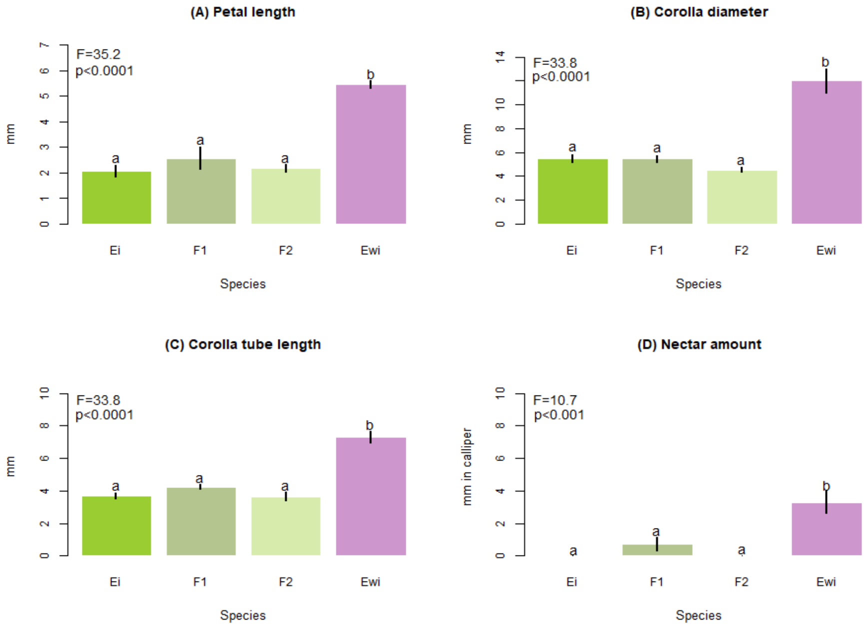

Figure 2.8. Differences in traits related to floral size as (A) the petal length, (B) the corolla diameter and (C) the corolla tube length, and (D) nectar production among *E. incanum*, *E. wilczekianum* and the first (F1) and second (F2) hybrid generation from *E. incanum*. Letters indicate significant differences.

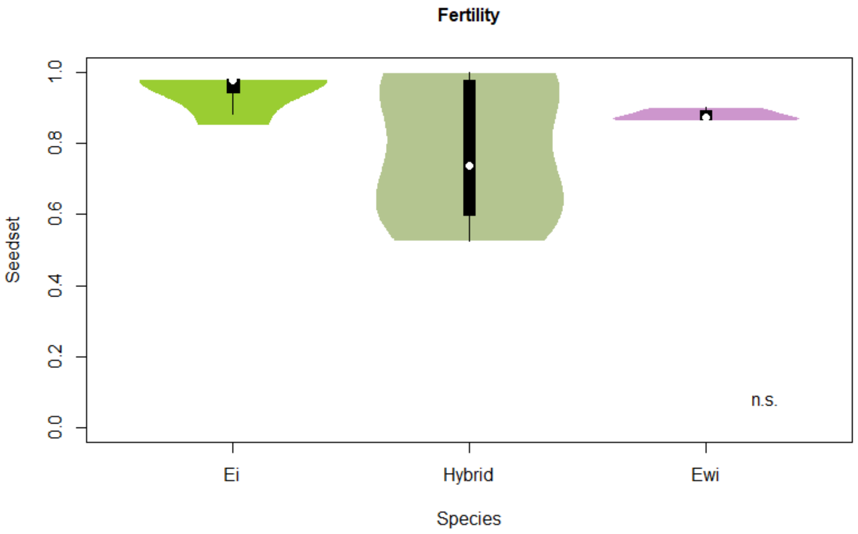

Figure 2.9. Differences in the produced seedset among *E. incanum* and *E. wilczekianum* and their hybrid from the first generation.

DISCUSSION

The higher magnitude of pre-pollination barriers compared to the weaker strength of post-pollination barriers suggest that the studied species are found in an early stage of ecological speciation (Nosil, 2012). A similar pattern is shown in other plant systems between divergent species (Lowry et al., 2008b; Briscoe Runquis et al., 2014). The strongest pre-pollination components of reproductive isolation are the ecogeography and pollinator preferences. Phenology would not deeply reduce the genetic exchange although reduce the time of both species being receptive to mate with each other. In addition, we have observed *E. wilczekianum* flowers were long-lasting and their life cycle was less advanced than *E. incanum* during our pollinator observations in the wild. This difference in flowering time is derived from the contrasting mating system (Murawski and Hamrick, 1992; Stenström and Molau, 1992; Fox, 2003).

Overlooking the ecogeographical barrier, the almost complete reproductive isolation found between *E. incanum* and *E. wilczekianum* relied on pollinator preferences. We found a clear tendency in insects for visiting large flowers of *E. wilczekianum* and, in fact, we only observed a few sporadic visits to the small flowers of *E. incanum*. Floral shaping the flower size seems to act as magic traits here because they strongly limit the gene flow between both species and thus contributing to the reproductive isolation (Haller et al., 2014). These traits are also highly related to the mating system. Indeed, we suggest that the found ecogeographical barrier is the product of the difference in mating system as well. Although we focused for this study in the close populations, *E. incanum* occupies a broad range, from stable to more stressful habitats, while *E. wilczekianum* populations are more restricted to a narrow distribution with more stable conditions. Colonization potential and the ability to manage with hard environments where mates and pollinators are scarce is enhanced by the reproductive assurance in selfing species (Pannell 2015; Grossenbacher et al. 2017).

The contrast in the mating system between *E. incanum* and *E. wilczekianum* has consequences also at molecular level on the pollen-stigma interaction once pollination between both species occur. Pollen grains from *E. wilczekianum* were able to grow in the stigma of the *E. incanum* but not vice versa (see Supplementary Material, Figure S2.1). The SIxSC rule (the cross between self-incompatible and self-compatible species cross fails, but the reciprocal cross is possible; Murfett et al., 1996) predicts this result, which has been tested by several studies (Hiscock and Dickinson, 1993; Hiscock et al., 1996; Onus and Pickersgill, 2004; Baek et al., 2015). In this sense, the genetic mechanisms underlying self-incompatibility have been also identified influencing the results of the pollen-stigma interactions (McClure and Franklin-Tong 2006; Koelling and Mauricio 2010; McClure et al. 2011). The WISO hypothesis is summed to this outcome regarding the strong forces derived from the high parental conflict in the outcrossing species *E. wilczekianum* compared to its selfing relative. Mating system also influences the pollen-stigma interaction from an ecological perspective (Swanson et al. 2004). Mechanisms of recognition and rejection of foraging pollen would be more developed in *E. wilczekianum*, which in addition present generalist pollinators. These mechanisms would be relaxed in E. incanum due to the low probability of foraging pollen arrival (Cruzan, 1990; Smith-Huerta, 1996; Kerwin and Smith-Huerta, 2000; Distefano et al., 2009).

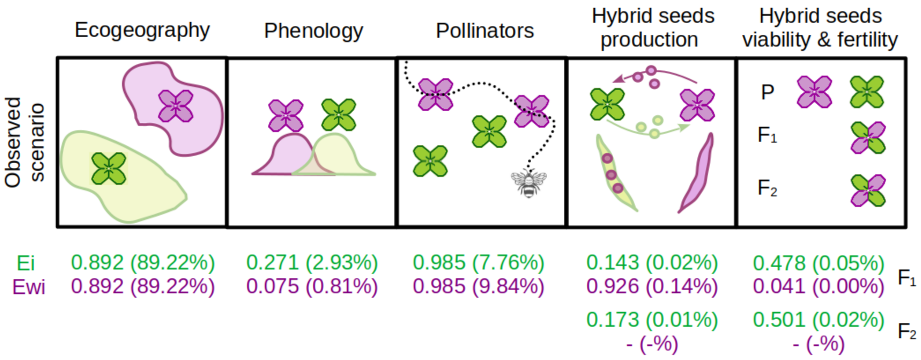

Figure 2.10. Summary of pre-pollination (ecogeography, phenology and pollinators) and post-pollination (hybrid seeds production, hybrid seeds viability and fertility) reproductive barrier values found in each species and their relative contribution (%) to total reproductive isolation.

Genetics and molecular incompatibilities when the stigma interacts with the pollen of a species with a different mating system lead to the strong asymmetry that we found in the first tested post-pollination barrier in this system (Tiffin et al. 2001). The lack of enough hybrid seeds from *E. wilczekianum* did not allow us to accurately evaluate the following barriers for both types of hybrids. Although germination of hybrids was lower than *E. incanum*, the growth of the hybrids was higher compared to both parental species in the two obtained hybrid generations, suggesting a certain degree of hybrid vigor although it did not influence in higher fertility. Likewise, similar values of germination and growth between first and second hybrid generation suggested that hybrids did not suffer the hybrid breakdown. The extent of hybrids in the wild is not tested in this study although further work is necessary to assess changes in the mating system after hybridization. Surprisingly, we found an increase in nectar production which is a novelty in this species, which could be caused by the input of genetic diversity from cross-pollination. We could expect, thus, a change in the mating system of individuals showing higher levels of genetic diversity. Indeed, the divergence in selfing rates within a species and thus, a shift in the mating system, has been identified as a force promoting reproductive isolation (Wendt et al., 2002; Palma-Silva et al., 2015).

Asymmetry in reproductive isolation is common in plant systems where species differ in mating system (Tovar-Méndez et al., 2014; Li and Chetelat, 2015). In addition, experimental works in *Mimulus* showed how the interaction among floral traits and mating systems strongly influence the degree of reproductive isolation (see Martin and Willis (2007) and references therein). We found a similar pattern in *Erysimum* because the factors strongly involved in reproductive isolation were characteristic of the reproductive strategy presented in each species. The consideration of the mating system as a reproductive barrier itself, however, has been widely discussed (Coyne and Orr, 1998; Fishman and Stratton, 2004; Lowe and Abbott, 2004;

Martin and Willis, 2007). Nevertheless, several pollination mechanisms reduce the chance for gene flow as for example prior selfing and cleistogamy (Martin and Willis, 2007). In this sense, we consider the selfing of *E. incanum* a strong reproductive barrier because the traits related to selfing syndrome are the main responsible for preventing gene flow through pollinators which are the drivers of ecological speciation.

CONCLUSIONS

In this study, we identify pollinator preference as the main responsible reproductive barrier contributing to the almost complete reproductive isolation between *E. incanum* and *E. wilczekianum.* We found a strong asymmetry in post-pollination barriers in controlled conditions. However, the pollinator preference and the outcome of the pollen-stigma interactions are shown as the consequence of the difference in mating system exhibited by each species. The differentiation in floral phenotype shapes the pollen transfer among individuals mediated by pollinators, isolating the populations showing small flowers. The mechanisms underlying the foreign pollen recognition and exclusion are expected to be more developed in *E. wilczekianum*, which translates to the asymmetry in the reproductive barrier at hybrid seed formation stage. Overall, reproductive barriers with higher influence in the reproductive isolation seem to be the product of the shift in mating system in this clade, which can be driven by an increase in trait values promoting the isolation of the incipient species with an outcrossing reproductive strategy. A better understanding of the evolutionary meaning of the reproductive barriers requires knowing the ecological context, the genetics underlying reproductive isolation, the timing of appearance and the time necessary of each individual reproductive barrier to evolve and contribute to the total isolation. Reproductive barriers play an essential role in maintenance species integrity and in the origin and diversification of new species but also are important for supporting phylogenetic results and predict the level of genetic differentiation between two divergent species and the stage in which they can be found in the ecological speciation continuum.

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AUTHOR CONTRIBUTIONS

AGM, AJMP, CF, EO and MA thought and designed the experiments. AGM, CF, EO and MA conducted the greenhouse experiments. AGM, AJMP and MA made the statistical analyses and designed the tables and figures. AGM wrote the first draft of this manuscript and all the rest of authors made significant contributions to the draft. AJMP and MA got the funds to develop this study. AJMP and MA supervised all the study.

DATA AVAILABILITY STATEMENT

Data is stored in XXX for review purposes, and will make it available in a public repository upon acceptance.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the following pages.

Appendix S2.1:

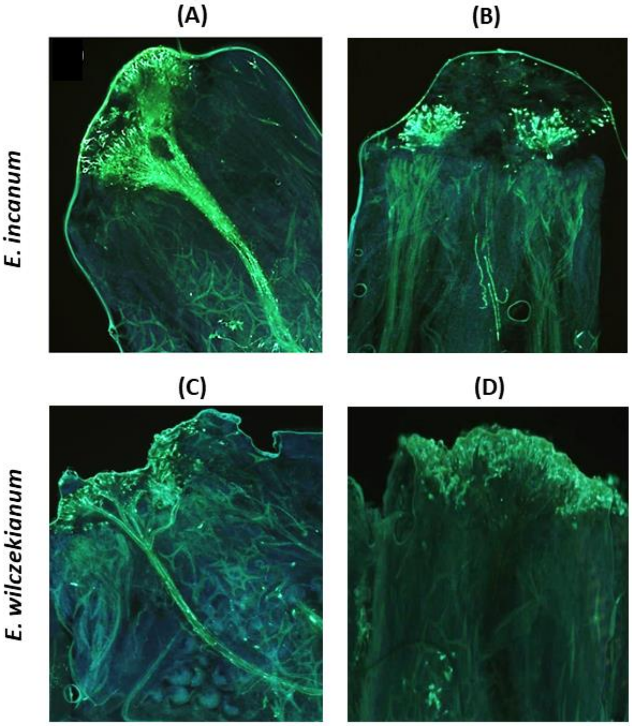

Figure S2.1. Visualization of the interaction between pollen and stigma observing the growth of pollen tubes by UV luminescence as proposed in ***Chapter 1*** for: (A) intraspecific and (B) interspecific cross in *E. incanum*; (C) intraspecific and (D) interspecific cross in *E. wilczekianum*.

Chapter 3

Ploidy effects on the relationship between floral phenotype, reproductive investment and fitness exhibited by an autogamous species complex

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ABSTRACT

The relationships between reproductive investment, phenotype and fitness have been broadly studied in cross-pollinated plants in contrast to selfing species, which are considered less interesting in this area because they are supposed to be a dead-end in any evolutionary pathway. Still, selfing plants are unique systems to study these questions since the position of reproductive structures and traits related to flower size play an important role in female and male pollination success. *Erysimum incanum s.l.* is a selfing species complex exhibiting three levels of ploidy: diploids, tetraploids and hexaploids. This species complex shows traits typically associated with the selfing syndrome. Here, we used 1609 plants belonging to these three ploidies to characterize floral phenotype and spatial configuration of reproductive structures, reproductive investment (pollen and ovule production) and plant fitness. Then, we explored the relationship between all these variables using structural equation modelling across ploidy levels. An increase in ploidy level leads to bigger flowers with more exerted anthers and a greater amount of pollen and ovules. In addition, hexaploid plants exhibit higher absolute values for herkogamy which is positively correlated with fitness. Ovule production mediated significantly the natural selection acting on different phenotypic traits and pollen production- This pattern is maintained across ploidies. Changes in floral phenotypes, reproductive investment and fitness with the ploidy level suggests that genome duplication can be a driver for the reproductive strategy transitions by modifying the investment in pollen and ovules and linking them with plant phenotype and fitness.

Key words Anther exertion, *Erysimum*, floral phenotype, herkogamy, natural selection, pollen-ovule ratio, self-pollination

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INTRODUCTION

The importance of male and female reproductive investment (i.e., pollen and ovules, respectively) on plant reproduction have been broadly explored in flowering plants (Knight et al., 2005; Morales and Traveset, 2008; Breed et al., 2012; Wong & Frank, 2013). However, most studies have focused mainly on outcrossing plants whose fertilization depends on different pollination mechanisms, leading to a broader understanding of biotic and abiotic interactions in these species. In contrast, selfing plant species have been overlooked in this ecological area, even when a high frequency of self-pollinated hermaphroditic plants occurs in the wild (Jarne and Charlesworth, 1993).

Reproductive strategies based on self-pollination facilitate pollen grains to reach the stigma within the same flower, ensuring fertilization (Lloyd, 1992; Ashman et al., 2004; Eckert et al., 2006). Female reproductive investment is the limiting factor for reproductive success in selfing plants. This causes the reduction of resource allocation to pollen compared to ovules (Lloyd, 1987; Michalski and Durka, 2009). A trade-off between pollen and ovules has been identified, and the pollen/ovule ratio (hereon, P/O ratio) was developed as an index to characterize different mating systems (Cruden, 1977). High values of P/O ratio are expected in outcrossing plant species, where male reproductive investment is a significant component of the plant's total fitness. Conversely, selfing plant species are expected to show lower P/O ratio as pollen is only used to fertilize the ovules in their own flower. The P/O ratio has been broadly used to explore plant mating system evolution and was positively correlated to floral traits driving the evolution of mating systems, such as corolla size or relative spatial position of sexual organs, such as herkogamy (Galloni et al., 2007).

Flower morphology and size were demonstrated long ago to be traits strongly selected in plants as they control the frequency and efficiency of plant reproduction in different taxa (Herrera, 1993; Caruso et al., 2003; Gómez et al., 2006; Caruso et al., 2019). Morphology and size of flowers can also have a significant effect on the mating system by their relationship with the relative spatial position of sexual organs (i.e., herkogamy; Lloyd and Schoen 1992; Herlihy and Eckert 2004; Larrinaga et al. 2009). Positive herkogamy avoids self-pollination and exposes the stigma surface to pollinators. However, self-pollination is facilitated when herkogamy is negative (reverse herkogamy) or near zero (Larrinaga et al., 2009; Brys et al., 2011). Experimental studies showed how selfing plants often exhibit a lower degree of herkogamy together with a low pollen amount because there is a high possibility of own pollen grains reaching the stigma (Johnston et al., 2009; Sicard and Lenhard, 2011). Apart from herkogamy, the anther exertion can also affect the mating system, which enhances the pollen export by exposing the anthers outside the flower (Barret, 2003, Medano et al., 2005). Ultimately, the balance between pollen and ovule development per flower is the major characteristic describing the mating system (Cruden, 2000) and is closely related to fitness. Thus, male and female reproductive investments are able to mediate indirectly the selection on the rest of traits mentioned above, connecting them with fitness and constraining the possible selective scenarios.

Another level of complexity in plants is the one produced by whole genome duplications. Last studies revealed that approximately 35% of vascular plants have a polyploid origin, evidencing the importance of polyploidy promoting speciation (Wood et al., 2009). Indeed, polyploidization events play an important role in the evolutionary history of angiosperms (Soltis and Soltis, 1999; Otto and Whitton, 2000; Soltis et al.; 2004; Van der Peer et al.; 2009, Leebens-Mack et al., 2019). Furthermore, ploidy can alter floral phenotypes in different dimensions (Jürgens et al., 2002) and several studies have demonstrated its influence on flower size and position of sexual organs, which, in turn, might affect pollinator preferences (te Beest et al., 2012; Moghe, 2014). Surprisingly, studies often pay poor attention to the effect of ploidy variation on the reproductive output of selfing species, despite it is widely accepted that ploidy level is able to alter floral phenotype in many dimensions (Jürgens et al., 2002).

Erysimum incanum is a self-compatible species complex showing small flowers and sharing a similar life form: monocarpic annual plants. The species in the complex differ in their ploidy level (including diploids, tetraploids and hexaploids; Faverger et al., 1979; Galland, 1988; Luque and Lifante, 1991), but they exhibit identical self-pollination mechanisms (Abdelaziz et al., 2019). Due to its self-reproductive strategy, *Erysimum incanum* is expected to increase the allocation of resources to female function compared to male function in its flowers (Charnov, 1982). Because of its short and monocarpic life history, it is possible to measure the entire life production of pollen, ovules and seed output as fitness estimates. Using this singular study system due to its reproductive strategy and its multiploidy condition, the main aims of this study were: (1) to quantify the selective pressures shaping the adaptive trajectories in populations with different ploidy levels; (2) to evaluate the effect of ploidy on the flower phenotype resulting from the selective scenarios; (3) to explore the role of P/O ratio in constraining the total selection regimes occurring through the variation exhibited by the species complex; and (4) evaluate the effects of polyploidization to decouple variation among traits in *E. incanum* species complex.

MATERIALS AND METHODS

Study system

Genus *Erysimum* L. is one of the most diverse in the Brassicaceae family, and their species can be found in Eurasia, North and Central America and North Africa (Al-Shehbaz et al., 2006). Recently, mechanisms promoting local adaptation and hybridization between lineages were identified for different species in the genus (Gómez et al., 2009; Abdelaziz, 2013). *Erysimum incanum* can be considered a species complex, including annual and monocarpic species and subspecies inhabiting the Western Mediterranean basin (Nieto-Feliner, 1993; Abdelaziz et al., 2019). The complex harbors three ploidy levels: diploids ($2n = 2x = 16$ chromosomes), tetraploids ($2n = 4x = 32$; Nieto-Feliner, 1993) and hexaploids ($2n = 6x = 48$; ***Chapter 1***). Diploids of *E. incanum* present a vicariant distribution in the Rif and the Pyrenees mountains and tetraploids present a similar distribution in southwest Iberian Peninsula and the Middle Atlas Mountains (Nieto-Feliner, 1993; Fennane et al., 1999). In contrast, hexaploid *E. incanum* plants were only found in the most southern ranges in Morocco (High Atlas and Antiatlas; Abdelaziz et al., in prep.) (Figure 3.1A and 3.1B).

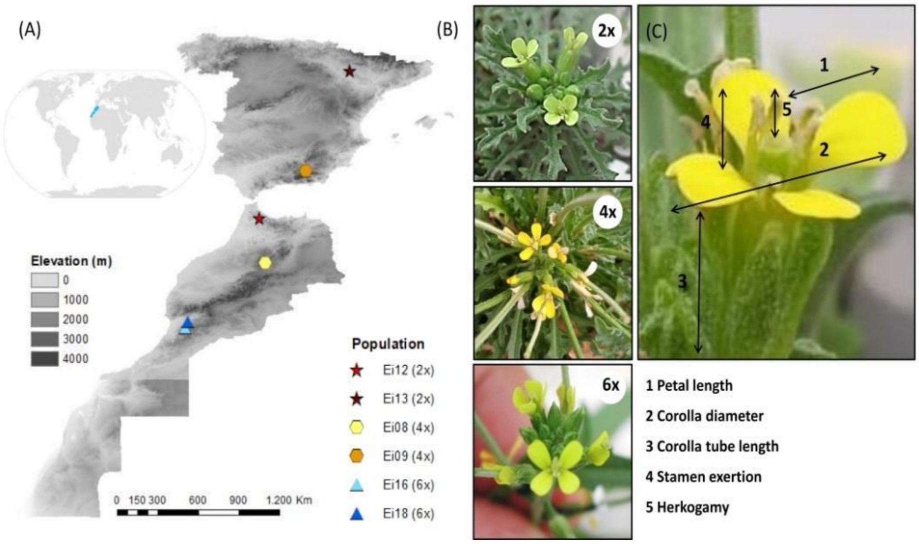


Figure 3.1. (A) Map of geographic locations of the six studied populations in Morocco and Spain from *Erysimum incanum*. **(B)** Flowers from each ploidy level of *E. incanum* individuals. **(C)** Flower diagram showing the measured floral traits.

The predominant phenotype shown by *E. incanum* species complex (but *E. wilczekianum*) is associated with selfing syndrome, including small, hermaphroditic and self-compatible flowers (Feliner, 1991). Indeed, we have recently described the *anther rubbing* mechanism (Abdelaziz et al., 2019) that promotes these plants' prior selfing.

Experimental design and phenotypic trait measurements

Seeds collected in two natural populations per ploidy (2*x*: Ei12 and Ei13; 4*x*: Ei08 and Ei09; 6*x*: Ei16 and Ei18; Figure 3.1A, Table 3.1) were sowed for three consecutive generations in greenhouse conditions at the University of Granada grow facilities to remove any local effect. A total of 640 seeds per population were sowed in groups of four seeds in square plastic pots (11x11x11 cm^3) filled with Gramoflor™ potting soil mixture.

During the blooming period of the plants, we measured a series of floral phenotypic traits on one flower per plant using a digital calliper on the 1622 plants which reached the flowering stage (Table 3.1, Figure 3.1C) Three of these traits were related to floral size, namely petal length (distance between the edge of the petal and the point where the petal starts to curve, shaping the corolla), corolla diameter (distance between the edge of the petal and the diametrically opposite one, in the flower) and corolla tube length (distance between the basis of the sepals and the corolla aperture point). In addition, we also measured the long stamen length (distance between the long filament insertion point and the anther) and the style height (distance between the style basis and the stigma) to estimate the herkogamy value (difference between the style height and the long stamen length) and the anther exertion (difference between the long stamen length and the corolla tube length). Herkogamy indicates the relative easiness for self-pollination to occur, while anther exertion denotes how much the anthers (and so the pollen grains) are exposed to flower visitors.

Ploidy level	Population	N phenotype	N genome size	Genome size	Geographical origin
2x	Ei 12	289	21	0.35 ± 0.01	Morocco
	Ei 13	116	20	0.34 ± 0.01	Spain
4x	Ei 08	513	25	0.78 ± 0.10	Morocco
	Ei 09	250	21	0.78 ± 0.03	Spain
6x	Ei 16	230	21	1.10 ± 0.03	Morocco
	Ei 18	224	20	1.13 ± 0.03	Morocco

Table 3.1. Genome size estimations of each population. The number of plants phenotyped by population and ploidy, the number of plant samples used for genome size analysis, the genome size per population and ploidy (mean ± SE values are shown) and the geographic origin of each studied population are also given.

Genome size measurements

Preliminary, we estimated the genome size from young leaves and infer the ploidy level using flow cytometry (FCM). We carried out the genome size analyses as described by Muñoz-Pajares et al. (2018). At least 20 individuals were analyzed per population (Table 3.1). As the ploidy level was homogeneous in each population studied, we assumed that each population was mainly composed of a single cytotype. According to our previous knowledge of genome size variation in the *Erysimum* genus (Nieto-Feliner et al., 1993; Fennane et al., 1999) and knowing the three ploidy levels previously described for the complex, it was possible to assign each individual and population to a specific ploidy level (Table 3.1) in order to be consistent with the main objectives of this study.

Reproductive investment measurements We collected half of the stamens (i.e., two long stamens and one short stamen) from the same flower in which we measured floral and conserved in alcohol 70% to estimate male reproductive investment. Next, we counted the number of pollen grains per flower (Pollen) using the Multisizer Coulter Counter 3™ particle counter provided by the morphometric lab of the Centro de Investigación, Tecnología e Innovación (CITIUS) at the University of Seville (Spain).

Once the plant was dried, we collected four matured fruits of each individual to quantify the number of viable and aborted seeds and the number of unfertilized ovules per fruit. The sum of these three measurements was the total number of produced ovules per flower, i.e., the female reproductive investment (Ovules). The P/O ratio was calculated as the fraction of pollen grains produced per flower divided by the number of ovules produced per plant.

Fitness estimation To estimate fitness, we counted the number of fruits per plant and multiplied it by the mean number of seeds estimated using four random fruits from the same plant. This way, we obtained the total seed production per individual plant used in the experiment. This value is an unbiased estimate of the individual fitness due to our plants' monocarpic character, which completes their life cycle in only 10-12 weeks.

Overall, we considered eight variables grouped into four categories: floral size (petal length, corolla diameter and corolla tube length), the spatial position of reproductive structures (anther exertion, herkogamy), reproductive investment (pollen, ovules) and fitness. A total of 1622 plants reached the final of their life cycle. On 1581 of these plants we were able to measure floral size and spatial position traits, male and female reproductive investment traits on 1380 and 1088, respectively, and finally we estimated the seed production of 943 plants as fitness component (Appendix S3.1; see Supplementary Data with this article).

Statistical analysis

Phenotypic traits selection

We used structural equation modelling (SEM) because we were interested in dealing with relationships among multiple variables. This statistical technique is a powerful multivariate analysis tool which has gained importance in ecological research in the last two decades (Fan et al., 2016). In addition, SEM incorporates path analysis techniques which are able to identify when a variable effect could be mediated by another different variable. We used SEM to estimate the selection of traits related to floral size, the relative position of reproductive structures, and male and female reproductive investment. Pathway analysis allowed us to evaluate the direct and indirect effects of a series of traits that act simultaneously on fitness.

Based on a series of hypothesized mechanisms (Table S3.2; Appendix S3.2) we built an a priori model (Figure S3.1; Appendix S3.2). We tested several hypothesized models before we found a consensus model with a good fit for all the populations, regarding the p-value and AIC values (Appendix S3.3). In our model, fitness is directly connected to traits related to floral traits, the relative position between sexual structures and the male and female reproductive investment. Floral size and position of sexual structures can promote or avoid self-pollination, which is why we connected them to fitness. These traits are linked to reproductive investment to test their influence on pollen and ovule production, which are directly affecting fitness. Moreover, we included a relevant covariance between pollen and ovule production, based on the trade-off between male and female sexual allocation (broadly described in flowering plants e.g. Charlesworth and Charlesworth, 1981; Lloyd, 1984; Charnov, 1987), and on the P/O ratio hypothesis, which suggests that autogamous plant species reallocate resources to increase the production of ovules in detriment of pollen grain production (Cruden, 1977). The rest of covariances are detailed in Appendix S3.2.

Checking multicollinearity issues is important before performing statistics based on regression models, specifically when variables are potentially correlated. We tried to deal with strong correlation among variables choosing these measurements that were taken independently and avoiding including derived traits in our model. However, we inspected multicollinearity among all the variables by the variance influence factors (VIF) using the *vif* function from the '*car'* package in R (Fox et al., 2012). It is an indicator of how much the variance of a coefficient in a regression model is inflated due to multicollinearity and it is obtained by regressing one of our variables on all of the other variables (Miles, 2014). A VIF score lower than 5 means that variables are not strongly correlated among them (Joshi et al., 2012; Table S3.4).

We evaluated the fit of the model for each population using Fisher's C statistic and the *P*-value, which must be higher than 0.05 for accepting the hypothetical proposed model. Fisher's C statistic is a fit index, analogous to the χ^2 *statistic*, which is useful to construct the AIC scores (Shipley, 2009; Shipley, 2013; Lefcheck, 2016). To analyse the effect of ploidy variation on trait selection, we fitted the same model to each population and ploidy level separately and compared the resulting different significant relationships between variables. Before these statistical analyses, we applied the *scale* function in R and performed some transformations (log) in the case of *Pollen* and *Fitness* variables to fulfil normality assumptions.

Trait effects on fitness

Using the relationships of the traits with fitness in the SEM analysis, we calculated the direct, indirect and total values of natural selection acting on each measured phenotypic and reproductive trait. Direct effects result from the unmediated relationship of the traits with fitness. Indirect effects were estimated as the product of different components of a path connecting any trait to fitness mediated by other traits. Finally, we added the indirect and direct effects to obtain the total value of natural selection.

Effect of P/O ratio on selection regimes

We evaluated the effect of the pollen-ovule ratio on the total selection experienced by each of the phenotypic and reproductive traits, considering the total natural selection regimes experienced by the traits. For this, we performed a regression of the total fitness effect per population on the P/O ratio.

Effect of ploidy level on phenotypic and reproductive investment values

We performed linear models using the ploidy level as fixed factor to evaluate possible differences in phenotypic traits, reproductive investment and P/O ratio between the three levels of ploidy exhibited by *E. incanum* species complex. In addition, we performed linear models using the population as a nested factor within the ploidy level in order to assess the effect of the ploidy level and the population on the measured variables and did multiple comparisons using '*lsmeans'* package (Lenth & Lenth, 2018).

Phenotypic correlation among traits across ploidies

Phenotypic correlations between each pair of *E. incanum* phenotypic traits for each studied ploidies were calculated using Pearson correlation for estimating relationships independently between each pair of traits. In addition, we calculated the correlation pooling of the plants by ploidy. Since we carried out multiple comparisons, we considered applying the Bonferroni correction, with a new significance threshold of 0.0025 in the three ploidies. All the statistical analyses described in this section were conducted in R version 4.0.3, using the package '*piecewiseSEM'* version 1.2.0 (Lefcheck et al., 2016) for the structural equation modelling, and *stats* for the rest of analyses.

RESULTS

Variation in phenotypic correlation among traits

The structural equation modelling comparing populations from each ploidy level showed heterogeneity in the relationships established among traits. However, some patterns are maintained across populations (Figure 3.2). We found a significant negative relationship between corolla tube length and anther exertion in every population, probably because of anatomic coherence due to anthers could be hidden within a very long corolla tube while anthers would exceed a short corolla tube and appear more exerted in the flower Similarly, we found a negative covariance between anther exertion and herkogamy across most populations from each ploidy. Since herkogamy exhibits negative values (i.e., it is the difference between the style height and the long stamen length) in this species complex, we must keep in mind that a lower degree of herkogamy translates into a greater separation of male and female structures and a greater exertion of anthers above the corolla.

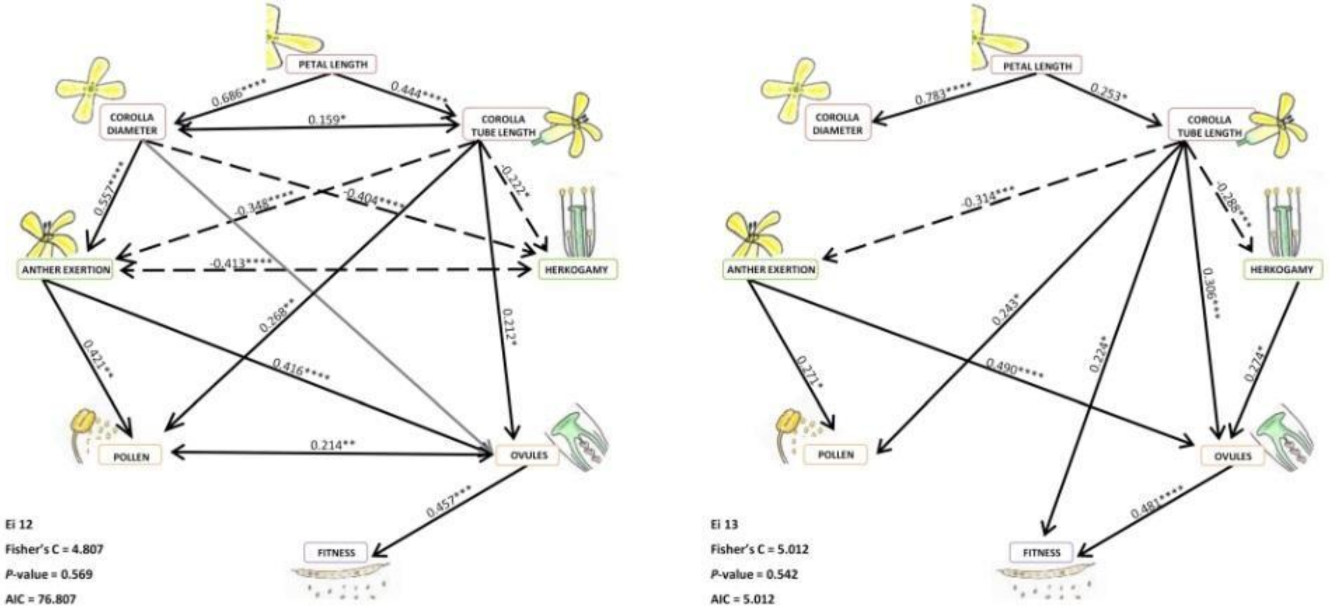
Apart from anther exertion, we found that phenotypic traits related to flower size (petal length, corolla diameter and corolla tube length) are commonly negatively connected with herkogamy in all populations except for Ei18, where the relationship is positive. In addition, corolla tube length is positively linked to female reproductive investment (ovules) across all populations. For the male reproductive investment (pollen), we only found a significant link with a floral trait for a diploid population (Ei12) and the tetraploid populations (Figure 3.2A and 3.2B).

Apart from anther exertion, we found that phenotypic traits related to flower size (petal length, corolla diameter and corolla tube length) are commonly negatively connected with herkogamy in all populations except for Ei18, where the relationship is positive. In addition, corolla tube length is positively linked to female reproductive investment (ovules) across all populations. For the male reproductive investment (pollen), we only found a significant link with a floral trait for a diploid population (Ei12) and the tetraploid populations (Figure 3.2A and 3.2B).

Anther exertion is linked to male and female reproductive investment across all populations, so flowers with more exerted anthers might produce more pollen and ovules. On the other hand, herkogamy is only connected with female reproductive investment. This means that flowers exhibiting lower distance between male and female organs could produce more ovules (Figure 3.2).

Figure 3.2 (in the following pages)**.** Path diagrams describing the effects of floral phenotypic traits (petal length, corolla diameter, and corolla tube length), the spatial position of reproductive structures (anther exertion and herkogamy) and reproductive investment (pollen and ovules) on fitness for **(A)** diploids, **(B)** tetraploids and **(C)** hexaploids. Solid and dashed arrows indicate positive and negative relationships, respectively. For visual simplicity, only significant relationships are represented accompanied by the mean values of path coefficients and asterisks indicating the degree of significance. Grey lines indicate marginally significant relationships. (*P-value<0.05; **P-value<0.01; ***P-value<0.001; ****P-value<0.0001).

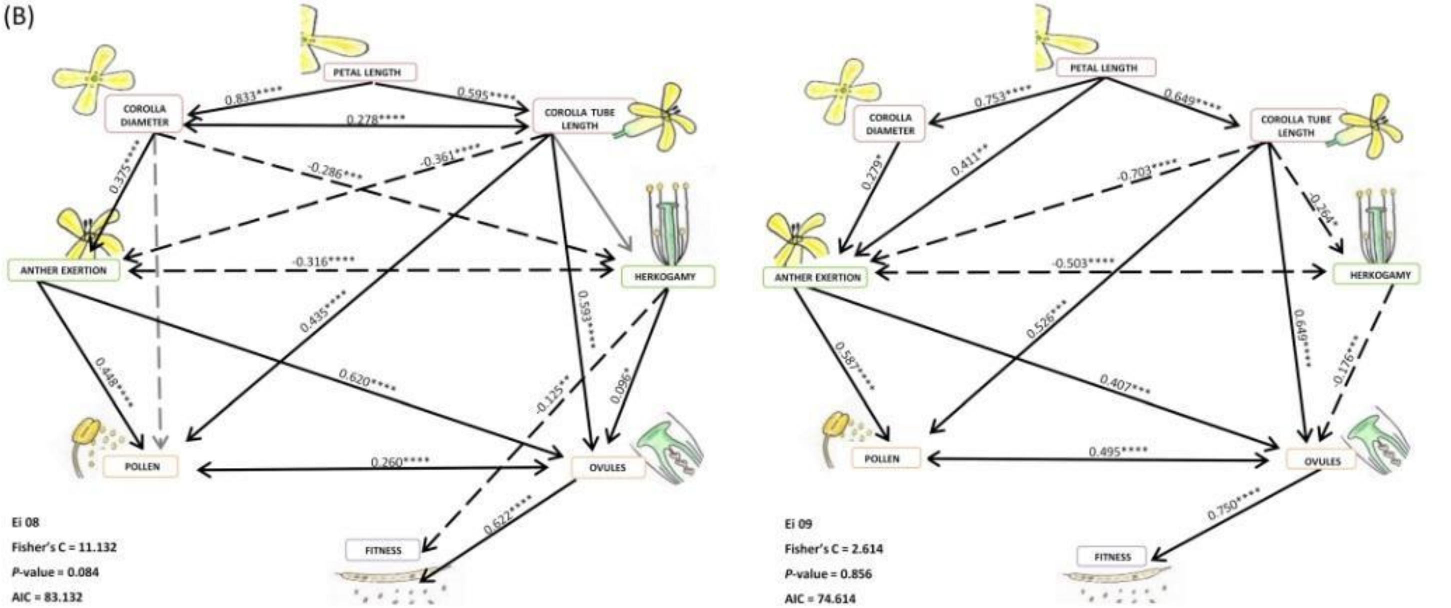
(A)



Ei 12
Fisher's C = 4.807
***P*-value = 0.569**
AIC = 76.807

Ei 13
Fisher's C = 5.012
***P*-value = 0.542**
AIC = 5.012

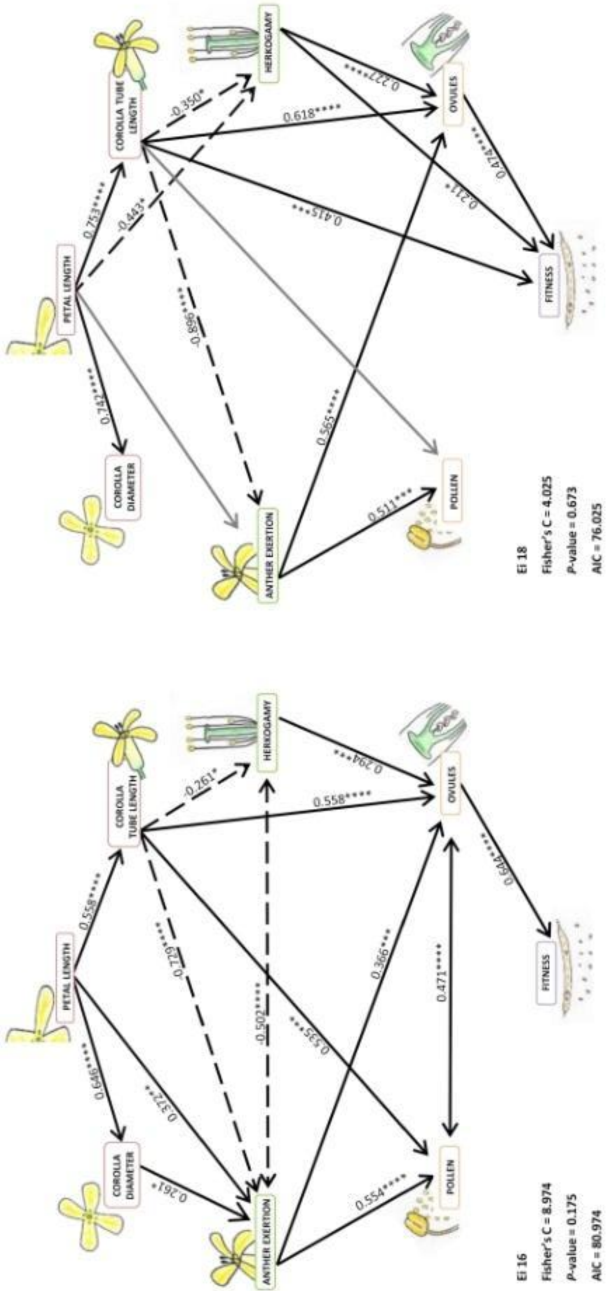
(B)



Ei 08
Fisher's C = 11.132
P-value = 0.084
AIC = 83.132

Ei 09
Fisher's C = 2.614
P-value = 0.856
AIC = 74.614

(C)



Ei 16
Fisher's C = 8.974
P-value = 0.175
AIC = 80.974

Ei 18
Fisher's C = 4.025
P-value = 0.673
AIC = 76.025

In general, we found similar relationships patterns among traits across populations although the number of significant connections changes. For instance, the lowest number of relationships among traits is shown by the diploid population Ei13 while the number of related traits increases with ploidy level, being maximum for the the populations Ei08 and Ei16 which are both polyploid populations. The population Ei18, hexaploid as well, shows less number of links among variables but we found several variables directly connected with fitness (Figure 3.2C).

Variation in selection on phenotypic and reproductive traits

Patterns of trait selection across populations indicate that ovule amount is the only trait directly linked with fitness in all the studied populations (Figure 3.2). Due to this ovule-fitness relationship, traits linked with the female reproductive investment are indirectly selected. Male reproductive investment is directly (and negatively) selected in hexaploid Ei18 only (Figure 3.2C). Nevertheless, we found indirect selection acting on pollen mediated by its covariance with ovule amount.

Corolla tube length is under indirect selection in all populations because it is the trait with more established relationships with other traits directly linked with fitness (e.g., the ovules). The other flower size traits are indirectly selected for at least one population from each ploidy level (Figure 3.2). Herkogamy is directly selected, while anther exertion is selected indirectly though ovules and by its covariance with herkogamy (Figure 3.2). The estimated parameters and their standard errors resulting from the path analyses per population and ploidy are given in Appendix S3.5. The mean values of the direct, indirect and total effect of each studied attribute on fitness are shown in Appendix S3.6.

Effect of P/O ratio on selection regimes

We found a significant relationship between the P/O ratio and selective regimes on herkogamy across populations (Figure 3.3). The proportion of variance of selective regimes explained by the P/O ratio ($R^2 = 0.73$) was significant ($P = 0.03$) even when the number of populations included in the analysis was low. In populations where the P/O ratio was low, the approximation between anthers and stigma (this is, values of herkogamy closer to zero) is selected. While in populations where the P/O ratio is higher, the separation between anther and stigma (this is, more negative values of herkogamy) is positively selected (Figure 3.3 and Table 3.2).

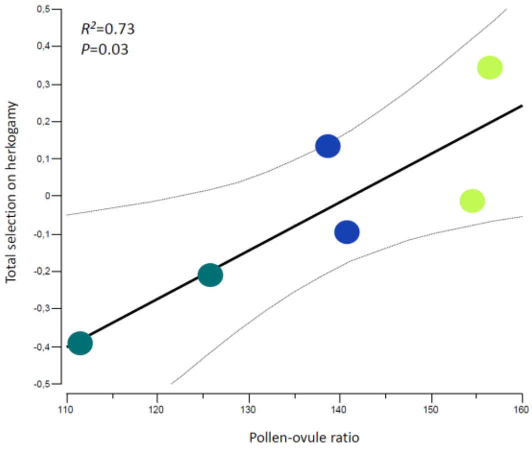


Figure 3.3. Effect of P/O ratio on selective strength. Response curves show the P/O ratio effect on the total selection on herkogamy. Each circle represents a single population: green circles refer to hexaploid populations, blue circles to diploid populations and ocean circles to tetraploid populations. Grey lines represent 95% confidence intervals.

Effect of ploidy level on phenotypic and reproductive traits

Ploidy level and the interaction between ploidy and the population had a significant effect for all the measured variables but not for P/O ratio in the case of the interaction ploidy-population (Appendix S3.7, Appendix S3.8). More specifically, we found significant among-ploidy differences in all traits related to floral size (petal length, corolla diameter, corolla tube length) separately (Figure 3.4). Plants with higher ploidy showed bigger corollas, and this pattern is maintained for the corolla tube length.

Flowers of hexaploid plants have significantly more exerted anthers above the corolla compared with diploid and tetraploid plants. We found significant differences among diploid and polyploid plants. Diploid plants exhibit reduced values of herkogamy compared to tetraploid and hexaploid plants, which present more negative values of herkogamy (Figure 3.4).

Traits	2n		4n		6n	
	Ei12	Ei13	Ei08	Ei09	Ei16	Ei18
Petal length	0.175	0.147	0.449	0.815	0.310	0.130
Corolla diameter	0.171	0	0.313	0.212	0.081	0
Corolla tube length	0.173	0.259	0.316	0.302	0.247	0.357
Anther exertion	0.231	0.236	0.479	0.590	0.309	0.268
Herkogamy	-0.095	0.132	-0.210	-0.295	-0.013	0.342
Pollen	0.098	0	0.162	0.371	0.303	0
Ovules	0.349	0	0.622	0.586	0.644	0.474

Table 3.2. Total selective effects on traits result from the sum of significant direct and indirect effects on fitness. The indirect effect results from the product of mediated on fitness according to SEM for each population.

Regarding reproductive investment, we found significant differences among ploidy levels but not the same pattern for pollen and ovules. Hexaploid plants produce a significantly higher amount of pollen and diploids showed the lowest value, with the tetraploids presenting an intermediate value (Figure 3.4). However, hexaploids exhibited significantly higher values of ovules per flower than diploids and tetraploids, which did not show significant differences for this trait (Figure 3.4). When we evaluated the P/O ratio, hexaploids presented the highest values. Still, diploids showed intermediate values and tetraploids the lowest ones (Figure 3.4).

Phenotypic correlation among traits across ploidies

We found significant differences between the phenotypic correlations among traits across ploidy levels. Diploids exhibited significant correlations between every pair of measured traits. However, the number of significant correlations decreased in tetraploids and dropped even more in number when we analyzed hexaploids (Table 3.3). There was a significant positive phenotypic correlation among all the attributes except for herkogamy, which negatively correlated with all the other traits. In tetraploids, most correlations were significant and positive; however, any correlation with herkogamy and the correlation between corolla tube length and exertion were negative. The correlations between corolla diameter-exertion and corolla tube length-herkogamy were non-significant. Finally, we found significant positive correlations in hexaploids between petal length, corolla diameter and corolla tube length. These floral attributes also showed significant positive correlations with ovules. Corolla tube length and herkogamy, exertion and pollen grains, and pollen grains and ovules were also significant and positively correlated. There were significant negative correlations of exertion with petal length, corolla tube length and herkogamy, and between herkogamy and pollen production. The rest of phenotypic correlations were non-significant in hexaploid plants (Table 3.3).

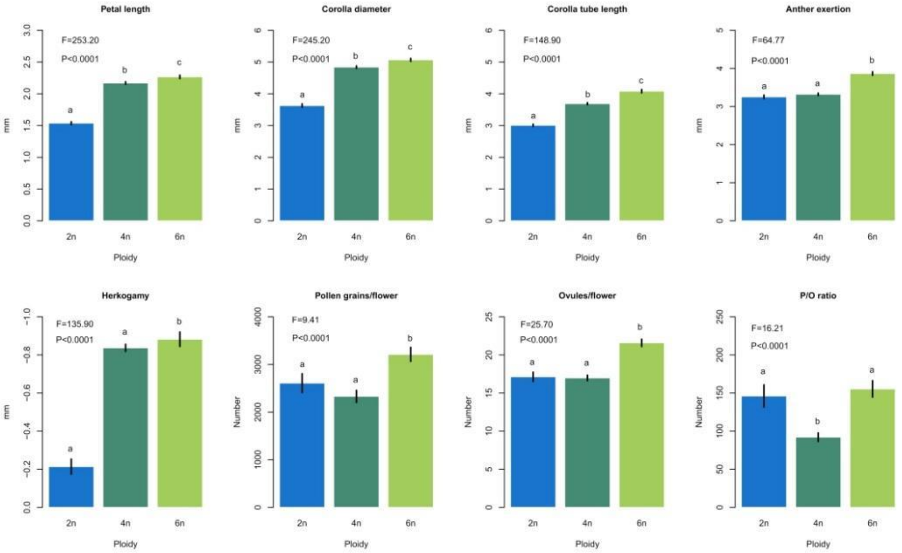


Figure 3.4. Mean and SE values for petal length, corolla diameter, corolla tube length, anther exertion, herkogamy, pollen and ovules amount per flower and P/O ratio for each ploidy level based on linear models. Letters refer to these groups that are significantly different according to a pairwise test (*P*-value < 0.05).

DISCUSSION

The efficacy of natural selection acting on a selfing plant species has been explored at a molecular level (Arunkumar et al., 2015) but received less attention than outcrossing species, mainly when the focus was natural selection acting on phenotypic traits or reproductive investment. By combining the study of relationships among traits, reproductive investment, and their direct and indirect effects on fitness across different ploidy levels, this work shows that changes in the DNA amount significantly affect the configuration of the relationships established between the studied floral traits, modifying the phenotypic integration of these traits across ploidies. In this group of selfing species, the effect of flower traits on reproductive investment and self-pollination plays a vital role in plant fitness, shaping the floral pattern we observe across ploidies. However, the strong selection of traits related to pollen export makes them key attributes driving changes in the evolution of reproductive strategies within a selfing multiploidy species complex.

Path analyses made it possible to detect the direct, indirect and total effects of traits on fitness. This is essential because indirect effects frequently pass undetected, as traits interact with fitness and several selective agents simultaneously, which are not always under our control. Often, traits are mediated by natural selection on other traits, modifying the selective pressure. For example, Gómez et al. (2009), using a SEM approximation on *Erysimum mediohispanicum* populations, described significant effects of plant phenotype by indirect selection on the total selection experienced by the plants. This work demonstrated that this indirect selection played a crucial role in the rise of the geographic mosaic of selection in *E. mediohispanicum* in the Sierra Nevada Mountains.

2n (405)	**Corolla diameter**	**Corolla tube length**	**Anther exertion**	**Herkogamy**	**Pollen**	**Ovules**
Petal length	**0.7908******	**0.6473******	**0.3244******	**-0.3493******	**0.2091******	**0.4619******
Corolla diameter		**0.6140******	**0.4343******	**-0.4103******	**0.1770**	**0.5513******
Corolla tube length			**0.1965******	**-0.3872******	**0.1677**	**0.5558******
Anther exertion				**-0.4908******	**0.3563******	**0.6124******
Herkogamy					**-0.2787******	**-0.4195******
Pollen						**0.3263******
4n (763)	**Corolla diameter**	**Corolla tube length**	**Anther exertion**	**Herkogamy**	**Pollen**	**Ovules**
Petal length	**0.6534******	**0.5491******	**0.1013**	**-0.1011****	**0.2703******	**0.3490******
Corolla diameter		**0.5109******	0.0874	**-0.1023****	**0.2420******	**0.3122******
Corolla tube length			**-0.2622******	-0.0214	**0.1480*****	**0.4095*******
Anther exertion				**-0.3482******	**0.4420******	**0.3418******
Herkogamy					**-0.1906******	**-0.1887******
Pollen						**0.6691******
6n (454)	**Corolla diameter**	**Corolla tube length**	**Anther exertion**	**Herkogamy**	**Pollen**	**Ovules**
Petal length	**0.6956******	**0.5492******	**-0.1456**	0.0132	0.0544	**0.2755******
Corolla diameter		**0.3668******	-0.0109	0.0417	-0.0614	**0.2134**
Corolla tube length			**-0.5936******	**0.1248**	-0.0509	**0.2855******
Anther exertion				**-0.2226******	**0.2622******	0.1174
Herkogamy					**-0.1408**	0.0627
Pollen						**0.2861******

Table 3.3. Phenotypic correlations among *Erysimum incanum* phenotypic traits for each ploidy level. Positive correlations are highlighted in blue, while negative correlations are highlighted in red. The sample size is shown by ploidy. Significant values are shown in bold after Bonferroni corrections (P-value < 0.0025) while asterisks indicate significance levels without Bonferroni correction (***P-value<0.001; ****P-value<0.0001).

In our case, we found that traits related to corolla size are scarcely under direct selection but more frequently under indirect selection. Cost production and maintenance of the structure could explain the negative selection acting on some of these traits (Ashman and Schoen, 1994). However, bigger flowers enhance the exertion of the anthers, which has a positive effect on fitness (Gómez et al., 2009; Sahli and Conner, 2011). Evaluating the total effect on fitness, we can observe an existing counterbalance between different selective gradients. Previous studies have already shown that opposite selective pressures acting on the same trait are important to maintain phenotypic variation within a population (Siepielski and Benkman, 2010), which is essential for evolution. Additionally, differential responses to selection by the same trait could favour diverse evolutionary trends within a species (Zhou et al., 2020). Consequently, if the selected traits are related to the reproductive strategy, a transition in the mating system might be possible in the *E. incanum* complex.

Polyploidization has the potential to produce changes in flower morphology, size and physiology (Müntzing, 1936; Stebbins, 1971; Levin, 2002; Clo and Kolář, 2021). Recent works comparing diploid and polyploid populations showed that higher ploidy levels exhibit not only larger flowers but also larger pollen grains (Oliveira et al., 2022). In addition, studies in *Heuchera grossularifolia* (Segraves and Thompson, 1999) demonstrated that tetraploid populations showed larger flowers conducting to a different pollinator assemblage than diploid populations. We also found a tendency for bigger flowers and higher levels of pollen grain production as ploidy increases in *E. incanum.* Although we did not test the existence of changes in pollinator visits, larger flowers associated with higher pollen production could increase floral advertisement and attraction, modifying pollinator interactions. Changes in metabolic routes could accompany these changes in ploidy level and flower size. An interesting example of this has been described in the *Dianthus broteri* species complex, where an increasing floral scent variation was associated with polyploidization (Picazo-Aragonés et al., 2020). In *E. incanum*, it would be interesting to evaluate changes in metabolites related to this increment in flower size and pollen grain production as a potential mechanism to attract flower visitors.

Stamen's length plays an important role in the exposure (and subsequent pollen removal) of self-pollen and the reception of outcrossing pollen grains by attracting pollen vectors. In autogamous plants, Rosas-Guerrero et al. (2010) demonstrated a high degree of integration among floral traits that promote self-pollination, such as the style and stamens lengths. In addition, higher values of stamens and pistil length are associated with greater pollen exposure and stronger competition between self-pollen and the incoming outcrossing pollen, as shown by classical studies performed in animal-pollinated plants (Campbell, 1989). Yet, stamen’s length does not necessarily relate with flower size, as observed here. According to our results, and contrasting with other autogamous mating systems, in *E. incanum* high values of floral traits, pollen amount and anther exertion in polyploids could suggest outcrossing reproduction might be gaining importance with increased ploidy level. Despite the theoretical expectations that polyploids should self-fertilize more on average than their diploid progenitors to reduce frequency-dependent selection, autopolyploid species have been found to self-fertilize less, on average than their diploid progenitors (Husband et al., 2008). Recent theoretical studies confirmed that avoiding selfing in neopolyploids promotes establishment, notably when polyploids suffer an initial decrease in fitness (Clo et al., 2022). In this sense our results provide more evidences to confirm that the association between ploidy and selfing is probably less clear than previously thought.

Herkogamy is one of the primary mechanisms reducing selfing and driving mating system transitions in plants (Opedal, 2017). *Erysimum incanum* exhibits reverse or negative herkogamy, i.e., the anthers are above the stigma surface to ensure self-pollen deposition in the stigma. However, reverse herkogamy is more accentuated when ploidy increases, with the biggest distances among sexual organs being observed in the hexaploids. Increasing values of herkogamy together with higher anther exertion would promote pollen exportation more easily after pollinator visits, even when an anther-rubbing mechanism occurs in *E. incanum* (Abdelaziz et al., 2019) and self-pollination is always assured.

It is worth noting that reproductive investment indirectly mediates most traits' selection. This is especially clear when we focus on ovule production, as the most significant selection of traits was exclusively mediated via female function. In addition, we found variability in pollen production associated with different phenotypes and significant differences between ploidy levels. So, to understand the full scope of these changes in sexual effort, we considered focusing on the P/O ratio and its evolutionary consequences. According to the sex allocation theory (Charnov, 1987; Brunet, 1992; Campbell, 2000), we expect a trade-off between male and female reproductive investment. Cruden (1977) described a trade-off mechanism between pollen and ovule production by proposing that low values in the P/O ratio are associated with autogamous mating systems in plants, while high values would be characteristic of outcrossing species. This pattern has been described in *Silene* and *Dianthus* species, where lower levels of P/O ratio were associated with self-compatible species (Jürgens et al. 2002). However, the positive covariance between pollen and ovule production we found in our system means that plants investing more in male reproduction also invest more in female reproduction. Our results demonstrate that a trade-off between male and female reproductive investment does not exist in *Erysimum incanum*. This finding is unexpected for a selfing species.

We also detected a positive effect of the P/O ratio on the natural selection intensity exhibited on herkogamy across the analyzed populations. This result suggests that in populations with a lower P/O ratio, a reduction in herkogamy is favored. Nevertheless, in populations where the P/O ratio is higher, the separation between male and female reproductive organs is favored by selection. This outcome agrees with previous results in other species from the genus *Melochia* (Faife-Cabrera et al., 2018) and Fabaceae species (Galloni et al., 2007), where a positive correlation was described between P/O ratio and herkogamy. Our findings show that in populations where the proximity between male and female reproductive organs is selected, the P/O ratio decreases, probably because self-pollination is more efficient. Conversely, in a population where the separation between sexual organs is strongly selected, the P/O ratio increases, likely to increase the success of outcrossing. These findings support and give a mechanistic explanation to Cruden's conclusion that the P/O ratio decreases with an efficient degree of self-fertilization (Cruden, 1977).

Studying multiple traits affecting fitness allowed us to have a global view of the different selective pressures acting on phenotypes. But it also allowed us to explore the possible relationship between the measured traits and their integration into the phenotype. In this sense, the gradual reduction of significant correlations from the diploid to hexaploid plants suggests that the increasing ploidy level significantly affects the covariation between plant traits (i.e., phenotypic integration). Phenotypic integration was pointed out as adaptive when it results from convergent evolution in the values of functional traits to develop a common or independent function in the organism. However, we can also think of phenotypic integration as a constraint for future evolution of each trait's covariance (Pigliucci, 2003). For example, sets of vegetative or reproductive attributes sharing common functions would strongly covary and hence limit the independent variation of each other. Polyploidy was described as contributing to modify covariation among traits in *Dianthus broteri* species complex (Balao et al., 2011) and *Brassica* allopolyploid species (Baker et al., 2017) and now in *E. incanum* complex as well (results herein). Reduced covariation values between traits may reduce and overcome the constraints imposed by phenotypic integration, allowing the new polyploid species to explore new trait spaces and potentially reach new or higher adaptive peaks unreached by their diploids counterparts (Hansen et al., 2019).

These findings in ecological variation between populations differing in ploidy level might shed light on the role of genetic mechanisms as drivers of the evolutionary trajectory followed even by a selfing species that generally have been considered with a reduced ability to evolve (Igic & Busch, 2013). However, in recent years new empirical and simulation studies have focused on discussing this traditional association between selfing species and a reduced adaptive potential (Clo and Opedal, 2021; Opedal et al., 2023). Our study provides new results consistent with these last studies and highlights the importance of genetics as a key factor enhancing the evolutionary dynamics within a selfing clade.

CONCLUSIONS

Changes in flower size, spatial position of sexual organs, and reproductive investment seem to be a direct consequence of genome duplications, with hexaploid plants showing the highest values for every studied attribute. No trade-off was found between pollen and ovule production. Still, the number of ovules per flower was the trait under significant selective pressure in every population independently of the ploidy. This fact evidences the limiting role of ovules as a fitness component for these plants. In addition, ovules also significantly affected adaptive trajectories by their relation with pollen production (P/O ratio), which constrains the selective pressures acting on important traits such as herkogamy. Finally, increasing ploidy values contributed significantly to decoupling variation among traits in *E. incanum* species complex, reducing phenotypic integration as ploidy increases. So, even working with a selfing species complex, our results evidence that the variation promoted by polyploidization is a key factor in the reproductive strategy transitions by modifying the investment in pollen and ovules and linking them with plant fitness and phenotype evolution.

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AUTHOR CONTRIBUTIONS

AGM, CF, AJMP and MA thought and designed the experiments. AGM, CF and CVB conducted the greenhouse experiments. JL and SC conducted the ploidy analyses. AGM, AJMP and MA made the statistical analyses and designed the tables and figures. CVB made the hand-made figures. AGM wrote the first draft of this manuscript and all the rest of the authors made significant contributions to the draft. AJMP, SC and MA got the funds to develop this study. AJMP and MA supervised all the study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included at the end of the article. Further inquiries can be directed to the corresponding author/s.

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SUPORTING INFORMATION

Additional Supporting Information can be found online in the supporting information section at the end of the article (published paper in American Journal of Botany, **DOI:10.1002/ajb2.16197**) or in the following pages for the thesis version.

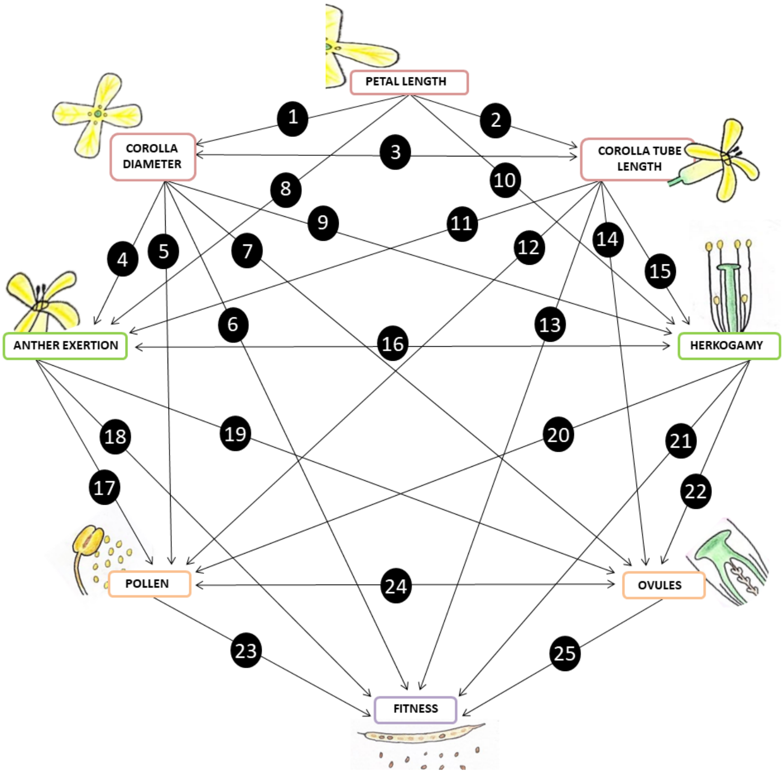
Appendix S3.1:

Table S3.1. Number of plant individuals per population on which we measured floral traits (petal length, corolla diameter and corolla tube length), spatial position between reproductive structures (anther exertion and herkogamy), reproductive investment (pollen and ovules) and seed production or fitness.

Ploidy level	**Population**	**Number of phenotyped plants**					
		Floral traits	Anther exertion	Herkogamy	Pollen	Ovules	Fitness
2x	Ei 12	286	286	286	229	174	176
	Ei 13	114	114	114	108	85	79
4x	Ei 08	513	513	513	402	402	330
	Ei 09	248	248	248	110	176	161
6x	Ei 16	230	230	230	197	137	103
	Ei 18	221	221	221	194	106	94

Appendix 3.2:

Figure S3.1. A-priori model structural equation model evaluating the effect of floral traits, spatial position between reproductive structures and male and female reproductive investment on plant fitness. Numbers indicate the proposed paths based on hypothesized mechanisms detailed and supported by literature in Table S3.2.



Path number	Path	Justification	Reference
1,2	Petal L. → Corolla D. Petal L. → Corolla T.L.	Floral pieces are closely related during flower formation due to development and evolutionary history	Stanton & Preston, 1988; Benitez-Vieyra et al., 2019 (and references therein)
3	Corolla D. ←→ Corolla T.L.	Pollinators select flowers with both large corollas and deep corolla tubes	Nishino, 1983; Fenster, 1991
4,8,11	Corolla D. → An. Exertion Petal L. → An. Exertion Corolla T.L. → An. Exertion	The pollinator effectiveness is higher in larger flowers facilitating the pollen exposure.	Galloni et al., 2007
5,12	Corolla D. → Pollen Corolla T.L. → Pollen	Bigger flowers are expected to be more visited and export more pollen, which can be a reward as well for pollinators.	Herrera, 1996; Gómez et al., 2008
6,13	Corolla D. → Fitness Corolla T.L. → Fitness	Bigger flowers increases the chance for mating and thus, the fitness	Schoen, 1982; Poveda et al., 2005
7,14	Corolla D. → Ovules Corolla T.L. → Ovules	A reduction in flower size is associated to selfing species which allocate more resources to ovules production	Herrera, 1996; Rosas-Guerrero et al., 2010; Tedder et al., 2015
9,10,15	Corolla D. → Herkogamy Corolla T.L. → Herkogamy Petal L. → Herkogamy	Larger flowers are associated to cross-pollination and herkogamy avoids self-pollination.	Brunet & Eckert, 1998
16	An. Exertion ←→ HK	Both the herkogamy and pollen exposition enhance cross-pollination.	Webb & Lloyd, 1986 Lloyd & Webb, 1986
17	An. Exertion → Pollen	Anther exertion increases the pollen amount which can be exported.	Campbell, 1989 Barret, 2003;
18	An. Exertion → Fitness	Pollen exposure is important to increase the chance for cross-pollination	Galloni et al., 2007; Medano et al., 2005
19	An. Exertion → Ovules	Higher pollen exposure is proper of out-pollinated plants, which allocate less resources to ovules	Campbell, 1989
20	Herkogamy → Pollen	A higher herkogamy reduces self-pollination and improve the pollen export for cross-pollination.	Webb & Lloyd, 1986 Lloyd & Webb, 1986; Faife-Cabrera et al., 2018
21	Herkogamy → Fitness	Herkogamy affects the fitness outcome facilitating pollen deposition on stigma	Medrano et al., 2005; Luo & Widmer, 2013
22	Herkogamy → Ovules	A reduced herkogamy improve self-pollination, increasing the ovules production.	Webb & Lloyd, 1986 Lloyd & Webb, 1986; Brys et al., 2013
23	Pollen → Fitness	Pollen grains limit the amount of ovules that can be fertilized.	Campbell & Halama, 1993; Ashman et al., 2004
24	Pollen ←→ Ovules	A trade-off between the allocation to male and female has been broadly described.	Cruden, 1977 Lloyd, 1984; Charnov, 1987
25	Ovules → Fitness	The potential for fitness depends on the ovules to be fertilized.	Campbell, 1989: Galloni et al., 2007

Table S3.2. Hypothesized mechanisms explaining the proposed links among measured variables in Figure S3.1. Abbreviations: Petal length (Petal L.), Corolla diameter (Corolla D.), Corolla tube length (Corolla T.L.), Anther exertion (An. Exertion).

Appendix S3.3:

Table S3.3. Comparison among proposed models based on *P*-value and AIC scores. We removed or added links until we found a model with a good fit index for each population (Model 5). Removed links in the other tested models that we added in our a-priori model: *Corolla diameter* ←→ *Corolla tube length* and *Herkogamy* ←→ *Anther exertion* in Model 1; Herkogamy ←→ Anther exertion in Model 2; *Corolla diameter* ←→ *Corolla tube length* and *Anther exertion* → *Ovules* in Model 3*; Anther exertion* → *Ovules* in Model 4.

Model	Pop 12		Pop 13		Pop 08		Pop 09		Pop 16		Pop 18	
	AIC	***P-value***	**AIC**	***P-value***	**AIC**	***P-value***	**AIC**	***P-value***	**AIC**	***P-value***	**AIC**	***P-value***
Model 1	91.473	0	67.534	0.473	97.192	0	104.421	0	100.335	0	33.836	0.586
Model 2	108.580	0	82.727	0.347	134.763	0	117.068	0	112.639	0	81.226	0.515
Model 3	103.460	0	100.976	0.001	215.710	0	92.164	0.014	92.206	0.014	94.217	0.007
Model 4	98.353	0	97.783	0.001	190.139	0	91.517	0.006	91.902	0.005	91.827	0.005
Model 5	76.807	0.569	77.012	0.542	83.132	0.084	76.614	0.856	80.974	0.175	76.025	0.673

Appendix S3.4:

Table S3.4. Variance inflation factors (VIF) calculated from multiple linear regressions in order to test multicollinearity among variables. Values higher than 5 indicate strong correlation between variables.

Trait	Petal length	Corolla diameter	Corolla tube length	Anther exertion	Herkogamy	Pollen	Ovules	Fitness
Petal length	-	1.674	2.266	1.982	1.294	1.467	2.552	1.739
Corolla diameter	1.839	-	2.519	1.966	1.293	1.456	2.534	1.728
Corolla tube length	2.957	2.99	-	1.395	1.232	1.454	2.334	1.724
Anther exertion	3.302	2.982	1.782	-	1.151	1.355	2.287	1.740
Herkogamy	3.338	3.035	2.433	1.782	-	1.466	2.555	1.725
Pollen	3.339	3.016	2.535	1.850	1.294	-	2.403	1.738
Ovules	3.329	3.008	2.332	1.790	1.292	1.377	-	1.284
Fitness	3.337	3.018	2.533	2.003	1.283	1.465	1.888	-

Appendix S3.5:

Table S3.5. Estimate parameters and standard error of direct effects on reproductive structures position (Anther exertion and Herkogamy) and male and female reproductive investment (Pollen and Ovules, respectively) based on Structural Equation Modelling for each ploidy level. Values in italics refer to covariance estimates. Significant relationships are shown in bold. Asterisks indicate the significance degree and a point indicates marginally significant relationships (**P-value*<0.05; ***P-value*<0.01; ****P-value*<0.001; *****P-value*<0.0001).

2x	Corolla diameter	Corolla tube length	Anther exertion	Herkogamy	Pollen	Ovules	Fitness
Petal length	**0.797 ± 0.032******	**0.623 ± 0.044******	**0.237 ± 0.071****	0.026 ± 0.109	-	-	-
Corolla diameter		**0.156*****	**0.476 ± 0.118*****	**-0.304 ± 0.101****	-0.006 ± 0.077	**0.193 ± 0.066****	0.054 ± 0.077
Corolla tube length			**-0.203 ± 0.080***	**-0.306 ± 0.074*****	**0.277 ± 0.073*****	**0.357 ± 0.063******	0.149 ± 0.079
Anther exertion				***-0.382*******	**0.363 ± 0.066******	**0.488 ± 0.057******	0.074 ± 0.078
Herkogamy					-0.108 ± 0.074	0.073 ± 0.062	0.076 ± 0.070 ·
Pollen						*0.115*	-0.010 ± 0.071
Ovules							**0.446 ± 0.082******

4x	Corolla diameter	Corolla tube length	Anther exertion	Herkogamy	Pollen	Ovules	Fitness
Petal length	**0.797 ± 0.033******	**0.622 ± 0.044******	**0.237 ± 0.081****	0.018 ± 0.094	-	-	-
Corolla diameter		***0.156******	**0.181 ± 0.076***	**-0.194 ± 0.088***	-0.047 ± 0.055	-0.065 ± 0.052	**0.111 ± 0.043***
Corolla tube length			**-0.453 ± 0.056******	0.095 ± 0.065	**0.478 ± 0.055******	**0.655 ± 0.051******	**-0.017 ± 0.051**
Anther exertion				***-0.375*******	**0.504 ± 0.051******	**0.525 ± 0.048******	**-0.121 ± 0.047***
Herkogamy					-0.015 ± 0.045	-0.017 ± 0.042	**-0.92 ± 0.035****
Pollen						***0.361*******	-0.046 ± 0.042
Ovules							**0.686 ± 0.045******

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6x	**Corolla diameter**	**Corolla tube length**	**Anther exertion**	**Herkogamy**	**Pollen**	**Ovules**	**Fitness**
Petal length	**0.685 ± 0.061******	**0.635 ± 0.082******	**0.283 ± 0.104****	-0.170 ± 0.129		-	-
Corolla diameter		*-0.033*	**0.244 ± 0.089****	0.183 ± 0.110 ·	**-0.203 ± 0.091***	0.053 ± 0.088	0.073 ± 0.070
Corolla tube length			**-0.814 ± 0.066******	0.023 ± 0.083	**0.426 ± 0.097******	**0.524 ± 0.094******	**0.274 ± 0.082 ****
Anther exertion				***-0.276******	**0.516 ± 0.090******	**0.395 ± 0.088******	**0.113 ± 0.076**
Herkogamy					-0.005 ± 0.074	**0.238 ± 0.072*****	**0.131 ± 0.058 ***
Pollen						***0.327*******	-0.030 ± 0.062
Ovules							**0.566 ± 0.063******

Appendix S3.6:

Table S3.6. Values of each trait's direct and indirect and total effect on fitness for each population. Indirect effects are calculated from the mediated effects on fitness product, based on significant estimates of structural equation modelling. The total effect is the sum of direct and indirect effects.

Traits	Ei12			Ei13		
	Direct effect	Indirect effect	Total effect	Direct effect	Indirect effect	Total effect
Petal length	-	0.175	0.175	-	0.147	0.147
Corolla diameter	0	0.171	0.171	0	0	0
Corolla tube length	0	0.173	0.173	0.224	0.035	0.259
Anther exertion	0	0.231	0.231	0	0.236	0.236
Herkogamy	0	-0.095	-0.095	0	0.132	0.132
Pollen	0	0.098	0.098	0	0	0
Ovules	0.349	0	0.349	0.615	0	0

Traits	Ei08			Ei09		
	Direct effect	Indirect effect	Total effect	Direct effect	Indirect effect	Total effect
Petal length	-	0.452	0.449	-	0.815	0.815
Corolla diameter	0	0.313	0.313	0	0.212	0.212
Corolla tube length	0	0.316	0.316	0	0.302	0.302
Anther exertion	0	0.479	0.479	0	0.590	0.590
Herkogamy	-0.125	-0.085	-0.210	0	-0.395	-0.395
Pollen	0	0.162	0.162	0	0.371	0.371
Ovules	0.622	0	0.622	0.586	0	0.586

Traits	Ei16			Ei18		
	Direct effect	Indirect effect	Total effect	Direct effect	Indirect effect	Total effect
Petal length	-	0.310	0.310	-	0.130	0.130
Corolla diameter	0	0.081	0.081	0	0	0
Corolla tube length	0	0.247	0.247	0.415	-0.058	0.357
Anther exertion	0	0.309	0.309	0	0.268	0.268
Herkogamy	0	-0.013	-0.013	0.211	0.131	0.342
Pollen	0	0.303	0.303	0	0	0
Ovules	0.644	0	0.644	0.474	0	0.474

Appendix S3.7:

Table S3.7. Effect of ploidy and population on measured traits evaluated by linear models. Population appears as a factor nested within ploidy. Significant *P*-values are shown in bold and asterisks indicate the significance degree (**P*-value<0.05; ***P*-value<0.01; ****P*-value<0.001; *****P*-value<0.0001).

	Ploidy			**Ploidy : Population**		
Trait	*df*	*F-value*	*P-value*	*df*	*F-value*	*P-value*
Petal length	2	273.38	**<0.0001******	3	43.01	**<0.0001******
Corolla diameter	2	294.70	**<0.0001******	3	107.30	**<0.0001******
Corolla tube length	2	156.90	**<0.0001******	3	29.33	**<0.0001******
Anther exertion	2	68.21	**<0.0001******	3	28.85	**<0.0001******
Herkogamy	2	144.30	**<0.0001******	3	33.60	**<0.0001******
Pollen	2	9.55	**<0.0001******	3	7.72	**<0.0001******
Ovules	2	28.89	**<0.0001******	3	45.95	**<0.0001******
P/O ratio	2	16.22	**<0.0001******	3	1.09	0.349

Appendix S3.8:

Table S3.8. Multiple comparisons of traits values between populations from the three ploidy levels based on linear models nesting Population within Ploidy. Significant estimates are in bold accompanied by the significance level according to the number of asterisks. (*P-value<0.05; **P-value<0.01; ***P-value<0.001; ****P-value<0.0001).

Population	Traits							
	Petal length	Corolla diameter	Corolla tube length	Anther exertion	Herkogamy	Pollen	Ovules	P/O ratio
Ei12 – Ei13	**-0.491******	**-1.300******	**-0.804******	**-0.740******	**0.637******	**-0.462*****	**-1.422******	0.004
Ei12 – Ei08	**-0.847******	**-1.879******	**-0.990******	**-0.160**	**0.803******	-0.002	**-0.430******	**0.396****
Ei12 – Ei09	**-0.610******	**-0.945******	**-0.731******	**-0.512******	**0.798******	-0.197	**-0.461******	0.214
Ei12 – Ei16	**-0.953******	**-1.894******	**-1.179******	**-0.885******	**0.996******	**-0.399*****	**-0.987******	-0.052
Ei12 – Ei18	**-0.776******	**-1.713******	**-1.422******	**-0.752******	**0.693**	-0.268	**-0.914******	-0.063
Ei13 – Ei08	**-0.356******	**-0.580******	**-0.186******	**0.581******	0.165	**0.459*****	**0.992******	**0.0391***
Ei13 – Ei09	-0.119	**0.550***	**0.074******	0.228	0.160	0.265	**0.961******	0.210
Ei13 – Ei16	**-0.462******	**-0.595******	**-0.374******	-0.145	**0.358******	0.062	**0.435****	-0.056
Ei13 – Ei18	**-0.285******	**-0.413****	**-0.617******	-0.012	0.055	0.193	**0.508****	-0.068
Ei08 – Ei09	**0.237******	0.935	**0.259******	**-0.352******	-0.005	-0.194	-0.031	-0.181
Ei08 – Ei16	-0.106	**-0.015******	**-0.189******	**-0.725******	**0.193****	**-0.397******	**-0.055******	**-0.448*****
Ei08 – Ei18	0.071	0.166	**-0.431******	**-0.592******	-0.109	**-0.266***	**-0.483******	**-0.460****
Ei09 – Ei16	**-0.343******	**-0.950******	**-0.448******	**-0.337*****	**0.197***	-0.202	**-0.525******	-0.266
Ei09 – Ei18	**-0.166*****	**-0.768******	**-0.691******	**-0.239***	-0.105	-0.071	**-0.452****	-0.278
Ei16 – Ei18	**0.177*****	0.181	**-0.243******	0.133	**-0.303******	0.131	0.073	-0.011

Chapter 4

Natural selection makes heritable phenotypic plasticity adaptive but constrained by trait function

Ana García Muñoz[1], Camilo Ferrón[1,2], Mohammed Bakkali[1], A. Jesús Muñoz-Pajares[1,3], Mohamed Abdelaziz[1]

ABSTRACT

Phenotypic plasticity is the ability of a single genotype to change its phenotype according to environmental conditions, which draws attention in the current global change context where plastic individuals would be able to successfully buffer perturbations. However, costs derived from phenotypic plasticity have also been identified. Regarding the effect on fitness, natural selection could act on phenotypic plasticity itself if this variation is heritable. In addition, other factors such as the ploidy and the modularity are able to affect the ability to change. We evaluated the requirements for natural selection action in the selfing species *Erysimum incanum*, which exhibits three levels of ploidy. We subjected individuals to two different treatments (indoor and outdoor growing conditions) and we measured a total of 13 traits grouped by floral, vegetative and reproductive modules on over 700 plants. We also quantified several fitness components and reproductive investment to assess the outcomes of natural selection on plastic responses. Finally we estimated the heritability of phenotypic plasticity exhibited by each trait. Our results revealed genetic variation in phenotypic plasticity and we found the highest phenotypic plasticity value in reproductive traits while floral traits were the most canalized, which exhibited a positive and negative effect on fitness, respectively. These results together with a higher heritability of phenotypic plasticity in increased ploidies suggest that the phenotypic plasticity could be considered as an evolutionary trait itself, leading to an adaptive or maladaptive scenario depending on the modularity.

Key words *Erysimum incanum*, fitness, genetic correlations, heritability, modularity, reproductive investment, ploidy

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INTRODUCTION

Phenotypic plasticity is defined as the ability of a single genotype to produce different phenotypes in response to changes in the environment (Bradshaw, 1965; Pigliucci et al., 2006). This mechanism has facilitated expansion and colonization events in several species (Yeh and Price, 2004; Geng et al., 2007, 2016; Lande, 2015) and has been identified as an important speciation and diversification promotor (Price et al., 2003; Schlichting, 2004; Soltis et al., 2009; Fitzpatrick, 2012). Recently, the capacity of responding to environmental changes has gained importance to cope with the even more frequent environmental perturbations within the current scenario of global climate change, especially in sessile organism such as plants (Potvin and Tousignant, 1996; Crispo et al., 2010; Matesanz et al., 2010; Nicotra et al., 2010; Reed et al., 2010, 2011; Richardson et al., 2017). Despite the wide literature about phenotypic plasticity, its adaptive potential has been an arguing topic for decades since adaptive and maladaptive scenarios resulting from plastic response have been identified (Schlichting, 1986; Via, 1993; Dudley and Schmitt, 1996; Aubret et al., 2007). Costs associated with plastic genotypes would constrain the adaptive potential of phenotypic plasticity (Dewitt et al., 1998; Auld et al., 2010). Within a scenario where plastic genotypes are strongly penalized, natural selection would favor canalization (Waddington and Robertson, 1966; Flatt, 2005), which is defined as the ability of a genotype to produce the same phenotype independently of the environmental conditions (Waddington, 1942).

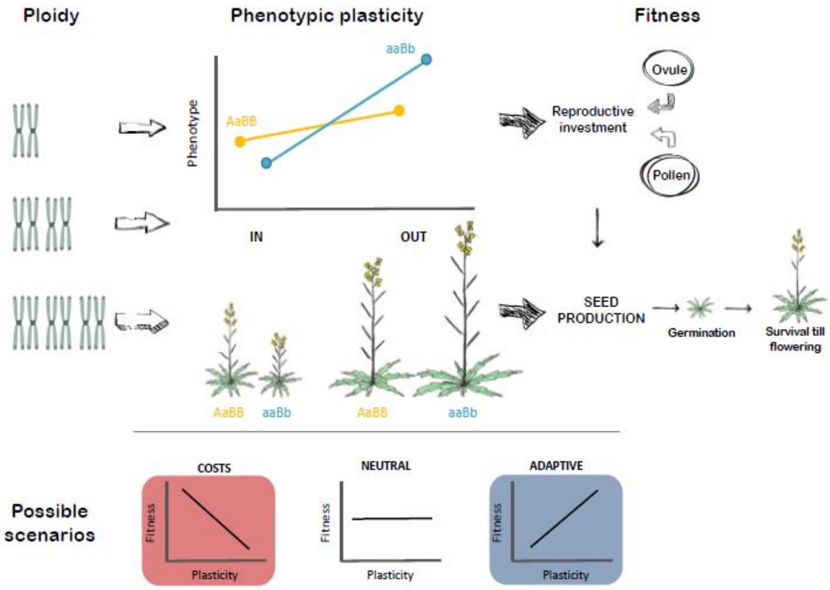

Figure 4.1. Experimental design scheme. Phenotypic plasticity, or the ability of a single genotype to express different phenotypes in different environments, can be quantified in different traits in a given organism. In our case the phenotypic plasticity is associated with families as the species present a selfing mating system. We can also estimate the fitness values exhibited by these genotypes quantifying different components of fitness, reproductive investment, seed production, germination and survival to blooming, which compose the whole life cycle of the plant. Significant heritability of phenotypic plasticity, variation on this plasticity and significant relationship between this variation and fitness means that plasticity is under natural selection regimes. Depending on the sign of the relationship we can conclude that phenotypic plasticity is an adaptive, maladaptive (cost) or neutral trait. Finally, the role of the variation in ploidy on the rise of different scenarios makes it very interesting to carry out this study in a multiploidy species complex.

Phenotypic plasticity costs could be buffered by the multiple genome copies provided by an increase in the ploidy level. Phenotypic variation resulting from a change in the ploidy is associated with the overall increase of fitness in polyploid populations (Sun et al., 2011; Yao et al., 2011; Corneillie et al., 2019; Ahmed et al., 2020), allowing them for establishment (Van de Peer et al., 2017; Čertner et al., 2019) and with its ability to shape and even constrain adaptive trajectories by modifying the interaction among traits and fitness (García-Muñoz et al., 2023 *in press*). The extra genomic diversity level provided by higher ploidies (Soltis and Soltis, 1995; Bennett et al., 2014) suggests that it could be involved in phenotypic plasticity processes through a potentially higher metabolic diversity (Chen, 2010; Roulin et al., 2013). In fact, the ability shown by polyploids to colonize new habitats and to be associated with stressing environments has been justified because they could respond to environmental changes to optimize their fitness by fitting their phenotype (Shimizu-Inatsugi et al., 2017). Polyploidization as well as phenotypic plasticity are broadly present in the evolutionary history in plants (Grant, 1981; Leitch and Bennett, 1997; Otto and Whitton, 2000; Price et al., 2003; Soltis, 2005; Aubret et al., 2007 Wood et al., 2009) but experimental studies linking ploidy and phenotypic plasticity are scarce (Bretagnolle and Thompson, 2001; Sánchez Vilas and Pannell, 2017; Wei et al., 2019).

Moreover, plants are modular organisms which means that traits can differ in their response to these mechanisms of phenotypic variation (Diggle, 2014; Ottaviani et al., 2017), especially to phenotypic plasticity (de Kroon et al., 2005; Niinemets, 2020). Plasticity in traits more related to fitness is expected to be adaptive (Madec et al., 2000)but the relative importance of traits for individual fitness depends on the predominant reproductive strategy of the plant. So, the traits more relevant for fitness would be those related to floral display and pollen production in plants with a reproduction that strongly relies on pollinators while ovules and a herkogamy degree facilitating self-pollination would be the selected traits in highly self-compatible species (Cruden, 1977; Barrett et al., 1997; Jürgens et al., 2002). Plants showing hermaphroditic flowers are able to show different phenotypic plasticity according to the allocation of resources to male and female reproductive functions (Li et al., 2019). Additionally, self-pollination in hermaphroditic flowers allows an accurate estimation of phenotypic plasticity and heritability because they produce clonal genotypes.

Here, we used the species *Erysimum incanum* to explore the ability of phenotypic plasticity to evolve by natural selection. For that, we had to estimate its variability, its heritability and its effect on fitness. Simultaneous evaluation of all these conditions is not straightforward although autogamous study systems, as the presented one here, stand as accurate models to explore the evolutionary extent of phenotypic plasticity. The species *Erysimum incanum*, besides being self-compatible, constitutes a multiploidy species complex which allows us to evaluate the effect of ploidy on the adaptive potential exhibited by phenotypic plasticity across different functional modules of traits and estimating different components of fitness in terms of plant reproductive investment and seed production. As it is a selfing plant, the production of ovules and pollen implies an indirect measurement of plant fitness. Three possible scenarios are expected according to the magnitude and sign of the link between fitness and phenotypic plasticity: maladaptive when a plastic response would impose a cost, adaptive when a plastic response would lead to a benefit or neutral when plastic responses have not a significant effect on plant fitness (Figure 4.1). The short and monocarpic life form of our study system would facilitate achieving this aim because it enables us to manage a large number of clonal individuals with an overall fitness estimated by several components.

MATERIALS AND METHODS

Study system

Erysimum L. is one of the largest genera of Brassicaceae, characterized by recent polyploidization and speciation events resulting in species complexes and cryptic species (Clot, 1991; Abdelaziz et al., 2011, 2014). The genus contains more than 200 species and subspecies inhabiting Eurasia, Central and North America and North Africa with a hot-spot diversification in the Mediterranean basin (Al-Shehbaz et al., 2006; Nieto Feliner, 2014). Three different ploidy levels have been identified in *E. incanum,* occupying different geographical distributions (Nieto-Feliner and Clot, 1993; Fennane and Ibn-Tattou, 1999; Abdelaziz et al., 2014). Diploids ($2n = 2x = 16$ chromosomes) and tetraploid populations ($2n = 4x = 32$ chromosomes) show a vicariant distribution in Iberian Peninsula and Morocco (Nieto-Feliner and Clot, 1993; Fennane and Ibn-Tattou, 1999) while hexaploid populations ($2n = 6x = 48$ chromosomes) are recently found in the southern ranges of Morocco (***Chapter 1***). The species *E. incanum* is monocarpic, so we could obtain the total seed production in 10-12 weeks when plants finish their life cycle under greenhouse conditions. Spontaneous self-pollination exhibited by this species allowed us to easily obtain pure lines after five years and to test phenotypic plasticity and ploidy effects among clonal individuals with an unexpected reduced genetic diversity.

Experimental design

Prior to the experiments, plants coming from two natural populations per ploidy level were grown under controlled conditions for seed multiplication in the greenhouse facility at University of Granada in order to remove local effects and to obtain inbred lines, ensuring reduced genetic diversity. Then, we sowed 16 seeds coming from 10 different mother plants belonging to each population and each ploidy level, this is, 3 ploidies x 10 mother plants x 2 populations x 16 seeds. The two populations per ploidy were the same that we previously used in ***Chapter 3***. This number was replicated because plants would be subjected to two different treatments, so 1920 seeds were sowed in square plastic pots (11x11x11 cm^3) filled with Gramoflor™ potting soil mixture. All seeds were sown inside the greenhouse until seedlings reached the rosette stage to obtain a similar germination and survival ratio. Then, half of the replicates were subjected to different treatments in order to test phenotypic plasticity.

The “indoor” (IN) treatment consisted in growing plants under controlled greenhouse conditions, with 28 °C average temperature, a photoperiod of 14 hours (from 8:00 to 22:00), and watering by irrigation. Plants growing under the “outdoor” (OUT) treatment were subjected to natural conditions in a common garden where only 100 ml of water were added per pot every two days.

Flowering started in February inside the greenhouse while the flowering and a month later outside. Then, we measured a series of traits grouped by functional modules. We measured traits related to the floral module using a digital caliper and they were: (1) **petal length**, distance between edge of a petal and the point where petal starts to curve shaping the corolla; (2) **corolla diameter**, distance between edge of a petal and the diametrically opposite one in the flower; (3) **corolla tube length**, distance between sepals basis and the corolla aperture point; (4) **long stamen length**, distance between long filament insert point and anther; (5) **short stamen length**, distance between short filament inset point and anther; (6) **style height**, distance between the style basis and stigma; and (7) **herkogamy**, difference between style height and long stamen length. After fruit maturation, we measured the following traits related to the vegetative module: (8) **plant height** and (9) **stalk diameter**, as the longitude and diameter of the tallest flowering stalk of the plant, respectively; (10) **number of stalks,** counting the number of flowering stalks; and (11) **number of flowers**, quantifying the total number of flowers in the plant. Finally, we measured the traits relative to the reproductive module: (12) **number of pollen grains** and (13) **number of ovules** per flower. Floral traits and pollen amount were estimated in a flower per plant while the number of ovules were the mean of four different fruits per plant. In total, over 730 phenotype plants were obtained for 13 phenotypic traits related to flower, vegetative body and male and female reproduction.

We also calculated five different component of plant fitness, including: (a) **male reproductive investment** per plant was obtained multiplying the number of pollen grains per flower by the total number flower per plant (total pollen grains per plant); (b) **female reproductive investment** was calculated multiplying the mean number of ovules per flower by the number of flowers per plant (total ovules per plant); (c) **seed production** was estimated multiplying the mean number of seeds in four ripe fruits by the number of ripe fruits produced by the plant; (d) **seed germination**, was the proportion of germinated plants by sown seeds per mother plant; and (e) **plant survival,** as the proportion of sowed seeds that reached flowering. It was possible to estimate the total fitness per plant and male and female reproductive investment simultaneously in the same plant, since *E. incanum* are monocarpic and their flowers are hermaphroditic, respectively.

Statistical analyses

Phenotypic plasticity

We estimated phenotypic plasticity for every trait by computing the relative distance plasticity index (**RDPI**; (Valladares et al., 2006). This index quantifies phenotypic plasticity based on phenotypic distances among genotypes grown in different environments, and ranges from 0 (no plasticity) to 1 (maximum plasticity; see Valladares et al., 2006 for further details). Since our plant families were composed of identical sister seeds (full-sibs), we could associate a RDPI value to each mother plant. We calculated RDPI significance by bootstrapping with 1000 permutations using the package *boot* v. 1.3-28 in R (Canty and Ripley, 2017). The obtained 95% confidence interval indicated if the family RDPI values were significantly different from zero.

Ploidy and phenotype module effect on phenotypic plasticity

We compared the difference in phenotypic plasticity between trait modules across the three ploidy levels. We performed generalized linear mixed models (GLMM) to analyze the effect of ploidy level and trait module on phenotypic plasticity using the package *lme4* v. 1.1.32 in R (Bates et al., 2009; Zuur et al., 2009). In these models, we included the mother plant as a random factor and the ploidy and the trait module as fixed factors. We built a last model including the interaction between ploidy and trait module. Differences among models were tested by comparing the values of their Akaike information criterion (AIC), Bayesian information criterion (BIC), log-likelihood (logLink) and chi-squared test (χ^2).

Heritability of the phenotypic plasticity

We estimated the narrow-sense heritability (h^2) of phenotypic plasticity for each measured trait per ploidy level deduced from mixed model ANOVA (Lynch et al., 1998), in which environment was included as fixed factor, and using the formula:

$$h^2 = \frac{\sigma_i^2}{\sigma_f^2+\sigma_i^2+\sigma_r^2} \qquad \textbf{(4.1)}$$

where, σ_i^2 is the variance component of the seed family-by-environment interactions; σ_f^2 is the variance component due to differences among seed families; and σ_r^2 is the residual variance (see Scheiner and Lyman, 1989 for further details). For this measurement, we considered that our plant families were composed of full-sib individuals because the seeds came from an autogamous fruit. We calculated heritability significance by bootstrapping with 1000 permutations using the package *boot* v. 1.3-28 in R (Canty and Ripley, 2017). The obtained 95% confidence interval indicated if the heritability values were significantly different from zero.

Selection gradients on phenotypic plasticity

Finally, direction and magnitude of selection for phenotypic plasticity of each trait per ploidy level was estimated by means of selection gradients. This is a multivariate technique that measures the selection acting on each trait plasticity independently of any other trait plasticity value (Lande and Arnold, 1983). Here we explore two types of selective gradients: (1) linear selection gradients, β, computed from the standardized partial-regression coefficients of a linear regression of relativized values of each fitness component on all character *i* phenotypic plasticities; and (2) direct nonlinear selection gradients for each trait *i* phenotypic plasticity, γ_{ii}, which was computed from the second-order standardized coefficient in quadratic regression of relativized values of each fitness component on the trait *i* phenotypic plasticity (Lande and Arnold, 1983). The multivariate models of selection gradients were built introducing as independent variables the standardized values of phenotypic plasticity per family (RDPI) and as dependent variables the relative estimate of the lifetime fitness components (male and female reproductive investment, seed production, seed germination, plant survival).

Phenotypic correlations

Finally, in order to assess the accuracy of the suggested functional modules, we performed pairwise correlations between each pair of traits independently of the module that we have established. We obtained a correlation matrix using *corrplot* (v.0.92; Wei et al. 2017). All analyses were performed by the packages mentioned above and the *stats* package implemented in R Statistical Software (v4.2.1; Team, 2021).

RESULTS

Phenotypic plasticity

Finally, we obtained RDPI values from 45 mother plants with individuals growing in both treatments, which showed inter-individual variation. However, GLMM results showed that the trait module was the variable affecting more strongly RDPI values while ploidy itself did not influence phenotypic plasticity (Table 4.1).

Specifically, reproductive traits showed the highest values of RDPI while the floral module was the least plastic (Figure 4.2). The module composed by vegetative traits exhibited intermediate values of RDPI (Figure 4.2). We found this same pattern of RDPI among trait modules across the three levels of ploidy (Figure 4.2).

	Phenotypic plasticity (RDPI)						
Model	df	AIC	BIC	LogLik	χ^2	df	*P-value*
Intercept	3	4388.1	4412.1	-2191.1			
Model 1	5	2456.8	2496.7	-1223.4	1935.3	2	**<0.0001**
Model 2	10	4392.5	4472.4	-2186.2	0.0	5	1
Model 3	16	2439.7	2567.6	-1203.8	1964.8	6	**<0.0001**

Table 4.1. Outcome of the GLMM testing the effect of ploidy level and trait module on phenotypic plasticity expressed as RDPI indices. Plant mother was introduced as a random effect nested within the ploidy level. Significant models showed p-values in bold.
Model 1: RDPI ~ Module + (1 | Plant mother)
Model 2: RDPI ~ Ploidy + (Ploidy | Plant mother)
Model 3: RDPI ~ Module * Ploidy + (Ploidy | Plant mother)

Specifically, reproductive traits showed the highest values of RDPI while the floral module was the least plastic (Figure 4.2). The module composed by vegetative traits exhibited intermediate values of RDPI (Figure 4.2). We found this same pattern of RDPI among trait modules across the three levels of ploidy (Figure 4.2).

Heritability of phenotypic plasticity

We calculated the heritability of phenotypic plasticity of each trait in each ploidy level. We only found two heritable plastic traits in diploids, the plant height and the number of ovules per flower (Table 4.2). In contrast, phenotypic plasticity was heritable in polyploid individuals for most of the measured traits across the three studied modules. Unlike diploids, we found a significant heritability of pollen plasticity instead of the ovule amount in tetraploids (Table 4.2). All the traits except the ovule amount and the herkogamy exhibited significant heritabilities for their phenotypic plasticity in hexaploids (Table 4.2). In addition, heritabilities in this ploidy showed stronger significance levels. Thus, the amount of traits with heritable phenotypic plasticity seems to increase with the ploidy level.

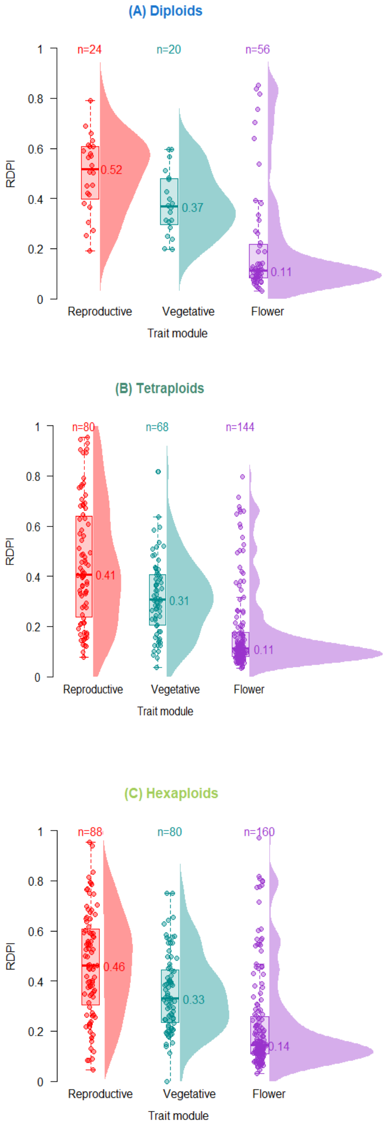

Figure 4.2. RDPI values exhibited by each trait module in (A) diploides, (B) tetraploids and (C) hexaploids plants from *E. incanum*. Shadows represent the data dispersion and the median value and the sample size are shown.

Mod	**Traits**	**2x**		**4x**		**6x**	
		h^2	**95% IC**	**h^2**	**95% IC**	**h^2**	**95% IC**
Fl.	Petal length	0.22	[0.19-0.25]	**0.35***	[0.33-0.36]	**0.60*******	[0.58-0.61]
	Corolla diameter	0.05	[0.03-0.08]	0.17	[0.15-0.19]	**0.31***	[0.30-0.32]
	Corolla tube length	0.14	[0.12-0.16]	**0.50****	[0.48-0.51]	**0.76*******	[0.75-0.78]
	Long stamen	0.06	[0.04-0.08]	**0.42***	[0.41-0.44]	**0.71*******	[0.70-0.73]
	Short stamen	0.05	[0.02-0.07]	**0.51****	[0.49-0.52]	**0.75*******	[0.74-0.77]
	Stigma height	0.16	[0.14-0.18]	0.41	[0.39-0.42]	**0.63*******	[0.61-0.64]
	Herkogamy	0.20	[0.17-0.23]	0.27	[0.25-0.28]	0.27	[0.25-0.29]
Veg.	Plant height	**0.29******	[0.27-0.32]	**0.46*******	[0.45-0.48]	**0.25*******	[0.24-0.27]
	No. stalk	0.28	[0.24-032]	**0.42***	[0.41-0.44]	**0.56*******	[0.54-0.57]
	Stalk diameter	0.29	[0.27-0.31]	**0.48****	[0.46-0.50]	**0.46******	[0.44-0.47]
	No. flowers	0.25	[0.22-0.28]	0.39	[0.38-0.41]	**0.57*******	[0.56-0.59]
Rep.	No. pollen grns/flower	0.22	[0.20-0.25]	**0.53*******	[0.52-0.55]	**0.54*******	[0.53-0.56]
	No. ovules/flower	**0.31***	[0.29-0.34]	0.30	[0.28-0.32]	0.36	[0.34-0.37]

Table 4.2. Heritability of the phenotypic plasticity exhibited by each trait from different trait modules (Mod.) and ploidy level. Values of heritability are shown accompanied by the 95% interval confidence. Significant values of heritability are shown in bold. Modules are indicated as Fl. (floral), Veg. (vegetative) and Rep. (reproductive). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Effects of phenotypic plasticity on fitness and reproductive investment

We did not find any significant nonlinear selection gradient between traits phenotypic plasticity and fitness components while significant linear selective gradients were found in both directions, positive and negative. Within the floral module, we found that all traits were negatively correlated with fitness across all the ploidies (Table 4.3). In diploides, we only found negative relationships among phenotypic traits and fitness. We did not find any significant effect of the reproductive module while negative significant influence on fitness occurred in traits from the vegetative module besides the floral traits. The only fitness component that was significantly affected was the seed germination (Table 4.3). In contrast, tetraploid individuals showed several positive relationships among traits plasticity and fitness. All reproductive and vegetative traits but the stalk diameter had a positive effect on at least one fitness component (Table 4.3). Finally, the number of significant relationships among trait plasticity and fitness decreased in hexaploids, with only two significant relationships found in stalk number and pollen amount per flower, both positive. In the case of stalk number plasticity, it affected all the tested fitness components (Table 4.3).

Fitness / **RDPI traits**	2x					4x					6x				
	Pollen	Ovules	Seeds	Germ	Surv	Pollen	Ovules	Seeds	Germ	Surv	Pollen	Ovules	Seeds	Germ	Surv
Petal length	-0.68	0.41	-1.25	0.34	0.26	2.71	0.95	2.75	0.31	-0.20	-1.58	-1.87	-1.34	-0.33	-0.31
Corolla diameter	-0.60	-5.32	-1.68	0.60	-0.35	0.78	0.12	0.60	-0.05	0.10	-0.35	-0.42	0.18	-0.01	**-0.63***
Corolla tube length	1.48	1.61	1.06	**-0.91 ****	0.64	1.11	-1.06	1.16	0.36	-0.11	-0.19	-1.02	-0.22	-0.14	-0.44
Long stamen	2.07	0.81	2.76	-0.49	-0.03	**-4.16***	-3.99	**-4.42***	0.18	-0.36	-2.61	**-3.92***	-2.38	-0.48	-0.46
Short stamen	0.33	2.16	0.27	**-0.88 ****	0.08	-2.49	**-3.77***	-2.53	**0.50***	-0.35	-1.51	-1.97	-1.31	-0.22	-0.23
Stigma height	2.58	-1.09	0.76	0.11	0.05	-2.43	-3.42	-2.52	0.27	-0.19	-2.36	**-2.63***	-1.70	-0.39	-0.32
Herkogamy	0.16	-0.38	-0.07	-0.51	0.42	-0.51	-0.37	-0.57	-0.05	0.39	-0.39	**-1.06 ****	-0.25	**-0.62***	-0.08
Plant height	0.38	-0.03	0.19	-0.88	0.85	1.29*	1.11*	1.33*	0.20	0.39	-0.32	-0.01	-0.42	-0.12	-0.02
No. stalk	0.38	1.64	0.33	**-0.97 ****	0.42	**0.83 *****	**1.06 ******	**0.78 ****	0.02	**0.70 ****	**0.99 ****	**0.74***	**1.00 ****	**0.61***	**0.58***
Stalk diameter	0.61	2.86	0.18	-0.88*	0.36	1.11	0.16	1.16	0.10	-0.39	0.71	0.25	0.73	0.28	-0.11
No. flowers	0.34	-0.16	0.47	-0.93*	0.72	**1.18***	**1.41 ****	**1.14***	-0.31	**0.65 ****	0.60	-0.09	0.48	0.15	-0.40
Pollen/flower	0.03	-0.81	-0.06	-0.03	0.66	**0.67***	**0.75 ****	0.70	0.03	0.17	0.78	0.70	0.70	**0.54 ***	0.22
Ovules/flower	0.52	-1.78	0.68	-0.33	0.92	1.12	**1.53***	-0.54	-0.03	0.16	-0.68	0.09	-0.54	-0.15	-0.15

Table 4.3. Selective gradients on trait phenotypic plasticity. Linear correlations (β) between phenotypic plasticity of traits measured on *E. incanum* and different components of plant fitness: total number of pollen grains per plant (Pollen), total number of ovules per plant (Ovules), total number of seeds (Seeds), germination of seeds produced per family (Germ) and survival of the offspring till flowering (Surv). Significant values are shown in bold. Red and blue colors indicate negative and positive correlations, respectively, and the color intensity indicates the level of significance. Nonlinear selection gradients (γ) are not shown since they were not significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

In summary, phenotypic plasticity exhibited by vegetative and reproductive traits had a positive effect on fitness in polyploid levels, while more plastic traits seemed to negatively affect fitness in diploids. Likewise, plasticity shown by floral traits were penalized in all ploidies.

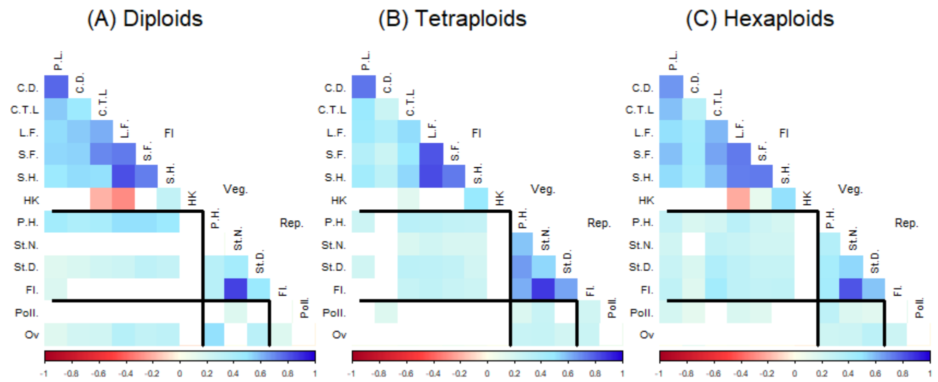

Figure 4.3. Pairwise correlations among traits. Gradient color indicates the sign of the correlation from negative (red) to positive (blue) and the color intensity refers to the significance level. Abbreviations refer to the traits (floral module: P.L. = Petal Length, C.D. = Corolla Diameter, C.T.L. = Corolla tube length, L.F. = Long Filament, S.F. = Short Filament, S.H. = Stigma height, HK = Herkogamy. Vegetative module: P.H. = Plant height, St. N. = Stalk Number, St. D = Stalk Diameter, FL. = Flowers Number. Reproductive module: Poll. = Pollen grains per flower, Ov. = Ovules per flower).

Phenotypic correlations

We found strong correlations between pairs of traits composing the floral module, which are maintained across all the ploidies (Figure 4.3). Likewise, vegetative traits maintained a high correlation in all ploidies (Figure 4.3). Reproductive traits consistently showed a lack of correlation. The number of correlations between vegetative and floral traits increases with ploidy (Figure 4.3B and 4.3C). In diploids, pollen amount only was correlated with a vegetative trait while the ovule production was linked to all floral traits and almost all vegetative traits (Figure 4.3A). In higher ploidies, ovules were correlated only with vegetative traits but not with the floral module (Figure 4.3A and 4.3B). In contrast, pollen production was correlated to corolla size and some vegetative traits in tetraploids (Figure 4.3B) while its correlations with floral traits increased in hexaploids (Figure 4.3C).

DISCUSSION

The benefits and costs of phenotypic plasticity have gained interest in recent years because of the ecological and evolutionary consequences of phenotypic plasticity. However, the benefits and costs of plasticity on fitness are not easy to demonstrate (Dewitt et al., 1998). After proving the existence of variability, we assume that phenotypic plasticity could be under natural selection when it is significantly affecting fitness components and, moreover, it is heritable. An adaptive scenario would be considered when this relationship between phenotypic plasticity and fitness was positive while a maladaptive phenotypic plasticity would occur when this relationship was detrimental. The neutral scenario would occur in absence of significant relationships between phenotypic plasticity and fitness (Figure 4.1). In addition, our study of how phenotypic plasticity can be influenced by three different levels of ploidy highlights the effect of the number of genome copies on the ability of changing the phenotype.

In this study we grouped plant traits in three categories according to their function in shaping the flower (floral module), the vegetative body of the plant (vegetative module) or the resource allocation to sexual function within the flower (reproductive module). The low values of phenotypic plasticity found in the floral module imply that these traits are more canalized, that is, they are less sensitive to the environment. A stronger canalization of floral traits compared to vegetative parts could be explained by their different developmental context (Mal and Lovett-Doust, 2005). It means that floral and vegetative traits differ in the time they were exposed to the environment. Vegetative parts of the plants are mainly all their life cycle accumulated from the effect of environmental influence while flowers show a short life compared to the rest of the plant body (Ledyard Stebbins, 1950; Bradshaw, 1965). Within the reproductive module, the pollen amount per flower was more plastic than ovules, suggesting that it is more sensitive to the environment (Srinivasan et al., 1999; Jiang et al., 2019). In addition, plasticity not only in the amount of pollen but also in its performance and composition has been previously described (Casper and Niesenbaum, 1993; Marshall et al., 2010; Zimmermann et al., 2017). Plasticity in ovules has been also identified in hermaphroditic flowers (Bishop et al., 2010) although it seems to be less extended. This fact could be influenced by the higher costs associated with female reproductive investment in terms of resource allocation compared to male effort (Willson, 1979).

Apart from the trait module, we tested the ploidy effect on phenotypic plasticity because our study system exhibits three ploidy levels which is not common in most study systems. Diploid populations of *E. incanum* are often found in more benevolent environments compared to the habitat of hexaploids, which are also more isolated. For this reason, we could expect that their polyploid condition allowed them to face environmental adversities as it was found in other Brassicaceae species (del Pozo and Ramirez-Parra, 2015). However, contrary to these predictions, we did not find significant differences on phenotypic plasticity values between ploidy levels (Figure 4.2). Therefore, the increase in genome copies is not affecting the phenotypic plasticity in this study system, which is also supported by other studies (Sánchez Vilas and Pannell, 2017; Wei et al., 2019; Harms et al., 2021).

To evaluate the adaptive potential of those traits showing phenotypic plasticity, we had to obtain the heritability of this phenotypic plasticity. Even though heritability is essential to infer the evolutionary potential of phenotypic plasticity, poor attention has been paid to methods to estimate it (but see (Scheiner and Lyman, 1989). Still, heritability of phenotypic plasticity is expected when it is found affecting fitness although not always it is proved (Delph et al., 1997; Delph, 2003). However, recent studies on phenotypic plasticity started to evaluate it and found significant levels of heritability in plastic response (Nussey et al., 2005; Zeng et al., 2017; Goldstein and Ehrenreich, 2021). Our results suggest that higher ploidies showed a greater heritability on plastic traits as previously shown by genetics of quantitative traits (Kopp and Matuszewski, 2014). However, one of the few studies using several ploidies revealed that heritability was more dependent on the growth environment than the ploidy level (Zlonis and Etterson, 2019).

It is worth noting that the trait module was the most influential factor on phenotypic plasticity and its heritability, which highlights the important aspect of modularity in plants (de Kroon et al., 2005). Trait pairwise correlations suggest that the traits involved in the floral module development are highly correlated between them. Vegetative traits are also correlated although this correlation appears to be stronger in polyploids. On the other hand, reproductive traits do not seem to be correlated between them, contrary to the expected trade-off in sex allocation hypothesis (Cruden, 1977). However, the phenotypic plasticity that we found in these two traits are so different from the other modules (Figure 4.2) that we chose to maintain them grouped as an independent module. Polyploidy effect on phenotypic correlations points to significant alterations in trait evolution (Baker et al., 2017). The evolution of plastic traits, thus, could lead to a change in the evolutionary trajectories of the traits highly correlated. In our study system, the even more frequent correlation between pollen and other traits in polyploids suggest that phenotypic changes on other modules (such as floral traits) could be promoted by changes in the number of pollen grains (which in addition shows the highest plasticity) in polyploids. Surprisingly, the correlation of the number of ovules with the rest of traits is absent in polyploid despite female function must be the key factor in selfing species (Cruden, 1977). This is consistent with the tendency to outcrossing observed in polyploid *E. incanum* individuals (***Chapter 2***).

We highlight that most traits showing heritable plastic responses are positively correlated with both fitness and reproductive investment, indicating a possible adaptive value of plasticity. However, there are some floral traits whose plasticity was heritable but negatively correlated with fitness. This could be due to the risks of departure of the trait value from its optimal value for reproductive success. In this sense, a mismatch in traits strongly related to pollination success could be more penalized by natural selection. Since the diversity of reproductive strategies in plants, importance of each trait for fitness can be relative among species. In fact, high values of phenotypic plasticity in floral traits are often expected in species showing a highly dependent on pollinators (Dai et al., 2017). However, Pélabon et al. (2011) found that traits related to pollinator attractiveness were less plastic than vegetative traits because a mismatch between floral traits and pollinators preferences would imply a failure in reproductive success. Here, the negative effect of plasticity shown by floral traits on fitness supports this state taking into account the autogamy exhibited by *E. incanum*. We have grouped traits related to floral size and reproductive structures as the herkogamy, which is quite relevant for self-pollination and, thus, the reproductive organs must be well coupled to ensure fertilization (Brys et al., 2013; Toräng et al., 2017). In this case, we would thus talk about natural selection on canalization of floral traits instead of phenotypic plasticity (Svensson et al., 2020).

Higher genomic diversity and metabolic diversity related to polyploidy could be buffering the costs associated with phenotypic plasticity or even could be masked by advantages of the plastic responses of other traits. In fact, recent studies suggested a possible trade-off between costs of plasticity in some traits and its benefits in other traits (Cotado and Munné-Bosch, 2020). In our case, most of the heritable plastic responses seem to have an advantage on fitness as ploidy level increases, so any or both of these explanations might apply. In addition, plasticity in reproductive investment could play an important role, in turn, on the plasticity of the reproductive strategy due to the strong relationship between sex allocation and mating system (Cruden, 1977; Schemske, 1978; Santos-Del-Blanco et al., 2013). Even, the adjustment of resource allocation to male or female function according to environmental changes could be shaping the wide existence of mixed mating systems in the wild (Dai et al., 2017).

Evolution of phenotypic plasticity is proposed as a mechanism used by organisms to buffer the adversity of changing environmental conditions. Plastic responses are expected in functional vegetative and reproductive traits in order to compensate for any reduced individual performance. In the plant species complex studied here, phenotypic plasticity in the number of stalks, plant size and number of flowers is mainly selected because indirectly these traits could be expected to have a positive effect on reproductive success, when they suppose a greater amount of fruits and seeds. Although this has been intuitive in trees, such correlation traditionally has been observed in herbaceous plants as well (Solbrig, 1981). Our study highlights that the adaptive value of phenotypic plasticity depends on the traits module and provides a proof that it can be selected as a trait itself in the cases that plastic traits are heritable and have a significant effect on fitness. However, the genetic correlations that intrinsically exist among traits can shape the evolutionary extent of plastic responses. So further experiments are needed to better understand how the interplay of phenotypic plasticity and phenotypic integration among traits are affecting the natural selection outcomes on plastic responses, which will increase our understanding of the ecological and evolutionary role of phenotypic plasticity in the wild.

CONCLUSIONS

The results obtained in this study demonstrated that phenotypic plasticity varied depending on the functional trait module. Reproductive module was the most plastic followed by vegetative module while floral traits were the most canalized. The same pattern is maintained across the three ploidy levels explored. We demonstrated the existence of heritable variation of phenotypic plasticity which seems to increase with the ploidy. Phenotypic plasticity in reproductive and vegetative traits showed a positive effect on fitness and male and female reproductive investment, so they can be favored by natural selection, with a major force of natural selection in the male function. Conversely, plastic floral traits exhibit a negative effect on reproductive success, and they may be penalized by natural selection. This pattern is more evident in tetraploids. Interaction among traits from different modules suggest that changes in more plastic traits could influence changes in other traits. In specific, the number of pollen grains and its plasticity seem to be the drivers of change on the rest of measured traits in polyploids while the effect of the number of ovules on other traits lost strength in higher ploidies, an unexpected result in a selfing species. Overall, our results highlight that phenotypic plasticity can be considered as a trait exposed to natural selection and, thus, able to evolve.

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AUTHOR CONTRIBUTIONS

AGM, CF, AJMP, MB and MA thought and designed the experiments. AGM and CF conducted the greenhouse experiments. AGM, AJMP and MA made the statistical analyses and designed the tables and figures. AGM wrote the first draft of this manuscript and all the rest of authors made significant contributions to the draft. AJMP, MB and MA got the funds to develop this study. AJMP and MA supervised all the study.

DATA AVAILABILITY STATEMENT

Data is stored in XXX for review purposes, and will make it available in a public repository upon acceptance.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the following pages.

Appendix S4.1:

Table S4.1. Values of RDPI of each trait per ploidy. Significant values are shown in bold accompanied by 95% interval confidence estimated by bootstrapping.

Module	Trait	2x		4x		6x	
		RDPI	95% C.I.	RDPI	95% C.I.	RDPI	95% C.I.
Floral	Petal	**0.146**	[0.143-0.150]	**0.119**	[0.117-0.120]	**0.140**	[0.139-0.141]
	Corolla diameter	**0.098**	[0.094-0.101]	**0.094**	[0.093-0.096]	**0.105**	[0.104-0.107]
	Corolla tube lenght	**0.099**	[0.096-0.101]	**0.118**	[0.117-0.119]	**0.186**	[0.184-0.189]
	Long stamen	**0.074**	[0.054-0.094]	**0.073**	[0.058-0.088]	**0.098**	[0.082-0.114]
	Short stamen	**0.109**	[0.092-0.126]	**0.104**	[0.092-0.117]	**0.151**	[0.137-0.164]
	Stigma height	**0.097**	[0.082-0.112]	**0.102**	[0.091-0.113]	**0.120**	[0.108-0.132]
	Herkogamy	**0.286**	[0.272-0.299]	**0.279**	[0.270-0.289]	**0.366**	[0.355-0.377]
Vegetative	Plant height	**0.441**	[0.429-0.453]	**0.213**	[0.204-0.222]	**0.245**	[0.236-0.255]
	No. stalk	**0.381**	[0.370-0.393]	**0.357**	[0.349-0.365]	**0.374**	[0.365-0.383]
	Stalk diameter	**0.308**	[0.297-0.318]	**0.301**	[0.294-0.309]	**0.302**	[0.293-0.310]
	No. flowers	**0.392**	[0.382-0.402]	**0.390**	[0.383-0.340]	**0.483**	[0.475-0.491]
Reproductive	No. pollen grains/flower	**0.523**	[0.514-0.533]	**0.471**	[0.464-0.478]	**0.526**	[0.518-0.534]
	No. ovules/flower	**0.331**	[0.322-0.340]	**0.187**	[0.180-0.193]	**0.184**	[0.177-0.192]

Chapter 5

Phenotypic integration does constrain phenotypic plasticity driven by natural selection and heritability

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ABSTRACT

Phenotypic plasticity is the ability of a given genotype to express different phenotypes in response to environmental changes. The environment can also affect complex correlation patterns among traits, shaping the phenotypic integration. The relationship existing between phenotypic plasticity and phenotypic integration has been described in several study systems but the mechanistic underlying this link is unknown. Here, we evaluated how phenotypic plasticity affects the phenotypic integration exhibited by traits under two different environments and across three different ploidy levels. These traits were grouped into floral, vegetative and reproductive modules and their heritability and the heritability of their plasticity were estimated. We analyzed the indirect selection acting on correlated traits and we found that it was more intense than direct selection on phenotypic plasticity. This finding together with the stronger heritability shown by traits themselves compared to their plasticity suggest that natural selection and the heritable genetic component are the mechanisms responsible for the constraining effect that phenotypic integration has on plasticity. The main contribution of this work is a first empirical demonstration of the underlying mechanisms driving the constraining effect of phenotypic integration on plasticity.

Key words Correlated selection, *Erysimum*, functional modules, genetic correlation, heritability, indirect selection, modularity, ploidy, selection response

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INTRODUCTION

Phenotypic plasticity is the ability of the same genotype to produce different phenotypes in different environments (Bradshaw, 1965; Pigliucci et al., 2006), while phenotypic integration refers to the pattern and amount of correlations that are established among the traits of an organism (Pigliucci, 2003). How phenotypic plasticity and integration affect each other is a question that has been debated for decades (Schlichting, 1989; Gianoli, 2001; Gianoli and Palacio-López, 2009). This relationship is of special interest when it comes to understanding the evolution of organisms, their ability to diversify, as well as their ability to deal with new or changing environments. The functional trade-off between phenotypic integration and plasticity has been accepted for years (Zimmermann et al., 2016; Vázquez et al., 2017) and even explored in different environments (Matesanz et al., 2021). However, the biological mechanisms underlying this trade-off and the causal relationship between phenotypic integration and plasticity have never been previously demonstrated.

It has been postulated that the relationship between phenotypic plasticity and phenotypic integration can occur in three ways. First, the relationship can be positive, so that characters whose plasticity is greater are, in turn, more integrated. In this case, plasticity could be a modular character, and the phenotypic changes affect the entire module whose integration would be increased from one environment to another. This would mean that the integration would somehow have some plasticity. Second, the relationship between plasticity and integration could be negative, so that increasing the plasticity of a character would reduce its integration. This could occur because the plasticity of one character is independent or significantly different to the other traits, so that they would lose their correlation when they are subjected to disparate environments. Third, it could also happen because a high integration between characters constrains the plastic capacity of some traits when they are subjected to different environments.

Despite the fact that the relationship between plasticity and phenotypic integration has been repeatedly demonstrated (Gianoli and Palacio-López, 2009; Matesanz et al., 2021), the mechanisms underlying this relationship have hardly been theoretically explored (Via et al., 1995; Ghalambor et al., 2007; Auld et al., 2010). In this sense, natural selection must play an important role modulating this relationship. Natural selection has been shown to shape adaptive trajectories of phenotypic plasticity in different organisms (Van Buskirk and Steiner, 2009). It has also been shown that it can modulate the interaction between different phenotypic characters, maintaining genetic correlation (Lande, 1984). But its effect on the causal relationship between plasticity and phenotypic integration had never been empirically demonstrated and quantified. Recent works exploring the relationship between phenotypic plasticity and integration call our attention to the importance of understanding the mechanisms that govern it (Matesanz et al., 2021).

Evolution is expected to occur when selection acts on a trait that has a heritable basis of phenotypic variation (Wilson et al., 2006). The quantification of this selection on characters of organisms in nature was already exposed by Lande and Arnold (1983) in their already classic work. However, natural selection acts simultaneously on different morphological and physiological traits (Relyea, 2001; Ghalambor et al., 2003) transforming the adaptation in a multivariate process (Lande, 1979; Blows, 2007; Damián et al., 2020). We refer to fitness epistasis when heritable traits are encoded by different sets of loci that interactively affect fitness (Whitlock et al., 1995). Correlational selection occurs when fitness is affected by the interaction among traits (Lande and Arnold, 1983; Phillips and Arnold, 1989). Hence, natural selection is able to alter the correlation among traits and thus shape the genetic structure (Sinervo and Svensson, 2002). These changes have consequences at both genomic and ecological level because they affect the natural selection outcomes on correlated traits. In addition, correlational selection at phenotypic level can occur when traits are heritable built on fitness epistasis at genotypic level, but also without any genetic basis. Hence, correlational selection and fitness epistasis are connected but not the same phenomena (Brodie, 2000; Wolf et al., 2000; Sinervo and Svensson, 2002). García-Muñoz et al. (***Chapter 4***) have already shown that phenotypic plasticity can be considered an adaptive trait in the Brassicaceae species *Erysimum incanum*. Thus, characters related to the size of the plant and the reproductive investment presented an adaptive variability. But the plasticity of the characters related to the flowers were negatively selected. In turn, we can guess in *E. incanum* a modular pattern for the correlation between phenotypic plasticity and fitness (***Chapter 4***).

Patterns resulting from natural selection on a trait not only depends on phenotypic selection but also on the level of heritability of that trait (Endler, 1986; Falconer, 1989). Heritability is influenced by different factors. Scheiner and Lyman (1989) suggested that heritability for phenotypic plasticity will be lower compared to heritability of phenotypic traits. But heritability was also demonstrated to be lower in natural conditions than under controlled conditions (Galloway, 1995) and increasing levels of heritability were found as level of ploidy increases in the salmon *Oncorhynchus tshawytscha* (Johnson et al., 2007). So, for an accurate understanding and quantification of the role of natural selection on the evolution of plant phenotype and the rising interaction it is essential to explore heritability and its effect on evolutionary change. In this sense, the quantification of response to selection (R), a parameter directly related to selection gradient and heritability, could be a useful tool to compare the effects of natural selection on different traits and their interaction.

Ploidy level, or the number of genome copies carried by an organism, may alter both the phenotypic integration and the phenotypic plasticity (Shimizu-Inatsugi et al., 2017; Van de Peer et al., 2017). Integration between phenotypic traits would be altered as ploidy can modify the genetic correlations between different traits (de Bem Oliveira et al., 2019). Changes in ploidy modify the genetic variability and dosage, which also modify the outcome of genotype-environment interaction (Baniaga et al., 2020). However, empirical results do not always support the effect of ploidy variation on phenotypic plasticity changes. In this sense, different authors have demonstrated how ploidy variation can alter the patterns of phenotypic plasticity (Shimizu-Inatsugi et al., 2017; Van de Peer et al., 2017), but some others just did not find this relationship between ploidy variation and plastic responses (Sánchez Vilas and Pannell, 2017; Wei et al., 2019). The effect of the ploidy variation on the relationship between phenotypic integration and plasticity has been scarcely explored, as neither were the mechanisms underlying this relationship.

Erysimum incanum is a monocarpic annual self-compatible species complex in the Brassicaceae family. The species in the complex differ in their ploidy level (including diploids, tetraploids and hexaploids; Faverger et al., 1979; Galland, 1988; Luque and Lifante, 1991; ***Chapter 1***), but they exhibit identical self-pollination mechanisms (Abdelaziz et al., 2019). Using this species complex García-Muñoz et al. (***Chapter 4***) have already demonstrated the significant patterns of phenotypic plasticities exhibited by the different ploidies. Levels of adaptive phenotypic plasticity were similar across the ploidy levels but significantly different between traits belonging to different plant modules (***Chapter 4***). Using this singular study system the main aims of this study were: (1) to explore the relationship between phenotypic plasticity and phenotypic integration across ploidy levels and environments (Figure 5.1A and 5.1B); (2) to quantify the heritability for phenotypic plasticity and phenotypic traits; (3) to quantify the strength of natural selection on phenotypic plasticity and interaction between traits promoting integration; and (4) to evaluate the response to selection of integrated trait and phenotypic plasticity to discern the constraining effect that phenotypic plasticity and integration have on each other (Figure 5.1C).

MATERIALS AND METHODS

Study system

Erysimum L. is one of the largest genera of Brassicaceae, characterized by recent speciation and polyploidization events resulting in species complexes and cryptic species (Clot, 1991; Abdelaziz et al., 2011, 2014). The genus contains more than 200 species and subspecies inhabiting Eurasia, Central and North America and North Africa with a hot-spot diversification in the Mediterranean basin (Al-Shehbaz et al., 2006; Nieto Feliner, 2014). Within the *E. incanum* complex, we can identify three different ploidy levels (Nieto-Feliner and Clot, 1993; Fennane and Ibn-Tattou, 1999; Abdelaziz et al., 2014). Diploids ($2n = 2x = 16$ chromosomes) and tetraploid populations ($2n = 4x = 32$ chromosomes) show a vicariant distribution in Iberian Peninsula and Morocco (Nieto-Feliner and Clot, 1993; Fennane and Ibn-Tattou, 1999) while hexaploid populations ($2n = 6x = 48$ chromosomes) have been recently found in the southern ranges of Morocco (***Chapter 1***). In addition, we are able to easily obtain pure lines because of the spontaneous self-pollination exhibited by this species, which result in an optimal study system for testing phenotypic plasticity and ploidy effects among clonal individuals.

Experimental design

Prior to the experiments, plants coming from two natural populations per ploidy level were grown under controlled conditions for seed multiplication in the greenhouse facility at University of Granada in order to remove local effects and to obtain inbred lines, ensuring a reduced genetic diversity. Following the experimental set up presented in ***Chapter 4,*** we sowed 16 seeds coming from 10 different mother plants belonging to each population and each ploidy level (3 ploidies x 10 mother plants x 2 populations x 16 seeds). This number was replicated because plants would be subjected to two different treatments, so a total of 1920 seeds were sowed in square plastic pots (11x11x11 cm^3) filled with Gramoflor™ potting soil mixture. All seeds were sown inside the greenhouse until seedlings reached the rosette stage to obtain a similar germination and survival ratio. Then, half of the replicates were subjected to different treatments in order to test phenotypic plasticity. The “indoor” (IN) treatment consisted in growing plants under controlled greenhouse conditions, with 28 °C average temperature, a photoperiod of

14 hours (from 8:00 to 22:00), and watering by irrigation. Plants growing under the “outdoor” (OUT) treatment were subjected to natural and unpredictable conditions in a common garden where only 100 ml of water were added per pot every two days.

Flowering started in February inside the greenhouse while the flowering and a month later outside. Then, we measured a series of traits grouped by functional modules. We measured traits related to the *floral module* using a digital caliper and they were: (1) **petal length**, distance between edge of a petal and the point where petal starts to curve shaping the corolla; (2) **corolla diameter**, distance between edge of a petal and the diametrically opposite one in the flower; (3) **corolla tube length**, distance between sepals basis and the corolla aperture point; (4) **corolla tube width**, difference between corolla diameter and twice the petal length; (5) **long stamen length**, distance between long filament insert point and anther; (6) **short stamen length**, distance between short filament inset point and anther; (7) **style height**, distance between the style basis and stigma; and (8) **herkogamy**, difference between style height and long stamen length. After fruit maturation, we measured the following traits related to the *vegetative module*: (9) **plant height** and (10) **stalk diameter**, as the longitude and diameter of the tallest flowering stalk of the plant, respectively; (11) **number of stalks,** counting the number of flowering stalks; and (12) **number of flowers**, quantifying the total number of flowers in the plant. Finally, we measured the traits relative to the *reproductive module*: (13) **number of pollen grains** and (14) **number of ovules** per flower. Floral traits and pollen amount were estimated in a flower per plant while the number of ovules were the mean of four different fruits per plant. In total, over 730 phenotype plants were obtained for 14 phenotypic traits.

Although fitness presents several components, for sake of clarity in this chapter we focused on the total of seeds produced by each individual (Figure 5.1C). The seed production was measured by multiplying the mean number of seeds in four ripe fruits by the number of fruits produced by the plant. In addition, the overall seed production is an accurate estimation of plant fitness due to the monocarpic condition of *E. incanum*.

Statistical analyses

Phenotypic plasticity

We estimated phenotypic plasticity for every trait by computing the relative distance plasticity index (**RDPI**; Valladares et al., 2006). This index quantifies phenotypic plasticity based on phenotypic distances among genotypes grown in different environments, and ranges from 0 (no plasticity) to 1 (maximum plasticity; see Valladares et al., 2006 for further details). Since our plant families were composed of identical sister seeds (full-sibs), we could associate a RDPI value to each mother plant. We calculated RDPI significance by bootstrapping with 1000 permutations using the package *boot* v. 1.3-28 in R (Canty and Ripley, 2017). The obtained 95% confidence interval showed the family RDPI values significantly different from zero.

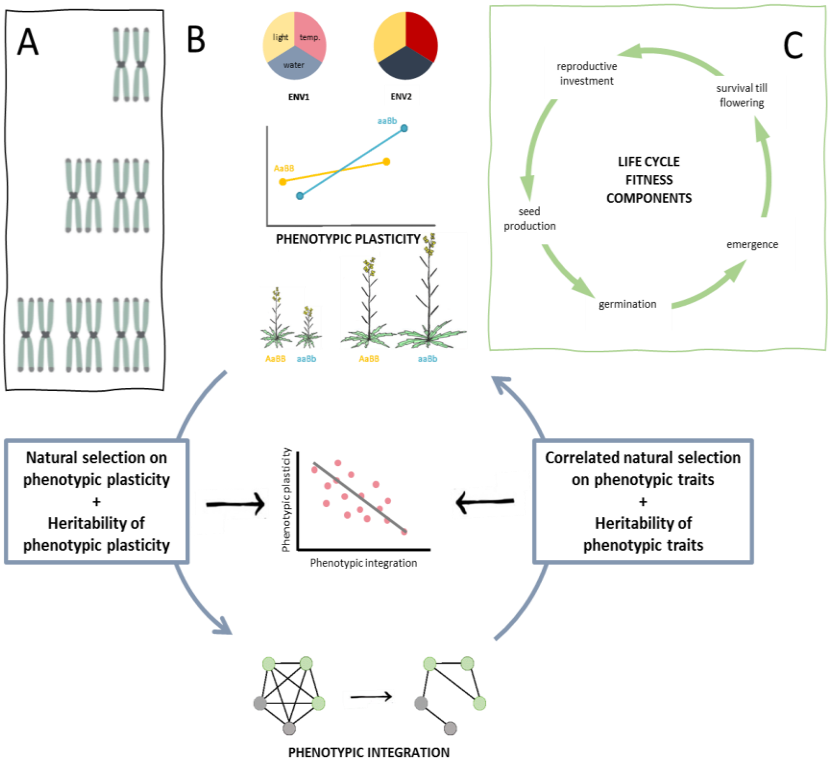

Figure 5.1. Experimental design scheme. (A) The ploidy could have an effect on phenotypic plasticity, which is the ability of a given genotype to alter its phenotype in response to the environment. (B) Environmental changes may also alter the patterns of phenotypic integration, this is, trait covariation. Integration among traits, quantified in this work as the overall number of significant correlations of one trait to all others in a given environment, may limit trait plasticity if the linkage among traits is constraining their trait range of variation. It often results in lower plasticity of highly integrated traits. Significant heritability, variation and a strong relationship between this variation and fitness are mandatory to talk about natural selection acting on a trait of its phenotypic plasticity. (C) Fitness could be estimated using several components but we focused on seed production. Estimating the intensity of natural selection acting on trait interaction or their independent phenotypic plasticity would help us to understand the causal relationship between phenotypic integration and plasticity.

Phenotypic correlation among traits across ploidies and environments

The phenotypic integration of a given trait was estimated as the number of significant correlations ($P<0.05$; Pearson product-moment correlation) with other traits as proposed by Gianoli and Palacio-López (2009) and Matesanz et al.

(2021). Phenotypic integration was obtained for both treatments separately. From these pairwise correlations we obtained a correlation matrix using the ‘corrplot’ package (v.0.92; Wei et al., 2017) in R. The rest of analyses were performed using the *‘stats’* package implemented in R Statistical Software (v4.2.1; Team, 2021).

Relationship between phenotypic plasticity and phenotypic integration

We performed linear regression analyses considering the mean value of phenotypic plasticity as the independent variable and phenotypic integration as the dependent variable. In this way, we correlated the RDPI value of each trait with the number of correlations that this trait established with others. These regressions were calculated for the three ploidies and separately for IN and OUT treatments since traits can be differently correlated in each environment.

Heritability of phenotypic traits and their plasticities

We calculated the heritability of phenotypic traits (h^2) for the three ploidies exhibited in *E. incanum* as:

$$h^2 = \frac{\sigma_f^2}{\sigma_f^2+\sigma_r^2} \qquad \textbf{(5.1)}$$

where σ_f^2 is the variance component due to differences among seed families; and σ_r^2 is the residual variance (Falconer 1989). Since these plants are autogamous, we considered that plant families were composed of full-sib individuals. We calculated heritability significance by bootstrapping with 1000 permutations using the package *boot* v. 1.3-28 in R (Canty and Ripley, 2017). The obtained 95% confidence interval indicated if the heritability values were significantly different from zero.

We estimated the narrow-sense heritability (h^2) of phenotypic plasticity for each measured trait per ploidy level deduced from mixed model ANOVA (Lynch et al., 1998), in which environment was included as fixed factor, and using the formula:

$$h^2 = \frac{\sigma_i^2}{\sigma_f^2+\sigma_i^2+\sigma_r^2} \qquad \textbf{(5.2)}$$

where, σ_i^2 is the variance component of the seed family-by-environment interactions; σ_f^2 is the variance component due to differences among seed families; and σ_r^2 is the residual variance (see Scheiner and Lyman (1989) for further details). As mentioned above, we considered full-sib individuals within each plant family and we estimated heritability significance by bootstrapping with 1000 permutations using the package *boot* v. 1.3-28 in R (Canty and Ripley, 2017). Again, the obtained 95% confidence interval indicated if the heritability values were significantly different from zero.

Selection gradients on phenotypic plasticity

Finally, direction and magnitude of selection for phenotypic plasticity of each trait per ploidy level was estimated by means of selection gradients. This is a multivariate technique that measures the selection acting on each trait plasticity independently of any other trait plasticity value (Lande and Arnold 1983). Here, we explored two types of selective gradients: (1) linear selection gradients, β, computed from the standardized partial-regression coefficients of a linear regression of relativized values of fitness (in this case, seed production) on all character *i* phenotypic plasticities (Figure 5.2); and (2) direct nonlinear selection gradients for each trait *i* phenotypic plasticity, γ_{ii}, which was computed from the second-order standardized coefficient in quadratic regression of relativized values of each fitness component on the trait *i* phenotypic plasticity (Figure 5.2; Lande and Arnold 1983). The multivariate models of selection gradients were built introducing as independent variables the standardized values of phenotypic plasticity per family (RDPI) and as dependent variables the relative estimate of the fitness (seed production).

Correlational selection on traits

Correlational selection gradients for traits *i* and *j*, termed as γ_{ij}, were computed using standardized coefficient in a quadratic regression of relative fitness on the product of *i* and *j* character value, describing the selection acting on the correlation between both *i* and *j* character (Lande & Arnold, 1983). As done before, the multivariate models of selection gradients were built introducing as independent variables the interaction between standardized values of original phenotypic trait per plant and as dependent variables the relative estimate of the fitness, also per plant which was the seed production in this case.

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1983). As done before, the multivariate models of selection gradients were built introducing as independent variables the interaction between standardized values of original phenotypic trait per plant and as dependent variables the relative estimate of the fitness, also per plant which was the seed production in this case.

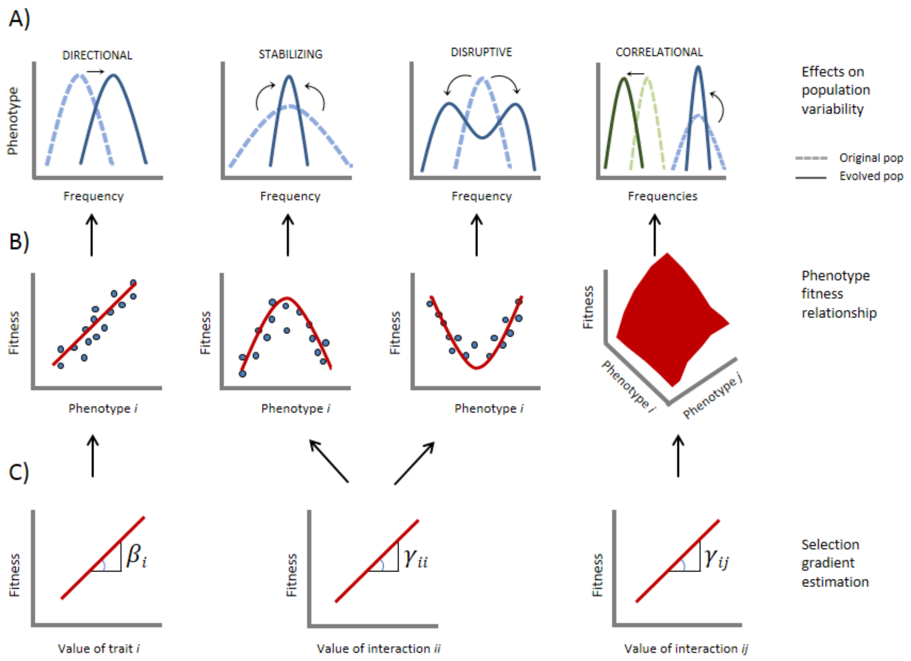

Figure 5.2. A) Outcomes of natural selection effects on distribution of population phenotypic variability. Original distribution of populations are showed in discontinuous lines, while evolved populations after selection action is showed with continuous lines. B) Functions fitting the relationship between phenotypic trait *i* and fitness values. C) Estimation of selection gradients using standardized phenotypic values of trait *i* and normalized fitness in case of directional selection and the interaction between the standardized values of traits *ii* or *ij* in case of quadratic and indirect selection, respectively. The arrows show the relationship between panels: estimation of selection gradient (C) informs us about how the natural selection is acting on a trait or group of traits (B) and this produce changes in the distribution and structure of the trait/s value/s in the population (A).

Correlational selection on traits

Correlational selection gradients for traits *i* and *j*, termed as γ_{ij}, were computed using standardized coefficient in a quadratic regression of relative fitness on the product of *i* and *j* character value, describing the selection acting on the correlation between both *i* and *j* character (Lande & Arnold, 1983). As done before, the multivariate models of selection gradients were built introducing as independent variables the interaction between standardized values of original phenotypic trait per plant and as dependent variables the relative estimate of the fitness, also per plant which was the seed production in this case.

Response to natural selection

The selection response (R) is the change produced by selection on the population mean in the offspring. The response to selection for a single trait is related to the heritability of the trait and the selection differential (S), which is the difference between the mean phenotypic value of the individuals selected as parents and the mean phenotypic value of all the individuals in the parental generation before selection (Falconer, 1989). Response to selection for a single trait is calculated as:

$$R = h^2 S \qquad \textbf{(5.3)}$$

For estimating the selection response, we can also use the selection gradients (β) and variance of the trait *i* (σ_i^2), following the equation:

$$R = \sigma_i^2 \beta \qquad \textbf{(5.4)}$$

We used this equation to estimate the response to selection of phenotypic plasticity considering only those traits exhibiting significant heritability and significant selection gradients acting on their phenotypic plasticity. However, the response to natural selection can be affected by the interaction between traits. In this sense, the selection response is affected by the selection acting directly on the trait *i*, but also the indirect selection acting on trait *i* by its correlation with trait *j* (Lande, 1979; Lande & Arnold, 1983). For correlated selection on different traits, Lande and Arnold (1983) generalized a matrix method to predict the change of a given trait considering its correlation with the rest of traits and fitness as:

$$R = Gb \qquad \textbf{(5.5)}$$

where *G* is the variance-covariance matrix among traits and *b* is the selective gradients vector (Rúa 2017). This equation illustrates how genetic correlations among traits can be limiting the response of a given trait to respond to natural selection (Kirkpatrick 2009). We used a modification of the equation 5.5, transforming the G matrix so that the values of the diagonal of variances were converted into zeroes. In this way, we estimated the selection response using only the correlational response but not the direct response of the trait to the natural selection. The response to selection was calculated considering only those traits that showed significant heritability and significant correlated selection gradients.

RESULTS

Phenotypic correlation among traits across ploidies and environments

We found a strong correlation among traits related to the flower across the three ploidies and treatments (Figure 5.3). However, these correlations seem to be weaker in hexaploids. Interesently, herkogamy appears more correlated with the rest of traits as ploidy increases. In tetraploids, herkogamy is negatively correlated with the male reproductive investment under the OUT treatment, suggesting that flowers with a higher separation between sexual organs are producing more pollen. In fact, the pollen production is correlated with other traits in polyploids while the ovule amount showed the contrary pattern. Female reproductive investment is correlated with most traits in diploids but it lost correlations in tetraploid and hexaploids. In these ploidies, ovule amount is correlated with traits related to the plant body instead of flower. A correlation between pollen and ovules has not been found except a light positive correlation in tetraploids under OUT conditions, suggesting that a trade-off in reproductive investment is not occurring in these plants (Figure 5.3).

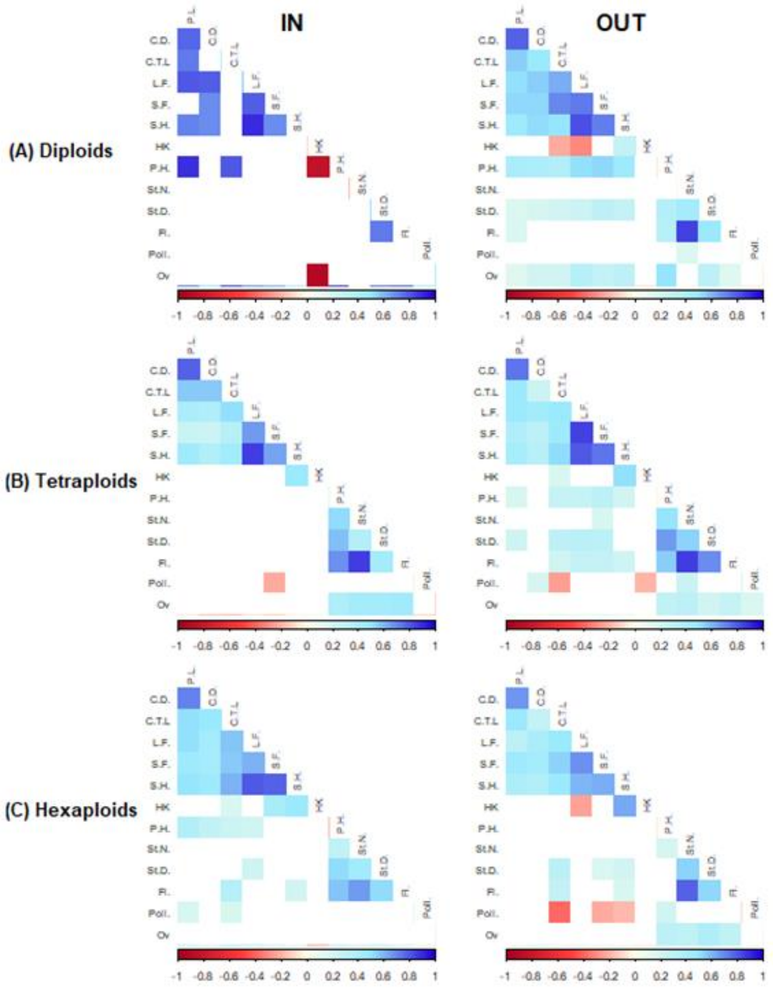

Figure 5.3. Pairwise correlations among traits. Gradient color indicates the sign of the correlation from negative (red) to positive (blue) and the color intensity refers to the significance level. Abbreviations refer to the traits (P.L. = Petal Length, C.D. = Corolla Diameter, C.T.L. = Corolla tube length, L.F. = Long Filament, S.F. = Short Filament, S.H. = Stigma height, HK = Herkogamy, P.H. = Plant height, St. N. = Stalk Number, St. D = Stalk Diameter, FL. = Flowers Number, Poll. = Pollen grains per flower, Ov. = Ovules per flower).

Relationship between phenotypic plasticity and integration

We obtained RDPI values as estimations of phenotypic plasticity, which were similar across ploidies (Table 5.1). In addition, these values were lower for traits from the floral module and increased within the vegetative and reproductive module (Table 5.1). The phenotypic integration of a trait was estimated as the number of traits with which were correlated for both types of treatments. Diploids showed the greater differences in phenotypic integration between IN and OUT treatment, with more integrated traits in plants subjected to the OUT treatment. Integration was similar between both treatments in tetraploids and quite different in hexaploids, where traits tended to be more integrated under the IN treatment, following a pattern just contrary to diploids (Table 5.1). For the three ploidies, floral traits showed the highest integration because of the modularity, due to the fact that we measured a higher number of floral traits compared to traits belonging to the other two modules (Table 5.1).

We found a strong negative relationship between phenotypic plasticity and phenotypic integration. More plastic traits were correlated with less traits showing, thus, a lower degree of phenotypic integration. Therefore, strongly integrated traits exhibited low values of phenotypic plasticity (RDPI). This same pattern was found across the three ploidies (Figure 5.4).

Mod.	Trait	2x				4x				6x			
		PP		PI		PP		PI		PP		PI	
		RDPI	95% C.I.	In	Out	RDPI	95% C.I.	In	Out	RDPI	95% C.I.	In	Out
Flor.	Petal length	**0.146**	[0.143-0.150]	5	8	**0.119**	[0.117-0.120]	5	7	**0.140**	[0.139-0.141]	7	6
	Corolla diameter	**0.098**	[0.094-0.101]	4	7	**0.094**	[0.093-0.096]	5	6	**0.105**	[0.104-0.107]	6	5
	Corolla tube length	**0.099**	[0.096-0.101]	2	8	**0.118**	[0.117-0.119]	5	10	**0.186**	[0.184-0.189]	9	8
	Long stamen	**0.074**	[0.054-0.094]	4	8	**0.073**	[0.058-0.088]	5	8	**0.098**	[0.082-0.114]	7	6
	Short stamen	**0.109**	[0.092-0.126]	3	7	**0.104**	[0.092-0.117]	6	9	**0.151**	[0.137-0.164]	6	7
	Stigma height	**0.097**	[0.082-0.112]	4	8	**0.102**	[0.091-0.113]	6	8	**0.120**	[0.108-0.132]	7	9
	Herkogamy	**0.286**	[0.272-0.299]	2	3	**0.279**	[0.270-0.289]	1	3	**0.366**	[0.355-0.377]	3	2
Veg.	Plant height	**0.441**	[0.429-0.453]	3	8	**0.213**	[0.204-0.222]	4	7	**0.245**	[0.236-0.255]	7	2
	No. stalk	**0.381**	[0.370-0.393]	0	3	**0.357**	[0.349-0.365]	4	5	**0.374**	[0.365-0.383]	3	3
	Stalk diameter	**0.308**	[0.297-0.318]	1	10	**0.301**	[0.294-0.309]	4	7	**0.302**	[0.293-0.310]	4	5
	No. flowers	**0.392**	[0.382-0.402]	1	4	**0.390**	[0.383-0.340]	4	7	**0.483**	[0.475-0.491]	5	4
Rep.	No. pollen grains/flower	**0.523**	[0.514-0.533]	0	1	**0.471**	[0.464-0.478]	1	4	**0.526**	[0.518-0.534]	2	4
	No. ovules/flower	**0.331**	[0.322-0.340]	1	1	**0.187**	[0.180-0.193]	4	0	**0.184**	[0.177-0.192]	0	1

Table 5.1. Phenotypic plasticity (PP) shown as RDPI mean values per ploidy and trait accompanied by their 95% confidence interval estimated by bootstrapping. Phenotypic integration (PI) was calculated quantifying the number of traits correlated significantly with a single trait at each environment (IN and OUT treatment). Traits are grouped by floral vegetative and reproductive modules.

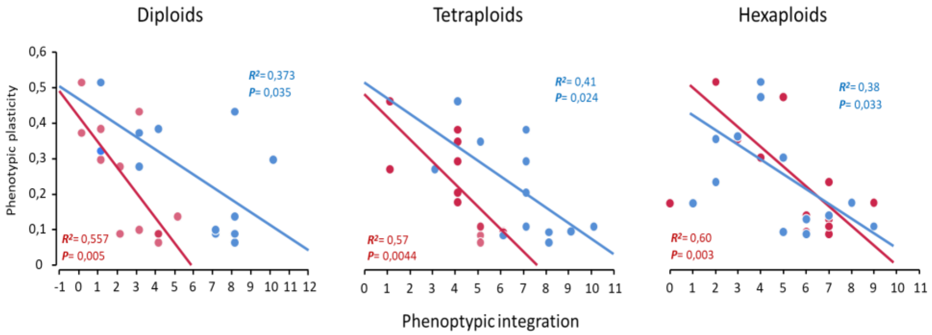

Figure 5.4. Regression of phenotypic plasticity on phenotypic integration for each ploidy level. Blue and red lines indicate IN and OUT treatment, respectively.

Heritability of phenotypic traits and their plasticities

The amount of heritable traits was different between treatments. Under IN treatment, a maximum of four traits showed a significant heritability in hexaploids. (Table S5.1; Appendix 1). Indeed, diploids and tetraploids only showed two heritable traits. We also found that heritability values were higher as ploidy increased (Table S5.1; Appendix 5.1). However, we found a greater amount of heritable traits under OUT treatment in the three ploidies (Table S5.2; Appendix 5.1). In addition, all these heritability values were similar among ploidies and higher than in the other treatment (Table S5.2; Appendix 5.1).

On the other hand, we calculated the heritability of phenotypic plasticity of each trait in each ploidy level. We only found two heritable plastic traits in diploids while most of the traits showed heritable plasticity in polyploids (Table S5.3; Appendix 5.2). In fact, all the traits except the ovule amount and the herkogamy exhibited significant heritabilities for their phenotypic plasticity in hexaploids

(Table S5.3; Appendix 5.2). Heritability values were also higher in polyploids (Table S5.3; Appendix 5.2).

When we compared the strength of the heritability exhibited by traits and the heritability exhibited by phenotypic plasticity, we found that traits showed higher heritability values than their plasticities (Figure 5.5).

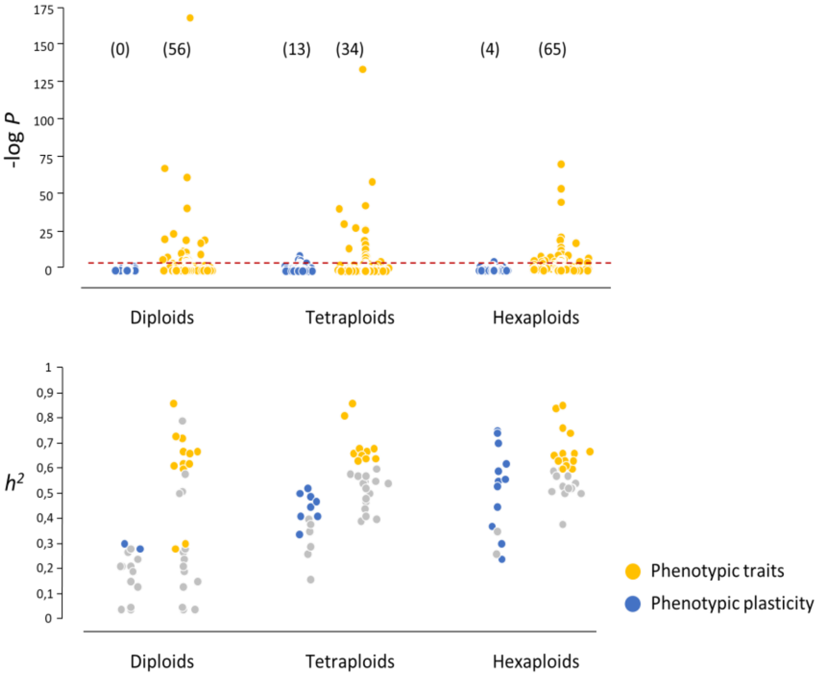

Figure 5.5. Values of –log of the significance associated to selective gradients on phenotypic plasticity (blue) and trait interaction (yellow). Red discontinuous lines indicate the significance threshold -log (0.05). Below, the heritability of phenotypic traits in yellow and heritability of the phenotypic plasticity of traits blue. Gray points represent non-significant heritability values.

Selection gradients on phenotypic traits and their plasticities

Several trait pairs exhibited significant selective gradients across ploidies. Most of these correlational selective gradients were between traits belonging to the same functional module. For example, vegetative and floral traits exhibited strong selective gradients with other traits in the same module in all the ploidies and across the environments. However, also significant correlational selective values were found for traits belonging to different modules (Table S5.5-S5.10; Appendix 5.4).

We did not find any significant nonlinear selection gradient between traits phenotypic plasticity and fitness while significant linear selective gradients were found in both directions, positive and negative. Within the floral module, we found that all traits were negatively correlated with fitness across all the ploidies (Table S5.4; Appendix 5.3). In diploids, we did not find any significant relationships between phenotypic plasticity and fitness. In tetraploid plants we found a significant and negative relationship between floral plasticity and fitness, while the plasticity of vegetative traits exhibited positive selective gradients (Table S5.4; Appendix 5.3). In hexaploid plants we found only a vegetative trait under significant and positive selection. In summary, phenotypic plasticity exhibited by vegetative and reproductive traits had a positive effect on fitness in polyploid levels.

Response to natural selection

We found a stronger response to selection exhibited by phenotypic integration of the traits compared to the exhibited one by their phenotypic plasticity for the three ploidies (Table 5.2). In addition, the response to selection was weaker under the IN treatment although it was increasing in polyploids within this treatment (Table 5.2).

Mod.	Trait	R in 2x			R in 4x			R in 6x		
		PP	PC		PP	PC		PP	PC	
		RDPI	In	Out	RDPI	In	Out	RDPI	In	Out
Floral	Petal length	0	0,374	0,037****	0	0	0,056**	0	0	0,038
	Corolla diameter	0	0,341	0,203	0	0*	0,233**	0	0	0,213*
	Corolla tube length	0	0,526	0,100***	0	0	0,338	0	0,102	0,438**
	Long stamen	0	0,322	0,107***	-1,85	0	0,080	0	0	0,142**
	Short stamen	0	0,494	0,197****	0	0	0,173	0	0	0,240**
	Stigma height	0	0,092	0,149**	0	0	0,159	0	0	0,249*
	Herkogamy	0	-0,230	0	0	0	0*	0	0	0
Veget.	Plant height	0	0,933	0,102****	0,61	0,093****	0,042****	0	0,151****	0,072****
	No. stalk	0	0,044	0,397	0,33	0,357	0,420*	0,56	0,146	0,345****
	Stalk diameter	0	0,609***	1,127*	0	0,750	0,655**	0	1,049	0,667*
	No. flowers	0	0,179	0,429*	0,44	0,577	0,427	0	0,540	0,330*
Rep.	No. pollen grains/flower	0	1,182	0	0	0	0**	0	0**	0
	No. ovules/flower	0	0,941*	0,084*	0	0,078	0,064*	0	0,058*	0,347**

Table 5.2. Response to selection (R) for phenotypic plasticity (PP) and for phenotypic correlations (PC) in the experimental environments (IN and OUT treatments) across the three different ploidies exhibited by *E. incanum*.

DISCUSSION

The main contribution of this work is a first empirical demonstration of the underlying mechanisms driving the constraining effect of phenotypic integration and plasticity. We found a significant negative relationship between phenotypic plasticity and integration across all the *Erysimum incanum* ploidies and environments where they grow. Significant patterns of heritabilities were found for phenotypic traits and their plasticities across ploidies, being the former more numerous and intense. Also significant values of natural selection were found acting on phenotypic plasticity of some traits in different ploidies, but even more intense and frequent across ploidies were the correlational selection acting on traits and thus promoting phenotypic integration. We decided to use the response to selection to evaluate the causal relationship between phenotypic plasticity and integration. A modification of the covariance matrix G method was done to evaluate the response to correlational selection proposed by Kirkpatrick (2009), in order to compare it with the response to selection of phenotypic plasticity. Higher values of heritability and selection acting on phenotypic integration produce also higher values of response to selection and leads us to conclude that phenotypic integration constrains phenotypic plasticity and not the other way around.

The negative relationship that we found between plasticity and phenotypic integration has been previously described in other organisms such as *Convolvulus chilensis* and *Lippia alba* (Gianoli and Palacio-López, 2009). There, they described how the plasticity of the measured traits affected the number of traits with which each trait was significantly correlated. But the correlation between plasticity and integration is not always negative. For example, Matesanz et al. (2021) described a positive relationship between the values of plasticity and integration of phenotypic characters in *Lepidium subulatum*. Although these two works extensively explored and discussed the relationship between phenotypic plasticity and integration, they did not analyze the mechanism underlying it. In our case, the relationship that we found in *E. incanum* was negative and constant across ploidies and environments. The maintenance of this pattern across the three ploidies suggests that ploidy does not affect the relationship between plasticity and integration and thus the mechanisms underlying it. This fact points to the conserved role of modularity and organs in the function and viability of plants (de Kroon et al., 2005; Damián et al., 2020).

In our system, the number of phenotypic traits with significant heritability were similar between ploidies and environments. However, the number of traits with significant heritabilities varied drastically from one environment to another. In addition, the heritability of plasticity exhibited by the traits showed an increase with ploidy, increasing their number and intensity. Similar patterns of variance and heritability have been described for phenotypic traits in Salmonids associated with artificially increased ploidy (Johnson et al., 2007). Evolution is expected when selection acts on heritable and variable traits (Wilson et al., 2006), being the evolutionary change on a trait directly proportional to the heritability and strength of selection (Rúa, 2017). In our work we found higher heritability values for the phenotypic traits than for their plasticities. This pattern was already predicted by (Scheiner and Lyman, 1989) in their work proposing the method to calculate the heritability of phenotypic plasticity.

Much has been discussed about the adaptive and non-adaptive nature of plasticity to environmental changes, trying to generate conceptual frameworks that predict the importance of phenotypic plasticity in evolutionary change (Via et al., 1995; Ghalambor et al., 2007; Auld et al., 2010). The adaptive character of phenotypic plasticity has been demonstrated in different organisms (Nussey et al., 2005; Svensson et al., 2020). In our system (***Chapter 4***), we describe how the plasticity of some characters is subject to positive selection, but others to negative selection. These differences in selection depend on the functional module of the plant in which the character develops its function. Thus, floral characters present a maladaptive plasticity, while characters associated with the size of the plant or the reproductive investment present values of positive selection and therefore adaptive (***Chapter 4***). In these circumstances, it is inevitable to think about the limiting effect of trait function and modularity on phenotypic plasticity (de Kroon et al., 2005).

The effect of natural selection on several characters simultaneously will generate the appearance of integration of these characters and the modular functioning of different organisms (Damián et al., 2020). In addition to pleiotropic phenomena, natural selection will be one of the most important phenomena in maintaining and even promoting covariance between characters (Lande and Arnold, 1983; Phillips and Arnold, 1989; Sinervo and Svensson, 2002) optimizing its function. In fact, recent studies have shown that this integration can be subject to selection and be adaptive (Damián et al., 2020). In our case, and with a larger sample size, we have shown that the integration of different characters is subject to selection. Such selection together with significant heritability patterns of the interacting traits would generate solid functional modules. The fact that natural selection acts more intensely on the appearance and maintenance of functional modules could explain why phenotypic integration constraints plasticity and not vice versa.

To the best of our knowledge, this is the first work that compares the effects of natural selection on phenotypic plasticity and on the promotion of phenotypic integration. Using matrix techniques of quantitative genetics (Lande and Arnold, 1983; Kirkpatrick, 2009) we estimated the response to selection on the plasticity of traits or their integration and compared them. *Erysimum incanum* showed higher values of response to correlational selection between pairs of characters, both in number and intensity. This means that natural selection is reinforcing phenotypic integration patterns more intensely than it does with phenotypic plasticity. Phenotypic plasticity is related to the response that an organism gives to a changing environment (Bradshaw, 1965; Pigliucci et al., 2006), while phenotypic integration will be related to the every organ and structure function of the plants, even when the environment is stressful or changing (Pigliucci, 2003; Damián et al., 2020; Matesanz et al., 2021). Therefore, according to our results, natural selection seems to favor the coordinated function of characters over their ability to respond to environmental changes. However, it must be taken into account that in our experimental design we have studied some very particular environmental changes. It would be very interesting to explore how natural selection, both on the integration of traits and on their plasticity, is altered when plants are subjected to different and, above all, more stressful environments.

CONCLUSIONS

The main contribution of this work is a first empirical demonstration of the underlying mechanisms driving the constraining relationship between phenotypic integration and plasticity. We demonstrate the negative relationship that exists between the integration level of a given phenotypic trait and its plasticity in *Eyrimum incanum*. This negative relationship is maintained in all the environments where the plants were grown and throughout all its ploidy levels. The significant relationship between phenotypic plasticity and integration of characters had already been described in other systems. How natural selection and heritability could be involved in the link between plasticity and integration is explored for the first time in this work. Heritabilities of the traits considered are significantly higher in number and intensity than the heritabilities of the trait phenotypic plasticities. Direct selection acting on phenotypic plasticity has already been described in this study system (***Chapter 4***). But an intense indirect selection, which is one of the main mechanisms controlling the rise of genetic correlation between traits, has also been found in this work. It has led to a higher response selection on trait correlations than on the phenotypic plasticity. Therefore, we propose this difference as a mechanistic demonstration for the constraining effect of phenotypic integration on plasticity. However, and in the absence of methods to quantify the response to the selection of correlated characters, development of methodological tools able to quantify differences in response to selection is necessary. It would allow us to predict the level of phenotypic plasticity would be limiting the evolution of the phenotypic plasticity in scenarios where it is especially important, such as changing environments.

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AUTHOR CONTRIBUTIONS

AGM, CF, AJMP, MB and MA thought and designed the experiments. AGM, CV and CF conducted the greenhouse experiments. AGM, AJMP, CF, CV and MA made the statistical analyses and designed the tables and figures. AGM and MA wrote the first draft of this manuscript and all the rest of authors made significant contributions to the draft. AJMP, MB and MA got the funds to develop this study. AJMP, MB and MA supervised all the study.

DATA AVAILABILITY STATEMENT

Data is stored in XXX for review purposes, and will make it available in a public repository upon acceptance.

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SUPPORTING INFORMATION

Additional Supporting Information can be found in the following pages.

Appendix S1:

Table S5.1. Heritability of the phenotypic traits from different trait modules and ploidy level under IN treatment. Values of heritability are shown accompanied by the 95% interval confidence. Significant values of heritability are shown in bold (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Modules	Traits	2x		4x		6x	
		h²	95% C.I.	*h²*	95% C.I.	*h²*	95% C.I.
Flor.	Petal length	0,22	[0.19-0.25]	0,59	[0.27-0.75]	0,51	[0.22-0.74]
	Corolla diameter	0,80	[0.32-0.66]	**0,65***	[0.28-0.77]	0,55	[0.28-0.68]
	Corolla tube length	0,14	[0.12-0.16]	0,55	[0.25-0.75]	0,54	[0.27-0.68]
	Long stamen	0,06	[0.04-0.08]	0,56	[0.25-0.72]	0,60	[0.23-0.69]
	Short stamen	0,05	[0.02-0.07]	0,55	[0.24-0.71]	0,58	[0.30-0.70]
	Stigma height	0,16	[0.14-0.18]	0,41	[0.26-0.70]	0,53	[0.25-0.70]
	Herkogamy	0,59	[0.30-0.68]	0,42	[0.26-0.71]	0,53	[0.16-0.71]
Veg.	Plant height	0,29	[0.27-0.32]	**0,87******	[0.28-0.69]	**0,86******	[0.29-0.70]
	No. stalk	0,52	[0.25-0.84]	0,58	[0.26-0.76]	0,58	[0.27-0.73]
	Stalk diameter	**0,29*****	[0.27-0.31]	0,57	[0.27-0.75]	0,53	[0.28-0.69]
	No. flowers	0,25	[0.22-0.28]	0,53	[0.23-0.80]	0,52	[0.26-0.78]
Rep.	No. pollen grains/flower	0,51	[0.29-0.74]	0,61	[0.19-0.75]	**0,67****	[0.28-0.74]
	No. ovules/flower	**0,31***	[0.29-0.34]	0,58	[0.21-0.74]	**0,64***	[0.26-0.77]

Table S5.2. Heritability of the phenotypic traits from different trait modules and ploidy level under OUT treatment. Values of heritability are shown accompanied by the 95% interval confidence. Significant values of heritability are shown in bold (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Modules	**Traits**	**2x**		**4x**		**6x**	
		h^2	**95% C.I.**	h^2	**95% C.I.**	h^2	**95% C.I.**
Flor.	Petal length	**0,73******	[0.29-0.68]	**0,68****	[0.26-0.70]	0,51	[0.23-0.73]
	Corolla diameter	0,05	[0.03-0.08]	**0,67****	[0.28-0.75]	**0,61***	[0.28-0.68]
	Corolla tube length	**0,68*****	[0.33-0.75]	0,51	[0.27-0.73]	**0,66****	[0.23-0.73]
	Long stamen	**0,68*****	[0.32-0.66]	0,45	[0.25-0.71]	**0,67****	[0.26-0.70]
	Short stamen	**0,74******	[0.32-0.64]	0,42	[0.25-0.71]	**0,68****	[0.27-0.68]
	Stigma height	**0,63****	[0.31-0.66]	0,49	[0.26-0.74]	**0,62***	[0.26-0.70]
	Herkogamy	0,20	[0.17-0.23]	**0,65***	[0.24-0.74]	0,39	[0.28-0.69]
Veg.	Plant height	**0,87******	[0.32-0.68]	**0,82******	[0.26-0.75]	**0,85******	[0.25-0.71]
	No. stalk	0,28	[0.24-032]	**0,69***	[0.25-0.81]	**0,77******	[0.25-0.80]
	Stalk diameter	**0,62***	[0.31-0.69]	**0,69****	[0.26-0.73]	**0,64***	[0.27-0.68]
	No. flowers	**0,63***	[0.26-0.82]	0,56	[0.24-0.76]	**0,61***	[0.24-0.67]
Rep.	No. pollen grains/flower	0,22	[0.20-0.25]	**0,66****	[0.27-0.73]	**0,75******	[0.27-0.68]
	No. ovules/flower	**0,67****	[0.26-0.74]	**0,64***	[0.25-0.73]	**0,67****	[0.27-0.71]

Appendix S2:

Table S5.3. Heritability of the phenotypic plasticity exhibited by each trait from different trait modules and ploidy level. Values of heritability are shown accompanied by the 95% interval confidence. Significant values of heritability are shown in bold (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Modules	**Traits**	**2x**		**4x**		**6x**	
		h^2	**95% C.I.**	***h^2***	**95% C.I.**	***h^2***	**95% C.I.**
Flor.	Petal length	0.22	[0.19-0.25]	**0.35***	[0.33-0.36]	**0.60******	[0.58-0.61]
	Corolla diameter	0.05	[0.03-0.08]	0.17	[0.15-0.19]	**0.31***	[0.30-0.32]
	Corolla tube length	0.14	[0.12-0.16]	**0.50****	[0.48-0.51]	**0.76******	[0.75-0.78]
	Long stamen	0.06	[0.04-0.08]	**0.42***	[0.41-0.44]	**0.71******	[0.70-0.73]
	Short stamen	0.05	[0.02-0.07]	**0.51****	[0.49-0.52]	**0.75******	[0.74-0.77]
	Stigma height	0.16	[0.14-0.18]	0.41	[0.39-0.42]	**0.63******	[0.61-0.64]
	Herkogamy	0.20	[0.17-0.23]	0.27	[0.25-0.28]	0.27	[0.25-0.29]
Veg.	Plant height	**0.29*****	[0.27-0.32]	**0.46******	[0.45-0.48]	**0.25******	[0.24-0.27]
	No. stalk	0.28	[0.24-032]	**0.42***	[0.41-0.44]	**0.56******	[0.54-0.57]
	Stalk diameter	0.29	[0.27-0.31]	**0.48****	[0.46-0.50]	**0.46*****	[0.44-0.47]
	No. flowers	0.25	[0.22-0.28]	0.39	[0.38-0.41]	**0.57******	[0.56-0.59]
Rep.	No. pollen grains/flower	0.22	[0.20-0.25]	**0.53******	[0.52-0.55]	**0.54******	[0.53-0.56]
	No. ovules/flower	**0.31***	[0.29-0.34]	0.30	[0.28-0.32]	0.36	[0.34-0.37]

Appendix 5.3.

Traits	Ploidy		
	2x	4x	6x
	β_{seeds}	β_{seeds}	β_{seeds}
Petal length	-1.25	2.75	-1.34
Corolla diameter	-1.68	0.60	0.18
Corolla tube length	1.06	1.16	-0.22
Long stamen	2.76	**-4.42***	-2.38
Short stamen	0.27	-2.53	-1.31
Stigma height	0.76	-2.52	-1.70
Herkogamy	-0.07	-0.57	-0.25
Plant height	0.19	**1.33***	-0.42
No. stalk	0.33	**0.78****	**1.00****
Stalk diameter	0.18	1.16	0.73
No. flowers	0.47	**1.14***	0.48
No. pollen grains/flower	-0.06	0.70	0.70
No. ovules/flower	0.68	-0.54	-0.54

Table S5.4. Selective gradients on trait phenotypic plasticity. Linear correlations (β) between phenotypic plasticity of traits measured on *E. incanum* and the total number of seeds (Seeds) as a component of fitness. Significant values are shown in bold. Red and blue colors indicate negative and positive correlations, respectively, and the color intensity indicates the level of significance. Nonlinear selection gradients (γ) are not shown since they were not significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Appendix 5.4.

Diploids (2x) IN

Traits	Corolla diameter	Corolla tube length	Long filament	Short filament	Stigma height	Herkogamy	Plant height	No. stalk	Stalk diameter	No. flowers	No. Pollen grains/flower	No. ovules/flower
Petal length	-0.061	-0.180	-0.060	-0.614	0.025	-0.128	-0.078	0.281	0.165	0.147	0.372	-0.019
Corolla diameter		0.044	-0.810	-0.024	0.867	**-0.347***	0.173	0.088	0.211	0.086	0.352	0.045
Corolla tube length			-0.013	0.034	-0.742	0.030	0.257	**0.358***	**0.199***	0.196	0.319	-0.080
Long filament				-0.002	0.903	-0.470	0.156	0.302	0.223	0.107	0.298	0.068
Short filament					0.385	**0.567****	0.677	0.390	0.103	0.177	0.583	0.251
Stigma height						-0.537	0.312	-0.079	0.414	0.136	0.895	0.153
Herkogamy							-0.370	-0.320	-0.180	-0.194	-0.254	-0.106
Plant height								0.305	0.209	0.261	0.554	-0.119
No. stalk									**-0.300***	-0.171	0.105	0.038
St. diameter										-0.193	0.187	0.089
No. flowers											0.330	0.140
No. pollen grains/flower												-0.017

Table S5.5. Correlational selective regimes on traits pairwise exhibited by diploid plants exposed to IN treatment. Significant values are shown in bold (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Diploids (2x) OUT

Traits	Corolla diameter	Corolla tube length	Long filament	Short filament	Stigma height	Herkogamy	Plant height	No. stalk	Stalk diameter	No. flowers	No. Pollen grains/flower	No. ovules/flower
Petal length	0.001	0.034	0.039	0.016	0.032	**0.047***	0.015	**0.035***	**0.036***	0.019	**0.046***	0.029
Corolla diameter		0.051	**0.053***	0.023	**0.046***	**0.057****	0.022	**0.052*****	**0.042*****	**0.033*****	**0.057*****	0.023
Corolla tube length			**0.082****	0.036	**0.051***	**0.082*****	0.012	**0.057*****	**0.038**	**0.042*****	**0.046***	0.034
Long filament				-0.048	0.002	**0.049***	-0.022	**0.060*****	**0.035***	**0.043*****	0.038	0.005
Short filament					**0.073****	**0.080******	0.018	**0.080******	**0.050****	**0.055******	**0.073*****	**0.042***
Stigma height						**0.047***	-0.003	**0.056*****	**0.033***	**0.044*****	0.044*	0.010
Herkogamy							0.020	0.007	0.001	0.011	0.006	0.015
Plant height								**0.121******	**0.086******	**0.067******	**0.107******	**0.082******
No. stalk									**0.076******	**-0.074****	**0.125******	**0.200******
St. diameter										**0.043*****	**0.108******	**0.097******
No. flowers											**0.176******	**0.213******
No. pollen grains/flower												0.009

Table S5.6. Correlational selective regimes on traits pairwise exhibited by diploid plants exposed to OUT treatment. Significant values are shown in bold (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Tetraploids (4x) IN

Traits	Corolla diameter	Corolla tube length	Long filament	Short filament	Stigma height	Herkogamy	Plant height	No. stalk	Stalk diameter	No. flowers	No. Pollen grains/flower	No. ovules/flower
Petal length	0.038	0.021	-0.013	-0.004	-0.014	0.002	-0.025	-0.007	-0.006	-0.008	0.001	0.002
Corolla diameter		0.004	-0.045	-0.030	-0.051	-0.021	-0.019	-0.008	-0.020	-0.002	-0.015	-0.005
Corolla tube length			**-0.083***	-0.053	-0.066·	-0.051	-0.005	-0.001	-0.024	0.003	-0.019	-0.010
Long filament				0.024	0.001	-0.003	-0.013	0.008	-0.003	-0.005	-0.002	0.003
Short filament					-0.022	-0.008	-0.004	0.008	-0.020	-0.006	-0.008	0.011
Stigma height						0.002	-0.016	0.003	-0.008	0.001	0.003	-0.004
Herkogamy							-0.008	-0.001	-0.008	0.001	0.003	-0.004
Plant height								**0.046****	**0.094*****	-0.003	**0.140******	**0.084******
No. stalk									**0.102****	0.025	**0.134******	**0.101******
St. diameter										**0.018****	**0.104******	0.016
No. flowers											**0.120******	**0.097******
No. pollen grains/flower												-0.009

Table S5.7. Correlational selective regimes on traits pairwise exhibited by tetraploid plants exposed to IN treatment. Significant values are shown in bold (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Tetraploids (4x) OUT

Traits	Corolla diameter	Corolla tube length	Long filament	Short filament	Stigma height	Herkogamy	Plant height	No. stalk	Stalk diameter	No. flowers	No. Pollen grains/flower	No. ovules/flower
Petal length	0.055·	0.004	0.009	0.004	0.021	0.021	-0.014	**0.027***	-0.022	0.006	0.017	0.022
Corolla diameter		-0.015	-0.025	-0.026	-0.009	-0.007	-0.002	**0.031***	-0.011	0.003	-0.014	-0.012
Corolla tube length			0.028	0.011	**0.072***	**0.068****	-0.008	0.020	-0.021	-0.001	0.046	0.032·
Long filament				0.022·	0.064	0.037	-0.001	0.009	-0.011	-0.014	0.044·	0.041·
Short filament					**0.079****	**0.056***	0.012	0.020	0.003	0.003	**0.062****	0.035·
Stigma height						0.047	-0.011	-0.014	-0.006	**0.025***	0.019	0.015
Herkogamy							-0.008	**0.024***	0.007	-0.013	-0.013	-0.011
Plant height								**0.030***	0.026	**0.027***	**0.115******	**0.089******
No. stalk									**0.100******	**0.0561****	**0.149******	**0.120******
St. diameter										0.021	**0.134******	**0.110******
No. flowers											**0.157******	**0.134******
No. pollen grains/flower												0.009

Table S5.8. Correlational selective regimes on traits pairwise exhibited by tetraploid plants exposed to OUT treatment. Significant values are shown in bold (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Hexaploids (6x) IN

Traits	Corolla diameter	Corolla tube length	Long filament	Short filament	Stigma height	Herkogamy	Plant height	No. stalk	Stalk diameter	No. flowers	No. Pollen grains/flower	No. ovules/flower
Petal length	0.082	0.029	0.074	0.089	0.088	0.109·	0.030	0.084·	0.083·	0.043	0.092	0.053
Corolla diameter		-0.010	0.064	0.069	0.072	0.093·	0.051	0.055	0.047	0.026	0.1021·	0.081·
Corolla tube length			**0.168****	**0.177****	**0.178****	**0.173****	**0.133****	**0.064****	**0.097***	0.064·	**0.140****	**0.119****
Long filament				0.062	0.076	0.081·	0.067·	0.058·	0.042	0.031	0.066	0.029
Short filament					0.047	0.071	**0.080***	0.038	0.047	0.019	0.049	0.028
Stigma height						0.072	**0.081***	0.042	0.026	0.017	0.052	0.039
Herkogamy							0.064	-0.024	0.020	-0.012	-0.009	0.045
Plant height								**0.120*****	**0.106****	**0.060***	**0.140*****	**0.121******
No. stalk									**0.129****	**0.103****	**0.136******	**0.108******
St. diameter										**0.059***	**0.167******	**0.129******
No. flowers											**0.194******	**0.215******
No. pollen grains/flower												0.017

Table S5.9. Correlational selective regimes on traits pairwise exhibited by hexaploid plants exposed to IN treatment. Significant values are shown in bold (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Hexaploids (6x) OUT

Traits	Corolla diameter	Corolla tube length	Long filament	Short filament	Stigma height	Herkogamy	Plant height	No. stalk	Stalk diameter	No. flowers	No. Pollen grains/flower	No. ovules/flower
Petal length	0.043	0.026	0.044	0.049	0.031	**0.068***	0.058·	0.049·	0.017	0.016	0.053·	0.037
Corolla diameter		0.036	0.034	0.042	0.014	0.049·	**0.055***	0.042·	0.024	0.028·	**0.053***	0.012
Corolla tube length			0.048	0.052	0.048	**0.075****	**0.075****	**0.064****	0.031	0.026	**0.091****	**0.049****
Long filament				0.045	0.005·	**0.080****	**0.051***	**0.041***	0.026	0.018	**0.050***	**0.042***
Short filament					-0.019	**0.053***	0.047·	0.037·	0.014	0.020	0.037	0.038·
Stigma height						**0.101*****	**0.091*****	**0.071*****	**0.057***	**0.043****	**0.088*****	**0.056****
Herkogamy							**0.053***	**0.043***	0.039·	**0.030***	0.047·	0.015
Plant height								**0.046***	0.034	**0.030***	**0.063***	0.021
No. stalk									**0.088*****	-0.027	**0.129******	**0.094******
St. diameter										0.028	**0.126******	**0.079******
No. flowers											**0.191******	**0.129******
No. pollen grains/flower												**-0.038***

Table S5.10. Correlational selective regimes on traits pairwise exhibited by hexaploid plants exposed to OUT treatment. Significant values are shown in bold (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Chapter 6

Effects of ploidy and genetic diversity on competitive outcomes

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ABSTRACT

Genetic diversity affects evolutionary trajectories but their ultimate effects on ecological interactions and community dynamics remain poorly understood. It has been hypothesized that phenotypic novelties produced by ploidy and heterozygosity modify the ecological interactions between novel genotypes and more ancient locally adapted ones, and therefore, their opportunities to coexist. We performed a greenhouse competition experiment with three taxa of the *Erysimum incanum* species complex differing in ploidy (2x, 4x and 6x) and heterozygosity (high and low). This experiment allows us to parameterize a population model to test the effect of genetic diversity on modulating the ecological forces that determine the outcome of competition, niche and fitness differences. Depending on whether ploidy variation and the level of heterozygosity made interspecific competition greater or smaller than intraspecific competition, we predicted either priority effects or coexistence. Such competitive outcome differences were explained by the phenotypic expression in the number of stalks (plant size surrogate) with genotypes under priority effects showing more stalks. Altogether, our results show that non-polyploid plants can coexist with polypoids contravening theoretical expectations of polyploidy dominance under stable conditions. However, historical contingency such as order of arrival promotes priority effects when adaptive phenotypic optimums strongly compete for space.

Key words: competition, coexistence, *Erysimum incanum*, heterozygosity, priority effects, polyploidy.

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INTRODUCTION

Progress in ecological theory during the last decades has substantially rendered a mechanistic understanding of the rules governing the maintenance of species diversity. These advances, formally named as modern coexistence theory (MCT; Chesson, 2000), posit that there are two species differences that determine the outcome of competitive interactions. On the one hand, niche differences occur when intraspecific competition exceeds interspecific competition. It stabilizes the population dynamics of interacting species by limiting their population growth when they are abundant but buffering them from extinction when they are rare (Adler et al., 2007). These stabilizing niche differences can arise from a wide variety of ecological factors such as differences in phenology (Godoy & Levine, 2014), differences in natural enemies (Petry et al., 2018), or differences in nutrient requirements (Harpole et al., 2016). On the other hand, fitness differences drive competition dominance and, in the absence of niche differences, determine the superior competitor. Fitness differences, understood within an ecological context, occur when good light competitors grow at the expense of other species (DeMalach et al., 2017) or when species are able to draw down common resources faster than their competitors (Tilman, 1982). This competition ability is the result of two components. The first one is the species demographic differences, which arise from different ability of species to produce viable offspring, and the second component is the competitive response differences, which arise when species show different responses to competition. At the extremes, a species can be a superior competitor either because it produces a great amount of viable offspring or has a low sensitivity to competition (i.e. offspring production is not reduced when neighbors density increases), although a combination of both strategies is also possible.

Importantly, theory predicts a variety of outcomes depending on the relationship between niche and fitness differences (Ke & Letten, 2018). Under negative density-dependence (i.e., population growth rates decrease as the density of a population increases), species are predicted to coexist when niche differences overcome fitness differences. On the other hand, if fitness differences are overwhelming, the inferior competitor species are predicted to be excluded. It can also be the case that species are experiencing positive density-dependence (i.e., population growth rates increase as the density of a population also increases). In such cases, priority effects are expected to occur. This means that contingency processes such as order of arrival influence community assembly and the species that arrives first dominates the community and excludes the other (Fragata et al., 2022).

Modern coexistence theory was developed within an ecological context and as such most of its application has been done within this domain. This implies that the role of evolution in determining the outcome of ecological interaction is still poorly understood. Empirical work at the macroevolutionary scale has shown that disparate evolutionary processes among species poorly predict the outcome of ecological interactions and they can either determine coexistence or competitive exclusion Narwani et al., 2013; Godoy et al., 2014; Germain et al., 2016). Moreover, Germain et al. (2016) showed that the scaling of niche and fitness differences with phylogenetic relatedness depends on whether species have evolved in sympatry or allopatry, being allopatric species less likely to coexist based on phylogenetic distances. At the microevolutionary scale, some examples have documented that rapid evolution ameliorates the negative effect of competition and ultimately can favor the coexistence of competing species (Lankau et al., 2009; Hart et al., 2019), whereas others have documented the opposite result (Qin *et al.*, 2013). This dependency context calls for further studies to better mechanistically understand the effect of evolution on ecological interactions. In that regard, processes affecting genetic diversity have been long thought to be an important driver of ecological interactions, and it has been amply discussed that common processes should control the maintenance of both genetic and species diversity (Dempster, 1955; Ayala & Campbell, 1974; Hughes et al., 2008).

Two evolutionary processes are expected to modulate the degree of genetic diversity. The first and most important one is polyploidization, which has played an essential role in plants evolutionary history (Grant 1981;

Soltis and Soltis 1999; Soltis et al. 2009; Wood et al. 2009) and diversification (Leebens-Mack et al., 2006). Complete genome duplication stimulates the neofunctionalization of duplicated, redundant genes, potentially leading to novel and innovative traits promoted by natural selection (Otto & Whitton, 2000; Parisod et al., 2010). Furthermore, it is broadly known that genome duplications lead to variation in plant phenotype (Jürgens et al., 2002), involving changes in the rest of ecological interactions within a community. For example, an increment in flower size exhibited by higher ploidies might modify pollinator preferences between co-occurring individuals differing in ploidy level (te Beest et al., 2012; Moghe & Shiu, 2014). Ultimately, polyploidization events are able to change the resources usage and, thus, how diploids and their polyploid counterparts are spatially located (Levin, 1981; Raabová et al., 2008; Kolář et al., 2013). The overall increasing fitness in polyploid species is suggested as a potential driver for ecological adaptation to colonize novel habitats and face a major diversity of environmental conditions compared to diploids, which would explain the diversification patterns shaped by polyploids, especially in islands (Meudt et al., 2021). In sum, these previous findings suggest that ploidy is a driver of both fitness and niche differences but empirical assessments that explicitly explore how these differences determine the outcome of competition are lacking.

Together with polyploidization, a second important characteristic is the degree of heterozygosity.

Heterozygosity is key in understanding the ecological consequences of competing genotypes because, just like polyploidy, it also increases raw material in the long term by novel allele combinations for evolution to act upon (Nieto Feliner et al., 2020). The allelic diversity effect is shown through changes in phenotype and even in the individual performance. An example is the classical heterosis event exhibited by the offspring originated by outbreeding (Hayes & Others, 1952; Bomblies & Weigel, 2007). This occurs when the heterozygotic offspring resulting from outbreed crosses exhibited a major performance compared with homozygotic parents. However, heterosis has been well documented in crop plants because heterozygotic phenotypes are commonly accompanied by a higher performance and adaptive ability (Fridman, 2015). Studies in heterosis help to understand the genotype-phenotype relationship due to the presence of different alleles resulting in phenotypes that, ultimately, could be able to drive evolutionary processes. However, both polyploidization and heterozygosity have been also shown to produce an immediate effect on the individual fitness within a single generation (Ramsey & Schemske, 2002). For this reason, comparisons of fitness between homozygotes and heterozygotes or diploids and polyploids are commonly investigated to explain their coexistence or spatial segregation (Sonnleitner et al., 2010; te Beest et al., 2012; Ramsey & Ramsey, 2014).

If we summarize all these previous findings on genetic diversity affecting ecological dynamics, we can posit that they have been mostly focused on understanding what processes drive the fitness differences among genotypes that determine competitive exclusion (Ramsey & Schemske, 2002). Within this perspective, coexistence has been considered a spatial process in which different genotypes persist under different locations thanks to being locally adapted. However, MCT predicts that the persistence of genetic diversity can be also achieved within the same location by promoting niche differences that stabilize the dynamics of competing genotypes. Yet, information on how genetic diversity promotes these niche differences is currently missing (Rey et al., 2017). Including the axis of niche differences is critical to understand when new variants are able to coexist with their ancestors within the same location when genome duplication occurs, or when the new variant excludes (or it is excluded by) the ancestor. This is well illustrated in the case of many species such as the strawberry, which has evolved their genome in response to arid or stressful conditions (Liston et al., 2020). We can hypothesize that if genome duplication has served to cope with stressful conditions, then, it would be likely to observe niche differences among genotypes with different ploidy due to niche segregation. Likewise, genetic diversity can also promote niche differences by phenotypic changes that allow new variants to explore different resources (Kolář et al., 2013; Hernández-Leal et al., 2019). Overall, we have expectations that genetic diversity promotes both niche and fitness differences and the study of the effect of genetic diversity on the drivers of competitive outcomes can allow us to obtain a better understanding of how microevolutionary processes maintain genetic diversity, or if this does not occur, it allows to identify which genotypes are excluded by deterministic processes because they become inferior competitors after evolutionary processes or by contingency due to the order or arrival (i.e., priority effects) (Figure 6.1). Fortunately, there are tools readily available to explore these questions by combining population models with detailed experiments in which it is possible to measure fitness and density dependent processes (Narwani et al., 2013; Godoy & Levine, 2014; Germain et al., 2016).

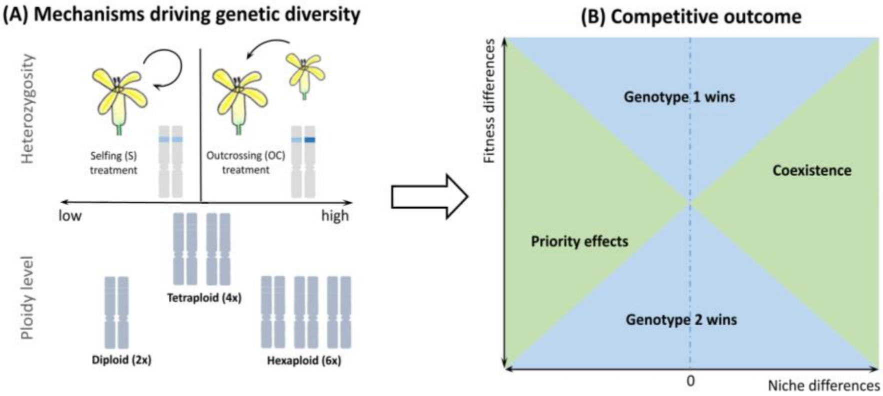

Figure 6.1. (A) Mechanisms affecting the genetic diversity of the studied individuals and (B) competitive outcomes expected in our experiments according to niche and fitness differences. The genetic diversity of the study system is mainly affected by the ploidy level (including diploid, tetraploid and hexaploid individuals) and by the heterozygosity level (the studied individuals have been produced after performing selfing and outcrossing manual pollination on parental individuals). Genetic diversity is expected to increase with ploidy level and outcrossing treatments. Such genetic diversity is expected to influence in turn the outcome of competition, depending on how they promote niche and fitness differences. Three different outcomes are expected. Coexistence (right green area) in which any genotype does not exclude each other, competitive exclusion (blue area) in which the genotype with higher fitness excludes the other, and finally, priority effects (left green area) in which the genotype that arrives first excludes the other.

Here, we focus on the annual multiploidy species complex *Erysimum incanum* (Brassicaceae) to address how genetic diversity determined by the degree of polyploidization and heterozygosity influences ecological interactions and competitive outcomes between contrasted genotypes. This variation in genetic diversity was obtained by combining different genotypes from diploids to tetraploids and hexaploids with crosses among individuals within the same level of ploidy to increment the degree of heterozygosity (Figure 6.1). With this experimental set up, we were able to answer the following questions: 1) Does ploidy determine differences in fitness among genotypes?, 2) How does the interaction between ploidy and heterozygosity influence the niche and fitness differences that determine the outcome of competition between genotypes and the maintenance of genetic diversity?, 3) Is a particular character able to summarize variation in competitive outcomes among genotypes?

MATERIALS AND METHODS

Study system and experimental set-up

We focus our study in the genus *Erysimum L.*, which is one of the most diverse in the Brassicaceae family, with species inhabiting Eurasia, North Africa and North and Central America (Al-Shehbaz et al., 2006). In particular, we studied the species complex *Erysimum incanum*. This complex includes annual monocarpic species and subspecies inhabiting the Western Mediterranean basin, which is a main diversification center of the genus (Abdelaziz et al., 2011;

Nieto Feliner, 2014). Within this complex and using flow cytometry, we found three ploidy levels: diploids (2x = 16 chromosomes), tetraploids (4x = 32 chromosomes) and hexaploids (6x = 48 chromosomes) (Nieto Feliner, 2014; García-Muñoz et al., ***Chapter 1***) with dissimilar geographic distribution. Diploids of *E. incanum* (*Erysimum incanum subsp. mairei*), present a vicariant distribution between the Rif and the Pyrenees mountains while tetraploids (*E. incanum subsp. incanum*) present a similar distribution in southwest Iberian Peninsula and the Middle Atlas Mountains (Fennane & Ibn-Tattou, 1999). In contrast to these ploidies occurring in both continents, hexaploid plants (*Erysimum meridionalis sp. nov.*) are only found in the High Atlas and Antiatlas mountains (Abdelaziz et al., *in prep.*). Most species of the *E. incanum* complex exhibit autogamy as the predominant reproductive strategy, showing hermaphroditic flowers with the specific characteristics of the selfing syndrome. This reproductive system results in full-sib individuals within the same family.

Using this species complex as a baseline, we removed any local effects before performing experiments by obtaining pure lines from more than five generations in controlled conditions. Once these pure lines were obtained, we further modified their degree of genetic diversity within each ploidy level by crossing individuals in order to maintain or remove the homozygosity exhibited by pure lines. To do so, the first treatment consisted in selfing hand-made crosses that allow obtaining seeds in which the homozygosity level is assumed to be complete within the pure line. The second treatment consisted in intra-population allogamous crosses, where some flowers of each plant were emasculated before first opening and pollinated with pollen of individuals from a different individual within the same population. This latter treatment resulted in seeds in which homozygosity was replaced by a full degree of heterozygosity (OC), except for these loci where alleles were identical by state. Overall, this procedure led to three “selfing (S)” and three “outcrossing (OC)” plant families according to low or high degree of heterozygosity, respectively, which were factorially combined with the three ploidy levels (Figure 6.1A). Therefore, a total of 18 plant families, three per combination of 2xS, 2xOC, 4xS, 4xOC and 6xS, 6xOC were used for evaluating experimentally the role of genetic diversity in coexistence outcomes.

Theoretical approach

Our greenhouse experiments were designed to experimentally parameterise a mathematical model describing the population dynamics of interacting species (Levine & HilleRisLambers, 2009), which here was extended to genotypes. With this model, it is possible to quantify stabilizing niche differences and average fitness differences between interacting organisms from plants to animals (Fragata et al., 2022; Godoy & Levine, 2014). The model is described as follows:

$$\frac{N_{i,t+1}}{N_{i,t}} = (1 - g_i)\, s_i + g_i F_{i,t} \qquad \textbf{(6.1)}$$

where $\frac{Ni,t+1}{Ni,t}$ is the per capita population rate, and $N_{i,t}$ is the number of seeds of genotype *i* in the soil prior to germination in winter of year *t*. In addition, the germination rate of species *i*, s_i, can be viewed as a weighting term for an average of two different growth rates: the annual survival of ungerminated seed in the soil (g_i) and the viable seeds produced per germinated individual (F_i). We assume that genotypes affect the performance of one another when germinated individuals limit the fecundity of competitors. Thus, the per-germinant fecundity, F_i, can be expanded into a function including the density of competing individuals in the system:

$$F_i = \frac{\lambda_i}{1 + \alpha_{ii} g_i N_i + \alpha_{ij} g_j N_j} \qquad \textbf{(6.2)}$$

where λ_i is the per-germinant fecundity in the absence of competition. It is reduced by germinated individuals of its own and other species, which are multiplied by interaction coefficients, α_{ij}, that describes the per capita effect of genotype *j* on genotype *i*. The model ignores the potential for age-dependent survival of ungerminated seeds, because prior works in annual plants showed that seed bank survival has negligible influence on the competitive outcomes (Godoy & Levine, 2014).

With the dynamics of competition among genotypes described by this population model, we followed the approach of Chesson (2012) to determine fitness and niche differences between species pairs. Following (Godoy &

Levine, 2014) method, niche overlap between pair of genotypes, ρ, was calculated as:

$$\rho = \sqrt{\frac{\alpha_{ij}}{\alpha_{jj}} \cdot \frac{\alpha_{ji}}{\alpha_{ii}}} \qquad \textbf{(6.3)}$$

Niche overlap describes the degree to which competition among individuals of the same genotype (α_{ii}, α_{jj}) impacts more than competition among individuals of different genotypes (α_{ij}, α_{ji}). Niche overlap spans from zero (i.e., no niche overlap) to one (i.e., complete niche overlap). With (ρ) defining niche overlap between a pair of genotypes, their stabilizing niche difference is expressed as $1-\rho$. As hypothesized, we expect that genetic differences in ploidy and in heterozygosity and their combination will reduce niche overlap among genotypes, and therefore, will increase niche differences.

As an opposing force to stabilizing niche differences, average fitness differences drive competitive dominance and, in the absence of niche differences, determine the competitive superiority between a pair of genotypes. Following previous methodologies (Godoy & Levine, 2014), we define average fitness differences between the competitors ($\frac{\kappa_j}{\kappa_i}$) as:

$$\frac{\kappa_j}{\kappa_i} = \frac{\eta_{j-1}}{\eta_{i-1}} \cdot \frac{\sqrt{\alpha_{ij}\,\alpha_{ii}}}{\sqrt{\alpha_{ji}\,\alpha_{jj}}} \qquad \textbf{(6.4)}$$

where η_i and η_j are the annual seed production for both genotypes and α_{ji} and α_{jj} are the per capita effect of a genotype i and genotype j on the seed production of a genotype j, respectively. It is worth noting that we did not explicitly estimate the germination rates (g_i) and soil survival rates (s_i) but we consider them to be equal to one. Therefore, in this particular study η_i and η_j are equal to λ_i and λ_j respectively. According to equation 4, average fitness differences can be decomposed in two different expressions. On the one hand $\frac{\eta_{j-1}}{\eta_{i-1}}$ describes the "demographic difference" (i.e., the extent to which genotype i produces more seeds per germinant than genotype j). On the other hand $\frac{\sqrt{\alpha_{ij}\,\alpha_{ii}}}{\sqrt{\alpha_{ji}\,\alpha_{jj}}}$ describes the "competitive response ratio" (i.e, the extent to which genotype i is more sensitive to competition than genotype j).

From the expression of average fitness difference (equation 4), we can describe the genotype competitive ability (Hart et al., 2018) as:

$$\kappa_i = \frac{(\lambda_i - 1)}{\sqrt{\alpha_{ij}\,\alpha_{ii}}} \qquad \textbf{(6.5)}$$

The competitive ability (κ_i) describes the ability of a genotype to be a superior competition as a function of two possibilities. Either because it can produce a high amount of viable seeds ($\lambda_i - 1$) or the genotype is not sensitive to competition with other genotypes ($\sqrt{\alpha_{ij}\,\alpha_{ii}}$), that is, the amount of viable seeds produced is not reduced as the density of the competitor increases.

Importantly, the greater the ratio between genotypes j and i, the greater the fitness advantage of genotype j over i. If this ratio is one, genotypes are equivalent competitors. Coexistence requires both genotypes to invade when rare (Chesson, 2012). Then we established coexistence condition as (Godoy & Levine, 2014):

$$\rho < \frac{\kappa_j}{\kappa_i} < \frac{1}{\rho} \qquad \textbf{(6.6)}$$

This condition allows us to distinguish three coexistence outcomes. The first outcome is *stable coexistence* that occurs when niche differences are larger than fitness differences. The second outcome is *competitive exclusion* that occurs when fitness differences are larger than niche differences. And finally, the third outcome is *priority effects* that occur when niche differences are negative, which indicates that species are experiencing positive density-dependence. In that final outcome it is predicted that the species that arrives first to the community excludes its competitor (Figure 6.1B).

Competition experiment and character measurements

To empirically parameterize the population model with which we can determine the competitive outcomes between genotypes, we conducted greenhouse experiments to estimate per germinant fecundities in the absence of neighbors (λ_i) and all pairwise interaction coefficients (α_{ij}). In March 2020, we displayed pots of 4.2 L (0.18x0.18x0.13cm), which were filled with Gramoflor™

potting soil mixture and watered every two days. The overall design involved sowing each genotype as focal individuals into a density gradient of each competitor genotype (including itself). To create this gradient, we followed a spatial explicit design within each pot proposed by Bartomeus et al. (2021), in which focal genotypes of the same family experienced a density gradient from 1 to 4 individuals of a different genotype family. In order to calculate all pairwise interaction coefficients (α_{ij}), this density gradient was created for each pairwise combination of families. We also grew individuals alone to accurately estimate the fecundities in the absence of neighbors (λ_i). To estimate such fecundity that we understand in an ecological context is the “demography performance” of the genotype and in the evolutionary context is the “fitness” of an individual, we counted the number of fruits per plant and multiplied it by the mean number of viable seeds estimated from four random fruits in the same plant. In this way, we obtained the total viable seed production per individual plant. This value is an unbiased estimate of the individual fitness due to the monocarpic life form of the study system.

Together with the competition experiment, we measured a series of characters for each family at the peak of individual biomass. These characters were overall related to vegetative body plants. Specifically, for every focal individual that produced seeds we measured its plant height, the number of flowering stalks and the diameter of the main stalk. This was done for a total of 210 individuals with 35 individuals per family.

Statistical analyses We used maximum likelihood techniques to parameterise the population model following a nested approach. That is, we first created a single model for which we estimate the intrinsic growth rate in absence of competitors (λ_i), and then we used this information as prior for subsequent more complex models that include an overall term of competition in the second step and intra and interspecific competitive interactions (the α's) in the third final step (Matías et al., 2018). λ_i were considered fixed per genotype family species but competition varied across genotype pairs. Finally, we used a one-way ANOVA in order to test whether coexistence outcomes between genotype pairs could be explained by a particular plant character. All analyses were done using R (R Core Team, 2021). To predict coexistence outcomes we used the package *'cxr'* (García-Callejas et al., 2020). Plots were done using *'ggplot2'* (Wickham, 2016) and *'cowplot'* (Wilke, 2020) packages.

RESULTS

Our results show that the genetic diversity of the different genotypes contribute to promote differences in viable seed production as well as competitive interactions. When we decomposed average fitness differences into its demographic and competitive response components, we found that diploids (2x) tended to be the most competitive genotypes followed by tetraploids (4x), and finally hexaploids (6x) under the experimental conditions we imposed with no drought treatment (Figure 6.2A) although these differences were not significant (F=2.784, p-value=0.14). This competitive superiority of the diploids would be due to a higher viable seed production in the absence of competition (F=3.896, p-value=0.08) as well as a lower sensitivity to reduce viable seed production in the presence of neighbors translated to the response to the competition ratio (F=0.343, p-value=0.723; Figure 6.2B and 6.2C). Conversely, the low competitive ability of the hexaploids were due to a combination of lower viable seed production and higher sensitivity to competition. The amount of niche differences between the diploids and the other ploidy levels was not enough to overcome their observed differences in average fitness. These results overall indicate that diploid genomes are the superior competitor. However, they were not able to competitively exclude the other ploidy families (Figure 6.3A). This outcome is driven by the low differences in fitness and response to competition (Figure 6.2), which lead diploids to share the scenario of strong priority effects with the two other ploidies.

Such strong priority effects were not predicted when considering tetraploid and hexaploid genotypes in combination with their degree of heterozygosity. For these two levels of ploidy, we found two contrasted clusters of outcomes when we added more resolution by explicitly accounting for the degree of heterozygosity. One cluster in which priority effects among the pairs of genotypes were predicted to occur and another cluster in which coexistence was predicted between three other different pairs of genotypes (Figure 6.3B). This result was not predicted by theoretical expectations and suggests that genetic diversity produces a wider variation of ecological outcomes than previously expected.

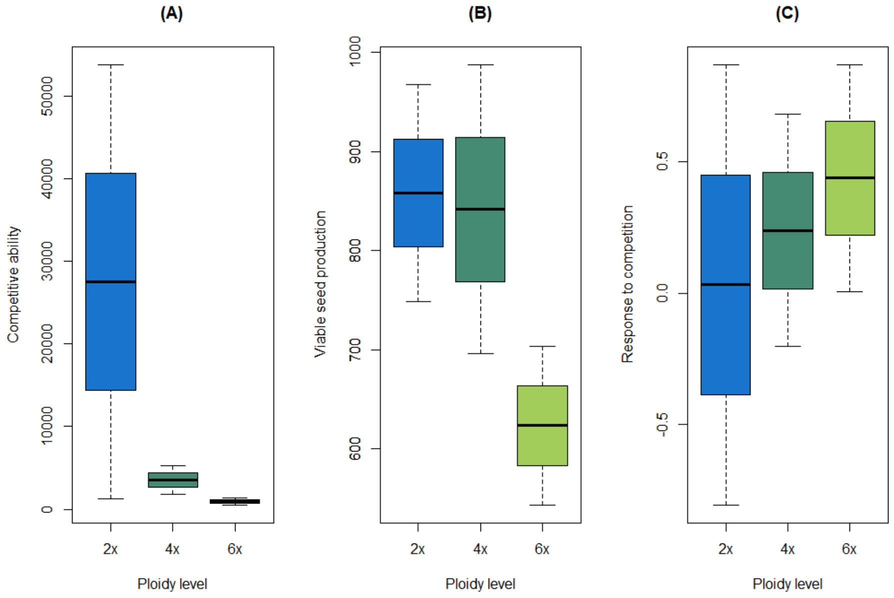

Figure 6.2. Competitive ability (A), viable seed production (B) and response to competition (C) for each one of the three ploidy levels tested in *E. incanum* system.

Priority effects occur under positive density dependence when interspecific competition is stronger than intraspecific competition, whereas, coexistence occurs under negative density dependence when intraspecific competition is stronger than interspecific competition. Therefore, the change from one location of the coexistence map to another can be due to a change in intraspecific competition, interspecific competition or a combination of both. In our experiment, detailed analysis revealed that changes in the strength of interspecific interactions rather than intraspecific interactions were the main driver of switches from priority effects to coexistence regions (Figure 6.4).

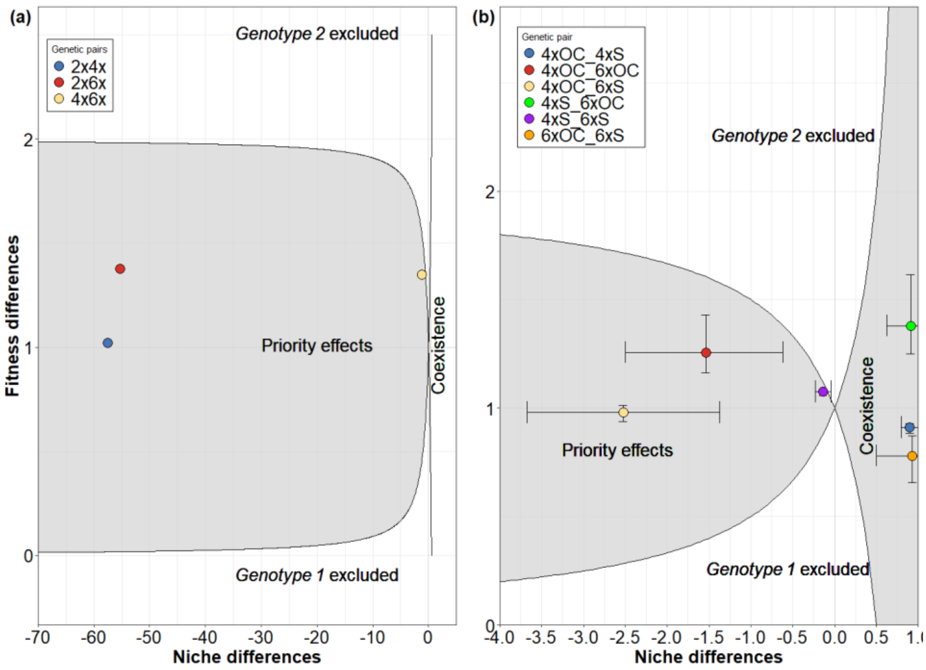

Figure 6.3. Relationship between fitness difference and niche difference (A) for different combinations of ploidy (2x, 4x and 6x) in *E. incanum* system and relationship between fitness difference and niche difference (B) for different combinations of ploidy (4x and 6x) and levels of heterozygosity (low, S and high, OC) in *E. incanum* system (B). The two solid black lines represent the coexistence condition and its symmetrical for each ploidy level tested and define the space in which genotypes could coexist and in which there could be priority effects. Error bars show coexistence outcomes at the 95% confidence interval.

Priority effects occur under positive density dependence when interspecific competition is stronger than intraspecific competition, whereas, coexistence occurs under negative density dependence when intraspecific competition is stronger than interspecific competition. Therefore, the change from one location of the coexistence map to another can be due to a change in intraspecific competition, interspecific competition or a combination of both. In our experiment, detailed analysis revealed that changes in the strength of interspecific interactions rather than intraspecific interactions were the main driver of switches from priority effects to coexistence regions (Figure 6.4).

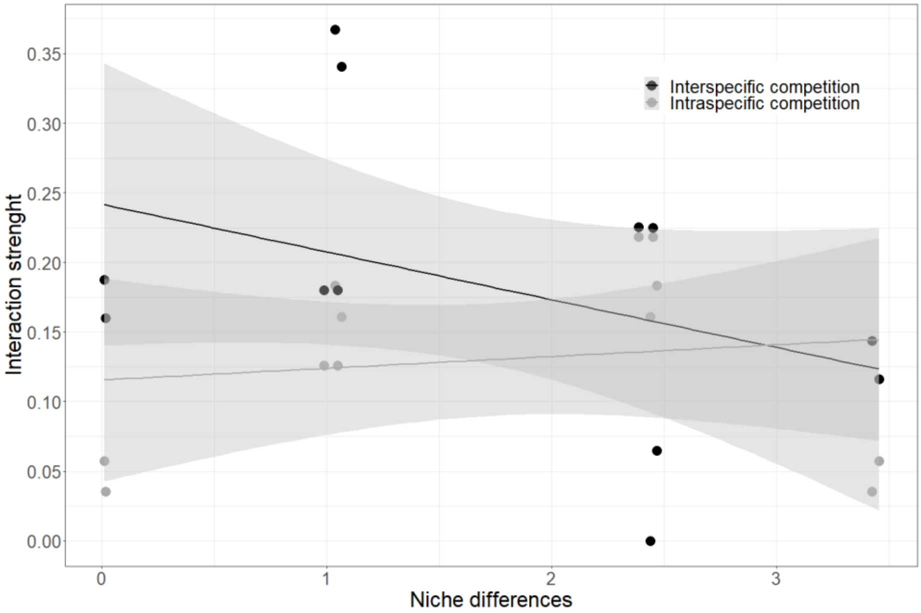

Figure 6.4. The effect of intra and interspecific interactions in driving variation in niche differences between genotype pairs.

We did not observe that differences between these two clusters were attributable to a particular genotypic difference. For both tetraploid and hexaploid groups, we observed pairs of genotypes that differed in the ploidy level as well as their degree of heterozygosity (e.g. see interaction between 4xOC and 6xS in priority effect group and 6xOC with 4xS in coexistence group) and others that only differed in one aspect (e.g. see interaction between 4xOC and 4xS in coexistence group and 4xS and 6xS in priority effects group; Figure 6.3). Despite this variability, we found that a particular vegetative character allows differentiating these two groups of pairs of genotypes. Specifically, the number of stalks, which is a character related to the size of the plant, predicted differences observed in competitive outcomes between coexistence and priority effects. Genotypes with larger numbers of stalks also showed priority effects (Figure 6.5), suggesting that space is a critical resource for which these genotypes compete.

DISCUSSION

Understanding the ecological consequences of genetic diversity is key to explain observed patterns of sympatric and allopatric genetic populations in nature. However, this understanding has been seldom explored because there is a lack of connection between ecological theory that describes the dynamics of interacting organisms and genetic material that can be manipulated to assess competitive interactions. In this study, we show how genetic diversity mechanisms provide a wide range of ecological outcomes based on the strength of competitive interactions between genotypes. Contrary to our expectations, low ploidy level showed higher competitive ability, compared with tetraploid and hexaploid plants of *E. incanum,* mainly due to a lower response to competition and to a higher seed production to a lesser extent. That is, the intrinsic ability of diploids to produce seeds was little affected by increases in the density of individuals of the same and different genotypes within the community. Although theory commonly predicts that an increase in ploidy should confer an increase in fitness, these competitive advantages tend to occur under changing or extreme environments due to aridity or cold (López-Jurado et al., 2016; Liu et al., 2021). Instead, in our experiment, conditions were stable and non-stressful (i.e. no drought treatment). Under such conditions recent computational work simulating biological evolution suggested that non-polyploid perform better than polyploids (Yao et al., 2019; Carretero-Paulet & Van de Peer, 2020). This phenomenon can be explained by the amplification of the effect of random mutations on their gene regulatory networks because of the rise of complexity linked to whole genome duplication (multiplying the number of nodes and interaction in the gene regulatory network). Random mutation, often maladaptive or detrimental, under stable environments will propagate widely. In contrast, a stressful or unstable environment may provide substantive variation for survival (Yao et al., 2019; Carretero-Paulet & Van de Peer, 2020; Van de Peer et al., 2021).

The reason for why polyploids were more sensitive to reduce their fitness in terms of seed production in the presence of neighbors can be due to the fact that they incur in a trade-off between being adapted to stressful conditions and tolerating competition from neighbors (López-Jurado et al., 2016, 2019). In fact, it has been found under natural conditions that polyploids adapted to arid conditions present in general low densities (Manzaneda et al., 2012; Penner et al., 2020). Nevertheless, the higher competitive ability we observed in non-polyploids was not translated to predict competitive exclusion between diploids and the rest of genotypes (tetraploids and hexaploids). The structure of intra and interspecific interactions between genetic pairs modulate this expectation to produce strong priority effects instead, meaning that the historical contingency such as the genotype that arrives first to the community excludes the other.

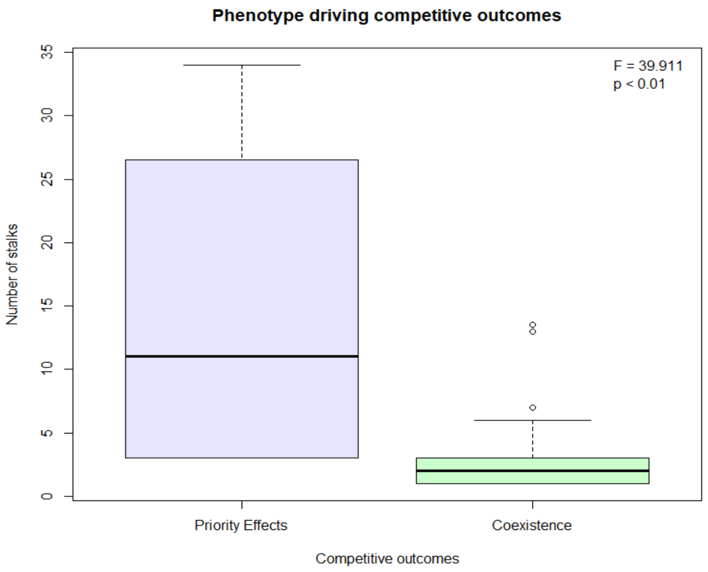

Figure 6.5. Boxplot represents median and quartiles of number of stalks for both coexistence outcomes we predicted when combining ploidy level and the degree of heterozygosity. Priority effects correspond to the combination of the three following genetic pairs: 4xS with 6xS, 4xOC with 6xOC and 4xOC with 6xS, while Coexistence groups the other three genetic pairs: 4xS with 6xOC, 4xOC with 4xS and 6xOC and 6xS.

Our results, therefore, suggest that certain evolutionary theories conveying a competitive advantage to more diverse genotypes should be revisited. In that sense, current theory puts a major role in the effect of the environment on driving an increase of ploidy and associated competitive advantages and the ability to colonize and dominate novel stressful environments. However, this association between polyploids and extreme conditions does not always occur, as exemplifies diploids of *Erysimum mediohispanicum* (another species belonging to *Erysimum*), which have been associated with hard conditions at high altitudes (Muñoz-Pajares et al., 2018). Even if they occur, such advantages seem to be related to species vital rates such as seed production or survival but it comes at the trade-off to tolerate heterospecific competition. This lack of ability to tolerate competition might compromise the successful colonization of new habitats to contingency events as we observed in the priority effects case, or it might be also the case of being excluded from communities that are well established with high local abundances. Further experiments across environmental gradients are needed to reveal the consequences of increases in ploidy for determining trade-off between seed production and tolerance to competition, and therefore, to tease apart the role of the abiotic component (stress conditions) from the biotic component (competition) in driving polyploids advantages to colonize novel environments.

Although most of the work on evolutionary biology has studied the role of ploidy variation in promoting differences in fitness between species, much less has been explored its effect in promoting niche differences between genotypes (Balao et al., 2011; Hülber et al., 2018; Alonso-Marcos et al., 2019). Our results strongly suggest that ploidy variation promotes the demographic consequences of niche differences which stabilize the population dynamics of competing genotypes and further indicate that this effect is in turn strongly influenced by the degree of heterozygosity. Specifically, we found that both levels of heterozygosity can coexist within the same level of ploidy (this is true for tetraploids and hexaploids) as well as between levels of ploidy. This result, which goes against predictions that heterozygote should exclude homozygous genotypes as well as higher ploidy should exclude lower ploidy genotypes, is very important because it suggests that both sources, ploidy and heterozygosity, are critical to maintain genetic diversity within and across genotypes. Moreover, with our ability to link the strength of genotype interactions with their likelihood to coexist, we found that genotypes were not weakly differentiated, as we might expect from closely related evolutionary units within the *E. incanum* system. That is, in those genetic pairs predicted to coexist, we did not observe that weak niche differences overcome small fitness differences. Instead, they presented strong niche differentiation. Such high niche differences indicate that genotypes experience greater intraspecific than interspecific competition. Although with our experiment it is not possible to know the ultimate sources of these axes promoting niche differences, we were not expecting these results taking into account that the greenhouse experiment was settled in relatively small pots where there were few environmental axes compared with natural conditions.

Besides coexistence, we also predicted that several of these tetra and hexaploids genotypes would incur in priority effects according to our experiment conditions. These priority effects mean that genotypes experienced positive rather than negative density dependence. That is, genotypes favor themselves rather than limit their competitors and the genotype exclusion is not due to deterministic processes but rather due to historical contingency such as order of arrival. At a single location, this contingency promotes the dominance of the genotype that arrives first, but at larger scales coexistence can occur if both genotypes arrive first to different locations. If we take a closer look at these genotypes incurring in priority effects, we observed that tetraploids with high levels of heterozygosity (4xOC, see Figure 6.3) are able to exclude any hexaploid plant if they arrive first. This result is very interesting as these species belong to a selfing clade (Abdelaziz et al., 2019) and the mating system transition theories predict that once selfing populations have purged its genetic load, no advantage of high heterozygous plants is expected (Goodwillie et al., 2005). However, the overdominance genetic model predicts that heterozygotes would be superior to homozygotes at loci affecting fitness (Khotyleva et al., 2017).

Based on independent information of either the level of ploidy or the degree of heterozygosity, we could not differentiate those genetic pairs predicted to coexist from those incurring in priority effects. However, we found that the phenotypic expression of these genotypes, measured as the number of stalks, differentiated these contrasted coexistence outcomes. Presenting a high number of stalks is an important feature that equally favors competitive ability by promoting low response to competition and high plant performance in terms of seed production. Therefore, genotypes with greater number of stalks can incur in priority effects for the following reasons. On one hand, the production of more flowering stalks or developing them faster allows genotypes to occupy more space and compete better by monopolizing more resources and by shadowing other surrounding neighbors (Craine & Dybzinski, 2013). On the other hand, since plants are modular organisms, the bigger they are, the more reproductive organs they develop. Thus, the number of flowering stalks has a direct effect on plant fitness by its effect on the number of flowers produced. This is the case of different species which use the size of plants to attract pollen vectors (Klinkhamer et al., 1989; Klinkhamer & de Jong, 1990), including species in the *Erysimum* genus (Gómez et al., 2009; Alonso-Marcos et al., 2019). But the production of more flowers also means higher fitness values when the plant has the ability to self-pollinate

(Gerber, 1985). However, why genotypes with high number of stalks incur in positive density dependence (priority effects) and why those with low number of stalks do the opposite (coexistence) is unclear. Unfortunately, there is no prior study in the literature that has found a single trait driving the strong variation in niche differences from negative to positive as we found in our experiment. It might be the case that the number of stalks correlate with other traits promoting niche differences such as differences in phenology (Navas & Violle, 2009; Godoy & Levine, 2014) or the ability to make photosynthesis at different light irradiances (Pérez-Ramos et al., 2019). Further studies need to explore more in depth the multiple and correlated phenotypic expression and associated mechanisms that underlie these switches between positive and negative density-dependence processes, but our results are the first to highlight that variation in the number of stalks, as a surrogate of plant size, is critical to predict opposed coexistence outcomes by varying niche differences among taxa.

CONCLUSIONS

Our study provides strong evidence that genetic diversity plays a critical role in determining ecological outcomes between closely related genotypes. Contrary to expectations, diploids showed greater competitive ability than tetra and hexaploids. However, this competitive advantage did not translate to competitive dominance and the exclusion of the inferior competitors, rather, we predicted that the winner of competition depends on contingency such as the genotype that arrives first to a location. This is an interesting result that needs further consideration in future work because priority effects is not an ecological outcome considered by current theories in evolution describing the advantage of polyploids and the consequences of polyploidization for describing allopatric and sympatric populations. Moreover, we found that ploidy interacts with the degree of heterozygosity to reverse the competitive outcomes from priority effects to coexistence, which highlights the importance of keeping a diverse genetic background within genotypes to maintain in turn genetic diversity across genotypes. Although linking genetic diversity with competition outcomes can be difficult for logistical and methodological limitations, our results strongly suggest that the phenotypic expression of an easy-to-measure trait, the number of stalks, can predict such variation in ecological outcomes. In particular, pairs of genotypes that show on average more stalks incur in priority effects, whereas those with low stalks number are predicted to coexist. This study does not explore why the number of stalks, considered a surrogate of competition for space, can change competition from positive to negative density dependence (negative versus positive niche differences). Yet, our results highlight the importance of exploring the effects of genetic diversity on the interactions among genotypes because they can strongly modify their ecological dynamics compared to expectations from only responses to the environment.

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AUTHOR CONTRIBUTIONS

O.G and M.A conceptualized the experiment. A.G-M, AJMP, EO and M.A contributed to conduct the experiment and data collection. J.A.P-R led modeling and statistical analyses. J.A.P-R and O.G wrote the first draft of the manuscript and all authors provided substantial revisions to the manuscript.

DATA AVAILABILITY STATEMENT

Data is storage in XXX for review purposes, and will make it available in a public repository upon acceptance

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SÍNTESIS GENERAL

En plantas la evolución y diversificación de sistemas reproductivos es central (Ornduff, 1969; Harder and Barrett, 2006; Barrett, 2008; Barrett and Harder, 2017). Las plantas presentan una batería de complejos caracteres y estrategias que favorecen su reproducción de formas muy dispares. Pero estos cambios evolutivos y las transiciones que vemos asociadas generalmente no están controlados por un solo mecanismo, sino que es la interacción de diferentes causas la que generalmente controla los patrones que podemos ver en la naturaleza (Nosil, 2012; Nosil et al., 2016). En la presente tesis, se han analizado los mecanismos que dirigen los efectos que la ploidía ejerce sobre la diversificación y especiación en un complejo de taxones que componen un clado monofilético y mayoritariamente autógamo (Figura conceptual 2). En este contexto de estrategias reproductivas autógamas, los eventos de diversificación y especiación se consideran mínimos debido a la reducida diversidad genética que presentan estas especies. Esto ha llevado a considerar a las especies autógamas como un callejón sin salida evolutivo, generando las hipótesis de *la autogamia como callejón sin salida evolutivo* (SEDE, de sus siglas en inglés; (Takebayashi and Morrell, 2001; Igic and Busch, 2013). Esta hipótesis considera que la autogamia es un estado final obligatorio en las transiciones evolutivas entre sistemas reproductivos en plantas ya que la autofecundación favorece la transmisión de la totalidad del material genético de padres a descendientes, además de un seguro reproductivo al no tener que buscar o competir por parejas (Darwin, 1877; Fisher, 1949; Lloyd, 1979, 1992). Esto generaría una ventaja tan alta a los individuos capaces de autofecundarse que la transición hacia la exogamia sería una evolución inesperada en la naturaleza y, por tanto, la aparición de un grupo alógamo también. Hasta ahora, la ausencia de ejemplos en la naturaleza demostrando la transición desde la autogamia hacia la alogamia cimenta la aceptación por parte de la comunidad científica de que estas transiciones son imposibles.

El sistema de estudio de esta tesis es el complejo de especies *Erysimum incanum*, compuesto por taxones que habitan desde el sudeste de Francia hasta el Antiatlas marroquí (Nieto-Feliner and Clot, 1993; Fennane and Ibn-Tattou, 1999). En este complejo se han identificado especies como *Erysimum incanum,* que da nombre al grupo; *E. aurigeratum*, endémico del Pirineo; *E. wilczekianum*, endemico del Medio Atlas y la única especie con características entomófilas del grupo; y *E. repandum,* una especie ampliamente distribuida pero nativa de mediterraneo occidental, que forman un clado monofilético y autógamo. Este complejo de especies incluyen poblaciones con hasta tres niveles distintos de ploidía (diploides, tetraploides y hexaploides). Previos análisis filogenéticos basados en el cloroplasto realizados en este grupo sugieren la reciente aparición de un grupo tetraploide, *E. wilczekianum*, dentro de este clado. En esta tesis se ha evaluado el potencial de esta aparición a partir de una especie autógama desde el punto de vista de la interacción entre mecanismos genéticos y ecológicos como es el efecto del incremento de copias genéticas sobre el fenotipo y el escenario reproductivo que puede estar dirigiendo esta divergencia entre especies.

La evaluación de las barreras reproductivas entre ambas especies realizadas en este estudio apoyan esta reciente diversificación (***Capítulo 2***). La mayor intensidad de las barreras pre-polinización en comparación con las posteriores a la polinización inter-específica sugieren un estado aún incipiente del proceso de especiación (Nosil, 2012). Se ha encontrado también una fuerte asimetría (Tiffin et al., 2001; Brandvain & Haig, 2005) en la producción de semillas híbridas, cuya formación es viable principalmente cuando *E. incanum* actúa como planta receptora del polen procedente de *E. wilczekianum*. Los mecanismos genéticos y moleculares que subyacen a esta interacción entre polen y estigma también han debido verse sometidos a una transición hacia la auto-incompatibilidad (Kerwin & Smith-Huerta, 2000; Koelling & Mauricio, 2010; Broz & Bedinger, 2021). Sin embargo, la mayor contribución al aislamiento reproductivo total es la preferencia de los polinizadores, recalcando el importante papel de dinámicas ecológicas en la divergencia entre ambas especies (Nosil & Crespi, 2006; Agrawal et al., 2011; Cardona et al., 2020). Estas interacciones bióticas están mayormente influenciadas por el tamaño floral, lo que sugiere una gran influencia del sistema reproductivo en la interrupción del flujo génico entre especies (Wendt et al., 2002; Martin & Willis, 2007). Por ello, además del tamaño floral se exploraron otros atributos ampliamente relacionados en la predicción de las estrategias reproductivas predominantes en cada especie en el ***Capítulo 1***.

Se ha caracterizado el sistema reproductivo (***Capítulo 1***)de cada una de estas especies, estableciendo importantes diferencias en cuanto a depresión por endogamia, inversión reproductiva masculina y femenina tanto en valores absolutos como la comparación relativa en forma de P/O ratio, performance de la germinación de los tubos polínicos y rasgos florales estrechamente relacionados con la polinización, como es el tamaño floral y el grado de hercogamia (Goodwillie et al., 2010; Pannell, 2015). A pesar del marcado contraste que manifiesta la predominante alogamia en *E. wilczekianum*, los resultados de los rasgos mencionados mostraron variación entre ploidías e incluso entre poblaciones dentro de la especie autógama *E. incanum*. La variación inter-poblacional fue más acentuada en hexaploides, alcanzando valores positivos de depresión por endogamia así como un incremento en el tamaño floral, en la inversión reproductiva tanto masculina como femenina y en la separación entre anteras y estigma (hercogamia).

La predicción de una mayor probabilidad de eventos de polinización cruzada en poliploides es reforzada por los altos niveles de heterocigosidad encontrados en los niveles poliploides (***Capitulo 1***), así como la depresión por endogamia mostrada en hexaploides que indican una purga incompleta de los alelos recesivos deletereos mediante la reproducción autógama (Allard, 1975). Los cambios observados en ploidías mayores sobre tamaño floral e inversión reproductiva aumentan, a su vez, la probabilidad de eventos de polinización cruzada. Los resultados encontrados respecto a la cantidad relativa entre polen y óvulos (referido como P/O ratio a lo largo del texto) llaman especialmente la atención por ser un rasgo ampliamente descrito en la literatura por su veracidad como predictor del sistema reproductivo predominante de una especie (Cruden, 1977). Al contrario de lo que cabría esperar, hemos encontrado altos valores de este ratio en *E. incanum* **(*Capítulo 3*)**, lo que indicaría que la transferencia de polen no es tan eficiente en poliploides como debería tratándose de una especie autógama. Además de la variación en la magnitud del ratio P/O, este parece tener un efecto positivo sobre los gradientes de selección que actúan sobre la hercogámia. El resultado de este proceso de selección se traduce en poblaciones cuyas flores muestran una mayor separación entre anteras y estigma y, por tanto, dificultado la auto-polinización mientras invierten una mayor cantidad de recursos en la producción de polen en comparación con los óvulos (***Capítulo 3***).

Los individuos procedentes de los eventos de polinización cruzada deben estar sometidos a mecanismos que permitan su establecimiento y mantenimiento durante el proceso de divergencia ecológica. El incremento de diversidad genética tras la reproducción cruzada puede permitir un mayor potencial adaptativo al medio, especialmente a las perturbaciones ambientales (Baker, 1955; Hiscock, 2000; Pannell, 2015; Grossenbacher et al., 2017). La plasticidad fenotípica juega un papel importante en la mitigación de los cambios ambientales y en los procesos de colonización de nuevos hábitats además de aportar diversidad fenotípica sin cambios en el genotipo (Yeh and Price, 2004; Ghalambor et al., 2007; Nicotra et al., 2010). No se encontró un efecto de la ploidía sobre la magnitud de la plasticidad fenotípica pero sí sobre su heredabilidad (***Capítulo 4***). Sin embargo, la plasticidad fenotípica se vio influenciada por el módulo funcional en el que agruparon los caracteres (de Kroon et al., 2005), siendo mayor en aquellos módulos relacionados con la inversión reproductiva mientras que los rasgos florales se mostraron mayormente canalizados. Los costes y beneficios de la plasticidad fenotípica se evaluaron mediante su efecto sobre la eficacia biológica (Dewitt et al., 1998; Van Buskirk and Steiner, 2009; Auld et al., 2010), revelando un efecto positivo y por tanto, un mayor potencial adaptativo en los módulos de carácteres más plásticos mientras que la plasticidad de los carácteres florales tuvieron un efecto negativo sobre la eficacia biológica. Este patrón se acentuó en poliploides, recalcando el potencial adaptativo de la plasticidad sobre la inversión reproductiva, especialmente en la producción de polen, la cual tiene un efecto importante en la trayectoria evolutiva del sistema reproductivo (Delesalle et al., 2008). Sin embargo, la plasticidad fenotípica queda constreñida por la integración entre rasgos fenotípicos (Gianoli and Palacio-López, 2009). Esta limitación estuvo dirigida por una mayor heredabilidad de los caracteres fenotípicos que de su plasticidad y por una mayor intensidad de selección sobre la correlación de estos que sobre sus plasticidades (***Capítulo 5***). Esto sugiere que la heredabilidad y selección natural podrían ser parte de los mecanismos que subyacen a la relación inversa entre plasticidad e integración fenotípica. La elevada heredabilidad de los rasgos reproductivos además de variación intra-específica y los efectos positivos sobre la eficacia biológica permite la actuación de la selección natural como agente promotor de diversificación dentro de la especie *E. incanum* (Thompson, 1991; Via, 1993; Nussey et al., 2005; Ghalambor et al., 2007).

Por último, la diversidad observada en la naturaleza es el resultado de dinámicas poblacionales y de interacción entre especies que permiten la coexistencia o los procesos de competencia y exclusión entre especies (Chesson, 2000). Las poblaciones diploides de *E. incanum* mostraron una mayor capacidad competidora debido a una mayor producción de descendencia y una menor sensibilidad a la competencia (***Capítulo 6***), lo cual, junto a los beneficios derivados de la auto-polinización, ha podido permitir una mayor expansión reflejada en la amplia distribución de esta ploidía a lo largo de dos continentes en contraposición a la estrecha distribución de las poblaciones hexaploides. Estos resultados se obtuvieron en condiciones estables que coinciden con las condiciones naturales de las poblaciones diploides en la naturaleza, mientras que las poblaciones hexaploides habitan en zonas con condiciones más extremas. Sin embargo, la diferencia en la habilidad competidora no se traduce en dinámicas de competencia y exclusión entre ploidías. Al contrario de lo que cabría esperar, los experimentos de interacción entre individuos de distinta ploidía revelaron la existencia de efectos prioritarios, lo que permite el establecimiento de poblaciones con cierta ploidía por eventos azarosos como el orden de llegada (Fragata et al., 2022). En cuanto a la interacción de individuos con niveles diferentes de heterocigosidad, se encontraron también efectos prioritarios y dinámicas de coexistencia en cualquier combinación entre ploidía y nivel de heterocigosidad (***Capítulo 6***). Estos resultados manifiestan la posibilidad de establecimiento de los individuos producto de eventos de la polinización cruzada, los cuales pueden ser más probables en poliploides debido al incremento de los atributos relacionados con la atracción de polinizadores y transferencia alógama de polen (Alonso-Marcos et al., 2019; Yao et al., 2019; Liu et al., 2021).

La combinación de los resultados expuestos en cada capítulo de esta tesis apoyan la viabilidad de alternativas a la reproducción autógama en *E. incanum* promovida por los efectos del incremento cromosómico sobre un amplio abanico de atributos relacionados con la polinización en plantas, y sobre la interacción de estos entre sí. Los resultados aquí descritos podrían ser la base ecológica de las primeras etapas de divergencia dentro de *E. incanum*, rompiendo con el paradigma del escaso potencial evolutivo de sistemas autógamos. Los mecanismos genéticos y ecológicos tienen sin duda un efecto promotor en la trayectoria evolutiva de este sistema de estudio, ayudando a entender las consecuencias evolutivas que resultan en los procesos de especiación y divergencia que alimentan la diversidad de especies que se observan en la naturaleza.

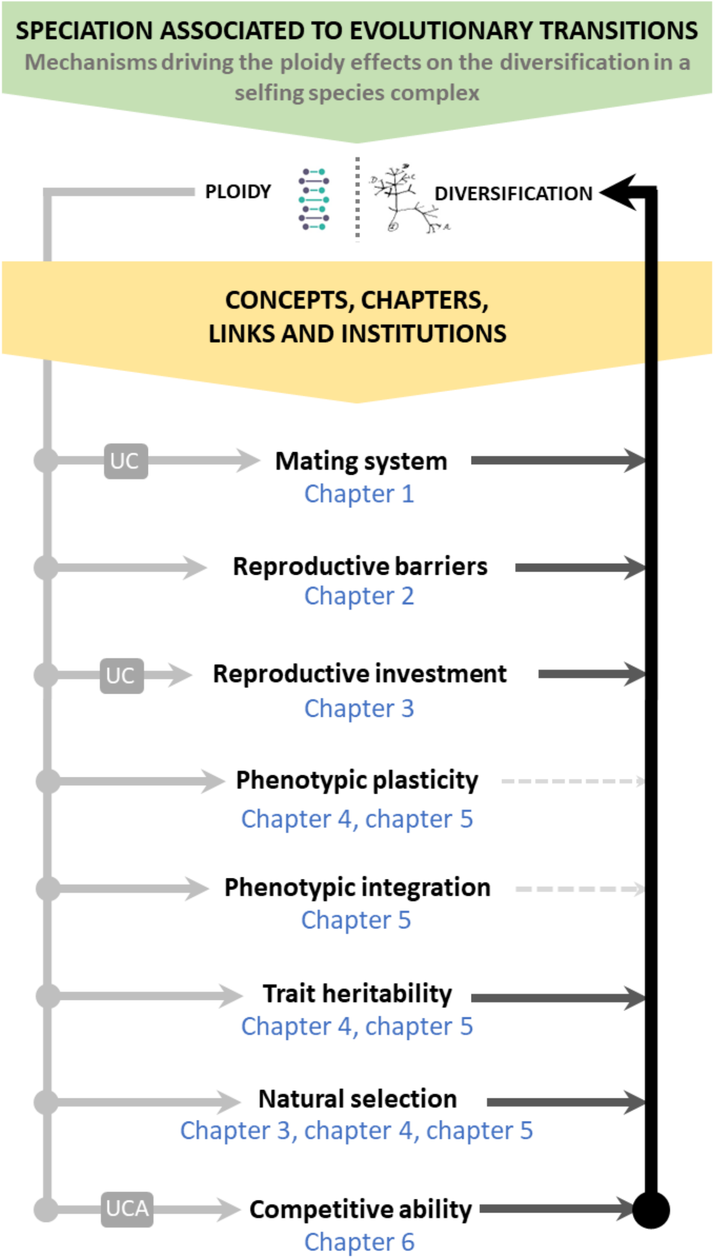

Figura S.3: Representación esquemática de los conceptos, capítulos y conexiones establecidas entre ellos en la presente tesis. En negro se destacan los mecanismos y fenómenos que tienen un efecto significativo sobre la divergencia y diversificación, mientras que en gris con linea discontinua los que no. También se indican las instituciones con las que se ha colaborado para el desarrollo de alguno de los capítulos: Universidade de Coimbra (UC) y Universidad de Cádiz (UCA).

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CONCLUSIONES GENERALES

1. El complejo de especies *Erysimum incanum* presenta además de los dos niveles de ploidía descritos hasta ahora (diploide y tetraploide) un tercer nivel hexaploide endémico de las cordilleras del Alto Atlas y el Antiatlas marroquí. Asimismo, hemos identificado que la ploidía de *E. wilczekianum* coincide con la ploidía del nivel tetraploide en el complejo *E. incanum*.

2. En el mencionado complejo hemos caracterizado el sistema reproductivo de *Erysimum wilczekianum* como eminentemente alógamo. Conjuntamente, hemos identificado un gradiente desde sistemas reproductivos autógamos a otros alógamos mixtos asociado al aumento de ploidía en *E. incanum* y ligado a cambios en caracteres reproductivos primarios y secundarios.

3. Dentro del nivel de ploidía en el que se ha dado el fenómeno de divergencia evolutiva más evidente y destacado (la aparición de *E. wilczekianum*) se han identificado y cuantificado barreras asimétricas a la reproducción entre ambas especies, a pesar de que estas se encuentran en peripatría, estrechamente ligadas al contraste en sistema reproductivo.

4. Los híbridos obtenidos entre *E. incanum* y *E. wilczekianum* presentan un fenotipo similar al de *E. incanum*, lo cual sugiere que los alelos particulares del genoma de *E. incanum* son dominantes o que existe un efecto materno significativo asociado al genoma de *E. incanum*.

5. Existe un cambio significativo en la inversión reproductiva masculina a medida que aumenta la ploidía, pero sin embargo no existe un *trade-off* entre inversión masculina y femenina. Además de producir más polen, las poblaciones hexaploides mostraron un mayor grado de hercogamia, lo que también sugiere la existencia de estrategias reproductivas alternativas a la autogamia en ploidía superiores.

6. Las diferencias en ploidía no tienen efecto sobre la plasticidad fenotípica exhibida por los caracteres vegetativos, reproductivos y florales medidos sobre las plantas, aunque sí sobre la heredabilidad de dicha plasticidad, que aumenta con la ploidía. Los caracteres reproductivos fueron los más plásticos mientras que los pertenecientes al módulo floral fueron los más canalizados.

7. Existe una relación negativa entre la integración fenotípica de los caracteres y su plasticidad, la cual no se ve afectada por la ploidía ni por el tipo de ambiente en el que las plantas crecieron.

8. Los caracteres fenotípicos considerados en la presente Tesis mostraron una heredabilidad mayor que la heredabilidad de la plasticidad de estos cuando se expresan en ambientes diferentes. Esto hace que la respuesta a la selección por dichos caracteres fenotípicos sea mayor que la respuesta a la selección por parte de la plasticidad fenotípica.

9. La selección natural actúa con mayor intensidad sobre la correlación de los caracteres fenotípicos que sobre sus plasticidades, lo que sugiere el papel de la selección natural como uno de los principales mecanismos involucrados en el efecto limitante de la integración fenotípica sobre la plasticidad.

10. Los patrones de selección natural cuantificados sobre la plasticidad fenotípica de poliploides indican que ésta tiene un potencial adaptativo, al menos para caracteres relacionados con los módulos vegetativo y reproductivo, mientras que es neutro o maladaptativo para los caracteres florales y en general para las plantas diploides.

11. Los caracteres sometidos a selección y que por tanto afectan al éxito reproductivo de las plantas parecen diferir entre las ploidías descritas en *E.*

incanum. Dichos patrones selectivos están limitados por caracteres como la inversión relativa en óvulos y polen.

12. Las plantas diploides tendieron a mostrar una habilidad competitiva superior a las demás ploidías, principalmente asociada a una mayor capacidad de producir escapos florales, aunque esta no se tradujo en dinámicas de exclusión de las demás ploidías. De hecho, se encontraron escenarios de efectos prioritarios y coexistencia entre plantas de cualquier ploidía y nivel de heterocigosidad.

13. Finalmente, en el presente trabajo de Tesis demostramos que un complejo de especies autógamo, como el de *Erysimum incanum*, presenta un importante potencial evolutivo, incluyendo fenómenos de divergencia evolutiva y especiación que tradicionalmente no se han asociado a especies o clados autógamos. Seguir explorando dichos fenómenos en este complejo de especies podría ayudarnos a entender mejor los mecanismos de subyacen a las dinámicas de especiación asociadas a transiciones evolutivas en plantas, incluso aquellas que no son predichas por el paradigma evolutivo vigente.

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