Mario Blanco Sánchez
Tesis doctoral

Genetic variation and phenotypic plasticity in Mediterranean gypsum specialists: insights into climate change responses

Universidad Rey Juan Carlos

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Mario Blanco Sánchez

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Adrián Escudero Alcántara

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RESUMEN

Antecedentes:

El cambio climático está alterando los patrones de temperatura y precipitación, siendo una gran amenaza para las poblaciones de plantas a nivel global. Se ha predicho que los efectos del cambio climático serán especialmente acusados en la región mediterránea, afectando intensamente a sus poblaciones de plantas. Una respuesta bien documentada para hacer frente al cambio climático es la migración hacia condiciones favorables. Sin embargo, dicha migración puede estar limitada en especies con alta especificidad por el sustrato, poca capacidad dispersiva y/o distribuciones fragmentadas, como es el caso de los gipsófitos estrictos (plantas restringidas a suelos de yeso). Cuando la migración es limitada, las poblaciones deben responder localmente para hacer frente a las nuevas condiciones ambientales impuestas por el cambio climático, y así evitar la extinción. En consecuencia, los procesos evolutivos *in situ* —evolución adaptativa y plasticidad fenotípica— son fundamentales para garantizar la supervivencia y persistencia de las poblaciones de gipsófitos en un contexto de cambio climático. Las respuestas adaptativas futuras de las poblaciones al cambio climático están influenciadas por procesos evolutivos que ocurrieron en el pasado, tanto neutrales como adaptativos. Además, estas repuestas futuras también dependen de la fuerza y dirección de la selección natural y el potencial evolutivo de los rasgos funcionales y la plasticidad fenotípica. Por lo tanto, esta tesis estudia la ecología evolutiva de los gipsófitos ibéricos, para comprender cómo responderán sus poblaciones al cambio climático.

Objetivos:

El objetivo general de esta tesis es proporcionar una visión global sobre la ecología evolutiva de las plantas mediterráneas endémicas de yeso (gipsófitos), para conocer el papel relativo de los diferentes factores que afectan a las respuestas de sus poblaciones al cambio climático.

Específicamente, el capítulo 1 evalúa los procesos filogeográficos asociados con el origen, la diversidad y la estructura genética del gipsófito *Lepidium subulatum* en poblaciones a lo largo de su rango de distribución. El capítulo 2 identifica las huellas de la selección pasada sobre los rasgos funcionales y su plasticidad, y las presiones selectivas que impulsaron la diferenciación poblacional adaptativa a lo largo del rango de distribución de *L. subulatum*, lo que permitió la identificación de poblaciones potencialmente vulnerables al cambio climático. El capítulo 3 caracterizó los patrones de selección en condiciones naturales en *Centaurea hyssopifolia* y *Helianthemum squamatum*, considerando la fuerte variación espaciotemporal de los ecosistemas de yeso. Finalmente, el capítulo 4 evalúa la potencial respuesta a la selección de rasgos funcionales y su plasticidad en *C. hyssopifolia* y *H. squamatum* bajo condiciones que simulan el incremento de aridez asociado con el cambio climático. Los resultados de esta tesis aportan información clave para comprender cómo los procesos evolutivos *in situ*, evolución adaptativa y plasticidad fenotípica, pueden mitigar los efectos negativos del cambio climático en las poblaciones de especies gipsófitas.

Métodos:

Usando un enfoque multidisciplinar que combina estudios de selección fenotípica en condiciones naturales, experimentos de jardín común, aproximaciones de genética cuantitativa, y análisis moleculares, obtuvimos una amplia visión sobre la ecología evolutiva de los gipsófitos mediterráneos. Esto nos permitió predecir las respuestas de las poblaciones de gipsófitos y su persistencia futura en un contexto de cambio climático. En concreto, esta tesis se centró en tres especies gipsófitas dominantes de la Península Ibérica, *Lepidium subulatum* L. (Brassicaceae), *Helianthemum squamatum* (L.) Dum. Cours (Cistaceae) y *Centaurea hyssopifolia* Vahl. (Asteraceae).

En concreto, el uso de jardines comunes que simulan condiciones realistas de cambio climático ha sido clave a lo largo de la tesis. Estos jardines comunes al aire libre nos han permitido detectar la presencia de diferenciación poblacional adaptativa, los patrones de selección y plasticidad de estas especies, y la presencia de variación genética para los rasgos y su plasticidad en dichos ambientes. Gracias a esta robusta aproximación experimental, esta tesis proporciona información fiable acerca de las consecuencias ecológicas y evolutivas del cambio climático para las poblaciones de gipsófitos estrictos.

Resultados:

Capítulo 1:

Lepidium subulatum surge ~3 millones de años, asociado a los procesos de aridificación y al origen de los suelos de yeso durante el Plio-Pleistoceno. *Lepidium subulatum* muestra una estructura genética más acusada en marcadores cloroplásticos que en marcadores nucleares, lo que indica la baja capacidad de dispersión a través de semillas de esta especie. En cambio, el flujo de polen no parece estar limitado entre las diferentes poblaciones y regiones. A pesar de ser un endemismo edáfico, *L. subulatum* posee una gran diversidad genética, probablemente asociada con su origen relativamente antiguo y al elevado y constante número de individuos dentro de cada población durante la historia evolutiva de la especie.

Capítulo 2:

Nuestros resultados mostraron diferenciación genética cuantitativa entre las poblaciones de *Lepidium subulatum* a consecuencia de selección divergente pasada. Dichos eventos de selección pasada estuvieron asociados a las diferencias climáticas entre las poblaciones. Los individuos de poblaciones más cálidas y secas mostraron fenotipos asociados tanto a una estrategia conservadora como a una estrategia adquisitiva de recursos, lo que sugiere que la

evolución de un síndrome adaptativo en las poblaciones más duras no se ha visto limitado por correlaciones genéticas entre rasgos. Además, los individuos de poblaciones climáticamente más húmedas y frías mostraron consistentemente una menor biomasa reproductiva, indicando la vulnerabilidad de estas poblaciones frente al cambio climático. Sorprendentemente, la diferenciación poblacional no se asoció con diferencias en la composición del suelo, demostrando que el clima, y no el contenido en yeso, ha sido un fuerte motor para la evolución de la especie. Por último, observamos que los patrones de plasticidad fenotípica fueron muy similares entre las poblaciones para todos los rasgos evaluados, probablemente fruto de selección homogeneizadora en plasticidad debido a la alta heterogeneidad ambiental experimentada en todas las poblaciones.

Capítulo 3:

En condiciones naturales, los rasgos bajo selección no variaron en gran medida entre años ni entre las diferentes laderas de los cerros de yeso, pese a las condiciones climáticas y ambientales tan contrastadas que experimentaron las plantas. No obstante, la magnitud de la selección fue más fuerte en el año climáticamente más restrictivo, no encontrando prácticamente variación entre laderas. La selección natural a través de la reproducción favoreció a aquellas plantas con fenología adelantada, baja eficiencia en el uso del agua, alta área foliar específica y altos valores de N foliar, en ambos años, laderas y especies. Nuestros resultados mostraron que, en contra de nuestras expectativas, la selección natural a través de la reproducción favoreció consistentemente una estrategia adquisitiva para escapar de la sequía, en lugar de una estrategia conservadora de uso de recursos, incluso en condiciones de mayor estrés abiótico. Esta estrategia adquisitiva podría permitir un desarrollo rápido al maximizar la asimilación de recursos, que aseguraría la reproducción antes de las condiciones climáticas más limitantes de mediados y final del verano. Sin embargo, la supervivencia de los individuos no se vio afectada

ni por el fenotipo de los individuos ni por las condiciones microambientales que experimentaron.

Capítulo 4:

En ambas especies, *H. squamatum* y *C. hyssopifolia*, observamos un mayor número de rasgos bajo selección en el tratamiento de sequía que simulaba el aumento de aridez producido por el cambio climático. Sin embargo, encontramos diferencias entre especies en las estrategias adaptativas de uso de recursos y en su variación genética. En *H. squamatum*, una estrategia de escape a la sequía, caracterizada por una fenología reproductiva adelantada y una mayor tasa de crecimiento, se asoció positivamente con el éxito reproductivo en condiciones de sequía. Además, la mayoría de los rasgos adaptativos mostraron variación genética. En *C. hyssopifolia*, la selección natural favoreció una estrategia de tolerancia a la sequía, con hojas más gruesas y fenologías duraderas en condiciones de baja disponibilidad hídrica. Sin embargo, todos los rasgos carecieron de variación genética, lo que sugiere su limitado potencial evolutivo. Para ambas especies, la mayoría de los rasgos exhibieron plasticidad fenotípica en respuesta a la sequía y variación genética para la plasticidad, lo que indica que la plasticidad puede ser un mecanismo muy importante para hacer frente a la sequía. Nuestros resultados mostraron que la plasticidad puede evolucionar independientemente de la evolución de los valores medios de los rasgos, lo que contribuye a nuestra comprensión de las posibles respuestas al cambio climático en estas especies.

Conclusiones:

Los resultados de esta tesis proporcionan información clave acerca de los procesos y factores relacionados con el origen y la diferenciación poblacional de las plantas endémicas de yeso de la Península Ibérica. Concretamente, mostraron la importancia de los procesos geológicos y

paleoclimáticos que ocurrieron durante el Plio-Pleistoceno en la cuenca mediterránea para la especiación y expansión de los gipsófitos ibéricos. Además, la reducida dispersión de semillas encontrada puede limitar la migración de las poblaciones de gipsófitos, destacando la importancia de los procesos evolutivos *in situ*, evolución por selección natural y plasticidad fenotípica, para hacer frente al cambio climático. Los diferentes gipsófitos estudiados en esta tesis mostraron altos niveles de variación genética neutral y cuantitativa dentro y entre poblaciones, mostrando la habilidad de sus poblaciones para responder a presiones selectivas pasadas y futuras. Por tanto, la especialización al yeso no ha resultado en "callejones sin salida evolutivos". Encontramos que eventos de selección pasada relacionados con diferencias climáticas entre poblaciones han creado un patrón de diferenciación poblacional adaptativa en *Lepidium subulatum*. Concretamente, las poblaciones mostraron notables diferencias tanto en sus rasgos como en su eficacia biológica, con poblaciones húmedas y frías mostrando bajo éxito reproductivo, especialmente en condiciones de sequía. Estos resultados mostraron la vulnerabilidad de estas poblaciones en un contexto de cambio climático. Además, esta tesis también proporciona información clave acerca de los patrones de selección y la presencia de variación genética para los rasgos y su plasticidad en gipsófitos a nivel intrapoblacional. En condiciones naturales, la selección natural favoreció consistentemente una estrategia adquisitiva de escape a la sequía, permitiendo el rápido desarrollo de los individuos y su reproducción antes de las condiciones climáticas más restrictivas del verano. Bajo condiciones que simulan el aumento de aridez del cambio climático, el potencial evolutivo de los rasgos varió entre especies. En cambio, la plasticidad fenotípica siempre mostró un amplio potencial evolutivo, destacando su importancia en las futuras respuestas evolutivas. En resumen, los resultados de esta tesis proporcionan información crucial para entender cómo la evolución adaptativa y la plasticidad fenotípica van a interactuar para modelar las respuestas de los gipsófitos al cambio climático.

SUMMARY

Background:

Climate change is altering global patterns of temperature and precipitation, being a major threat for plant populations worldwide. The negative effects of climate change are expected to be greater in the Mediterranean region, intensely affecting plant populations. A well-documented response to cope with climate change is migration to find suitable conditions. However, migration may be particularly challenging for species with specific edaphic requirements, poor dispersal ability, and fragmented distributions such as gypsophiles, plants restricted to gypsum soils. When migration is limited or unfeasible, populations need to respond locally to cope with the new environmental conditions imposed by climate change and avoid extinction. Consequently, *in situ* evolutionary processes, i.e., adaptive evolution and phenotypic plasticity, are critical to guarantee the survival and persistence of gypsophile populations in a climate change context. Future adaptive responses of populations to climate change are not only influenced by past neutral and adaptive evolutionary processes, but also depend on the strength and direction of natural selection and the evolutionary potential of functional traits and their plasticity. Therefore, this thesis studies the evolutionary ecology of Iberian gypsophiles, to understand how the populations of these species will respond to climate change.

Objectives:

The main objective of this thesis is to provide a comprehensive picture of the evolutionary ecology of endemic Mediterranean gypsum plants (gypsophiles), to predict how their populations will respond to climate change, and understand the relative role of the different factors affecting such responses. Specifically, chapter 1 evaluates the phylogeographic processes associated with the origin, genetic diversity, and genetic structure of populations of the dominant gypsophile *Lepidium subulatum* throughout its entire distribution range. Chapter

2 identifies footprints of past selection on functional traits and their plasticity, and the selection pressures driving adaptive population differentiation throughout the distribution range of *L. subulatum*, allowing the identification of potentially vulnerable populations to climate change. Chapter 3 assesses the patterns of selection under natural conditions in *Centaurea hyssopifolia* and *Helianthemum squamatum*, considering the high spatiotemporal variation of gypsum ecosystems. Finally, chapter 4 evaluates the potential response to selection of functional traits and their plasticity in *C. hyssopifolia* and *H. squamatum* under conditions that simulate the increment of aridity predicted with climate change. The results of this thesis provide crucial information to understand how *in situ* evolutionary processes, adaptive evolution and phenotypic plasticity, can mitigate the negative effects of climate change on gypsophile populations.

Results:

Chapter 1:

The origin of *Lepidium subulatum* was dated ~3 Mya, likely associated with the geological paleoclimatic events of the Mediterranean basin around the Plio-Pleistocene. This species showed a higher genetic structure in chloroplast markers than in nuclear markers, indicating the limited dispersal capacity of the species via seeds. In contrast, pollen flow was not limited across different populations and regions. In spite of being an edaphic endemism, *Lepidium subulatum* showed high levels of genetic diversity, likely related to its relatively old age, the high effective number of individuals per population, and the lack of demographic changes during the evolutionary history of the species throughout its range.

Chapter 2:

Our results showed significant quantitative genetic differentiation among populations of *Lepidium subulatum* due to past divergent selection. Importantly, past selection events were associated with climatic differences between populations. Individuals from climatically harsher populations showed phenotypes associated with both a conservative and an acquisitive resource-use strategy, suggesting that the evolution of an adaptive syndrome has not been genetically constrained. In addition, individuals from wetter and colder populations consistently showed lower reproductive fitness, suggesting the vulnerability of milder populations in a climate change context. Surprisingly, population differentiation was not associated with differences in soil chemical composition, highlighting that climate, but not gypsum content, was the main selective pressure driving the evolution of this species. Finally, patterns of phenotypic plasticity were surprisingly similar between the populations for all traits evaluated, probably due to homogenizing selection in plasticity related with high environmental heterogeneity across populations.

Chapter 3:

In natural conditions, selection patterns did not greatly vary between years or slopes of gypsum hills in both species (*Centaurea hyssopifolia* and *Helianthemum squamatum*), despite the high spatiotemporal variation in the climatic and environmental conditions experienced by plants. However, the magnitude of selection was stronger in the most climatically restrictive year. Natural selection through reproduction favored plants with advanced phenology, low water use efficiency, high specific leaf area, and high leaf N content in both years, slopes, and species. In contrast to our expectations, natural selection through reproduction consistently favored an acquisitive, drought-escape strategy, even under highly-restrictive environmental conditions. Such acquisitive strategy allowed the rapid development of individuals by maximizing resource

assimilation, favoring reproduction before the most limiting conditions of mid-late summer. However, individual survival was not affected by either their phenotypes or the microenvironmental conditions experienced.

Chapter 4:

In both species, *H. squamatum* and *C. hyssopifolia*, we found a higher number of traits under selection in the drought treatment that simulated the increase of aridity of climate change. However, we found differences between species in their adaptive strategies, and more importantly, in the presence of quantitative genetic variation of adaptive traits. In *H. squamatum*, a drought escape strategy, characterized by an advanced phenology and higher growth rates, was positively associated with reproductive fitness under drought conditions. Furthermore, most adaptive traits showed significant genetic variation. In contrast, natural selection favored a drought-tolerance strategy in *C. hyssopifolia*, with thicker leaves and longer phenologies associated with higher fitness under drought conditions. However, all traits lacked quantitative genetic variation, suggesting their limited evolutionary potential. For both species, most traits exhibited phenotypic plasticity in response to drought and genetic variation for plasticity, indicating that plasticity may be a very important mechanism to cope with drought. Our results showed that phenotypic plasticity may evolve independently of the evolution of traits in gypsophiles, contributing to our understanding of potential responses to climate change in these species.

Conclusions:

The results of this thesis provide crucial information on the processes and factors related with the origin and population differentiation of Iberian gypsum endemic plants. Specifically, geological and paleoclimatic events that occurred in the Plio-Pleistocene within the

Mediterranean basin were crucial for the speciation and expansion of Iberian gypsophiles. Furthermore, the limited seed dispersal found may constrain migration to suitable habitats in these gypsophile populations, highlighting the importance of *in situ* evolutionary processes, adaptive evolution and phenotypic plasticity, to cope with climate change. The different gypsophile species studied showed high neutral and quantitative genetic variation both within and across populations, indicating their ability to respond to past and future selective pressures. Therefore, gypsum specialization has not led to evolutionary dead-end species in Iberian gypsophiles. Past selection events related with climatic differences among populations have driven adaptive population differentiation in *Lepidium subulatum*. Populations showed significant phenotypic differences. Specifically, cold and humid populations showed very low reproductive output, especially under drought conditions, highlighting the vulnerability of these populations in a climate change context. This thesis also provides crucial information about the patterns of selection and the presence of genetic variation for traits and their plasticity in gypsophiles at the intrapopulation level. In natural conditions, natural selection consistently favored an acquisitive, drought-escape strategy, allowing the rapid development of individuals and their reproduction before the most limiting conditions of mid-late summer. Under experimental conditions that simulated the increment of aridity of climate change, the evolutionary potential of ecophysiological traits varied across gypsophile species. In contrast, phenotypic plasticity consistently showed high evolutionary potential, highlighting its importance in future evolutionary responses. Overall, the results of this thesis provide crucial information to understand how adaptive evolution and phenotypic plasticity interact to shape the evolutionary responses of gypsophiles to climate change.

General Introduction:

GENERAL INTRODUCTION

Responses of plant populations in a climate change context

Earth is experiencing anthropogenic changes at unprecedented rates, and global change drivers have been identified as major threats to biodiversity worldwide (Sage, 2020; Sala et al., 2000). Global change involves all human-mediated impacts that alter the global composition and functioning of ecosystems (Matesanz et al., 2010; Sage, 2020; Sala et al., 2000; Vitousek, 1992). Mediterranean ecosystems are especially vulnerable to biodiversity loss due to their high sensitivity to several global change drivers such as land use and climate change (Matesanz and Valladares, 2014; Sala et al., 2000). Specifically, climate change is altering the global patterns of temperature and precipitation, but this alteration is expected to be greater in areas with dry summers and semiarid climatic conditions such as the Mediterranean Region (IPCC, 2022). Furthermore, climate change in Mediterranean ecosystems not only involves changes in mean temperature and precipitation, but also higher climatic variability, unpredictability, and frequency of extreme events such as heatwaves and severe droughts (Giorgi and Lionello, 2008; IPCC, 2022; Sage, 2020). Since climate change will impose novel environmental conditions, populations might likely not express an adaptive phenotype in these new climatic conditions (Franks et al., 2014; Shaw and Etterson, 2012). Therefore, assessing the active responses of populations to cope with climate change and avoid extinction is of paramount importance nowadays (Bonamour et al., 2019; Fox et al., 2019; Jump and Peñuelas, 2005; Shaw and Etterson, 2012).

A well-documented response to cope with climate change is migration in latitude or altitude, in order to find suitable living conditions (Fig. 1; Aitken et al., 2008; Jump and Peñuelas, 2005; Nicotra et al., 2010; Shaw and Etterson, 2012). Several studies have reported changes in the distribution range of a wide variety of species as a consequence of climate change (reviewed in Parmesan, 2006; Parmesan and Hanley, 2015; Parmesan and Yohe, 2003; Shaw

and Etterson, 2012). However, migration may be particularly challenging for sessile organisms like plants, especially in species with poor dispersal ability, long life cycles, and/or those inhabiting fragmented and isolated habitats (Berg et al., 2010; Chevin and Hoffmann, 2017; Kremer et al., 2012; Leimu et al., 2010). In these cases, migration is expected to be slower than the rate of environmental change and hence, insufficient to track suitable environmental conditions (Davis and Shaw, 2001; Dullinger et al., 2012; Thuiller et al., 2005). When migration is limited or unfeasible, populations need to respond locally to cope with the new environmental conditions and avoid extinction (Chevin and Hoffmann, 2017; Franks et al., 2014; Jump and Peñuelas, 2005; Meyers and Bull, 2002; Van Kleunen and Fischer, 2005). Therefore, *in situ* evolutionary processes, i.e., adaptive evolution and phenotypic plasticity (Fig. 1), are crucial to guarantee the survival and persistence of populations with limited dispersal ability in a climate change context (Aitken et al., 2008; Anderson et al., 2012a; Chevin and Hoffmann, 2017; Franks et al., 2014; Gienapp et al., 2008; Jump and Peñuelas, 2005). Importantly, despite some of these responses may occur at the individual level (and even other hierarchical levels; see a deeper discussion in section III in Losos, 2014), populations are the entities that ultimately persist or perish.

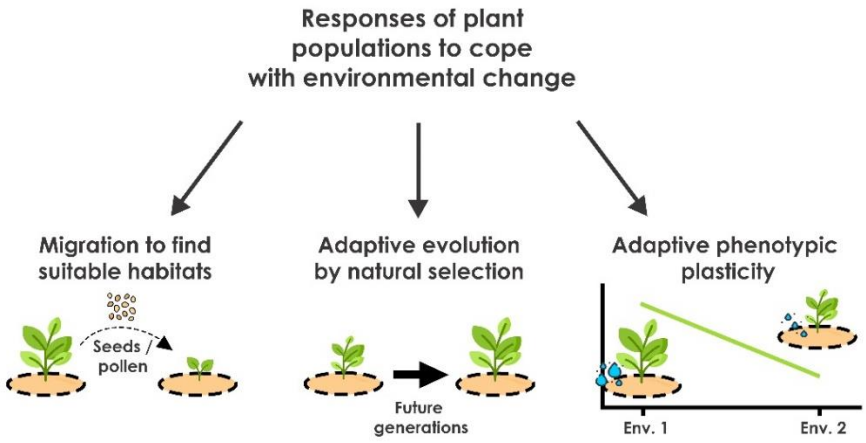

Figure 1: Potential responses of plant populations to cope with environmental changes, including climate change, and avoid extinction. Importantly, migration, adaptive evolution, and phenotypic plasticity are non-exclusive processes, and populations can respond to climate change through a complex combination of these mechanisms.

In the new environmental conditions imposed by climate change, populations can locally persist through adaptive evolution by natural selection, phenotypic plasticity, or a combination of both processes (Franks et al., 2014; Gienapp et al., 2008; Nicotra et al., 2010). Adaptive evolution is defined as a genetically-based phenotypic change driven by natural selection, and may be critical to cope with environmental change (Gienapp et al., 2008; Leimu et al., 2010; Merilä, 1997). Particular trait values are favored by natural selection (i.e., they are adaptive) when they have a significant effect on individual fitness (Ackerly et al., 2000; Dobzhansky, 1956; Kremer et al., 2012). In addition, for evolution by natural selection to occur, there must be genetic variation in adaptive traits (heritable phenotypic variation). In other words, phenotypic differences must be related with genetic differences between individuals, being the presence of both intraspecific phenotypic and genetic variation prerequisites for evolution by natural selection (Conner and Hartl, 2004; Endler, 1986; Lenormand, 2002; Sih, 2004). Consequently, adaptive evolution can maintain or enhance survival and/or reproductive output (i.e., fitness) in response to environmental changes, including climate change (Leimu et al., 2010; Morrissey et al., 2012). Furthermore, growing evidence supports that rapid adaptive evolution in response to climate change is occurring in plant populations, highlighting its importance in the adaptation to such novel conditions (Franks et al., 2007; Hoffmann and Sgró, 2011; Kingsolver et al., 2012; Kruuk et al., 2003; Matesanz and Valladares, 2014; Shaw and Etterson, 2012).

Phenotypic plasticity is the ability of a genotype to produce different phenotypes in different environments, and is considered the main mechanism to cope with environmental heterogeneity (Ackerly et al., 2000; Fox et al., 2019; Pigliucci, 2005, 2001; Valladares et al., 2007; Van Kleunen and Fischer, 2005). Although plasticity is ubiquitous in nature, not all plastic changes might be considered adaptive (Ackerly et al., 2000; Dorn et al., 2000; Nicotra et al., 2010; Nicotra and Davidson, 2010; Pigliucci, 2001; Valladares et al., 2007; Van Kleunen

and Fischer, 2005). Phenotypic plasticity is adaptive when it maintains or increases individual fitness (Kelly, 2019; Matesanz et al., 2010; Nicotra et al., 2010; Sultan, 2000; Via et al., 1995). In contrast, some plastic responses are merely inevitable responses attributed to resource limitation, and others can even be maladaptive (Chevin and Hoffmann, 2017; Ghalambor et al., 2007; Matesanz and Valladares, 2014; Nicotra et al., 2010; Pigliucci, 2001; Sultan, 2000; Valladares et al., 2007; Van Kleunen and Fischer, 2005). Since adaptive plasticity can cause rapid phenotypic changes, it is likely crucial in the short term of an environmental change, and consequently, may be of paramount importance to buffer climate change (Fig. 1; Anderson et al., 2012a; Fox et al., 2019; Matesanz et al., 2010; Nussey et al., 2007; Van Kleunen and Fischer, 2005). Furthermore, genotypes may differ in their phenotypic responses across environments (i.e., genetic variation for plasticity; often measured and referred to as a genotype-by-environment interaction or G × E), indicating that phenotypic plasticity is a genetically-based trait (Ghalambor et al., 2007; Pigliucci, 2001; Van Kleunen and Fischer, 2005). Consequently, if there is significant genetic variation for plasticity and different plastic responses result in fitness differences (i.e., plasticity is adaptive), phenotypic plasticity might evolve by natural selection (Matesanz et al., 2010; Matesanz and Valladares, 2014; Pfennig, 2021; Pigliucci, 2001; Weijschedé et al., 2006). Therefore, adaptive phenotypic plasticity can be also an important mechanism to deal with long-term environmental change (Matesanz et al., 2010; Nicotra et al., 2010; Pfennig, 2021). Although adaptive plasticity is favored by natural selection, it occurs less often than expected in nature, suggesting the presence of trade-offs or costs of plasticity (Tonsor et al., 2013; Valladares et al., 2007). The costs of plasticity often depend on the environment and their causes are not totally understood, but costs are predicted to be greater in stressful environments (Dorn et al., 2000; Valladares et al., 2007). Overall, since phenotypic plasticity can be an important source of phenotypic variation to cope with environmental variation, assessing the patterns of plasticity and the presence of genetic

variation for plasticity across the distribution range of species is crucial to fully understand the responses of populations to climate change (Nicotra et al., 2010; Valladares et al., 2007; West-Eberhard, 2005, 2003).

Although adaptive evolution and phenotypic plasticity are not mutually exclusive processes that can interact to shape the evolutionary responses of populations, whether phenotypic plasticity favors or constrains adaptive evolution is still debated (Berg et al., 2010; Fox et al., 2019; Franks et al., 2014; Ghalambor et al., 2015, 2007; Lande, 2009; Merilä, 2015; Nicotra et al., 2010). Since plastic responses can occur within a generation and at the individual level, plasticity may be crucial to buffer against rapid environmental changes and "buy time" for adaptive evolution (Fox et al., 2019; Franks et al., 2014; Jump and Peñuelas, 2005; Kelly, 2019; Matesanz et al., 2010; Nicotra et al., 2010; Pfennig, 2021; Sih, 2004). Plastic responses can also result in optimum phenotypes to contrasting environmental conditions. In this case, plasticity may be enough to cope with environmental variation, shielding the evolution of functional traits (Fox et al., 2019; Ghalambor et al., 2007; Matesanz et al., 2010; Schlichting and Pigliucci, 1998; Sultan and Spencer, 2002), as has been observed in multiple species (e.g., Oplaat and Verhoeven, 2015; Ross et al., 2009; Sultan and Matesanz, 2015). Therefore, adaptive evolution of traits is more frequent in species with limited plasticity, which fail to express ideal plasticity across populations (Fox et al., 2019; Ghalambor et al., 2007; Kawecki and Ebert, 2004; Pfennig, 2021; Sultan, 2000; Van Kleunen and Fischer, 2005). Overall, plant populations can cope with climate change and avoid extinction with a non-exclusive, complex combination of migration, adaptive evolution, and phenotypic plasticity, although whether these processes can mitigate the negative effects of climate change and their relative importance is far from resolved (Aitken et al., 2008; Berg et al., 2010; Csilléry et al., 2020; Davis and Shaw, 2001; de Lafontaine et al., 2018; Franks et al., 2014; Gienapp et al., 2008; Hoffmann and Sgró, 2011; Kelly, 2019; Nicotra et al., 2010; Shaw and Etterson, 2012).

Intraspecific phenotypic variation among populations: Causes and consequences

Importantly, species are not uniform entities. In contrast, it is well-known that species generally show substantial intraspecific phenotypic variation both within and among populations (Auld and Morrison, 1992; Bolnick et al., 2011; Mehrhoff and Turkington, 1990; Valladares et al., 2014; Westerband et al., 2021). Evaluating the processes that have shaped such phenotypic variation is crucial in a climate change context, since it allows to identify potentially resilient and vulnerable populations and predict their evolutionary responses to face further environmental change. Intraspecific phenotypic variation results from the interaction between genetic differentiation caused by both adaptive and neutral evolutionary processes, and differences in environmentally induced phenotypic responses (i.e., differences in phenotypic plasticity) among populations (Fig. 2; Gianoli and Valladares, 2012; Gienapp et al., 2008; Nicotra et al., 2010; Pigliucci, 2001; Westerband et al., 2021). Genetic differentiation among populations is caused by differences in allele frequencies due to the action of neutral processes such as mutation, genetic drift and gene flow, and adaptive evolutionary processes such as past natural selection (de Lafontaine et al., 2018; Hartl and Clark, 1997; Shaw and Etterson, 2012). Genetic drift is defined as changes in the allele frequencies of populations due to random sampling (Barrett and Schluter, 2008; Shaw and Etterson, 2012). Although these changes are random, genetic drift tends to reduce genetic variation and consequently, adaptive potential (Blanquart et al., 2013; Ellegren and Galtier, 2016; Shaw and Etterson, 2012). The negative effects of genetic drift are inversely related to population size, being smaller populations more prone to lose adaptive alleles (i.e., those encoding adaptive trait values in a particular environment; Barrett and Schluter, 2008; Ellegren and Galtier, 2016; Franks et al., 2014; Shaw and Etterson, 2012). Although effective gene flow between populations can mitigate the negative consequences of genetic drift, the size and spatial configuration of populations

influence how genetic material is moved across the distribution range of species. Specifically, habitat fragmentation may limit the genetic admixture of different populations and therefore, fragmented populations are especially vulnerable to suffer the negative effects of genetic drift (Aguilar et al., 2008; Honnay and Jacquemyn, 2007).

Gene flow is a process that transfers and exchanges genetic material among populations and, in plants, it occurs via seeds and pollen dispersal (Franks et al., 2014; Robledo-Arnuncio, 2011). Due to this genetic exchange, gene flow influences the degree of neutral and quantitative genetic differentiation among populations caused by genetic drift or selection (De-Lucas et al., 2008; Hoffmann and Sgró, 2011; Robledo-Arnuncio, 2011). Indeed, gene flow and natural selection are usually seen as opposing evolutionary forces, with extensive gene flow able to prevent adaptive population differentiation (frequently named as swamping effects; Blanquart et al., 2013; Franks et al., 2014; Hoffmann and Sgró, 2011; Kawecki and Ebert, 2004; Latta, 2003; Lenormand, 2002; Sork, 2016). However, other studies support that adaptive evolution may occur even in the presence of gene flow across populations when selection is strong and constant (Jump and Peñuelas, 2005; Latta, 2003; Rajakaruna, 2018; Thompson, 2005). Gene flow can also have beneficial consequences for populations, affecting their potential future evolutionary responses. Specifically, gene flow can increase genetic variation within populations, facilitate adaptation due to the introduction of preadapted alleles, and reduce inbreeding effects, being a particularly important process in small and isolated populations (Anderson et al., 2012b; Davis and Shaw, 2001; Franks et al., 2014; Hoffmann and Sgró, 2011; Jump and Peñuelas, 2005; Kremer et al., 2012). Therefore, to fully understand how neutral evolutionary processes may have influenced both past and future evolutionary responses of plant populations, quantitative estimates of gene flow and genetic structure among populations are needed (Kawecki and Ebert, 2004). Gene flow can be indirectly assessed using neutral molecular markers by evaluating the geographical patterns of population genetic structure

(Ennos, 1994; Kawecki and Ebert, 2004; Petit et al., 2005; Sork, 2016). Phylogeographic studies are useful to understand the processes and historical events that promoted the origin of species and shaped the patterns of neutral genetic diversity and genetic structure across the distribution range of species (Avise, 2000; Avise et al., 2017; Beheregaray, 2008; Hickerson et al., 2010; Sork, 2016).

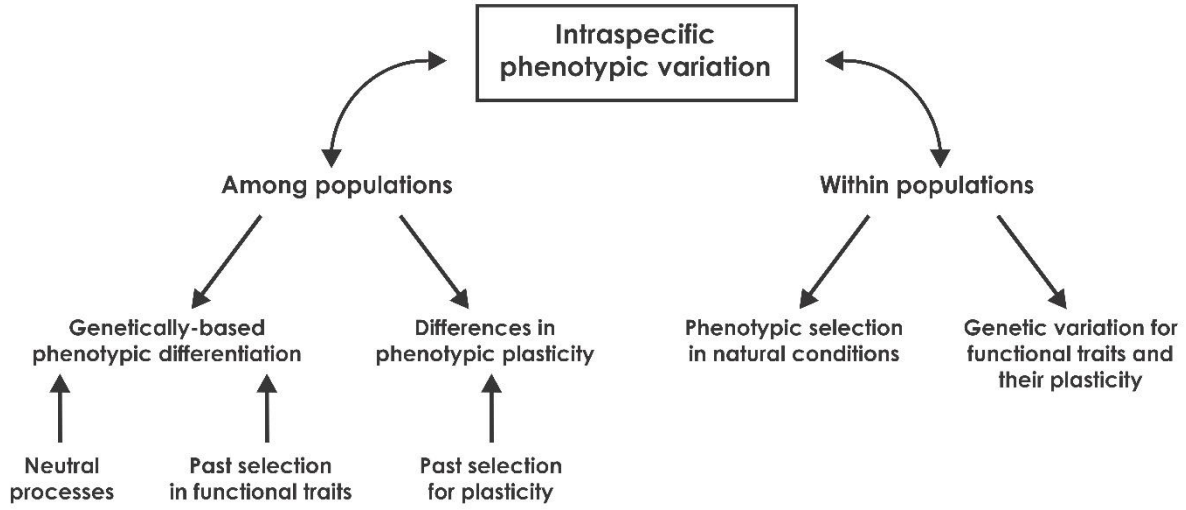

Figure 2: Conceptual framework of processes affecting intraspecific phenotypic variation, both among and within populations. Note that all these processes can interact to shape the phenotypic variation observed in nature. Assessing intraspecific variation is of paramount importance in a climate change context, since it provides crucial information about how populations may respond to cope with the new environmental conditions. Specifically, the presence of intraspecific phenotypic variation among populations provides insights on the ecological and evolutionary processes that may have shaped such genetic differentiation, which may affect in turn their future responses. Intraspecific phenotypic variation within populations provides insight on the trait values that have an influence on individual fitness, and the evolutionary potential of adaptive traits and phenotypic plasticity.

Phenotypic differentiation among populations may be also caused by adaptive evolutionary processes, such as adaptive evolution by natural selection (Fig. 2; Franks et al., 2014; McKay and Latta, 2002; Siepielski et al., 2013). Different populations often experience contrasting abiotic and biotic environmental conditions, which likely impose differential selective pressures that favor different phenotypes across populations (Blanquart et al., 2013; Pigliucci, 2001; Ramírez-Valiente et al., 2010; Siepielski et al., 2013). In the presence of sufficient adaptive genetic variation within populations, divergent natural selection will act,

enhancing genetic and phenotypic differences among populations and generating a pattern of adaptive population differentiation (Blanquart et al., 2013; Kawecki and Ebert, 2004). Therefore, spatiotemporal heterogeneity in selective pressures acting on genetically-based phenotypic traits may be responsible for the presence of phenotypic variation across natural populations (Merilä and Crnokrak, 2001). To evaluate whether quantitative genetic differentiation among populations has been caused by natural selection or neutral processes, measures of population differentiation in quantitative traits can be compared with that in neutral loci (McKay and Latta, 2002; Merilä, 1997; Merilä and Crnokrak, 2001; Whitlock, 2008). A robust approach to assess the role of past natural selection and neutral evolutionary processes in the genetic differentiation of populations is $Q_{ST} - F_{ST}$ comparisons (Leinonen et al., 2013; Merilä and Crnokrak, 2001; Spitze, 1993; Whitlock, 2008). F_{ST} and Q_{ST} quantify population differentiation caused by only neutral evolutionary processes, and by both neutral processes and natural selection, respectively (Leinonen et al., 2013; Whitlock, 2008; Wright, 1951). Therefore, significant differences between both parameters (Q_{ST} and F_{ST}) can be attributed to past natural selection (Leinonen et al., 2013; Merilä and Crnokrak, 2001; Whitlock, 2008).

In addition, assessing the ecological factors underlying adaptive population differentiation (i.e., the selective pressures, also named agents of selection) is key to draw relevant conclusions on how natural selection have acted in the past (Franks et al., 2014; Kawecki and Ebert, 2004; Wadgymar et al., 2022). Stressful climatic conditions related with water availability have been identified as major selective pressures in Mediterranean and semiarid ecosystems, and several studies have shown their power to drive adaptive genetic differentiation among populations (Aitken and Bemmels, 2016; Alberto et al., 2013; Blondel et al., 2010; Gómez, 2004; Jump and Peñuelas, 2005; Ramírez-Valiente et al., 2022a, 2022b). To identify the selective pressures that have driven adaptive phenotypic differentiation among populations, assessing whether there is an association between the phenotypes expressed by

populations under common conditions and the local environmental conditions that potentially have shaped adaptive differentiation is crucial (Blanquart et al., 2013; Brouillette et al., 2014; Keller et al., 2011; Ramírez-Valiente et al., 2022a). A gradual change in the phenotypes of populations associated with an environmental gradient suggests past adaptive evolution in response to such environmental variation. For instance, in species experiencing a gradient of water availability across their range, natural selection often favors low specific leaf area to minimize evapotranspiration in harsher populations and higher specific leaf area in mesic populations, to maximize photosynthesis when conditions are less restrictive (e.g., Lázaro-Nogal et al., 2016; Ramírez-Valiente et al., 2014; Solé-Medina et al., 2022). This generates a pattern of quantitative population differentiation in a key functional trait due to natural selection. Importantly, adaptive population differentiation may also evidence the capacity of populations to face further environmental changes, since it allows the identification of vulnerable and resilient populations to particular selective pressures (Franks et al., 2014; Shaw and Etterson, 2012). Overall, assessing whether climate or other important selective pressures have shaped quantitative population differentiation is crucial to understand the process of adaptive evolution and robustly predict the persistence of populations in a climate change context (Franks et al., 2014; Ramírez-Valiente et al., 2022b; Shaw and Etterson, 2012).

Finally, phenotypic differentiation among populations can result from differences in the responses of populations to the environment, i.e., differences in phenotypic plasticity among populations (Fig. 2; Aitken et al., 2008; Franks et al., 2014; Pigliucci, 2001; Valladares et al., 2007). A central goal in evolutionary ecology is to determine to what extent adaptive phenotypic differentiation among populations results from adaptive evolution, differences in phenotypic plasticity, or a combination of both processes (Conner and Hartl, 2004; Franks et al., 2014; Ghalambor et al., 2007). Indeed, since phenotypic plasticity may have a genetic component, differences in plasticity across populations can be the result of past differential

selection on plasticity patterns (Valladares et al., 2007). Differential selection on plasticity may be the consequence of differences in environmental heterogeneity among populations (Baythavong, 2011; Valladares et al., 2007). Populations with higher phenotypic plasticity are expected to have higher ability to cope with environmental changes such as those associated with climate change (Aitken et al., 2008; Chevin and Hoffmann, 2017).

Future evolutionary responses of populations to climate change

Past neutral and adaptive evolutionary processes affect the standing genetic variation within populations, and consequently, the future adaptive responses of populations to climate change. These adaptive responses ultimately depend on the strength and direction of natural selection within populations and the evolutionary potential of functional traits and their plasticity (Etterson, 2004; Teplitsky et al., 2014). Therefore, to understand how adaptive evolution may alleviate the negative effects of climate change, information about the within-population patterns of natural selection and the presence of genetic variation is needed (Fig. 2; Alberto et al., 2013; Hoffmann and Sgró, 2011; Janzen and Stern, 1998). Since natural selection favors particular phenotypes that affect individual fitness, significant trait-fitness associations provide evidence of the adaptive value of functional traits (Ackerly et al., 2000; Dobzhansky, 1956; Kremer et al., 2012). Despite the importance on quantifying the relationship between traits and fitness variation, it is not until the 1980s that trait-based ecology and the development of phenotypic selection analyses allowed identifying traits under selection (Lande and Arnold, 1983; Phillips and Arnold, 1989). Furthermore, since natural selection is a dynamic force that can vary in magnitude, direction and form depending on the environmental conditions (Kingsolver et al., 2001; Siepielski et al., 2009; Thompson, 2005; and references therein), experimental approaches that account for spatiotemporal environmental variation in natural conditions and simulate the increase of aridity predicted with climate change are needed to fully

understand the evolution of functional traits. Mediterranean plants have evolved functional adaptations both within and across species to cope with climatic stress (Blondel et al., 2010; Matesanz and Valladares, 2014; Thompson, 2005). These adaptations are mainly related to cope with drought and occur along a continuum of adaptive strategies: tolerance, escape, and avoidance (reviewed in Volaire, 2018). Tolerant plants often have more sclerophyllous leaves, high water use efficiency, low photosynthetic rates and leaf nutrient concentrations, and consequently, slow growth rates and a conservative resource-use strategy. In contrast, to avoid the most stressful conditions of the season, plants with an escape strategy advance reproduction, owing to their higher resource acquisition and growth rates through acquisitive leaves, low water use efficiency, high leaf nutrient concentrations, etc. (Kooyers, 2015; Pérez-Ramos et al., 2013; Volaire, 2018; Welles and Funk, 2021). Finally, plants with an avoidance strategy avoid cavitation by reducing transpiration and/or tapping onto more reliable water sources under severe drought conditions (McKay et al., 2003; Pérez-Ramos et al., 2013). Although assessing the patterns of natural selection and the adaptive strategies of Mediterranean plant populations is crucial to understand their future evolutionary responses, we lack information about the functional traits and trait values under selection in both natural conditions and experimental settings that simulate climate change.

Evolution by natural selection not only requires adaptive phenotypic variation within populations, but also genetically-based phenotypic variation among individuals; i.e., heritable variation (Fig. 2; Ackerly et al., 2000; Blows and Hoffmann, 2005; Etterson and Shaw, 2001; Gomez, 2000; Janzen and Stern, 1998). Indeed, since genetic variation is the substrate for adaptive evolution, high levels of quantitative genetic variation within populations are associated with the ability to adapt to environmental-driven changes, including climate change (Jump et al., 2009; Nicotra et al., 2010). Several factors and processes that affect the presence and maintenance of such trait variation within populations have been identified. First, new

mutations may generate quantitative genetic variation (Blows and Hoffmann, 2005; Donelson et al., 2019; North et al., 2011). However, adaptation to fast environmental changes such as climate change will likely depend on standing genetic variation, not only because mutations usually occur at slower rates than climate change, but also because adaptive alleles often start at higher frequencies from pre-existing genetic variation (Barrett and Schluter, 2008; Jump et al., 2009; Olson-Manning et al., 2012). Second, constant strong selection (or particular cases of fluctuating selection; reviewed in Kawecki, 2000) may favor the same phenotype over time, leading to genetic (and phenotypic) canalization and consequently reducing within-population genetic variation even in the presence of gene flow among populations (Blows and Hoffmann, 2005; Endler, 1986; Rajakaruna, 2018; Sork, 2016; Thompson, 2005; Wagner et al., 1997). Third, the expression of quantitative genetic variation and covariation (i.e., phenotypic integration, the correlation among different traits) depends on the environmental conditions (Ackerly et al., 2000; Falconer and Mackay, 1996; Fischer et al., 2021; Hoffmann and Merilä, 1999; Matesanz et al., 2020a; Oostra et al., 2018). Consequently, the evolutionary potential of populations depends on the environmental conditions and should be assessed in ecologically-meaningful environments that simulate climate change conditions (Charmantier and Garant, 2005; Diamond and Martin, 2016; Ghalambor et al., 2007; Hoffmann and Merilä, 1999; Oostra et al., 2018; Ramírez-Valiente et al., 2018). Finally, even in the presence of quantitative genetic variation, adaptive evolution may be constrained by the presence of genetic correlations among traits in the opposite direction of the direction of selection (Ackerly et al., 2000; Etterson and Shaw, 2001). Therefore, understanding the patterns of selection, the presence of standing genetic variation for adaptive traits, and the potential genetic constraints to adaptive evolution in ecologically-meaningful environments is key to determine the capacity of populations to future adaptation to climate change (Ackerly et al., 2000; Bradshaw, 1991; Franks et al., 2014; Jump and Peñuelas, 2005).

Finally, genotypes within populations may vary in their phenotypic responses to different environmental conditions, which reflects the presence of genetic variation for phenotypic plasticity or G × E (Fig. 2; Ackerly et al., 2000; Josephs, 2018; Pigliucci, 2005, 2001; Sultan, 2000). Importantly, the presence of genetic variation for plasticity in natural plant populations indicates the existence of evolutionary potential for plasticity in response to new selective pressures (Ackerly et al., 2000; Matesanz et al., 2010; Pigliucci, 2005). Again, high levels of within-population genetic variation for plasticity are often correlated with a higher capacity to cope with environmental changes such as those caused by climate change (Kelly, 2019; Matesanz et al., 2010). Despite its importance, our knowledge on the presence of the evolutionary potential for phenotypic plasticity in natural populations is still limited (Matesanz et al., 2010). Therefore, assessing the presence of genetic variation for traits and their plasticity is key to evaluate the role that adaptive evolution will have in the evolutionary responses of populations to cope with climate change.

Climate change and selective pressures within the Mediterranean region

Climate change will exacerbate the harsh climatic conditions already experienced in the Mediterranean Region, and the Iberian Peninsula is especially prone to suffer its negative consequences. Specifically, the latter climatic projections for the Iberian Peninsula predict a significant increase between 2-4 ºC and a 10-20% reduction in the mean annual precipitation by the end of the 21st century, being climatic changes more intense in areas with arid and semiarid conditions (Cardoso Pereira et al., 2020; Viceto et al., 2019). Therefore, plants inhabiting semiarid Iberian ecosystems are expected to be particularly vulnerable to climate change.

A particularly interesting example are gypsophiles, plants restricted to gypsum soils *sensu* Meyer (1986). Gypsum (calcium sulfate dihydrate, $CaSO_4 \cdot 2H_2O$) is a mineral rock that

can also occur in the composition of soils (Escudero et al., 2015). Gypsum ecosystems harbor rich plant communities with many endemic and endangered species, some exclusive to gypsum soils (i.e., gypsophiles; Escudero et al., 2015; Moore et al., 2014; Mota et al., 2011). Furthermore, gypsum ecosystems are characterized by their discontinuous spatial configuration. Gypsum outcrops are often immersed in other substrates, forming an island-like configuration (Escudero et al., 2015). This natural fragmentation has been intensified due to long-term anthropogenic practices such as agriculture and livestock grazing (Pueyo et al., 2008). The fragmentation of gypsum soils, together with the predicted lack of effective dispersal mechanisms of gypsophiles (Escudero et al., 2015; Moore and Jansen, 2007) may limit the migration ability of these species to track suitable environmental conditions. In addition, this spatial configuration may have caused losses of genetic diversity and a strong genetic structure due to potentially limited effective gene flow across populations. Consequently, habitat fragmentation may limit the occurrence of *in situ* evolutionary processes to alleviate the negative effects of climate change and guarantee the persistence of gypsophile populations.

Importantly, Iberian gypsophile populations have experienced several strong and potentially differential selective pressures during their evolutionary history. Among these pressures, the presence of high calcium and sulfate concentrations of gypsum soils imposes nutrient imbalances that are restrictive for plant growth and development (Escudero et al., 2015; Rajakaruna, 2018; and references therein). Therefore, differences in soil gypsum and nutrient contents may have driven adaptive differentiation among populations. In addition, although gypsum ecosystems are restricted to arid and semiarid conditions, populations of widespread gypsophiles also experience substantial variation in precipitation and temperature along species' ranges. These climatic differences may have also shaped adaptive differences among populations (Fig. 3; Escudero et al., 2015; Matesanz et al., 2020b). Alternatively, to deal with

such restrictive soil chemical composition and climatic conditions, natural selection may have favored a uniform, optimum, canalized phenotype across populations (i.e., an stress resistance syndrome *sensu* Rajakaruna, 2018; Lamy et al., 2012; Ramírez-Valiente et al., 2022a). However, whether past selection in gypsophile populations has been driven by soil chemical composition or climatic conditions is, to date, unknown.

In addition, the stressful climatic conditions of gypsum ecosystems are not only characterized by the presence of a semiarid climate but also by remarkable spatiotemporal heterogeneity (Escudero et al., 2015; Palacio et al., 2007). While temporal heterogeneity is associated to high seasonal and interannual climatic variation, spatial heterogeneity stems from significant environmental differences between slope aspects in gypsum hills (Escudero et al., 2015; Olano et al., 2011). South-facing slopes in the Iberian Peninsula receive greater insolation, leading to higher evapotranspiration rates and lower water availability, which affect the composition and structure of plant communities in each slope (Fig. 3; Aragón et al., 2007). Such high environmental variation may alter the patterns of selection within gypsophile populations, i.e., the suite of traits and trait values related to fitness, and consequently favoured by natural selection. Due to the stressful conditions of Mediterranean ecosystems, plant species inhabiting Mediterranean ecosystems are predicted to show a tolerant strategy (Matesanz and Valladares, 2014; Volaire, 2018). However, whether adaptive traits vary across spatiotemporal heterogeneous conditions and whether the predicted stress-tolerant syndrome is adaptive in gypsum ecosystems is unknown (Escudero et al., 2015; Siepielski et al., 2009; Thompson, 2005). Finally, despite the standing phenotypic and genotypic variation of gypsophile populations has been affected by the interaction of past neutral and adaptive evolutionary processes, little is known about the influence of these processes on the evolutionary potential of adaptive traits and phenotypic plasticity, and the evolutionary consequences for gypsophile populations.

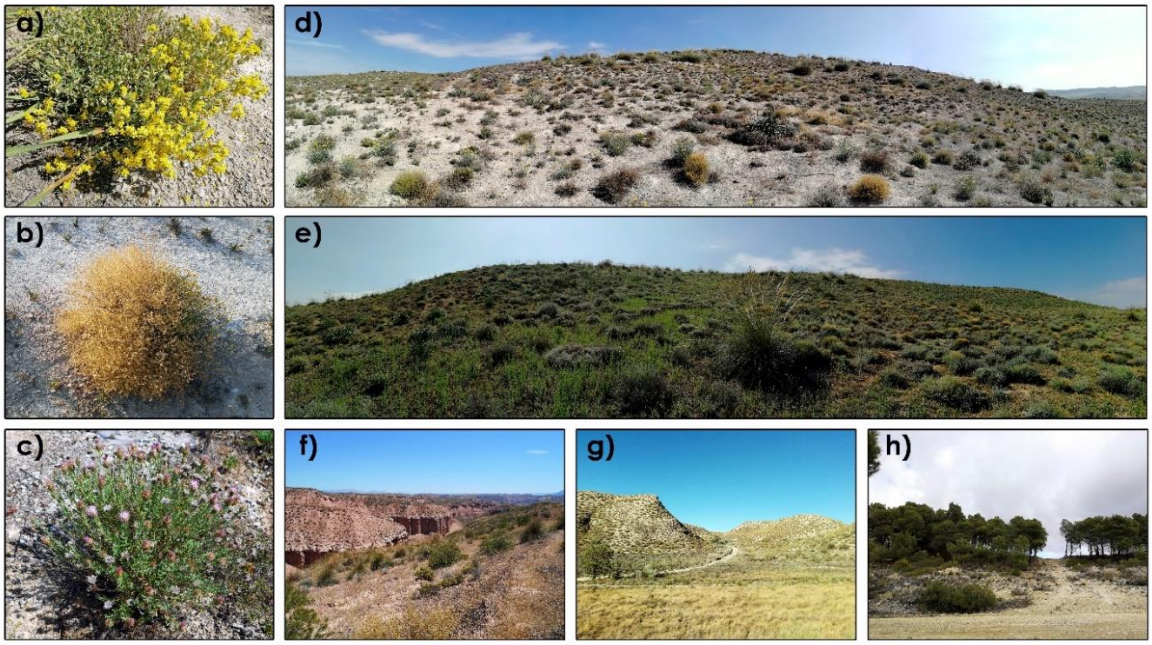

Figure 3: a), b) and c) Individuals of the three study species of this thesis, *Helianthemum squamatum*, *Lepidium subulatum* and *Centaurea hyssopifolia*, respectively, growing in natural conditions (Belinchón, Cuenca, Spain); d) and e) Typical configuration of south-facing and north-facing slopes of gypsum hills, respectively (Belinchón, Cuenca, Spain); f), g), and h) Landscape view of gypsum plant communities near Altiplano Granadino (Granada, Spain), Aranjuez (Madrid, Spain) and Peralta (Navarra, Spain), populations studied in this thesis.

Methodological overview

Using a multidisciplinary approach that combines phenotypic selection studies in natural conditions, common garden experiments and molecular analyses, this thesis provides a comprehensive picture on the evolutionary ecology of Mediterranean gypsophiles, to predict the responses of gypsophile populations and their persistence in a climate context. Specifically, this thesis focuses on three of the most dominant and widespread gypsophile species in the Iberian Peninsula, *Lepidium subulatum* L. (Brassicaceae), *Helianthemum squamatum* (L.) Dum. Cours (Cistaceae), and *Centaurea hyssopifolia* Vahl. (Asteraceae).

Common garden experiments are powerful tools to answer relevant questions on quantitative genetics and evolutionary ecology. To detect adaptive population differentiation, genotypes from different populations must be compared under the same environmental conditions, since it allows decomposing the phenotypic variance of a trait into its genetic and

environmental components (de Lafontaine et al., 2018; Falconer and Mackay, 1996; Kawecki and Ebert, 2004; Shaw and Etterson, 2012). Furthermore, when common gardens experiments recreate more than one test environment, they allow to evaluate not only the patterns of selection and the presence of genetic variation for adaptive traits, but also patterns of phenotypic plasticity and the presence of genetic variation for plasticity (De Villemereuil et al., 2016; Hoffmann and Sgró, 2011; Solé-Medina et al., 2022). Since the environment has a major effect on the phenotypic expression of individuals, common garden experiments should simulate realistic and ecologically meaningful environmental conditions (Pigliucci, 2001; Shaw et al., 2015; Shaw and Etterson, 2012). Therefore, the long-term, outdoor common garden experiments performed in this thesis provide robust insights on the ecological and evolutionary consequences for gypsophile species (Fig. 4). Furthermore, because drought is the main selective pressure within the Mediterranean region and it is expected to increase due to ongoing climate change, watering treatments that simulate an increment of aridity were implemented across experiments in this thesis.

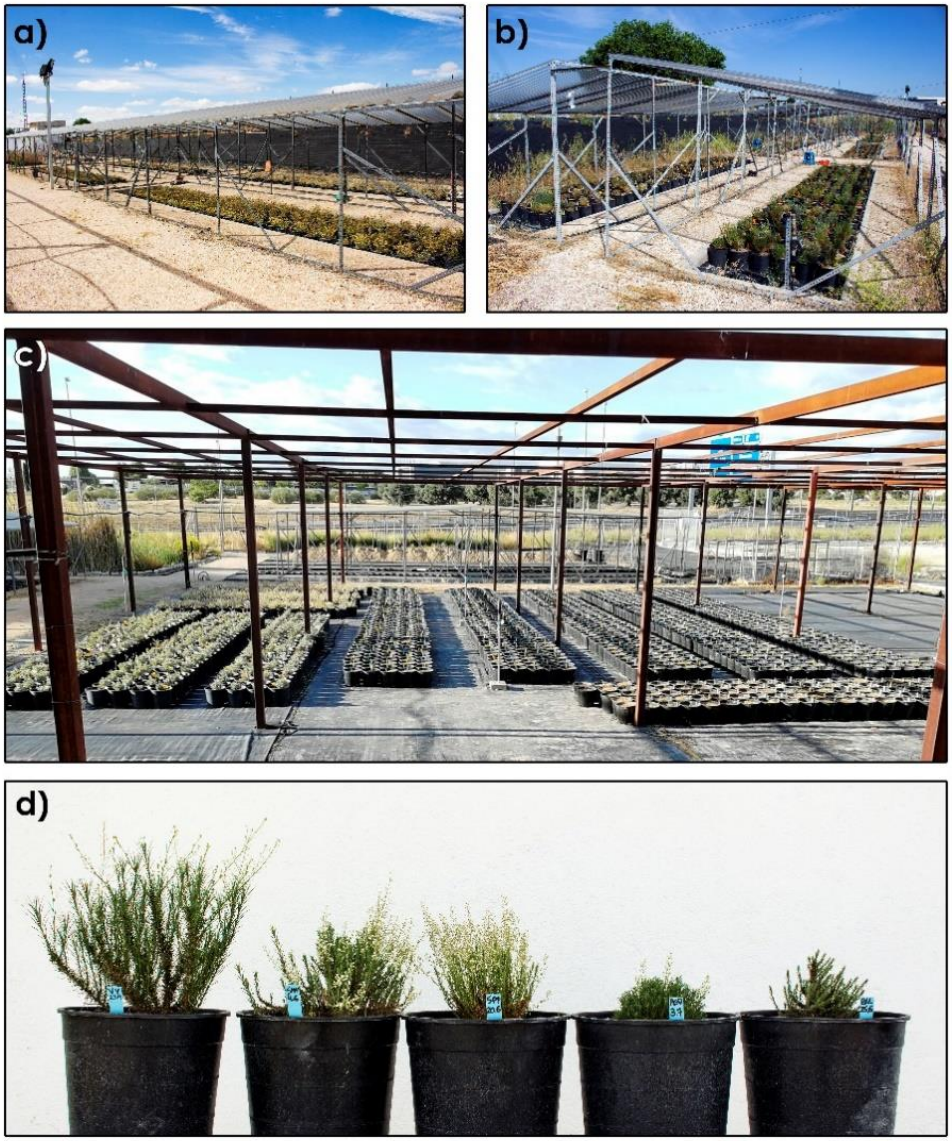

Figure 4: Common garden experiments performed in this thesis. a) and b) Rain exclusion structures employed to avoid all natural precipitation and robustly control the applied watering treatments; c) Plants growing under outdoor, common conditions, until individuals reach the reproductive stage (i.e., in their second growing season), when experimental treatments were applied; d) phenotypic differences among populations of *Lepidium subulatum* growing under common, well-watered conditions. Individuals from five different populations that follow a climatic gradient of temperature and rainfall are shown, with harsher populations at the left of the picture.

Objectives:

This PhD thesis addressed the following specific objectives (Fig. 5):

1.- To study the phylogeographic processes that determined the origin, genetic diversity, and genetic structure of the dominant gypsophile *Lepidium subulatum* across its entire distribution range.

2.- To assess the role of past natural selection and neutral evolutionary processes in quantitative genetic differentiation among populations and phenotypic plasticity patterns of the dominant gypsophile *L. subulatum* across its distribution range.

3.- To identify the traits under selection (i.e., those that have an influence on fitness, adaptive traits) in natural conditions in two dominant gypsophiles species, *Centaurea hyssopifolia* and *Helianthemum squamatum*, and how they vary under contrasting spatiotemporal environmental conditions.

4.- To evaluate the potential response to selection of functional traits and their plasticity in two dominant gypsophiles species, *C. hyssopifolia* and *H. squamatum*, under ecologically meaningful conditions that simulated climate change conditions.

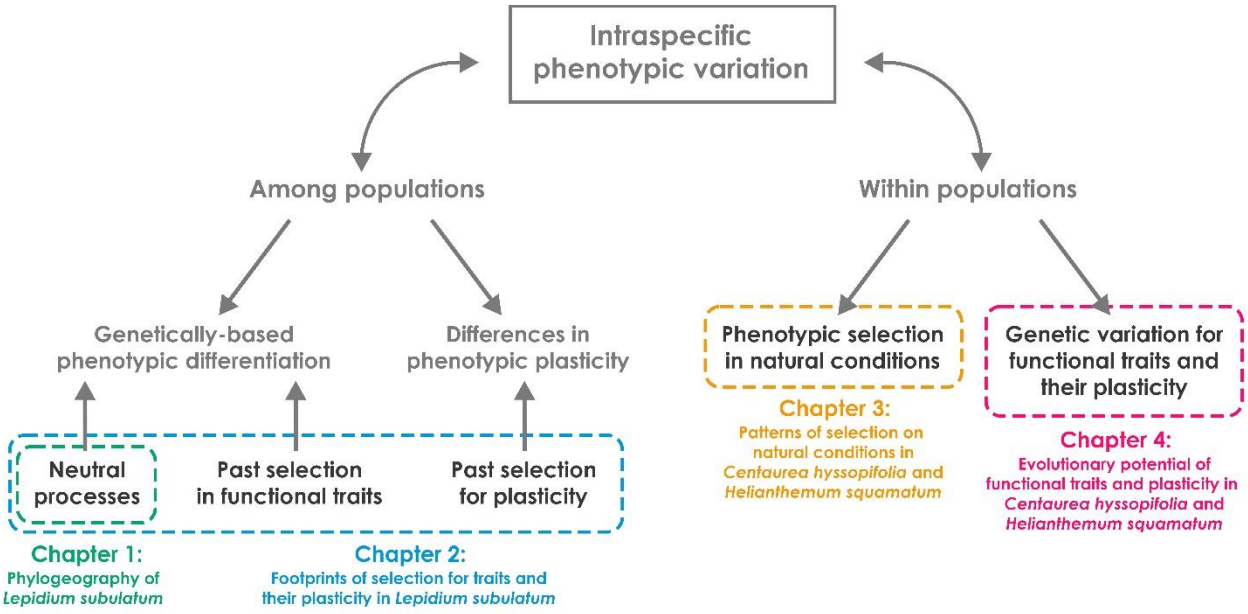

Figure 5: Specific objectives of this thesis, within the theoretical framework outlined in the General Introduction.

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Chapter 1: **Phylogeography of a gypsum endemic plant across its entire distribution range in the western Mediterranean**

Mario Blanco-Sánchez, Michael J. Moore, Marina Ramos-Muñoz, Beatriz Pías, Alfredo García-Fernández, María Prieto, Lidia Plaza, Ignacio Isabel, Adrián Escudero and Silvia Matesanz

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Abstract

Gypsum soils in the Mediterranean Basin house large numbers of edaphic specialists that are adapted to stressful environments. The evolutionary history and standing genetic variation of these taxa have been influenced by the geological and paleoclimatic complexity of this area and the long-standing effect of human activities. However, little is known about the origin of Mediterranean gypsophiles and the factors affecting their genetic diversity and population structure. Using phylogenetic and phylogeographic approaches based on microsatellites and sequence data from nuclear and chloroplast regions, we evaluated the divergence time, genetic diversity and population structure of 27 different populations of the widespread Iberian gypsophile *Lepidium subulatum* throughout its entire geographic range. *Lepidium subulatum* diverged from its nearest relatives ~3 Mya, and the ITS and *psbA/matK* trees supported the monophyly of the species. These results suggest that both geological and climatic changes that occurred in the region around the Plio-Pleistocene promoted its origin, compared to other evolutionary processes. We found high genetic diversity in both nuclear and chloroplast markers, but a greater population structure in the chloroplast data. This suggests that while seed dispersal is limited, pollen flow may be favored by the presence of numerous habitat patches that enhance the movement of pollinators. Despite being an edaphic endemic, *L. subulatum* possesses high genetic diversity probably related to its relatively old age and high population sizes across its range. Our study highlights the value of using different markers to fully understand the phylogeographic history of plant species.

Keywords: Phylogeography, gypsophiles, genetic diversity, population structure, nuclear microsatellites, cpDNA, pollen flow, seed dispersal, *Lepidium subulatum*

Introduction

The genetic diversity of plant populations and how it is distributed geographically across species ranges depends on processes that operate at different spatial and temporal scales. The Mediterranean Basin has experienced a highly complex geological and paleoclimatic history. Past changes in its geological, climatic and ecological conditions, especially during the Pliocene and Pleistocene (5.33-0.01 Mya), have been decisive in shaping the genetic composition of Mediterranean plants (Blondel et al., 2010; Nieto Feliner, 2014). More recently, humans have profoundly transformed Mediterranean ecosystems through long-standing, yet dynamic activities (Blondel et al., 2010; Nieto Feliner, 2014), further contributing to modulate the genetic diversity and structure of plant populations in this region (Thompson, 2005).

A particular example within Mediterranean taxa are gypsophiles, defined as plants that are restricted to gypsum (calcium sulfate dihydrate) soils (Meyer, 1986). In the Mediterranean Basin, these soils harbor rich plant communities with large proportions of endemic species adapted to arid and semiarid conditions (Escudero et al., 2015). Iberian gypsum outcrops have been dated to as old as the Cambrian, but more than two thirds of the gypsum soils in this area appeared in the Cenozoic, mostly during the Neogene (Escavy et al., 2012). Different events that occurred in this period favored the formation of gypsum outcrops. First, geological events like the Alpine Orogeny allowed the accumulation of salts and sediments in basins (Escavy et al., 2012). Furthermore, the tectonic uplift of the Gibraltar arc reduced water flow from the Atlantic Ocean to the Mediterranean Sea, resulting in the Messinian Salinity Crisis (~6 - 5.3 Mya). This, together with global changes in sea level, produced the desiccation of the Mediterranean Sea by evaporation processes that favored gypsum precipitation (Garcia-Castellanos and Villaseñor, 2011). Second, changes in the paleoclimatic conditions of the Mediterranean region further accelerated evaporation by rainfall reduction and prevented the loss of precipitated gypsum by leaching (Parsons, 1976). The progressive aridification and

seasonality of precipitation that started 9.5-8 Mya (van Dam, 2006) led to the appearance of the Mediterranean climate 3.2 Mya, characterized by high seasonality and marked summer drought (Suc, 1984). Although both the availability of gypsum soils and the increasingly drier climatic conditions of the late Miocene and Pliocene likely determined the origin of Iberian gypsophiles, it is yet not established whether these species originated in gypsum environments or, alternatively, in other stressful habitats from which they colonized gypsum soils (Escudero et al., 2015).

A remarkable feature of gypsum environments is their discontinuous spatial configuration. Not only are gypsum soils naturally fragmented into island-like outcrops surrounded by other substrates (Escudero et al., 2015), but also anthropogenic practices like agriculture and livestock grazing have exacerbated the natural patchiness of these habitats in the Mediterranean region for centuries (Pueyo et al., 2008). Both natural and human-induced fragmentation may affect the genetic diversity and structure of gypsophile populations due to neutral processes such as genetic drift, demographic changes, inbreeding and reduced gene flow (Aguilar et al., 2008). This unique spatial configuration may be even more critical in species that lack effective seed dispersal mechanisms, as is the case in most widely-distributed gypsophiles (Escudero et al., 2015). However, livestock practices like transhumance and grazing could enhance gene flow between populations if they promote seed movement (Pueyo et al., 2008; Azcárate et al., 2013). Consequently, genetic diversity and population structure of gypsophiles may be determined by a complex interaction between landscape configuration and land use, among others.

Phylogeography provides a useful framework to assess the origin and evolutionary history of species and closely related species groups (Avise, 2000). Combining markers with different mutation rates enables phylogeographic studies to elucidate how past and present processes have modulated the genetic diversity and structure of populations (Wang, 2011).

Furthermore, the use of markers with different modes of transmission such as chloroplast DNA (maternally inherited and dispersed only by seeds in most angiosperms) and nuclear DNA (biparentally inherited and dispersed by both seeds and pollen) allows for the quantification of the relative contribution of seed and pollen flow to the genetic structure of populations (Ennos et al., 1999; Petit et al., 2005).

In this study, we used a phylogeographic approach based on nuclear microsatellite loci and chloroplast and nuclear sequence data to assess the origin, genetic diversity and population structure of the gypsophile *Lepidium subulatum* L. (Brassicaceae) throughout its entire distribution range. *Lepidium subulatum* is a regionally dominant gypsophile endemic to the Iberian Peninsula and North Africa and is the most geographically widespread gypsophile in the western Mediterranean (Romão and Escudero, 2005). Because of its high substrate specificity, dominance and life-history traits common to other gypsophiles, *L. subulatum* provides a compelling study system to evaluate the genetic diversity, structure and date of origin of gypsophiles. We studied 27 different populations that represent the current geographical and climatic distribution of the species, to address the following questions: 1) When did the evolutionary divergence of *L. subulatum* occur and how was it influenced by the complex geological and paleoclimatic history of the Mediterranean Basin? 2) Do populations of the species show different levels of genetic diversity? 3) Are populations genetically structured, and if so, is this structure explained by their geographical location and/or by historic demographic changes? and 4) Is genetic variation and population structure inferred by either microsatellites or chloroplast markers different, and if so, how does it relate to pollen and seed flow? This is the first study to estimate the date of origin of this species and the distribution of genetic diversity across its entire geographical and climatic range. We expect that the complex historical events experienced by Mediterranean plants had a major role in the origin and evolution of *L. subulatum*. We also hypothesize that *L. subulatum* populations show substantial

genetic structure due to the spatial configuration of gypsum soils and the reproductive attributes of the species.

Materials and methods

Habitat and species description

Gypsum plant communities in the Iberian Peninsula are mostly composed by chamaephytes and ephemeral annual plants, with a large proportion of endemic species. In these systems, plants form discrete patches immersed in a matrix of bare ground and biological soil crusts (BSC) formed by cyanobacteria, lichens and mosses (Escudero et al., 2015).

The genus *Lepidium* L. is one of the largest in the Brassicaceae, with approximately 175 widespread plant species. Most of them are edaphic generalists, but two species, *Lepidium subulatum* and *Lepidium cardamines*, are restricted to the gypsum soils of the Iberian Peninsula. *Lepidium subulatum* L. (Brassicaceae) is one of the most common and widely distributed gypsophiles in Iberian gypsum habitats (Romão and Escudero, 2005). It is a non-clonal perennial shrub (20–60-cm high) endemic to the Iberian Peninsula and North Africa. This species is mainly outcrossing with partial self-compatibility, as supported by both field experiments (Gómez et al., 1996) and low inbreeding coefficients inferred from molecular markers (Gómez-Fernández et al., 2016; Matesanz et al., 2018). It has entomophilous pollination, being pollinated by a very rich community of generalist species from seven different orders of insects (Santamaría et al., 2018). Seeds are released from very numerous small fruits (silicles), lack obvious long-distance dispersal mechanisms and have a mucilage that enhances seed adhesion to the soil (Romão and Escudero, 2005).

Population sampling

We sampled 27 populations in the Iberian Peninsula and North Africa, spanning the worldwide geographic and climatic distribution of *L. subulatum* (Table 1 and Fig. 1). Each population was assigned to one of five different geographic zones that roughly match different river basins in the Iberian Peninsula (Fig. 1) and are related to the main gypsum vegetation habitats described in the region (Mota et al., 2011). Elevations of sampled populations varied from 219 (ALF) to 1157 m asl (TOP). The closest sampled populations (SMV and CHI) were 15 km apart and the furthest populations (ARGL and BAL) were separated by 972 km. At each population, fresh leaves of 20 individuals were collected and stored in paper bags, except for the Moroccan population (MAR, 10 individuals), and the Peralta population (PER, 14 individuals). Leaves were air-dried and stored until DNA extraction. Voucher specimens (one per sampled population) were deposited at the herbarium of the Universidad Rey Juan Carlos (Móstoles, Spain; Appendix S1, see Supplemental Data with this article). Additionally, 14 samples from the same locality in Algeria (Chott Ech Chergui region) dating from 1884 to 1952 were obtained from herbarium specimens (Muséum National d'Histoire Naturelle, Paris, France; Appendix S1). A total of 508 samples were included in the study. At each site, sampled individuals were collected in a ≈ 20 × 20 m area at S – SE aspect to homogenize microenvironmental conditions experienced by individuals. The south-oriented slopes of gypsum hills in the study region receive more insolation and have lower water availability compared to north-oriented slopes. Furthermore, gypsophiles are dominant and more abundant on slopes with S-SE aspects. All populations had moderate to large size, from several hundred to several thousand individuals. Individuals within populations were separated at least one meter from each other, to avoid sampling closely-related individuals.

Climatic information of each population was extracted from CHELSA Bioclim layers (Karger et al., 2017) using ArcMap 10.2.2 (ArcGIS Desktop, ESRI, Redlands, California,

USA). A 2 km buffer around each population was created to account for the within-site climatic heterogeneity. Sampled populations spanned a wide climatic range: mean annual temperature ranged from 11.4 to 16.9 ºC and mean annual precipitation ranged from 254.7 to 647.8 mm (Table 1).

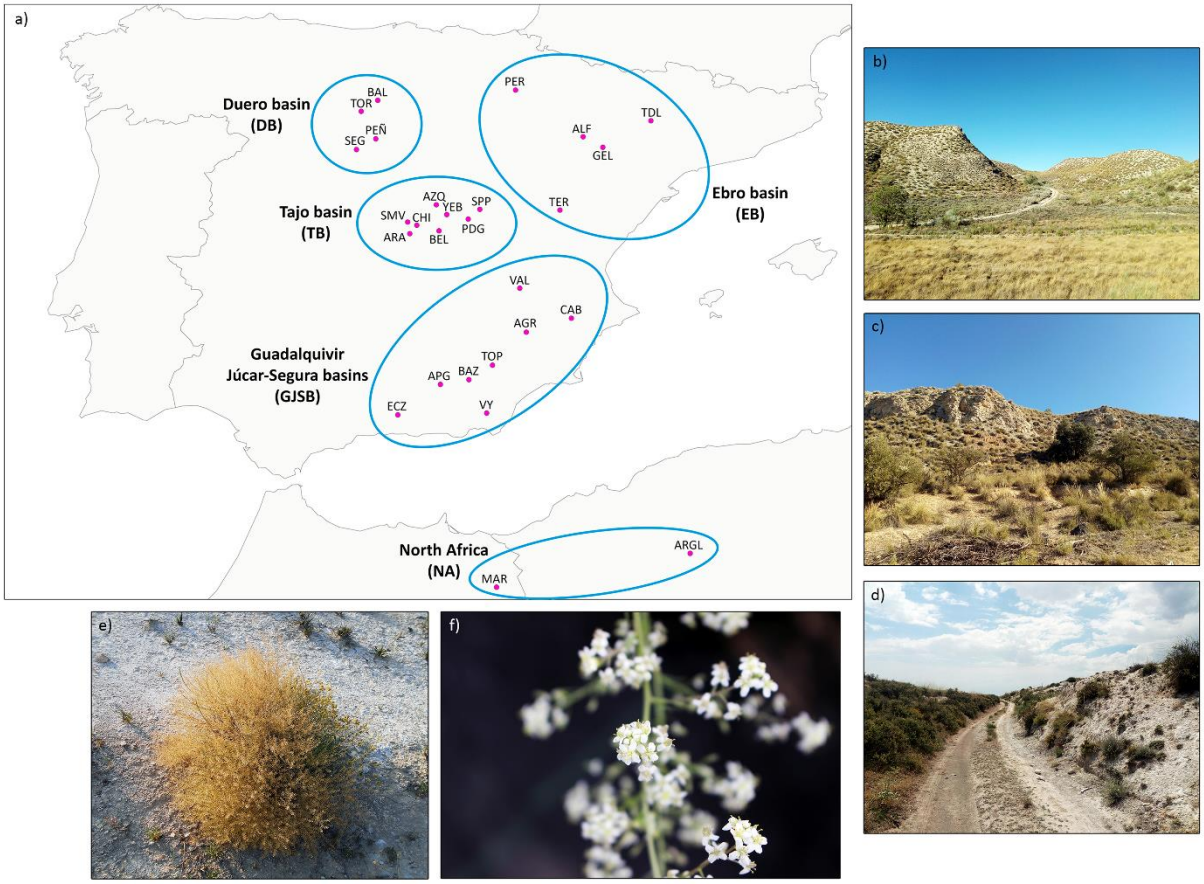

Figure 1: a) Map of the Iberian Peninsula and North Africa showing all sampled populations and their assignment to regions for analyses of population structure (these regions are related to the main gypsum vegetation habitats described; Mota, Sánchez-Gómez, & Guirado, 2011); b), c) and d) Gypsum environments at ARA, SPP and TOP, respectively; e) Individual of *Lepidium subulatum* at the end of its fruiting period (mid-June); f) Flowers of *L. subulatum.*

Table 1: Population code, location, geographical coordinates, elevation, and geographical region of the 27 populations of *Lepidium subulatum* L. used in this study.

Geographic region	Population code	Population location	Geographical coordinates		Altitude (m asl)	T. mean (ºC)	T. min. (ºC)	T. max. (ºC)	Prec. (mm)
Duero Basin (DB)	BAL	Los Balbases (Burgos, Spain)	42º 13' 20.3" N	4º 4' 30.9" W	851	11.4	4.2	19.8	467.7
	PEÑ	Peñafiel (Valladolid, Spain)	41º 35' 25.0" N	4º 6' 30.1" W	815	12.6	4.5	22.0	432.3
	SEG	Vallelado (Segovia, Spain)	41º 24' 48.5" N	4º 25' 30.3" W	818	12.7	4.7	22.2	510.7
	TOR	Torquemada (Palencia, Spain)	42º 2' 26.6" N	4º 20' 49.3" W	833	12.1	4.6	20.8	443.6
Ebro Basin (EB)	ALF	Alfajarín (Zaragoza, Spain)	41º 37' 25.5" N	0º 41' 52.3" W	219	15.7	7.7	25.2	363.2
	GEL	Gelsa (Zaragoza, Spain)	41º 27' 5.3" N	0º 22' 24.6" W	254	15.7	7.6	25.3	367.3
	PER	Peralta (Navarra, Spain)	42º 23' 22.5" N	1º 48' 38.5" W	385	13.6	6.1	21.9	589.9
	TDL	Tamarite de Litera (Huesca, Spain)	41º 53' 9.5" N	0º 24' 58.5" E	418	13.8	6.0	23.0	490.0
	TER	Villalba Baja (Teruel, Spain)	40º 25' 9.7" N	1º 4' 48.2" W	954	12.0	4.2	21.5	339.7
Guadalquivir and Júcar-Segura Basins (GJSB)	AGR	Agramón (Albacete, Spain)	38º 24' 51.2" N	1º 37' 56.1" W	388	16.9	8.9	26.4	300.6
	APG	Altiplano granadino (Granada, Spain)	37º 33' 23.5" N	3º 2' 47.9" W	738	15.7	6.8	25.9	523.5
	BAZ	Hoya de Baza (Granada, Spain)	37º 38' 0.8" N	2º 34' 37.1" W	903	14.4	6.0	24.2	459.9
	CAB	Cabezo Redondo (Alicante, Spain)	38º 38' 32.9" N	0º 53' 33.5" W	533	15.7	8.2	24.4	370.7
	ECZ	Escúzar (Granada, Spain)	37º 3' 20.2" N	3º 44' 41.5" W	927	14.2	6.0	23.7	520.3
	TOP	Topares (Almería, Spain)	37º 52' 18.4" N	2º 11' 22.0" W	1157	12.4	4.4	21.9	395.1
	VAL	Valdeganga (Albacete, Spain)	39º 8' 10.4" N	1º 44' 26.7" W	632	15.2	7.1	25.1	346.7
	VY	Venta de Yesos (Almería, Spain)	37º 5' 2.3" N	2º 17' 7.3" W	539	16.3	9.4	24.7	254.7
Tajo Basin (TB)	ARA	Aranjuez (Madrid, Spain)	40º 1' 51.5" N	3º 32' 54.4" W	595	15.8	6.4	26.8	406.9
	AZQ	Aranzueque (Guadalajara, Spain)	40º 30' 23.7" N	3º 6' 47.1" W	742	13.6	5.3	24.2	414.9
	BEL	Belinchón (Cuenca, Spain)	40º 4' 43.5" N	3º 4' 3.7" W	706	15.1	5.8	26.0	419.2
	CHI	Chinchón (Madrid, Spain)	40º 10' 13.2" N	3º 25' 59.4" W	676	15.1	5.9	26.0	465.3
	PDG	Portalrubio de Guadamejud (Cuenca, Spain)	40º 16' 15.8" N	2º 35' 14.7" W	794	13.9	5.2	24.5	508.6
	SMV	San Martín de la Vega (Madrid, Spain)	40º 13' 19.2" N	3º 35' 3.3" W	551	15.6	6.4	26.6	376.3
	SPP	San Pedro Palmiches (Cuenca, Spain)	40º 25' 51.9" N	2º 23' 51.1" W	850	13.6	5.0	24.0	647.8
	YEB	Yebra (Guadalajara, Spain)	40º 20' 43.0" N	2º 56' 27.2" W	718	14.3	5.6	25.1	419.8
North Africa (NA)	ARGL	Chott Ech Chergui (Algeria)	34° 17' 59.3" N	0° 40' 33.5" E	989	16.4	6.8	27.9	257.3
	MAR	Yerada (Morocco)	34º 13' 29.4" N	2º 7' 21.2" W	944	16.5	8.1	26.5	282.2

DNA extraction, microsatellite markers, cpDNA markers and PCR conditions

Genomic DNA was extracted from 30 mg of air-dried leaf tissue, using a commercial kit (DNeasy Plant Minikit; QIAGEN, California, USA) with minor changes to the manufacturer's extraction protocol to improve the process. DNA extraction success was checked using 1% agarose gels stained with GreenSafe Premium (NZYTech, Lisbon, Portugal). Ten species-specific, nuclear polymorphic microsatellite markers previously described in Martínez-Nieto, Merlo, Mota, Salmerón-Sánchez, & Segarra-Moragues (2012) were used to assess neutral genetic diversity. Detailed information concerning microsatellite markers used and PCR reactions is found in Appendix S2 and Appendix S3, respectively. Amplified DNA was analyzed using an ABI 3730 (Applied Biosystems, Madrid, Spain) at "Unidad de Genómica y Proteómica" of Universidad Complutense (Madrid, Spain), employing the GS500 size standard. For phylogeographic analyses, we performed a preliminary screening with ten nuclear, chloroplast, and mitochondrial loci widely used in phylogeographic studies (Appendix S4). From this screening we selected the chloroplast *matK* gene and the *psbA-trnH* intergenic spacer region because they showed relatively high variability at the population level. We sequenced *matK* and *psbA-trnH* (henceforth referred to simply as *psbA*) from 8-10 individuals of each study population. Additionally, we also sequenced the same regions in four individuals from two different populations (Orusco de Tajuña and Portalrubio de Guadamejud, in the center of the Iberian Peninsula) of *Lepidium cardamines* L., an Iberian gypsophile species that is a close relative of *L. subulatum* (Mummenhoff et al., 2009), to evaluate whether the two species share haplotypes indicative of processes like hybridization, chloroplast capture, and/or incomplete lineage sorting that might confound interpretation of phylogeographic data (Schaal et al., 1998). Finally, to test the monophyly and to date the origin of *L. subulatum*, we sequenced the nuclear internal transcribed spacer (ITS) region and the chloroplast *trnT-trnL*, *trnL* intron and *trnL-trnF* regions (Appendix S4), respectively, of one individual from four different *L. subulatum*

populations (BAL, ECZ, TDL, SMV) and one individual from one population (Orusco de Tajuña) of *L. cardamines*. Detailed information concerning PCR conditions is found in Appendix S3. Amplified DNA was sequenced at Macrogen DNA Sequencing Service (Madrid, Spain).

Microsatellite genotyping and alignment of chloroplast sequences

Microsatellite scoring was performed using GeneMarker v2.2.0 (SoftGenetics, State College, Pennsylvania, USA). Each sample was manually checked by three different researchers to guarantee a robust scoring process. Different sizes of the amplified DNA fragments were considered as different alleles. Every microsatellite locus exhibited polymorphic patterns, yielding one (homozygous) or two alleles (heterozygous) per individual at each locus, consistent with the ploidy level of the species. We repeated five percent of the samples to ensure the repeatability of the scoring process. For population ARGL, only four individuals were successfully genotyped. Therefore, this population was excluded from analyses of microsatellite genetic diversity and population structure. We only considered in our analyses the individuals for which at least 9 of 10 loci were successfully genotyped, representing 98.6% of all individuals.

DNA sequences were manually trimmed, edited and cleaned using SEQUENCHER 5.4.6 (Gene Codes Corporation, Ann Arbor, Michigan, USA). A total of 207 individuals were successfully sequenced for *matK* and 219 for *psbA*. We were able to concatenate *matK* and *psbA* regions from 204 individuals, which were used in all downstream analyses. Sequence alignment was performed in AliView (Larsson, 2014), with manual adjustments.

Statistical analyses

Phylogenetic analyses

To assess the evolutionary relationships between *L. subulatum* and other species of *Lepidium* and to test the monophyly of *L. subulatum* (see other phylogenies in Beilstein, Nagalingum, Clements, Manchester, & Mathews, 2010; Mummenhoff et al., 2009), we estimated phylogenies of *Lepidium* using newly generated sequences of *L. subulatum* and *L. cardamines* as well as publicly available sequences of other species of *Lepidium*, for the nuclear ITS region and the chloroplast *matK* gene and *psbA* spacer region. For ITS, we included one individual each from 4 populations of *L. subulatum* (BAL, ECZ, TDL, SMV) that covered the entire geographic range of the species. We also included one individual from one population (Orusco de Tajuña) of *L. cardamines*. We added these to all *Lepidium* ITS sequences available on GenBank (Clark et al., 2016), which yielded 90 species from the genus in total (including *L. subulatum* and *L. cardamines*). As outgroups, we downloaded GenBank ITS sequences for seven *Arabidopsis* species and three *Cardaria* species. The total data set included 408 accessions, with 1-59 individuals per species (Appendix 1). Sequences were aligned as described above, excluding the ambiguous regions for downstream analyses. Maximum Likelihood (ML) analyses were conducted in RAxML (Stamatakis, 2014) using the CIPRES Science Gateway v. 3.3 (Miller et al., 2010), selecting 1000 replicates, the GTRCAT model, and rapid bootstrapping. We undertook similar ML analyses on the *matK* and *psbA* data generated for phylogeographic analyses (described above). However, sequences of other taxa of *Lepidium* beyond *L. subulatum* and *L. cardamines* were not available for inclusion.

To understand the temporal divergence of *L. subulatum*, we performed a molecular dating analysis using the *trnT-trnL*, *trnL* intron and *trnL-trnF* regions for 68 different species of *Lepidium* and 12 outgroup species (*Brassica napus*, *Cochlearia pyrenaica*, and ten species of *Arabidopsis*; see Appendix 1). Sequence alignment was performed in Aliview, excluding the

ambiguous regions for further analysis. The dating analysis was performed in BEAST v1.10.4 (Suchard et al., 2018) using the CIPRES Science Gateway v. 3.3 (Miller et al., 2010). We selected three different unlinked partitions with the HKY substitution model (Hasegawa et al., 1985) for each partition. We used an uncorrelated lognormal relaxed clock model, which allows uncorrelated rates of molecular evolution across the tree, and a birth-death process as tree prior (Gernhard, 2008). We calibrated the tree at the basal node (the split point *Lepidium-Arabidopsis*), using the date obtained by Guo et al., (2017) for the crown clade "A": 16.9-20.3-24 Mya, constraining the calibration point with a normal distribution with mean = 20.3 and standard deviation = 2.0. Then, we ran a relaxed log-normal clock with default priors to estimate prior distributions to be used in a second analysis that was used to estimate priors for the final analysis. BEAST analyses were run for 40 million generations, logging parameters and trees every 1000 generations. Convergence, mixing, and effective sample sizes (ESS) of parameters were checked using Tracer v1.5.0 (Rambaut and Drummond, 2009). A burn-in of 1000 trees was removed from each analysis. The remaining trees were used to generate a maximum clade credibility tree with TreeAnnotator v1.8.2 (Rambaut and Drummond, 2014).

Phylogeographic analyses

To evaluate phylogeographic patterns within *L. subulatum*, a haplotype network using the concatenated *psbA* and *matK* sequences of each individual was estimated using PopART (Leigh and Bryant, 2015) and employing the TCS method, which is appropriate to estimate genealogies among populations (Clement et al., 2002). We also performed a ML phylogeny estimated from RAxML (Stamatakis, 2014), using 10000 replicates, the GTRCAT model, and rapid bootstrapping (Appendix S5).

To test the existence of historical demographic changes, Tajima's *D* (Tajima, 1989), Fu and Li's *F** (Fu and Li, 1993) and Fu's F_S (Fu, 1997) statistics were calculated for each

population using DnaSP6 (Rozas et al., 2017). These tests were originally designed to assess the neutrality of markers, but their combination is also useful to test departures from population equilibrium due to historical demographic changes, bottlenecks or genetic hitchhiking (Fu, 1997). Thus, these tests allow for distinguishing the relative role of demographic changes or other processes (like gene flow or mutation) in shaping the allele frequencies of populations. While significant and positive Tajima's *D* values can inform us about the admixture of two different populations, significant and negative Tajima's *D* values indicate a recent bottleneck in a population (Tajima, 1989; Aris-Brosou and Excoffier, 1996). Fu's F_S is also used to test for demographic expansion and it is described as more sensitive to the growth of the populations than Tajima's *D* (Chávez-Pesqueira and Núñez-Farfán, 2016). These tests may be performed only if the populations possess more than one haplotype.

Intrapopulation genetic diversity

We checked the presence of null alleles and genotyping errors such as allele dropouts or false positive alleles due to stuttering in the nuclear microsatellites dataset, using Micro-Checker 2.2.3 (Van Oosterhout et al., 2004). No genotyping errors or null alleles were detected. For each population, we calculated the following genetic diversity indices: *P*, proportion of polymorphic loci; *A*, allele richness (mean number of alleles per locus); A_{rare}, mean number of rarefied alleles per locus; A_e, mean number of effective alleles; H_o, observed heterozygosity ($H_o = 1 - \sum_k \sum_i Pkii/np$, Nei, 1987); H_e, expected heterozygosity ($\mathrm{H_e} = \tilde{n}/(\tilde{n}-1)[1 - \sum_i \bar{p}_i^2 - \mathrm{H}_o/2\tilde{n}]$, Nei, 1987); F_{IS}, inbreeding coefficient; β, neutral genetic differentiation between populations (from 0 to 1; Weir & Hill, 2002); the number of private alleles and the number of multilocus genotypes. *A*, A_{rare}, H_o, H_e, F_{IS} (and their confidence intervals) and β were calculated using the functions *nb.alleles*, *allelic.richness*, *basic.stats*, *boot.ppfis* and *betas*, respectively, from the package hierfstat (Goudet and Jombart, 2015) as implemented in R (R Core Team,

2018). Rarefaction allowed for calculating the mean number of alleles per locus (A_{rare}) considering equal sample sizes in all populations (note that MAR only had 10 individuals sampled). A_e was calculated using the function *genetic_diversity* (package gstudio; Dyer, 2016), the number of private alleles was calculated using the function *private_alleles* and the number of multilocus genotypes was calculated using function *poppr*, both in package poppr (Kamvar et al., 2014).

Genetic diversity of chloroplast markers was assessed using DnaSP6 (Rozas et al., 2017). For each population, we calculated the number of segregating sites, the number of haplotypes, haplotype diversity (Hd) and nucleotide diversity (π).

Population structure

To assess population differentiation we calculated a pairwise F_{ST} matrix based on microsatellite markers using the *genet.dist* function (package hierfstat, Goudet & Jombart, 2015). The matrix of pairwise Nei's (D) differences between populations from chloroplast markers was calculated using the *pairnei* function (package haplotypes; Aktas, 2015). We also calculated a Euclidean geographical distance matrix between populations, performed with ecodist package (Goslee and Urban, 2007), using the UTM coordinates of each population.

To assess the distribution of genetic variation of microsatellite and chloroplast markers across regions and populations, we used Analysis of Molecular Variance (AMOVA, Excoffier, Smouse, & Quattro, 1992). AMOVAs were performed using the *poppr.amova* function (package poppr, Kamvar et al., 2014), with 99999 permutations and excluding within-individual variation. We performed two different AMOVAs: 1) Non-hierarchical AMOVA, considering all populations within the same region; 2) Hierarchical AMOVA, assigning each population to each of five geographical zones (Fig. 1).

To assess whether closer populations are more genetically similar, we tested for isolation by distance (IBD). We performed two different Mantel correlograms (Legendre and Legendre, 2012), using the pairwise genetic distance matrix calculated from microsatellite markers and from chloroplast markers and the pairwise geographical distance matrix between populations. While Mantel tests show the overall relationship between the genetic and the geographic matrix, a Mantel correlogram compares the pairwise genetic distance matrix (F_{ST} in our case) and the pairwise geographical distance matrix (Euclidean distance), which allow for finding significant correlations between them at different distance classes. Each distance class includes all pairs of points that are included within a specific distance. A correlation index (Mantel statistic, rM) between genetic and geographical distance matrices is calculated for each distance class. The size and number of distance classes was set using Sturge's rule (Legendre and Legendre, 2012). Significance was tested using 99999 permutations. Mantel correlograms were generated using the *mantel.correlog* function (package vegan, Oksanen et al., 2019).

Population genetic structure from microsatellite markers was further evaluated using the Bayesian clustering algorithm in STRUCTURE v. 2.3. (Pritchard et al., 2000). This method evaluates the membership of each individual to a specific genetic cluster (*K*). We performed 10 independent runs for each *K* (from $K = 1$ to $K = 30$), with a burn-in period of 10^5 iterations and 10^6 MCMC iterations after the burn-in period, using the admixture model, where individuals from different *K* values could have a common ancestry (Falush et al., 2003), as recommended for microsatellites. We ran STRUCTURE assuming correlated and independent allele frequencies (Pritchard et al., 2000; Falush et al., 2003) and both methods provided very similar clustering results. STRUCTURE results were extracted using Structure Harvester (Earl and vonHoldt, 2012), which were then used to generate CLUMPP input files. Then, using CLUMPP 1.1.2 (Jakobsson and Rosenberg, 2007), results from 10 runs of each *K* were combined, using the Greedy algorithm. Membership of each individual to a specific genetic cluster was

visualized using DISTRUCT 1.1 (Rosenberg, 2004). To ensure the assignment performed by STRUCTURE, we repeated the clustering assignment with rMavericK (Verity and Nichols, 2016), obtaining virtually the same assignment results.

Some recent work has drawn attention to the problems related to determining the appropriate number of genetic clusters (*K*) (Meirmans, 2015; Janes et al., 2017). To determine this, we first considered the average log probability (L_K) of the data for each *K*, and determined the value of *K* for which this probability is maximized (Pritchard et al., 2000). We also calculated the optimum value of *K* using the Evanno method (Evanno et al., 2005) implemented in Structure Harvester (Earl and vonHoldt, 2012). This *ad hoc* method is based on changes in the mean values of log probability of data at successive *K* values. The Evanno method and L_K simplify model assumptions because these methods obtain the value of *K* for each assignment model, so estimating the optimum value of *K* requires comparison between models, which is not straightforward (Verity and Nichols, 2016). Thus, we also calculated *K* using rMavericK. This software uses generalized thermodynamic integration (GTI), which has been hypothesized to be more accurate and precise (Verity and Nichols, 2016). Therefore, *K* was calculated using rMavericK, although L_K and Evanno methods provided similar results (Appendix S6).

Results

Analyses of sequence data

Information on lengths and sequence variation for all sequence alignments is provided in Appendix S7. In both the ITS (Appendix S8) and chloroplast (*trnT-trnL*, *trnL* intron and *trnL-trnF*; Fig. 2) trees, *Lepidium subulatum* was sister to the Iberian gypsophile *L. cardamines* (ITS bootstrap support = 97%; chloroplast Bayesian posterior probability = 95%). In the ITS tree, all sequences of *L. subulatum* formed a clade with high support (bootstrap support = 98%). In the *matK/psbA* tree (Appendix S5), haplotypes of *L. subulatum* and *L. cardamines* were relatively distant from each other and none were shared between the two species. The molecular dating analysis based on the chloroplast loci dated the evolutionary divergence of *L. subulatum* from 5.08 – 1.33 Mya (mean = 3.01 Mya; Fig. 2). Furthermore, the divergence of the gypsophile clade of *L. subulatum* and *L. cardamines* was dated to 5.96 – 2.05 Mya (mean = 3.86 Mya).

Haplotype analyses recovered 22 different haplotypes and 19 segregating sites (*S*). Total nucleotide (π) and total haplotype diversity (Hd) across populations was 0.0038 and 0.747, respectively. Twelve populations possessed more than one haplotype, while 15 populations possessed one fixed haplotype for all sampled individuals (Table 2).

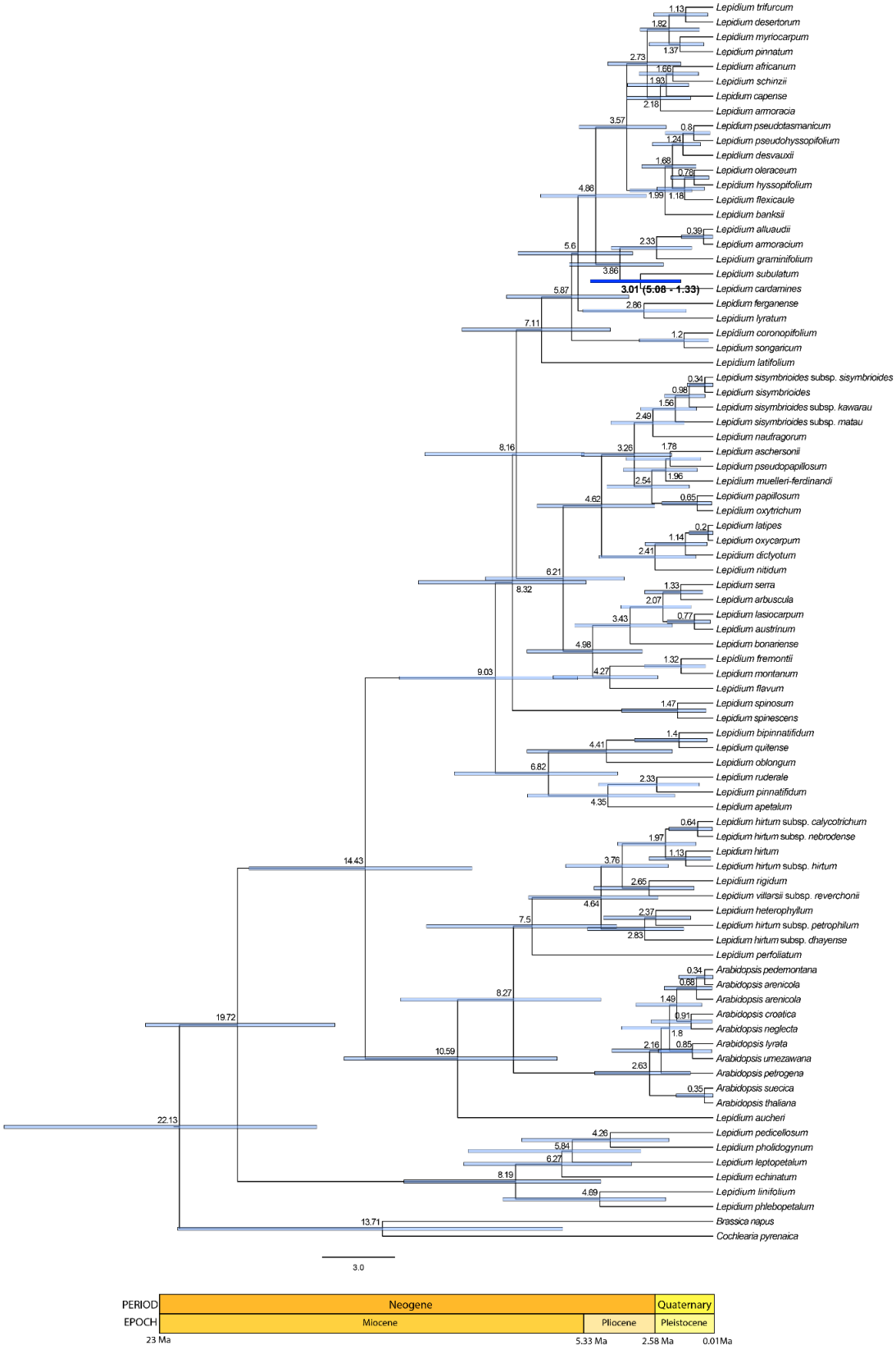

Figure 2: Maximum clade credibility (MCC) tree obtained from the BEAST analysis based on concatenated *trnT-trnL*, *trnL* intron and *trnL-trnF* sequences. Blue bars show highest posterior densities (HPD) credibility intervals and numbers above branches show mean estimated divergence time (Mya). HPD and dates for *L. subulatum* are in bold and dark blue (Bayesian posterior probability of this node = 95%).

Table 2: Genetic diversity indices based on concatenated chloroplast *matK* and *psbA* regions for the 27 populations of *Lepidium subulatum*. Significant values for Tajima's *D*, Fu and Li's *F** and Fu's F_S tests are in bold.

Population code	**N. of sequences**	**N. of segregating sites**	**N. of haplotypes**	**Haplotype diversity (Hd)**	**Nucleotide diversity (π)**	**Tajima's *D***	**Fu and Li's *F****	**Fu's F_S**
AGR	7	0	1	0	0	-	-	-
ALF	7	1	2	0.571	0.00080	1.342	1.102	0.856
APG	8	0	1	0	0	-	-	-
ARA	8	0	1	0	0	-	-	-
ARGL	2	0	1	0	0	-	-	-
AZQ	7	0	1	0	0	-	-	-
BAL	7	0	1	0	0	-	-	-
BAZ	7	0	1	0	0	-	-	-
BEL	8	2	2	0.250	0.00070	-1.310	-1.514	0.762
CAB	8	0	1	0	0	-	-	-
CHI	7	1	2	0.286	0.00040	-1.237	-1.374	0.856
ECZ	8	0	1	0	0	-	-	-
GEL	8	6	4	0.750	0.00334	0.215	0.881	0.869
MAR	10	0	1	0	0	-	-	-
PDG	8	3	3	0.607	0.00185	0.585	0.401	0.723
PEÑ	8	2	2	0.536	0.00150	1.449	1.297	2.083
PER	8	2	2	0.571	0.00160	1.794	1.384	2.216
SEG	8	0	1	0	0	-	-	-
SMV	8	3	2	0.571	0.00239	**1.982**	1.541	3.149
SPP	7	1	2	0.286	0.00040	-1.237	-1.374	0.856
TDL	8	1	2	0.250	0.00035	-1.310	-1.514	0.762
TER	8	0	1	0	0	-	-	-
TOP	7	0	1	0	0	-	-	-
TOR	8	0	1	0	0	-	-	-
VAL	8	0	1	0	0	-	-	-
VY	8	2	3	0.464	0.00070	-1.310	-1.514	-0.999
YEB	8	2	2	0.250	0.00070	-1.310	-1.514	0.762
Overall	204	19	22	0.747	0.00382	-0.399	-1.640	-5.931

The haplotype network showed that *L. subulatum* was connected to its closest relative *L. cardamines* by three mutation steps, with no shared haplotypes between the two species (Fig. 3). The network was complex, with one loop and three extinct or unsampled haplotypes. Despite the complexity of the network, we identified four common haplotypes. The most frequent haplotype (haplotype A, in blue), was found in 16 populations (in nine of them it was the only haplotype present) and was distributed broadly across the Iberian Peninsula (in the north-west, the center and the south). The second most common haplotype (haplotype B, in red) was separated from haplotype A by one mutational step and was found mainly in 4 populations of the eastern Iberian Peninsula: CAB, PDG, VAL and YEB, and one individual from VY population. The third most common haplotype was haplotype C (in purple), which was restricted to the Ebro River Valley. This haplotype was the most divergent, being separated by many mutational steps from all other main haplotypes. The fourth most common haplotype was haplotype D (in yellow), which was restricted to North Africa (ARGL and MAR) and was the only haplotype found in this region. Overall, the center of the Iberian Peninsula showed the highest haplotype diversity.

We obtained very similar results from the phylogenetic tree based on *psbA* and *matK*. Although some of the groups were identical between the tree and the network, the low support values of some branches in the tree showed the uncertainty of relationships among some haplotypes (*e.g.*, see green haplotypes in Fig. 3 and Appendix S5).

The Mantel Correlogram based on the chloroplast markers showed that the closest populations (first distance class, 62.15 km) were significantly similar (Fig. 4a; $R_M = 0.181$, p-value = 0.010), and populations separated by ~350 km were statistically different (Fig. 4a; $R_M = -0.176$, p-value = 0.041), confirming the presence of isolation by distance.

The non-hierarchical AMOVA of chloroplast loci performed with all individuals and populations showed a variation of 46.08% among populations and 53.92% within them (p-value

< 0.001; Table 3). In the hierarchical AMOVA with populations grouped by their geographic location, 32.82% of the variation was explained by the geographic region (p-value < 0.001; Table 3).

In the populations with more than one haplotype, overall Tajima's *D*, Fu and Li's *F** and Fu's F_S were not statistically different from 0 (p-value > 0.05), except for SMV, which showed a significantly positive Tajima's *D* value (i.e., a higher average pairwise differences observed than expected; Table 2). Therefore, our results suggest the admixture of two distant populations in SMV population; and we did not detect demographic changes in the other populations.

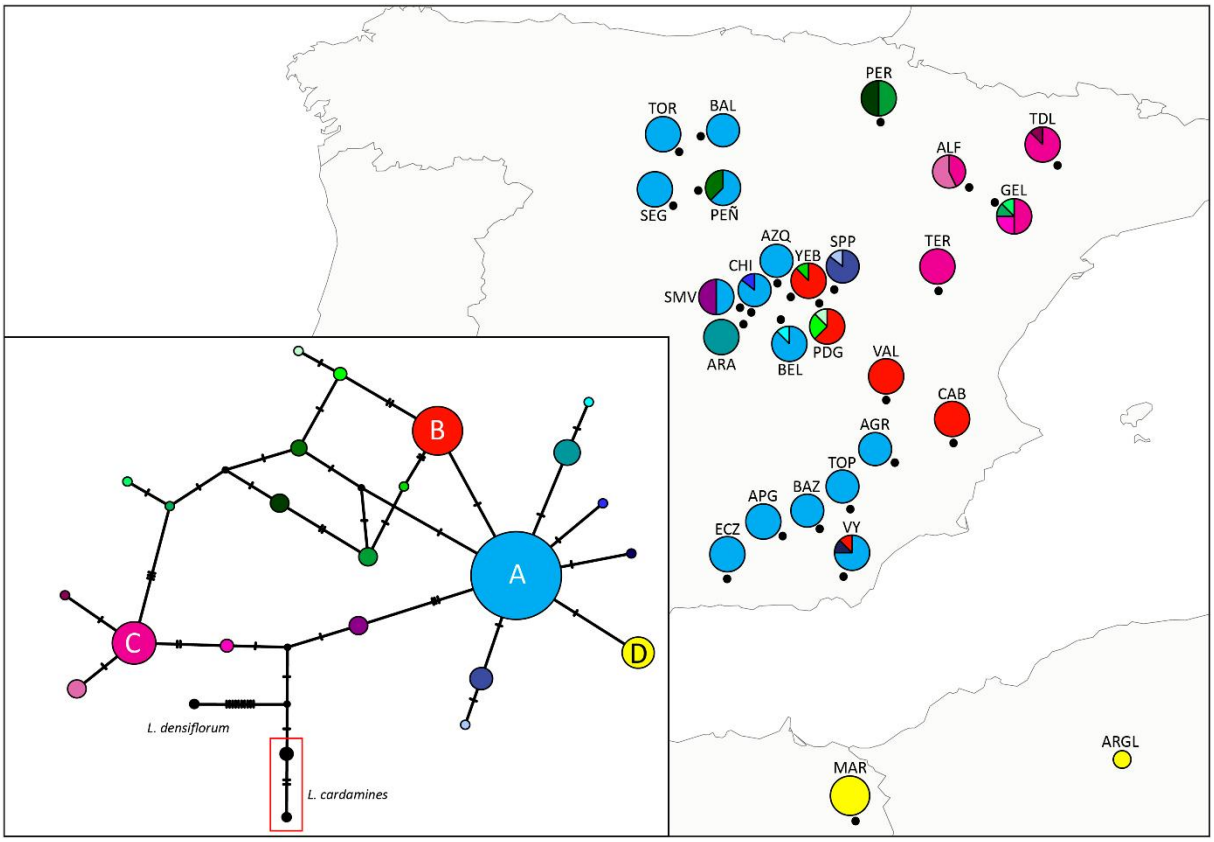

Figure 3: Haplotype network for 27 populations of *Lepidium subulatum*, based on concatenated *matK* and *psbA* sequences of 204 individuals. The main groups (A, B, C and D) are shown in the haplotype network. Three missing haplotypes (extinct or unsampled) are represented by small black dots in the haplotype network. The size of the different haplotypes in the network is proportional to the number of individuals with each haplotype. The size of the pie charts in the map is proportional to the number of samples in each population. Note that the location of the ARGL population is approximate. See Appendix S10 for haplotype networks for each locus.

Table 3: Results of two different AMOVA tests for microsatellite and chloroplast markers: 1) non-hierarchical AMOVA; 2) hierarchical AMOVA considering the geographic location (regions) of the populations; df = degrees of freedom.

		Nuclear DNA					**Chloroplast DNA**				
AMOVA type	Source of variation	df	Sum of squares	Variance	Percentage of variation	p-value	df	Sum of squares	Variance	Percentage of variation	p-value
1) Non – hierarchical AMOVA	Among populations	25	1544.746	1.419	18.627 %	< 0.001	26	1389.277	6.129	46.080 %	< 0.001
	Within populations	982	6074.267	6.201	81.373 %		177	1269.484	7.172	53.920 %	
	Total	1007	7619.012	7.621	100 %		203	2658.760	13.302	100 %	
2) Populations grouped by geographic location	Among regions	4	368.845	0.227	2.955 %	< 0.001	4	831.532	4.691	32.815 %	< 0.001
	Among populations within regions	21	1175.901	1.245	16.230 %		22	557.745	2.432	17.015 %	
	Within populations	982	6074.267	6.201	80.815 %		177	1269.484	7.172	50.170 %	
	Total	1007	7619.012	7.673	100 %		203	2658.760	14.296	100 %	

Microsatellite analyses

We found 145 different alleles among the 504 individuals, for an average of 14.5 alleles per locus. The number of alleles per locus ranged from six (Locus 10 and 11) to 24 (Locus 4). Microsatellite genetic diversity was high for all populations (Table 4). Most populations possessed 100% polymorphic loci, except for AGR, SMV, SPP and YEB, with 90% polymorphic loci. Expected heterozygosity ranged from 0.452 (SEG) to 0.681 (BAZ; Table 4). Observed heterozygosity varied from 0.415 (SPP) to 0.718 (PER; Table 4). The fixation index F_{IS} was low for all populations, ranging from -0.193 (PER, showing a heterozygote excess) to 0.237 (BAZ) and none of the populations had a F_{IS} statistically different from 0. Population-specific F_{ST} (β) varied from 0.064 (BAZ) to 0.379 (SEG; Table 4). The average number of alleles per locus (*A*) ranged from 3.3 (SEG) to 7.1 (BAZ), with an overall average of 5.15 alleles per locus. The rarefied mean number of alleles per locus (A_{rare}) ranged from 2.62 (CAB population) to 5.65 (BAZ population), with an overall average of 4.40 alleles per locus. The mean number of effective alleles per locus (*Ae*) varied from 1.93 (SEG) to 3.89 (BEL), with an overall average of 3.06 effective alleles per locus. The number of multilocus genotypes matched the number of individuals sampled in each population, except for ARA and SPP, where there were two individuals with the same genotype. We found a total of 23 private alleles in 15 of the 26 populations, ranging from one to three per population.

Microsatellite population structure

Pairwise F_{ST} values were generally low, ranging from very low (0.030) between populations CHI and SMV to high (0.440) between populations SEG and SPP (Appendix S9). Results from rMavericK clearly supported the presence of three different genetic clusters (K=3). L_K and the Evanno method supported K=2 but also K=3 (Appendix S6). Thus, we selected K=3 that

allowed a clearer interpretation of the data. Based on the *K*=3 solution, most of the populations included admixed individuals assigned to more than one genetic cluster. Four populations from the Tajo river basin (ARA, BEL, SPP and YEB) and one from the Guadalquivir-Júcar-Segura basins (CAB) contained individuals that were mostly assigned to one genetic cluster (blue, Fig. 5). Individuals from AGR, BAL, ECZ, PDG, TDL, TOR and VAL were mostly assigned to a different genetic cluster (yellow, Fig. 5). Individuals from APG, BAZ, PER, TOP and VY were mostly assigned to the magenta genetic cluster (Fig. 5). Finally, individuals from ALF, AZQ, CHI, GEL, MAR, PEÑ, SEG, SMV and TER belonged to two or even three different genetic clusters (frequently a mixture of the magenta and yellow genetic clusters; Fig. 5).

The non-hierarchical AMOVA with all individuals and populations showed that 81.37% of variation was found within populations and 18.63% between populations (p-value < 0.001; Table 3). There was a small but significant population structure explained by the geographical location of the populations. In the geographic AMOVA, 2.95% of the variation was explained by the geographic region (p-value < 0.001; Table 3).

The Mantel correlogram based on microsatellites did not show evidence of isolation by distance (IBD). Only the closest populations (first distance class, 58.55 km) were significantly similar (Fig. 4b; R_M = 0.151, p-value = 0.023).

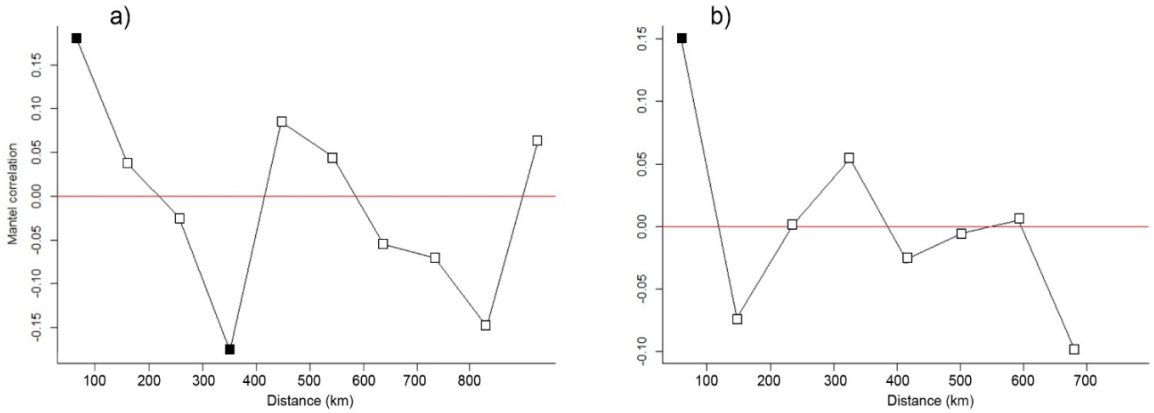

Figure 4: Mantel Correlograms calculated from a) chloroplast markers and b) microsatellite. In both cases, solid squares indicate that the Mantel statistic is different from zero at the 95% confidence level. Distance classes were calculated using Sturge's rule (Legendre & Legendre, 2012).

Table 4: Genetic diversity indices of the 26 populations (excluding ARGL) of *Lepidium subulatum* using 10 microsatellite loci. *N*: Number of individuals sampled; *N eff.*: Effective number of individuals sampled; *P*: percentage of polymorphic loci; *A*: Mean number of alleles per locus; A_{rare}: Rarefied number of alleles per locus (10 individuals, 20 genes); *Ae*: Mean number of effective alleles per locus; H_o: Observed heterozygosity; H_e: Expected heterozygosity; F_{IS}: Inbreeding coefficient; β: Population-specific F_{ST} coefficient.

Population code	***N***	***N eff.***	***P***	***A***	A_{rare}	A_e	H_o	H_e	F_{IS}	β	**Nb. of private alleles**	**Nb. of genotypes**
AGR	20	18.9	90.0%	3.8	3.47	2.54	0.528	0.548	0.060	0.245	0	20
ALF	20	19.7	100.0%	5.4	4.68	3.35	0.587	0.605	0.065	0.169	0	20
APG	20	19.9	100.0%	6.1	4.87	3.50	0.574	0.580	0.024	0.203	2	20
ARA	20	19.8	100.0%	5.9	4.91	3.29	0.621	0.618	-0.002	0.150	2	19
AZQ	20	19.9	100.0%	6.0	5.11	3.80	0.669	0.662	-0.009	0.090	2	20
BAL	20	19.5	100.0%	4.8	4.11	2.56	0.463	0.515	0.171	0.292	0	20
BAZ	20	19.8	100.0%	7.1	5.65	3.86	0.520	0.681	0.270	0.064	1	20
BEL	20	19.9	100.0%	7.0	5.57	3.89	0.538	0.647	0.178	0.111	1	20
CAB	20	19.6	100.0%	3.4	2.92	2.08	0.500	0.497	0.026	0.317	0	20
CHI	20	19.9	100.0%	6.8	5.54	3.71	0.557	0.627	0.111	0.138	1	20
ECZ	20	19.1	100.0%	4.5	3.76	2.32	0.470	0.505	0.074	0.306	0	20
GEL	20	19.9	100.0%	5.6	4.78	3.41	0.594	0.608	0.049	0.165	0	20
MAR	10	9.7	100.0%	4.5	4.50	3.05	0.479	0.602	0.258	0.149	1	10
PDG	20	19.6	100.0%	5.5	4.61	3.32	0.572	0.631	0.145	0.132	0	20
PEÑ	20	20	100.0%	6.0	5.10	3.53	0.570	0.667	0.144	0.084	1	20
PER	14	13.8	100.0%	4.0	3.79	2.79	0.718	0.601	-0.093	0.164	2	14
SEG	20	19.7	100.0%	3.3	3.01	1.93	0.471	0.452	0.000	0.379	0	20
SMV	20	19.3	90.0%	5.5	4.78	3.38	0.514	0.599	0.204	0.176	1	20
SPP	20	19.7	90.0%	4.1	3.49	2.49	0.415	0.483	0.153	0.335	1	19
TDL	20	20	100.0%	5.1	4.15	2.47	0.485	0.493	0.019	0.323	3	20
TER	20	19.5	100.0%	5.3	4.41	3.07	0.561	0.602	0.066	0.172	1	20
TOP	20	19.8	100.0%	4.8	4.17	2.89	0.548	0.594	0.134	0.184	0	20
TOR	20	20	100.0%	5.6	4.83	3.38	0.640	0.635	0.008	0.127	0	20
VAL	20	19.9	100.0%	4.4	4.03	3.14	0.663	0.619	-0.043	0.150	0	20
VY	20	19.8	100.0%	5.1	4.39	3.15	0.479	0.585	0.194	0.196	2	20
YEB	20	19.8	90.0%	4.2	3.75	2.64	0.479	0.521	0.094	0.283	2	20
Overall	504	19.096	98.5%	5.15	4.40	3.06	0.547	0.584	0.089	0.196	23	502

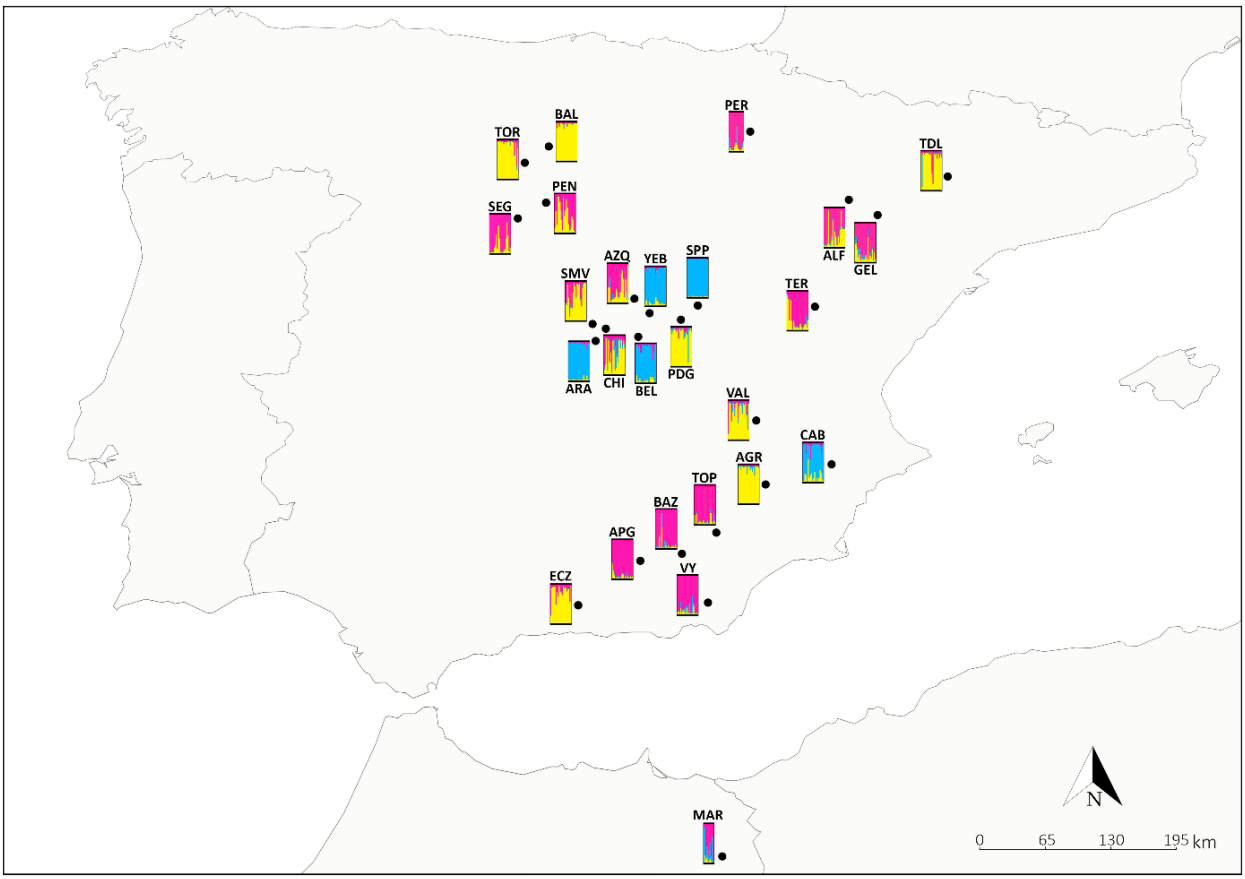

Figure 5: Population structure (*K*=3) inferred by Bayesian cluster analyses (STRUCTURE) for 504 *L. subulatum* individuals from 26 populations. Each individual is represented by a vertical bar in each population. The size of the boxes is proportional to the number of individuals sampled in each population.

Discussion

Our molecular dating results suggest that the Iberian gypsophilic clade composed by *Lepidium subulatum* and *L. cardamines* originated ~ 3.86 Mya (5.96-2.05 Mya) and the stem lineage of *L. subulatum* diverged ~3.01 Mya (5.08-1.33 Mya). Thus, it is likely that the specialization to gypsum soils (gypsophily) in this group appeared at some point from the latest Miocene to the early Pleistocene, in the ancestor of both species. Furthermore, these dates for the divergence of the study species in the Plio-Pleistocene also suggest that the paleoclimatic and geological events that occurred in the Mediterranean Basin around this period could be associated with the origin and further expansion of this gypsophile. First, the massive emergence at the surface of gypsum soils during the Neogene consequence of evaporitic processes in the region (Escavy et

al., 2012) increased the probability of colonizing a novel edaphic habitat by chance (chance dispersal *sensu* Rajakaruna, 2017; Escudero et al., 2015; Moore & Jansen, 2007), likely facilitating the evolution of gypsum-restricted taxa. Second, the progressive aridification of the Mediterranean basin before and during the Messinian salinity crisis (~6-5 Mya) not only favored the creation of gypsum soils, but also probably acted as an evolutionary force promoting the evolution of *L. subulatum* and other gypsophiles in the new climatic conditions (Thompson, 2005). It has been hypothesized that certain gypsophiles may have been preadapted to the global aridification that started in the mid-Miocene that subsequently colonized gypsum soils (Escudero et al., 2015 and references therein). However, the availability of gypsum soils in the Iberian Peninsula prior to our estimated date of origin (Escavy et al., 2012) suggests that this is not the case for *L. subulatum*. The relatively old date of origin of the species would provide enough time to colonize isolated gypsum patches and is congruent with its widespread distribution in the Iberian Peninsula, even moreso when its inefficient dispersal ability is considered (Escudero, Iriondo, Olano, Rubio, & Somolinos, 2000; see Moore & Jansen 2007 for similar patterns). Our results also agree with the estimated age of other Iberian and non-Iberian gypsophiles. The clade that includes *Helianthemum squamatum* (L.) Dum. Cours. started its diversification 4.37 Mya (8.57 – 1.65 Mya; Aparicio et al., 2017) and the clade formed by *Ferula loscosii* (Lange) Willk. and its sister species diverged from their common ancestor 4 Mya (6.4 – 1.6 Mya; Pérez-Collazos et al., 2009). Other North American gypsophiles such as *Tiquilia hispidissima* (Torr. & A. Gray) A.T. Richardson split from its nearest relatives in the early/mid-Pliocene (5 – 3.5 Mya; Moore and Jansen, 2007).

The evolutionary distinctiveness of *Lepidium subulatum* and *L. cardamines* in both the haplotype network and the ITS phylogeny is important because it reinforces the idea that past edaphic and climatic changes could be important in the origin of *L. subulatum*, compared to other evolutionary processes. The two species did not share haplotypes, which is consistent

with a lack of hybridization between both species that could have resulted in chloroplast capture (Schaal et al., 1998). Nevertheless, it is important to note that existing population sampling of *L. cardamines* is limited and additional sampling may reveal shared haplotypes between the two species. Some authors have noted the importance of hybridization in the origin of edaphic specialists (Rajakaruna, 2017 and references therein), but Ellstrand, Whitkus, & Rieseberg, (1996) reported that Brassicaceae taxa are not particularly prone to natural hybridization. Several aspects, including differences in their reproductive phenology (Hernández Bermejo and Clemente, 1993) may have served to minimize potential hybridization between them.

Based on the haplotype analysis, *L. subulatum* may have originated in the center of the Iberian Peninsula. This region shows the highest haplotype diversity (see also individual *psbA* and *matK* haplotype networks in Appendix S10), suggesting that populations in this region have had enough time to reach such high diversity. Furthermore, gypsum outcrops of the Tajo Valley present the greatest climatic variation of the entire distribution range, which could also explain the high genetic diversity found in this region. The populations of the Ebro Valley (purple shades in Fig. 3) possessed the most distantly related haplotypes, which indicates that gene flow via seeds between the Ebro Valley and the rest of the Iberian Peninsula has likely been limited during the evolutionary history of *L. subulatum* (see Fig. 1 and Appendix S10).

Interestingly, North Africa populations (MAR and ARGL) are fixed for a single haplotype that is closely related to the most common one. It is thus likely that the colonization of North Africa occurred via a recent, long-distance dispersal event from the Iberian Peninsula. Several pieces of evidence support this claim. First, our analysis estimated the mean date of origin of the species after the Messinian Salinity Crisis, when the Iberian Peninsula and North Africa were disconnected again by the Mediterranean Sea. Second, if *L. subulatum* had been isolated in North Africa for at least 6-5 My (during the Messinian Salinity Crisis, when the Mediterranean Sea was desiccated) we would expect a greater haplotype diversity in North

Africa or, alternatively, only one, much more divergent haplotype. Our results match those of other studies that have found that populations from both sides of the Mediterranean Sea were closely related as a consequence of long-distance dispersal events between the Iberian Peninsula and North Africa (Terrab et al., 2008), suggesting that these events have not been rare within the Mediterranean region (see Nieto Feliner, 2014 and references therein).

In our analyses, we did not detect significant demographic changes at the species level, although 15 populations showed a fixed haplotype. These fixed populations likely experienced bottlenecks caused by founder effects, likely reflecting the poor seed dispersal ability of the species. However, *L. subulatum* showed high chloroplast genetic diversity at the species level. This high haplotype diversity observed across populations in the chloroplast markers was also coupled with high overall genetic diversity in microsatellite markers. Furthermore, *L. subulatum* also exhibited high microsatellite intrapopulation diversity in all populations. These high values of genetic diversity are congruent with the current high number of individuals at each population, which may reach up to several thousand plants (personal observation). The effective population size of organelle genes is lower than that of nuclear genes (Petit et al., 2005), which could explain the slightly higher values of genetic diversity found in microsatellites markers in some populations. Some authors have reported that edaphic specialists may be composed of genetically depauperate populations due to the specialization to the substrate (see Rajakaruna, 2017 for a deeper discussion), and as such, they may constitute evolutionary dead-ends. However, our results for this species show that this is not necessarily the case, and agree with other studies that also found high levels of genetic diversity at the landscape level in both Iberian (Matesanz et al., 2019) and non-Iberian gypsophiles (Aguirre-Liguori et al., 2014).

Even though genetic variation was high regardless of the type of marker, we found contrasting results for the population genetic structure inferred by microsatellites and

chloroplast markers. We observed significant genetic structure in both markers, but greater geographic structure in chloroplast loci. We are aware that comparing markers with different number of alleles and/or different mutation rates (e.g. nuclear microsatellites and chloroplast sequence data) could bias the comparison of genetic differentiation among populations (Meirmans, 2006; Jost, 2008; Verity & Nichols 2014). Jost (2008) proposed D_{est} as a nearly unbiased estimator to assess genetic differentiation between populations accounting for different allele numbers. The calculated D_{est} values of nuclear and chloroplast markers for our populations are virtually identical to the computed F_{ST} values (see Appendix S11), suggesting that the large difference in population structure is not an artifact due to the choice of markers, but rather, it is due to eco-evolutionary processes. Because chloroplast DNA is maternally inherited and nuclear DNA is biparentally inherited in our species, it is likely that the greater genetic structure observed in the chloroplast data indicates that gene flow via pollen is higher than via seeds in *L. subulatum*. Indeed, using the indices of population differentiation (F_{ST} and G_{ST}) calculated for both markers types, and applying Ennos' equation (see Appendix S12), we estimated an effective gene flow via pollen between ~2-10 times higher than via seeds, agreeing with studies reporting that pollen flow is usually higher than seed flow (Petit et al., 2005).

However, we did not expect such large restrictions to the movement of seeds among populations in this system. In a field study assessing the role of grazing in gypsum plant communities, Pueyo et al., (2008) found that livestock act as effective seed dispersal agents between fragments. Accordingly, livestock practices, which often involve the movement of cattle across different geographical regions in the Iberian Peninsula (Azcárate et al., 2013), could have favored the movement of seeds between different *L. subulatum* populations, reducing the high genetic structure found in chloroplast markers. However, our results show limited seed dispersal, particularly between geographical regions. Specifically, the populations from the Ebro Valley and North Africa were strongly different from all other populations, as

shown by the isolation by distance among different regions, suggesting that animals are likely not playing a key role in the movement of seeds in our system, at least when long distances are considered. Despite the importance of transhumance in the Iberian Peninsula, drove roads of the Ebro Valley never have been connected to all others main drove roads (see Fig. 1 in Manzano & Casas, 2010), which could have increased the differences between this region and the rest of the Iberian Peninsula. Furthermore, several nearby populations in the same geographic region did not share haplotypes (see Tajo Valley in Fig. 3), which shows limited seed dispersal even across short distances. These results may also be explained by the fact that, similar to other gypsophiles, seeds of *L. subulatum* lack obvious long-distance dispersal mechanisms (Escudero et al., 2000; Moore and Jansen, 2007). Therefore, our results also suggest that seed movement between distant areas may only be possible by chance long-distance dispersal events. The Iberian Mountain Range, which separates the Ebro Basin populations from all others, could restrict seed movement between the populations from the Ebro Basin and all the other populations, accounting for the high genetic differences observed between this region and the rest. Similarly, the presence of the Mediterranean Sea may also block seed dispersal from the Iberian Peninsula to North Africa, explaining the distinctive haplotype in these populations.

Conversely, pollen movement does not appear to have been strongly limited between populations or geographical regions, as shown by the assignment of individuals from populations from different geographical regions to the same genetic cluster in microsatellite analyses (Fig. 5). High pollen flow among populations and regions could be favored by the presence of numerous patches of gypsum habitat among populations that would increase their connectivity, allowing an efficient movement of different pollinators between populations (Santamaría et al., 2018; Matesanz et al., 2019). *Lepidium subulatum* presents an advanced phenology compared to other species of gypsum ecosystems (Hernández Bermejo and

Clemente, 1993; Matesanz et al., 2018) and it is possible that pollinators could actively seek the flowering plants at this early season, facilitating pollen flow to further distances. Interestingly, in a few instances populations within the same region (sometimes located less than 50 km apart) possessed individuals that were assigned to different genetic clusters (Fig. 5). Although we cannot pinpoint the exact processes that modulate this complex pattern, several factors, including uneven pollen flow between populations, differential barriers to pollen flow at small scales, differences in connectivity among populations and population size could be responsible for this pattern (Aguilar et al., 2008).

Conclusions

Our results show how paleoclimatic and geological changes in Plio-Pleistocene could be important in the origin and evolution of *L. subulatum*. The contrasting pattern of genetic structure found in the nuclear and chloroplast markers, suggesting lower seed flow among populations compared to pollen flow, also highlight the importance of using both maternally and biparentally inherited markers to fully understand the phylogeography of plant species. Furthermore, the species exhibited high values of genetic diversity in both markers, especially in microsatellites. Our results suggest that regionally dominant gypsophiles like *L. subulatum* have had broad distributions and maintained high effective population sizes during their evolutionary history, suggesting that these gypsophilic taxa are relatively old. Although the markers used in this study inform us about the neutral genetic diversity of the populations, if neutral genetic diversity and quantitative genetic diversity were correlated in populations of *L. subulatum*, our results would suggest the existence of adaptive potential to cope with changing conditions. In this context, further studies should focus on the levels of quantitative genetic variation of populations and whether it is influenced by the geographical location or the evolutionary history of the populations.

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APPENDIX 1

List of: a) GenBank accession numbers for ITS sequences used in this study (individuals with only one accession number included sequence for both ITS regions). b) GenBank accession numbers for *trnT-trnL*, *trnL* intron and *trnL-trnF* regions used in this study (hyphens indicate missing sequences).

a) ***Arabidopsis arenicola,*** GQ922906; ***Arabidopsis arenosa* 1,** AAU52182; ***Arabidopsis arenosa* 2,** AAU43231; ***Arabidopsis arenosa* 3,** AAU43230; ***Arabidopsis arenosa* 4,** AAU43232; ***Arabidopsis arenosa* 5,** AAU43233; ***Arabidopsis arenosa* 6,** AAU43229; ***Arabidopsis arenosa* 7,** AAU52181; ***Arabidopsis croatica* 1,** DQ528930; ***Arabidopsis croatica* 2,** DQ528949; ***Arabidopsis croatica* 3,** DQ528826; ***Arabidopsis croatica* 4,** DQ528825; ***Arabidopsis halleri* 1,** DQ528887; ***Arabidopsis halleri* 2,** DQ528882; ***Arabidopsis halleri* 3,** DQ528881; ***Arabidopsis halleri* 4,** DQ528884; ***Arabidopsis halleri* 5,** DQ528883; ***Arabidopsis halleri* 6,** DQ528885; ***Arabidopsis halleri* 7,** DQ528886; ***Arabidopsis lyrata* 1,** DQ528819; ***Arabidopsis lyrata* 2,** DQ528815; ***Arabidopsis lyrata* 3,** DQ528820; ***Arabidopsis lyrata* 4,** DQ528814; ***Arabidopsis lyrata* 5,** DQ528817; ***Arabidopsis lyrata* 6,** DQ528816; ***Arabidopsis lyrata* 7,** DQ528821; ***Arabidopsis lyrata* 8,** DQ528818; ***Arabidopsis pedemontana,*** DQ914842; ***Arabidopsis thaliana* 1,** KM892649; ***Arabidopsis thaliana* 2,** DQ528813; ***Cardaria chalepensis,*** AJ628275, AJ628276; ***Cardaria draba,*** AJ628277, AJ628278; ***Cardaria pubescens,*** AJ628279, AJ628280; ***Lepidium affghanum,*** DQ780948; ***Lepidium africanum,*** AJ582441, AJ582498; ***Lepidium aletes,*** FM178548, FM178549; ***Lepidium alluaudii,*** AJ582436, AJ582493; ***Lepidium alyssoides* 1,** KX646435; ***Lepidium alyssoides* 2,** KF022714; ***Lepidium angustissimum,*** KC174369; ***Lepidium apetalum* 1,** AJ582466, AJ582514; ***Lepidium apetalum* 2,** JF976762; ***Lepidium apetalum* 3,** JF976761; ***Lepidium apetalum* 4,** JF976760; ***Lepidium apetalum* 5,** JF976759; ***Lepidium apetalum* 6,** JF976758; ***Lepidium apetalum* 7,** JF976757; ***Lepidium apetalum* 8,** JF976756; ***Lepidium apetalum* 9,** JF976755; ***Lepidium apetalum* 10,** JF976770; ***Lepidium apetalum* 11,** JF976767; ***Lepidium apetalum* 12,** MF785672; ***Lepidium apetalum* 13,** FJ980405; ***Lepidium apetalum* 14,** JF976769; ***Lepidium apetalum* 15,** JF976754; ***Lepidium apetalum* 16,** DQ310525; ***Lepidium apetalum* 17,** KM892613; ***Lepidium apetalum* 18,** JF976768; ***Lepidium apetalum* 19,** JF976766; ***Lepidium apetalum* 20,** JF976765; ***Lepidium apetalum* 21,** JF976764; ***Lepidium apetalum* 22,** JF976763; ***Lepidium arbuscula,*** AJ582451, AJ582517; ***Lepidium armoracia,*** AJ582454, AJ582502; ***Lepidium aschersonii,*** AJ582426, AJ582483; ***Lepidium aucheri* 1,** AJ582443, AJ582525; ***Lepidium aucheri* 2,** KF850569; ***Lepidium austrinum,*** AJ582467, AJ582515; ***Lepidium banksii* 1,** AJ582433, AJ582490; ***Lepidium banksii* 2,** KC109332; ***Lepidium banksii* 3,** KC109331; ***Lepidium bidendatum,*** AJ582468, AJ582516; ***Lepidium bipinnatifidum,*** AJ582446, AJ582522; ***Lepidium biplicatum,*** FM178550, FM178551; ***Lepidium bonariense* 1,** AJ582458, AJ582506; ***Lepidium bonariense* 2,** HM134831; ***Lepidium campestre* 1,** AJ582412, AJ582469; ***Lepidium campestre* 2,** AF055197; ***Lepidium capense,*** AJ582452, AJ582500; ***Lepidium capitatum,*** FM178552, FM178553; ***Lepidium cardamines* 1,** FM178554, FM178555; ***Lepidium cardamines* 2,** MW058062; ***Lepidium chalepense,*** KX646446; ***Lepidium crenatum,*** KX646437; ***Lepidium davisii* 1,** KX774365; ***Lepidium davisii* 2,** FJ541491; ***Lepidium davisii* 3,** FJ541492; ***Lepidium davisii* 4,** FJ541493; ***Lepidium davisii* 5,** FJ541494; ***Lepidium densiflorum,*** KX646438; ***Lepidium desertorum,*** AJ582453, AJ582501; ***Lepidium desvauxii* 1,** AJ582429, AJ582486; ***Lepidium desvauxii* 2,** KC109334; ***Lepidium dictyotum,*** AJ582415, AJ582472; ***Lepidium didymum* 1,** KM892610; ***Lepidium didymum* 2,** KM892632; ***Lepidium didymum* 3,** KM892647; ***Lepidium divaricatum,*** AJ582437, AJ582494; ***Lepidium draba* 1,** KJ623487; ***Lepidium draba* 2,** FM164554, FM164555; ***Lepidium draba* 3,** EF367913; ***Lepidium draba* 4,** KU746329; ***Lepidium draba* 5,** KX774361; ***Lepidium draba* 6,** KX646439; ***Lepidium draba* 7,** KX646440; ***Lepidium draba* 8,** KX646441; ***Lepidium draba* 9,** KX646444; ***Lepidium draba* 10,** KX646445; ***Lepidium draba* 11,** KF022715; ***Lepidium fasciculatum,*** AJ582428, AJ582485; ***Lepidium ferganense* 1,** AJ582449, AJ582519; ***Lepidium ferganense* 2,** KM892614; ***Lepidium flavum,*** AJ582444, AJ582524; ***Lepidium flexicaule* 1,** AJ582430, AJ582487; ***Lepidium flexicaule* 2,** AF100685; ***Lepidium flexicaule* 3,** KC109335; ***Lepidium flexicaule* 4,** KC109337; ***Lepidium flexicaule* 5,** KC109336; ***Lepidium foliosum* 1,** KC109339; ***Lepidium foliosum* 2,** KC109338; ***Lepidium fremontii,*** AJ582456, AJ582504; ***Lepidium fremontii* subsp. *fremontii,*** KX646447; ***Lepidium graminifolium,*** FN821616; ***Lepidium heterophyllum,*** KX646448; ***Lepidium hirtum subsp. hirtum,*** AJ582413, AJ582470; ***Lepidium huberi,*** KX646451; ***Lepidium hyssopifolium,*** AJ582435,

AJ582492; ***Lepidium kirkii,*** EF109738, EF109739; ***Lepidium lacerum* 1,** FN821519; ***Lepidium lacerum* 2,** FN821675; ***Lepidium lacerum* 3,** FN821676; ***Lepidium lasiocarpum,*** AJ582455, AJ582503; ***Lepidium latifolium,*** AJ582447, AJ582521; ***Lepidium latipes,*** AJ582416, AJ582473; ***Lepidium lyratum,*** AJ582448, AJ582520; ***Lepidium meyenii* 1,** AJ582445, AJ582523; ***Lepidium meyenii* 2,** KX646452; ***Lepidium montanum* 1,** AJ582457, AJ582505; ***Lepidium montanum* 2,** EF367921; ***Lepidium montanum* 3,** EF367922; ***Lepidium montanum* 4,** EF367923; ***Lepidium montanum* 5,** EF367924; ***Lepidium montanum* 6,** EF367925; ***Lepidium montanum* 7,** EF367926; ***Lepidium montanum* 8,** EF367927; ***Lepidium montanum* 9,** EF367928; ***Lepidium montanum* 10,** EF367929; ***Lepidium montanum* 11,** EF367930; ***Lepidium montanum* 12,** KX646453; ***Lepidium montanum* 13,** EF367931; ***Lepidium montanum* 14,** EF367932; ***Lepidium montanum* 15,** EF367933; ***Lepidium montanum* 16,** EF367934; ***Lepidium montanum* 17,** EF367935; ***Lepidium montanum* 18,** EF367936; ***Lepidium montanum* 19,** EF367937; ***Lepidium montanum* 20,** EF367938; ***Lepidium montanum* 21,** EF367939; ***Lepidium montanum* 22,** EF367940; ***Lepidium montanum* 23,** EF367914; ***Lepidium montanum* 24,** EF367941; ***Lepidium montanum* 25,** EF367942; ***Lepidium montanum* 26,** EF367943; ***Lepidium montanum* 27,** EF367944; ***Lepidium montanum* 28,** EF367945; ***Lepidium montanum* 29,** EF367946; ***Lepidium montanum* 30,** EF367947; ***Lepidium montanum* 31,** EF367948; ***Lepidium montanum* 32,** EF367949; ***Lepidium montanum* 33,** EF367950; ***Lepidium montanum* 34,** EF367915; ***Lepidium montanum* 35,** EF367951; ***Lepidium montanum* 36,** EF367952; ***Lepidium montanum* 37,** EF367953; ***Lepidium montanum* 38,** EF367954; ***Lepidium montanum* 39,** EF367955; ***Lepidium montanum* 40,** EF367956; ***Lepidium montanum* 41,** EF367957; ***Lepidium montanum* 42,** EF367958; ***Lepidium montanum* 43,** EF367959; ***Lepidium montanum* 44,** EF367960; ***Lepidium montanum* 45,** EF367916; ***Lepidium montanum* 46,** EF367961; ***Lepidium montanum* 47,** EF367962; ***Lepidium montanum* 48,** EF367963; ***Lepidium montanum* 49,** EF367964; ***Lepidium montanum* 50,** EF367965; ***Lepidium montanum* 51,** EF367966; ***Lepidium montanum* 52,** EF367967; ***Lepidium montanum* 53,** EF367968; ***Lepidium montanum* 54,** EF367969; ***Lepidium montanum* 55,** EF367970; ***Lepidium montanum* 56,** EF367917; ***Lepidium montanum* 57,** EF367918; ***Lepidium montanum* 58,** EF367919; ***Lepidium montanum* 59,** EF367920; ***Lepidium muelleri ferdinandi,*** AJ582427, AJ582484; ***Lepidium myriocarpum,*** AJ582442, AJ582499; ***Lepidium naufragorum* 1,** AJ582422, AJ582479; ***Lepidium naufragorum* 2,** AF100686; ***Lepidium navasii* 1,** KU213888; ***Lepidium navasii* 2,** KU213880; ***Lepidium navasii* 3,** KU213881; ***Lepidium navasii* 4,** KU213882; ***Lepidium navasii* 5,** KM201470; ***Lepidium navasii* 6,** KM201471; ***Lepidium navasii* 7,** KM201472; ***Lepidium navasii* 8,** KM201473; ***Lepidium navasii* 9,** KM201474; ***Lepidium navasii* 10,** KU213883; ***Lepidium navasii* 11,** KU213884; ***Lepidium navasii* 12,** KM201477; ***Lepidium navasii* 13,** KU213885; ***Lepidium navasii* 14,** KU213886; ***Lepidium navasii* 15,** KU213887; ***Lepidium navasii* 16,** KM201478; ***Lepidium navasii* 17,** KM201479; ***Lepidium navasii* 18,** KM201480; ***Lepidium navasii* 19,** KM201481; ***Lepidium navasii* 20,** KM201482; ***Lepidium navasii* 21,** KU213889; ***Lepidium navasii* 22,** KU213890; ***Lepidium navasii* 23,** KM201465; ***Lepidium navasii* 24,** KU213891; ***Lepidium navasii* 25,** KU213892; ***Lepidium navasii* 26,** KU213893; ***Lepidium navasii* 27,** KU213894; ***Lepidium navasii* 28,** KU213895; ***Lepidium navasii* 29,** KU213896; ***Lepidium navasii* 30,** KU213897; ***Lepidium navasii* 31,** KU213898; ***Lepidium navasii* 32,** KU213899; ***Lepidium navasii* 33,** KU213900; ***Lepidium navasii* 34,** KM201466; ***Lepidium navasii* 35,** KU213901; ***Lepidium navasii* 36,** KU213902; ***Lepidium navasii* 37,** KU213903; ***Lepidium navasii* 38,** KM201475; ***Lepidium navasii* 39,** KM201476; ***Lepidium navasii* 40,** KU213904; ***Lepidium navasii* 41,** KU213905; ***Lepidium navasii* 42,** KU213906; ***Lepidium navasii* 43,** KU213907; ***Lepidium navasii* 44,** KU213908; ***Lepidium navasii* 45,** KM201467; ***Lepidium navasii* 46,** KU213909; ***Lepidium navasii* 47,** KU213910; ***Lepidium navasii* 48,** KU213911; ***Lepidium navasii* 49,** KU213912; ***Lepidium navasii* 50,** KU213913; ***Lepidium navasii* 51,** KM201468; ***Lepidium navasii* 52,** KM201469; ***Lepidium navasii* 53,** KU213878; ***Lepidium navasii* 54,** KU213879; ***Lepidium nesophilum* 1,** KC109342; ***Lepidium nesophilum* 2,** KC109343; ***Lepidium nitidum,*** AJ582414, AJ582471; ***Lepidium oblongum* 1,** AJ582462, AJ582510; ***Lepidium oblongum* 2,** KX646454; ***Lepidium obtusatum,*** KC109344; ***Lepidium obtusum* 1,** MH507026; ***Lepidium obtusum* 2,** MH507027; ***Lepidium obtusum* 3,** MH507028; ***Lepidium obtusum* 4,** MH507029; ***Lepidium obtusum* 5,** MH507030; ***Lepidium obtusum* 6,** MH507031; ***Lepidium oleraceum* 1,** AJ582434, AJ582491; ***Lepidium oleraceum* 2,** KC109352; ***Lepidium oleraceum* 3,** KC109359; ***Lepidium oleraceum* 4,** KC109347; ***Lepidium oleraceum* 5,** KC109349; ***Lepidium oleraceum* 6,** KC109361; ***Lepidium oleraceum* 7,** KC109345; ***Lepidium oleraceum* 8,** KC109355; ***Lepidium oleraceum* 9,** KC109360; ***Lepidium oleraceum* 10,** KC109356; ***Lepidium oleraceum* 11,** KC109357; ***Lepidium oleraceum* 12,** AF100687; ***Lepidium oleraceum* 13,** KC109354; ***Lepidium oleraceum* 14,** KC109350; ***Lepidium oleraceum* 15,** KC109351; ***Lepidium oleraceum* 16,** KC109358; ***Lepidium oleraceum* 17,** KC109348; ***Lepidium oleraceum* 18,** KC109353; ***Lepidium oleraceum* 19,** KC109346; ***Lepidium oxycarpum,*** AJ582417, AJ582474; ***Lepidium oxytrichum,*** AJ582424,

AJ582481; ***Lepidium paniculatum,*** FM164556, FM164557; ***Lepidium papilliferum* 1,** FJ541497; ***Lepidium papilliferum* 2,** EF367976; ***Lepidium papilliferum* 3,** EF367977; ***Lepidium papilliferum* 4,** EF367978; ***Lepidium papilliferum* 5,** EF367979; ***Lepidium papilliferum* 6,** EF367980; ***Lepidium papilliferum* 7,** EF367981; ***Lepidium papilliferum* 8,** EF367982; ***Lepidium papilliferum* 9,** EF367983; ***Lepidium papilliferum* 10,** EF367984; ***Lepidium papilliferum* 11,** EF367985; ***Lepidium papilliferum* 12,** FJ541498; ***Lepidium papilliferum* 13,** EF367986; ***Lepidium papilliferum* 14,** EF367987; ***Lepidium papilliferum* 15,** EF367988; ***Lepidium papilliferum* 16,** EF367989; ***Lepidium papilliferum* 17,** EF367990; ***Lepidium papilliferum* 18,** EF367991; ***Lepidium papilliferum* 19,** EF367992; ***Lepidium papilliferum* 20,** EF367993; ***Lepidium papilliferum* 21,** EF367994; ***Lepidium papilliferum* 22,** EF367995; ***Lepidium papilliferum* 23,** FJ541495; ***Lepidium papilliferum* 24,** EF367996; ***Lepidium papilliferum* 25,** EF367997; ***Lepidium papilliferum* 26,** EF367998; ***Lepidium papilliferum* 27,** EF367999; ***Lepidium papilliferum* 28,** EF368000; ***Lepidium papilliferum* 29,** EF368001; ***Lepidium papilliferum* 30,** EF368002; ***Lepidium papilliferum* 31,** EF368003; ***Lepidium papilliferum* 32,** EF368004; ***Lepidium papilliferum* 33,** EF368005; ***Lepidium papilliferum* 34,** FJ541496; ***Lepidium papilliferum* 35,** EF368006; ***Lepidium papilliferum* 36,** EF367971; ***Lepidium papilliferum* 37,** EF367972; ***Lepidium papilliferum* 38,** EF367973; ***Lepidium papilliferum* 39,** EF367974; ***Lepidium papilliferum* 40,** EF367975; ***Lepidium papillosum,*** AJ582425, AJ582482; ***Lepidium perfoliatum* 1,** DQ399120; ***Lepidium perfoliatum* 2,** EF368007; ***Lepidium perfoliatum* 3,** KJ623470; ***Lepidium perfoliatum* 4,** KJ623469; ***Lepidium perfoliatum* 5,** KJ623472; ***Lepidium perfoliatum* 6,** KJ623471; ***Lepidium perfoliatum* 7,** JF976773; ***Lepidium perfoliatum* 8,** JF976772; ***Lepidium perfoliatum* 9,** JF976771; ***Lepidium phlebopetalum* 1,** FM178556, FM178557; ***Lepidium phlebopetalum* 2,** AY254528; ***Lepidium pinnatifidum,*** AJ582464, AJ582512; ***Lepidium pinnatum,*** AJ582439, AJ582496; ***Lepidium platypetalum,*** DQ780949; ***Lepidium pseudohyssopifolium,*** AJ582431, AJ582488; ***Lepidium pseudopapillosum,*** AJ582423, AJ582480; ***Lepidium pseudotasmanicum,*** AJ582432, AJ582489; ***Lepidium quitense,*** AJ582463, AJ582511; ***Lepidium rotundum,*** DQ780950; ***Lepidium rubtzovii* 1,** FN821677; ***Lepidium rubtzovii* 2,** FN821520; ***Lepidium ruderale* 1,** AJ582465, AJ582513; ***Lepidium ruderale* 2,** KX646455; ***Lepidium ruderale* 3,** JF976777; ***Lepidium ruderale* 4,** KJ623528; ***Lepidium ruderale* 5,** KJ623529; ***Lepidium ruderale* 6,** KJ623527; ***Lepidium ruderale* 7,** JF976776; ***Lepidium ruderale* 8,** JF976775; ***Lepidium ruderale* 9,** JF976774; ***Lepidium sativum* 1,** AJ582459, AJ582507; ***Lepidium sativum* 2,** AF283494, AF283495; ***Lepidium sativum* 3,** AY662279; ***Lepidium sativum* 4,** LC090011; ***Lepidium schinzii,*** AJ582440, AJ582497; ***Lepidium serra,*** AJ582450, AJ582518; ***Lepidium sisymbrioides* 1,** DQ997559; ***Lepidium sisymbrioides* 2,** DQ997564; ***Lepidium sisymbrioides* 3,** DQ997560; ***Lepidium sisymbrioides* 4,** DQ997568; ***Lepidium sisymbrioides* 5,** DQ997562; ***Lepidium sisymbrioides* 6,** DQ997561; ***Lepidium sisymbrioides* 7,** DQ997570; ***Lepidium sisymbrioides* 8,** DQ997565; ***Lepidium sisymbrioides* 9,** DQ997569; ***Lepidium sisymbrioides* subsp. *matau,*** AJ582418, AJ582475; ***Lepidium sisymbrioides* subsp. *kawarau* 1,** AJ582419, AJ582476; ***Lepidium sisymbrioides* subsp. *kawarau* 2,** AF100688; ***Lepidium sisymbrioides* subsp. *sisymbrioides,*** AJ582420, AJ582477; ***Lepidium solandri* 1,** DQ997567; ***Lepidium solandri* 2,** DQ997566; ***Lepidium solandri* 3,** DQ997553; ***Lepidium solandri* 4,** DQ997556; ***Lepidium solandri* 5,** DQ997558; ***Lepidium solandri* 6,** DQ997557; ***Lepidium solandri* 7,** DQ997554; ***Lepidium solandri* 8,** DQ997555; ***Lepidium solandri* 9,** DQ997563; ***Lepidium solandri* 10,** DQ997571; ***Lepidium spinescens,*** AJ582461, AJ582509; ***Lepidium spinosum* 1,** AJ582460, AJ582508; ***Lepidium spinosum* 2,** KX646456; ***Lepidium subcordatum,*** FN821674; ***Lepidium subulatum* 1,** MW067154; ***Lepidium subulatum* 2,** MW067155; ***Lepidium subulatum* 3,** MW067156; ***Lepidium subulatum* 4,** MW067157; ***Lepidium tenuicaule,*** AJ582421, AJ582478; ***Lepidium tiehmii,*** FM164558, FM164559; ***Lepidium trifurcum,*** AJ582438, AJ582495; ***Lepidium vesicarium,*** KX646458; ***Lepidium virginicum* 1,** AF283496, AF283497; ***Lepidium virginicum* 2,** AY662280; ***Lepidium virginicum* 3,** LC090012; ***Lepidium virginicum* 4,** HM134830; ***Lepidium virginicum* 5,** AF128109; ***Lepidium virginicum* 6,** KM892658; ***Lepidium virginicum* 7,** GQ478095; ***Lepidium virginicum* 8,** KP214507;

b) ***Arabidopsis arenicola,*** DQ914838, GQ244583, - ; ***Arabidopsis croatica,*** DQ529064, AY665580, - ; ***Arabidopsis lyrata,*** DQ529095, GQ244585, - ; ***Arabidopsis neglecta,*** FJ477707, LN610061, - ; ***Arabidopsis pedemontana,*** KF547407, KF547039, - ; ***Arabidopsis petrogena,*** DQ529090, DQ313520, - ; ***Arabidopsis suecica,*** LN610047, AY167921, - ; ***Arabidopsis suecica,*** LN610047, AY167921, - ; ***Arabidopsis thaliana,*** KP191402, KX668047, - ; ***Arabidopsis umezawana,*** LN610051, LN610063, - ; ***Brassica napus,*** EF426775, -, - ; ***Cochlearia pyrenaica,*** HQ268698, -, - ; ***Lepidium africanum,*** AY015921, AY015833, AY015703; ***Lepidium alluaudii,*** AY015922, AY015834, AY015706; ***Lepidium apetalum,*** DQ821406, -, - ; ***Lepidium***

arbuscula, AY015924, AY015836, AY015707; ***Lepidium armoracia,*** AY015925, AY015837, AY015709; ***Lepidium aschersonii,*** AY015926, AY015838, AY015711; ***Lepidium aucheri,*** AY015927, AY015839, AY015713; ***Lepidium austrinum,*** AY015928, AY015840, AY015715; ***Lepidium banksii,*** AY015929, AY015841, AY015717; ***Lepidium bipinnatifidum,*** AY015931, AY015843, AY015721; ***Lepidium bonariense,*** MK261665, AY015844, AY015723; ***Lepidium capense,*** AY015933, AY015846, AY015728; ***Lepidium cardamines,*** -, MW048749, MW048751; ***Lepidium desertorum,*** AY015934, AY015847, AY015730; ***Lepidium desvauxii,*** AY015935, KC109371, AY015731; ***Lepidium dictyotum,*** AY015936, AY015849, AY015734; ***Lepidium echinatum,*** AY015937, AY015850, AY015735; ***Lepidium ferganense,*** AY015938, AY015851, AY015738; ***Lepidium flavum,*** AY015908, AY015852, AY015739; ***Lepidium flexicaule,*** AY015939, AY015853, AY015741; ***Lepidium fremontii,*** AY015940, AY015854, AY015815; ***Lepidium heterophyllum,*** AY015941, AY015855, AY015816; ***Lepidium hirtum subsp. calycotrichum,*** AY015942, AY015856, AY015817; ***Lepidium hirtum subsp. dhayense,*** AY015943, AY015857, AY015818; ***Lepidium hirtum subsp. hirtum,*** AY015819, AY015858, - ; ***Lepidium hirtum subsp. nebrodense,*** AY015945, AY015859, AY015820; ***Lepidium hirtum subsp. petrophilum,*** AY015946, AY015860, AY015821; ***Lepidium hyssopifolium,*** AY015947, AY015861, AY015743; ***Lepidium lasiocarpum,*** AY015948, EF367912, AY015745; ***Lepidium latifolium,*** MH507041, MH507043, AY015747; ***Lepidium latipes,*** AY015950, AY015864, AY015749; ***Lepidium leptopetalum,*** AY01595, AY015865, AY015751; ***Lepidium linifolium,*** AY015952, AY015866, AY015753; ***Lepidium lyratum,*** AY015953, AY015867, AY015755; ***Lepidium montanum,*** FJ541504, EF367772, AY015760; ***Lepidium muelleri-ferdinandi,*** AY015956, AY015870, AY015761; ***Lepidium myriocarpum,*** AY015957, AY015871, AY015764; ***Lepidium naufragorum,*** AY015958, AY015872, AY015765; ***Lepidium nitidum,*** AY015959, AY015873, AY015767; ***Lepidium oblongum,*** AY015960, AY015874, AY015769; ***Lepidium oleraceum,*** AY015961, AY015875, AY015771; ***Lepidium oxycarpum,*** AY015962, AY015876, AY015773; ***Lepidium oxytrichum,*** AY015963, AY015877, AY015776; ***Lepidium papillosum,*** AY015964, AY015878, AY015777; ***Lepidium pedicellosum,*** AY015965, AY015879, AY015779; ***Lepidium perfoliatum,*** KJ623396, KJ623328, - ; ***Lepidium phlebopetalum,*** AY015966, AY015881, AY015783; ***Lepidium pholidogynum,*** AY015967, AY015882, AY015785; ***Lepidium pinnatifidum,*** AY015968, AY015883, AY015787; ***Lepidium pinnatum,*** AY015969, AY015884, AY015827; ***Lepidium pseudohyssopifolium,*** - , AY015885, AY015789; ***Lepidium pseudopapillosum,*** AY015971, AY015886, - ; ***Lepidium pseudotasmanicum,*** AY015972, AY015887, AY015826; ***Lepidium quitense,*** AY015973, AY015888, AY015794; ***Lepidium rigidum,*** AY015974, AY015889, AY015828; ***Lepidium ruderale,*** KJ623452, KJ623383, AY015795; ***Lepidium schinzii,*** AY015976, AY015892, AY015797; ***Lepidium serra,*** AY015977, AY015893, AY015799; ***Lepidium sisymbrioides,*** DQ997054, - , - ; ***Lepidium sisymbrioides subsp. kawarau,*** AY015978, AY015894, AY015801; ***Lepidium sisymbrioides subsp. matau,*** AY015979, AY015895, AY015803; ***Lepidium sisymbrioides subsp. sisymbrioides,*** AY015980, AY015896, AY015805; ***Lepidium spinescens,*** AY015981, AY015897, AY015807; ***Lepidium spinosum,*** AY015914, AY015898, AY015824; ***Lepidium subulatum,*** - MW048753, MW048756; ***Lepidium trifurcum,*** AY015983, AY015900, AY015811; ***Lepidium villarsii,*** AY015916, AY015901, AY015825.

Supporting information — Blanco-Sánchez et al. — Chapter 1

Phylogeography of a gypsum endemic plant across its entire distribution range in the western Mediterranean

Mario Blanco-Sánchez, Michael J. Moore, Marina Ramos-Muñoz, Beatriz Pías, Alfredo García-Fernández, María Prieto, Lidia Plaza, Ignacio Isabel, Adrián Escudero and Silvia Matesanz

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Appendix S1: Voucher specimens deposited at URJC herbarium and obtained from Muséum National d'Histoire Naturelle (Paris) herbarium. All the Algerian samples belonged to the same locality (Chott Ech Chergui region).

Accession number	Population code	Population location
URJC-153	AGR	Agramón (Spain)
URJC-154	ALF	Alfajarín (Spain)
URJC-155	APG	Altiplano granadino (Spain)
URJC-156	ARA	Aranjuez (Spain)
URJC-157	AZQ	Aranzueque (Spain)
URJC-158	BAL	Los Balbases (Spain)
URJC-159	BAZ	Hoya de Baza (Spain)
URJC-160	BEL	Belinchón (Spain)
URJC-161	CAB	Cabezo Redondo (Spain)
URJC-162	CHI	Chinchón (Spain)
URJC-163	ECZ	Escúzar (Spain)
URJC-164	GEL	Gelsa (Spain)
URJC-165	MAR	Yerada (Morocco)
URJC-166	PDG	Portalrubio de Guadamejud (Spain)
URJC-167	PEÑ	Peñafiel (Spain)
URJC-168	PER	Peralta (Spain)
URJC-169	SEG	Vallelado (Spain)
URJC-170	SMV	San Martín de la Vega (Spain)
URJC-171	SPP	San Pedro Palmiches (Spain)
URJC-172	TDL	Tamarite de Litera (Spain)
URJC-173	TER	Villalba Baja (Spain)
URJC-174	TOP	Topares (Spain)
URJC-175	TOR	Torquemada (Spain)
URJC-176	VAL	Valdeganga (Spain)
URJC-177	VY	Venta de Yesos (Spain)
URJC-178	YEB	Yebra (Spain)
MNHN-P-P06649387	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P06649388	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P06649389	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P04627181	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P04627183	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P04627184	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P04627185	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P04627186	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P05099808	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P05352647	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P05352649	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P05352660	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P05352664	ARGL	Chott Ech Chergui (Algeria)
MNHN-P-P05352669	ARGL	Chott Ech Chergui (Algeria)

Appendix S2: Microsatellite loci used in this study. For each locus, the following information is provided: primer sequences (all developed by Martínez-Nieto, Merlo, & Mota, 2012), observed sized ranges, repeat motif, and number of alleles recovered.

Locus	Primer Sequence (5' – 3')	Size range (bp)	Repeat	Nb. of alleles
Locus 1	F: CTTTCTCGCTGAGCTGTCAA R: TTGTCTCTGCCGAAATCCAT	183 - 215	GA	16
Locus 2	F: GGATTTAATTCGTGGACAGCA R: CACCGACTACTCCGATCCTC	195 - 233	AG	20
Locus 3	F: CAAATGAAAGCAGATCAAGCA R: TGGATCAATTTCCTGTTGGA	169 - 205	AG	19
Locus 4	F: TCCATTGATATTCCGAGCAA R: GGGTTACGTGATTTAGGGAACA	157 -214	TCA	24
Locus 5	F: GGGTTTGTCCCACAAGAAGA R: CAGGTCAATCGCGTGTTCTA	285 - 312	GA	14
Locus 7	F: CCAATCAATACCATCTCCCAAG R: TGTCGTTAGAATCTTGCTGAATGT	158 - 181	TG	15
Locus 8	F: GCCAACGTACAACGGAGAAT R: ATCCGATTTCGTCACTCTGC	182 - 204	GA	12
Locus 10	F: TGGTGGAGAGGACAAAGGAT R: TCAACGTAAAGCAACCCAAA	270 - 280	GA	6
Locus 11	F: ACTCCGATAAATTGGGCATC R: CAAATCTCCATTTCTCGACCA	176 - 185	AG	6
Locus 12	F: AGCTGGAGATCCGAAGAACA R: TCCATTGAAACCTCAACGTG	163 - 202	GAA	13

Appendix S3: Supporting Methods.

Details of PCR reactions.

All PCRs were performed in a S1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). Eight microsatellite markers were amplified in two multiplexed PCR reactions (two groups of four markers) and the other two markers were amplified individually. For multiplexed markers, PCR reactions were performed using a QIAGEN Multiplex PCR Kit (QIAGEN Inc., Chatsworth, California, USA), in a final volume of 12.7 µL containing 2.75 µL MiliQ H_2O, 6.25 µL PCR Master Mix, 1.25 µL Primer mix, 1.25 µL Q-solution and 1.2 µL of extracted DNA. Primer mix was made using 1 µL of forward primer and 1 µL of reverse primer (10 µM) for each marker (8 µL in total) and 42 µL of MiliQ H_2O. Thermocycler conditions for multiplexed markers were as follows: an initial denaturation step of 15 min at 95ºC followed by 30 cycles of 95ºC for 45s, 55ºC for 45s, 72ºC for 1 min, and a final extension of 72ºC for 7 min. For markers amplified individually, PCR reactions were performed in a final 20.2 µL volume containing 13.6 µL MiliQ H_2O, 2 µL 10X PCR Buffer with $MgCl_2$ (BIOTOOLS, B&M Labs, S.A., Madrid, Spain), 0.8 µL dNTPs, 0.8 µL of each primer (10 µM), 1 µL of DNA Polymerase (BIOTOOLS, B&M Labs, S.A., Madrid, Spain) and 1.2 µL of extracted DNA. Reaction conditions for the two markers performed individually were exactly the same, only modifying the first step of 15 min at 95ºC for 4 min at 95ºC.

PCR reactions for nuclear and chloroplast loci were performed in a final 20.2 µL volume containing 13.6 µL MiliQ H_2O, 2 µL 10X PCR Buffer with $MgCl_2$ (BIOTOOLS, B&M Labs, S.A., Madrid, Spain), 0.8 µL dNTPs, 0.8 µL of each primer (10 µM), 1 µL of DNA Polymerase (BIOTOOLS, B&M Labs, S.A., Madrid, Spain) and 1.2 µL of extracted DNA. Reaction conditions slightly differed for each marker, as follows:

For *psbA*: 2 min at 95°C followed by 30 cycles of: 94°C for 1 min, 45s at 54°C and 1 min at 72°C, and a final extension step at 72°C for 8 min.

For *matK*: 2.5 min at 94ºC followed by ten cycles of: 94ºC for 30s, 54ºC for 30s, 72ºC for 30s, and 25 cycles of: 88ºC for 30s, 54ºC for 30s, 72ºC for 30s, and a final extension at 72ºC for 10 min.

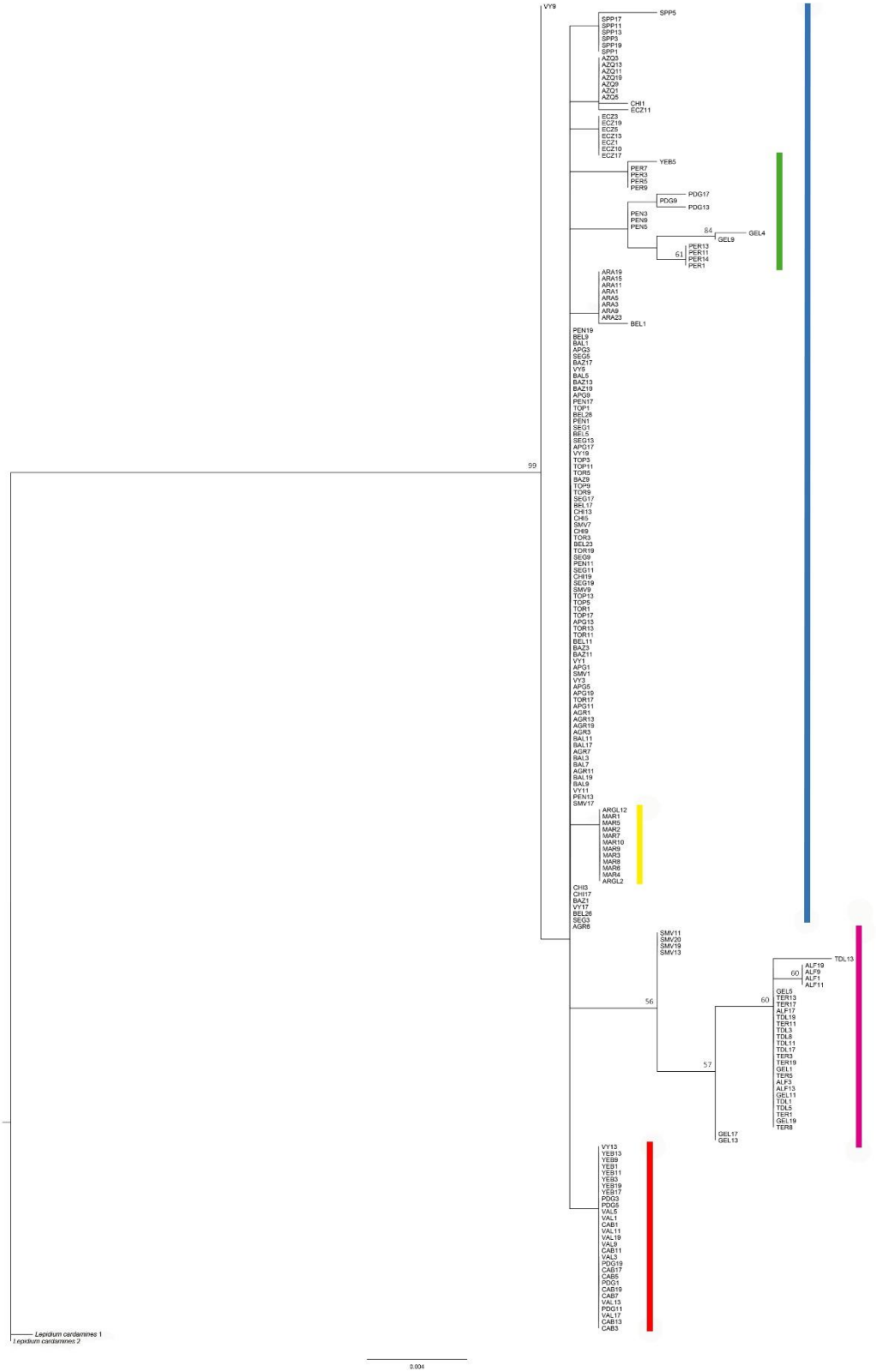
For ITS: 2.5 min at 94°C followed by 42 cycles of: 94°C for 30s, 30s at 40°C and 1 min at 70°C, and a final extension step at 72°C for 4 min.

For *trnT-trnL*, *trnL* intron and *trnL-trnF*: 3 min at 94°C followed by 35 cycles of: 94°C for 1 min, 1 min at 50°C and 2 min at 72°C, and a final extension step at 72°C for 10 min.

Appendix S4: Molecular markers used in the preliminary screening. Markers in bold were selected for our study. Used in: MD=Molecular dating; GD&PS=Genetic diversity and population structure; TM=Testing monophyly.

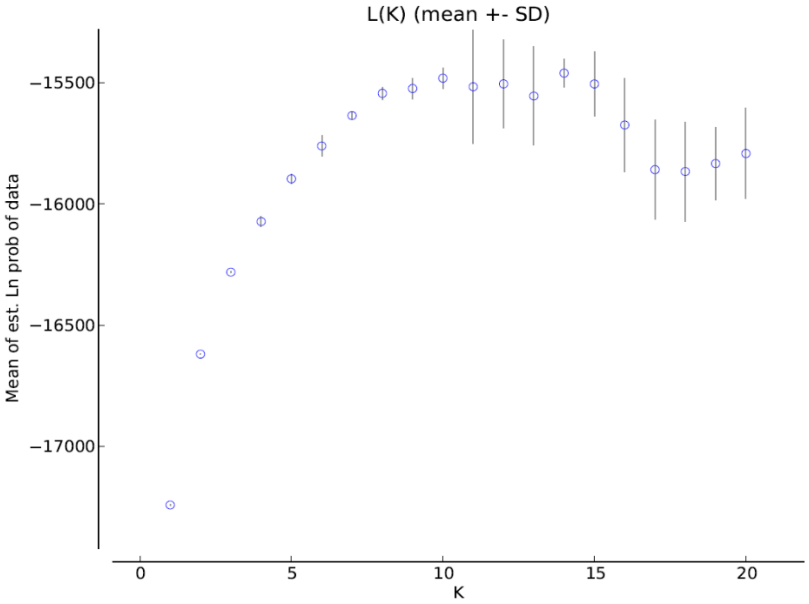
Marker	Primer Sequence (5' – 3')	Developed by:	Used in:
***psbA-trnH* spacer region**	F: CGAAGCTCCATCTACAAATGG R: ACTGCCTTGATCCACTTGGC	Bergh & Linder, 2009	GD&PS
***matK* 3F – 1R**	F: CGTACAGTACTTTTGTGTTTACGAG R: ACCCAGTCCATCTGGAAATCTTGGTTC	Jeanson, Labat, & Little, 2011	GD&PS
ITS 1 – 4	F: GGAAGGAGAAGTCGTAACAAGG R: TCCTCCGCTTATTGATATGC	Mummenhoff, Franzke, & Koch, 1997	TM
***trnT-trnL* spacer region**	F: CATTACAAATGCGATGCTCT R: TCTACCGATTTCGCCATATC	Taberlet, Gielly, Pautou, & Bouvet, 1991	MD
***trnL* intron**	F: CGAAATCGGTAGACGCTACG R: GGGGATAGAGGGACTTGAAC	Taberlet, Gielly, Pautou, & Bouvet, 1991	MD
***trnL-trnF* spacer region**	F: GGTTCAAGTCCCTCTATCCC R: ATTTGAACTGGTGACACGAG	Taberlet, Gielly, Pautou, & Bouvet, 1991	MD
rbcL 1F – 742R	F: ATGTCACCACAAACAGAAAC R: TCGCATGTACCTGCAGTAGC	Lledo, Crespo, Cameron, Fay, & Chase, 1998	-
nad7 2 – 3R	F: GCTTTACCTTATTCTGATCG R: TGTTCTTGGGCCATCATAGA	Dumolin-Lapegue, Pemonge, & Petit, 1997	-
atp6 IRD700 – IRD800	F: GGAGATTTATAGCATCATTCAAG R: ATTGTCCCATTGATTCCTAT	Zeng et al., 2012	-
PISTILLATA ITF – ITR	F: GAAATTATCTGGCAAGAAACTTTGGG R: TCCTATCAATCTCATTGCTGAGGTTC	Lee, Mummenhoff, & Bowman, 2002	-

Appendix S5: Maximum Likelihood (ML) phylogenetic tree based on concatenated *matK* and *psbA* sequences (N=204). Colored bars indicated the main haplotypes found in the haplotype network (Fig. 3). Support values higher than 50% are shown.

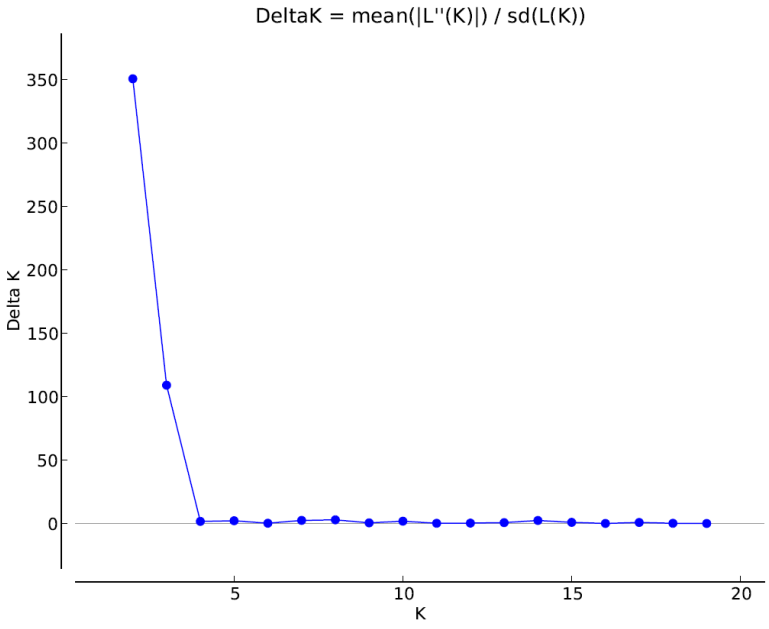


Appendix S6: a) Mean log probability of the data at each *K*. Error bars represent standard deviation; b) ΔK, changes in the log probability of data in successive K values (Evanno et al., 2005); c) Rate of change of mean log probability of the data at each *K* and ordinary probability of *K* values [calculated with rMaverick, Verity & Nichols, (2016)].

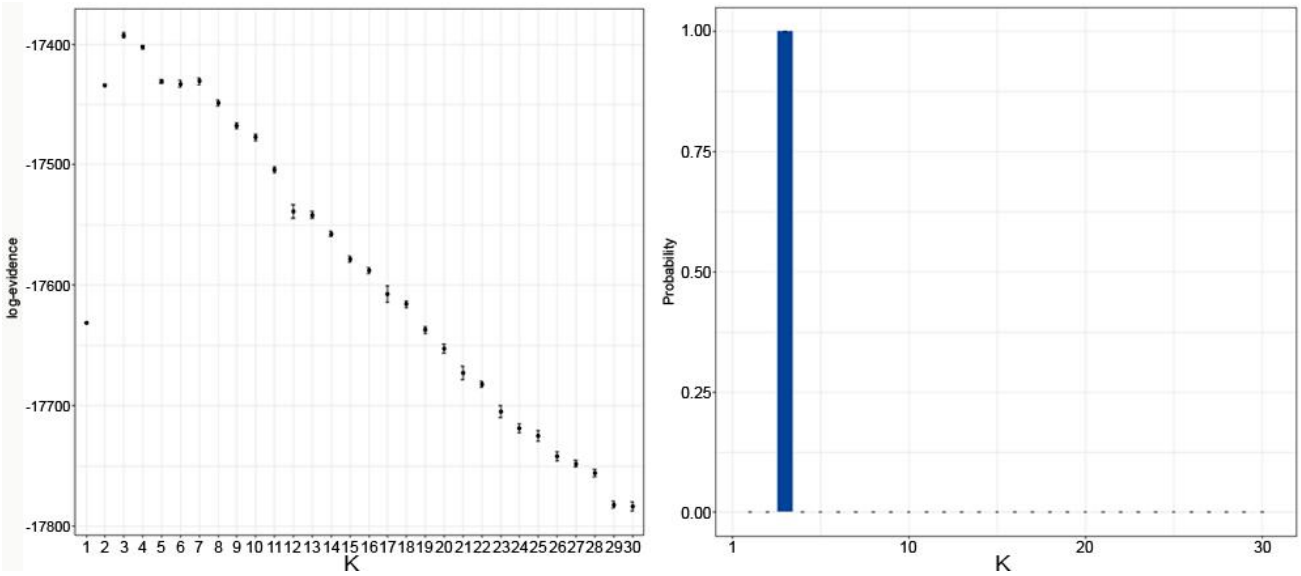
a)



b)



c)


log-evidence
-17400
-17500
-17600
-17700
-17800
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
K
Probability
1.00
0.75
0.50
0.25
0.00
1
10
20
30
K

Appendix S7: Final length of the alignments used in this study. Used in: MD=Molecular dating; CHN=Corroborating haplotype network; TM=Testing monophyly

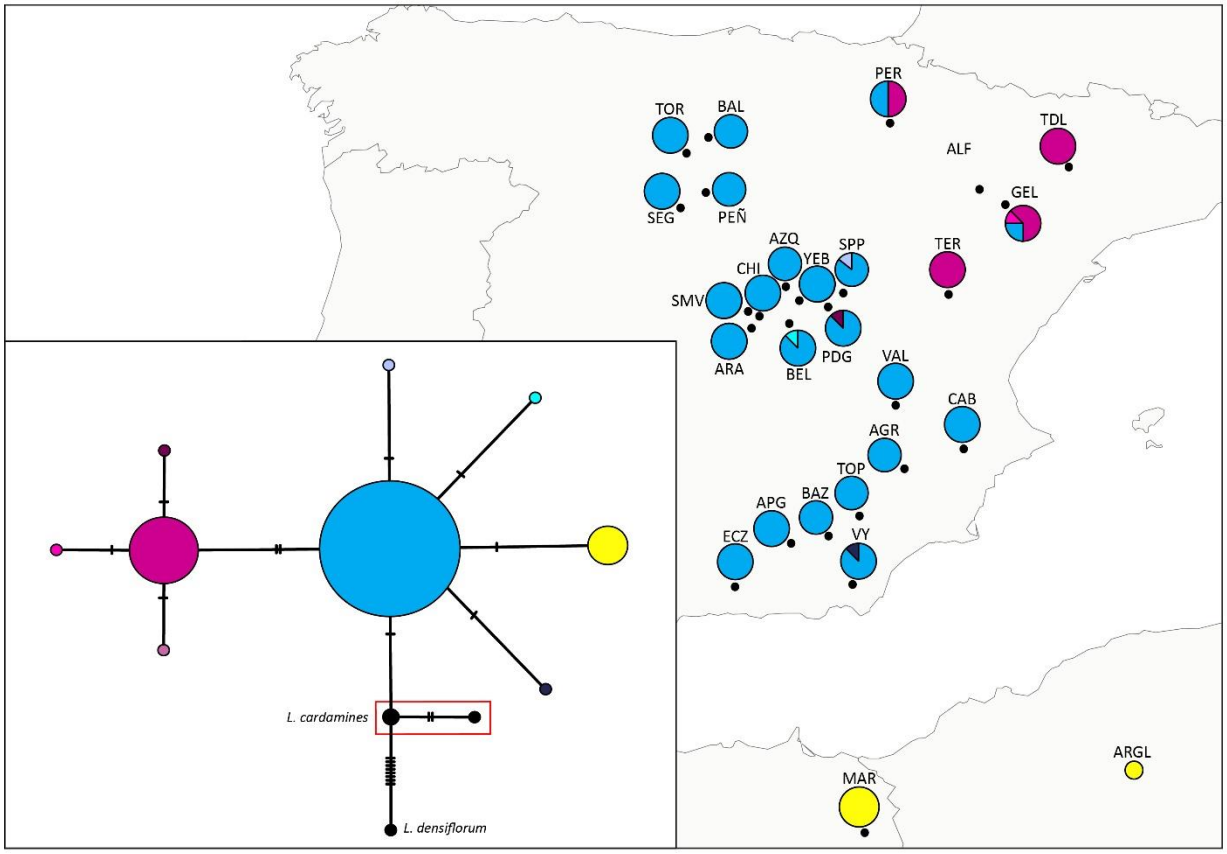
Alignment:	Final length (bp):	Sequence variation:	Used in:
psbA & *matK* regions	904	12 parsimony informative sites and 7 singleton variable sites	CHN
ITS region	561	86 parsimony informative sites and 21 singleton variable sites	TM
trnT-trnL, *trnL* intron and *trnL-trnF* regions	1561	-	MD

Appendix S9: Pairwise F_{ST} values from nuclear microsatellites for all sampled populations.

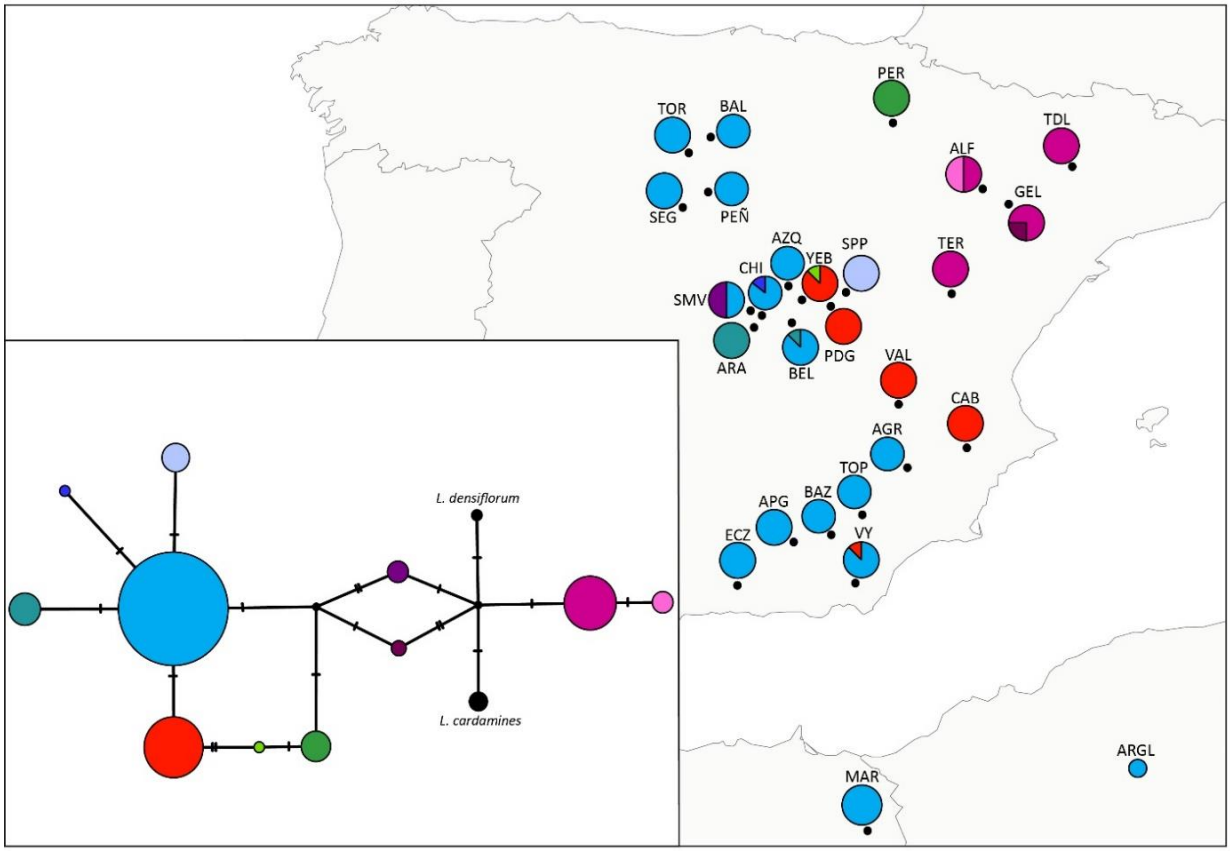
	AGR	ALF	APG	ARA	AZQ	BAL	BAZ	BEL	CAB	CHI	ECZ	GEL	MAR	PDG	PEN	PER	SEG	SMV	SPP	TDL	TER	TOP	TOR	VAL	VY	YEB
AGR	0	0.1516	0.2434	0.2279	0.1216	0.2069	0.2148	0.1623	0.2468	0.1191	0.212	0.1521	0.2599	0.0846	0.1765	0.2227	0.2789	0.1271	0.2804	0.2125	0.1754	0.2083	0.1483	0.1433	0.1958	0.2107
ALF	0.1516	0	0.0939	0.1655	0.1043	0.1786	0.0978	0.1543	0.2148	0.0939	0.1216	0.0398	0.227	0.1148	0.1146	0.1324	0.172	0.0879	0.2653	0.1499	0.1075	0.103	0.1164	0.1312	0.0707	0.2009
APG	0.2434	0.0939	0	0.2312	0.1343	0.2734	0.0639	0.2392	0.2723	0.1664	0.2201	0.1515	0.2844	0.1757	0.1251	0.1477	0.2124	0.1723	0.3187	0.234	0.0919	0.1272	0.1747	0.1741	0.1479	0.3117
ARA	0.2279	0.1655	0.2312	0	0.2071	0.2938	0.1686	0.0476	0.2011	0.1612	0.287	0.1867	0.1646	0.1833	0.1996	0.2255	0.329	0.1917	0.1981	0.2785	0.2044	0.2025	0.2269	0.2028	0.2259	0.1261
AZQ	0.1216	0.1043	0.1343	0.2071	0	0.1933	0.1233	0.165	0.2466	0.0857	0.1835	0.1096	0.2286	0.0926	0.1046	0.1455	0.2041	0.109	0.264	0.1989	0.1031	0.1623	0.1349	0.1288	0.1231	0.2249
BAL	0.2069	0.1786	0.2734	0.2938	0.1933	0	0.2383	0.2591	0.2957	0.2022	0.1867	0.1803	0.3239	0.1511	0.176	0.2343	0.2497	0.1544	0.3922	0.2392	0.2431	0.2357	0.0965	0.2184	0.2177	0.2694
BAZ	0.2148	0.0978	0.0639	0.1686	0.1233	0.2383	0	0.1647	0.2503	0.1546	0.2148	0.1424	0.1766	0.1525	0.1041	0.1309	0.1521	0.1571	0.2864	0.2177	0.1015	0.0891	0.1598	0.1616	0.1529	0.2386
BEL	0.1623	0.1543	0.2392	0.0476	0.165	0.2591	0.1647	0	0.1459	0.1474	0.2517	0.1463	0.142	0.1515	0.1747	0.1976	0.3126	0.1649	0.1344	0.2253	0.1832	0.1875	0.2002	0.1949	0.1978	0.1109
CAB	0.2468	0.2148	0.2723	0.2011	0.2466	0.2957	0.2503	0.1459	0	0.2403	0.2848	0.2288	0.2573	0.2044	0.2488	0.2526	0.363	0.2215	0.2675	0.3134	0.2508	0.279	0.2228	0.2126	0.2555	0.2401
CHI	0.1191	0.0939	0.1664	0.1612	0.0857	0.2022	0.1546	0.1474	0.2403	0	0.2106	0.061	0.229	0.085	0.1088	0.1385	0.2571	0.0298	0.2415	0.1314	0.1493	0.1587	0.1218	0.1128	0.1162	0.2179
ECZ	0.212	0.1216	0.2201	0.287	0.1835	0.1867	0.2148	0.2517	0.2848	0.2106	0	0.1627	0.3338	0.1676	0.1693	0.2269	0.2002	0.1952	0.3767	0.2785	0.2071	0.241	0.1729	0.1902	0.1965	0.3059
GEL	0.1521	0.0398	0.1515	0.1867	0.1096	0.1803	0.1424	0.1463	0.2288	0.061	0.1627	0	0.2376	0.1011	0.1095	0.1381	0.2345	0.0641	0.2242	0.1229	0.1562	0.1241	0.118	0.1514	0.0589	0.1998
MAR	0.2599	0.227	0.2844	0.1646	0.2286	0.3239	0.1766	0.142	0.2573	0.229	0.3338	0.2376	0	0.232	0.2459	0.2409	0.3488	0.2431	0.2938	0.3165	0.2384	0.2367	0.2517	0.2148	0.2568	0.2466
PDG	0.0846	0.1148	0.1757	0.1833	0.0926	0.1511	0.1525	0.1515	0.2044	0.085	0.1676	0.1011	0.232	0	0.1292	0.1478	0.2262	0.0653	0.2395	0.1475	0.1278	0.1596	0.0854	0.0954	0.1428	0.2071
PEN	0.1765	0.1146	0.1251	0.1996	0.1046	0.176	0.1041	0.1747	0.2488	0.1088	0.1693	0.1095	0.2459	0.1292	0	0.1426	0.1947	0.1181	0.2912	0.1832	0.1313	0.1232	0.089	0.1393	0.1509	0.23
PER	0.2227	0.1324	0.1477	0.2255	0.1455	0.2343	0.1309	0.1976	0.2526	0.1385	0.2269	0.1381	0.2409	0.1478	0.1426	0	0.2984	0.1146	0.2763	0.1968	0.1358	0.1651	0.1574	0.1752	0.1994	0.3148
SEG	0.2789	0.172	0.2124	0.329	0.2041	0.2497	0.1521	0.3126	0.363	0.2571	0.2002	0.2345	0.3488	0.2262	0.1947	0.2984	0	0.2456	0.4403	0.3212	0.2054	0.2211	0.1977	0.2296	0.2273	0.3268
SMV	0.1271	0.0879	0.1723	0.1917	0.109	0.1544	0.1571	0.1649	0.2215	0.0298	0.1952	0.0641	0.2431	0.0653	0.1181	0.1146	0.2456	0	0.2512	0.1274	0.1422	0.1307	0.0937	0.1227	0.1273	0.2452
SPP	0.2804	0.2653	0.3187	0.1981	0.264	0.3922	0.2864	0.1344	0.2675	0.2415	0.3767	0.2242	0.2938	0.2395	0.2912	0.2763	0.4403	0.2512	0	0.3104	0.3045	0.3121	0.3143	0.3026	0.2826	0.2826
TDL	0.2125	0.1499	0.234	0.2785	0.1989	0.2392	0.2177	0.2253	0.3134	0.1314	0.2785	0.1229	0.3165	0.1475	0.1832	0.1968	0.3212	0.1274	0.3104	0	0.2071	0.2393	0.1385	0.1807	0.1822	0.2897
TER	0.1754	0.1075	0.0919	0.2044	0.1031	0.2431	0.1015	0.1832	0.2508	0.1493	0.2071	0.1562	0.2384	0.1278	0.1313	0.1358	0.2054	0.1422	0.3045	0.2071	0	0.1119	0.1475	0.1278	0.1712	0.2803
TOP	0.2083	0.103	0.1272	0.2025	0.1623	0.2357	0.0891	0.1875	0.279	0.1587	0.241	0.1241	0.2367	0.1596	0.1232	0.1651	0.2211	0.1307	0.3121	0.2393	0.1119	0	0.1579	0.1931	0.1531	0.2563
TOR	0.1483	0.1164	0.1747	0.2269	0.1349	0.0965	0.1598	0.2002	0.2228	0.1218	0.1729	0.118	0.2517	0.0854	0.089	0.1574	0.1977	0.0937	0.3143	0.1385	0.1475	0.1579	0	0.1214	0.137	0.2246
VAL	0.1433	0.1312	0.1741	0.2028	0.1288	0.2184	0.1616	0.1949	0.2126	0.1128	0.1902	0.1514	0.2148	0.0954	0.1393	0.1752	0.2296	0.1227	0.3026	0.1807	0.1278	0.1931	0.1214	0	0.1709	0.2565
VY	0.1958	0.0707	0.1479	0.2259	0.1231	0.2177	0.1529	0.1978	0.2555	0.1162	0.1965	0.0589	0.2568	0.1428	0.1509	0.1994	0.2273	0.1273	0.2826	0.1822	0.1712	0.1531	0.137	0.1709	0	0.2232
YEB	0.2107	0.2009	0.3117	0.1261	0.2249	0.2694	0.2386	0.1109	0.2401	0.2179	0.3059	0.1998	0.2466	0.2071	0.23	0.3148	0.3268	0.2452	0.2826	0.2897	0.2803	0.2563	0.2246	0.2565	0.2232	0

Appendix S10: TCS haplotype networks for 27 populations (204 individuals) of *L. subulatum*, based on a) *matK* and b) *psbA*. The size of the different haplotypes in both networks is proportional to the number of individuals with each haplotype. The size of the pie charts is also proportional to the number of samples in each population. Note that the location of the ARGL population is approximate.

a)



b)


PER
TOR
BAL
TDL
ALF
GEL
SEG
PEÑ
AZQ
SPP
TER
YEB
CHI
SMV
ARA
BEL
PDG
VAL
CAB
AGR
TOP
APG
BAZ
ECZ
VY
L. densiflorum
L. cardamines
ARGL
MAR

Appendix S11: D_{est} values and F_{ST} values of nuclear and chloroplast markers. Note that both estimators provided virtually the same results. D_{est} values were calculated using mmod package in R (Winter, 2012).

	Nuclear microsatellites markers	**Chloroplast DNA sequences**
D_{est}	0.1901	0.5038
F_{ST}	0.1862	0.4608

Appendix S12: a) Population differentiation indices calculated from chloroplast and nuclear markers. b) Ennos' equation used to calculate the ratio between pollen flow and seeds flow.

a)

Chloroplast DNA differentiation indices
G_{ST} = 0.739 F_{ST} =0.461
Nuclear DNA differentiation index
F_{ST} = 0.186

b)

Ennos' equation (Ennos, 1994):

pollen flow / seed flow = $[(1/F_{ST\ (biparentally\ inherited)} - 1) - 2(1/ F_{ST\ (maternally\ inherited)} - 1)] / (1/F_{ST\ (maternally\ inherited)} - 1)$

Chapter 2: **Range-wide intraspecific variation reflects past adaptation to climate in a gypsum Mediterranean shrub**

Mario Blanco-Sánchez, José Alberto Ramírez-Valiente, Marina Ramos-Muñoz, Beatriz Pías, Steven J. Franks, Adrián Escudero and Silvia Matesanz

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Abstract

Assessing the existence of adaptive population differentiation is key to detect footprints of past selection, identify potentially vulnerable and resilient populations to climate change, and understand their evolutionary trajectories. Phenotypic differentiation between populations can be influenced by both genetic and environmental factors, being genetic differentiation the result of a complex combination of adaptive and neutral evolutionary processes. We evaluated the role of past adaptation and neutral evolutionary processes (gene flow, mutation, and drift) in the phenotypic differentiation and plasticity patterns of 11 populations of the dominant gypsophile *Lepidium subulatum*, throughout a wide geographic and climatic range. Using a common garden experiment with two contrasting watering treatments, we measured a large set of ecophysiological and fitness traits in each experimental plant (N=1100) and assessed neutral population differentiation (F_{ST}) through 10 nuclear polymorphic microsatellite loci. We performed F_{ST} - Q_{ST} comparisons in both watering conditions and assessed the ecological factors associated with population trait variation. Our results showed adaptive plastic responses to drought, which were surprisingly similar across populations, suggesting homogenizing past selection in plasticity. F_{ST} was significantly lower than Q_{ST} for several traits, suggesting that divergent selection has played a key role in the phenotypic differentiation among populations. Rather than soil chemical composition, population differentiation was related to local climate, with colder and humid populations showing higher specific leaf area and leaf nitrogen, lower water use efficiency and lower fitness under drought conditions. Overall, our results reveal the presence of adaptive phenotypic differentiation across populations of a gypsum specialist due to past natural selection, which was in turn mainly driven by climatic conditions. Our study also highlights the vulnerability of colder and humid populations to climate change.

Keywords: $Q_{ST} - F_{ST}$ comparisons, adaptive intraspecific variation, gypsophiles, natural selection, phenotypic plasticity, divergent evolution, adaptive population differentiation, *Lepidium subulatum.*

Introduction

Intraspecific variation in functional traits and fitness is widely found across species, and studying its causes and consequences is key to understand the evolutionary trajectories of their populations (Bolnick et al., 2011; Merilä and Crnokrak, 2001; Mitchell-Olds et al., 2007; Moreira et al., 2012). Phenotypic differentiation among populations can be influenced by both genetic and environmental factors, being quantitative genetic differentiation the result of natural selection, neutral evolutionary processes, or a combination of both (Leinonen et al., 2013; Merilä and Crnokrak, 2001; Mitchell-Olds et al., 2007; Westerband et al., 2021). Specifically, differences in the intensity of selection across environmental gradients may lead to adaptive genetic differentiation among populations, but distinguishing the effect of natural selection from a neutral process such as genetic drift is not straightforward (Merilä and Crnokrak, 2001).

A particularly useful approach to determine the importance of past natural selection and neutral evolutionary processes in the genetic differentiation of populations is Q_{ST} – F_{ST} comparisons (Lamy et al., 2012; Leinonen et al., 2013; Merilä and Crnokrak, 2001; Spitze, 1993; Whitlock, 2008). F_{ST} (Wright, 1951) is a metric that quantifies the divergence between populations in neutral markers, caused by neutral evolutionary processes such as migration and genetic drift (Leinonen et al., 2013; Whitlock, 2008). Analogous to $F_{ST,}$ Q_{ST} (Spitze, 1993) measures population genetic differentiation in phenotypic traits by assessing the proportion of variation that occurs between and within populations. Since quantitative genetic differentiation is affected by both natural selection and neutral evolutionary processes, significant differences between Q_{ST} and F_{ST} can be attributed to natural selection (Leinonen et al., 2013; Merilä and Crnokrak, 2001; Whitlock, 2008). When $Q_{ST} > F_{ST}$, trait divergence among populations is significantly higher than expected by neutral processes, suggesting that different genetically-based phenotypes have been favored in different populations by past natural selection (spatially divergent selection). In contrast, when $Q_{ST} < F_{ST}$, genetically-based phenotypic differentiation

among populations is significantly lower than expected by neutral processes, suggesting that similar phenotypes have been favored in different populations (spatially homogenizing selection). Finally, when $Q_{ST} \approx F_{ST}$, population differentiation has been likely caused only by neutral processes (Lamy et al., 2012; Leinonen et al., 2013; Merilä and Crnokrak, 2001; Whitlock, 2008).

In addition, phenotypic plasticity is an equally important adaptive process that may also affect the phenotypic differentiation of populations (Mitchell-Olds et al., 2007; Pigliucci, 2001; Westerband et al., 2021). Particularly, different populations may express differential phenotypic plasticity (i.e., P×E interactions), which may increase or decrease phenotypic differentiation under particular environmental conditions (Pujol et al., 2008). Although it is well known that the expression of both within and among population genetic variation may vary across environmental conditions (Fischer et al., 2021; Pigliucci, 2001; Pujol et al., 2008; Ramírez-Valiente et al., 2018; Sherrard et al., 2009), few studies have tested the environmental effect on Q_{ST} - F_{ST} comparisons (Ramírez-Valiente et al., 2018). Therefore, studying quantitative genetic differentiation among populations under different, ecologically-meaningful environmental conditions is needed to interpret Q_{ST} - F_{ST} comparisons robustly and make future predictions for the conservation of species, especially in species inhabiting heterogeneous habitats.

Plants restricted to gypsum soils (gypsophiles *sensu* Meyer, 1986) are excellent models to evaluate the importance of neutral and adaptive processes on population differentiation, since they have been subjected to strong selective pressures during their evolutionary history. Despite being restricted to semiarid and heterogeneous habitats, populations of widespread gypsophiles can experience substantial variation in precipitation and temperature (Escudero *et al.*, 2015; Matesanz *et al.*, 2020b). Furthermore, gypsum soils impose restrictive conditions for plant development due to soil chemical and nutrient imbalances (Cera et al., 2021; Escudero et al.,

2015; Palacio et al., 2022). In a climate change scenario, assessing adaptive population differentiation of gypsophiles could be particularly important. Insights on adaptive evolution due to climatic differences among populations are essential to make accurate predictions on species dynamics by the identification of potentially vulnerable and resilient populations to climate change. However, the role of natural selection and other evolutionary processes, and the precise selective pressures that have promoted adaptive evolution across gypsophile populations are unknown.

In our study, we assessed the role of past natural selection and neutral evolutionary processes on quantitative genetic differentiation and patterns of phenotypic plasticity across populations in the Mediterranean gypsum shrub *Lepidium subulatum*. Furthermore, we evaluated whether the possible past selection events were likely associated with differences in latitude, climate and/or soil composition across populations. We sampled 11 populations throughout the entire distribution range of the species, genotyped them using ten species-specific nuclear microsatellites, and characterized genetically-based phenotypic differentiation across populations in an outdoor common garden experiment with two ecologically-meaningful watering treatments, well-watered and drought. Specifically, we addressed the following questions: i) Was there significant quantitative genetic differentiation across populations of *L. subulatum* as a consequence of past selection?; ii) If so, what was the selective pressure behind this adaptive intraspecific differentiation?; iii) Did *L. subulatum* express phenotypic plasticity to drought in key functional traits? Did plastic responses vary among populations?; and iv) Were phenotypic plasticity patterns likely shaped by natural selection? We hypothesized that climatic differences across populations may have driven adaptive population differentiation due to differential past selection. Alternatively, since gypsum soils impose strong constraints to plant growth, soil chemical composition could have played a more important role than climate in the evolution of gypsophiles, favoring a common stress resistance syndrome across

populations (i.e., $Q_{ST} < F_{ST}$). Finally, because all populations likely experienced environmental heterogeneity during their evolutionary history, we expected the presence of high phenotypic plasticity in all populations, and similar results of Q_{ST}-F_{ST} comparisons across treatments.

Materials and methods

Species description and sampled populations

Lepidium subulatum L. (Brassicaceae) is a small perennial shrub (20–60 cm high) endemic to the Iberian Peninsula and North Africa, where it forms large populations. It is one of the most dominant and widespread gypsophiles in the Iberian gypsum habitats, has low seed dispersal ability, and is predominantly outcrossing with partial self-compatibility, as supported by low inbreeding coefficients throughout its range (Blanco-Sánchez et al., 2021).

In June 2017, we sampled 11 populations across the Iberian Peninsula. Selected populations covered the worldwide climatic distribution of *L. subulatum*, spanning a wide gradient of climatic conditions among populations (Table 1; Fig. 1; Supp. 1), with remarkable differences in annual precipitation (328-580 mm) and annual mean temperature (11.6-17.1 ºC). Climatic data were extracted from WorldClim bioclimatic layers (Fick and Hijmans, 2017) and Trabucco & Zomer (2010) soil-water balance layers, using ArcMap 10.5 (ArcGIS Desktop, ESRI, CA, USA). To account for within-site climatic heterogeneity, a 2 km buffer around each population sampling location was used to extract climatic data. At each population, we collected mature seeds and fresh leaves from 20 maternal plants separated by at least three meters from each other to avoid sampling closely-related individuals. Leaves and seeds from each maternal plant were stored separately in paper bags at room temperature until DNA extraction and the beginning of our common garden experiment, respectively (see below). To characterize soil properties, we also randomly collected three soil cores (0-20 cm depth) within a 20×20 m plot at each study population. From soil samples, we determined: total S (as a proxy of gypsum content), C, and N concentrations, using an elemental analyzer (TruSpec CHNS, LECO, MI, USA), organic matter content, estimated by chromic acid digestion, and available Olsen P, using standardized protocols with $NaHCO_3$ (Olsen, 1954), of each population. Soil samples were analyzed at IPE-CSIC (Zaragoza, Spain).

Table 1: Location, geographical coordinates, elevation, climatic conditions, and gypsum content of the 11 sampled populations of *Lepidium subulatum* L. Climatic data were extracted from WorldClim bioclimatic layers using a 2km buffer. Detailed climatic data and soil composition in each population can be found in Supp. 1.

Population code	Population location	Geographical coordinates (WGS84)		Altitude (m asl)	T. mean (ºC)	T. min. (ºC)	T. max. (ºC)	Prec. (mm)	Gypsum content (%)
BAL	Los Balbases (Burgos, Spain)	42º 13' 20.3" N	4º 4' 30.9" W	851	11.47	4.36	19.52	467.7	56.67
PEÑ	Peñafiel (Valladolid, Spain)	41º 35' 25.0" N	4º 6' 30.1" W	815	11.97	4.46	20.37	432.3	85.69
ALF	Alfajarín (Zaragoza, Spain)	41º 37' 25.5" N	0º 41' 52.3" W	219	15.17	7.46	23.59	363.2	52.00
GEL	Gelsa (Zaragoza, Spain)	41º 27' 5.3" N	0º 22' 24.6" W	254	15.36	7.42	24.01	367.3	76.22
CAB	Cabezo Redondo (Alicante, Spain)	38º 38' 32.9" N	0º 53' 33.5" W	533	15.13	7.62	23.69	370.7	76.57
ECZ	Escúzar (Granada, Spain)	37º 3' 20.2" N	3º 44' 41.5" W	927	14.78	7.42	23.37	520.3	80.22
TOP	Topares (Almería, Spain)	37º 52' 18.4" N	2º 11' 22.0" W	1157	12.40	4.26	22.10	395.1	86.93
VY	Venta de Yesos (Almería, Spain)	37º 5' 2.3" N	2º 17' 7.3" W	539	15.95	8.67	24.36	254.7	80.98
PDG	Portalrubio de Guadamejud (Cuenca, Spain)	40º 16' 15.8" N	2º 35' 14.7" W	794	13.54	5.68	22.77	508.6	26.21
SMV	San Martín de la Vega (Madrid, Spain)	40º 13' 19.2" N	3º 35' 3.3" W	551	14.73	6.76	23.83	376.3	67.84
SPP	San Pedro Palmiches (Cuenca, Spain)	40º 25' 51.9" N	2º 23' 51.1" W	850	13.50	5.75	22.60	647.8	87.92

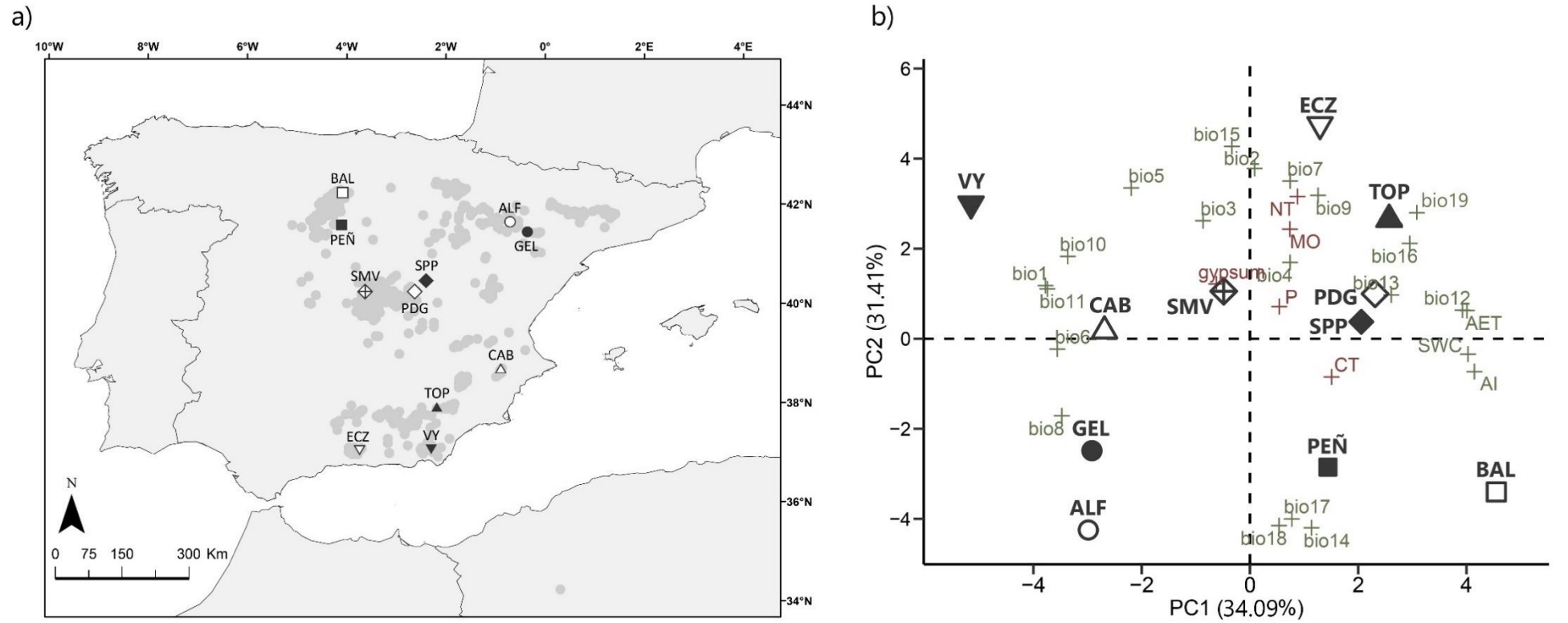

Figure 1: a) Map of the Iberian Peninsula showing all sampled populations. The grey-colored area indicates the worldwide distribution of *L. subulatum* extracted from GBIF records. Each studied population is represented with a different symbol, and population codes are those in Table 1; b) Principal component analysis (PCA) used to summarize environmental variables (climate and soil composition) of populations. The proportion of variance explained by the two first PCA axes is shown in parenthesis. Loadings and names of climatic and soil composition variables are shown in green and maroon, respectively. Abbreviations: bio1: annual mean temperature; bio2: mean diurnal range; bio3: isothermality; bio4: temperature seasonality; bio5: maximum temperature of warmest month; bio6: minimum temperature of coldest month; bio7: temperature annual range; bio8: mean temperature of wettest quarter; bio9: mean temperature of driest quarter; bio10: mean temperature of warmest quarter; bio11: mean temperature of coldest quarter; bio12: annual precipitation; bio13: precipitation of wettest month; bio14: precipitation of driest month; bio15: precipitation seasonality; bio16: precipitation of wettest quarter; bio17: precipitation of driest quarter; bio18: precipitation of warmest quarter; bio19: precipitation of coldest quarter; SWC: soil water content; AI: aridity index; AET: actual evapotranspiration; CT: soil total C; NT: soil total N; P: available Olsen P; OM: organic matter (%); gypsum: soil gypsum content (%).

Molecular analyses

To assess population differentiation in neutral markers (F_{ST}), we first extracted genomic DNA from air-dried field-collected leaves, using a commercial kit (DNeasy Plant Minikit; QIAGEN, Germany). Then, individuals were genotyped using ten species-specific nuclear polymorphic microsatellite markers (Martínez-Nieto et al., 2012). Detailed information about DNA extractions, PCR reactions, and microsatellite genotyping can be found in Blanco-Sánchez *et al.*, 2021.

Set-up of common garden experiment

The experiment was performed in the CULTIVE facilities at URJC (Móstoles, Madrid, Spain). Before sowing, 10 different maternal plants per population were randomly selected, and 10 seeds per plant were individually weighed using a Mettler Toledo MX5 microbalance (1 μg precision; Mettler Toledo, Columbus, OH, USA) to obtain a family-level seed mass. In mid-July 2018, seeds from each maternal plant and population were sown in 0.5 L pots (Alpifer, Valencia, Spain) filled with soil extracted from a gypsum quarry close to the experimental site (Yesos Ibéricos-Algiss S.A., Valdemoro, Madrid, Spain). Since *L. subulatum* pollination is mostly outcrossing, individuals from the same maternal plant constituted a maternal family and were considered half-siblings. To maximize seed germination and establishment, pots were placed in a greenhouse and maintained in optimum conditions for ~3 months. To confirm that gypsum substrate did not contain seeds of *L. subulatum*, control pots were also filled and placed in the greenhouse, showing no germination from the seed bank. In October 2018, seedlings were individually transplanted into 6 L pots (22 × 20 cm; Alpifer, Valencia, Spain) filled with the same substrate, and moved to the outdoors cultivation facility. Experimental individuals were grown in common, optimum conditions until the implementation of watering treatments.

To minimize potential maternal effects, which are expected to be greater in early phases of the growth cycle (Bischoff and Müller-Schärer, 2010 and references), and because most individuals of *L. subulatum* reach their reproductive stage in their second growing season, watering treatments were applied after two years of plant growth, when we characterized the phenotypic and fitness traits of experimental individuals. In late-February 2020, plants were haphazardly assigned to two contrasting watering treatments, well-watered and drought (N = 1100 plants; 11 populations × 10 families / population × 5 individuals / family / treatment × 2 watering treatments). To successfully implement our watering treatments, pots were moved under six purposefully-built rain exclusion structures (three per treatment) that eliminated natural precipitation without affecting other environmental conditions (see details of the structures in Supp. 2). Treatments were implemented using a drip-irrigation system with pressure-compensating emitters (Rain Bird XB05PC; Rain Bird Corporation, CA, USA), adjusting the number and duration of watering events to reach ecologically-meaningful levels of soil water content (SWC hereafter) in the experimental pots, that simulated the spatiotemporal heterogeneity of gypsum habitats (Blanco-Sánchez et al., 2022). In the well-watered treatment, plants were kept at field capacity (~25% of SWC for our substrate), simulating conditions experienced in climatically milder populations or periods when soil moisture is high (e.g., in early spring). In contrast, SWC in the drought treatment was gradually reduced and then maintained at ~50% of field capacity (12-14% of SWC), simulating the climatic conditions of harsher populations or periods when soil moisture is lower (e.g., in early summer). During the experiment, we monitored SWC of 30 pots per treatment (10 per rain exclusion structure) every 2-4 days, using an HH2 Moisture Meter with an ML3 Sensor (Delta-T devices, Cambridge, UK; see Supp. 3). The watering treatments were set for ~3 months, ending when plants in the well-watered treatment showed senescent leaves (June 2020).

Phenotypic and fitness characterization of populations

We measured the height, maximum diameter, and perpendicular diameter to the latter in all plants at the onset and the end of the watering treatments. From these, we calculated initial and final plant volume as the volume of a hemispheroid, $\frac{2}{3}\ \pi\ r_1\ r_2\ h$, where r_1 is the maximum radius, r_2 is the perpendicular radius to maximum radius and h is the height of the plant; and volume-based relative growth rate (RGR) as $\frac{(\ln V_2 - \ln V_1)}{T_{2-1}}$, where m_1 and m_2 are initial and final measurements of plant volume, respectively, and $T_{2\text{-}1}$ is the time elapsed between the two measurements (≈ 100 days).

During the experiment, we monitored the reproductive phenology of all plants every ~3 days (26 censuses). Following the phenological events described in Palacio & Montserrat-Martí (2005), we recorded the onset of flower bud formation, open flowers and fully-developed fruits for each plant, and the proportion of reproductive individuals in each census. At harvest, we visually estimated the percentage of senescent leaves in each plant. Phenological censuses were always performed by the same researcher.

In late-May 2020, we randomly collected eight non-senescent leaves per plant, storing them in zipper plastic bags with moisturized filter papers. Leaves were rehydrated for 12h, scanned using an Epson Perfection V370 Photo scanner (Seiko Epson Corporation, Japan), and oven-dried at 60ºC for 48h. Dried leaves were weighed using a microbalance, and leaf area (LA) and specific leaf area (SLA hereafter; one-sided area of water-saturated leaves divided by their oven-dry mass) were calculated. Using these leaves, we also determined leaf carbon and nitrogen content, and carbon and nitrogen stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$; a proxy of water use efficiency and N assimilation, respectively) in 3 half-siblings per population, maternal family, and treatment (N = 660). These analyses were conducted at UC Davis Stable Isotope Facility (Davis, CA, USA). We also measured the midday maximum photochemical efficiency (F_v/F_m), using a Handy PEA+ chlorophyll fluorimeter (Hansatech, UK) during three

consecutive full-sun days (from 13:00 to 17:30, UTC + 2). Measurements were taken after adapting leaves to the dark for 30 minutes.

Before harvesting, we counted the number of inflorescences in all plants and, when possible, measured the length of three randomly-selected inflorescences. Reproductive biomass was collected, weighed in a microbalance, and thoroughly cleaned to separate viable seeds. Ten viable seeds per plant were individually weighed using a microbalance. Finally, above-ground tissues were harvested, oven-dried and weighed in a Kern ABJ 120-4M analytical balance (1 mg precision; Kern & Sohn GmbH, Germany).

Statistical analyses

Population differentiation in quantitative traits and phenotypic plasticity patterns

To assess genetically-based population differentiation in quantitative traits, the effect of watering treatments on trait expression (i.e., phenotypic plasticity) and whether populations differed in their plastic responses (i.e., P×E), we fitted linear mixed models with restricted maximum likelihood (REML), including individual trait values as the dependent variable, population, treatment and the population-by-treatment interaction as fixed factors, and maternal family as a random factor. Furthermore, to consider potential factors affecting trait expression, family-level seed mass and the identity of rain-exclusion structures as fixed covariates. The significance of fixed factors was assessed using function *Anova* (package car; Fox et al., 2012) with type III sum of squares and the Kenward–Roger approach. Marginal and conditional R^2 (i.e., the proportion of variance explained by fixed factors, and by all factors in the models, respectively), were calculated for each model using function *r.squaredGLMM* (package MuMIn; Barton, 2020). To avoid issues caused by multiple testing, *P-values* were corrected by false discovery rate (FDR; Benjamini & Hochberg, 1995) using function *p.adjust*. A significant effect of population indicated genetically-based differences among populations in quantitative

traits; a significant effect of treatment indicated significant phenotypic differences across treatments (phenotypic plasticity); and a significant population-by-treatment interaction indicated differences in plasticity among populations (i.e., differential plasticity; P×E).

Then, we calculated within-treatment quantitative genetic differentiation among populations for each trait (Q_{ST}) by partitioning the total additive genetic variance into the between- and within-population components (σ_B^2 and σ_W^2, respectively), using the following formula (Spitze, 1993):

$$Q_{ST} = \frac{\sigma_B^2}{(\sigma_B^2 + 2 \cdot \sigma_W^2)} = \frac{V_P}{V_P + 2 \cdot (4 \cdot V_F)}$$

Variance components for the calculation of mean Q_{ST} and Q_{ST} distributions for each trait and treatment were estimated using Bayesian mixed models with MCMCglmm package (Hadfield et al., 2019). We ran 5000000 iterations of Markov chain Monte Carlo (MCMC), with a burning period of 500000 and a thinning interval of 5000 iterations, and non-informative inverse Wishart priors were set (V = 1, nu = 0.002; Hadfield, 2010). Population and maternal family were included in the models as random factors. Within-population variance components (σ_W^2) were calculated multiplying by four the variance among families (V_F), since individuals from the same family were considered half-siblings (Ramírez-Valiente et al., 2018; Whitlock and Guillaume, 2009). To minimize potential maternal effects and environmental differences across rain exclusion structures affecting the phenotypic expression of individuals, family-level seed mass and the identity of rain exclusion structures were included in the models as fixed covariates. However, because experimental individuals came from seeds collected in populations with contrasting environmental conditions, we cannot exclude that the estimates of within-population genetic variance (σ_W^2) were upwardly biased due to maternal effects, being our Q_{ST} estimates potentially smaller than the actual values (Ramírez-Valiente et al., 2018; Whitlock, 2008).

Population differentiation in neutral markers and $Q_{ST} - F_{ST}$ comparisons

To assess population differentiation in neutral markers, we calculated the F_{ST} distribution from microsatellite data. Briefly, we generated 10000 bootstrapped F_{ST} values across loci using GDA 1.1 (Lewis and Zaykin, 2002), and these values were multiplied by Lewontin–Krakauer χ^2 distribution (Lewontin and Krakauer, 1973) to account for potential deviations in F_{ST} among loci caused by demographic factors (Whitlock, 2008; Whitlock & Guillaume, 2009; see similar approaches in Hernández-Serrano *et al.*, 2014; Ramírez-Valiente *et al.*, 2018).

Then, to quantify the relative importance of neutral evolutionary processes and natural selection in the quantitative genetic differentiation of populations, we compared the Q_{ST} distribution for each trait in each treatment with the F_{ST} distribution inferred from neutral microsatellites. Since F_{ST} estimates are extremely variable across loci, Whitlock (2008) highlighted that the distributions of Q_{ST} and F_{ST} should be compared, rather than their mean values, to robustly interpret the results obtained from $Q_{ST} - F_{ST}$ comparisons. We first compared the 95% CIs of both parameters, and considered that Q_{ST} for a particular trait was not statistically different from F_{ST} when their CIs overlapped (Marin et al., 2020). Then, we compared the distributions of Q_{ST} and F_{ST} using Kruskal–Wallis nonparametric tests (Ramírez-Valiente et al., 2018).

Associations between population trait means and their plasticity, and environmental conditions of populations.

To test whether population differentiation in quantitative traits was associated with local climatic conditions or soil composition of populations, we performed both univariate and multivariate associations between: a) populations' trait means in each treatment, and b) their plasticity, with the climatic conditions and soil chemical composition of populations. First, to obtain within-treatment populations' trait means, we fitted within-treatment linear mixed

models with individual trait values as the dependent variable, population, family-level seed mass, and rain-exclusion structure ID as fixed factors, and maternal family as a random factor. Within-treatment populations' trait means were extracted from these models using emmeans package (Lenth, 2021). Furthermore, for traits that showed significant variation for plasticity among populations (i.e., P×E), we calculated a plasticity index (RDPI; Valladares *et al.*, 2006) for each population. For univariate associations, we selected three climatic variables that were associated with the two first axes of the PCA used to summarize the climatic conditions and soil chemical composition of populations (see results of Fig. 1b, environmental PCA hereafter): mean annual temperature (bio1), annual precipitation (bio12), and precipitation seasonality (bio15). Finally, we explored univariate associations between populations' trait means in each treatment and trait plasticity with the local climate, latitude, and soil chemical composition of populations by calculating pairwise Pearson correlations.

We also assessed whether: a) multivariate phenotypic differentiation of populations in each watering treatment and, b) multivariate plasticity, were associated with multivariate differences among populations in climatic conditions and soil chemical composition. First, we summarized the multivariate phenotypic differences across populations in quantitative traits performing two PCAs, one per treatment, using the population means of all traits (phenotypic PCA in well-watered and drought hereafter). Then, we summarized climatic and soil chemical composition differences across populations performing one PCA with the values of all climatic variables and the soil chemical composition variables of populations (environmental PCA; Fig. 1b). Furthermore, multivariate plasticity was calculated by performing one PCA including the population trait means in each watering treatment, which allowed to draw a vector of phenotypic change that connected the multivariate phenotype of each population in each treatment in a multivariate phenotypic space (i.e., a multivariate reaction norm for each population; Collyer & Adams, 2007; Solé-Medina *et al.*, 2022). From this PCA, we calculated

the magnitude and direction of the multivariate plasticity vector for each population. Differences between populations in the length of this vector indicated differences in the magnitude of plasticity, while differences in direction showed a shift in the traits involved in the multivariate phenotypic change. All variables were scaled and centered before performing all PCAs. Finally, we explored multivariate associations between: a) the multivariate quantitative population differentiation in each treatment and, b) multivariate plasticity of populations, with their multivariate environmental differences by calculating pairwise Pearson correlations between the two first axes of each PCA (vector length and vector direction in the plasticity PCA).

To avoid multiple testing issues, *p-values* of both univariate and multivariate associations were corrected again using FDR. We performed all analyses using R v4.0.5 (R Core Team, 2018).

Results

Overall genetic differentiation among populations and phenotypic plasticity patterns

We detected significant differences among populations (significant effect of Population; Table 2) and between treatments in the phenotypic expression of most traits (i.e., phenotypic plasticity, significant effect of Treatment; Table 2), but plastic responses were remarkably similar across populations (i.e., no P×E, parallel norms of reaction; Table 2, Supp. 4). Only in δ13C, leaf C and N content, final plant volume, number of inflorescences and individual seed mass, populations expressed significant differential plasticity (Table 2). Conditional R^2 was higher than marginal R^2 in the models of all traits except RGR, indicating substantial variation across families within populations. The effect of maternal seed size was not significant for most functional traits (Table 2).

Table 2: Results from the linear mixed models used for testing the effect of population (df = 10), treatment (i.e., phenotypic plasticity; df =1) and their interaction (P×E; df = 10) in the phenotypic expression, including maternal seed size (df = 1) and rain exclusion structure ID (df = 2) as covariates and maternal family as a random factor. *F-statistics* (X^2 for the generalized mixed models performed with italicized traits), *P-values*, marginal, and conditional variance (R^2_M and R^2_C, respectively) for each model are shown. Significant ($P < 0.05$) and marginally significant ($0.05 < P < 0.1$) terms after FDR correction are presented in bold and italic, respectively.

	Population		**Treatment**		**Population × Treatment**		**Maternal seed size**		**Rain exclusion structure**		**R^2_m**	**R^2_c**
	F / X^2	*P*	*F / X^2*	*P*	*F / X^2*	*P*	*F / X^2*	*P*	*F / X^2*	*P*		
F_v/F_m	6.143	**<0.001**	95.178	**<0.001**	1.031	0.414	0.282	0.597	6.094	**0.004**	0.144	0.150
δ13C	5.589	**<0.001**	148.935	**<0.001**	2.454	**0.039**	0.035	0.852	2.419	0.113	0.280	0.361
δ15N	4.567	**<0.001**	257.602	**<0.001**	1.945	0.116	1.151	0.286	3.797	**0.033**	0.340	0.346
Leaf C content	3.157	**0.002**	139.380	**<0.001**	7.508	**<0.001**	0.160	0.690	2.252	0.106	0.289	0.314
Leaf N content	19.805	**<0.001**	0.008	0.931	3.717	**<0.001**	0.187	0.666	8.551	**<0.001**	0.334	0.357
Leaf area	40.461	**<0.001**	420.358	**<0.001**	0.979	0.460	2.298	0.133	16.106	**<0.001**	0.503	0.521
SLA	106.895	**<0.001**	109.245	**<0.001**	1.362	0.193	6.130	*0.075*	3.769	**0.033**	0.608	0.624
Initial plant volume	30.961	**<0.001**	0.030	0.863	1.025	0.420	0.080	0.777	20.566	**<0.001**	0.330	0.361
Final plant volume	41.063	**<0.001**	189.742	**<0.001**	2.318	**0.040**	0.981	0.324	40.453	**<0.001**	0.457	0.488
RGR	4.390	**<0.001**	300.916	**<0.001**	0.482	0.902	0.011	0.919	10.263	**<0.001**	0.274	0.274
Aerial biomass	12.981	**<0.001**	99.789	**<0.001**	1.264	0.246	0.916	0.341	39.563	**<0.001**	0.255	0.281
Flower bud onset	33.290	**<0.001**	1.178	0.278	0.296	0.982	5.185	0.100	0.384	0.681	0.371	0.399
Flowering onset	26.092	**<0.001**	0.134	0.715	0.758	0.669	8.737	**0.027**	0.073	0.929	0.354	0.401
Fruiting onset	39.860	**<0.001**	3.041	0.109	0.404	0.945	9.887	**0.020**	1.661	0.191	0.434	0.462
Proportion of reproductive plants	113.854	**<0.001**	0.032	0.859	5.959	0.819	0.370	0.543	10.471	**0.008**	0.690	0.709
Senescence	7.129	**<0.001**	426.913	**<0.001**	1.580	0.107	0.403	0.527	9.334	**<0.001**	0.330	0.363
Reproductive biomass	20.045	**<0.001**	28.763	**<0.001**	1.081	0.374	1.695	0.197	8.046	**<0.001**	0.280	0.292
Number of inflorescences	291.428	**<0.001**	3.789	*0.074*	87.441	**<0.001**	0.885	0.347	158.811	**<0.001**	0.799	0.965
Inflorescence size	17.950	**<0.001**	239.970	**<0.001**	1.685	0.220	3.951	0.167	6.312	**0.004**	0.478	0.504
Individual seed mass	15.070	**<0.001**	25.638	**<0.001**	2.351	**0.040**	45.064	**<0.001**	3.366	**0.047**	0.562	0.591

Genetic differentiation and F_{ST} - Q_{ST} comparisons

Genetic differentiation in neutral markers (F_{ST}) was 0.187 (CI = 0.145 - 0.224; Fig. 2). We found significant quantitative genetic differentiation across populations (Q_{ST}) for morphological, physiological, phenological and fitness traits in both treatments. In all cases, Q_{ST} distributions were significantly higher than F_{ST} distribution (Fig. 2). Specifically, Q_{ST} distributions were significantly higher than F_{ST} distribution for SLA, initial and final plant volume, RGR, flower bud, flowering and fruiting onset, and reproductive biomass in both treatments; for aboveground biomass, senescence, number of inflorescences and inflorescences size in the well-watered treatment; and for leaf N in the drought treatment (Fig. 2).

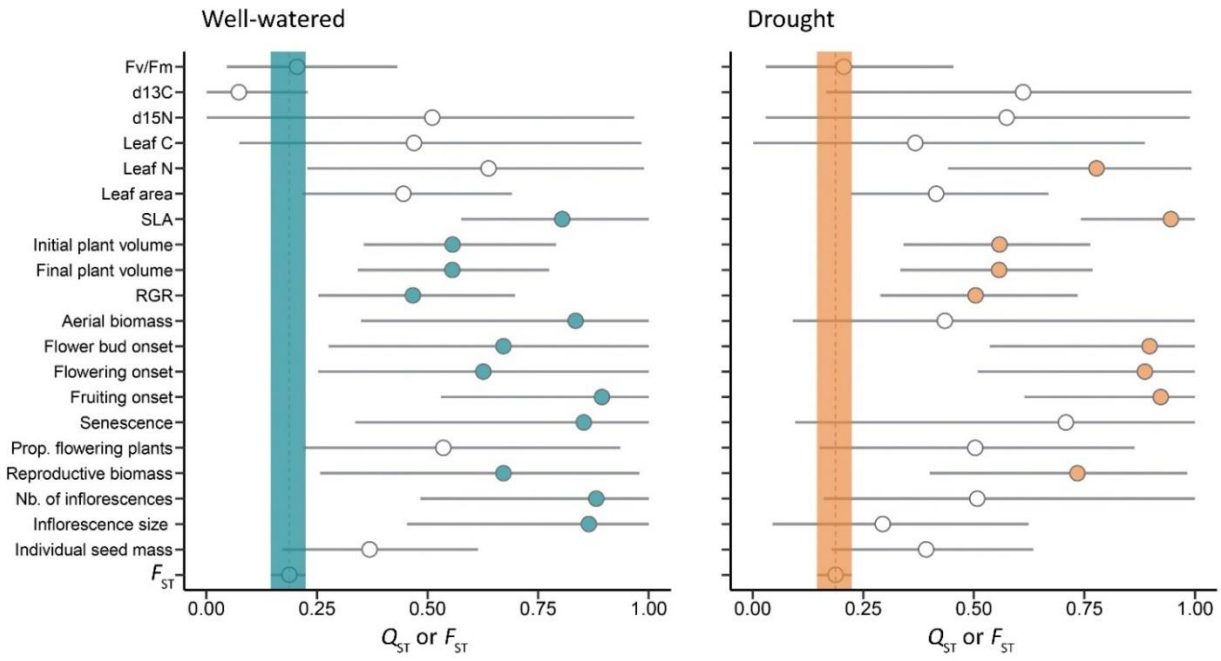

Figure 2: F_{ST} and Q_{ST} distributions (estimates and 95% CIs) for all phenotypic traits measured in 11 different populations of *L. subulatum* in the common garden experiment under well-watered and drought conditions. Colored symbols for a given trait indicate significant differences between F_{ST} and Q_{ST} distributions, while shaded areas show 95% CIs for F_{ST}.

Univariate associations between population trait means and their plasticity, and environmental conditions of populations.

We found significant univariate associations between quantitative genetic differentiation among populations in both treatments (extracted from the within-treatment models) and the local climate of populations (extracted from Worldclim bioclimatic layers; Fig. 3; Supp. 5). On average, individuals from hotter populations (higher annual mean temperature) expressed significantly higher leaf area and both initial and final plant volume, but lower SLA and leaf N content (i.e., more sclerophyllous leaves) than individuals from colder populations in both watering treatments (Fig. 3). Annual mean temperature of populations was also negatively associated with phenology and positively associated with the length and mass of inflorescences in both treatments, especially under well-watered conditions, indicating that individuals from hotter populations flowered significantly earlier and showed higher fitness (Fig. 3). Furthermore, we found a negative association between δ13C and annual mean precipitation of populations (only in the drought treatment), indicating that drier populations showed higher WUE under drought conditions (Fig. 3). In contrast, neither precipitation seasonality, latitude, nor soil composition of the sampled populations were associated with quantitative genetic differentiation among populations in either treatment (Supp. 5).

Finally, for several traits showing differentiation in population plasticity (i.e., significant P×E), we also found significant univariate associations between the plasticity of populations and their local climate. Differences in plasticity of WUE and leaf N content were positively and negatively associated with annual mean temperature and annual precipitation, respectively, indicating that more arid populations (i.e., those with higher temperature and lower precipitation) expressed higher plasticity in δ13C and leaf N (Fig. 4a).

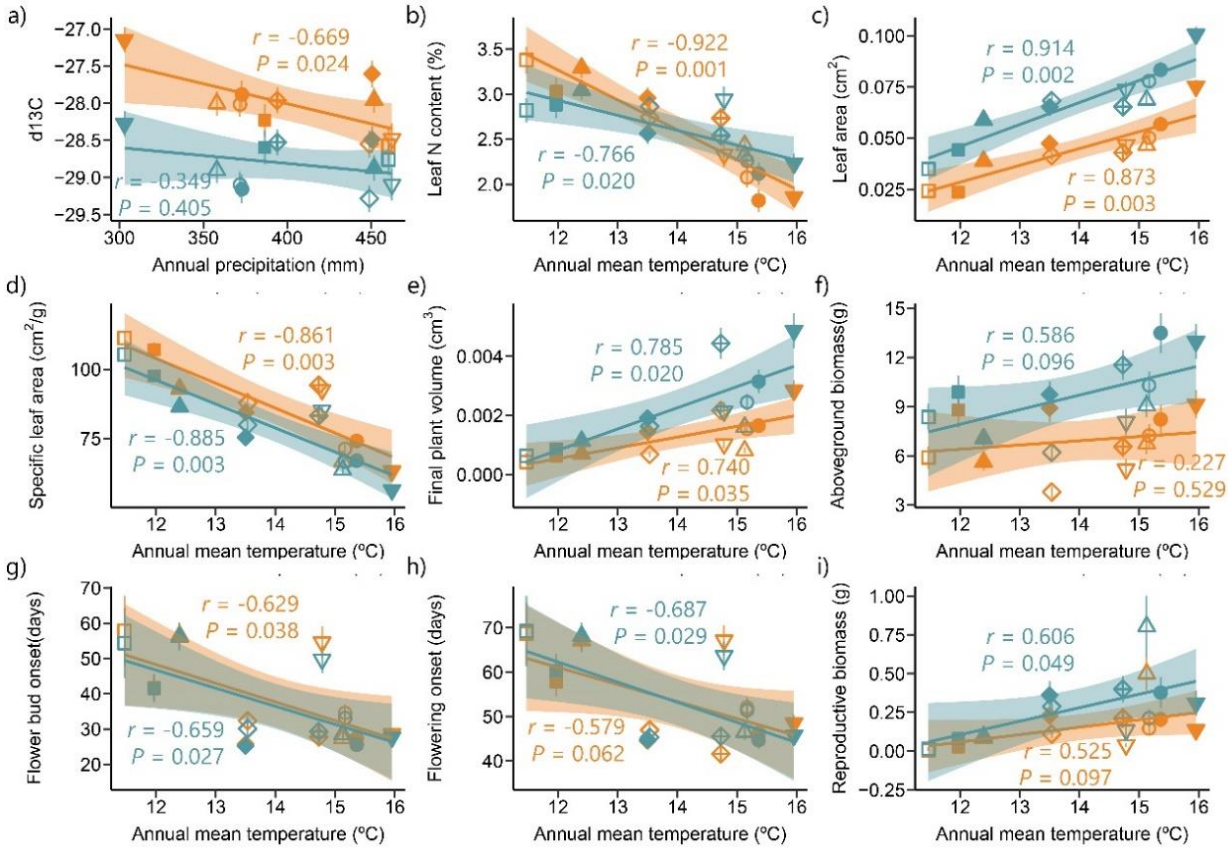

Figure 3: Univariate correlations between annual mean temperature and annual precipitation and populations' trait means in our common garden experiment (well-watered and drought treatment in blue and orange, respectively). Populations' trait means and SE are shown, using a different symbol for each population (matching those in Fig. 1). Pearson correlation results (*r* and *P*) and regression lines for each treatment are shown. Shaded areas represent 95% CIs.

Associations between multivariate phenotypes of population and multivariate plasticity, and environmental conditions of populations.

We found significant associations between the multivariate environmental PCA and the multivariate phenotypic PCAs performed to summarize quantitative genetic differentiation among populations in both treatments (Figs. 1b and 5). The first axis of the environmental PCA (Fig. 1b) explained 34.09% of the variance and was positively and negatively associated with mean temperature and mean precipitation, respectively. The second axis explained 31.41% of the environmental variance and was positively related with the seasonality of populations. Therefore, populations with higher and lower eigenvalues of PC1 and PC2, respectively,

experience lower climatic stress (i.e., higher precipitation, and lower temperature and seasonality). Finally, the third axis of the environmental PCA explained 14.38% of the variance and was positively associated with soil total N, C, P, and organic matter, and negatively associated with the gypsum content of populations (Fig. 1).

The phenotypic PCAs from both treatments showed similar trait loadings and explained variance (Fig. 5). PC1 in the well-watered and the drought phenotypic PCA explained 44.35% and 45.32% of the variance, respectively, and were positively associated with leaf N, δ15N, phenology, and SLA; and negatively with plant size (aboveground biomass, initial and final plant volume), fitness (length and mass of inflorescences), senescence, δ13C, and leaf area. PC2 explained 17.16% (well-watered) and 17.87% (drought) of the variance, and showed positive loadings for leaf C content, the number of inflorescences and the proportion of reproductive plants; and negative for F_v/F_m in both treatments. Furthermore, populations in both the well-watered and the drought phenotypic PCAs showed a similar position along the PC1, which also matched the order along the PC1 of the environmental PCA (compare population order along PC1 in Fig. 1a and Fig. 5c, d). Indeed, we found significant correlations between the PC1 of the environmental PCA and the PC1 of the both phenotypic PCAs (Fig. 5; Supp. 6). These results indicated that harsher (i.e., hotter and drier) populations showed larger but more sclerophyllous leaves (i.e., higher leaf area and lower SLA) with lower N content, higher WUE, aboveground biomass and fitness (i.e., higher length and mass of inflorescences), and earlier phenologies than those from populations with less restrictive climatic conditions. Conversely, we did not find any significant association between the PC1 of the environmental PCA and the PC2 of either phenotypic PCAs, or between either PC2 or PC3 of the environmental PCA (related to climatic seasonality and soil composition of populations, respectively) and the two first axes of either phenotypic PCAs ($P > 0.05$ in all cases; Supp. 6).

Finally, neither the magnitude nor the direction of multivariate plasticity was significantly correlated with the three first axes of the environmental PCA ($P > 0.05$ in all cases; Supp. 6). Both the magnitude and the direction of multivariate plasticity were very similar across populations (i.e., parallel multivariate reaction norms; Fig. 4), supporting the lack of P×E found in the analyses of phenotypic plasticity patterns (Table 2; Supp. 4).

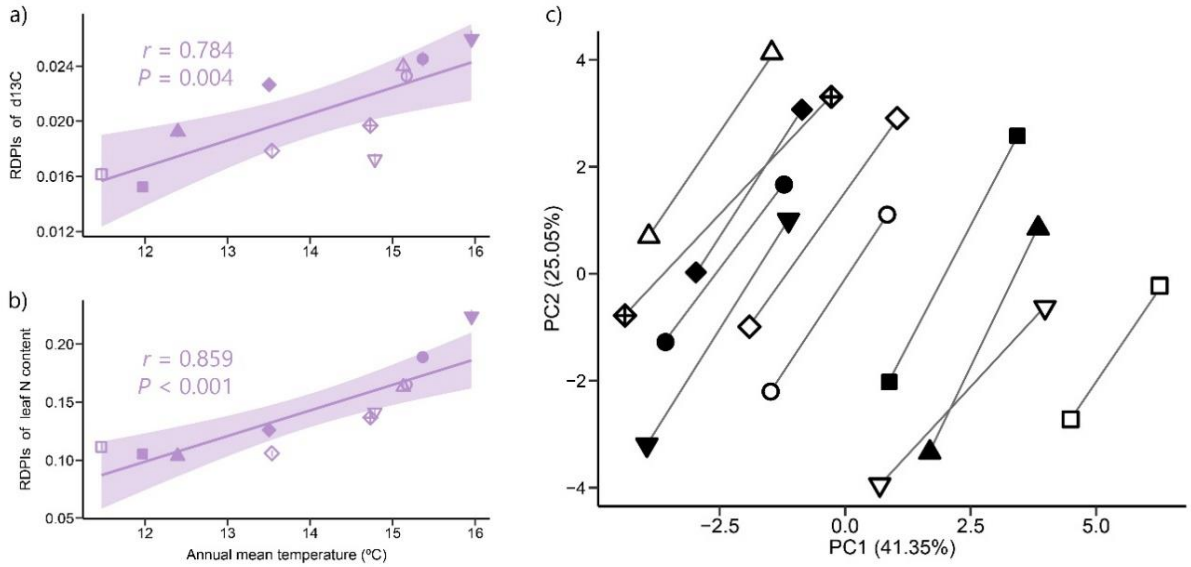

Figure 4: a) and b) Univariate correlation between annual mean temperature and phenotypic plasticity means (RDPIs) of populations for δ13C and leaf N content, respectively. Means of plasticity and SE are shown using a different symbol for each population (matching those in Fig. 1). Pearson correlation results (*r* and *P*) and regression lines are shown. Shaded areas represent 95% CIs. c) Results from the PCA used to calculate multivariate phenotypic plasticity. Population scores for each population in each treatment are shown using a different symbol for each population (matching those in Fig. 1). Multivariate reaction norms for each population are shown. Note that multivariate reaction norms of different populations are very similar in magnitude (i.e., length) and direction (i.e., angle), resulting in a pattern of parallel reaction norms.

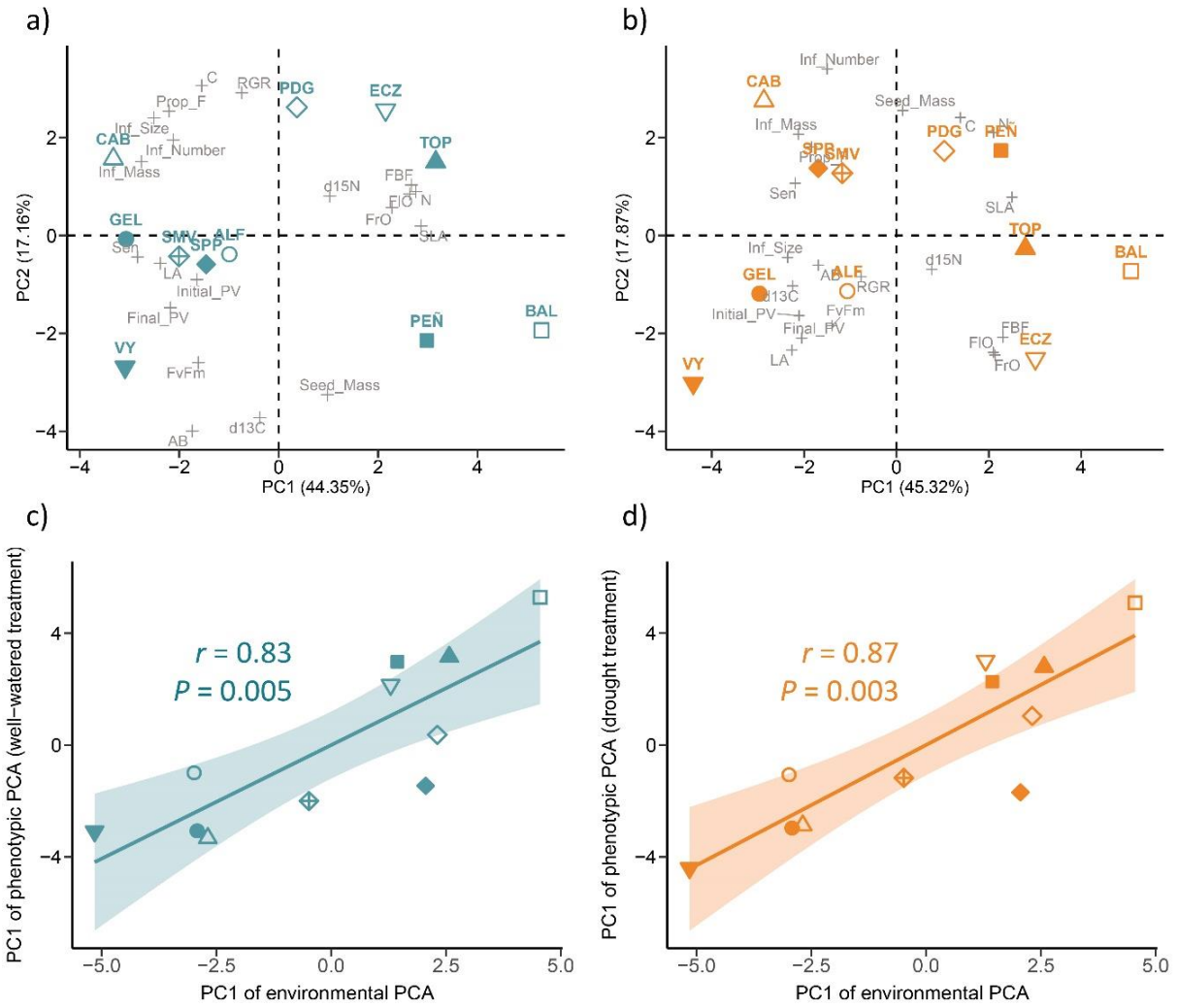

Figure 5: Principal component analysis (PCA) used to summarize quantitative trait expression of populations in the a) well-watered and b) the drought treatment; and multivariate associations between summarized climate (PC1) and summarized quantitative variation of populations (PC1) in the c) well-watered and d) the drought treatment. Each population is represented with a different symbol (matching those in Fig. 1), and population codes are those in Table 1. In the PCA, the proportion of variance explained by the two first PCA axes is shown in parenthesis. Loadings and names of functional traits are shown in grey. AB: aboveground biomass; Initial_PV: initial plant volume; Final_PV: final plant volume; RGR: relative growth rate; LA: leaf area; SLA: specific leaf area; C: leaf Carbon content; N: leaf Nitrogen content; d13C: leaf Carbon isotope ratio; d15N: leaf Nitrogen isotope ratio; FvFm: photochemical efficiency; FBF: flower bud formation; FlO: flowering onset; FrO: fruiting onset; Sen: senescence; Inf_Mass: mass of inflorescences; Inf_Number: number of inflorescences; Inf_Size: length of inflorescences; Prop_F: proportion of flowering plants; Seed_Mass: Individual seed mass. Multivariate associations show Pearson correlation results (*r* and *P*) after FDR correction, and regression lines for each treatment, with shaded areas representing 95% CIs.

Discussion

Our study assessed the importance of past natural selection and neutral evolutionary processes in the quantitative genetic differentiation among populations of the Mediterranean gypsum endemic shrub *Lepidium subulatum*. Population differentiation in neutral markers was moderate (mean F_{ST} = 0.187). In both watering treatments, Q_{ST} distributions were significantly higher than F_{ST} distributions, showing that quantitative genetic differentiation among populations has been driven by past divergent selection. Furthermore, all traits showed similar values of Q_{ST} across treatments, indicating that the expression of genetic variation of populations was not largely influenced by the watering treatment, which was supported by the absence of P×E interaction in the plasticity patterns. Furthermore, we found that intraspecific adaptive variation was associated with the climatic differences among populations. These results support that genetic differences among populations of *Lepidium subulatum* in ecophysiological traits were due to past adaptation to their local climate, rather than neutral evolutionary processes.

Gypsum habitats show several simultaneous selective pressures that impose restrictive conditions for plant growth and could have driven quantitative genetic differentiation among populations, including the presence of soil nutrient imbalances and semiarid climatic conditions among other abiotic and biotic stressors (Escudero *et al.*, 2015; Rajakaruna, 2018; and references therein). Specifically, gypsum soils have high calcium and sulfate but low nutrient concentrations, being S content is a nutritional requirement for gypsophiles (Cera et al., 2021; Palacio et al., 2022). Therefore, it could be expected that differences in soil gypsum content and nutrient composition could have promoted adaptive differentiation among populations (i.e., $Q_{ST} > F_{ST}$). Alternatively, natural selection could have favored a uniform optimum phenotype across populations able to deal with such restrictive soil chemical composition (i.e., an stress resistance syndrome *sensu* Rajakaruna, 2018), leading to phenotypic canalization (i.e., $Q_{ST} < F_{ST}$; Lamy *et al.*, 2012). However, adaptive intraspecific variation was not associated either

with gypsum or nutrient content of populations, suggesting that soil chemical composition has not shaped phenotypic differentiation in *L. subulatum*. In addition, it has been proposed that the evolution under isolated and poor-quality habitats such as special substrates often reduces within-population genetic variation, which may limit the macro and microevolution processes of edaphic specialists and leading to "evolutionary dead-ends" species (Anacker et al., 2011; Rajakaruna, 2018). However, we found significant footprints of selection and quantitative genetic variation both among and within populations, highlighting that this is not the case for this Iberian gypsophile.

Both univariate and multivariate trait-climate associations showed that quantitative genetic differentiation in *L. subulatum* was strongly related to climatic differences among populations (Figs. 3 and 5). These results suggest that past divergent selection was likely driven by heterogeneous climatic conditions across the species range, highlighting the importance of climate (mainly annual temperature and precipitation) as an evolutionary force capable to promote adaptive intraspecific differentiation in gypsum specialists. Specifically, individuals from populations with harsher climatic conditions showed earlier reproductive phenology (Figs. 3, 5), consistent with an adaptive evolutionary response to escape from drought in Mediterranean-type and semiarid ecosystems, agreeing with previous studies reporting population differentiation in phenology associated with climatic differences (Brouillette et al., 2014; Franks et al., 2007; Matesanz et al., 2020a). Furthermore, individuals from drier populations showed significantly higher water use efficiency (WUE; estimated from δ13C) than those from mesic populations in the drought treatment (Fig. 3a). In Mediterranean species, higher WUE in drier populations has been reported as an adaptation to minimize water loss, resulting in adaptive differences among populations (Lázaro-Nogal et al., 2016; Matesanz and Valladares, 2014; Ramírez-Valiente et al., 2010). Although our results contrast with the predicted trade-off between high WUE and fast reproductive phenology (Kooyers, 2015),

several recent studies have shown that populations can express both early phenologies and high WUE due to higher photosynthetic capacity, as an adaptation to lower water availability (Brouillette *et al.*, 2014; Kooyers, 2015; Kooyers *et al.*, 2015; and references therein).

Individuals from harsher populations also showed larger but more sclerophyllous leaves (i.e., higher leaf area but lower SLA) with lower leaf N content than those from climatically-milder populations (Figs. 3, 5). Since plant species often show a positive correlation between leaf area, SLA and leaf N and reduced values of these traits in harsher environments, our results contrasted again with the pattern predicted by the leaf economics spectrum (Wright et al., 2004) and reported in previous studies (Blumenthal et al., 2020; Kooyers et al., 2015). Nevertheless, a similar trait syndrome has been found in dry populations from Mediterranean species (Ramírez-Valiente et al., 2014, 2011). Previous studies have shown the adaptive value of more sclerophyllous leaves in populations inhabiting harsh environments (e.g. Ramírez-Valiente *et al.*, 2011, 2014), because robust leaves tend to have smaller cells, thicker walls, and other anatomical properties that minimize water loss and photoinhibition damage (Blumenthal et al., 2020; Solé-Medina et al., 2022). Furthermore, although high leaf area may be associated with high evapotranspiration, several studies showed that larger leaves may be adaptive for Mediterranean species even in dry populations (Donovan et al., 2007; Ramírez-Valiente et al., 2011). Larger leaf areas are sometimes associated with higher WUE (e.g., Dudley, 1996), suggesting that natural selection favored highly-efficient photosynthetic individuals with a maximized balance between carbon uptake and water loss in harsher populations. Indeed, the adaptive role of lower leaf N under drought has been discussed in terms of photosynthetic efficiency, since leaves with low SLA often lack an effective photosynthetic use of high leaf N due to CO_2 diffusion limitations (Ramírez-Valiente *et al.*, 2014 and references therein).

Although previous studies have reported that individuals from climatically hasher populations usually have conservative strategies to cope with drought (Ramírez-Valiente et al.,

2009; Solé-Medina et al., 2022), our results matched with those from studies that showed that intraspecific adaptive differentiation is not easily linked to a particular resource-use strategy, supporting that drought-tolerance and drought-escape are not mutually exclusive (Brouillette et al., 2014; Kooyers et al., 2015; Ramírez-Valiente et al., 2018, 2011). Since natural selection acts on multivariate phenotypes, selective pressures shaping the adaptive responses of populations may have contrasting or even opposing effects at the intraspecific level, which may cause discrepancies between the predicted and the observed trait values related with the resource-use strategies of species (Anderegg et al., 2021; Kooyers et al., 2015; Solé-Medina et al., 2022). The fact that individuals from hasher populations had both conservative (e.g., higher WUE, lower SLA and leaf N content) and acquisitive trait values (e.g., higher leaf area and RGR, advanced phenology) suggested that natural selection favored more intensely particular trait values to cope with drought in *L. subulatum*, instead of a specific strategy. Importantly, these results highlight that the evolution phenotypes related with contrasting resource-use strategies (acquisitive or conservative) was not genetically constrained in *L. subulatum*, matching with those from other Mediterranean species (Ramírez-Valiente et al., 2011) and agreeing with studies that discussed the smaller role of genetic constraints compared to selection in the evolution of traits (Donovan et al., 2011). More importantly, individuals from harsher populations showed consistently higher reproductive biomass in both watering treatments, indicating that adaptation to harsh environmental conditions was not coupled with a fitness trade-off across conditions (Hereford, 2009; Matesanz et al., 2020b). In contrast, individuals from populations with milder climatic conditions showed very limited fitness, especially under drought conditions, highlighting the vulnerability of these populations in a climate change context.

Phenotypic plasticity patterns showed significant and similar plastic responses to drought across populations. Since it has been hypothesized that maintaining high plasticity in

stressful environments often provides more costs than benefits and consequently, plasticity is selected against in such environments (Solé-Medina et al., 2022; Valladares et al., 2007), our results suggested that plastic responses of populations may have been also subjected to past selection in *L. subulatum*. Therefore, the strikingly similar patterns of phenotypic plasticity found suggested that natural selection has favored similar plasticity across populations (i.e., homogenizing selection on plasticity; Pigliucci & Kolodynska, 2002). Heterogeneous conditions within populations often promote the expression of phenotypic plasticity (Matesanz et al., 2020b; Van Kleunen and Fischer, 2005), being differences in environmental heterogeneity a major driver for differential plasticity across populations (Lázaro-Nogal et al., 2015; Matesanz et al., 2020b; Valladares et al., 2007). Therefore, similar levels of environmental heterogeneity across populations may have favored the presence of similar, highly plastic responses across populations, matching with previous results that revealed similar plasticity patterns across four central populations of *L. subulatum* associated with similar heterogeneity (Matesanz et al., 2020b). Such high plasticity across populations may have had relevant evolutionary consequences for gysophiles, since it likely allowed them to cope with the stressful and variable environments of gypsum habitats while maintaining high genetic variation within populations, which favored the adaptation to different climatic conditions (Gomez-Mestre and Jovani, 2013; Matesanz et al., 2020b).

Overall, past adaptive evolution was associated with climatic differences among populations in *L. subulatum*, highlighting the importance of climate rather than soil composition in adaptative intraspecific variation. Individuals from hasher populations were preadapted to drought and exhibited higher fitness in both watering treatments, highlighting the vulnerability of cold and humid populations of *L. subulatum* in a climate change context. Furthermore, all populations showed high plasticity to drought, which was likely subjected to past homogenizing

selection. The presence of past selection events on functional traits and their plasticity may be one of the main causes for the success of gypsophile species in such stressful habitats.

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Supporting information — Blanco-Sánchez et al. — Chapter 2

Range-wide intraspecific variation reflects past adaptation to climate in a gypsum Mediterranean shrub

Mario Blanco-Sánchez, José Alberto Ramírez-Valiente, Marina Ramos-Muñoz, Beatriz Pías, Steven J. Franks, Adrián Escudero and Silvia Matesanz

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Supp. 1: Mean values of environmental variables (climate and soil composition) of the 11 sampled populations of *Lepidium subulatum* L. Climatic data were extracted from WorldClim bioclimatic layers using a 2km buffer. Abbreviations: bio1: annual mean temperature; bio2: mean diurnal range; bio3: isothermality; bio4: temperature seasonality; bio5: maximum temperature of warmest month; bio6: minimum temperature of coldest month; bio7: temperature annual range; bio8: mean temperature of wettest quarter; bio9: mean temperature of driest quarter; bio10: mean temperature of warmest quarter; bio11: mean temperature of coldest quarter; bio12: annual precipitation; bio13: precipitation of wettest month; bio14: precipitation of driest month; bio15: precipitation seasonality; bio16: precipitation of wettest quarter; bio17: precipitation of driest quarter; bio18: precipitation of warmest quarter; bio19: precipitation of coldest quarter; SWC: soil water content; AET: actual evapotranspiration; AI: aridity index; NT: soil total N; CT: soil total C; P: available Olsen P; OM: organic matter (%); gypsum: soil gypsum content (%).

Pop.	bio1	bio2	bio3	bio4	bio5	bio6	bio7	bio8	bio9	bio10	bio11	bio12	bio13	bio14	bio15	bio16	bio17	bio18	bio19	SWC	AET	AI	NT	CT	P	Gypsum	OM
ALF	15.17	11.35	38.49	660.43	31.82	2.33	29.49	17.40	8.77	23.59	7.46	371.75	49.80	17.50	29.22	126.65	69.05	79.35	72.70	31.00	314.45	0.22	0.03	3.05	1.01	52.00	0.67
BAL	11.47	11.82	40.29	623.95	28.79	-0.54	29.33	7.87	19.52	19.52	4.36	460.15	52.85	18.10	29.65	144.70	66.45	66.45	131.35	49.58	456.80	0.36	0.05	4.13	0.74	56.67	0.93
CAB	15.13	13.12	41.49	662.11	32.77	1.16	31.61	15.92	23.69	23.69	7.62	358.00	47.80	9.45	37.64	118.70	48.70	48.70	78.05	34.50	354.85	0.21	0.06	2.47	1.20	76.57	0.94
ECZ	14.78	13.68	42.04	657.73	33.83	1.29	32.54	8.28	23.37	23.37	7.42	462.28	64.28	3.00	55.67	184.50	25.06	25.06	177.44	41.00	429.22	0.28	0.09	1.97	1.06	80.22	1.34
GEL	15.36	12.10	39.31	678.35	32.75	1.98	30.77	17.64	8.77	24.01	7.42	372.95	50.58	14.26	32.05	124.11	68.53	73.84	72.42	32.58	321.74	0.23	0.06	3.08	1.11	76.22	1.00
PDG	13.54	12.66	39.19	703.29	32.44	0.14	32.31	9.32	22.71	22.77	5.68	448.83	54.11	12.00	38.69	157.56	54.17	54.89	137.11	37.92	388.17	0.30	0.11	6.35	2.98	26.21	2.62
PEN	11.97	11.77	39.36	652.25	29.47	-0.44	29.90	8.10	20.24	20.37	4.46	386.45	46.30	14.05	32.67	123.65	53.35	65.20	109.75	40.00	377.95	0.29	0.02	0.48	1.75	85.69	0.53
SMV	14.73	12.17	38.43	699.12	32.85	1.17	31.68	10.49	23.82	23.83	6.76	393.95	50.68	9.47	40.51	142.32	46.11	46.16	119.89	35.92	360.47	0.26	0.10	2.20	1.97	67.84	1.46
SPP	13.50	12.05	38.32	692.72	31.81	0.37	31.44	9.33	22.60	22.60	5.75	450.32	56.53	12.32	39.98	162.63	54.42	54.42	137.68	39.17	386.21	0.30	0.09	4.02	1.88	87.92	1.77
TOP	12.40	14.40	40.92	734.85	33.22	-1.97	35.19	8.15	21.91	22.10	4.26	451.63	51.63	8.00	41.58	151.84	41.68	45.53	142.16	40.92	424.68	0.27	0.07	1.46	0.88	86.93	1.20
VY	15.95	13.32	42.44	647.92	33.76	2.36	31.39	12.37	24.36	24.36	8.67	303.00	37.47	3.00	48.55	110.84	21.21	21.21	102.74	27.67	283.00	0.18	0.07	1.02	1.43	80.98	1.61

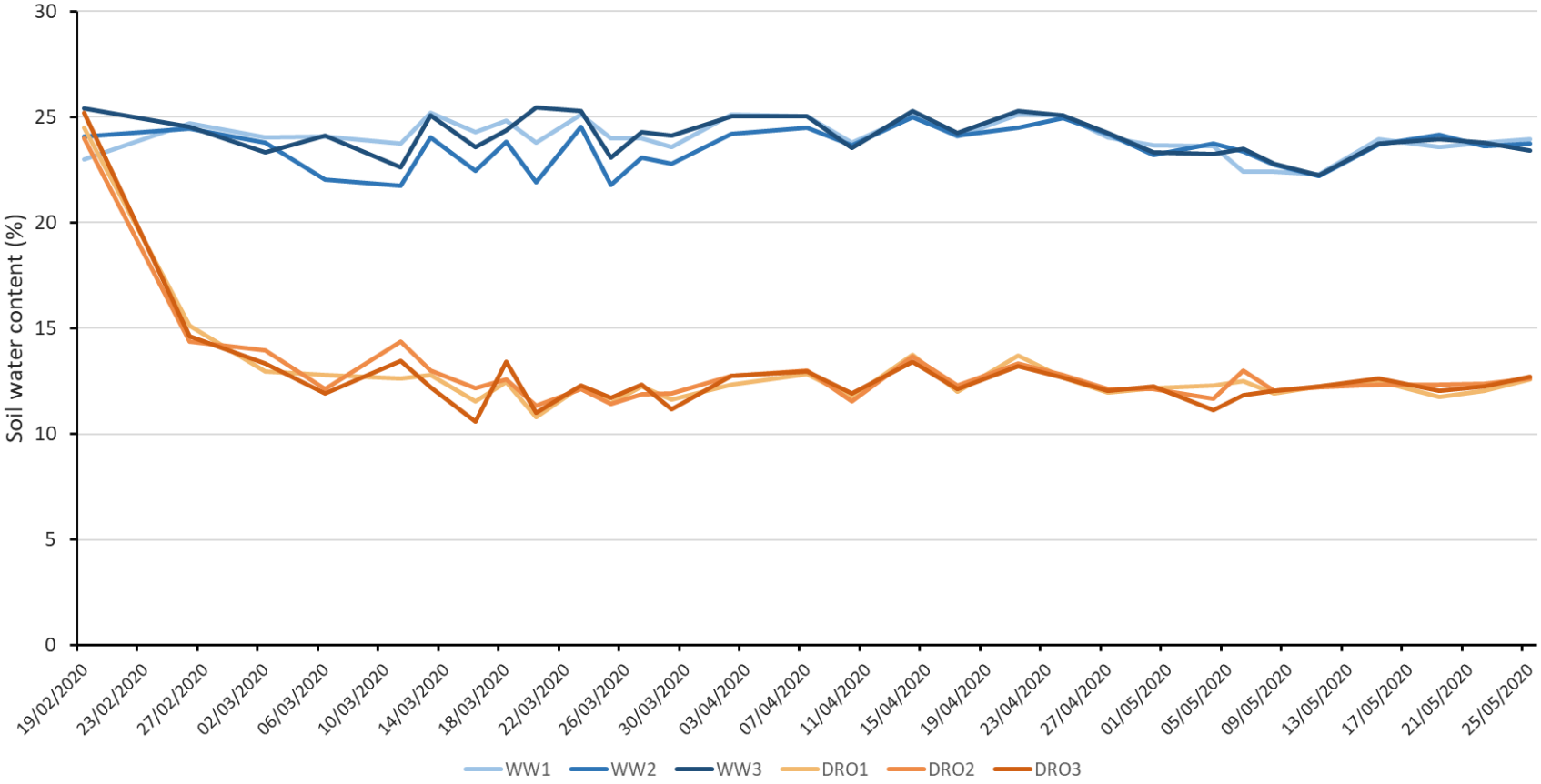
Supp. 2: a) Detailed description of rain exclusion structures and mean environmental conditions registered below and outside the structures. b) Experimental individuals of *Lepidium subulatum* growing below the rain exclusion structures.

a)

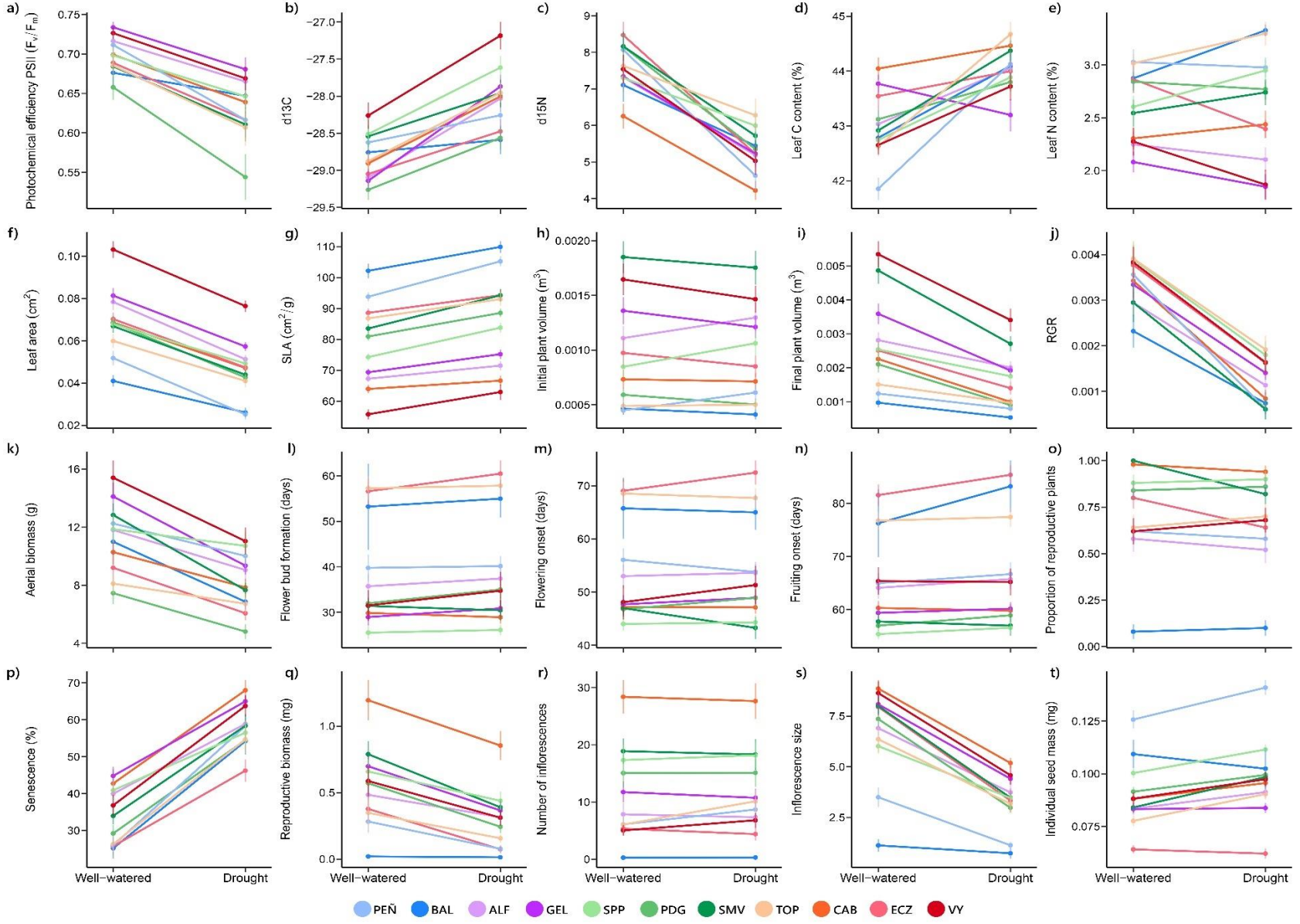
Rain exclusion structures were built using steel frameworks, covering the top of the roof with corrugated transparent polycarbonate sheets (Rooflite, Wetherill Park, Australia). Roofs had an inclination of ~10º to avoid the accumulation of natural precipitation above the structures. The height of the structures (2 and 1.5 meters on the tallest and the shortest side, respectively) and the transparent material of the roof assured a minimal effect of the structures on the conditions experienced below them. To compare the environmental conditions below and outside rain-exclusion structures, we set up two climatic HOBO H21 Micro Stations, one below and one outside the structures. Both stations recorded temperature, photosynthetic active radiation (PAR), and relative humidity every 10 minutes during the experiment. Average temperature and relative humidity below the structures were 16.98 ºC, and 54.27 %, respectively; and 16.29 ºC, and 57.93 % outside the structures, and midday PAR values exceeded 1800 μmol m^{-2} s^{-1} both below and outside the structures in full-sun days.

b)

Supp. 3: Soil water content (%) in each of the three rain exclusion structures for both watering treatments (well-watered and drought; WW and DRO, respectively). Soil water content was monitored every 2–4 days in 10 pots per structure, using an HH2 Moisture Meter with an ML3 Sensor (Delta-T Devices, Cambridge, UK). Plants in the well-watered treatment were kept at ~100% of field capacity for our gypsum soil (~30-25% of soil water content), while plants in the drought treatment were maintained at ~50% of field capacity (~15-12% of soil water content).

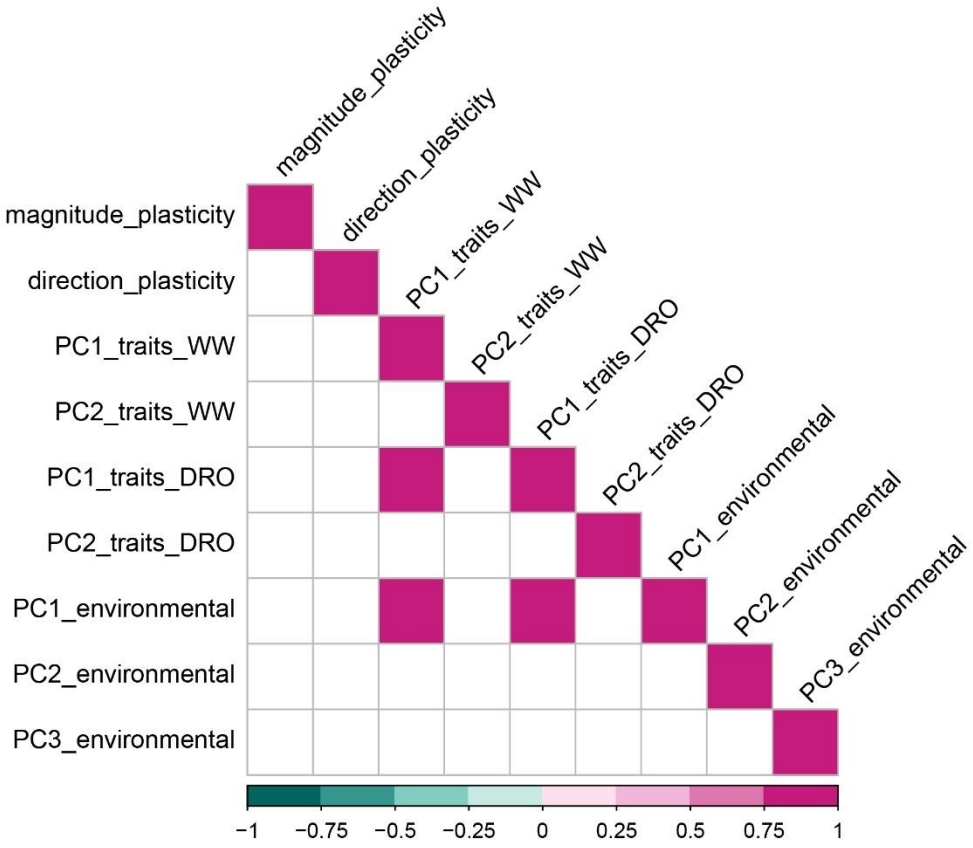


Supp. 4: Phenotypic variation of populations across watering treatments. Each line represents the norm of reaction of each population for a given trait. Mean values and standard error of each population in each treatment are shown (10 maternal families per population and five half-siblings per family and treatment). See Table 2 for results of linear mixed models for each trait.



Supp. 5: Results from univariate associations between quantitative genetic differentiation among populations in each treatment and the environmental conditions of populations (climate and soil composition). Only significant ($P < 0.05$) Pearson's pairwise correlations are coloured.

Supp. 6: Results from multivariate associations between the two first axes of the phenotypic PCA performed to summarize quantitative genetic differentiation among populations in both treatments (well-watered and drought; WW and DRO, respectively), the magnitude and direction of multivariate plasticity, and the environmental PCA used to summarize climatic and soil composition differences among populations. Only significant ($P < 0.05$) Pearson's pairwise correlations are coloured.



Chapter 3: **Natural selection favours drought escape and an acquisitive resource-use strategy in semiarid Mediterranean shrubs**

Mario Blanco-Sánchez, Marina Ramos-Muñoz, Beatriz Pías, José Alberto Ramírez-Valiente, Laura Díaz-Guerra, Adrián Escudero and Silvia Matesanz

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Abstract

Natural selection is the major force driving adaptive evolution in natural populations, varying in strength, direction, and form through space and time, especially in highly variable environments such as Mediterranean ecosystems. Although a conservative resource-use strategy has been hypothesized to be adaptive in Mediterranean taxa, patterns of selection at the intraspecific level, i.e., the suite of traits determining individual fitness, are largely unknown. Using a phenotypic selection experiment in natural semiarid conditions, we measured direct and indirect selection acting through two different fitness components (survival and reproduction), to assess the adaptive value of 20 ecophysiological traits on individuals of two gypsum endemic species, *Centaurea hyssopifolia* and *Helianthemum squamatum*, dwelling in environments with contrasting abiotic conditions (south- and north-facing slopes) during two climatically contrasting years (dry and mesic). This allowed quantifying the magnitude and direction of natural selection at different spatiotemporal scales. Our results revealed that different abiotic conditions did not alter selection patterns, being the magnitude of selection more strongly affected by temporal environmental variation. Selection through reproduction indicated consistent selection for early phenology, low water use efficiency, high specific leaf area, low leaf dry matter content, and high leaf N across slopes and years in both species. In contrast, phenotypic trait variation was not linked to survival in either species. Furthermore, while individual reproductive output was higher or similar in environments with higher abiotic stress in both species and years, survival was similar across environmental conditions, and it was neither affected by plant size nor reproductive output. Contrary to our expectations, natural selection via reproductive fitness consistently favoured a drought-escape, acquisitive resource-use strategy in Mediterranean semiarid plants, rather than a conservative resource-use strategy, even under conditions of higher abiotic stress (i.e., south slopes and dry year). Such acquisitive strategy could allow rapid development by maximizing resource assimilation and reproduction

before the most limiting climatic conditions of mid-late summer. Our results shed light on adaptive functional strategies of Mediterranean taxa at the intraspecific level, providing insight on future responses to environmental change, and highlight remarkable differences in selection acting through different fitness components.

Keywords: acquisitive strategy, gypsum specialists, individual fitness, Mediterranean, natural selection, phenotypic selection, selection differential, stress-escape

Introduction

Natural selection is the main process underlying adaptive evolution. Pioneer studies on phenotypic selection analyses indicated that variability in functional traits are strong drivers of fitness variation (Lande & Arnold, 1983; Phillips & Arnold, 1989; Kingsolver et al., 2001). Furthermore, environmental variation may affect both the phenotypic expression of individuals and the patterns of selection, because natural selection is a dynamic force that can vary in magnitude, direction and form through space and time (see Kingsolver et al., 2001; Siepielski et al., 2009), especially in highly variable environments. Therefore, identifying adaptive traits is key to unveil successful plant strategies in contrasting environments, which may in turn provide insight on evolutionary responses to further environmental change (Hoffmann & Sgrò, 2011).

Mediterranean ecosystems show large spatiotemporal environmental heterogeneity, with high climatic seasonality, marked summer drought, low resource availability and the occurrence of simultaneous and fluctuating abiotic stresses (Blondel et al., 2010). Plants in the Mediterranean region have evolved functional adaptations to cope with abiotic stress (Blondel et al., 2010; Matesanz & Valladares, 2014), many of which are drought-related and occur along a continuum of adaptive strategies: tolerance, escape, and avoidance (Volaire, 2018). Tolerant plants often have slow growth rates and a conservative resource-use strategy characterized by low specific leaf area (SLA), high leaf dry matter content (LDMC), high water use efficiency (WUE), low photosynthetic rates and leaf nutrient concentrations, etc. In contrast, plants with an escape strategy reproduce early to avoid the most stressful conditions of the growing season. This requires higher rates of resource acquisition and growth, which is often achieved through high SLA, low LDMC, low WUE, high leaf nutrient concentrations and photosynthetic rates, etc. (Kooyers, 2015; Pérez-Ramos et al., 2013; Volaire, 2018; Welles & Funk, 2021). Finally, plants with an avoidance strategy minimize the risk of hydraulic failure during the most stressful

part of the growth season by reducing transpiration and/or tapping onto more reliable water sources (McKay et al., 2003; Pérez-Ramos et al., 2013). Therefore, specific trait values are usually associated with contrasting resource acquisition rates and physiological strategies (Adler et al., 2014; Kooyers, 2015; Pérez-Ramos et al., 2013). Specifically, in stressful Mediterranean ecosystems, plants have often been catalogued as tolerant taxa (Matesanz & Valladares, 2014), but stress-escape strategies have also been reported (e.g., Franks, 2011; McKay et al., 2003). While these categories have proved useful to predict patterns of interspecific trait variation across resource gradients (e.g., Reich, 2014), how individuals of the same species differ in traits related to resource use and stress response and, importantly, how such among-individual variation is related to fitness is unknown for most Mediterranean plants (Bolnick et al., 2003; Welles & Funk, 2021).

A particular example within the Mediterranean are gypsum habitats, characterized by high soil gypsum content, semiarid climate, and remarkable spatiotemporal heterogeneity (Escudero et al., 2015; Palacio et al., 2007). Temporal heterogeneity is linked to high seasonal and interannual climatic variability (Escudero et al., 2015), while landscape-scale spatial heterogeneity is mostly associated with strong environmental differences between slope aspects (Fig. 1). In the northern hemisphere, south-facing slopes receive greater insolation, resulting in higher evapotranspiration and lower water availability, leading in turn to profound differences in the biotic structure, which altogether may impose different selection pressures. Given the adaptive value of conservative water and resource use often reported in water-limited Mediterranean environments (Blondel et al., 2010; Matesanz & Valladares, 2014) and their frequent late flowering phenology, gypsum endemics (gypsophiles) are predicted to be stress-tolerant (Escudero et al., 2015; Palacio et al., 2007). However, although several studies assessed adaptive traits in the wild in different habitats (Kingsolver et al., 2012, 2001), there is virtually no information on the suite of traits that determine the success of Mediterranean gypsophiles at

the intraspecific level, whether adaptive traits fit the predicted stress-response strategy, and how selection patterns vary across spatiotemporal scales.

In this study, we evaluated patterns of phenotypic selection on natural conditions on two dominant Mediterranean gypsum species, *Centaurea hyssopifolia* and *Helianthemum squamatum.* We measured a wide suite of functional traits related to resource-use and response to abiotic stress, and considered two different fitness components, survival and reproductive output, while accounting for the spatiotemporal variation inherent to these systems. Using phenotypic selection analyses (Lande & Arnold, 1983; Phillips & Arnold, 1989), we assessed which traits were under selection and how the direction and magnitude of selection for those traits varied in environments with contrasting abiotic conditions (south- and north-facing slopes; *a priori* higher and lower abiotic stress, respectively), during a dry and a mesic year. We addressed the following questions: 1) What functional traits determine reproductive fitness and survival in Mediterranean gypsum habitats? i.e., what traits are under selection?; 2) Are adaptive traits consistent with a stress-tolerance, avoidance or escape strategy?; 3) Are patterns of selection affected by spatiotemporal differences between slopes and years, and do they vary between species?; 4) Is selection acting through reproductive fitness consistent with selection acting through survival? Based on functional and life-history traits of these species, we predict that a conservative resource-use and stress-tolerant strategy characterized by more sclerophyllous leaves, higher WUE and lower leaf nutrient concentrations would be adaptive in scenarios where water availability is lower (i.e., south-facing slopes and dry year), whereas more mesophyllous leaves (higher SLA and nutrient concentrations), with lower WUE would be beneficial for fitness under more mesic conditions (i.e., north slopes and mesic year).

Materials and methods

Study site and species description

Our study was carried out in Belinchón, Spain (40º 04' N, 3º 04' W; ~700 m a.s.l). The area has a Mediterranean semiarid climate, with pronounced summer drought, high interannual variation (mean annual precipitation 419.2 mm; 192 – 504 mm), and mean annual temperature of 14.6°C (35-year climatic data, CHELSA; Karger et al., 2017). Massive gypsum outcrops in the form of gypsum hills harbour gypsophile populations from hundreds to thousands of individuals (Fig. 1). North-facing and south-facing slopes in gypsum hills at mid latitudes strongly differ in environmental conditions. While north-facing slopes show higher plant cover, south-facing slopes are characterized by a patchy shrub community dominated by endemic gypsum plants and higher cover of biological soil crusts (BSC hereafter).

Centaurea hyssopifolia Vahl. (Asteraceae) and *Helianthemum squamatum* (L.) Dum. Cours. (Cistaceae) are small chamaephytes (20-60 cm height) endemic to gypsum outcrops of the Iberian Peninsula (IP). *Helianthemum squamatum* is a widely distributed species, while *C. hyssopifolia* is a narrow endemism mostly found on central IP (Palacio et al., 2007). Both species are dominant in central Iberian gypsum habitats (Matesanz et al., 2018), accounting for 46% and 35%, respectively, of the total perennial cover in our study site. The reproductive period of *C. hyssopifolia* lasts from May to July, while *H. squamatum* shows delayed phenology, from late May to early-mid August (Matesanz et al., 2018). The studied species differ in their longevity, with *H. squamatum* showing a shorter life span (Supp. 1).

Experimental design

In both species, we quantified phenotypic selection on two environmentally contrasting slope aspects (south- and north-facing), and two climatically contrasting years (dry and mesic), accounting for the effect of the microenvironment on each plant (Fig. 1). We randomly selected

three different hills and established 20 x 20 m plots at the north and south slopes of each hill for each species (3 hills x 2 slopes x 2 species = 12 plots). In each plot, we randomly tagged 40 reproductive individuals that encompassed the size range of the species in the plot, for a total of 480 plants (Fig. 1). All plots were established within an area of ~1 km^2, to minimize climatic differences among them. The study was carried out over 2017 and 2018 (2017 was warmer and drier than 2018; Supp. 2). Indeed, 2017 had the highest number of heat waves in the IP, the second warmest summer temperature, and was the second year with an early heat wave (mid-June) recorded in a 46-year time series (Spanish meteorology agency; Supp. 2). Data were collected from late April to mid-August each year, ending when both the vegetative and reproductive season were completed for both species. Several plants died between the first and the second study years, likely due to the harsh climatic conditions during 2017. Therefore, we replaced 15 *C. hyssopifolia* (6.25%) and 109 *H. squamatum* plants (45.42%) in 2018. Note that new-tagged individuals were similar in size, reproductive output, phenotypic traits and microenvironmental conditions compared to surviving individuals (P>0.05 in all cases).

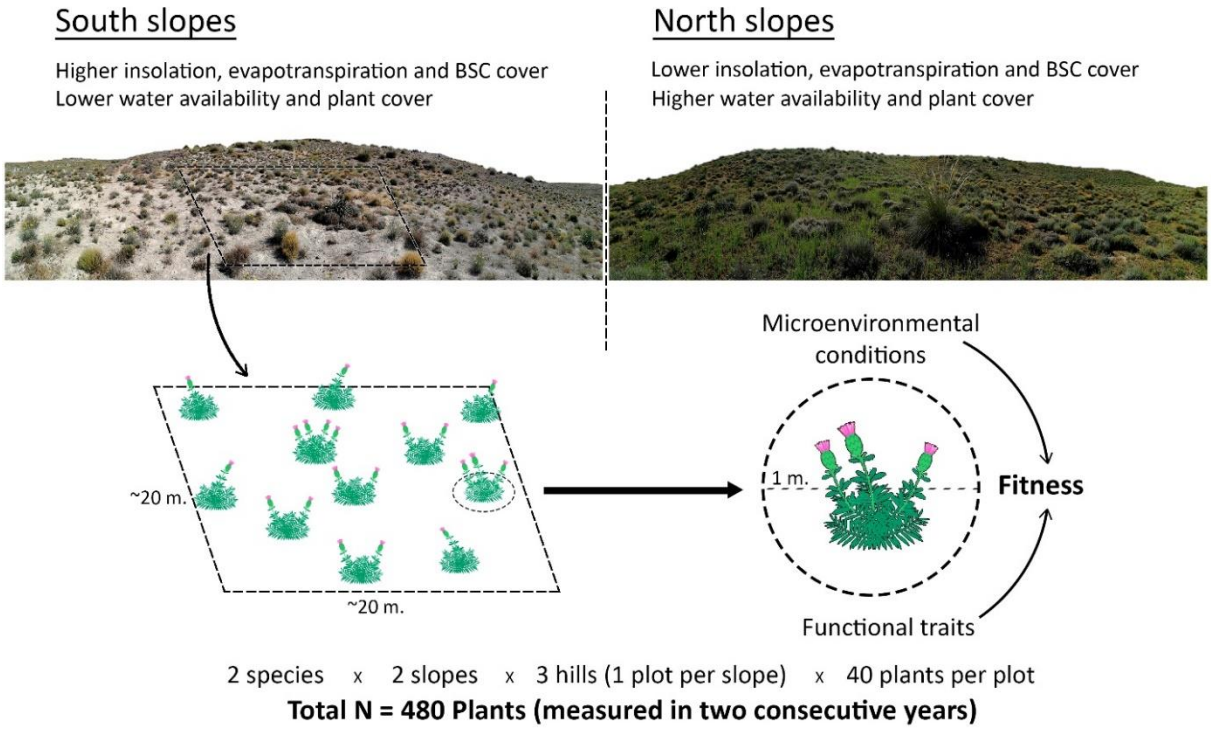

Figure 1: Diagram of the phenotypic selection study. Plots were established in south-facing and north-facing slopes in the study site. Three hills were randomly-selected and one 20 x 20 m plot was established at each slope and hill for each species (12 plots total). Forty reproductive individuals per species and plot were selected (N = 480 individuals). Phenotypic traits and fitness were measured at each plant in two consecutive years, and microenvironmental conditions surrounding each individual were characterized.

Collection of phenotypic and fitness traits

Our field study did not need permission for fieldwork and all traits were measured in all individuals in both study years. We measured height and maximum diameter before the onset of reproduction (end of April), and calculated plant volume (Supp. 1). At the reproductive peak of each species, we collected one primary branch per plant, wrapped it in moist paper in a zipper plastic bag, and stored in a portable cooler box, guaranteeing transportation to the laboratory in cool, water-saturated conditions (Pérez-Harguindeguy et al., 2013). Then, we followed Garnier et al., (2001) to ensure complete leaf rehydration (see Supp. 1). The next morning, five undamaged, non-senescent and fully-expanded leaves per plant were haphazardly selected. First, we weighed the fresh mass of all leaves using a microbalance (1 µg precision; Mettler

Toledo, Columbus, OH, USA). Second, we measured leaf thickness on three leaves (two measurements per leaf, one at each side of the leaf midrib) using a dial thickness gauge (Mitutoyo Corporation, Tokyo, Japan). Third, the five leaves were scanned, oven-dried at 60ºC for 48 h and weighed. Specific leaf area (SLA) was calculated as the one-side area of the leaves divided by their oven-dry mass. Leaf dry matter content (LDMC) was calculated as dry mass divided by water-saturated fresh mass.

We monitored reproductive phenology and leaf senescence every 12-14 days during 2017 (7 censuses), and every 6-8 days during 2018 (14 censuses). At each census, we visually estimated the percentage of closed inflorescences, inflorescences with open flowers, fully-developed fruits, dispersed inflorescences, and the percentage of senescent and green leaves. From these data, we calculated the onset, duration and peak of flowering, fruiting and dispersion, and plant senescence (see Supp. 1).

We measured midday maximum photochemical efficiency of photosystem II (F_v/F_m) with a portable fluorometer Handy PEA+ (Hansatech, UK) at the reproductive peak of each species. To test whether early-season ecophysiological status affected plant fitness, F_v/F_m was also measured before the onset of reproduction in 2018 (Supp. 1). Leaf chlorophyll content was measured twice in 2018 (early-season and flowering peak) in three leaves per plant, using a SPAD 502 chlorophyll meter (Konica Minolta, Tokyo, Japan). Leaf carbon and nitrogen content (leaf C and N hereafter), and carbon and nitrogen stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) were also determined, using leaves from the same branch collected for morphological traits (Supp. 1).

We randomly collected five closed and mature inflorescences per plant before seed dispersion and stored them separately. Inflorescences were dissected individually to separate viable seeds from those aborted/predated. Then, we calculated the mean number of viable seeds per inflorescence. Finally, five viable seeds per plant were randomly selected and individually

weighed to obtain mean seed mass per plant. At the end of the reproductive season (end of July) of each year, we counted the number of viable inflorescences (not predated/aborted). From this, we calculated two plant-level reproductive fitness traits for each plant: i) total seed number, the product of the number of viable inflorescences and the number of viable seeds per inflorescence, and ii) total seed mass, the product of the total seed number and the mean seed mass. Since an important proportion of *H. squamatum* individuals died between the first and second year, we also assessed survival at the beginning of 2018 in this species as an additional fitness trait.

Microenvironmental and environmental conditions

We measured local biotic and abiotic microenvironmental conditions around each plant. In early April 2017, we established a circle of one meter in diameter centred around each plant, and we visually estimated the percentage of perennial, annual and total cover, bare ground cover, BSC cover and litter cover. Cover estimations were always performed by the same observer. As a proxy of intraspecific competition, we counted the number of conspecifics within the circle and measured the distance of the closest conspecific to each tagged plant.

Soil water content was monitored in 2017 at the peak of flowering of each species, and twice in 2018, before the onset of reproduction and at the peak of flowering of each species. At each sampling point, four measurements were recorded around each plant (N = 1920 measurements in 2017 and 3840 in 2018), using an HH2 Moisture Meter (Delta-T devices, Cambridge, UK). Insolation was estimated through the Gandullo's method (Gandullo, 1974) based on the latitude, orientation, and micro-slope around each plant (see Supp. 1).

Statistical analyses

All analyses were performed in R (R Core Team, 2018; see a detailed description of models' specification in Supp. 3). We arranged the functional traits and fitness data into different datasets according to our research questions (see details in Supp. 1). Phenotypic selection analyses provide information on: i) the magnitude of phenotypic selection, i.e., the strength of the relationship between traits and fitness; ii) direction, whether positive or negative trait values are associated with higher fitness, and iii) form, the shape (linear or quadratic) of the relationship between traits and fitness.

To assess directional selection, we calculated for each species, year and slope: a) linear selection differentials (S' = Cov[w,z]), the covariance between relative fitness and a given standardized trait, which quantify direct and indirect selection, and b) linear selection gradients ($\beta' = P^{-1}S'$), the vector of partial regressions of multiple traits included in the same regression model, which estimate direct selection. To assess stabilizing or disruptive selection, we calculated: a) quadratic selection differentials (C' = Cov[w,(z-$\bar{z}$ (z-$\bar{z}$)^T) and b) quadratic selection gradients ($\gamma' = P^{-1}\ C'\ P^{-1}$), where w is the vector of relative fitness, z is the vector of standardized phenotypic values, and P is the phenotypic variance–covariance matrix (Lande and Arnold, 1983; Phillips and Arnold, 1989). Non-linear selection was considered only when both significant quadratic estimators (C' and γ') and an intermediate maximum or minimum in the fitness function were observed (see Ramírez-Valiente et al., 2021). For each species, traits were standardized and reproductive fitness was relativized (see Supp. 1 for details).

Selection differentials and gradients were calculated using generalized linear mixed models (GLMMs) with function *glmer* (package lme4; Bates et al., 2015). Relative reproductive fitness (and survival in *H. squamatum*) was included as the dependent variable, and the standardized trait (in selection differentials) or traits (in selection gradients) were included as fixed factors. Total seed mass integrated the information of total seed number, and both

reproductive fitness traits showed very similar selection patterns, so we present results using total seed mass (see Supp. 4 and 5 for results using total seed number, and Supp. 6 for results of *H. squamatum* using survival). Plant volume and microenvironmental data (see below) were also included in the models as covariates, and plot was included as a random factor (Supp. 3). As reproductive fitness did not follow a gaussian distribution, models were performed using family = "Gamma" and link = "log" (family = "binomial" and link = "logit" in the models using survival). To avoid potential multicollinearity issues, we computed both variance inflation factors (VIFs) and pairwise correlations for each trait in all datasets before performing selection analyses (see Supp. 1 and 7). To account for the consequences of multiple testing, results from selection models were corrected using false discovery rate (FDR) for each species, year and slope (Benjamini and Hochberg, 1995) using *p.adjust* function.

Microenvironmental data from each plant was summarized using Principal Component Analyses (PCA) using function *prcomp* (R Core Team, 2018) for each slope, year, and species (see Supp. 1). We selected the three first principal components of each PCA, which explained ~75% of the microenvironmental variance (Supp. 8). The eigenvectors of the three first principal component of each PCA were consistently associated with different microenvironmental variables: PC1 was in general related to total and perennial cover; PC2 to the number of conspecifics, the distance of the closest conspecific and BSC cover; and PC3 to soil water content, annual and litter cover (Supp. 8 and 9). Eigenvalues for each plant were included in the models to account for the microenvironmental conditions experienced by each plant.

We tested whether the relationship between a trait and reproductive fitness differed between slopes and/or years, i.e., variation in the magnitude and direction of selection for a given trait, by quantifying the interactions trait-by-year and trait-by-slope, respectively, using GLMMs. Plant volume and microenvironmental data (nested in slope or year) were included as

covariates and plot as a random factor. Significance levels were corrected using FDR. Significant trait × slope and trait × year indicates that the magnitude and/or the direction of selection for a given trait varied among slopes and years, respectively. Furthermore, to test the relative effect of environmental variation across space (slopes) and time (years) on selection patterns, we assessed whether selection was more similar between slopes in the same year or between years in the same slope. First, we performed pairwise correlations between selection differentials (and gradients) from each slope and year. Then, we evaluated the percentage of variation of the selection differentials and gradients explained by slope and year through commonality analysis (see Supp. 1, 10 and 11).

Finally, we tested if functional traits, fitness (reproductive output and survival) and environmental conditions varied across space (between slopes) and time (years) for each species, and the relationship between both fitness components. First, we performed GLMMs to test the effect of slope, year and their interaction on fitness and phenotypic traits, including plant volume as covariate. Then, we tested the differences between slopes in the same year with GLMMs.

Results

There were large differences in the environmental conditions between slopes and years (Fig. 2). Despite great biotic and abiotic differences between slopes, both the identity of the traits under selection and the direction of selection acting through reproductive fitness were consistent across slopes in both species, particularly for *H. squamatum* (Fig. 3; Supp. 12). Furthermore, plot explained less than 10% of the variance of the selection models in most of the cases (~80%; Supp. 9). Similarly, the direction of selection on adaptive traits based on the sign of selection differentials was similar in both years (Fig. 3; Supp. 12). However, the magnitude of selection differed between slopes mainly in *H. squamatum*, and between years

mainly in *C. hyssopifolia* (Table 1). In contrast, we did not detect any trait under selection through survival in *H. squamatum*. Overall, selection was stronger on *H. squamatum* than in *C. hyssopifolia*, especially in the south slopes and in 2018.

Spatiotemporal heterogeneity between slopes and years

North and south slopes differed in their environmental conditions in both years (Fig. 2). While total, perennial and bare soil cover was ~20% higher, and annual cover ~1.5 times higher in the north slopes (Fig. 2; $P<0.01$ in all cases), BSC cover was ~50% higher in the south slopes (Fig. 2; $P<0.001$). Biotic conditions within the same slope did not vary between years. Soil water content (SWC) varied between slopes and also between years. North slopes had higher SWC than south slopes in both years (Fig. 2; $P<0.001$). Furthermore, the higher rainfall in 2018 resulted in higher overall SWC in both slopes. In fact, SWC in the south slopes of 2018 was higher than those found in the north slopes of 2017 (Fig. 2; $P<0.001$).

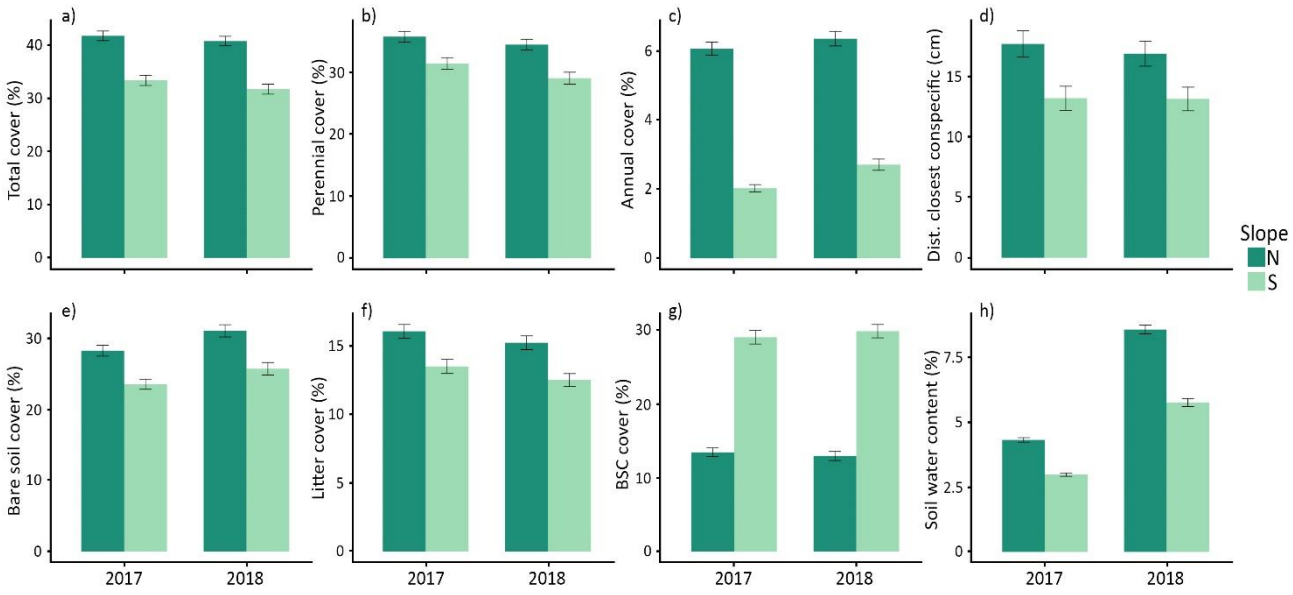

Figure 2: Differences in environmental conditions between slopes and years. Means and standard errors of a) total cover (%); b) perennial cover (%); c) annual cover (%); d) distance to the closest conspecific individual (cm); e) bare soil cover (%); f) litter cover (%); g) BSC cover (%) and h) SWC (%) are shown. Data from both species were grouped in these analyses. All variables were significantly different ($P<0.05$) between slopes but not between years within slopes, except for SWC (see text).

Linear and quadratic selection differentials and gradients

For *H. squamatum*, selection differentials showed that most phenological traits had a significant association with the reproductive output of individuals in both years and slopes (Figs. 3 and 4; Supp. 12). Specifically, individuals that flowered earlier, showed a shorter flowering period (only in the south slopes), an advanced and longer fruiting period, and a delayed onset of seed dispersion showed higher reproduction in 2017 in both slopes. Similarly, in 2018, early flowering, delayed fruiting peak, and delayed dispersion onset were also under selection in both north- and south-facing slopes, although, in contrast to 2017, long rather than short flowering periods were adaptive in the south slopes. Other morphological and ecophysiological functional traits were also under directional selection in this species. In 2017, SLA and LDMC had a positive and negative association with reproduction in both slopes, respectively, and both leaf N and $\delta^{13}C$ (a proxy of WUE) were negatively linked to fitness in the north slopes. In 2018, individuals with larger leaves and higher leaf chlorophyll content at the peak of flowering showed higher reproduction in both slopes. Early-season leaf chlorophyll content and LDMC had a positive and negative impact on fitness in the south slopes, respectively (Fig. 3; Supp. 12). Selection gradients showed that the duration of flowering and leaf N were under negative direct selection in the south and north slopes, respectively, in 2017. In 2018, leaf senescence was under negative direct selection only in the north slopes (Supp. 12). In contrast, the results performed using survival as fitness did not show any trait under selection in *H. squamatum*, except for leaf senescence, which was negatively linked to survival in the north slopes (Supp. 6).

For *C. hyssopifolia*, significant selection differentials for phenological traits were also found, but mostly in the north slopes in 2017. Individuals with longer flowering and earlier and longer fruiting and dispersal periods showed higher reproduction. There were virtually no functional traits under selection in the south slopes in 2017 and in either slope type in 2018

(Fig. 3; Fig. 4; Supp. 12). Selection gradients showed that lower WUE in 2017 and higher duration of flowering in 2018 were under direct selection in north slopes (Supp. 12).

Quadratic selection was less frequent than directional selection (Supp. 5, 13 and 14). Significant and marginally significant quadratic selection differentials and gradients were observed only in 2018, in both slopes and species.

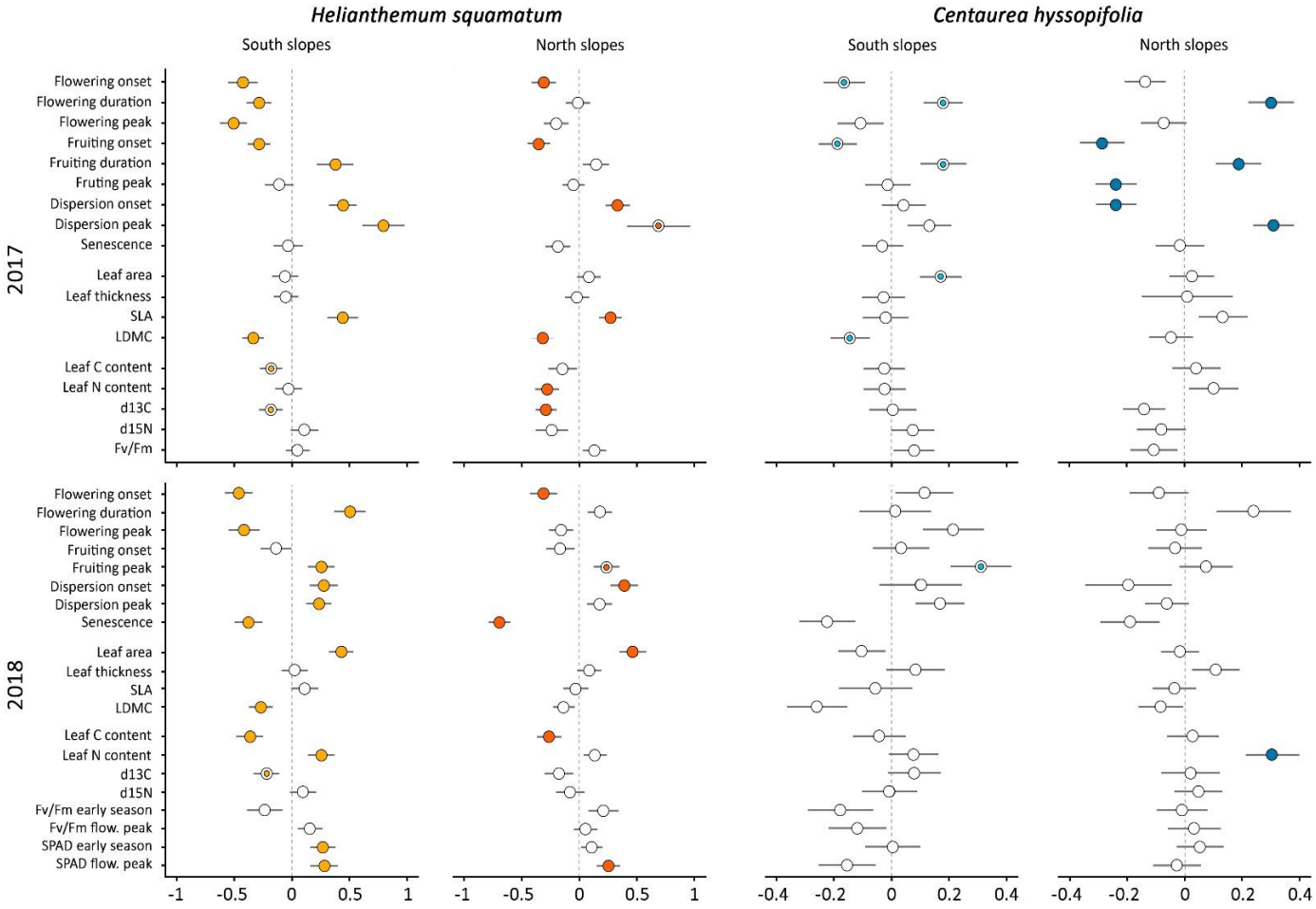

Figure 3: Directional selection differentials (*S'*) and their standard error for *H. squamatum* and *C. hyssopifolia* in both slopes and years using total seed mass as fitness variable. Significance levels after FDR corrections: coloured circle = $P<0.05$; small coloured dot inside circle = $0.05<P<0.1$; white = n.s. ($P>0.05$) selection differentials.

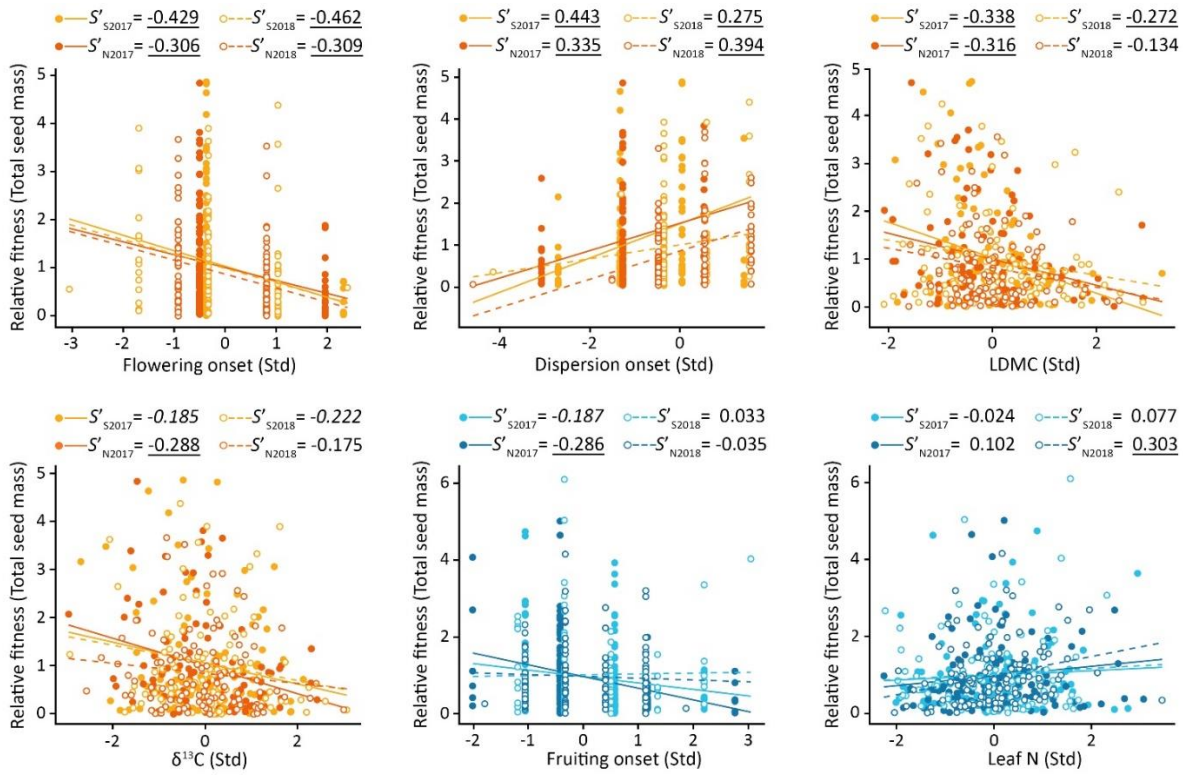

Figure 4: Relationship between relative reproductive fitness (total seed mass) and functional traits in both species, slopes and years, with orange shades for *H. squamatum* and blue shades for *C. hyssopifolia*. Estimated values of linear selection differentials (*S*') are shown in each plot. Subscripts indicate the slope and year. Significant ($P<0.05$) and marginally significant ($0.05<P<0.1$) selection differentials are underlined and italicized, respectively.

Differences in selection between years and slopes

Phenotypic selection analyses and GLMMs revealed that selection acting through reproductive fitness varied between years and slopes depending on the species, mainly as a result of differences in magnitude rather than direction. For *H. squamatum*, selection was stronger in the south slopes for all the traits under selection in 2017, except for leaf N, $\delta^{13}C$ and fruiting onset (Table 1), as shown by the significant trait-by-slope interaction, and the larger selection differentials in the south (Fig. 3; Supp. 12). In 2018, selection was also stronger in the south slopes, except for leaf area, dispersion onset and leaf senescence. Between years, the magnitude of selection was stronger for certain traits in 2017 than in 2018 within the same slope, and *vice versa*. For instance, late phenological phases were under selection more intensely in 2017, while there was stronger selection for flowering traits in 2018 in the south slopes. For *C. hyssopifolia*

the magnitude of selection was clearly stronger in the north slopes in 2017 (Table 1; Fig. 3; Supp. 12). Both correlation and commonality analyses showed more similar patterns of selection between slopes in the same year than for the same slope between years, especially in *H. squamatum* (Supp. 10 and 11).

Table 1: Results from the GLMMs testing differences in the magnitude and/or the direction of selection between slopes and years, using total seed mass fitness variable. χ^2-statistic and significance (in brackets) of each model are shown. A significant (P<0.05) value after FDR correction (trait:slope or trait:year columns) indicates that the magnitude and/or the direction of selection varied between slopes or years (significant trait:slope or trait:year interaction). In columns *2017 (S vs. N)* and *2018 (S vs. N)*, the magnitude and the direction of selection is compared between slopes within the same year. In columns *South slope* (*2017 vs. 2018)* and *North slope (2017 vs. 2018)*, the magnitude and the direction of selection is compared between years within the same slope. Significance levels: ***** P<0.05; ****** P<0.01; ******* P<0.001.

	H. squamatum				***C. hyssopifolia***			
	2017 S vs. N	**2018 S vs. N**	**South slope 2017 vs. 2018**	**North slope 2017 vs. 2018**	**2017 S vs. N**	**2018 S vs. N**	**South slope 2017 vs. 2018**	**North slope 2017 vs. 2018**
	trait:slope	trait:slope	trait:year	trait:year	trait:slope	trait:slope	trait:year	trait:year
Leaf area	1.018	**32.407(***)**	**17.049(***)**	**14.761(**)**	4.637	2.186	7.104	0.303
Leaf thickness	1.174	0.580	0.531	1.037	0.107	2.049	0.826	5.820
SLA	**17.746(***)**	1.117	**12.337(**)**	6.531	2.258	0.073	0.455	4.072
LDMC	**20.992(***)**	**10.754(*)**	**21.777(***)**	**11.858(**)**	5.305	9.001	**12.600(*)**	2.607
Flowering onset	**23.399(***)**	**20.014(***)**	**18.908(***)**	**15.988(**)**	**9.592(*)**	2.966	6.829	6.938
Flowering duration	**8.547(*)**	**17.732(***)**	**18.435(***)**	3.586	**22.133(***)**	4.098	5.256	**15.708(**)**
Flowering peak	**24.878(***)**	**10.233(*)**	**17.832(***)**	**7.050(*)**	2.505	5.077	6.596	1.943
Fruiting onset	**12.840(**)**	3.818	1.495	**15.771(**)**	**20.457(***)**	0.541	6.530	**12.535(*)**
Fruiting duration	**8.507(*)**	NA	3.795	5.855	**9.454(*)**	NA	4.825	7.471
Fruiting peak	2.774	**10.320(*)**	5.951	4.991	**10.177(*)**	8.359	**11.634(*)**	**11.505(*)**
Dispersion onset	**23.302(***)**	**13.708(**)**	**19.893(***)**	**12.219(**)**	**10.516(*)**	3.186	1.316	**16.548(**)**
Dispersion peak	**22.586(***)**	**8.106(*)**	**14.428(**)**	**8.481(*)**	**22.675(***)**	4.827	7.877	**11.347(*)**
Senescence	3.324	**55.285(***)**	**10.920(**)**	**52.495(***)**	0.187	9.034	6.721	4.669
Leaf Carbon content	5.610	**16.581(**)**	**10.465(**)**	**9.348(*)**	0.444	0.458	0.489	1.201
Leaf Nitrogen content	**8.274(*)**	6.721	4.142	**8.998(*)**	1.431	6.492	0.777	**11.724(*)**
Leaf $\delta^{13}C$	**14.374(**)**	5.673	**8.628(*)**	**12.443(**)**	4.015	0.570	0.786	2.280
Leaf $\delta^{15}N$	**7.442(*)**	1.651	1.007	**7.523(*)**	2.396	0.109	0.741	0.087
F_v/F_m	4.293	2.903	2.662	3.658	2.853	0.647	3.517	3.346

Microenvironment effect on individual fitness

We found significant effects of the microenvironmental conditions experienced by each individual on reproduction, evidenced by significant effects of PCA components in the selection models (Supp. 9). For *H. squamatum*, reproduction in 2017 was negatively associated with total, perennial and BSC cover (significant PC1 and PC2; Supp. 9), and positively associated with the number of conspecific individuals and SWC in the north slopes (significant PC2; Supp. 9). In 2018, reproduction was negatively associated with total and perennial cover in the north slopes (significant PC1; Supp. 9). For *C. hyssopifolia*, reproduction in 2017 was negatively associated with BSC cover in the north slopes (significant PC3; Supp. 9). In 2018, reproduction was marginally positively correlated to litter cover and SWC in the south slopes (PC3; Supp. 9).

In contrast, individual survival was not related to the microenvironmental conditions surrounding each plant in both slopes and study species (not significant effects of PC1, PC2 and/or PC3 on survival; Supp. 15).

Differences in fitness and mean trait values between slopes and years

Overall, reproduction was higher, or similar, in the south slopes compared to the north slopes in both species (Figure 5). There were also significant interannual differences in fitness, which were larger in *H. squamatum* than in *C. hyssopifolia* (Fig. 5). For *H. squamatum*, reproduction in 2018 was approximately one order of magnitude higher than in 2017, due to the higher number of viable seeds per inflorescence (ten-fold difference) in 2018. Fitness was also higher in the south compared to the north slopes in both years in this species (Fig. 5). For *C. hyssopifolia*, there were differences between slopes in reproduction in 2017, with higher fitness in south slopes. There were also differences between years in total seed mass, due to the higher mass of seeds in 2018 (Fig. 5).

In contrast, we found no differences in survival across slopes in both study species (*P*>0.05; Supp. 15). Furthermore, survival was not associated with either plant size (a proxy for plant age) or the reproductive output of individuals, i.e., there was no apparent trade-off between survival and reproduction (*P*>0.05 in both cases; Supp. 15).

Mean values of most functional traits varied significantly between years and slopes in both species. While phenology was delayed in 2018 with respect to 2017 for both species, leaf morphological and chemical composition traits varied in a species-specific manner (see Supp. 16 and 17 for details).

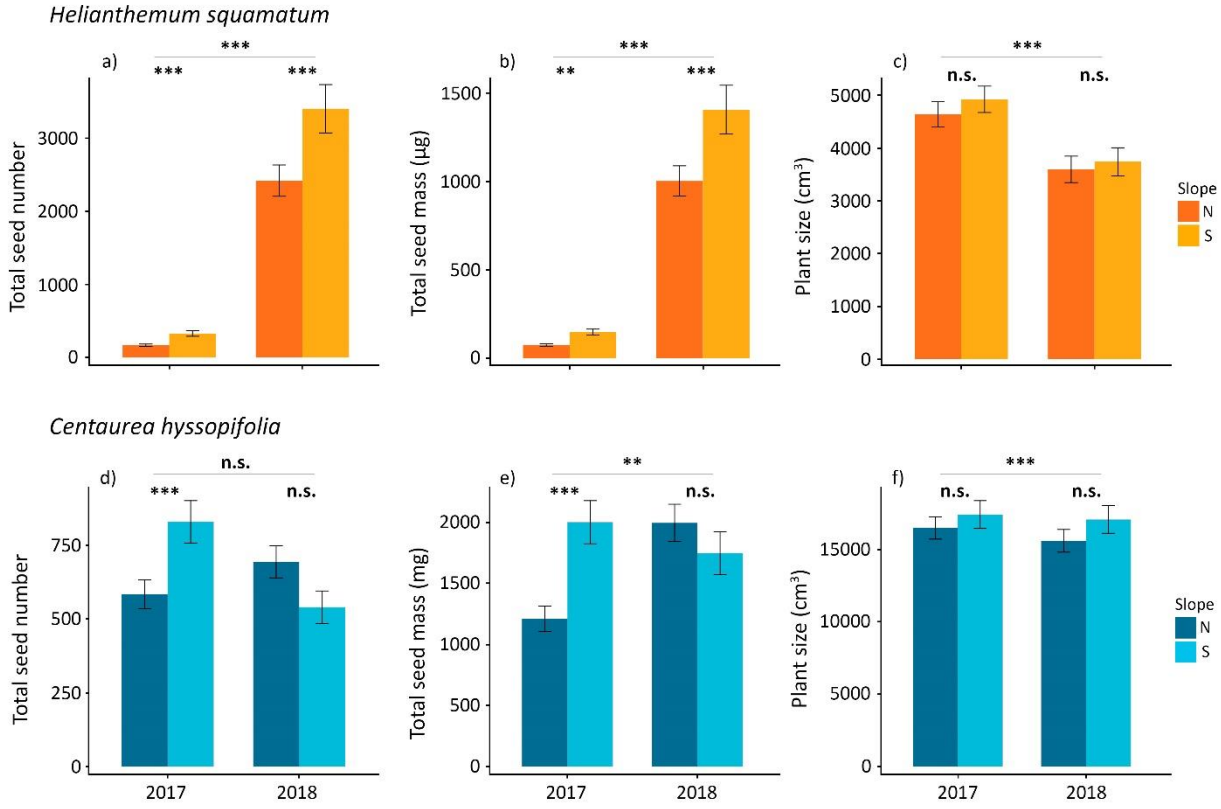

Figure 5: Means and standard errors for each slope and year of: a) total seed number in *H. squamatum*; b) total seed mass in *H. squamatum*; c) plant size (plant volume) in *H. squamatum*; d) total seed number in *C. hyssopifolia*; e) total seed mass in *C. hyssopifolia*; and f) plant size (plant volume) in *C. hyssopifolia*;. Significance levels: n.s. = not significant; ****** *P*<0.01; ******* *P*<0.001.

Discussion

Our results showed significant linear selection acting through reproduction in both species, years, and slopes. The adaptive value of traits related to reproductive phenology showed similar selection patterns across slopes and years, particularly in *H. squamatum*. Contrary to our hypotheses, and despite the contrasting environmental conditions found between north and south slopes, neither the direction of selection nor the identity of the traits under selection through reproduction varied between slopes within years in either study species, i.e., we generally observed a consistent adaptive strategy in both species across slopes. Indeed, selection was more similar between slopes within years than within slopes between years, suggesting that the climatic differences between years had a stronger effect on selection than the environmental differences found between slopes. Contrastingly, selection through survival was negligible. Interestingly, our results assessing selection through reproduction showed that, rather than the expected conservative use of resources, a drought-escape, acquisitive strategy was adaptive in Mediterranean gypsum plants, even in the most restrictive conditions.

The adaptive value of an acquisitive resource-use strategy in Mediterranean gypsum plants

We found that early phenology, lower WUE, higher SLA, lower LDMC, and, to a lower extent, higher leaf N, were associated with higher individual reproductive output (Fig. 3), consistent with a resource-acquisitive strategy. The phenological traits under selection and the direction of selection for those traits were highly consistent in both north- and south-facing slopes and years, particularly in *H. squamatum*. Specifically, an advanced phenology, where plants that reproduced earlier had higher reproductive output, was generally adaptive in *H. squamatum.* A similar pattern was found in *C. hyssopifolia* in the harsher year, although selection for early reproduction was weaker in the south slopes. Our results concur with a meta-analysis on 87 different species showing that selection consistently favours early flowering (Munguía-Rosas

et al., 2011) across biomes. Several factors have been discussed to explain selection on advanced phenology. First, early flowering may minimize water loss later in the season, when the negative effects of abiotic stress are more severe (Franks, 2011; Herrera, 1992; Sherrard & Maherali, 2006). Indeed, an advanced phenology is usually related to drought escape rather than to a drought-tolerance strategy (Franks, 2011; Volaire, 2018; Welles & Funk, 2021). Second, early flowering individuals could avoid competition for pollination (Herrera, 1992; Munguía-Rosas et al., 2011), and finally, an advanced flowering phenology may allow longer fruiting periods before seed dispersal, maximizing fruit maturation (Kudo, 2006). This could explain the adaptive value of earlier but longer fruiting periods and delayed dispersion found in our study. Although we cannot pinpoint the exact mechanism underlying selection on early phenology, our results clearly show that an advanced and extended reproductive phenology is adaptive in semiarid gypsum plants, even in the less environmentally restrictive conditions (milder year and north slopes).

Other leaf morphological, physiological, and chemical composition traits significantly impacted reproduction in both years and species, although again, we found stronger selection in *H. squamatum*. In this species, lower LDMC, higher SLA and leaf area (larger and less sclerophyllous leaves) were under selection in both slopes and years, consistent with an acquisitive strategy. This matches the pattern often observed at the species level, with high resource-use species showing large, high SLA leaves to maximize light capture, photosynthesis, and resource assimilation (Pérez-Ramos et al., 2013; Reich, 2014). Although plant species living in special substrates usually present morphological adaptations to minimize water loss (Damschen et al., 2012; Escudero et al., 2015), at the intraspecific level, selection favoured trait values associated with an acquisitive strategy, contrary to our expectations.

We also detected a negative relationship between WUE, estimated from leaf $\delta^{13}C$ isotopic composition, and reproduction in both species (Fig. 3 and significant selection

gradients in *C. hyssopifolia*; Supp. 12). Individuals with lower WUE, i.e., more negative $\delta^{13}C$, had higher reproductive output. Several selection studies have reported the adaptive value of high WUE under water stress, often associated with a tolerance strategy (e.g. Dudley, 1996; Heschel et al., 2002). However, our results showed that water conservation was not advantageous for either species, suggesting that stomatal regulation is optimized to maintain C gain. Furthermore, the adaptive value of WUE can vary depending on the timing of water stress throughout the growing season. For instance, Heschel and Riginos (2005) showed that lower WUE and earlier phenology were favoured when water stress occurred early in the season because it allowed rapid individual development through high water use (see also Agrawal et al., 2008). The climatic anomalies that occurred in 2017, e.g., heat waves and extremely high temperatures, could explain the observed adaptive value of lower WUE during the harsher year. However, our study also showed the adaptive value of lower WUE in the milder year. Selection on lower WUE has also been reported when water stress occurs late in the season in Mediterranean environments, which, again, has been interpreted as a drought-escape strategy (Franks, 2011).

Overall, patterns of selection for early reproduction and traits related to high resource acquisition observed in our study are consistent with a stress-escape strategy, where high C gain at the expense of fast water spending would allow rapid development and reproduction before water becomes critically limiting (e.g. Franks, 2011; Heschel & Riginos, 2005; Welles & Funk, 2021). Selection for drought escape and fast resource use has been similarly reported in other Mediterranean and semiarid ecosystems, favouring, for instance, early reproductive phenology in highly seasonal environments (Sherrard & Maherali, 2006; Stanton et al., 2000) and low WUE in plants living under Mediterranean and arid/semiarid climatic conditions (Donovan et al., 2007; Heschel & Riginos, 2005). Indeed, some studies have reported a positive association between early-flowering and lower WUE, as evidence of a drought-escape strategy (Franks,

2011; McKay et al., 2003; but see Sherrard & Maherali, 2006). The acquisitive strategy observed in our species could be favoured due to other unmeasured morphological traits, including those belowground, related to resource assimilation and nutrient uptake. For instance, several studies revealed a surprisingly diverse mycorrhizal community associated to gypsophiles, including *H. squamatum* (Palacio et al., 2012). Therefore, our study provides evidence of a mismatch between the tolerant strategy predicted for semiarid Mediterranean taxa and the acquisitive strategy observed at the intraspecific level.

Quadratic selection was consistently less frequent than directional selection in both slopes, years, and species. However, the results from non-linear selection should be interpreted cautiously due to relatively low sample size. Further analyses with larger sample sizes may provide a more robust test of the occurrence and relative importance of non-linear selection in these species (see Kingsolver et al., 2012, 2001).

In contrast with the results obtained for reproductive fitness, individual survival in *H. squamatum* was not associated with functional trait variation in either slope, indicating that the same selection pressures may act differently on the phenotype depending on the fitness component. Furthermore, we found no evidence of a trade-off between survival and reproductive fitness, which could have accounted for opposing selection patterns via different fitness components. Differences in selection among fitness components match previous studies reporting weaker estimates of linear selection via survival than those via reproduction (Kingsolver et al., 2012 and references therein). These results highlight that the phenotype expressed in the growing season prior to plant death was not a reliable predictor of individual survival in gypsum ecosystems.

Environmental effects on fitness components

Although traits under selection were generally consistent across slopes and years, we observed differences in reproduction between slopes and years (Fig. 5). Individual reproductive output was higher (or similar) in the south slopes compared to the north slopes for both species. This result indicates that, despite the lower SWC and the higher insolation, south slopes were not more stressful than north slopes for our study species. Differences in reproduction could be partly explained by the differential effect of the microenvironmental conditions at the plant level in north and south slopes. In the harsher year, BSC cover had a negative effect on reproduction in *C. hyssopifolia* in north slopes, and both total and perennial cover negatively influenced reproduction in *H. squamatum* in north slopes (Supp. 9). Similarly, in 2018, total and perennial cover had, again, a negative effect on reproduction in the north slopes in *H. squamatum*, and litter and soil moisture had a positive effect in the south slopes in *C. hyssopifolia*. These results suggest that although abiotic environmental conditions are harsher in the south slopes, higher competition —based on the negative effect of total and perennial cover— in the north slopes could have a larger negative effect on individual reproductive output than the abiotic conditions. Gypsophiles and other edaphic endemics growing on serpentines have been described as competition avoiders (Anacker et al., 2011; Harrison et al., 2009; Mota et al., 2011), which could explain the negative effect of total and perennial cover on fitness. Conversely, survival was not influenced either by the environmental differences between slopes, individual size, or the microenvironmental conditions experienced by each individual (Supp. 15).

Reproduction in the milder year, 2018, was higher than in the harsher year, but interannual differences varied between species. While *H. squamatum* showed a higher number of heavier seeds in the milder year, differences between years in *C. hyssopifolia* were exclusively due to higher seed mass (Supp. 16). These interspecific differences could be related

to the different longevity, life-history, and a potential trade-off between reproduction and survival (e.g. Harshman & Zera, 2007). Long and medium-lived species (like *C. hyssopifolia*) usually base their lifetime fitness on survival, in contrast to annual and short-lived perennials (like *H. squamatum*), which maximize their fitness by a high reproductive output that guarantees a persistent seed bank (Adler et al., 2014; García & Zamora, 2003). Indeed, reproduction has been reported in *H. squamatum* even under very stressful conditions and at the expense of individual survival (Aragón et al., 2009). However, we did not detect such trade-off between reproduction and survival at the intraspecific level as demonstrated by the lack of differences in reproductive output between dead and surviving individuals in both slopes and species (Supp. 15).

Similar to the contrasting selection patterns acting through reproduction and survival, fitness components were differentially affected by individual traits and environmental conditions. While reproductive output was determined by individual phenotypic expression and microenvironmental conditions in both species, survival was not associated with either functional trait variation, microenvironmental conditions, reproductive output, or plant size, suggesting that individuals progressively died due to differences in other unmeasured traits or environmental conditions (Supp. 6 and Supp. 15). Future studies are needed to understand the determinants of plant survival at the intraspecific level in gypsum specialists.

Conclusions

Our results provide evidence that a drought escape, acquisitive strategy was linked to higher reproductive fitness in our study system at the intraspecific level. This pattern was mostly consistent across species, environmentally contrasting slopes, and climatically contrasting years. Such acquisitive strategy may be adaptive in these stressful environments if it allows rapid individual development and reproduction before the most limiting climatic conditions

encountered in mid-late summer. Further studies should aim to identify the precise physiological mechanisms that maintain this strategy in highly stressful Mediterranean environments. Additionally, our study showed remarkable differences in patterns of selection depending on the fitness component used (reproduction or survival), emphasizing the importance of studies quantifying selection on the same trait, considering more than one fitness component, and spanning more than one year to fully understand the temporal and spatial dynamics of selection in natural populations. Finally, our study provides insights on phenotypic evolution and plant responses to rapid environmental change, and underlines that studies focused on the intraspecific level are key to unveil unexplored adaptive strategies.

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Supporting information — Blanco-Sánchez et al. — Chapter 3

Natural selection favours drought escape and an acquisitive resource-use strategy in semiarid Mediterranean shrubs

Mario Blanco-Sánchez, Marina Ramos-Muñoz, Beatriz Pías, José Alberto Ramírez-Valiente, Laura Díaz-Guerra, Adrián Escudero and Silvia Matesanz

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Supp. 1: Detailed methods.

Study site and species description

Centaurea hyssopifolia Vahl. (Asteraceae) and *Helianthemum squamatum* (L.) Dum. Cours. (Cistaeae) are small chamaephytes (20-60 cm height) endemic to gypsum outcrops of the Iberian Peninsula (IP). Both species are pollinated by a rich community of generalist insects (Santamaría et al., 2018) and are mainly outcrossers with partial self-compatibility (Matesanz et al., 2018 and references). The studied species differ in their longevity. While *H. squamatum* is a short-lived species that lives between 3 and 6 years (up to a maximum of 10 years), *C. hyssopifolia* is a medium-lived shrub, living a mean of 4-8 years (up to a maximum of 20 years; Eugenio et al., 2012; Olano et al., 2011). Therefore, our two years of study represent approximately between one quarter and a half of the entire lifetime of most of the sampled individuals.

Collection of phenotypic and fitness traits

Plant size—We measured the height and the maximum diameter of every plant. Using these measurements, we calculated the volume of each plant as the volume of a hemispheroid, $\frac{2}{3}\ \pi\ r^2\ h$ where r is the radius and h is the height of the plant.

Leaf morphology traits—In the laboratory, branches were unwrapped and placed in beakers filled with water up to the insertion of the lowest fully expanded leaf in the branch. Then, the end of the stem of the submerged branch was cut to restore the flux of water within the branch. Beakers were stored overnight (12h) in cool (4ºC) and dark conditions, maximizing the rehydration of leaves.

Phenological traits— We monitored the reproductive phenology and leaf senescence of each plant every 12-14 days during 2017 (7 censuses), and every 6-8 days during 2018 (14 censuses). At each phenology census, we visually estimated the percentage of closed inflorescences, inflorescences with open flowers, fully-developed fruits, dispersed inflorescences, and the percentage of senescent and green leaves. From these data, we calculated the following phenological traits for each individual: i) flowering, fruiting and dispersal onset, as the number of days elapsed between the first census (May 10th both years) and the appearance of the first fully open flower, fruit and dispersed inflorescence, respectively; ii) flowering, fruiting and dispersal peak, as the number of days between the first census and the day in which the percentage of flowers, fruits and dispersed inflorescences was highest; iii) flowering, fruiting and dispersal duration, as the number of days that each plant showed flowers, fruits and dispersed inflorescences, respectively; and plant senescence, as the mean percentage of senescent leaves across censuses.

Ecophysiological and leaf chemical composition traits— Midday photochemical efficiency (F_v/F_m) was measured from 13:00 to 16:30 (UTC + 2) during three consecutive sunny days. Prior to the measurement, a leaf clip was set for 30 min in one fully-expanded leaf from a primary branch to adapt it to the dark. $\delta^{13}C$ is an estimate of water use efficiency (WUE, the amount of C fixed per unit of water transpired), integrated over the lifetime of the leaf. More negative $\delta^{13}C$ values are associated with lower WUE (Farquhar et al., 1989). $\delta^{15}N$ provides information about plant nitrogen demand and assimilation capacity (Ariz et al., 2015). These analyses were carried out at UC Davis Stable Isotope Facility (Davis, CA, USA).

Microenvironmental and environmental conditions

Orientation and microslope were measured using the built-in GPS receiver of an Honor 9 smartphone, with the application GPS Status & Toolbox (MobiWIA Ltd.).

Statistical analyses

We arranged the functional traits and fitness data into different datasets. First, to perform phenotypic selection analyses, i.e. quantify selection differentials and selection gradients for each set of environmental conditions, we broke down our data by species, year and slope, obtaining eight different datasets (2 species x 2 years x 2 slopes = 8 datasets). Then, to test whether the magnitude and the direction of selection varied between slopes, we combined these datasets into four that contained the data from the same year and different slopes (two for each species). Similarly, to test whether the magnitude and the direction of selection varied among years, we grouped the data from the same slope and different years (two for each species). Finally, to assess whether the mean values of phenotypic traits and fitness varied among slopes and years for each species, we arranged two datasets containing all the information from each species. Environmental data from both species was arranged by slope and year, to test whether environmental conditions varied among slopes and years.

To avoid potential multicollinearity issues, we computed both variance inflation factors (VIFs) and pairwise correlations for each trait in all datasets before performing selection analyses We excluded from our models predictors with VIF > 10, as recommended (Dormann et al., 2013). Therefore, dispersal duration was not included in the models of the 2017 datasets in both slopes and species, and both fruiting duration and dispersal duration were excluded from models of the 2018 datasets in both slopes and species.

Before the calculation of selection differentials and gradients, traits were standardized, and fitness was relativized for each species. Traits were standardized as $\frac{X-\mu}{\sigma}$, where X is the trait value of an individual, μ is the mean value of the trait and year and σ is the standard deviation of the trait for a given slope and year. Relative fitness (w) was calculated as individual fitness divided by the mean value of fitness for a given slope and year. In the selection analyses performed using survival as fitness, selection differentials and gradients were "estimated" by multiplying the estimates from the models by (1- $\bar{w}$), since the regression coefficients from binomial models cannot be directly assigned to selection estimators (Gomez, 2004; Janzen and Stern, 1998).

To assess the percentage of variation explained by the fixed and random factors in our selection models, we calculated marginal and conditional R^2 of each model using *r.squaredGLMM* function in R (package MuMIn; Barton 2020). While marginal R^2 indicates the variance explained by fixed factors, conditional R^2 shows the variance explained by both fixed and random factors. Please note that marginal and conditional R^2 values were similar in most of the cases, indicating that "plot" explained a low percentage of variance and thus, confirming the universality of our results across plots (Supp. 9).

Finally, to test whether selection was similar within slopes between years or within years between slopes, we performed commonality analyses to evaluate the unique and common effect of slope and year on the values of selection differentials and gradients. This analysis assesses the percentage of variance (R^2) explained by the unique and common effects of predictors on the response variable (Ray-Mukherjee et al., 2014). Commonality analyses were performed using *regr* function in R (package yhat; Nimon et al., 2013). Results from commonality analysis are shown using Venn diagrams in Supp. 10.

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Supp. 2: Climatic data of both study years (2017 and 2018), climatic data extracted from the 35-year CHELSA climatic timeseries (Karger et al., 2017) and description of the climatic anomalies recorded in the harsher year (2017). Due to technical problems with the data from the closest climatic station (Tarancón, Cuenca, Spain), data from a close population with semiarid climate and massive gypsum habitats are shown (Aranjuez, Madrid, Spain; ~40 km far from Belinchón). It is worth mentioning that Aranjuez is slightly drier than Belinchón.

Climatic data extracted from an AEMET climatic station (Aranjuez, Madrid)

	Annual temperature (ºC)			Precipitation (mm)
Year	Mean	Mean of minimum	Mean of maximum	Total
2017	16.07	8.50	23.64	321.8
2018	15.00	7.62	22.39	389.8

Climatic data extracted from 35-year CHELSA climatic timeseries (Belinchón, Cuenca)

	Annual temperature (ºC)			Precipitation (mm)
Period	Mean (SD)	Mean of minimum (SD)	Mean of maximum (SD)	Total (SD)
1979-2013	14.60 (0.63)	9.43 (0.58)	19.73 (0.71)	419.2 (78.51)

The harsher year (2017) was characterized by the presence of climatic anomalies in the centre of the Iberian Peninsula. Indeed, it had the highest number of heat waves in the region (5), the higher maximum temperature during a heat wave (41.1°C), the second warmest year in summer temperature (24.6°C), and the second year with an earlier heat wave (June 13th-21st) since records have been made (data from the Spanish meteorology agency, AEMET).

Supp. 3: Detailed description of the models and the R code used for each analysis.

1.- Pairwise correlations of traits

```
# First, import data in R (8 different matrices: 2 species x 2 years x 2 slopes = 8 datasets)
data_hyssopifolia_2017_S <- read.table("clipboard", header = T, sep = "\t", dec=".")
# Analysis
M1 <- cor (data_hyssopifolia_2017_S, use = "pairwise.complete.obs")
res1 <- cor.mtest (data_hyssopifolia_2017_S, conf.level = .95)
p.mat <- cor.mtest (data_hyssopifolia_2017_S)$p
col4 <- colorRampPalette (c ("#01665e", "#35978f", "#80cdc1", "#c7eae5", "#fde0ef", "#f1b6da", "#de77ae", "#c51b7d"))
corrplot(M1, method = "color", type="lower", col = col4(8), p.mat = p.mat, sig.level = 0.05, insig = "blank", tl.col = "black", tl.srt = 45)
```

2.- Variance inflation factors (VIFs)

```
model1 <- lm (Fitness ~ . , data = data_hyssopifolia_2017_S)
predictions <- predict(model1)
data.frame (RMSE = RMSE (predictions, data_hyssopifolia_2017_S $Fitness, na.rm = TRUE), R2 = R2 (predictions, data_hyssopifolia_2017_S $Fitness , na.rm = TRUE))
car::vif (model1)
# If VIFs of any variable are higher than 10, build a model excluding the tax variable
model2 <- lm (Fitness ~ . -variable, data = data_hyssopifolia_2017_S)
predictions <- predict(model2)
data.frame (RMSE = RMSE (predictions, data_hyssopifolia_2017_S $Fitness, na.rm = TRUE), R2 = R2 (predictions, data_hyssopifolia_2017_S $Fitness , na.rm = TRUE))
car::vif (model2)
```

3.- PCAs to summarize the microenvironmental information and include it in the models

```
# First, import data in R (8 different matrices: 2 species x 2 years x 2 slopes = 8 datasets)
microenvironmental_data_hyssopifolia_2017_S <- read.table ("clipboard", header=T, sep = "\t", dec=".")
# Analysis
pca hyssopifolia_2017_S <- prcomp (microenvironmental_data_hyssopifolia_2017_S, scale = TRUE)
biplot(x = pca hyssopifolia_2017_S, scale = 0, cex = 0.6, col = c("blue4", "brown3"))
# To extract the eigenvectors and eigenvalues, respectively
write.table(pca hyssopifolia_2017_S$rotation, "clipboard", sep = "\t", dec=".")
write.table(pca hyssopifolia_2017_S$x, "clipboard",sep = "\t", dec=".")
# To assess the variance explained by each PC.
variance <- pca hyssopifolia_2017_S$sdev^2 / sum(pca hyssopifolia_2017_S$sdev^2)
variance
```

4.- Linear selection differentials

```
glmer1<- glmer (Relative_Fitness ~ Trait (Std.) + Plant_volume + PC1 + PC2 + PC3 + (1|plot), family="Gamma"(link=log), data= data_hyssopifolia_2017_S)
Anova (glmer1, type=3)
summary (glmer1)
```

5.- Linear selection gradients

glmer2<- glmer (Relative_Fitness ~ Trait_1 (Std.) + Trait_2 (Std.) + … + Trait_X (Std.) + Plant_volume + PC1 + PC2 + PC3 + (1|plot), family="Gamma"(link=log), data= data_hyssopifolia_2017_S)
Anova (glmer2, type=3)
summary (glmer2)

6.- Quadratic selection differentials

glmer3<- glmer (Relative_Fitness ~ Trait (Std.) + I((1/2)* Trait (Std.)^2) + Plant_volume + PC1 + PC2 + PC3 + (1|plot), family="Gamma"(link=log), data= data_hyssopifolia_2017_S)
Anova (glmer3, type=3)
summary (glmer3)

7.- Quadratic selection gradients

glmer4<- glmer (Relative_Fitness ~ Trait_1 (Std.) + I((1/2)* Trait_1 (Std.)^2) + Trait_2 (Std.) + I((1/2)* Trait_2 (Std.)^2) + … + Trait_X (Std.) + I((1/2)* Trait_X (Std.)^2) + Plant_volume + PC1 + PC2 + PC3 + (1|plot), family="Gamma"(link=log), data= data_hyssopifolia_2017_S)
Anova (glmer4, type=3)
summary (glmer4)

8.- Intensity of selection

# In this example is only shown the model used to test differences in the intensity of selection between slopes within the same year (2017). First, import data in R
data_hyssopifolia_2017 <- read.table("clipboard", header=T, sep = "\t", dec=".")
# Analysis
glmer5 <- glmer (Relative_Fitness ~ Trait (Std.) : slope + Plant_volume + PC1/slope + PC2/slope + PC3/slope + (1|plot), family="Gamma"(link=log), data= data_hyssopifolia_2017)
Anova (glmer5, type=3)
summary (glmer5)

9.- Commonality analysis

# First, import the results of selection differentials/gradients of each slope and year from each species in R
results_differentials_hyssopifolia <- read.table ("clipboard", header=T, sep = "\t", dec=".")
# Analysis
m.par <- lm (Selection differentials ~ slope+year, data= results_differentials_hyssopifolia)
partition.dat <- regr(m.par)
partition.dat

10.- Differences in Fitness or Trait values between slopes and years

# First, import data in R (the matrix with all the data from each species)
data_hyssopifolia <- read.table("clipboard", header=T, sep = "\t", dec=".")
# Analysis
glmer6 <- glmer (Fitness (or Trait) ~ slope*year + (1|plot), family="Gamma"(link=log), data= data_hyssopifolia)
Anova (glmer6, type=3)
summary (glmer6)

Supp. 4: Directional selection differentials (S') and gradients (β'), and their standard error (in brackets) of both species, slopes and years for total seed number fitness variable. Significant ($p < 0.05$) and marginally significant ($0.05 < P < 0.1$) values after FDR correction are in bold and italic, respectively. Significance levels: · $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (E.S.) and (P.F.) in Fv/Fm and chlorophyll content in 2018 indicates early-season and peak of flowering, respectively.

		***C. hyssopifolia*, south slope**		***C. hyssopifolia*, north slope**		***H. squamatum*, south slope**		***H. squamatum*, north slope**	
Year	Functional trait	S' (SE)	β' (SE)	S' (SE)	β' (SE)	S' (SE)	β' (SE)	S' (SE)	β' (SE)
2017									
	Leaf area	0.149 (0.070)	0.027 (0.083)	0.015 (0.075)	0.016 (0.073)	-0.114 (0.113)	**-0.372 (0.127)** *	0.059 (0.109)	-0.028 (0.126)
	Leaf thickness	-0.040 (0.071)	0.009 (0.074)	0.030 (0.081)	0.047 (0.078)	-0.053 (0.120)	0.226 (0.168)	-0.043 (0.113)	0.238 (0.177)
	SLA	-0.003 (0.079)	-0.171 (0.095)	0.147 (0.081)	0.099 (0.104)	**0.497 (0.152)** **	0.150 (0.218)	**0.314 (0.104)** **	0.326 (0.214)
	LDMC	-0.120 (0.065)	-0.204 (0.086)	-0.089 (0.074)	0.149 (0.104)	**-0.326 (0.102)** **	-0.192 (0.191)	**-0.354 (0.104)** **	-0.279 (0.200)
	Flowering onset	-0.151 (0.068)	-0.074 (0.106)	-0.131 (0.070)	0.077 (0.075)	**-0.515 (0.132)** ***	**-0.386 (0.141)** *	**-0.328 (0.106)** **	**-0.376 (0.120)** *
	Flowering duration	0.162 (0.068)	0.056 (0.089)	**0.277 (0.073)** ***	0.252 (0.100)	**-0.369 (0.119)** ***	**-0.555 (0.132)** ***	-0.036 (0.107)	-0.233 (0.132)
	Flowering peak	-0.089 (0.076)	-0.035 (0.082)	-0.106 (0.076)	-0.130 (0.079)	**-0.589 (0.108)** ***	0.379 (0.177)	*-0.210 (0.104)* ·	-0.075 (0.103)
	Fruiting onset	*-0.187 (0.063)* ·	-0.269 (0.107)	**-0.278 (0.071)** ***	-0.036 (0.108)	**-0.276 (0.113)** *	-0.234 (0.118)	**-0.357 (0.099)** **	-0.114 (0.121)
	Fruiting duration	0.185 (0.076)	0.084 (0.096)	**0.213 (0.071)** **	0.026 (0.092)	**0.562 (0.157)** **	0.187 (0.177)	*0.218 (0.109)* ·	0.159 (0.136)
	Fruiting peak	0.027 (0.077)	0.074 (0.088)	**-0.234 (0.069)** ***	-0.193 (0.082)	-0.131 (0.137)	0.041 (0.174)	-0.055 (0.101)	0.015 (0.106)
	Dispersion onset	0.024 (0.079)	0.188 (0.085)	**-0.246 (0.069)** ***	-0.099 (0.092)	**0.503 (0.133)** ***	0.264 (0.128)	**0.293 (0.111)** *	0.151 (0.108)
	Dispersion peak	0.130 (0.075)	0.086 (0.091)	**0.261 (0.067)** ***	0.115 (0.078)	**0.847 (0.108)** ***	**0.930 (0.246)** **	0.619 (0.341)	-0.159 (0.380)
	Senescence	-0.038 (0.070)	-0.006 (0.071)	-0.016 (0.079)	0.085 (0.072)	-0.046 (0.141)	-0.124 (0.117)	-0.198 (0.111)	**-0.358 (0.134)** *
	Leaf Carbon content	-0.025 (0.069)	0.132 (0.081)	-0.037 (0.076)	-0.129 (0.087)	-0.198 (0.108)	0.035 (0.145)	-0.148 (0.129)	0.155 (0.156)
	Leaf Nitrogen content	0.001 (0.074)	0.035 (0.074)	0.055 (0.085)	0.117 (0.080)	-0.026 (0.124)	-0.072 (0.107)	**-0.266 (0.111)** *	**-0.365 (0.115)** *
	Leaf δ13C content	-0.011 (0.082)	-0.069 (0.085)	-0.147 (0.068)	**-0.213 (0.070)** *	-0.188 (0.112)	-0.225 (0.142)	**-0.300 (0.095)** **	-0.044 (0.118)
	Leaf δ15N content	0.060 (0.075)	0.096 (0.077)	-0.086 (0.075)	0.034 (0.074)	0.096 (0.131)	-0.029 (0.135)	-0.181 (0.142)	0.054 (0.146)
	Fv/Fm	0.049 (0.068)	0.061 (0.071)	-0.093 (0.081)	-0.111 (0.069)	0.050 (0.115)	-0.174 (0.128)	0.109 (0.103)	0.060 (0.124)
2018									
	Leaf area	-0.126 (0.096)	-0.188 (0.137)	0.013 (0.071)	-0.066 (0.087)	**0.392 (0.108)** **	**0.389 (0.135)** *	**0.487 (0.127)** **	0.214 (0.104)
	Leaf thickness	0.053 (0.119)	-0.064 (0.146)	0.091 (0.088)	0.167 (0.117)	0.011 (0.116)	-0.179 (0.161)	0.113 (0.117)	-0.174 (0.127)
	SLA	-0.073 (0.147)	-0.252 (0.234)	0.006 (0.082)	0.003 (0.176)	0.089 (0.115)	0.194 (0.185)	-0.047 (0.127)	-0.238 (0.138)
	LDMC	-0.217 (0.126)	-0.304 (0.184)	-0.111 (0.085)	-0.130 (0.162)	*-0.225 (0.106)* ·	0.178 (0.172)	-0.105 (0.103)	-0.204 (0.095)
	Flowering onset	0.112 (0.116)	0.113 (0.333)	-0.027 (0.111)	0.383 (0.258)	**-0.530 (0.122)** ***	**-0.584 (0.186)** *	**-0.435 (0.122)** **	-0.437 (0.172)
	Flowering duration	-0.046 (0.149)	0.003 (0.227)	0.223 (0.145)	*0.418 (0.159)* ·	**0.478 (0.142)** **	- 0.317 (0.22)	*0.220 (0.107)* ·	-0.395 (0.183)
	Flowering peak	0.209 (0.119)	0.162 (0.152)	0.001 (0.093)	-0.068 (0.107)	**-0.422 (0.146)** *	-0.200 (0.137)	-0.185 (0.110)	-0.131 (0.097)
	Fruiting onset	0.038 (0.115)	-0.158 (0.294)	0.016 (0.101)	0.206 (0.187)	-0.173 (0.147)	-0.023 (0.115)	*-0.271 (0.124)* ·	-0.188 (0.105)
	Fruiting peak	0.228 (0.132)	0.127 (0.177)	0.101 (0.099)	0.082 (0.119)	*0.234 (0.121)* ·	0.456 (0.248)	*0.247 (0.112)* ·	0.298 (0.176)
	Dispersion onset	0.073 (0.166)	-0.208 (0.259)	-0.126 (0.165)	-0.615 (0.254)	**0.315 (0.129)** *	-0.042 (0.176)	**0.457 (0.124)** **	-0.066 (0.168)
	Dispersion peak	0.174 (0.106)	0.176 (0.125)	-0.047 (0.084)	0.057 (0.088)	**0.243 (0.106)** *	0.119 (0.111)	0.171 (0.104)	-0.008 (0.099)
	Senescence	-0.205 (0.112)	-0.035 (0.146)	-0.172 (0.110)	-0.085 (0.121)	**-0.384 (0.121)** **	-0.254 (0.122)	**-0.679 (0.102)** ***	**-0.731 (0.103)** ***
	Leaf Carbon content	-0.058 (0.109)	-0.087 (0.129)	0.050 (0.090)	-0.028 (0.107)	**-0.301 (0.121)** *	-0.259 (0.134)	*-0.271 (0.115)* ·	0.057 (0.090)
	Leaf Nitrogen content	0.088 (0.101)	0.047 (0.129)	**0.317 (0.100)** *	*0.356 (0.132)* ·	0.193 (0.122)	0.047 (0.106)	0.092 (0.110)	0.062 (0.113)
	Leaf δ13C content	0.080 (0.105)	0.064 (0.133)	0.000 (0.103)	-0.152 (0.124)	*-0.212 (0.109)* ·	0.108 (0.143)	-0.210 (0.135)	0.032 (0.114)
	Leaf δ15N content	-0.071 (0.115)	-0.194 (0.146)	0.068 (0.090)	-0.053 (0.096)	0.001 (0.099)	-0.065 (0.097)	-0.142 (0.108)	-0.248 (0.112)
	Fv/Fm (E.S.)	-0.121 (0.129)	-0.097 (0.158)	-0.009 (0.094)	0.011 (0.096)	-0.200 (0.159)	-0.339 (0.148)	*0.276 (0.141)* ·	0.170 (0.090)
	Fv/Fm (P.F.)	-0.074 (0.117)	0.003 (0.144)	0.035 (0.096)	-0.069 (0.103)	0.134 (0.112)	0.207 (0.098)	0.085 (0.104)	0.117 (0.074)
	Chlorophyll content (E.S.)	-0.088 (0.110)	0.084 (0.145)	0.064 (0.087)	0.031 (0.096)	**0.293 (0.114)** *	0.183 (0.092)	0.115 (0.104)	0.168 (0.076)
	Chlorophyll content (P.F.)	-0.236 (0.113)	-0.233 (0.161)	-0.043 (0.085)	-0.154 (0.091)	*0.252 (0.122)* ·	0.071 (0.105)	*0.256 (0.110)* ·	0.103 (0.091)

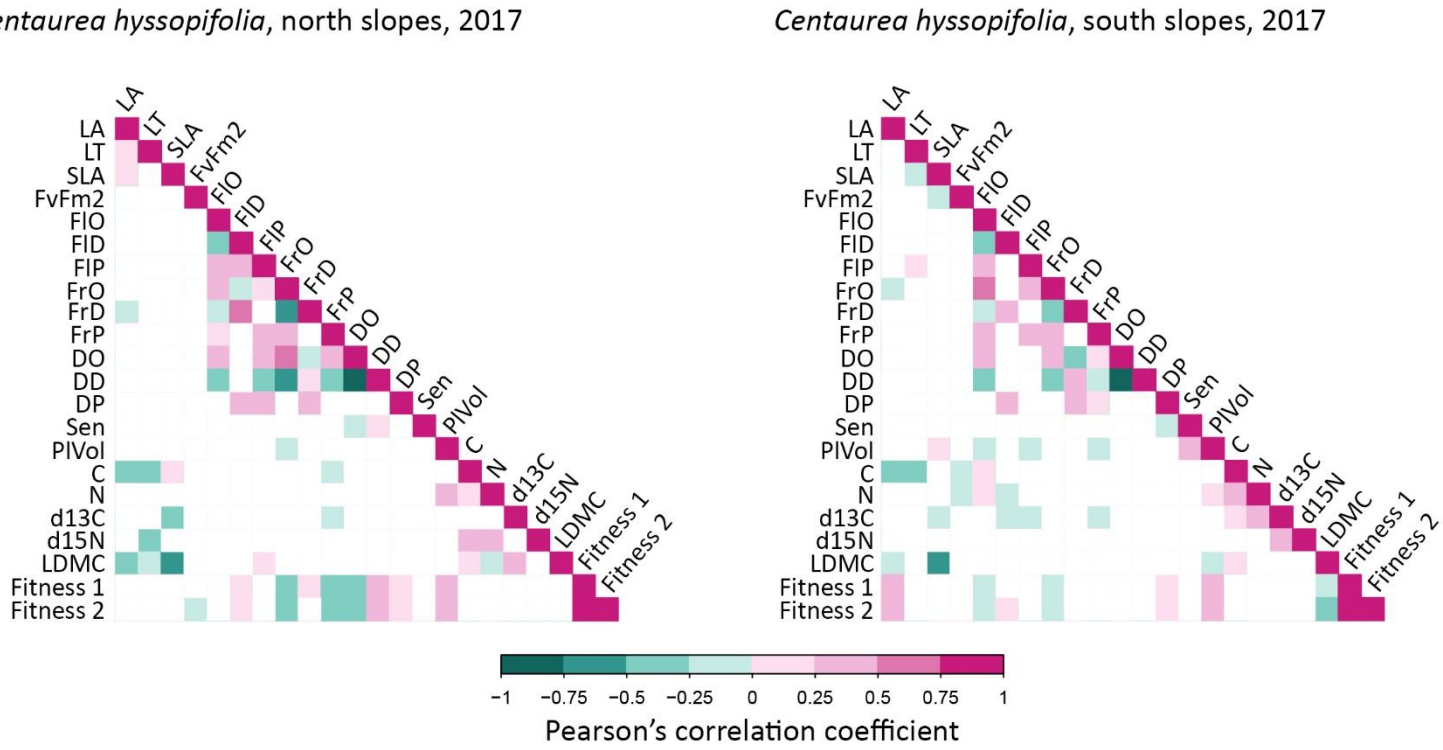
Supp. 5: Quadratic selection differentials (C') and gradients (γ'), and their standard error (in brackets) of both species, slopes and years for total seed number fitness variable. Significant ($P < 0.05$) and marginally significant ($0.05 < P < 0.1$) values after FDR correction are in bold and italic, respectively. Significance levels: · $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (E.S.) and (P.F.) in Fv/Fm and chlorophyll content in 2018 indicates early-season and peak of flowering, respectively.

		***C. hyssopifolia*, south slope**		***C. hyssopifolia*, north slope**		***H. squamatum*, south slope**		***H. squamatum*, north slope**	
Year	Functional trait	C' (SE)	γ' (SE)	C' (SE)	γ' (SE)	C' (SE)	γ' (SE)	C' (SE)	γ' (SE)
2017									
	Leaf area	0.102 (0.127)	-0.121 (0.139)	0.031 (0.094)	-0.023 (0.087)	-0.432 (0.204)	-0.162 (0.201)	-0.291 (0.161)	-0.239 (0.166)
	Leaf thickness	0.081 (0.124)	-0.109 (0.115)	0.185 (0.131)	-0.103 (0.143)	-0.085 (0.155)	-0.047 (0.187)	0.212 (0.166)	0.410 (0.206)
	SLA	-0.080 (0.068)	-0.024 (0.082)	-0.215 (0.123)	-0.245 (0.124)	*-0.261 (0.097)*·	-0.205 (0.159)	-0.065 (0.103)	-0.026 (0.199)
	LDMC	0.137 (0.103)	0.025 (0.125)	0.087 (0.114)	0.019 (0.112)	0.068 (0.084)	0.012 (0.103)	-0.106 (0.126)	0.020 (0.215)
	Flowering onset	0.059 (0.191)	0.115 (0.194)	0.088 (0.105)	0.115 (0.104)	-----	-----	------	------
	Flowering duration	0.018 (0.123)	0.004 (0.120)	-0.232 (0.106)	-0.215 (0.136)	-0.135 (0.257)	-0.057 (0.282)	-0.052 (0.149)	0.359 (0.166)
	Flowering peak	-0.146 (0.101)	-0.261 (0.100)	0.058 (0.142)	0.194 (0.143)	0.150 (0.141)	0.480 (0.206)	-0.288 (0.442)	-0.018 (0.362)
	Fruiting onset	0.278 (0.128)	0.373 (0.153)	-0.021 (0.089)	-0.107 (0.123)	-----	-----	------	------
	Fruiting duration	-0.031 (0.113)	-0.016 (0.140)	-0.117 (0.119)	0.157 (0.132)	*-0.801 (0.279)*·	0.210 (0.563)	-0.002 (0.253)	-0.419 (0.236)
	Fruiting peak	-0.129 (0.106)	-0.044 (0.118)	-0.044 (0.102)	-0.112 (0.147)	*-1.001 (0.422)*·	-0.474 (0.455)	-0.378 (0.278)	-0.307 (0.271)
	Dispersion onset	-----	-----	0.022 (0.079)	0.056 (0.093)	*-0.425 (0.172)*·	-0.125 (0.201)	-0.187 (0.130)	- 0.196 (0.13)
	Dispersion peak	0.265 (0.165)	0.120 (0.193)	-0.074 (0.132)	-0.035 (0.143)	0.125 (0.130)	0.266 (0.310)	0.313 (1.702)	-1.772 (1.554)
	Senescence	0.057 (0.032)	0.146 (0.094)	-0.045 (0.067)	-0.005 (0.079)	-0.090 (0.092)	-0.024 (0.093)	-0.153 (0.112)	-0.256 (0.148)
	Leaf Carbon content	0.052 (0.081)	0.049 (0.090)	0.233 (0.135)	0.073 (0.131)	0.061 (0.117)	-0.115 (0.132)	0.074 (0.201)	-0.058 (0.181)
	Leaf Nitrogen content	-0.010 (0.110)	0.040 (0.109)	-0.137 (0.107)	-0.082 (0.110)	-0.119 (0.190)	0.012 (0.191)	0.012 (0.130)	-0.137 (0.143)
	Leaf δ13C content	-0.205 (0.114)	-0.187 (0.121)	0.056 (0.093)	-0.019 (0.094)	0.032 (0.189)	0.455 (0.273)	0.153 (0.147)	0.233 (0.165)
	Leaf δ15N content	0.034 (0.082)	-0.102 (0.080)	0.002 (0.085)	-0.094 (0.081)	-0.235 (0.212)	-0.367 (0.232)	-0.091 (0.131)	-0.114 (0.127)
	Fv/Fm	0.089 (0.113)	0.045 (0.111)	-0.107 (0.087)	-0.096 (0.080)	-0.021 (0.245)	-0.201 (0.255)	0.020 (0.114)	0.214 (0.167)
2018									
	Leaf area	0.259 (0.159)	0.204 (0.228)	0.215 (0.095)	0.308 (0.111)	-0.057 (0.130)	0.048 (0.154)	**-0.313 (0.112)***	-0.045 (0.106)
	Leaf thickness	-0.052 (0.117)	-0.183 (0.227)	-0.055 (0.128)	-0.127 (0.131)	-0.326 (0.140)	**-0.382 (0.138)***	-0.199 (0.143)	-0.141 (0.188)
	SLA	**-0.552 (0.152)****	*-0.643 (0.217)*·	0.067 (0.123)	0.162 (0.122)	-0.162 (0.091)	0.106 (0.107)	-0.258 (0.126)	0.094 (0.150)
	LDMC	-0.264 (0.144)	-0.044 (0.207)	-0.005 (0.077)	-0.023 (0.089)	-0.080 (0.156)	-0.307 (0.163)	0.072 (0.067)	0.048 (0.083)
	Flowering onset	0.015 (0.161)	0.179 (0.362)	**-0.526 (0.146)****	-0.433 (0.220)	-0.272 (0.161)	*-0.501 (0.206)*·	-----	-----
	Flowering duration	-0.109 (0.281)	-0.448 (0.382)	-0.429 (0.238)	-0.340 (0.221)	**-0.452 (0.120)****	-0.098 (0.134)	-0.137 (0.143)	-0.039 (0.136)
	Flowering peak	-0.093 (0.129)	-0.193 (0.171)	0.022 (0.168)	-0.133 (0.177)	-0.188 (0.184)	-0.043 (0.184)	-0.139 (0.190)	-0.250 (0.170)
	Fruiting onset	0.274 (0.207)	-0.235 (0.378)	-0.630 (0.264)	-0.454 (0.313)	-0.211 (0.334)	0.630 (0.306)	**-0.524 (0.102)*****	-0.432 (0.269)
	Fruiting peak	-0.096 (0.172)	-0.099 (0.229)	-0.103 (0.160)	-0.082 (0.172)	-0.092 (0.161)	-0.066 (0.143)	-0.202 (0.112)	-0.114 (0.149)
	Dispersion onset	0.185 (0.345)	0.709 (0.474)	**-1.108 (0.275)****	-0.221 (0.374)	-0.070 (0.097)	**1.180 (0.344)****	**-0.216 (0.072)***	0.222 (0.296)
	Dispersion peak	-0.042 (0.286)	-0.062 (0.337)	0.032 (0.147)	-0.170 (0.145)	-0.012 (0.072)	**-0.701 (0.211)****	-0.256 (0.171)	-0.110 (0.168)
	Senescence	-0.033 (0.150)	0.085 (0.158)	-0.098 (0.178)	-0.201 (0.181)	-0.365 (0.251)	0.075 (0.232)	-0.374 (0.191)	0.094 (0.164)
	Leaf Carbon content	0.128 (0.184)	0.027 (0.208)	-0.028 (0.076)	-0.130 (0.087)	-0.036 (0.152)	0.123 (0.149)	-0.159 (0.163)	0.053 (0.142)
	Leaf Nitrogen content	0.210 (0.176)	0.120 (0.224)	-0.118 (0.127)	0.442 (0.189)	-0.365 (0.184)	0.049 (0.162)	0.069 (0.138)	-0.253 (0.153)
	Leaf δ13C content	0.110 (0.150)	0.029 (0.208)	-0.084 (0.079)	-0.234 (0.101)	-0.113 (0.125)	0.019 (0.110)	-0.256 (0.149)	-0.108 (0.118)
	Leaf δ15N content	0.066 (0.152)	-0.002 (0.185)	0.063 (0.127)	-0.023 (0.157)	0.045 (0.139)	0.131 (0.117)	0.325 (0.161)	0.057 (0.153)
	Fv/Fm (E.S.)	-0.081 (0.169)	-0.294 (0.232)	-0.103 (0.100)	-0.181 (0.106)	*-0.548 (0.205)*·	-0.290 (0.169)	-0.260 (0.197)	-0.078 (0.156)
	Fv/Fm (P.F.)	0.118 (0.200)	0.160 (0.245)	-0.198 (0.113)	-0.101 (0.120)	0.102 (0.269)	-0.037 (0.211)	0.051 (0.274)	-0.067 (0.200)
	Chlorophyll content (E.S.)	-0.029 (0.148)	-0.014 (0.205)	-0.101 (0.149)	-0.056 (0.152)	-0.117 (0.136)	0.072 (0.123)	-0.005 (0.158)	0.082 (0.127)
	Chlorophyll content (P.F.)	-0.047 (0.160)	0.253 (0.274)	0.000 (0.001)	-0.132 (0.128)	-0.126 (0.171)	-0.048 (0.126)	-0.065 (0.143)	-0.081 (0.128)

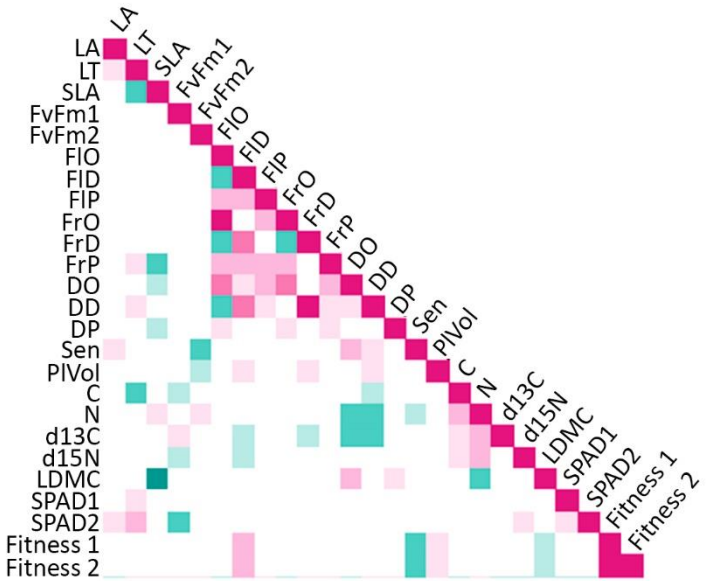
Supp. 6: Directional selection differentials (*S'*) and gradients (*β'*), quadratic selection differentials (*C'*) and gradients (*γ'*), and their standard error (in brackets) of *H. squamatum* in both slopes using survival as a fitness variable. Significant ($P < 0.05$) values after FDR correction are in bold. Significance levels: ****** $P < 0.01$.

		***H. squamatum*, south slope**				***H. squamatum*, north slope**			
Year	Functional trait	*S'* (SE)	*β'* (SE)	*C'* (SE)	*γ'* (SE)	*S'* (SE)	*β'* (SE)	*C'* (SE)	*γ'* (SE)
2017									
	Leaf area	-0.010 (0.088)	-0.075 (0.149)	-0.177 (0.133)	-0.207 (0.250)	0.082 (0.097)	0.076 (0.152)	-0.017 (0.145)	-0.117 (0.246)
	Leaf thickness	0.050 (0.089)	0.316 (0.217)	0.079 (0.125)	0.044 (0.259)	0.021 (0.121)	-0.129 (0.200)	0.288 (0.187)	0.557 (0.518)
	SLA	0.029 (0.093)	0.497 (0.267)	0.270 (0.151)	0.627 (0.455)	0.034 (0.094)	-0.134 (0.255)	0.131 (0.135)	0.529 (0.558)
	LDMC	-0.003 (0.092)	0.224 (0.217)	0.067 (0.072)	0.014 (0.131)	0.059 (0.123)	-0.082 (0.231)	0.058 (0.137)	-0.555 (0.490)
	Flowering onset	-0.244 (0.105)	-0.467 (0.189)	0.081 (0.107)	---	0.114 (0.103)	0.120 (0.143)	---	---
	Flowering duration	0.205 (0.093)	0.140 (0.136)	-0.038 (0.193)	0.186 (0.344)	0.080 (0.103)	-0.032 (0.154)	-0.112 (0.151)	-0.365 (0.276)
	Flowering peak	0.034 (0.092)	-0.207 (0.292)	- 0.041 (0.11)	0.339 (0.380)	-0.057 (0.096)	-0.192 (0.131)	-0.420 (0.468)	-0.893 (0.700)
	Fruiting onset	0.089 (0.087)	0.109 (0.097)	---	---	0.013 (0.096)	-0.023 (0.170)	---	---
	Fruiting duration	0.045 (0.089)	0.204 (0.209)	0.193 (0.148)	0.975 (0.777)	-0.039 (0.100)	-0.075 (0.186)	-0.018 (0.231)	-0.186 (0.507)
	Fruiting peak	0.066 (0.095)	0.138 (0.171)	0.322 (0.285)	0.181 (0.530)	0.071 (0.097)	-0.056 (0.127)	-0.668 (0.276)	-0.603 (0.625)
	Dispersion onset	-0.001 (0.091)	-0.232 (0.131)	-0.164 (0.133)	-0.340 (0.246)	0.016 (0.103)	-0.035 (0.125)	-0.426 (0.134)	-0.488 (0.301)
	Dispersion peak	0.030 (0.090)	0.133 (0.344)	0.142 (0.111)	-0.316 (0.302)	-0.058 (0.123)	-0.057 (0.240)	0.019 (0.093)	-0.225 (0.800)
	Senescence	-0.378 (0.146)	-0.526 (0.208)	0.008 (0.145)	-0.865 (0.799)	**-0.554 (0.143)****	**-0.651 (0.179)****	0.026 (0.202)	0.115 (0.391)
	Leaf Carbon content	0.109 (0.092)	-0.017 (0.162)	0.049 (0.114)	- 0.075 (0.26)	-0.225 (0.115)	-0.142 (0.183)	-0.084 (0.115)	-0.767 (0.439)
	Leaf Nitrogen content	0.112 (0.091)	0.167 (0.128)	0.371 (0.174)	0.952 (0.400)	0.049 (0.103)	0.007 (0.140)	0.086 (0.151)	0.157 (0.246)
	Leaf δ13C content	0.029 (0.089)	0.184 (0.168)	-0.097 (0.132)	-0.026 (0.283)	-0.186 (0.103)	-0.093 (0.154)	0.080 (0.155)	0.124 (0.291)
	Leaf δ15N content	-0.078 (0.096)	-0.018 (0.142)	0.063 (0.155)	0.130 (0.260)	0.059 (0.123)	0.044 (0.165)	0.026 (0.138)	0.543 (0.264)
	Fv/Fm (E.S.)	-0.031 (0.091)	-0.100 (0.139)	0.258 (0.201)	0.112 (0.324)	0.118 (0.107)	0.060 (0.150)	0.014 (0.106)	0.501 (0.407)

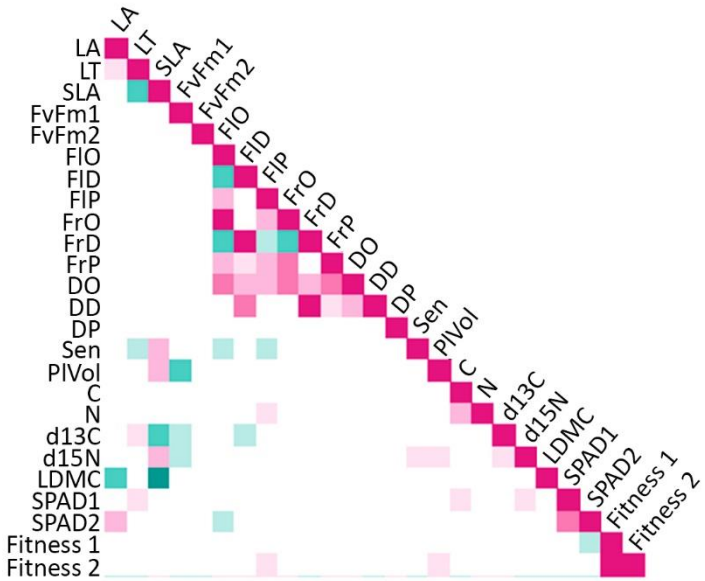
Supp. 7: Correlation matrices of functional traits for both species, slopes, and years. Only significant ($P < 0.05$) Pearson's pairwise correlations are coloured. LA: leaf area; LT: leaf thickness; SLA: specific leaf area; LDMC: leaf dry matter content; FlO: flowering onset; FlD: flowering duration; FlP: flowering peak; FrO: fruiting onset; FrD: fruiting duration; FrP: fruiting peak; DO: dispersion onset; DD: dispersion duration; DP: dispersion peak; Sen: senescence; PlVol: plant volume; C: leaf Carbon content; N: leaf Nitrogen content; d13C: leaf Carbon isotope ratio; d15N: leaf Nitrogen isotope ratio; FvFm1: early season photochemical efficiency; FvFm2: photochemical efficiency at the peak of flowering; SPAD1: early season chlorophyll content; SPAD2: chlorophyll content at the peak of flowering; Fitness1: Total seed number; Fitness2: Total seed mass.



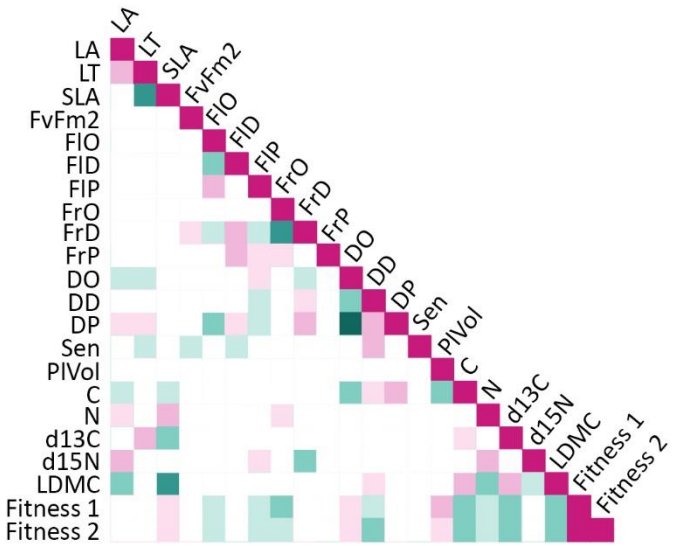
Centaurea hyssopifolia, north slopes, 2018



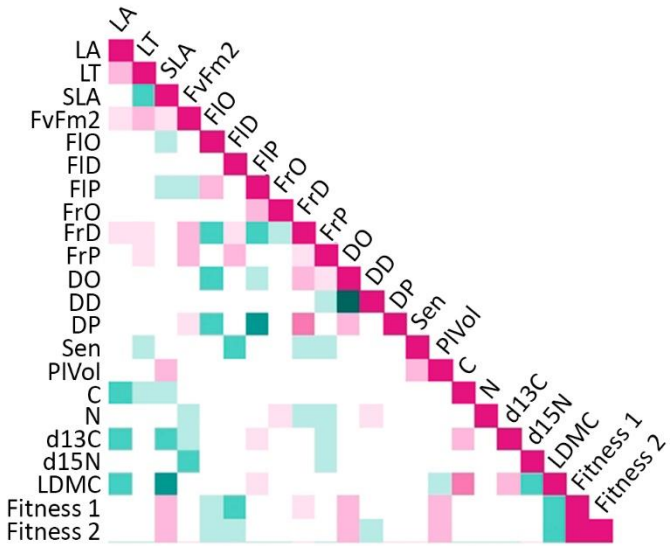
Centaurea hyssopifolia, south slopes, 2018

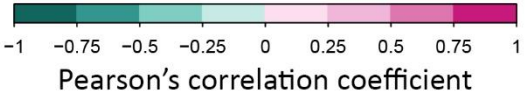


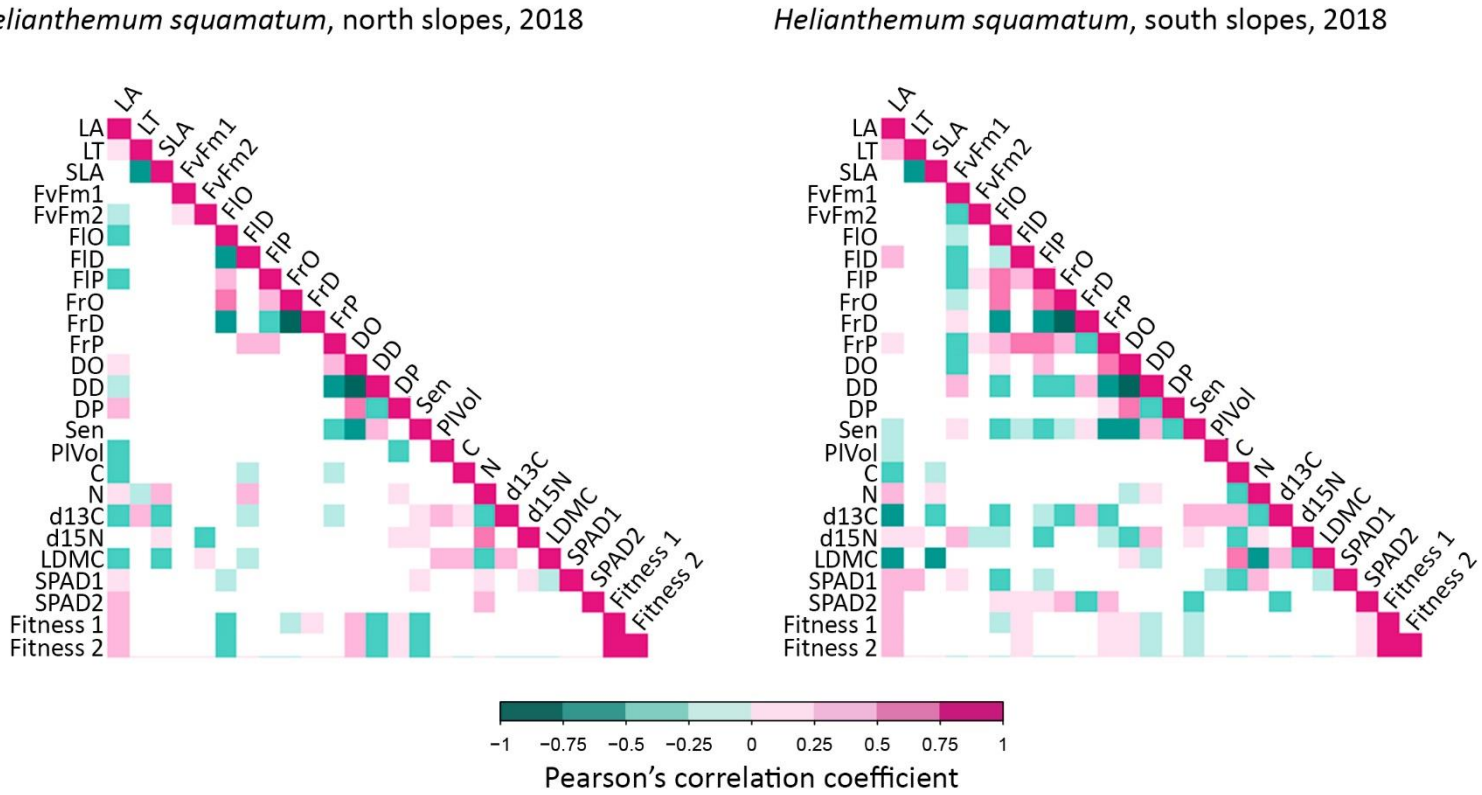
Helianthemum squamatum, north slopes, 2017



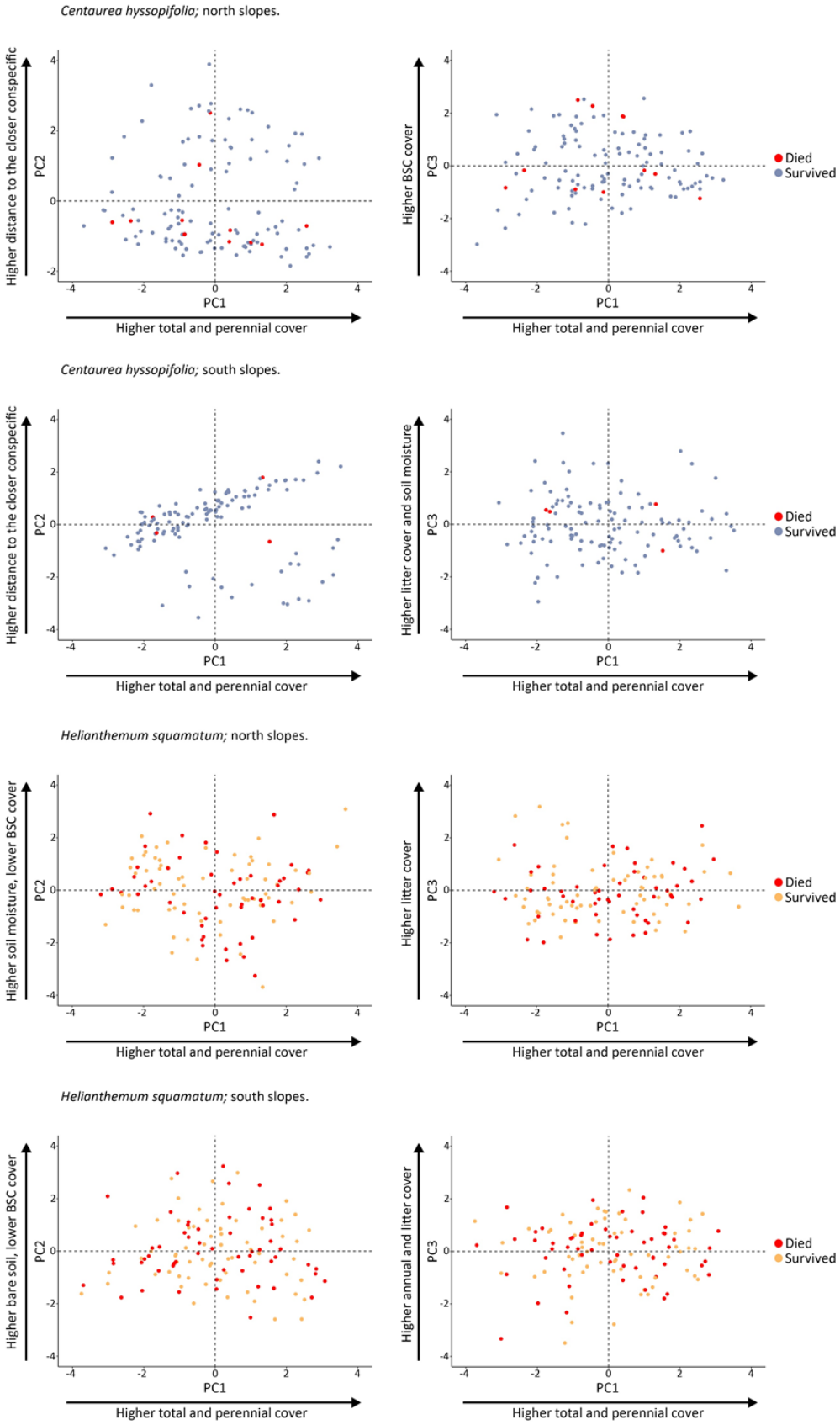
Helianthemum squamatum, south slopes, 2017






Helianthemum squamatum, north slopes, 2018
Helianthemum squamatum, south slopes, 2018
LA
LT
SLA
FvFm1
FvFm2
FIO
FID
FIP
FrO
FrD
FrP
DO
DD
DP
Sen
PlVol
C
N
d13C
d15N
LDMC
SPAD1
SPAD2
Fitness 1
Fitness 2
−1
−0.75
−0.5
−0.25
0
0.25
0.5
0.75
1
Pearson's correlation coefficient

Supp. 8: Plots of the principal component analyses (PCAs) used to summarize the microenvironmental conditions experienced by plants. For each species and slope, PC1 vs. PC2 and PC1 vs. PC3 are represented. In each plot, individuals that died and survived between the first and the second year are shown. The microenvironmental variables associated with the eigenvectors of each principal component (PC) are indicated in each plot.



Supp. 9: Results of the models used to calculate selection differentials. The effect of the environmental conditions on fitness was assessed by the significant effect of the different PCAs. The environmental variables associated with each PCA for each species, slope and year are shown below each table. The percentage of environmental variance explained by each PCA is shown. Marginal and conditional R^2 (R^2_m and R^2_c, respectively) of each model are shown (see Supp. 1 for details). LA: leaf area; LT: leaf thickness; SLA: specific leaf area; LDMC: leaf dry matter content; FlO: flowering onset; FlD: flowering duration; FlP: flowering peak; FrO: fruiting onset; FrD: fruiting duration; FrP: fruiting peak; DO: dispersion onset; DP: dispersion peak; Sen: senescence; C: leaf Carbon content; N: leaf Nitrogen content; d13C: leaf Carbon isotope ratio; d15N: leaf Nitrogen isotope ratio; FvFm1: early season photochemical efficiency; FvFm2: photochemical efficiency at the peak of flowering; SPAD1: early season chlorophyll content; SPAD2: chlorophyll content at the peak of flowering; Fitness1: total seed number; Fitness2: total seed mass; PlVol: plant volume. Significance levels: · $P < 0.1$; ***** $P < 0.05$; ****** $P < 0.01$; ******* $P < 0.001$. Significant and marginally significant terms are shown in bold.

Helianthemum squamatum, south slopes, 2017

	Fitness2	PlVol	PC1 (28.8%)	PC2 (17.7%)	PC3 (13.9%)	R^2_m	R^2_c
LA	-0.060	**141.260 (**)**	0.058	0.028	0.027	0.099	0.099
LT	-0.053	**136.653 (**)**	0.059	0.032	0.031	0.097	0.097
SLA	**0.440 (**)**	**83.150 (·)**	0.066	0.012	0.048	0.231	0.231
FvFm	0.048	**137.632 (**)**	0.056	0.029	0.034	0.098	0.098
FlO	**-0.429 (**)**	**130.970 (**)**	0.032	0.047	0.037	0.196	0.196
FlD	**-0.287 (**)**	**137.039 (**)**	0.051	0.071	-0.005	0.159	0.159
FlP	**-0.509 (***)**	**145.446 (**)**	0.072	0.014	0.009	0.199	0.199
FrO	**-0.288 (**)**	**135.101 (**)**	0.053	0.040	0.050	0.156	0.156
FrD	**0.376 (*)**	**137.376 (**)**	0.054	0.041	0.045	0.149	0.149
FrP	-0.111	**140.914 (**)**	0.040	0.039	0.039	0.108	0.108
DO	**0.443 (***)**	**155.642 (***)**	0.056	-0.023	0.058	0.199	0.199
DP	**0.794 (***)**	**151.083 (***)**	0.072	0.025	0.026	0.195	0.195
Sen	-0.033	**144.018 (**)**	0.055	0.034	0.035	0.097	0.097
C	**-0.182 (·)**	**142.052 (**)**	0.064	0.049	0.036	0.146	0.146
N	-0.029	**139.188 (**)**	0.053	0.027	0.033	0.094	0.094
d13C	**-0.185 (·)**	**140.402 (**)**	0.052	0.060	0.043	0.131	0.131
d15N	0.108	**134.913 (**)**	0.036	0.017	0.030	0.110	0.110
LDMC	**-0.338 (***)**	**119.027 (**)**	0.060	0.036	0.039	0.218	0.218

PC1: Total and perennial cover (positively related)
PC2: Bare soil (positively related) and BSC cover (negatively related)
PC3: Annual and litter cover (positively related)

Helianthemum squamatum, north slopes, 2017

	Fitness2	PlVol	PC1 (32.3%)	PC2 (18.5%)	PC3 (13.5%)	R^2_m	R^2_c
LA	0.085	**69.793 (·)**	**-0.187 (*)**	**0.158 (·)**	-0.018	0.127	0.259
LT	-0.018	**73.737 (·)**	**-0.187 (*)**	**0.179 (*)**	-0.021	0.116	0.245
SLA	**0.273 (*)**	**73.193 (*)**	**-0.226 (**)**	**0.172 (·)**	-0.005	0.171	0.335
FvFm	0.134	**75.055 (*)**	**-0.154 (·)**	**0.173 (*)**	-0.043	0.130	0.214
FlO	**-0.306 (**)**	**76.798 (*)**	**-0.194 (*)**	**0.183 (*)**	0.03	0.206	0.307
FlD	-0.011	**72.802 (*)**	**-0.186 (*)**	**0.181 (*)**	-0.025	0.115	0.235
FlP	**-0.200 (·)**	**68.873 (·)**	**-0.164 (·)**	**0.171 (·)**	-0.011	0.140	0.222
FrO	**-0.351 (**)**	**72.082 (*)**	**-0.195 (*)**	**0.177 (*)**	-0.021	0.212	0.313
FrD	0.148	**76.083 (*)**	**-0.199 (*)**	**0.186 (*)**	-0.020	0.134	0.243
FrP	-0.049	**71.416 (·)**	**-0.182 (*)**	**0.177 (*)**	-0.033	0.120	0.240
DO	**0.335 (**)**	**82.121 (*)**	**-0.203 (*)**	0.101	0.011	0.183	0.314
DP	**0.691 (·)**	**76.809 (*)**	**-0.188 (*)**	**0.187 (*)**	-0.035	0.157	0.269
Sen	-0.184	**69.797 (·)**	**-0.157 (·)**	**0.191 (*)**	-0.021	0.133	0.232
C	-0.143	58.547	**-0.168 (·)**	0.147	0.005	0.122	0.216
N	**-0.277 (*)**	**97.776 (**)**	**-0.233 (**)**	**0.162 (·)**	-0.022	0.181	0.271
d13C	**-0.288 (**)**	**103.826 (**)**	**-0.174 (*)**	0.116	-0.001	0.203	0.307
d15N	-0.238	**83.794 (*)**	**-0.195 (*)**	**0.214 (*)**	0.004	0.127	0.259
LDMC	**-0.316 (**)**	40.011	**-0.187 (*)**	0.117	-0.038	0.116	0.245

PC1: Total and perennial cover (positively related)
PC2: Soil moisture (positively related), BSC cover (negatively related)
PC3: Litter cover (positively related)

Helianthemum squamatum, south slopes, 2018

	Fitness2	PlVol	PC1 (24.3%)	PC2 (18.3%)	PC3 (13.3%)	R^2_m	R^2_c
LA	**0.427 (***)**	65.614	0.013	-0.091	**-0.176 (*)**	0.196	0.219
LT	0.023	21.436	0.029	-0.065	-0.043	0.016	0.028
SLA	0.111	16.477	0.029	-0.073	-0.054	0.029	0.037
FvFm1	-0.237	25.557	0.012	-0.093	-0.080	0.054	0.054
FvFm2	0.157	26.102	0.011	-0.085	-0.044	0.044	0.044
FlO	**-0.462 (***)**	-6.322	-0.031	-0.062	-0.084	0.187	0.319
FlD	**0.504 (***)**	-18.472	-0.009	-0.058	-0.058	0.203	0.219
FlP	**-0.417 (**)**	12.991	0.002	-0.096	-0.067	0.156	0.276
FrO	-0.138	18.548	-0.004	**-0.098 (·)**	-0.090	0.034	0.087
FrP	**0.256 (*)**	18.338	-0.005	-0.087	-0.036	0.073	0.073
DO	**0.275 (*)**	30.460	-0.013	-0.107	-0.035	0.073	0.073
DP	**0.235 (*)**	30.392	0.004	-0.089	0.004	0.071	0.073
Sen	**-0.378 (**)**	24.435	0.049	**-0.130 (·)**	0.031	0.118	0.118
C	**-0.354 (**)**	19.899	0.010	**-0.155 (·)**	**-0.164 (·)**	0.124	0.189
N	**0.229 (*)**	25.683	0.021	-0.119	-0.073	0.064	0.104
d13C	**-0.222 (·)**	40.324	0.066	-0.076	-0.063	0.073	0.073
d15N	0.096	12.931	0.020	-0.079	-0.067	0.026	0.056
LDMC	**-0.272 (**)**	18.549	-0.008	-0.099	-0.031	0.099	0.121
SPAD1	**0.267 (*)**	24.693	-0.004	-0.091	-0.099	0.077	0.134
SPAD2	**0.281 (*)**	16.176	0.025	0.005	-0.069	0.084	0.089

PC1: Total and perennial cover (positively related)
PC2: Bare soil (positively related) and BSC cover (negatively related)
PC3: Annual cover (positively related)

Helianthemum squamatum, north slopes, 2018

	Fitness2	PlVol	PC1 (25.7%)	PC2 (17.4%)	PC3 (12.8%)	R^2_m	R^2_c
LA	**0.466 (***)**	37.901	**-0.153 (*)**	0.121	0.026	0.288	0.381
LT	0.089	28.781	**-0.141 (*)**	**0.124 (·)**	-0.045	0.082	0.136
SLA	-0.03	34.248	**-0.138 (·)**	0.126	-0.038	0.076	0.128
FvFm1	0.211	53.114	**-0.156 (*)**	0.115	-0.071	0.104	0.174
FvFm2	0.057	33.072	-0.120	0.121	-0.031	0.066	0.111
FlO	**-0.309 (*)**	28.697	**-0.121 (·)**	0.040	-0.073	0.135	0.171
FlD	0.181	37.051	**-0.152 (*)**	0.087	-0.076	0.107	0.141
FlP	-0.157	21.464	-0.127	0.153	0.002	0.113	0.201
FrO	-0.165	31.137	-0.129	0.082	-0.035	0.093	0.125
FrP	**0.239 (·)**	58.876	**-0.147 (·)**	0.094	-0.084	0.128	0.151
DO	**0.394 (**)**	47.395	-0.099	0.150	-0.013	0.217	0.269
DP	0.179	41.316	-0.086	0.102	0.001	0.111	0.145
Sen	**-0.692 (***)**	66.772	**-0.135 (*)**	0.065	-0.043	0.424	0.424
C	**-0.228 (*)**	40.987	**-0.160 (*)**	0.165	-0.082	0.175	0.211
N	0.139	34.308	**-0.149 (·)**	0.152	-0.035	0.136	0.215
d13C	-0.175	64.773	**-0.131 (·)**	0.145	-0.030	0.094	0.136
d15N	-0.078	43.976	**-0.147 (·)**	0.111	-0.022	0.063	0.091
LDMC	-0.134	66.047	**-0.151 (·)**	0.152	-0.054	0.113	0.165
SPAD1	0.111	40.582	**-0.144 (·)**	0.135	-0.062	0.106	0.162
SPAD2	**0.256 (*)**	51.961	**-0.166 (*)**	0.099	-0.075	0.162	0.233

PC1: Total and perennial cover (positively related)
PC2: Soil moisture (positively related), BSC cover (negatively related)
PC3: Litter cover (positively related)

Centaurea hyssopifolia, south slopes, 2017

	Fitness2	PlVol	PC1 (32.8%)	PC2 (18.3%)	PC3 (13.8%)	R^2_m	R^2_c
LA	**0.172 (·)**	**43.565 (***)**	-0.004	0.013	-0.002	0.317	0.317
LT	-0.027	**45.377 (***)**	0.015	0.029	0.020	0.273	0.273
SLA	-0.020	**45.278 (***)**	0.019	0.037	0.023	0.271	0.271
FvFm	0.079	**44.081 (***)**	0.027	0.029	0.025	0.278	0.278
FlO	**-0.164 (·)**	**42.168 (***)**	0.023	0.034	0.019	0.303	0.303
FlD	**0.180 (·)**	**45.327 (***)**	0.023	0.029	0.009	0.328	0.328
FlP	-0.107	**43.267 (***)**	0.017	0.030	0.018	0.285	0.285
FrO	**-0.187 (·)**	**44.365 (***)**	0.017	0.026	0.037	0.310	0.310
FrD	**0.180 (·)**	**47.049 (***)**	-0.013	0.005	0.039	0.300	0.300
FrP	-0.012	**44.988 (***)**	0.017	0.034	0.019	0.273	0.273
DO	0.043	**44.510 (***)**	0.025	0.041	0.020	0.274	0.274
DP	0.132	**42.103 (***)**	0.024	0.022	0.010	0.283	0.283
Sen	-0.031	**45.532 (***)**	0.021	0.031	0.016	0.271	0.271
C	-0.025	**44.844 (***)**	0.020	0.036	0.016	0.275	0.275
N	-0.024	**45.638 (***)**	0.020	0.036	0.020	0.276	0.276
d13C	0.005	**45.055 (***)**	0.017	0.034	0.020	0.272	0.272
d15N	0.074	**46.164 (***)**	0.010	0.013	0.033	0.291	0.291
LDMC	**-0.144 (·)**	**43.182 (***)**	0.006	0.028	0.002	0.318	0.318

PC1: Total and perennial cover (positively related)
PC2: Distance to the closest conspecific individual (positively related)
PC3: Litter cover and soil moisture (positively related)

Centaurea hyssopifolia, north slopes, 2017

	Fitness2	PlVol	PC1 (29.4%)	PC2 (21.6%)	PC3 (15.4%)	R^2_m	R^2_c
LA	0.026	**42.585 (***)**	0.002	0.017	**-0.166 (**)**	0.214	0.233
LT	0.010	**43.215 (***)**	0.004	0.011	**-0.166 (**)**	0.217	0.234
SLA	0.134	**41.336 (***)**	-0.030	0.015	**-0.186 (**)**	0.224	0.252
FvFm	-0.106	**40.493 (***)**	0.007	0.020	**-0.139 (*)**	0.203	0.231
FlO	-0.137	**41.177 (***)**	0.013	0.025	**-0.151 (*)**	0.225	0.257
FlD	**0.302 (***)**	**40.282 (***)**	0.063	0.009	**-0.159 (**)**	0.330	0.330
FlP	-0.072	**43.674 (***)**	-0.003	0.021	**-0.169 (**)**	0.212	0.239
FrO	**-0.286 (**)**	**36.988 (***)**	0.011	-0.008	**-0.136 (*)**	0.332	0.334
FrD	**0.189 (*)**	**41.001 (***)**	0.007	-0.002	**-0.168 (**)**	0.252	0.270
FrP	**-0.238 (**)**	**38.523 (***)**	0.018	0.010	**-0.188 (**)**	0.320	0.329
DO	**-0.237 (**)**	**36.735 (***)**	0.004	-0.002	**-0.142 (*)**	0.281	0.315
DP	**0.309 (***)**	**44.209 (***)**	0.037	-0.004	**-0.181 (**)**	0.333	0.333
Sen	-0.015	**42.893 (***)**	0.008	0.009	**-0.166 (**)**	0.216	0.234
C	0.041	**42.673 (***)**	0.002	0.003	**-0.173 (**)**	0.216	0.239
N	0.102	**39.914 (***)**	-0.003	0.010	**-0.181 (**)**	0.228	0.257
d13C	-0.14	**44.849 (***)**	0.013	0.021	**-0.160 (**)**	0.244	0.257
d15N	-0.08	**43.561 (***)**	0.003	0.014	**-0.158 (*)**	0.232	0.244
LDMC	-0.046	**42.233 (***)**	-0.004	0.018	**-0.171 (**)**	0.216	0.236

PC1: Total and perennial cover (positively related)
PC2: Distance to the closest conspecific individual (positively related)
PC3: BSC cover (positively related)

Centaurea hyssopifolia, south slopes, 2018

	Fitness2	PlVol	PC1 (29.4%)	PC2 (19.2%)	PC3 (12.9%)	R^2_m	R^2_c
LA	-0.104	**25.578 (*)**	-0.017	-0.082	0.136	0.093	0.093
LT	0.083	**24.308 (*)**	-0.009	-0.080	**0.183 (·)**	0.090	0.090
SLA	-0.056	**25.483 (*)**	-0.015	-0.079	**0.163 (·)**	0.084	0.084
FvFm1	-0.178	**20.295 (·)**	-0.051	-0.080	**0.196 (*)**	0.111	0.111
FvFm2	-0.119	**25.585 (*)**	0.008	-0.074	**0.170 (·)**	0.112	0.112
FlO	0.114	**26.007 (*)**	-0.032	-0.072	**0.164 (·)**	0.092	0.092
FlD	0.013	**24.884 (*)**	-0.016	-0.078	**0.152 (·)**	0.084	0.084
FlP	0.214	**22.629 (*)**	-0.033	-0.048	**0.183 (*)**	0.114	0.114
FrO	0.033	**25.090 (*)**	-0.020	-0.073	**0.158 (·)**	0.083	0.083
FrP	**0.311 (·)**	**34.410 (*)**	-0.061	-0.067	**0.175 (*)**	0.183	0.183
DO	0.101	**25.700 (*)**	-0.022	-0.069	**0.151 (·)**	0.086	0.086
DP	0.168	**25.009 (*)**	-0.013	-0.089	**0.162 (·)**	0.118	0.118
Sen	-0.224	**24.912 (*)**	-0.035	-0.083	**0.158 (·)**	0.126	0.126
C	-0.160	**27.406 (*)**	-0.034	-0.078	0.113	0.085	0.085
N	0.046	**24.071 (*)**	-0.015	-0.078	**0.165 (·)**	0.096	0.096
d13C	0.079	**23.817 (*)**	-0.020	-0.070	**0.166 (·)**	0.090	0.090
d15N	-0.007	**24.848 (*)**	-0.015	-0.078	**0.152 (·)**	0.083	0.083
LDMC	-0.260	**29.201 (*)**	-0.025	-0.087	**0.177 (*)**	0.167	0.167
SPAD1	0.004	**24.980 (*)**	-0.016	-0.076	**0.153 (·)**	0.084	0.084
SPAD2	-0.155	**23.270 (*)**	-0.010	-0.090	0.110	0.097	0.097

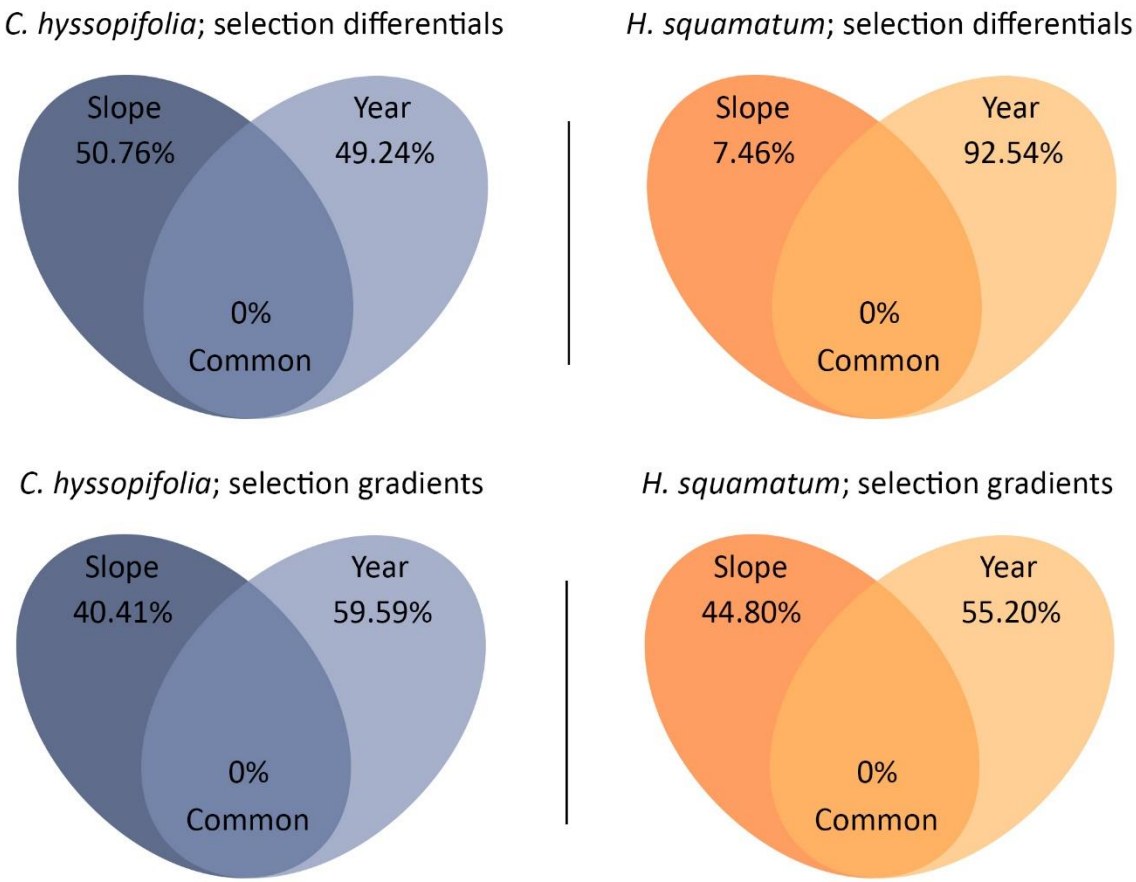
PC1: Total and perennial cover (positively related)
PC2: Distance to the closest conspecific individual (positively related)
PC3: Litter cover and soil moisture (positively related)

Centaurea hyssopifolia, north slopes, 2018

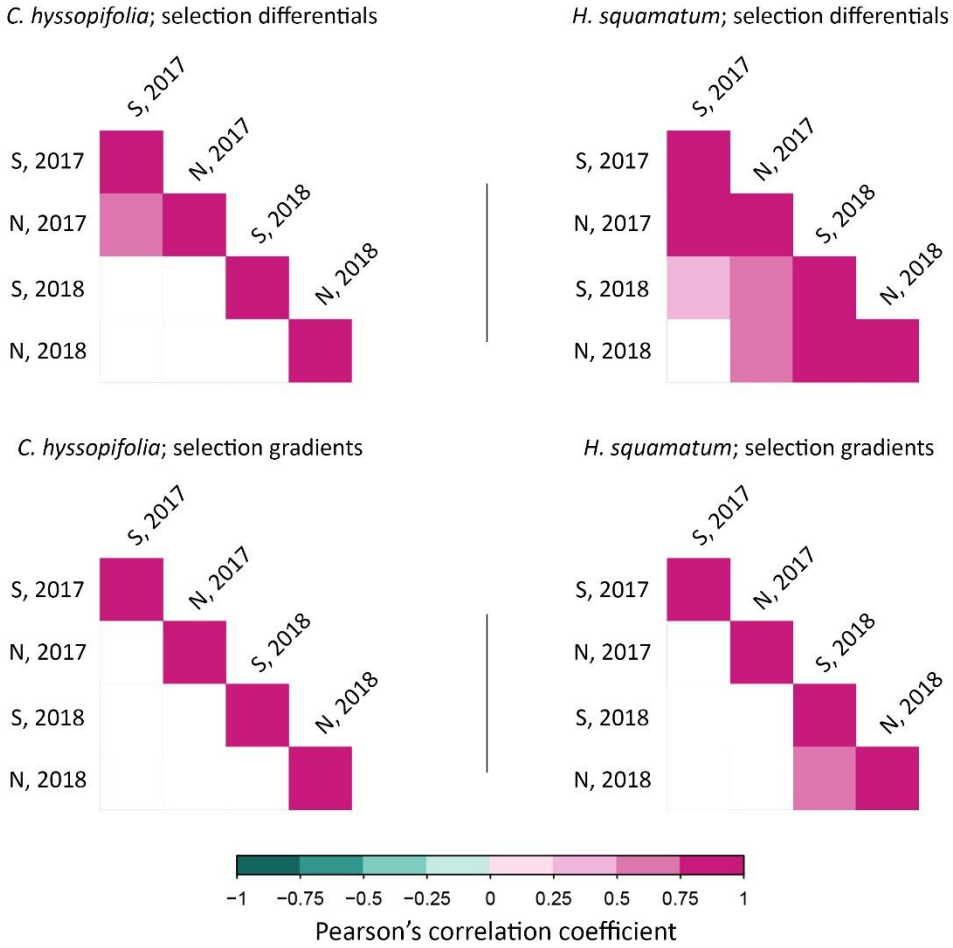
	Fitness2	PlVol	PC1 (29.5%)	PC2 (20.2%)	PC3 (14.8%)	R^2_m	R^2_c
LA	-0.018	**18.744 (*)**	-0.052	0.023	0.007	0.047	0.206
LT	0.106	**20.435 (*)**	-0.040	0.032	0.025	0.070	0.222
SLA	-0.037	**19.451 (*)**	-0.050	0.026	0.008	0.050	0.215
FvFm1	-0.011	**18.958 (*)**	-0.049	0.025	0.011	0.048	0.208
FvFm2	0.031	**20.100 (*)**	-0.051	0.024	0.012	0.051	0.207
FlO	-0.091	**19.402 (*)**	-0.038	0.023	0.012	0.057	0.206
FlD	0.238	**18.265 (*)**	-0.018	0.009	0.028	0.106	0.194
FlP	-0.013	**19.198 (*)**	-0.050	0.025	0.009	0.047	0.206
FrO	-0.035	**19.172 (*)**	-0.049	0.025	0.010	0.049	0.208
FrP	0.073	**20.169 (*)**	-0.053	0.022	0.022	0.057	0.210
DO	-0.198	**18.771 (*)**	-0.037	0.027	0.009	0.076	0.217
DP	-0.063	**19.626 (*)**	-0.063	0.030	0.016	0.056	0.224
Sen	-0.192	**17.554 (·)**	-0.055	0.018	-0.011	0.087	0.209
C	-0.040	**19.887 (*)**	-0.054	0.029	0.016	0.048	0.208
N	**0.303 (*)**	11.207	-0.027	-0.006	0.030	0.155	0.317
d13C	0.019	**18.995 (*)**	-0.052	0.025	0.013	0.048	0.208
d15N	0.046	**18.699 (*)**	-0.055	0.027	0.015	0.055	0.228
LDMC	-0.085	**18.912 (*)**	-0.050	0.026	0.020	0.057	0.200
SPAD1	0.052	**18.812 (*)**	-0.049	0.021	0.015	0.051	0.214
SPAD2	-0.028	**21.129 (*)**	-0.058	0.018	-0.018	0.052	0.215

PC1: Total and perennial cover (positively related)
PC2: Distance to the closest conspecific individual (positively related)
PC3: Annual cover, litter cover and soil moisture (positively related)

Supp. 10: Venn diagrams representing the results obtained from the commonality analysis using selection differentials for both study species. See Supp. 1 for details.



Supp. 11: Pairwise correlation matrices of selection differentials and gradients from both species, slopes, and years. Only significant ($P < 0.05$) Pearson's pairwise correlations are coloured. S and N indicate south slopes and north slopes, respectively.



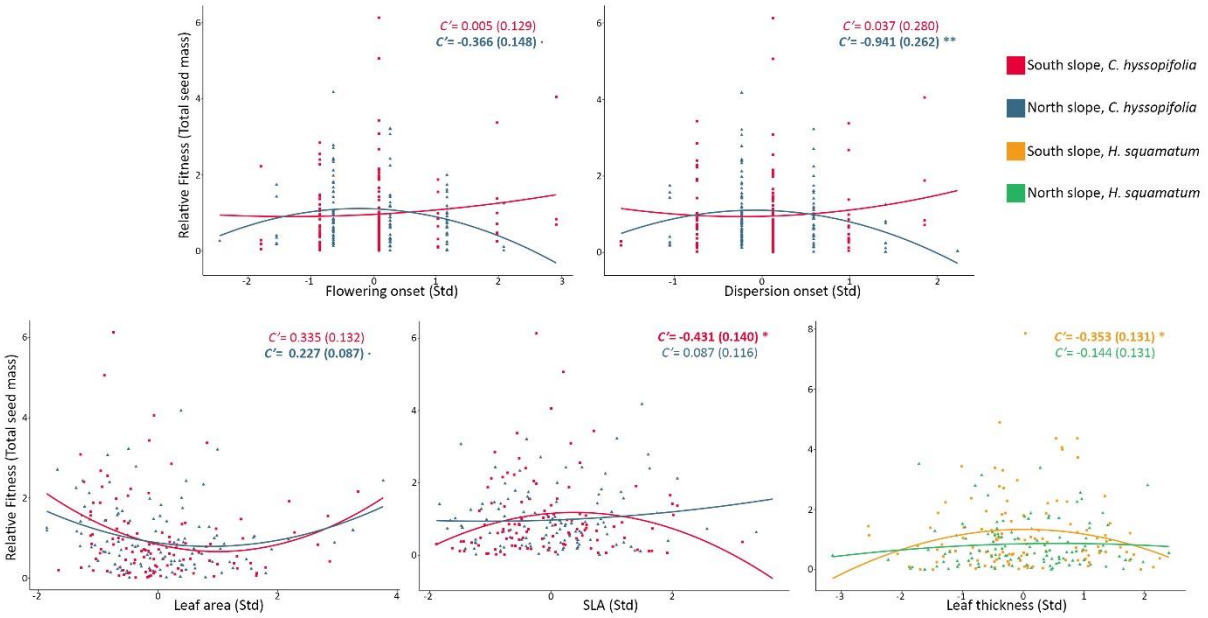
Supp. 12: Directional selection differentials (S') and gradients (β'), and their standard error (in brackets) for both species, slopes and years, using total seed mass as fitness trait. Significant ($P < 0.05$) and marginally significant ($0.05 < P < 0.1$) values after FDR correction are shown in bold and italic, respectively. Significance levels: · $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (E.S.) and (P.F.) in Fv/Fm and chlorophyll content in 2018 indicates early-season and peak of flowering, respectively.

		***C. hyssopifolia*, S**		***C. hyssopifolia*, N**		***H. squamatum*, S**		***H. squamatum*, N**	
Year	Functional trait	S' (SE)	β' (SE)	S' (SE)	β' (SE)	S' (SE)	β' (SE)	S' (SE)	β' (SE)
2017									
	Leaf area	*0.172 (0.071)* ·	0.060 (0.085)	0.026 (0.077)	0.046 (0.076)	-0.060 (0.113)	*-0.286 (0.115)* ·	0.085 (0.103)	0.089 (0.115)
	Leaf thickness	-0.027 (0.073)	0.025 (0.077)	0.010 (0.157)	0.089 (0.082)	-0.053 (0.105)	0.110 (0.147)	-0.018 (0.104)	0.275 (0.159)
	SLA	-0.020 (0.079)	-0.171 (0.096)	0.134 (0.083)	0.097 (0.107)	**0.440 (0.131)** **	0.079 (0.193)	**0.273 (0.096)** *	0.416 (0.195)
	LDMC	*-0.144 (0.067)* ·	-0.207 (0.087)	-0.046 (0.076)	0.182 (0.107)	**-0.381 (0.117)** **	-0.263 (0.164)	**-0.316 (0.097)** **	-0.134 (0.183)
	Flowering onset	*-0.164 (0.071)* ·	-0.085 (0.110)	-0.137 (0.071)	0.051 (0.078)	**-0.429 (0.126)** **	-0.256 (0.135)	**-0.306 (0.102)** **	*-0.288 (0.108)* ·
	Flowering duration	*0.180 (0.068)* ·	0.061 (0.091)	**0.302 (0.076)** ***	*0.267 (0.103)* ·	**-0.287 (0.107)** *	**-0.387 (0.120)** *	-0.011 (0.101)	-0.090 (0.119)
	Flowering peak	-0.107 (0.078)	-0.044 (0.083)	-0.072 (0.078)	-0.117 (0.083)	**-0.509 (0.116)** ***	0.273 (0.233)	-0.200 (0.103)	-0.013 (0.099)
	Fruiting onset	*-0.187 (0.066)* ·	-0.214 (0.109)	**-0.286 (0.077)** **	-0.124 (0.112)	**-0.288 (0.096)** **	-0.244 (0.118)	**-0.351 (0.095)** **	-0.182 (0.114)
	Fruiting duration	*0.180 (0.078)* ·	0.084 (0.099)	**0.189 (0.077)** *	-0.036 (0.095)	**0.376 (0.155)** *	0.090 (0.159)	0.148 (0.110)	-0.007 (0.128)
	Fruiting peak	-0.012 (0.078)	0.026 (0.090)	**-0.238 (0.071)** **	-0.161 (0.085)	-0.111 (0.122)	-0.072 (0.153)	-0.049 (0.094)	-0.012 (0.094)
	Dispersion onset	0.043 (0.075)	0.195 (0.087)	**-0.237 (0.069)** **	-0.039 (0.096)	**0.443 (0.118)** ***	*0.311 (0.114)* ·	**0.335 (0.104)** **	*0.244 (0.096)* ·
	Dispersion peak	0.132 (0.075)	0.088 (0.095)	**0.309 (0.069)** ***	0.163 (0.082)	**0.794 (0.178)** ***	0.733 (0.339)	*0.691 (0.311)* ·	0.046 (0.310)
	Senescence	-0.031 (0.071)	-0.005 (0.073)	-0.015 (0.084)	0.046 (0.074)	-0.033 (0.123)	-0.105 (0.103)	-0.184 (0.105)	-0.244 (0.122)
	Leaf Carbon content	-0.025 (0.071)	0.152 (0.082)	0.041 (0.082)	-0.052 (0.091)	*-0.182 (0.095)* ·	0.040 (0.129)	-0.143 (0.121)	0.211 (0.144)
	Leaf Nitrogen content	-0.024 (0.072)	0.006 (0.075)	0.102 (0.084)	0.116 (0.084)	-0.029 (0.112)	-0.061 (0.095)	**-0.277 (0.102)** *	**-0.379 (0.104)** **
	d13C	0.005 (0.081)	-0.048 (0.088)	-0.140 (0.072)	**-0.223 (0.072)** *	*-0.185 (0.098)* ·	-0.211 (0.128)	**-0.288 (0.087)** **	-0.021 (0.105)
	d15N	0.074 (0.074)	0.139 (0.079)	-0.080 (0.084)	0.026 (0.077)	0.108 (0.116)	-0.031 (0.121)	-0.238 (0.139)	-0.090 (0.133)
	Fv/Fm	0.079 (0.070)	0.098 (0.072)	-0.106 (0.081)	-0.103 (0.071)	0.048 (0.101)	-0.099 (0.111)	0.134 (0.098)	0.067 (0.120)
2018									
	Leaf area	-0.104 (0.080)	-0.182 (0.108)	-0.018 (0.065)	-0.123 (0.078)	**0.427 (0.104)** ***	*0.391 (0.142)* ·	**0.466 (0.112)** ***	0.241 (0.107)
	Leaf thickness	0.083 (0.100)	-0.078 (0.113)	0.106 (0.082)	0.166 (0.109)	0.023 (0.112)	-0.240 (0.163)	0.089 (0.104)	-0.191 (0.128)
	SLA	-0.056 (0.127)	-0.203 (0.183)	-0.037 (0.075)	-0.066 (0.158)	0.111 (0.116)	0.148 (0.189)	-0.030 (0.108)	-0.225 (0.140)
	LDMC	-0.260 (0.103)	**-0.439 (0.145)** *	-0.085 (0.077)	-0.153 (0.148)	**-0.272 (0.099)** **	0.120 (0.175)	-0.134 (0.089)	-0.228 (0.095)
	Flowering onset	0.114 (0.099)	0.286 (0.257)	-0.091 (0.101)	0.272 (0.240)	**-0.462 (0.115)** ***	*-0.523 (0.186)* ·	**-0.309 (0.114)** *	-0.324 (0.174)
	Flowering duration	0.013 (0.124)	0.164 (0.178)	0.238 (0.129)	**0.437 (0.143)** *	**0.504 (0.132)** ***	-0.273 (0.228)	0.181 (0.102)	-0.276 (0.184)
	Flowering peak	0.214 (0.105)	0.078 (0.120)	-0.013 (0.087)	-0.062 (0.097)	**-0.417 (0.131)** **	-0.209 (0.138)	-0.157 (0.103)	-0.157 (0.097)
	Fruiting onset	0.033 (0.097)	-0.235 (0.222)	-0.035 (0.093)	0.212 (0.174)	-0.138 (0.131)	-0.007 (0.117)	-0.165 (0.119)	-0.187 (0.106)
	Fruiting peak	*0.311 (0.108)* ·	0.193 (0.134)	0.073 (0.092)	0.065 (0.107)	**0.256 (0.114)** *	0.434 (0.254)	*0.239 (0.108)* ·	0.247 (0.178)
	Dispersion onset	0.101 (0.142)	-0.091 (0.205)	-0.198 (0.149)	*-0.581 (0.222)* ·	**0.275 (0.119)** *	-0.046 (0.178)	**0.394 (0.116)** **	-0.038 (0.170)
	Dispersion peak	0.168 (0.084)	0.131 (0.096)	-0.063 (0.076)	0.070 (0.080)	**0.235 (0.106)** *	0.129 (0.115)	0.179 (0.106)	-0.005 (0.100)
	Senescence	-0.224 (0.096)	-0.010 (0.109)	-0.192 (0.102)	-0.100 (0.115)	**-0.378 (0.117)** **	-0.267 (0.124)	**-0.692 (0.092)** ***	**-0.773 (0.106)** ***
	Leaf Carbon content	-0.043 (0.091)	-0.106 (0.101)	0.026 (0.089)	-0.051 (0.095)	**-0.366 (0.113)** **	-0.224 (0.137)	**-0.262 (0.103)** *	0.079 (0.091)
	Leaf Nitrogen content	0.077 (0.085)	-0.010 (0.101)	**0.303 (0.092)** *	**0.342 (0.116)** *	**0.255 (0.114)** *	0.054 (0.110)	0.139 (0.096)	0.083 (0.113)
	Leaf d13C	0.079 (0.090)	0.062 (0.104)	0.019 (0.101)	-0.112 (0.117)	*-0.222 (0.106)* ·	0.155 (0.145)	-0.175 (0.122)	0.094 (0.114)
	Leaf d15N	-0.007 (0.095)	-0.172 (0.115)	0.046 (0.083)	-0.070 (0.089)	0.096 (0.111)	-0.010 (0.100)	-0.078 (0.122)	-0.257 (0.112)
	Fv/Fm (E.S.)	-0.178 (0.112)	-0.226 (0.126)	-0.011 (0.087)	-0.035 (0.089)	-0.237 (0.151)	-0.338 (0.152)	0.211 (0.130)	0.156 (0.092)
	Fv/Fm (P.F.)	-0.119 (0.100)	-0.037 (0.116)	0.031 (0.090)	-0.046 (0.095)	0.157 (0.103)	0.235 (0.100)	0.057 (0.099)	0.137 (0.075)
	Chlorophyll content	0.004 (0.094)	0.152 (0.116)	0.052 (0.080)	-0.003 (0.090)	**0.267 (0.107)** *	0.180 (0.095)	0.111 (0.092)	0.150 (0.076)
	Chlorophyll content	-0.155 (0.098)	-0.140 (0.126)	-0.028 (0.081)	-0.118 (0.084)	**0.281 (0.116)** *	0.076 (0.108)	**0.256 (0.098)** *	0.109 (0.093)

Supp. 13: Quadratic selection differentials (C') and gradients (γ'), and their standard error (in brackets) of both species, slopes and years for total seed mass fitness variable. Significant ($p < 0.05$) and marginally significant ($0.05 < P < 0.1$) values after FDR correction are in bold and italic, respectively. Significance levels: · $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (E.S.) and (P.F.) in Fv/Fm and chlorophyll content in 2018 indicates early-season and peak of flowering, respectively.

		***C. hyssopifolia*, south slope**		***C. hyssopifolia*, north slope**		***H. squamatum*, south slope**		***H. squamatum* , north slope**	
Year	Functional trait	C' (SE)	γ' (SE)	C' (SE)	γ' (SE)	C' (SE)	γ' (SE)	C' (SE)	γ' (SE)
2017									
	Leaf area	0.121 (0.128)	-0.099 (0.142)	0.043 (0.099)	-0.012 (0.091)	-0.275 (0.198)	-0.029 (0.207)	-0.342 (0.147)	-0.228 (0.140)
	Leaf thickness	0.100 (0.129)	-0.124 (0.116)	0.221 (0.135)	-0.060 (0.149)	-0.057 (0.135)	0.027 (0.165)	0.159 (0.148)	0.265 (0.172)
	SLA	-0.073 (0.070)	0.018 (0.085)	-0.221 (0.125)	-0.234 (0.129)	*-0.244 (0.085)*·	-0.242 (0.134)	-0.066 (0.094)	-0.106 (0.166)
	LDMC	0.171 (0.105)	-0.013 (0.132)	0.059 (0.116)	0.022 (0.113)	0.053 (0.072)	0.001 (0.088)	-0.050 (0.120)	-0.007 (0.193)
	Flowering onset	0.018 (0.196)	0.056 (0.200)	0.131 (0.104)	0.077 (0.108)	------	------	------	------
	Flowering duration	0.087 (0.128)	0.006 (0.126)	-0.257 (0.108)	-0.195 (0.140)	-0.036 (0.233)	-0.037 (0.249)	-0.097 (0.139)	0.164 (0.148)
	Flowering peak	-0.178 (0.103)	-0.271 (0.106)	-0.044 (0.143)	0.121 (0.149)	0.159 (0.147)	0.969 (0.563)	-0.375 (0.410)	-0.226 (0.324)
	Fruiting onset	0.218 (0.133)	0.287 (0.155)	0.001 (0.099)	-0.054 (0.126)	------	------	------	------
	Fruiting duration	0.022 (0.117)	0.100 (0.143)	-0.138 (0.122)	0.153 (0.136)	-0.782 (0.388)	-0.597 (0.505)	0.016 (0.255)	-0.379 (0.231)
	Fruiting peak	-0.158 (0.107)	-0.048 (0.119)	-0.026 (0.110)	-0.141 (0.153)	*-0.948 (0.368)*·	-0.656 (0.383)	-0.486 (0.254)	-0.333 (0.234)
	Dispersion onset	-----	-----	0.079 (0.081)	0.089 (0.096)	*-0.365 (0.155)*·	0.017 (0.175)	-0.164 (0.120)	-0.184 (0.114)
	Dispersion peak	0.202 (0.170)	-0.012 (0.200)	-0.121 (0.134)	-0.135 (0.151)	0.263 (0.279)	-1.016 (1.092)	0.029 (1.538)	-1.365 (1.298)
	Senescence	0.058 (0.083)	0.138 (0.096)	-0.060 (0.069)	0.008 (0.081)	-0.101 (0.081)	-0.030 (0.080)	-0.165 (0.104)	-0.210 (0.134)
	Leaf Carbon content	0.043 (0.083)	0.108 (0.093)	0.189 (0.138)	0.052 (0.139)	0.073 (0.106)	-0.068 (0.112)	0.101 (0.190)	0.064 (0.173)
	Leaf Nitrogen content	0.015 (0.106)	0.105 (0.112)	-0.134 (0.109)	-0.116 (0.115)	-0.159 (0.164)	0.022 (0.163)	0.070 (0.123)	0.052 (0.130)
	Leaf d13C content	-0.206 (0.117)	-0.211 (0.123)	0.019 (0.100)	-0.029 (0.099)	0.114 (0.172)	0.604 (0.232)	0.131 (0.134)	0.225 (0.145)
	Leaf d15N content	0.080 (0.085)	-0.103 (0.083)	-0.043 (0.091)	-0.124 (0.086)	-0.236 (0.182)	-0.214 (0.198)	-0.127 (0.116)	-0.163 (0.108)
	Fv/Fm	0.094 (0.115)	0.067 (0.116)	-0.103 (0.088)	-0.078 (0.084)	-0.073 (0.215)	-0.246 (0.215)	-0.007 (0.102)	0.092 (0.155)
2018									
	Leaf area	0.335 (0.132)	0.295 (0.189)	*0.227 (0.087)*·	*0.269 (0.095)*·	-0.098 (0.124)	0.058 (0.157)	**-0.296 (0.099)***	-0.060 (0.106)
	Leaf thickness	-0.011 (0.145)	-0.024 (0.168)	-0.095 (0.117)	-0.176 (0.112)	**-0.353 (0.131)***	*-0.370 (0.140)*·	-0.144 (0.131)	-0.156 (0.185)
	SLA	**-0.431 (0.140)***	-0.334 (0.173)	0.087 (0.116)	0.128 (0.109)	-0.203 (0.087)	0.109 (0.109)	**-0.216 (0.053)****	0.122 (0.148)
	LDMC	-0.230 (0.118)	-0.129 (0.149)	0.026 (0.071)	-0.049 (0.081)	-0.014 (0.033)	-0.274 (0.167)	0.080 (0.056)	0.078 (0.084)
	Flowering onset	0.005 (0.129)	0.030 (0.252)	*-0.366 (0.148)*·	-0.422 (0.200)	-0.275 (0.149)	-0.442 (0.208)	------	------
	Flowering duration	-0.083 (0.235)	-0.063 (0.279)	*-0.528 (0.223)*·	-0.315 (0.185)	**-0.484 (0.109)****	-0.122 (0.137)	**-0.115 (0.041)***	-0.026 (0.136)
	Flowering peak	-0.120 (0.116)	-0.136 (0.129)	-0.011 (0.152)	-0.156 (0.156)	-0.173 (0.179)	-0.015 (0.189)	-0.001 (0.189)	-0.290 (0.170)
	Fruiting onset	0.289 (0.174)	0.141 (0.260)	-0.432 (0.249)	-0.258 (0.279)	-0.160 (0.307)	0.622 (0.313)	**-0.392 (0.060)****	-0.368 (0.269)
	Fruiting peak	-0.110 (0.141)	-0.194 (0.165)	0.052 (0.151)	0.258 (0.157)	-0.044 (0.156)	-0.074 (0.146)	-0.209 (0.105)	-0.116 (0.149)
	Dispersion onset	0.037 (0.280)	0.241 (0.332)	**-0.941 (0.262)****	-0.203 (0.323)	0.044 (0.128)	**1.327 (0.349)****	-0.170 (0.080)	0.239 (0.297)
	Dispersion peak	0.156 (0.230)	-0.165 (0.241)	0.061 (0.133)	0.035 (0.135)	-0.007 (0.071)	**-0.775 (0.214)****	-0.291 (0.169)	-0.157 (0.166)
	Senescence	0.058 (0.136)	0.253 (0.123)	-0.049 (0.174)	0.066 (0.167)	-0.270 (0.243)	0.058 (0.237)	-0.155 (0.193)	0.108 (0.162)
	Leaf Carbon content	0.233 (0.159)	0.172 (0.153)	0.026 (0.071)	0.022 (0.081)	0.054 (0.156)	0.058 (0.154)	-0.128 (0.146)	0.068 (0.14)
	Leaf Nitrogen content	0.206 (0.149)	0.199 (0.156)	-0.180 (0.115)	0.162 (0.171)	-0.363 (0.193)	0.045 (0.167)	0.070 (0.120)	-0.257 (0.151)
	Leaf d13C content	0.066 (0.126)	0.061 (0.151)	-0.141 (0.070)	-0.195 (0.089)	-0.137 (0.120)	-0.013 (0.112)	*-0.297 (0.132)*·	-0.111 (0.117)
	Leaf d15N content	0.157 (0.127)	-0.005 (0.143)	0.001 (0.115)	0.007 (0.138)	0.068 (0.146)	0.123 (0.119)	0.296 (0.146)	0.022 (0.152)
	Fv/Fm (E.S.)	-0.070 (0.146)	-0.183 (0.177)	-0.140 (0.096)	-0.078 (0.094)	**-0.584 (0.189)***	-0.280 (0.173)	-0.100 (0.190)	-0.131 (0.154)
	Fv/Fm (P.F.)	0.024 (0.164)	-0.143 (0.175)	*-0.231 (0.102)*·	-0.220 (0.103)	0.239 (0.249)	0.027 (0.215)	-0.020 (0.247)	-0.077 (0.202)
	Chlorophyll content (E.S.)	-0.057 (0.122)	-0.020 (0.151)	-0.137 (0.136)	-0.230 (0.133)	-0.116 (0.128)	0.031 (0.126)	0.036 (0.146)	0.112 (0.128)
	Chlorophyll content (P.F.)	-0.068 (0.136)	0.079 (0.204)	-0.079 (0.108)	-0.187 (0.117)	-0.149 (0.158)	-0.061 (0.131)	-0.065 (0.126)	-0.073 (0.129)

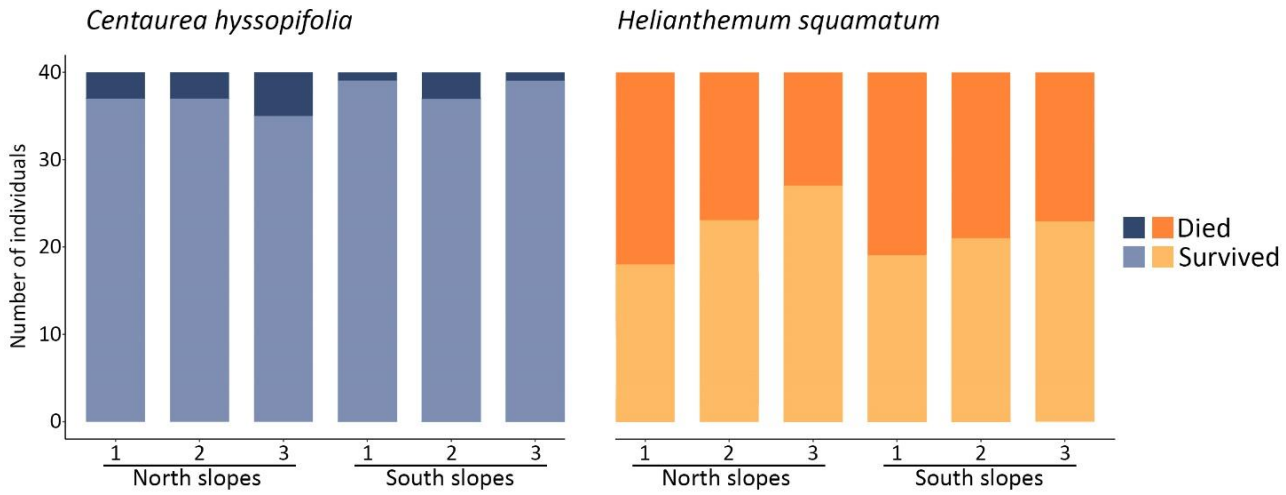
Supp. 14: Graphic representation of quadratic selection differentials (*C'*) for the traits under quadratic selection that showed a slight maximum/minimum in the fitness function in both species. Significant quadratic selection was observed only in 2018.



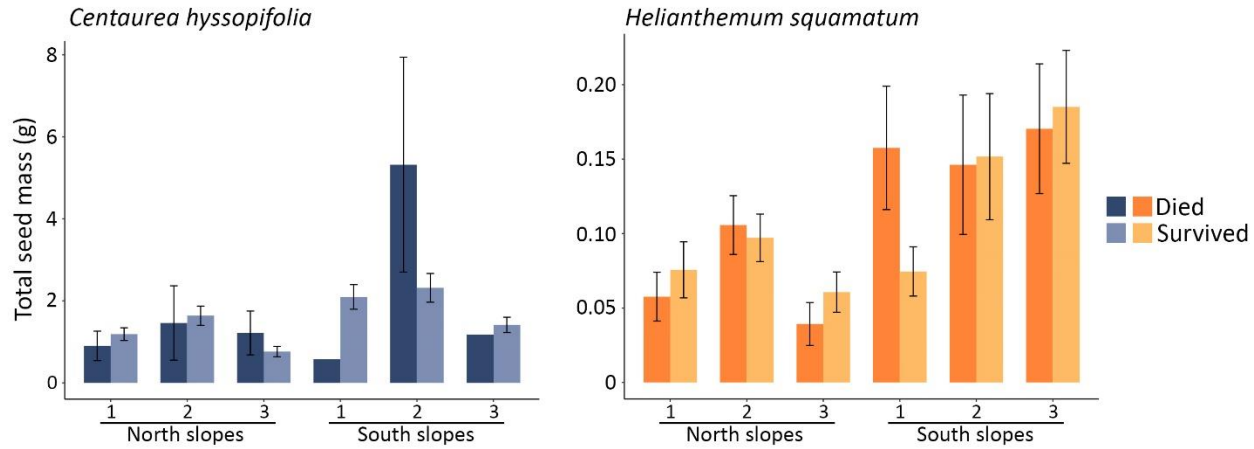
Although several functional traits showed significant *C'* and *γ'* coefficients (Supp. 13), most of them did not show a maximum or minimum on fitness associated with intermediate phenotypic values. For *H. squamatum*, leaf thickness in the south slopes showed a slight intermediate maximum (stabilizing selection) in the fitness function (Supp. 13). None of the significant correlational selection differentials showed a maximum or minimum in the fitness function. For *C. hyssopifolia*, only SLA in the south slopes, and flowering onset and dispersion onset in the north slopes were under stabilizing selection, i.e., individuals expressing intermediate trait values showed higher fitness, while leaf area in the north slopes was under disruptive selection, i.e., intermediate phenotypic values showed lower fitness (Supp. 13).

Supp. 15: a) Stacked plots representing the number of surviving and dead individuals between the first and the second study year in each slope and plot; b) Bar plots representing the reproductive output (total seed mass) of surviving and dead individuals between the first and the second study year in each slope and plot; c) Bar plots representing the size (plant volume; a proxy for age) of surviving and dead individuals between the first and the second study year in each slope and plot; d) Main results extracted from the models used to test differences in the reproductive output and size of surviving and dead individuals. Error bars represent standard errors.

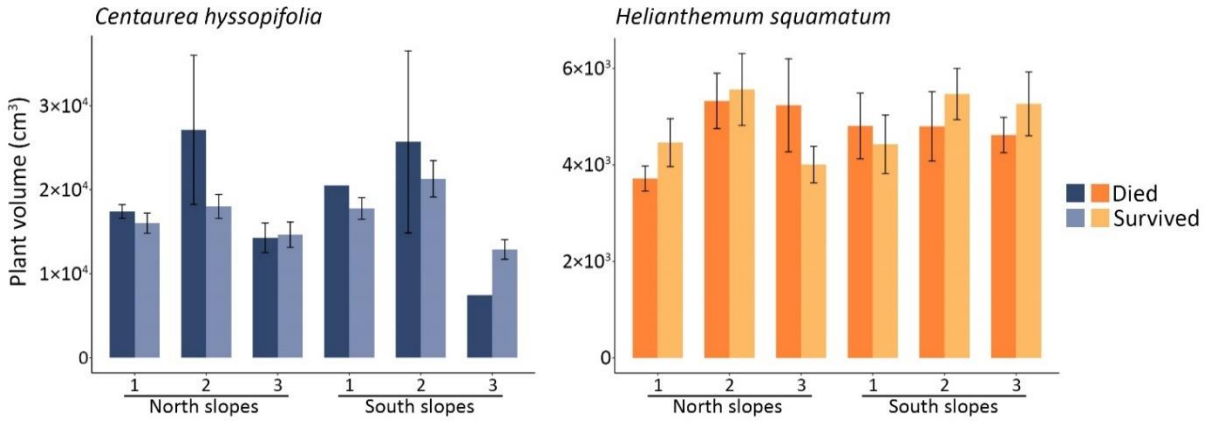
a)



b)



c)



d) To test if survival differed between plots and slopes, we have performed binomial GLMs for each species, including the survival between 2017 and 2018 as the dependent variable, and plot, slope, and their interaction as the independent variables. Likewise, we visually inspected these results using stacked plots that represent the number of surviving and dead individuals in each plot and slope (Supp. 15 a). These results showed that the survival of individuals was similar across slopes and plots in both species ($P > 0.05$ in all cases). Then, to test for differences in the reproductive output of plants that survived and died in each slope, we have performed GLMMs for each species, including total seed mass as the dependent variable; survival, slope, and their interaction as fixed factors; and plot as a random factor. Furthermore, we have represented these differences graphically using bar plots that contained the reproductive output of the individuals that survived and died in each plot and slope (Supp. 15 b). From these models, no significant ($P < 0.05$) values of Survival and/or Survival × Slope were obtained, indicating that the reproductive output of surviving and dead individuals was similar. Finally, we also tested whether the size (plant volume) of surviving and dead individuals differed (size of individuals can be used as a proxy for age; e.g., Connell et al., 2021; Lefkovitch, 1965; Throop & Archer, 2008). Additionally, the size of the individuals that survived and died in each plot and slope was represented using bar plots (Supp. 15 c). These models and plots revealed that the size of surviving and dead individuals was not statistically different, suggesting that the individuals that died were not older than those that survived.

Overall, in both species, dead and surviving individuals did not differ in their functional traits (see Supp. 6), microhabitat conditions (see Supp. 8), reproductive output, or size (a proxy for age), indicating that individuals progressively died due to unknown causes and/or differences in unmeasured traits. Therefore, we did not detect the frequently discussed trade-off between reproduction and survival at the intraspecific level, i.e., individuals with an acquisitive strategy and higher reproductive output did not present lower survival and *vice versa*.

Literature cited:

Connell, R. K., O'Connor, R. C., Nippert, J. B., & Blair, J. M. (2021). Spatial variation in soil microbial processes as a result of woody encroachment depends on shrub size in tallgrass prairie. *Plant and Soil*, *460*(1–2), 359–373. doi: 10.1007/s11104-020-04813-9

Lefkovitch, L. P. (1965). The study of population growth in organisms grouped by stages. *Biometrics*, *21*(1), 1–18.

Throop, H. L., & Archer, S. R. (2008). Shrub (Prosopis velutina) encroachment in a semidesert grassland: Spatial-temporal changes in soil organic carbon and nitrogen pools. *Global Change Biology*, *14*(10), 2420–2431. doi: 10.1111/j.1365-2486.2008.01650.x

Supp. 16: Mean values of the functional traits used in the study, their standard deviation (sd), and the coefficient of variation (CV) of each species, slope and year. LA: leaf area (cm^2); LT: leaf thickness (µm); SLA: specific leaf area ($cm^2\ g^{-1}$); LDMC: leaf dry matter content ($mg\ g^{-1}$); FlO: flowering onset; FlD: flowering duration; FlP: flowering peak; FrO: fruiting onset; FrD: fruiting duration; FrP: fruiting peak; DO: dispersion onset; DP: dispersion peak; Sen: senescence (%); C: leaf Carbon content (%); N: leaf Nitrogen content (%); $\delta^{13}C$: leaf Carbon isotope ratio; $\delta^{15}N$: leaf Nitrogen isotope ratio; FvFm1: early season photochemical efficiency; FvFm2: photochemical efficiency at the peak of flowering; SPAD1: early season chlorophyll content; SPAD2: chlorophyll content at the peak of flowering; Fitness1: total seed number; Fitness2: total seed mass; PlVol: plant volume (cm^3). Please note that we used the absolute values of means of $\delta^{13}C$ and $\delta^{15}N$ for CVs calculation.

Helianthemum squamatum, south slopes, 2017

	LA	LT	SLA	LDMC	FlO	FlD	FlP	FrO	FrD	FrP	DO	DP	Sen	C	N	d13C	d15N	FvFm2	Fitness1	Fitness2	PlVol
Mean	0.470	556.365	60.829	283.750	27.842	41.383	39.867	52.109	47.450	58.825	67.513	88.346	14.134	39.927	1.134	-29.384	-2.175	0.599	328.553	148904.161	4922.966
sd	0.187	111.126	10.766	33.539	4.854	13.481	3.946	1.192	9.630	10.384	9.465	9.250	8.909	2.209	0.133	1.102	1.393	0.217	404.997	164225.480	2735.720
CV	**0.399**	**0.200**	**0.177**	**0.118**	**0.174**	**0.326**	**0.099**	**0.023**	**0.203**	**0.177**	**0.140**	**0.105**	**0.630**	**0.055**	**0.117**	**0.037**	**0.640**	**0.362**	**1.233**	**1.103**	**0.556**

Helianthemum squamatum, north slopes, 2017

	LA	LT	SLA	LDMC	FlO	FlD	FlP	FrO	FrD	FrP	DO	DP	Sen	C	N	d13C	d15N	FvFm2	Fitness1	Fitness2	PlVol
Mean	0.530	551.091	59.567	288.597	28.708	44.308	41.492	53.733	46.800	64.350	64.017	89.538	11.611	39.596	1.008	-29.438	-2.040	0.713	168.620	75324.742	4640.239
sd	0.158	92.045	7.432	22.669	5.302	12.211	5.207	4.438	8.659	10.161	7.212	7.223	5.535	1.862	0.116	0.923	1.813	0.125	165.461	74003.482	2574.155
CV	**0.298**	**0.167**	**0.125**	**0.079**	**0.185**	**0.276**	**0.125**	**0.083**	**0.185**	**0.158**	**0.113**	**0.081**	**0.477**	**0.047**	**0.115**	**0.031**	**0.889**	**0.176**	**0.981**	**0.982**	**0.555**

Helianthemum squamatum, south slopes, 2018

	LA	LT	SLA	LDMC	FlO	FlD	FlP	FrO	FrP	DO	DP	Sen	C	N	d13C	d15N	FvFm1	FvFm2	SPAD1	SPAD2	Fitness1	Fitness2	PlVol
Mean	0.758	509.873	71.079	241.105	40.625	25.404	47.477	49.129	69.902	80.600	87.231	29.163	38.108	1.447	-31.002	-3.137	0.730	0.376	67.740	53.276	3404.078	1407354	1943.543
sd	0.293	78.783	9.635	23.570	4.785	6.417	4.177	3.453	5.445	6.867	4.749	24.406	1.899	0.194	1.050	1.346	0.133	0.254	6.729	7.505	3614.002	1499406	1557.714
CV	**0.387**	**0.155**	**0.136**	**0.098**	**0.118**	**0.253**	**0.088**	**0.070**	**0.078**	**0.085**	**0.054**	**0.837**	**0.050**	**0.134**	**0.034**	**0.429**	**0.182**	**0.674**	**0.099**	**0.141**	**1.062**	**1.065**	**0.801**

Helianthemum squamatum, north slopes, 2018

	LA	LT	SLA	LDMC	FlO	FlD	FlP	FrO	FrP	DO	DP	Sen	C	N	d13C	d15N	FvFm1	FvFm2	SPAD1	SPAD2	Fitness1	Fitness2	PlVol
Mean	0.705	492.446	70.861	250.543	42.496	23.651	48.231	50.471	70.080	81.168	87.094	25.890	38.046	1.322	-31.012	-3.047	0.697	0.292	68.704	52.165	2419.784	1003899	1882.250
sd	0.234	65.266	10.255	26.608	3.765	4.915	3.736	3.245	3.322	6.325	3.872	14.633	1.800	0.165	0.819	1.612	0.107	0.239	5.817	5.218	2343.304	927404	1223.818
CV	**0.332**	**0.133**	**0.145**	**0.106**	**0.089**	**0.208**	**0.077**	**0.064**	**0.047**	**0.078**	**0.044**	**0.565**	**0.047**	**0.125**	**0.026**	**0.529**	**0.153**	**0.817**	**0.085**	**0.100**	**0.968**	**0.924**	**0.650**

Centaurea hyssopifolia, south slopes, 2017

	LA	LT	SLA	LDMC	FlO	FlD	FlP	FrO	FrD	FrP	DO	DP	Sen	C	N	d13C	d15N	FvFm2	Fitness1	Fitness2	PlVol
Mean	0.499	301.404	124.579	234.905	9.442	61.750	30.171	16.350	68.575	41.817	28.708	87.100	22.758	43.293	2.298	-29.939	-3.048	0.683	829.130	2002.269	17402.933
sd	0.165	64.641	15.720	22.447	12.046	14.325	11.362	13.665	12.941	12.800	5.302	7.649	6.802	1.161	0.238	0.912	0.959	0.113	782.201	1919.602	10486.069
CV	**0.331**	**0.214**	**0.126**	**0.096**	**1.276**	**0.232**	**0.377**	**0.836**	**0.189**	**0.306**	**0.185**	**0.088**	**0.299**	**0.027**	**0.104**	**0.030**	**0.315**	**0.165**	**0.943**	**0.959**	**0.603**

Centaurea hyssopifolia, north slopes, 2017

	LA	LT	SLA	LDMC	FlO	FlD	FlP	FrO	FrD	FrP	DO	DP	Sen	C	N	d13C	d15N	FvFm2	Fitness1	Fitness2	PlVol
Mean	0.453	247.364	132.956	225.197	21.458	59.692	40.679	28.867	62.292	47.071	36.833	87.100	16.802	43.528	2.368	-29.500	-0.472	0.720	583.235	1211.590	16476.230
sd	0.145	76.237	15.867	21.998	11.628	16.209	13.257	9.707	14.090	12.327	7.784	7.317	5.406	1.372	0.259	0.737	1.287	0.083	528.190	1123.270	8417.361
CV	**0.321**	**0.308**	**0.119**	**0.098**	**0.542**	**0.272**	**0.326**	**0.336**	**0.226**	**0.262**	**0.211**	**0.084**	**0.322**	**0.032**	**0.109**	**0.025**	**2.728**	**0.115**	**0.906**	**0.927**	**0.511**

Centaurea hyssopifolia, south slopes, 2018

	LA	LT	SLA	LDMC	FlO	FlD	FlP	FrO	FrP	DO	DP	Sen	C	N	d13C	d15N	FvFm1	FvFm2	SPAD1	SPAD2	Fitness1	Fitness2	PlVol
Mean	0.348	267.345	118.697	244.905	25.618	63.744	47.548	35.450	60.883	45.335	90.284	39.404	43.494	2.243	-29.953	-3.531	0.710	0.695	43.420	33.085	540.123	1749.660	17060.164
sd	0.106	40.784	16.833	23.352	6.516	9.472	7.166	7.016	10.153	4.767	1.810	5.907	1.179	0.234	0.939	1.034	0.140	0.088	5.337	8.759	597.981	1834.790	10295.110
CV	**0.305**	**0.153**	**0.142**	**0.095**	**0.254**	**0.149**	**0.151**	**0.198**	**0.167**	**0.105**	**0.020**	**0.150**	**0.027**	**0.104**	**0.031**	**0.293**	**0.198**	**0.127**	**0.123**	**0.265**	**1.107**	**1.049**	**0.603**

Centaurea hyssopifolia, north slopes, 2018

	LA	LT	SLA	LDMC	FlO	FlD	FlP	FrO	FrP	DO	DP	Sen	C	N	d13C	d15N	FvFm1	FvFm2	SPAD1	SPAD2	Fitness1	Fitness2	PlVol
Mean	0.390	276.895	127.858	235.372	30.861	58.336	49.996	42.277	61.913	48.144	89.733	38.763	43.348	2.266	-29.719	-1.068	0.761	0.698	47.549	32.543	693.808	1996.162	15587.307
sd	0.111	42.303	16.007	24.167	6.621	10.719	7.516	8.067	9.597	4.973	2.168	4.957	1.255	0.267	1.022	1.137	0.110	0.099	5.365	8.352	586.816	1622.702	8467.639
CV	**0.286**	**0.153**	**0.125**	**0.103**	**0.215**	**0.184**	**0.150**	**0.191**	**0.155**	**0.103**	**0.024**	**0.128**	**0.029**	**0.118**	**0.034**	**1.064**	**0.145**	**0.141**	**0.113**	**0.257**	**0.846**	**0.813**	**0.543**

Supp. 17: Detailed differences mean trait values between slopes and years in both species.

Mean values of most functional traits varied significantly between years and slopes in both species. Phenology was delayed in 2018 with respect to 2017 for both species. However, morphological and leaf chemical composition traits varied in a species-specific manner. While *H. squamatum* showed higher leaf area and SLA and lower LDMC in 2018, *C. hyssopifolia* showed the opposite pattern, with higher leaf area and SLA and lower LDMC in 2017. Furthermore, individuals from the south slopes reproduced earlier in both species and years. In *H. squamatum*, leaf area was higher in the north slopes in 2017, and marginally higher in the south slopes in 2018. LDMC was significantly lower only in the south slopes in 2018. Finally, leaf N content was higher in the south slopes in both years. In *C. hyssopifolia*, $\delta^{13}C$ and leaf N content were lower and marginally lower; and higher and marginally higher, respectively, in the north slopes of each year. LDMC was higher in the south slopes in both years.

Chapter 4: **Contrasting adaptive trait variation in response to drought in two Mediterranean shrubs**

Mario Blanco-Sánchez, Steven J. Franks, Marina Ramos-Muñoz, Beatriz Pías, José Alberto Ramírez-Valiente, Adrián Escudero and Silvia Matesanz

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Abstract

Adaptive evolution and phenotypic plasticity are key mechanisms of climate change responses. However, we still lack a detailed understanding of the strategies different species use to cope with climatic changes such as increased droughts, particularly for species with special edaphic requirements and limited dispersal such as gypsum endemics. In this study, we assessed phenotypic and genotypic selection, phenotypic plasticity and genetic variation in traits potentially related to drought response in two dominant gypsum Mediterranean species, *Helianthemum squamatum* and *Centaurea hyssopifolia*. We established a common garden in which 524 plants from 79 maternal families from both species were grown under two contrasting watering treatments. Our results revealed that selection was stronger under drought than well-watered conditions for both species, but we found contrasting adaptive strategies and genetic variation. In *H. squamatum*, a drought-escape strategy with advanced reproductive phenology and faster growth rates was positively associated with fitness under dry conditions, and most adaptive traits exhibited quantitative genetic variation. In contrast, in *C. hyssopifolia*, selection under dry conditions favored a drought-tolerance strategy with thicker leaves and longer phenologies, but all traits lacked quantitative genetic variation, indicating that their evolutionary potential may be limited. Most traits exhibited significant plasticity in response to drought and genetic variation for trait plasticity in both species, indicating that trait plasticity can evolve independently of the evolution of trait means in these gypsophiles. Our results show that these gypsum endemic species vary in strategies and adaptive potential in response to drought, which contributes to our understanding of potential adaptive responses to climate change in such edaphic specialists.

Keywords: functional covariation, genetic variation for plasticity, gypsum endemics, phenotypic plasticity, potential response to selection, quantitative genetic variation.

Introduction

Climate change is a major threat to plant biodiversity due to the worldwide alteration of temperature and precipitation patterns (Hoffmann and Sgró, 2011; Matesanz and Valladares, 2014). The Mediterranean region is especially vulnerable to climate change due to the expected increase in aridity and environmental heterogeneity, particularly in areas with arid and semiarid conditions such as the Iberian Peninsula (Giorgi and Lionello, 2008; IPCC, 2022). A well-documented response to cope with climate change is migration to less climatically restrictive areas (Jump and Peñuelas, 2005; Parmesan, 2006; and references). However, migration may be limited for species with strict edaphic requirements, fragmented distributions, and/or low dispersal ability (Blanco-Sánchez et al., 2021; Jump and Peñuelas, 2005; Shaw and Etterson, 2012). In these cases, adaptive responses occurring *in situ* within populations, i.e. evolution by natural selection and adaptive phenotypic plasticity, may be key mechanisms to guarantee their long-term persistence (Chevin and Hoffmann, 2017; Franks et al., 2014; Gomez-Mestre and Jovani, 2013).

Adaptive evolution, a genetically-based shift in the mean phenotype of populations driven by natural selection, is a major force to cope with altering selection pressures, and mounting evidence shows that rapid evolution in response to climate change is occurring in plant populations (Franks et al., 2007; Giménez-Benavides et al., 2007; Hoffmann and Sgró, 2011; Matesanz and Valladares, 2014). In the new conditions imposed by climate change, populations may evolve by natural selection if fitness and fitness-related traits (i.e., adaptive traits) exhibit genetic variation within populations (Etterson, 2004; Jump et al., 2009; Lande and Arnold, 1983). However, evolution of adaptive traits may be constrained by a lack of quantitative genetic variation in some cases, or, even in the presence of quantitative genetic variation, by genetic correlations among traits if the direction of the correlation does not match the direction of selection (Arnold, 1992; Etterson & Shaw, 2001; Conner, 2012). Furthermore,

since both the adaptive value of traits and the expression of quantitative genetic variation are environment-dependent, testing the potential response to selection in relevant ecological environments that simulate climate change conditions is needed to make reliable predictions of the future evolutionary trajectories of plant populations (Chevin and Hoffmann, 2017; Shaw and Etterson, 2012).

Phenotypic plasticity, the ability of a genotype to express different phenotypes in different environments, is the main response mechanism to buffer rapid environmental changes, and is particularly favored in highly heterogeneous environments (Chevin and Hoffmann, 2017; Hoffmann and Sgró, 2011; Jump and Peñuelas, 2005; Matesanz et al., 2010; Stotz et al., 2021; Van Kleunen and Fischer, 2005). Furthermore, plasticity may also evolve by natural selection, and the evolution of adaptive plastic responses may play a major role in the persistence of plant populations in future environmental conditions (Chevin and Hoffmann, 2017; Jump and Peñuelas, 2005; Matesanz et al., 2010). Although phenotypic plasticity and adaptive evolution are complementary mechanisms that might act simultaneously, the relative importance of both mechanisms to cope with climate change and how adaptive evolutionary responses may differ between co-occurring species is far from resolved (Ghalambor et al., 2007; Merilä, 2015; Nicotra et al., 2010), particularly in Mediterranean semiarid plants (Franks et al., 2014; Matesanz and Valladares, 2014; Parmesan, 2006). Evaluating the contribution of both mechanisms could be especially important for gypsophiles —plants restricted to gypsum soils— since these species live under semiarid and highly heterogeneous conditions, have specific edaphic requirements, and lack long-distance dispersal mechanisms (Blanco-Sánchez et al., 2021; Escudero et al., 2015). To assess the potential response to selection of plant populations and patterns of adaptive plasticity, it is particularly useful to conduct quantitative genetics studies in which individuals of known family structure are grown in common gardens

under experimental conditions that simulate contrasting and realistic future environments (De Villemereuil et al., 2016; Franks et al., 2014).

In this study, we assessed the potential response to selection of traits and their plasticities in two dominant gypsophile species, *Helianthemum squamatum* and *Centaurea hyssopifolia*. Since drought is often the primary selection pressure in Mediterranean gypsum habitats (Blondel et al., 2010), we performed an outdoor common garden experiment with two contrasting watering treatments, well-watered and drought, to evaluate phenotypic plasticity, quantitative genetic variation and patterns of phenotypic selection in traits related to drought response. A recent phenotypic selection study in natural conditions revealed the adaptive value of earlier and longer phenologies, less sclerophyllous leaves, and lower water use efficiency associated with a drought-escape strategy for both species (Blanco-Sánchez et al., 2022), but the genetic basis of this strategy, and therefore, its potential to evolve, is to date unknown. Therefore, we predicted that a similar trait syndrome will be adaptive, especially under drought conditions (Blanco-Sánchez et al., 2022). Finally, we hypothesized that both species will show adaptive plasticity to drought and genetic variation for traits and their plasticity, since large populations evolving in highly-variable environments usually express high levels of plasticity and genetic variation for both traits and their plasticity (Chevin and Hoffmann, 2017; Hoffmann and Sgró, 2011; Kelly, 2019; Saltz et al., 2018; Stotz et al., 2021).

Materials and methods

Study species and seed collection

Centaurea hyssopifolia Vahl. (Asteraceae) and *Helianthemum squamatum* (L.) Dum. Cours (Cistaceae) are two of the most dominant gypsophiles of the center of the Iberian Peninsula (Matesanz et al., 2018). Both are small (20-60 cm of height), endemic chamaephytes of Iberian gypsum habitats. *Centaurea hyssopifolia* is restricted to the central Iberian Peninsula, while *H.*

squamatum is widely distributed in most Iberian gypsum outcrops (Matesanz et al., 2018). Furthermore, *C. hyssopifolia* shows an earlier reproductive period (from May to July), while the flowering and fruiting phenology of *H. squamatum* lasts from late May to early-mid August (Blanco-Sánchez et al., 2022).

In July 2017, we collected mature seeds from 45 maternal plants per species in a large population in the center of the Iberian Peninsula (Belinchón, Spain; 40º 04' N, 3º 04' W; ~700 m a.s.l). The site has a typical Mediterranean semiarid climate, with mean annual precipitation and temperature of 419.2 mm and 14.6°C, respectively, and pronounced summer drought (mean climatic data extracted from the 35-year climatic time series of CHELSA; Karger et al., 2017). In this site, the plant community is established in gypsum hills that harbour populations of thousands of individuals of both study species, forming discrete vegetation patches surrounded by bare soil and biological soil crust. To account for the high environmental variability of gypsum habitats (Blanco-Sánchez et al. 2022), maternal plants were sampled from south and north slopes at three different gypsum hills (22 and 23 maternal plants per species from north and south slopes, respectively). To avoid sampling closely-related individuals, maternal plants were separated by at least three meters from each other.

Common garden experiment

The experiment was performed in the outdoor CULTIVE facilities at URJC (Móstoles, Madrid, Spain). The climatic conditions of this area match those experienced by individuals in natural conditions, providing a realistic experimental environment, with similar climatic conditions and high light intensity typical of Mediterranean gypsum habitats (mean annual precipitation and temperature of 434.4 mm and 14.81°C, respectively; data extracted from CHELSA time series (Karger et al., 2017); PAR > 1600 μmol m^{-2} s^{-1}, Supp. 1). In August 2017, seeds from each maternal plant were sown in 6 L pots (22 × 20 cm; Alpifer, Valencia, Spain) filled with soil

extracted from a nearby gypsum quarry (Yesos Ibéricos-Algiss S.A., Valdemoro, Madrid, Spain). Since both species are mostly outcrossing, individuals from the same maternal plant constituted a maternal family and were considered half-siblings. Before sowing, ten seeds per maternal plant were individually weighed using a Mettler Toledo MX5 microbalance (1 μg precision; Mettler Toledo, Columbus, OH, USA) to obtain a family-level seed mass. To ensure that the substrate did not contain seeds of the study species, control pots were filled and watered for several weeks, showing no germination of either species. Eight pots per maternal family were placed in a greenhouse and maintained in well-watered conditions during germination and seedling establishment. Approximately three months after sowing, in November 2017, pots were moved from the greenhouse to the outdoor cultivation facility. Finally, in January 2018, pots were thinned out to one experimental individual per pot. Due to differences in germination success, the final size of the experiment was 221 plants for *C. hyssopifolia* (35 maternal families, 4-8 half-siblings per family) and 303 plants for *H. squamatum* (44 maternal families, 4-8 half-siblings per family).

To ensure that experimental plants reached the reproductive stage and to minimize potential maternal effects, which are larger in early stages of plant development (Bischoff and Müller-Schärer, 2010), we performed the plasticity experiment and collected phenotypic data in the second growing season. On March 15th, 2019, after ~2 years of growing in common, optimum conditions, 2-4 individuals per maternal family were randomly assigned to each of two contrasting watering treatments, well-watered and drought. Treatments were implemented by modifying the soil water content (SWC hereafter) of the experimental pots using a drip irrigation system with pressure-compensating emitters (Rain Bird XB05PC; Rain Bird Corporation, CA, USA) and adjusting the number and duration of watering events. In the well-watered treatment, plants were kept at field capacity (~25% of SWC for our substrate), simulating periods/years when SWC is high for several days, such as during a rainy spring or

in wet years. In contrast, SWC in the drought treatment was progressively reduced by decreasing the intensity and frequency of watering events and then maintained at ~50% of field capacity (12-14% of SWC), simulating periods later in the season (e.g., early summer) or springs drier than the average. Although similar SWC values to those imposed in our treatments have been registered in natural conditions at different time points in the study population (data not shown), climate change models for the semiarid Mediterranean region predict an increase in the frequency and duration of drought events (IPCC, 2022). Therefore, our drought treatment mimics conditions that will become more common in the near future, being key to assess the response of the study species to climate change. Treatments lasted for ~4 months, ending when plants in the well-watered treatment began to senesce (July 2nd, 2019). To guarantee the successful implementation of both watering treatments, pots were placed under rain exclusion structures that eliminated all natural precipitation and did not substantially affect other environmental conditions (see details of the structures on Supp. 2 and Matesanz et al. 2020). Furthermore, SWC was monitored every 3–5 days in 12 pots per treatment using an HH2 Moisture Meter with an ML3 Sensor (Delta-T devices, Cambridge, UK; see Supp. 3).

Phenotypic and fitness traits measurements

We measured a wide set of functional and fitness traits in all plants (see Supp. 2 for details).

Phenological traits— Reproductive phenology was monitored every three days during the experiment (24 censuses). At each census, we visually assessed the presence of inflorescences with open flowers, fully-developed fruits, and dispersed inflorescences. Using these data, we calculated flowering, fruiting, and dispersal onset and duration.

Leaf morphological traits— At the reproductive peak of each species, we randomly collected five non-senescent, fully-developed leaves per plant. After 12h of rehydration, the saturated fresh mass of all leaves was weighed using a Mettler Toledo MX5 microbalance. Then, we

measured leaf thickness using a dial thickness gauge (Mitutoyo Corporation, Japan). Next, leaves were scanned using an Epson Perfection V370 Photo scanner (Seiko Epson Corporation, Japan), and oven-dried at 60ºC for 48 h. Finally, dried leaves were weighed again. From these data, we calculated specific leaf area (SLA), leaf dry matter content (LDMC) and total estimated leaf area (TELA).

Ecophysiological traits— At the time of leaf collection, we also measured the midday maximum photochemical efficiency (F_v/F_m), and leaf chlorophyll content. Midday photochemical efficiency was measured from 13:00 to 16:30 (UTC + 2) during two consecutive sunny days using a Handy PEA+ chlorophyll fluorimeter (Hansatech, UK), adapting leaves to the dark for 30 minutes before the measurement. Leaf chlorophyll content was measured in three leaves per plant, using a SPAD 502 chlorophyll meter (Konica Minolta, Japan).

Plant size and growth traits— We measured the height of each plant, its maximum diameter, and the perpendicular diameter to the maximum diameter at the onset and at the end of the watering treatments. We calculated initial and final plant volume of each plant as the volume of a hemispheroid, and relative growth rate (RGR; Supp. 2 for details). Aboveground tissues were harvested and oven-dried, and leaves and stems from each individual were weighed using a Kern ABJ 120-4M analytical balance (1 mg precision; Kern & Sohn GmbH, Germany). From these data, we calculated aboveground biomass, as the sum of leaf and stem biomass, and the leaf:stem ratio.

Reproductive fitness traits— We haphazardly collected three mature inflorescences per plant before seed dispersal, storing them individually in paper bags. Then, inflorescences were thoroughly dissected, obtaining the mean number of viable seeds per inflorescence. To assess the mean seed mass, five viable seeds per plant were randomly selected and individually weighed. Finally, before the end of the experiment, we counted the number of viable inflorescences of all plants. From these data, we calculated two integrated plant-level fitness

variables: i) total seed number, as the product of the number of inflorescences and the number of seeds per inflorescence, and ii) total seed mass, as the product of total seed number and the mean seed mass.

Statistical analyses

Phenotypic and genotypic selection analyses— To identify traits under selection in each species and treatment, we calculated phenotypic (Lande and Arnold, 1983) and genotypic (Rausher, 1992) selection differentials and gradients. Selection differentials (S'), i.e., the covariance between relative fitness and a particular standardized trait, assess the total relationship between traits and fitness (total selection; including direct selection and indirect selection caused by correlations with other traits). Selection gradients (β'), i.e., the vector of partial regression coefficients of relative fitness on standardized traits, assess direct selection on the traits, removing the effect of correlations with other traits. First, trait values were standardized as $\frac{X-\mu}{\sigma}$, where X is the trait value of an individual, and μ and σ are the mean and the standard deviation, respectively, of the trait in each watering treatment. Then, reproductive fitness was relativized in each species and watering treatment as individual fitness divided by the mean value of fitness for a given treatment. To estimate genotypic selection differentials and gradients, we calculated the mean of each functional and fitness trait (standardized and relativized, respectively) for each maternal family in each treatment. To assess directional selection, we calculated linear selection differentials and gradients. To evaluate stabilizing and disruptive selection, we estimated quadratic selection differentials for each species and treatment (see Supp. 2). To avoid potential multicollinearity in our selection analyses, we computed both variance inflation factors (VIFs) and pairwise phenotypic correlations for each trait in both species and watering treatments (see Supp. 4). As recommended, we excluded from our models predictors with VIF > 10 (Dormann et al., 2013). Therefore, due to their high correlation with other traits (>0.7) and high VIFs,

TELA was not included in the models of either species, fruiting duration in the models of *H. squamatum*, and dispersion onset and duration in the models of *C. hyssopifolia*.

Phenotypic and genotypic selection analyses were performed using linear mixed models and generalized linear mixed models (LMMs and GLMMs) with functions *lmer* and *glmer* (package lme4; Bates et al., 2015). We included relative reproductive fitness as the dependent variable, and the standardized trait (in selection differentials) or traits (in selection gradients) as independent variables. To account for potential factors that may affect the relationship between traits and fitness, initial plant volume of each individual and the mean seed mass from each maternal family were also included as covariates, and the identity of each maternal family was included as a random factor (in phenotypic selection analyses). Selection analyses excluding the covariates and the random factor provided very similar results (Supp. 5), but the explained variance of the latter models was slightly lower. Since reproductive fitness did not follow a gaussian distribution in either treatment in *C. hyssopifolia* and in the drought treatment in *H. squamatum* (the distribution was positively skewed), models were performed using family = "Gamma" and link = "log" in these cases, and family = "gaussian" with link = "identity" in the models performed for *H. squamatum* in the well-watered treatment. Finally, to account for multiple testing, results from selection analyses were corrected using false discovery rate (FDR) in each species and treatment (Benjamini and Hochberg, 1995) using function *p.adjust*. Because the total seed mass fitness variable includes total seed number, and models using both fitness variables resulted in very similar selection patterns, we only show selection analyses using total seed mass in the main text (see Supp. 6 and 7 for results using total seed number).

Finally, to assess if genetic trade-offs between traits may constrain adaptive evolution, we assessed the genetic variance-covariance matrices (G-matrices) for all traits for each species and treatment (Supp. 8), which quantified the additive genetic variance of traits (diagonal) and the genetic covariance among traits, using function *MCMCglmm* (package MCMCglmm;

Hadfield et al., 2019) with scaled trait values and gaussian priors. Bayesian models were fitted including all the studied traits as dependent variables and maternal family as a random factor. Each model was run for one million iterations, with a burn-in period of 100000 iterations and a thinning interval of 1000.

Quantitative genetic variation— To quantify the degree to which phenotypic differences among individuals within each watering treatment were genetically-based, i.e., the presence of quantitative genetic variation in both species, we performed for each trait and treatment a mixed model including individual trait values as dependent variables, seed mass of each maternal family as covariate and the identity of maternal family as a random factor. We compared this model to the same model excluding the random term maternal family using a likelihood ratio test (Zuur et al., 2009), with function *lrtest* (package lmtest; Zeileis and Hothorn, 2002). A significant effect from a Chi-square test comparing these models ($P < 0.05$*)* indicated genetic differences among maternal families. Results were again corrected by FDR. Families with fewer than three replicate half-siblings in a given treatment were dropped from these analyses.

Plastic responses to drought, genetic variation and selection for plasticity— To test the effect of the watering treatments on phenotypic expression (phenotypic plasticity), we fitted linear mixed models for each species with the individual trait values as the dependent variable, treatment as a fixed factor, and maternal family and the family-by-treatment interaction as random factors. The significance of the fixed factor was assessed using function *Anova* (package car; Fox et al., 2012), with type III sum of squares and the Kenward–Roger approach, and the proportion of variance explained by each model (R^2) was calculated using function *summary* (R Core Team, 2018). A significant effect of treatment indicated phenotypic variation between watering conditions (phenotypic plasticity). Then, to quantify the specific response to

drought of each maternal family, assess the presence of genetic variation for plasticity for each trait, and estimate the selection differentials and gradients for plasticity, we calculated the relative phenotypic distances between individuals from the same maternal family in different treatments, which allows calculating an index of plasticity (RDPI index) for each maternal family and comparing them statistically (Valladares et al., 2006). To evaluate the presence of genetic variation for plasticity among maternal families, we performed two different models for the RDPIs of each trait with maternal seed mass as a covariate, including and excluding the random term maternal family, and compared them using likelihood ratio tests. Results were corrected by FDR. Significant differences between models ($P < 0.05$) indicated that maternal families responded differently to drought, i.e., the presence of genetic variation for plasticity. Finally, to assess if plastic responses were under selection, we estimated genotypic selection differentials and gradients (see above) including standardized RDPIs values for each trait (or traits) as fixed factors, maternal seed mass as a covariate, and two different relativized fitness variables as dependent variables (Caruso et al., 2006). We first assessed if plasticity was adaptive in the sense that the most plastic genotypes have the highest average fitness across environments (Van Kleunen and Fischer, 2005). To test this, selection analyses for plasticity were performed using mean fitness across treatments. Second, to evaluate whether plasticity could enhance fitness under stressful conditions, models were fitted using relativized fitness under the drought treatment.

All analyses were performed in R v4.0.5 (R Core Team, 2018).

Results

Selection patterns across watering conditions

In both species, we found a higher number of traits under linear selection in the drought treatment than in the well-watered treatment. The magnitude of selection, based on the larger selection differentials and gradients, was also higher under drought (Fig. 1; Supp. 9). We did not find evidence of stabilizing or disruptive selection for any trait in either treatment or species. Although a few functional traits showed significant C' and γ' coefficients (Supp. 10), there were not clear maxima or minima of fitness associated with intermediate phenotypic values.

Helianthemum squamatum and *Centaurea hyssopifolia* differed in both the number and the identity of the traits under selection. For *H. squamatum* in drought conditions, there were significant selection differentials for phenological traits, with individuals with earlier flowering and fruiting phenology and with longer flowering periods having higher fitness (Fig. 1; Supp. 9). Also, lower leaf chlorophyll content (SPAD), lower leaf:stem ratio, and higher relative growth rate (RGR) were associated with greater reproductive fitness under drought (Fig. 1; Supp. 9). Under drought conditions, flowering and fruiting onset, individual leaf chlorophyll content, and leaf:stem ratio were under negative direct selection, while flowering duration was under positive direct selection (Supp. 9). In well-watered conditions, leaf:stem ratio was under negative total and direct selection (Fig. 1; Supp. 9). In this species, genotypic selection analysis showed very similar results compared to those obtained using individual trait values, especially under drought conditions. In this treatment, families with advanced flowering and fruiting phenology, longer flowering periods, lower leaf:stem ratio and higher RGR showed higher fitness (Supp. 9). Conversely, there was no significant genotypic selection in well-watered conditions (Supp. 9).

In *C. hyssopifolia* under drought, we found that longer phenologies were associated with greater fitness, with significant selection differentials for flowering and fruiting duration and a

significant selection gradient for flowering duration (Fig. 1; Supp. 9). In well-watered conditions, selection differentials showed that only greater aboveground biomass was marginally associated with fitness (Fig. 1; Supp. 9). We did not find any trait under direct selection in *C. hyssopifolia* in the well-watered treatment (Supp. 9). Genotypic selection analyses showed no trait under total or direct selection in either the well-watered or the drought treatment (Supp. 9).

Quantitative genetic variation and genetic correlations

In *H. squamatum*, we found significant quantitative genetic variation for several traits in both watering conditions, some of which were under selection (Table 1). Specifically, in drought conditions, traits under selection that exhibited genetic variation were flowering and fruiting onset, leaf:stem ratio, and total seed number. In the well-watered treatment, flowering onset, and leaf:stem ratio showed genetic variation in *H. squamatum* (Table 1). Furthermore, G-matrices did not show any genetic correlation of the opposite sign to the direction of selection in this species (Supp. 8).

In contrast to *H. squamatum*, there was no significant genetic variation for any trait under selection in either treatment in *C. hyssopifolia* (Table 1).

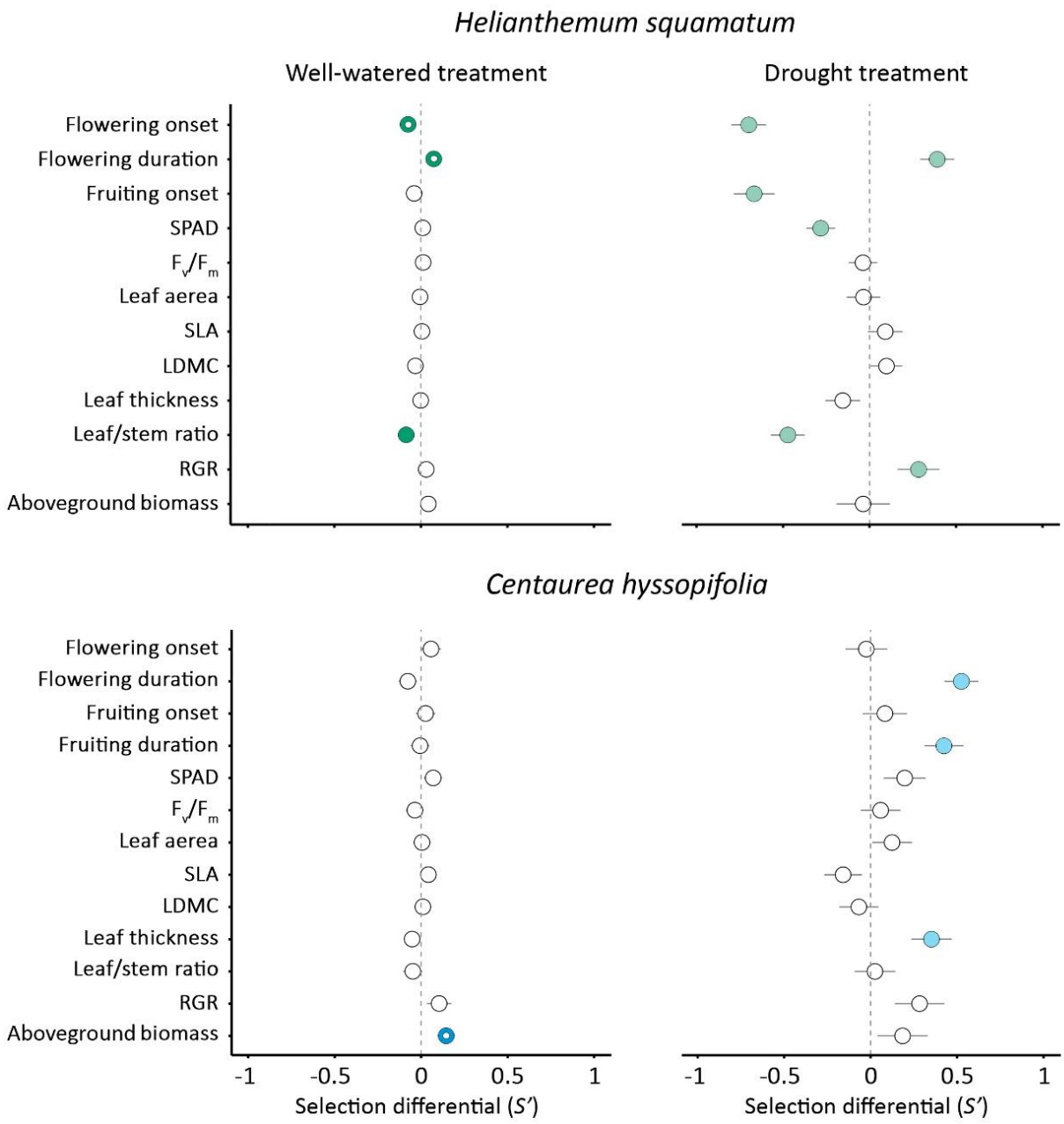

Figure 1: Linear phenotypic selection differentials (*S'*) and their standard error for *H. squamatum* and *C. hyssopifolia* in both watering conditions using total seed mass as fitness variable. Significance levels after FDR corrections: colored circle = $P < 0.05$; small white dot inside colored circle = $0.05 < P < 0.1$; white = n.s. ($P > 0.05$) selection differentials. Significant and marginally selection differentials in the drought treatment are shown in light green and light blue for *H. squamatum* and *C. hyssopifolia*, respectively, and in dark green and dark blue for *H. squamatum* and *C. hyssopifolia* under well-watered conditions, respectively.

Table 1: Results of Likelihood Ratio tests assessing the presence of genetic variation for functional traits (within treatments) and their plasticity (across treatments, using RDPIs values for each trait) for both study species. χ^2 statistics and *P*-values after FDR correction are shown. Traits with significant genetic variation within treatments, and significant genetic variation for plasticity are shown in bold. Traits under selection (as shown by linear selection differentials and gradients) are underlined. Traits in both bold type and underlined were traits under selection with genetic variation (i.e., with significant differences among maternal families).

	Genetic variation for functional traits				Genetic variation for plasticity	
H. squamatum	**Well-watered treatment**		**Drought treatment**		**Across treatments (RDPIs)**	
	χ^2	*P*	χ^2	*P*	χ^2	*P*
Flowering onset	**8.899**	**0.010**	**32.480**	**<0.001**	**72.228**	**<0.001**
Flowering duration	1.863	0.301	3.302	0.097	**95.878**	**<0.001**
Fruiting onset	0.358	0.592	**19.533**	**<0.001**	**55.253**	**<0.001**
SPAD	1.352	0.343	0.000	1.000	**26.366**	**<0.001**
F_v/F_m	1.432	0.343	**5.638**	**0.031**	**126.013**	**<0.001**
Leaf area	3.636	0.132	**9.387**	**0.008**	**62.165**	**<0.001**
SLA	**11.785**	**0.003**	**7.426**	**0.018**	**36.104**	**<0.001**
LDMC	**13.303**	**0.002**	1.318	0.293	**33.394**	**<0.001**
Leaf thickness	1.039	0.392	**5.642**	**0.031**	**6.850**	**0.009**
Leaf:stem ratio	**8.550**	**0.010**	**35.379**	**<0.001**	**65.447**	**<0.001**
RGR	2.705	0.200	2.109	0.186	**14.062**	**<0.001**
Aboveground biomass	**15.206**	**0.001**	0.564	0.488	**33.096**	**<0.001**
Total seed number	0.895	0.402	**6.373**	**0.027**	**110.818**	**<0.001**
Total seed mass	0.215	0.643	4.439	0.055	**113.400**	**<0.001**
C. hyssopifolia	**Well-watered treatment**		**Drought treatment**		**Across treatments**	
	χ^2	*P*	χ^2	*P*	χ^2	*P*
Flowering onset	2.874	0.211	-1.14E-13	1.000	**17.419**	**<0.001**
Flowering duration	0.846	0.580	1.14E-13	1.000	**6.696**	**0.010**
Fruiting onset	**8.008**	**0.034**	3.41E-13	1.000	0.790	0.374
Fruiting duration	**6.252**	**0.047**	0.000	1.000	**33.902**	**<0.001**
SPAD	2.729	0.211	6.287	0.182	**69.832**	**<0.001**
F_v/F_m	0.636	0.580	0.114	1.000	**33.895**	**<0.001**
Leaf area	**7.335**	**0.034**	2.286	0.792	**23.219**	**<0.001**
SLA	1.762	0.346	1.14E-13	1.000	**31.621**	**<0.001**
LDMC	0.200	0.755	1.14E-13	1.000	**40.664**	**<0.001**
Leaf thickness	4.786	0.086	0.000	1.000	**40.900**	**<0.001**
Leaf:stem ratio	**14.061**	**0.003**	0.080	1.000	**27.444**	**<0.001**
RGR	0.704	0.580	1.990	0.792	**42.923**	**<0.001**
Aboveground biomass	5.68E-14	1.000	0.732	1.000	**40.109**	**<0.001**
Total seed number	0.488	0.606	2.27E-13	1.000	**19.565**	**<0.001**
Total seed mass	0.135	0.764	0.735	1.000	**22.134**	**<0.001**

Plastic responses, genetic variation and selection for plasticity

We found significant plasticity (differences in phenotypic expression between treatments) for most traits in both species (Fig. 2; Supp. 11). Likelihood-ratio tests performed with RDPI values showed genetic variation for the plasticity of all traits except for fruiting onset in *C. hyssopifolia* (Table 1; Fig. 3).

Individuals of *H. squamatum* significantly advanced their flowering and fruiting onset (~1.5 and 4.2 days, respectively) in response to drought, and there was a significant reduction in the duration of flowering (~5.7 days) under drought conditions (Fig. 2). In response to drought, plants also showed significant changes in leaf morphology, producing smaller (~18.2% decrease in leaf area) and thicker leaves (~10.2% increase in leaf thickness), and increasing their leaf:stem ratio by ~9.5% (Fig. 2). Furthermore, in well-watered conditions, plants showed higher values of leaf chlorophyll content and photochemical efficiency (~9.25% increase for both traits). In addition, drought constrained plant growth, resulting in a 10-fold reduction in RGR and 1.25 times reduction in aboveground biomass (Fig. 2). Finally, water stress significantly reduced individual fitness. Plants in the well-watered treatment showed greater total seed number and total seed mass (12.8 and 10.4 times greater) than in the drought treatment (Fig. 2).

In *C. hyssopifolia*, the onset of fruiting was significantly advanced in response to drought (~2 days), and plants showed a significant reduction in flowering and fruiting duration (18.7 and 13.4 days, respectively; Fig. 2). Morphological traits were also significantly affected by drought. In the drought treatment, individuals produced smaller and thinner leaves (decrease of ~19.3% and 15.5%, respectively; Fig. 2). Furthermore, we also observed reduced leaf chlorophyll content and photochemical efficiency under water stress (~13.6 and 6.2%, respectively; Fig. 2). Finally, a reduction in size and fitness was also observed under drought conditions. In the well-watered treatment, plants had more aboveground biomass, total seed

number and total seed mass (~2, 9 and 10 times higher, respectively), compared to the drought treatment.

Finally, selection analysis performed on the plasticity indices showed that plasticity was under selection only in *H. squamatum*. Specifically, selection analyses performed using relativized mean fitness across environments showed that the plasticity of flowering duration was negatively related to fitness (S' = -0.122, P = 0.007), indicating that maternal families with a lower reduction in flowering duration under drought conditions (i.e., flatter reaction norms, lower plasticity) showed higher fitness. In the models performed using relativized fitness under drought, we also found that higher plasticity in leaf chlorophyll content (SPAD) and SLA indicating that also families with a higher reduction in leaf chlorophyll content and SLA in response to drought were associated with higher fitness in *H. squamatum* (Supp. 12).

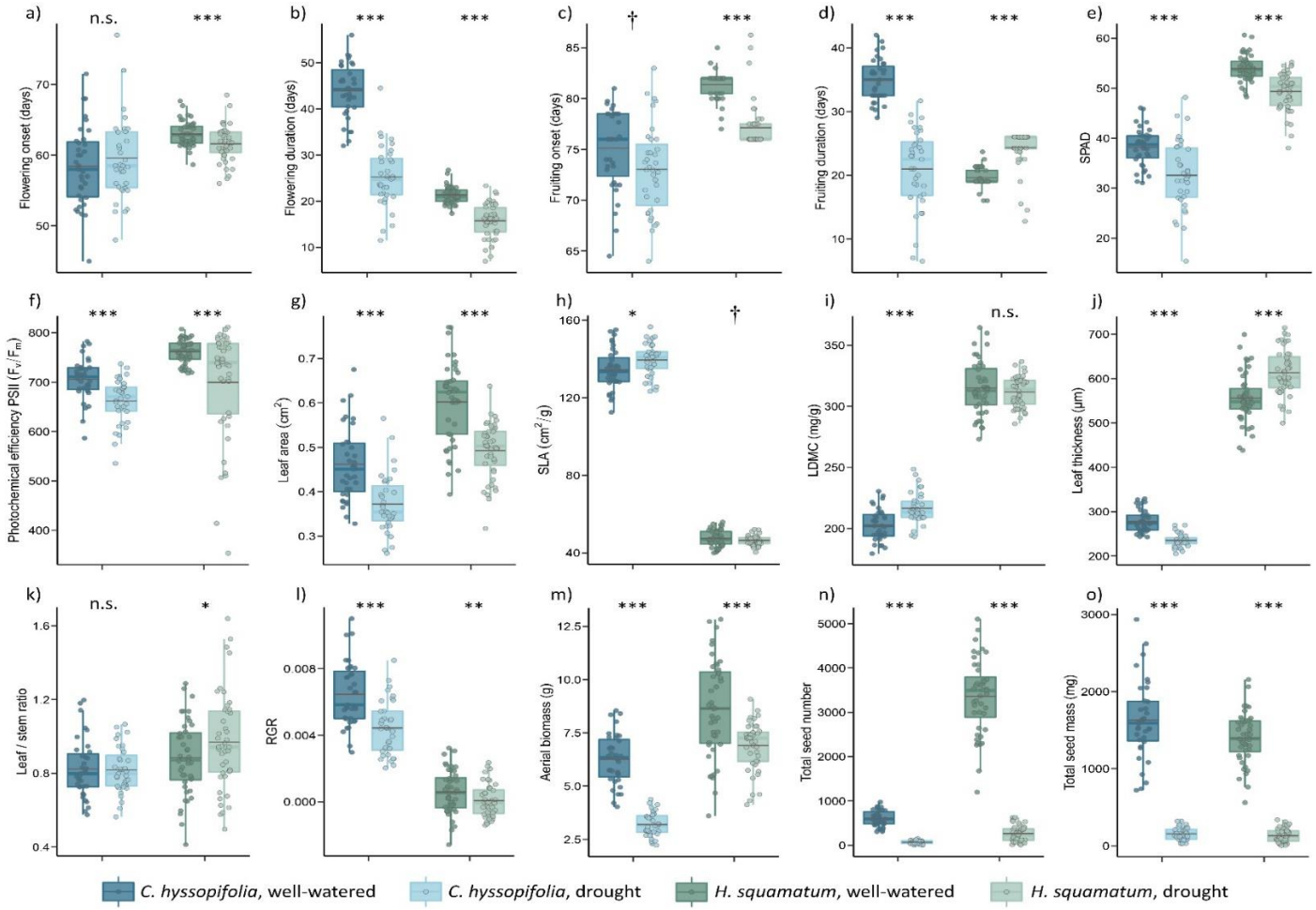

Figure 2: Phenotypic variation across watering treatments and study species. a) flowering onset; b) flowering duration; c) fruiting onset; d) fruiting duration; e) leaf chlorophyll content (SPAD); f) photochemical efficiency of PSII (F_v/F_m); g) leaf area; h) SLA; i) LDMC; j) leaf thickness; k) leaf/stem ratio; l) RGR; m) Aboveground biomass; n) total seed number; o) total seed mass, for each species in each watering treatment. Boxplots show median, first and third quartiles, and mean is represented using a grey line. Upper whiskers show 1.5 times the interquartile range (or the maximum value in case it is lower), while lower whiskers show 1.5 times the interquartile range (or the minimum value if it is higher). Dots represent mean trait values of each maternal family. Phenotypic differences between treatments (i.e., phenotypic plasticity) are shown. Significance levels: n.s. = not significant; † $0.05<P<0.1$; ***** $P<0.05$; ****** $P<0.01$; ******* $P<0.001$.

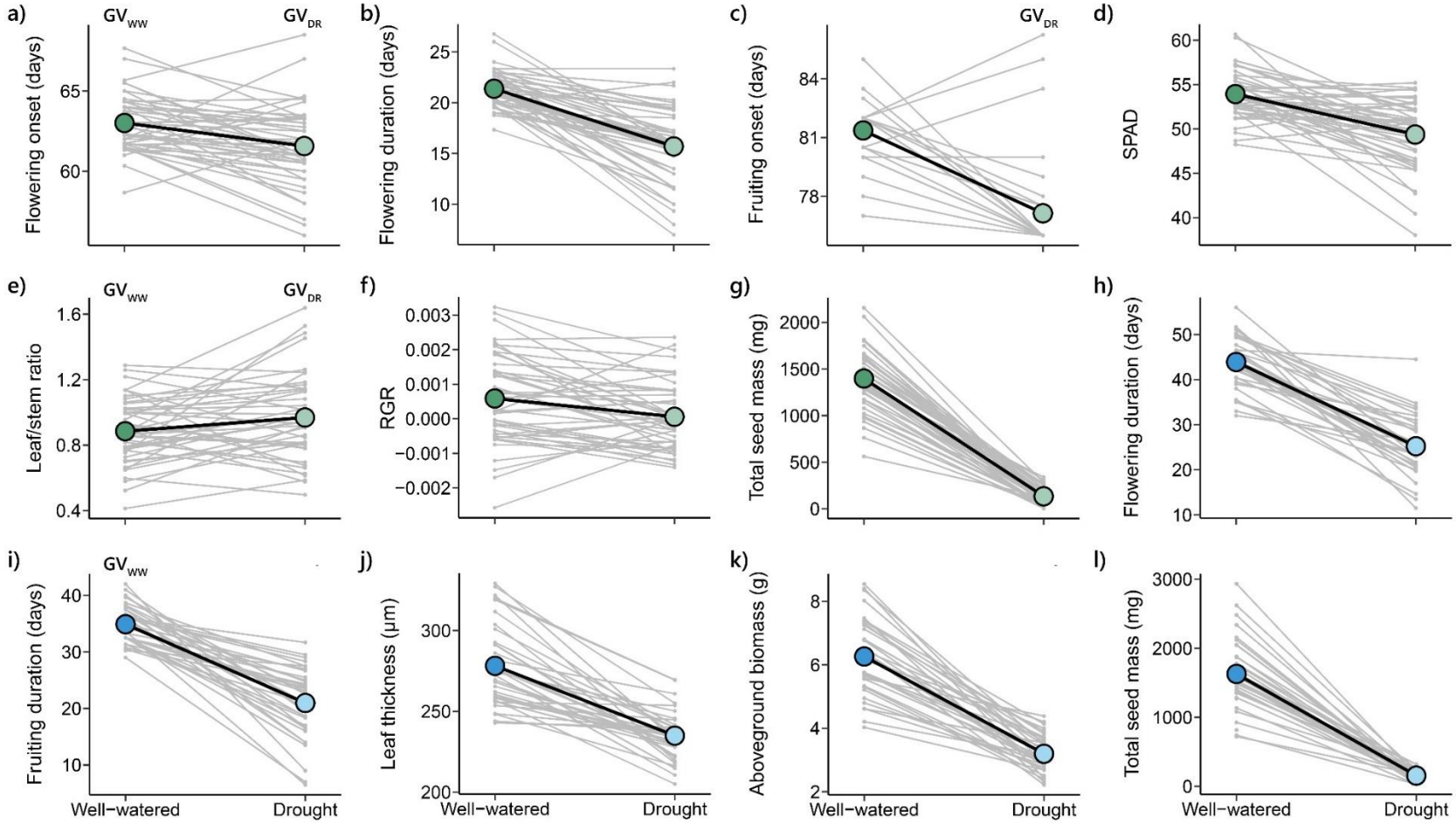

Figure 3: Phenotypic means (large dots), average reaction norms (black lines) and maternal families reaction norms (grey lines) for the traits under selection and total seed mass fitness variable in both species: a) flowering onset in *H. squamatum*; b) flowering duration in *H. squamatum* c) fruiting onset in *H. squamatum*; d) SPAD in *H. squamatum*; e) leaf/stem ratio in *H. squamatum*; f) RGR in *H. squamatum*; g) total seed mass in *H. squamatum*; h) flowering duration in *C. hyssopifolia*; i) fruiting duration in *C. hyssopifolia*; j) leaf thickness in *C. hyssopifolia*; k) aboveground biomass in *C. hyssopifolia*; l) total seed mass in *C. hyssopifolia*. Dark green and light green dots indicate phenotypic means for *H. squamatum* under well-watered and drought treatments, respectively. Dark blue and light blue dots indicate phenotypic means for *C. hyssopifolia* under well-watered and drought treatments, respectively. The presence of significant quantitative genetic variation within well-watered and drought conditions is shown with GV_{WW} and GV_{DR}, respectively. All traits showed plastic responses to drought and significant genetic variation for plasticity between families (i.e., non-parallel reaction norms).

Discussion

Our results showed strong selection and plasticity in response to drought in a large population of Mediterranean gypsophiles, but we found substantial differences between species in adaptive traits and in evolutionary potential as shown by quantitative genetic variation. In *H. squamatum*, selection favored earlier and longer reproductive phenology, higher RGR and lower leaf chlorophyll content, coupled with significant genetic variation for several traits and fitness, indicating that adaptive evolution may occur in this species in response to continuing climate change. In contrast, drought tolerance traits such as thicker leaves were favored by selection in *C. hyssopifolia*, but the lack of genetic variation suggested that these traits may be constrained in their evolution in response to further drought selection. Furthermore, plastic responses to drought and genetic variation for plasticity were found in both species, suggesting that plasticity may play a key role in buffering the climatic conditions imposed by climate change. Overall, our results showed differences in the potential evolutionary responses of two dominant gypsophile species, which may affect their persistence in a climate change context.

Selection patterns within watering treatments highlighted the importance of drought as a key selective pressure for Mediterranean plant species, agreeing with previous studies (Blanco-Sánchez et al., 2022; Ramírez-Valiente et al., 2021). In *H. squamatum*, earlier and longer reproductive phenology, higher RGR, and lower leaf chlorophyll content were significantly associated with individual fitness under drought conditions, a syndrome consistent with a drought-escape strategy (Franks, 2011; Volaire, 2018; Welles and Funk, 2021), which was also found to be adaptive in natural conditions (Blanco-Sánchez et al., 2022). Several studies have reported the adaptive value of an advanced phenology in Mediterranean taxa to escape from drought (Blanco-Sánchez et al., 2022; Franks, 2011). Furthermore, earlier phenologies could be favored by the adaptive value of higher RGR. Especially under drought conditions, the onset of reproduction depends on resource acquisition rate, which is often

correlated with individual growth rate (Segrestin et al., 2020; Welles and Funk, 2021), since more acquisitive individuals may complete their lifecycles earlier and escape the most stressful conditions. In addition, plants often show lower chlorophyll content under drought and/or high irradiance conditions (Dai et al., 2009; Letts et al., 2012; Matesanz et al., 2020b). This reduction may be adaptive in stressful habitats such as Mediterranean gypsum ecosystems, since it prevents damage in the photosynthetic system caused by photoinhibition (Dai et al., 2009; Letts et al., 2012). Importantly, we found genetic variation for several adaptive traits and for reproductive fitness in drought conditions in *H. squamatum*, and patterns of trait covariance suggest that genetic correlations will not likely constrain trait evolution. Such heritable variation indicates the potential of this species to evolve higher reproductive output under drought conditions, associated with the evolution of an acquisitive resource strategy, which may be crucial given the predicted increased aridity for the Mediterranean region.

In contrast to *H. squamatum*, selection favored individuals with thicker leaves and longer flowering and fruiting periods under dry conditions in *C. hyssopifolia*, but both traits and fitness lacked genetic variation. More sclerophyllous leaves often have smaller cells with thicker walls, and are usually associated with conservative resource-use and drought-tolerance strategies that minimize water loss (Blumenthal et al., 2020; Ramírez-Valiente et al., 2020; Solé-Medina et al., 2022). Accordingly, selection studies have previously reported the adaptive value of thicker leaves to tolerate drought (Etterson, 2004; Ramírez-Valiente et al., 2014, 2011). Indeed, sclerophyllous leaves are usually associated with longer periods of photosynthetic activity during the growing season (Ramírez-Valiente et al., 2011; and references therein), favoring longer reproductive phenologies (Blumenthal et al., 2020; Ocheltree et al., 2020). However, the observed selection on increased leaf thickness under dry conditions in our experiment differed from the results obtained in previous studies under natural conditions (Blanco-Sánchez et al., 2022). Volaire (2018) argued that shifts between drought-related

strategies may be a consequence of different levels of water availability. Differences in adaptive traits between studies might be also related to differences in the onset of drought. In natural conditions, gypsophiles encounter severe water stress mostly in the later stages of the season (Blanco-Sánchez et al., 2022; Escudero et al., 2015), while our common garden simulated the increment of aridity caused by climate change and individuals experienced drought conditions during the entire growing season.

Nevertheless, *C. hyssopifolia* lacked heritable variation for adaptive traits and fitness in the studied population, potentially constraining their evolution. The absence of within-population quantitative genetic variation in this species was not likely caused by past selection that could have eroded genetic variation of adaptive traits. In such a scenario, low phenotypic variation would be expected (Blows and Hoffmann, 2005; Matesanz et al., 2010), but this was not the case in this species. Quantitative genetic variation can also be reduced in small and isolated populations by stochastic processes such as genetic drift (Shaw and Etterson, 2012), but our studied population harbored hundreds of individuals. Although we cannot pinpoint the exact reason behind the lack of quantitative genetic variation, the contrasting levels of genetic variation between species have important implications for their future evolutionary responses, since adaptive evolution requires within-population quantitative genetic variation (Blows and Hoffmann, 2005; Jump et al., 2009; Shaw and Etterson, 2012). Our results highlight the fact that two dominant species co-occurring in semiarid Mediterranean habitats and that are subject to similar selection pressures may substantially differ in their potential to respond to selection at the population level, which will likely alter the dynamics of the plant community over time. Nevertheless, because quantitative genetic variation can vary across populations, i.e., populations of the same species may differ in their evolutionary potential (Matesanz et al., 2014; Matesanz and Valladares, 2014; Ramírez-Valiente et al., 2011), further studies with other

populations of *C. hyssopifolia* would be needed to assess the evolutionary potential of this species in a climate change scenario.

In contrast to the differences in adaptive strategies under drought between the two study species, both showed significant plasticity to drought and genetic variation for plasticity in most functional traits (Fig. 3). Some of these plastic responses were consistent with adaptive responses to drought based on previous evidence. For instance, individuals of both species reduced their leaf area under drought conditions, which minimizes evapotranspiration under water stress (Matesanz et al., 2020b; Matesanz and Valladares, 2014). However, selection analyses indicated that plasticity was not under selection in either species. Furthermore, in some instances, plastic responses were in the opposite direction to the direction of selection found within the drought treatment (cf. Fig. 1 and 2). Determining the adaptive value of plasticity is not straightforward, and statistical approaches that quantify the contribution of phenotypic change to fitness may fail because environmental conditions affect both phenotypic expression and fitness, making it impossible to isolate the effect on fitness of the phenotypic change across environments (Auld et al., 2010; Sultan, 2004, 2000). Our experiment shows the limitations of evaluating adaptive plastic responses based on selection patterns assessed both across and within environmental conditions, highlighting the need for a novel and robust approach to statistically assess the adaptive value of plasticity.

Although the precise drivers promoting high levels of plasticity and variation in norms of reaction (i.e., genetic variation for plasticity) are yet not fully understood, several factors have been discussed (reviewed in Saltz et al., 2018; see also Kelly, 2019), highlighting the role of fluctuating selection pressures and environmental heterogeneity. Gypsum habitats have high coarse- and fine-grained spatiotemporal environmental variation (Blanco-Sánchez et al., 2022; Escudero et al., 2015; Matesanz et al., 2020a), which may have favored the expression of phenotypic plasticity in gypsophile species (Matesanz et al., 2010, 2020a; Sultan and Spencer,

2002; Via et al., 1995). Indeed, the fine-grained heterogeneity of gypsum habitats may select for different norms of reaction within populations (Sih, 2004; Via et al., 1995), maintaining genotypes (or families) expressing differential plasticity if heterogeneous environmental conditions impose variable selective pressures that favor genotypes with contrasting plastic responses (i.e., highly plastic genotypes in highly-variables microsites, and those with lower plasticity in more stable microsites). In contrast, constant directional selection as a consequence of harsh and predictable environments may reduce genetic variation for plasticity (Blows and Hoffmann, 2005; Matesanz et al., 2010), resulting in similar response patterns across families. Therefore, it is likely that a particular plastic response has not evolved by natural selection during the evolutionary history of these species, with environmental heterogeneity having a critical role in promoting the presence of genetic variation for plasticity and maintaining it over time.

The presence of genetic variation for plasticity at the intrapopulation level has important evolutionary implications for these species. First, it could be advantageous for populations inhabiting gypsum ecosystems because it allows a wide variety of phenotypic responses in such stressful heterogeneous habitats. This diversity may be maintained if families expressing different response patterns are equally fit (i.e., if spatiotemporal heterogeneous conditions favored different phenotypic responses). Indeed, high levels of genetic variation for plasticity are often correlated with higher resistance of populations against environmental-driven changes such as those caused by climate change (Kelly, 2019; Matesanz et al., 2010), since genetic variation is the substrate for natural selection (Fisher, 1930; Matesanz and Valladares, 2014). Second, our results showed that, in both species, quantitative genetic variation of a particular trait and its plasticity might remarkably differ, and therefore both trait means and plasticities may evolve independently (see also Pigliucci, 2005; Weijschedé et al., 2006). Surprisingly, in contrast to previous results both within and among populations (Scheiner, 1993; Lázaro-Nogal

et al., 2015; Matesanz et al., 2017; Matesanz and Valladares, 2014), in some instances the evolutionary potential of plasticity in the study species was higher than the evolutionary potential of traits, especially in *C. hyssopifolia*. These results suggested that, particularly in *C. hyssopifolia*, the evolution of adaptive norms of reaction may play a more important role than the evolution of trait means in the adaptation to the changing environmental conditions driven by climate change.

Overall, our results indicate that traits and trait plasticities have the potential to evolve in gypsum endemics, with the evolutionary direction and evolutionary potential varying among species, traits, and environmental conditions. Thus, phenotypic plasticity and adaptive evolution can interact to shape adaptive responses in these habitat specialists, with implications for species responses to climatic changes more broadly.

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Supporting information — Blanco-Sánchez et al. — Chapter 4

Contrasting adaptive trait variation in response to drought in two Mediterranean shrubs

Mario Blanco-Sánchez, Steven J. Franks, Marina Ramos-Muñoz, Beatriz Pías, José Alberto Ramírez-Valiente, Adrián Escudero and Silvia Matesanz

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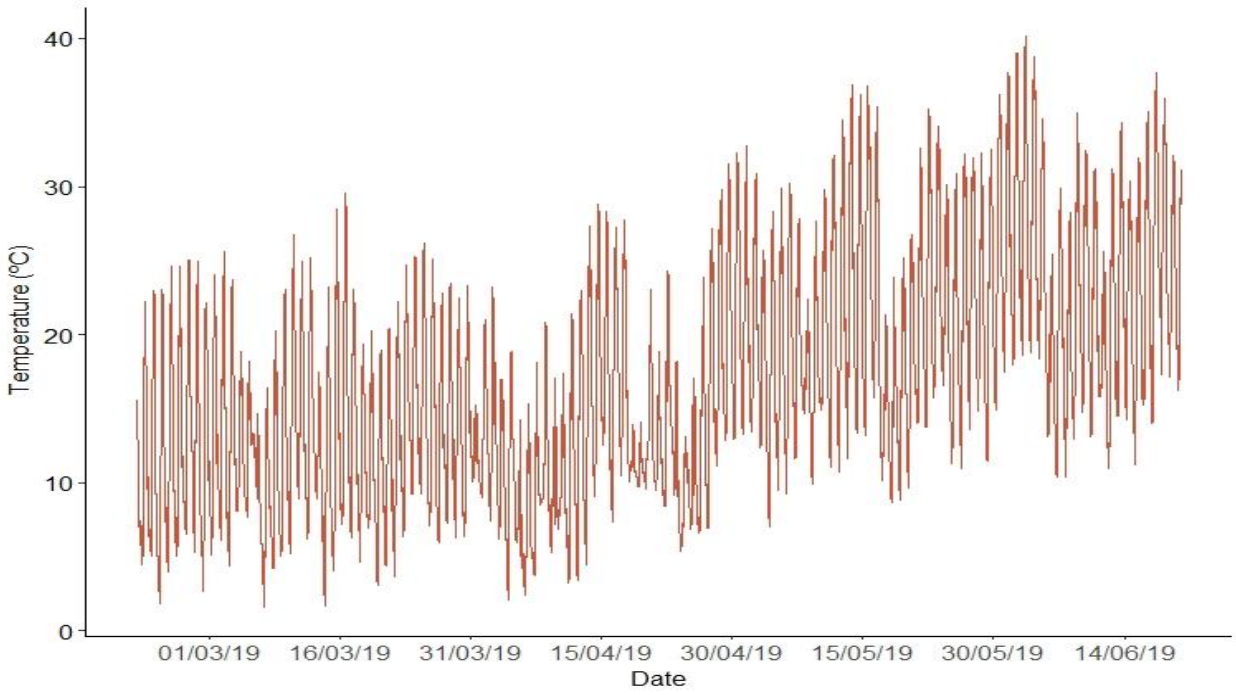
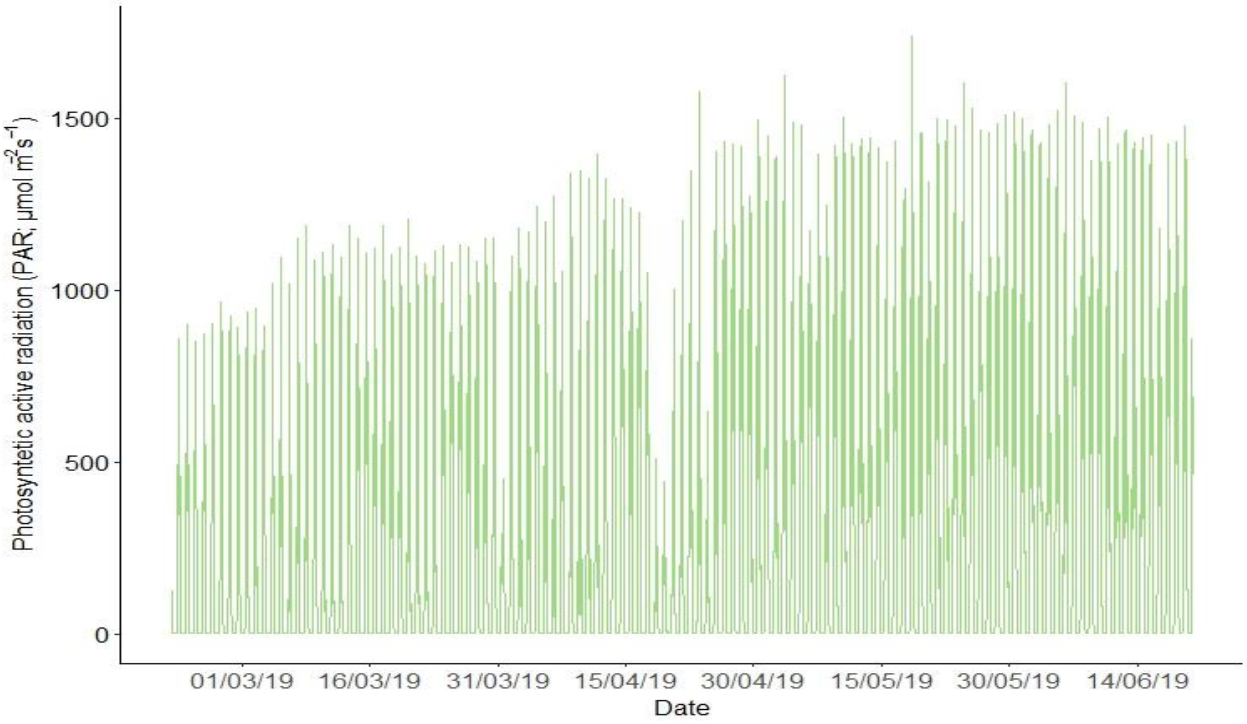
Supp. 1: a) Temperature, b) photosynthetic active radiation (PAR), and c) relative humidity, recorded below the rain exclusion structures throughout the experiment; d) Experimental individuals of *Helianthemum squamatum* growing below the rain exclusion structures.

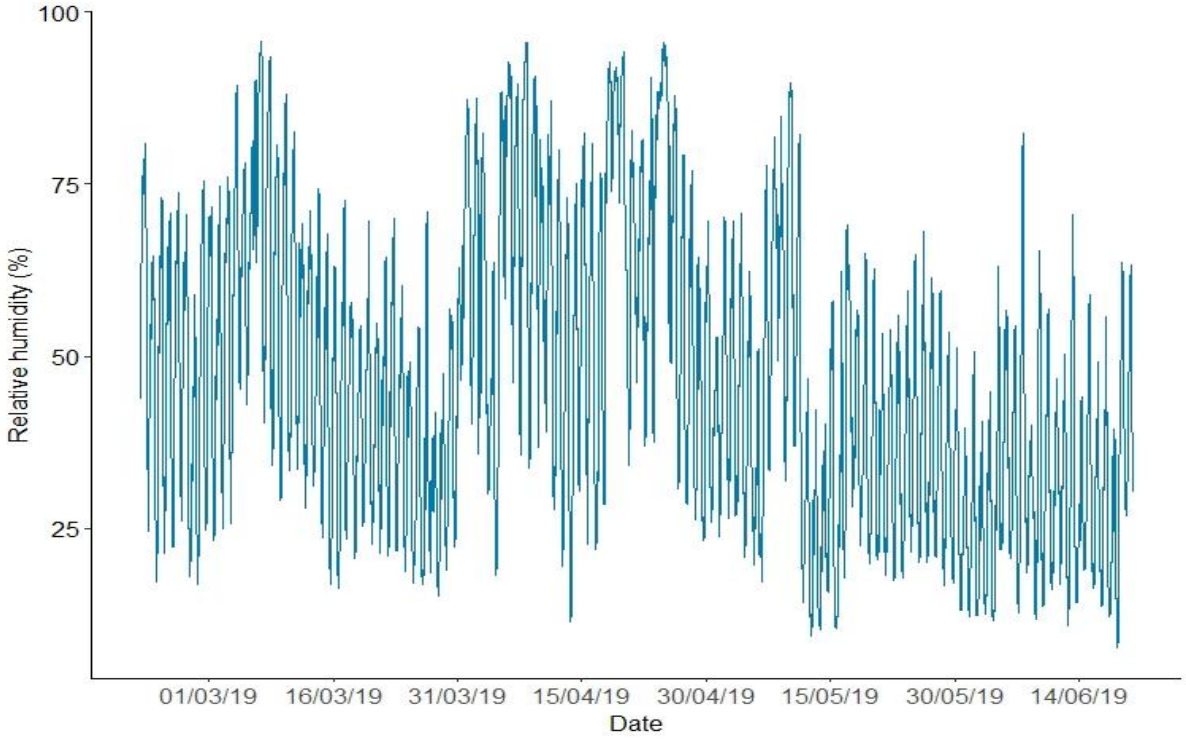
a)



b)


Photosyntetic active radiation (PAR; µmol m-2 s-1)
1500
1000
500
0
01/03/19
16/03/19
31/03/19
15/04/19
30/04/19
15/05/19
30/05/19
14/06/19
Date

c)


Relative humidity (%)
100
75
50
25
01/03/19
16/03/19
31/03/19
15/04/19
30/04/19
15/05/19
30/05/19
14/06/19
Date

d)

Supp. 2: Detailed methods.

Experimental set-up

Rain exclusion structures were built using steel frameworks, and the top of the roof was covered using corrugated transparent polycarbonate sheets (Rooflite, Wetherill Park, Australia), with an inclination of ~10º to avoid the accumulation of rainfall above the structures. The height of the structures (2 and 1.5 meters on the tallest and the shortest side, respectively) and the transparent material of the roof assured a minimal effect of the structures on the conditions below. To compare the microclimatic conditions below and outside the structures, two climatic HOBO H21 Micro Station were set up, one below the rain exclusion structures and one outside. Both stations recorded temperature, photosynthetic active radiation (PAR), and relative humidity every 10 minutes during the experiment. Average temperature and relative humidity below the structures were 16.87 ºC, and 46.63 %, respectively; and 16.21 ºC, and 48.56 % outside the structures, and midday PAR values exceeded 1600 μmol m^{-2} s^{-1} both below and outside the structures in full-sun days.

Collection of phenotypic and fitness traits

Phenological traits— We monitored the reproductive phenology of each plant every three days during the experiment (24 censuses in total). At each census, we visually assessed the presence of inflorescences with open flowers, fully-developed fruits, and dispersed inflorescences. Using these data, we calculated the following phenological variables: a) flowering, fruiting, and dispersal onset, as the number of days elapsed between the onset of the experimental watering treatments and the appearance of the first fully open flower, fully developed fruit, and dispersed inflorescence, respectively; b) flowering, fruiting, and dispersal duration, as the number of days that each plant showed open flowers, fully developed fruits, and dispersed inflorescences, respectively.

Leaf morphological traits— At the reproductive peak of each species (mid-May for *C. hyssopifolia*; early June for *H. squamatum*), we randomly collected five non-senescent, fully-

developed leaves per plant. To maximize complete leaf rehydration, leaves were wrapped in moist filter papers, placed in zipper plastic bags, and stored overnight (12h) in cool (4ºC) and dark conditions. After 12h, the fresh mass of all leaves was weighed using a Mettler Toledo MX5 microbalance. Then, we measured leaf thickness of three leaves per plant using a dial thickness gauge (Mitutoyo Corporation, Tokyo, Japan). Two measurements per leaf were taken, one at each side of the leaf midrib. Next, leaves were scanned using an Epson Perfection V370 Photo scanner (Seiko Epson Corporation, Tokyo, Japan), and oven-dried at 60ºC for 48 h until they were fully dehydrated. Finally, dried leaves were weighed again. From these data, we calculated: i) specific leaf area (SLA) as the one-side area of the scanned leaves divided by their oven-dry mass; ii) leaf dry matter content (LDMC), as the oven-dry mass of the leaves divided by their water-saturated fresh mass; and iii) total estimated leaf area (TELA), as the product of SLA and leaf biomass (see below).

Ecophysiological traits— At the time of leaf collection, we also measured the midday maximum photochemical efficiency (F_v/F_m), and leaf chlorophyll content. Midday photochemical efficiency (F_v/F_m) was measured from 13:00 to 16:30 (UTC + 2) during two consecutive sunny days using a Handy PEA+ chlorophyll fluorimeter (Hansatech, UK). One fully-expanded leaf per plant from a primary branch was adapted to the dark setting a leaf clip for 30 minutes before the measurement. Leaf chlorophyll content was measured in three fully-expanded, non-senescent leaves per plant, using a SPAD 502 chlorophyll meter (Konica Minolta, Tokyo, Japan).

Plant size and growth traits— We measured the height, maximum diameter, and the perpendicular diameter to the maximum diameter in each plant at the onset (March 15th, 2019), and at the end of the watering treatments (July 2nd, 2019). From these, we calculated initial and final plant volume of each plant as the volume of a hemispheroid, $\frac{2}{3}\,\pi\, r_1\, r_2\, h$, where r_1 is the maximum radius, r_2 is the perpendicular radius to maximum radius and h is the height of the

plant; and relative growth rate (RGR), as $(\ln h_2 - \ln h_1)/T_{2-1}$, where h_1 and h_2 are initial and final plant height, respectively, and $T_{2\text{-}1}$ is the time elapsed between the two measurements (108 days). Furthermore, above-ground tissues of each plant were harvested and oven-dried at the end of the experiment. Then, leaves and stems from each individual were manually separated, oven-dried and weighed using a Kern ABJ 120-4M analytical balance (1 mg precision; Kern & Sohn GmbH, Albstadt, Germany). From these data, we calculated aboveground biomass, as the sum of leaf and stem biomass; and leaf:stem ratio, dividing leaf by stem biomass.

Fitness traits— For both species, we haphazardly collected three mature inflorescences per plant before seed dispersion and stored them individually in paper bags. Then, inflorescences were thoroughly dissected to separate viable from aborted/predated seeds to obtain the mean number of viable seeds per inflorescence. To obtain the mean seed mass for each plant, five viable seeds per plant were randomly selected and individually weighed. Finally, before the end of the experiment, we counted the number of viable inflorescences of all plants. From these data, we calculated two integrated plant-level fitness variables: i) total seed number, as the product of the number of viable inflorescences and the number of viable seeds per inflorescence, and ii) total seed mass, as the product of total seed number and the mean seed mass.

Statistical analyses

To assess the adaptive traits of each species in each treatment, we calculated phenotypic and genotypic selection differentials and gradients. To assess directional selection, we estimated a) linear selection differentials (S' = Cov[w,z]), the covariance between relative fitness and a particular standardized trait, which quantify total selection (i.e., direct and indirect selection), and b) linear selection gradients ($\beta' = P^{-1}S'$), the vector of partial regressions of multiple traits included in the same model, which estimate direct selection on each trait. Furthermore, to assess quadratic selection (i.e., stabilizing or disruptive selection), we calculated: a) quadratic

selection differentials (C' = Cov[w,(z-$\bar{z}$ (z-$\bar{z}$)^T) and b) quadratic selection gradients ($\gamma' = P^{-1} C' P^{-1}$), where w is the vector of relative fitness, z is the vector of standardized phenotypic values, and P is the phenotypic variance–covariance matrix (Lande and Arnold, 1983; Phillips and Arnold, 1989). The presence of quadratic selection was considered only when both significant quadratic estimators (C' and/or γ') and an intermediate maximum or minimum in the fitness function were reported (see Ramírez-Valiente et al., 2021).

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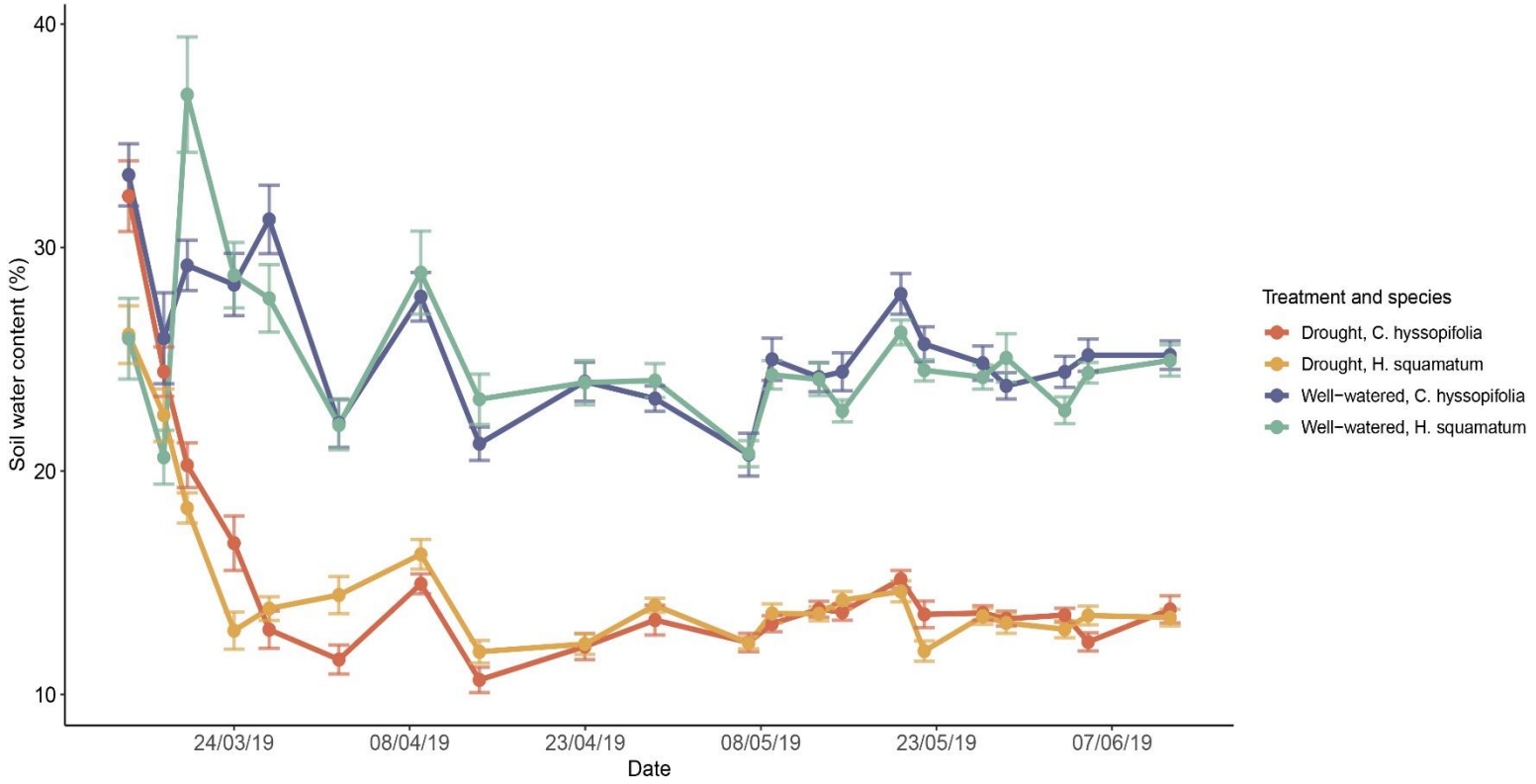
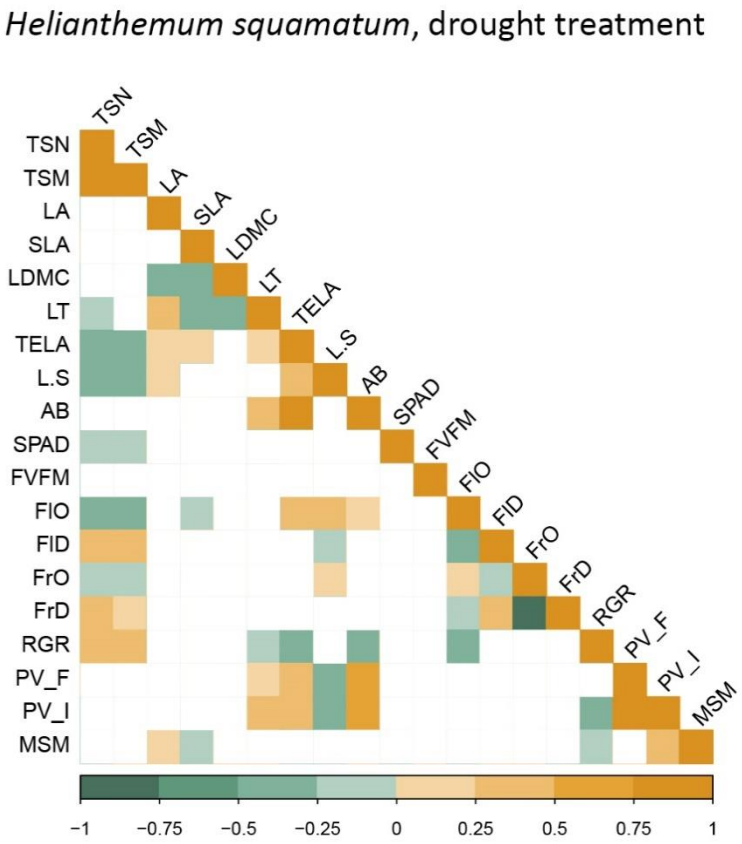
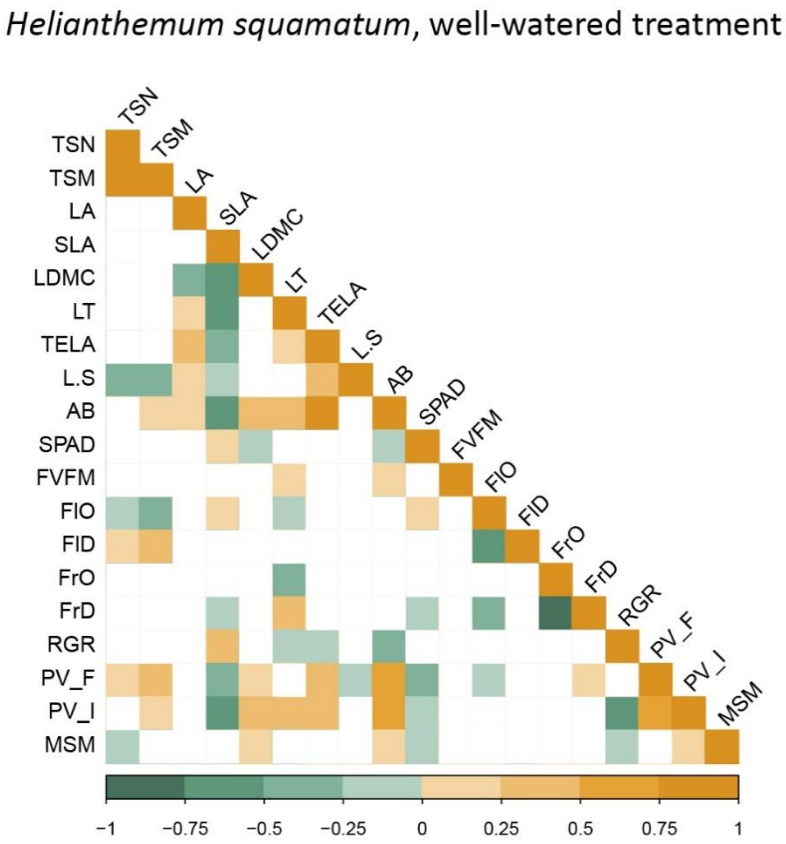
Supp. 3: Soil water content (%) for both species in the two watering treatments. Mean values and standard error in each treatment and species are shown throughout the experiment. Soil water content was monitored every 3–5 days in 12 pots per treatment, using an HH2 Moisture Meter with an ML3 Sensor (Delta-T Devices, Cambridge, UK). Plants in the well-watered treatment were kept at ~100% of field capacity for our gypsum soil (~30-25% of soil water content), and plants in the drought treatment were maintained at ~50% of field capacity (~15-12% of soil water content).

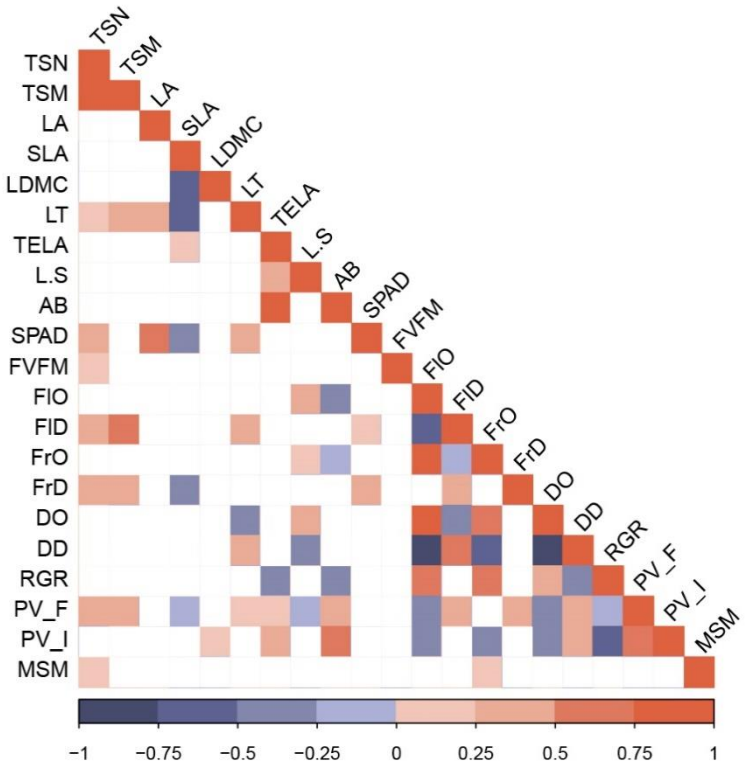


Supp. 4: Correlation matrices of functional traits for both species and treatments. Only significant ($P < 0.05$) Pearson's pairwise correlations are coloured. TSN: Total seed number; TSM: Total seed mass; LA: leaf area; SLA: specific leaf area; LDMC: leaf dry matter content; LT: leaf thickness; TELA: total estimated leaf area; L.S: leaf:stem ratio; AB: aboveground biomass; SPAD: leaf chlorophyll content; FVFM: photochemical efficiency; FlO: flowering onset; FlD: flowering duration; FrO: fruiting onset; FrD: fruiting duration; DO: dispersion onset; DD: dispersion duration; RGR: relative growth rate; PV_F: final plant volume; PV_I: initial plant volume; MSM: maternal seed mass.

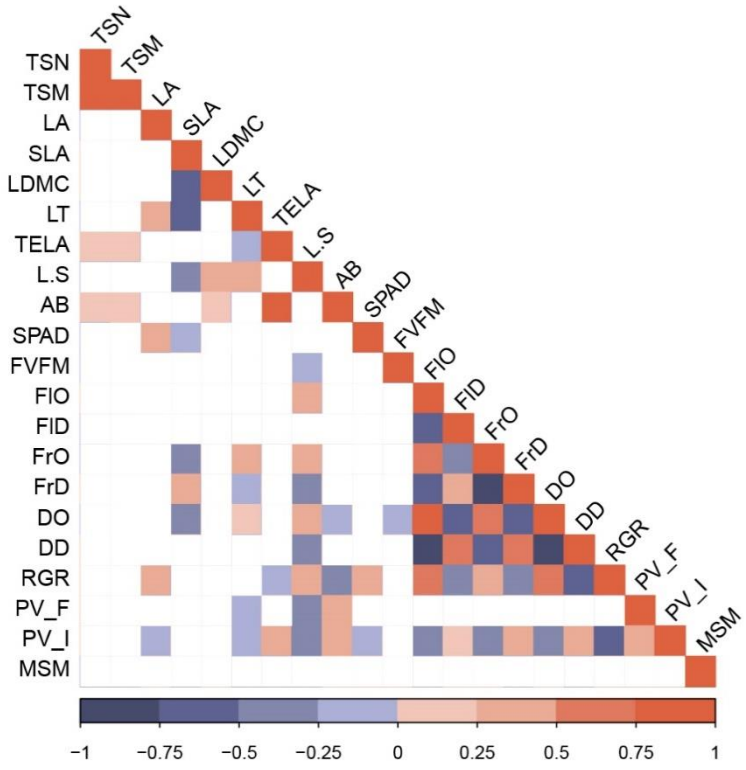




Centaurea hyssopifolia, drought treatment



Centaurea hyssopifolia, well-watered treatment



Supp. 5: Phenotypic directional selection differentials (*S'*) and gradients (*β'*), and their standard error (in parentheses) of both species and treatments using total seed mass fitness variable. Analyses were performed including and excluding covariates in the models, to corroborate that selection estimates were very similar. Significant ($p < 0.05$) and marginally significant ($0.05 < p < 0.1$) values after FDR correction are in bold and italic, respectively. Significance levels: · $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

	***H. squamatum*, drought treatment**		***H. squamatum*, well-watered treatment**		***C. hyssopifolia*, drought treatment**		***C. hyssopifolia*, well-watered treatment**	
	With covariates	**Without covariates**	**With covariates**	**Without covariates**	**With covariates**	**Without covariates**	**With covariates**	**Without covariates**
Selection differentials	*S'* (SE)	*S'* (SE)	*S'* (SE)	*S'* (SE)	*S'* (SE)	*S'* (SE)	*S'* (SE)	*S'* (SE)
Flowering onset	**-0.698 (0.101) *** **	**-0.705 (0.079) *** **	*-0.074 (0.029) ·*	**-0.090 (0.030) ***	-0.025 (0.121)	-0.044 (0.088)	0.057 (0.058)	0.030 (0.047)
Flowering duration	**0.391 (0.098) *** **	**0.294 (0.093) ** **	*0.074 (0.028) ·*	**0.090 (0.029) ***	**0.525 (0.097) *** **	**0.441 (0.079)**	-0.077 (0.052)	-0.060 (0.049)
Fruiting onset	**-0.667 (0.118) *** **	**-0.713 (0.115) *** **	-0.039 (0.029)	-0.034 (0.032)	0.083 (0.128)	0.083 (0.091)	0.026 (0.056)	0.014 (0.047)
Fruiting duration	-----	-----	-----	-----	**0.424 (0.112) ** **	**0.374 (0.086)**	-0.006 (0.055)	0.016 (0.049)
SPAD	**-0.282 (0.083) ** **	*-0.182 (0.083) ·*	0.011 (0.030)	-0.015 (0.030)	0.197 (0.121)	0.137 (0.095)	0.070 (0.052)	0.059 (0.049)
F_v/F_m	-0.038 (0.083)	-0.045 (0.078)	0.013 (0.030)	0.021 (0.031)	0.058 (0.115)	0.015 (0.085)	-0.036 (0.051)	-0.021 (0.049)
Leaf area	-0.035 (0.097)	-0.032 (0.082)	-0.005 (0.029)	-0.002 (0.030)	0.124 (0.115)	0.081 (0.096)	0.005 (0.050)	-0.008 (0.047)
SLA	0.091 (0.101)	0.053 (0.084)	0.006 (0.033)	-0.041 (0.030)	-0.159 (0.109)	-0.123 (0.089)	0.042 (0.048)	0.049 (0.047)
LDMC	0.098 (0.091)	0.020 (0.082)	-0.032 (0.031)	0.003 (0.030)	-0.068 (0.113)	-0.077 (0.087)	0.010 (0.050)	-0.001 (0.047)
Leaf thickness	-0.155 (0.100)	-0.149 (0.081)	-0.001 (0.030)	0.021 (0.030)	**0.352 (0.117) ***	**0.258 (0.092)**	-0.052 (0.049)	-0.055 (0.047)
Leaf:stem ratio	**-0.472 (0.098) *** **	**-0.345 (0.083) *** **	**-0.085 (0.029) ***	**-0.107 (0.029) ** **	0.025 (0.118)	0.011 (0.095)	-0.048 (0.055)	-0.060 (0.049)
RGR	**0.283 (0.121) ***	**0.286 (0.083) ** **	0.031 (0.034)	-0.025 (0.030)	0.283 (0.143)	0.097 (0.091)	0.104 (0.071)	0.042 (0.047)
Aboveground biomass	-0.037 (0.155)	*-0.160 (0.083) ·*	0.043 (0.045)	**0.081 (0.030) ***	0.185 (0.145)	0.178 (0.094)	*0.145 (0.052) ·*	0.106 (0.045)
Selection gradients	*β'* (SE)	*β'* (SE)	*β'* (SE)	*β'* (SE)	*β'* (SE)	*β'* (SE)	*β'* (SE)	*β'* (SE)
Flowering onset	**-0.442 (0.109) *** **	**-0.445 (0.095) *** **	-0.069 (0.048)	-0.069 (0.048)	0.059 (0.208)	0.191 (0.201)	0.079 (0.101)	0.099 (0.096)
Flowering duration	**0.229 (0.092) ***	**0.251 (0.088) ***	0.019 (0.045)	0.019 (0.046)	**0.609 (0.109) *** **	**0.568 (0.110) *** **	0.002 (0.074)	0.008 (0.072)
Fruiting onset	**-0.573 (0.107) *** **	**-0.615 (0.110) *** **	-0.019 (0.038)	-0.011 (0.038)	0.303 (0.155)	0.269 (0.143)	0.002 (0.124)	0.064 (0.103)
Fruiting duration	-----	-----	-----	-----	0.220 (0.106)	0.169 (0.098)	0.017 (0.133)	0.092 (0.106)
SPAD	**-0.195 (0.079) ***	-0.119 (0.079)	0.002 (0.032)	-0.013 (0.031)	-0.175 (0.121)	-0.011 (0.111)	0.046 (0.083)	0.058 (0.076)
F_v/F_m	-0.021 (0.075)	0.018 (0.071)	0.032 (0.031)	0.030 (0.031)	0.045 (0.090)	-0.016 (0.080)	-0.011 (0.055)	-0.011 (0.052)
Leaf area	0.114 (0.106)	0.089 (0.091)	-0.004 (0.033)	-0.010 (0.033)	0.204 (0.123)	0.084 (0.125)	-0.012 (0.071)	-0.028 (0.065)
SLA	0.011 (0.158)	-0.037 (0.147)	-0.127 (0.072)	-0.150 (0.073)	-0.307 (0.180)	-0.316 (0.175)	0.111 (0.141)	0.094 (0.131)
LDMC	0.232 (0.146)	0.187 (0.143)	-0.099 (0.066)	-0.115 (0.066)	-0.232 (0.176)	-0.383 (0.158)	0.102 (0.109)	0.085 (0.102)
Leaf thickness	0.007 (0.148)	0.032 (0.143)	-0.062 (0.057)	-0.078 (0.058)	0.174 (0.139)	0.025 (0.132)	0.055 (0.117)	0.048 (0.108)
Leaf:stem ratio	**-0.293 (0.102) ***	**-0.257 (0.084) ***	**-0.122 (0.035) ** **	**-0.135 (0.033) ** **	0.029 (0.090)	-0.007 (0.088)	-0.065 (0.072)	-0.082 (0.065)
RGR	0.098 (0.093)	0.142 (0.086)	0.038 (0.039)	0.028 (0.035)	0.110 (0.128)	0.025 (0.105)	0.029 (0.087)	0.027 (0.074)
Aboveground biomass	0.027 (0.125)	-0.149 (0.084)	0.029 (0.052)	0.059 (0.038)	0.002 (0.121)	0.125 (0.098)	0.132 (0.070)	0.117 (0.058)

Supp. 6: Phenotypic and genotypic directional selection differentials (S') and gradients (β'), and their standard error (in parentheses) of both species and treatments for total seed number fitness variable. Significant ($p < 0.05$) and marginally significant ($0.05 < p < 0.1$) values after FDR correction are in bold and italic, respectively. Significance levels: · $p < 0.1$; ***** $p < 0.05$; ****** $p < 0.01$; ******* $p < 0.001$.

	***H. squamatum*, drought treatment**		***H. squamatum*, well-watered treatment**		***C. hyssopifolia*, drought treatment**		***C. hyssopifolia*, well-watered treatment**	
Phenotypic selection analysis	S' (SE)	β' (SE)	S' (SE)	β' (SE)	S' (SE)	β' (SE)	S' (SE)	β' (SE)
Flowering onset	**-0.678 (0.099)*****	**-0.427 (0.110)****	-0.061 (0.032)	-0.062 (0.051)	0.056 (0.133)	0.229 (0.218)	0.050 (0.054)	0.051 (0.093)
Flowering duration	**0.456 (0.099)*****	**0.243 (0.096)***	0.061 (0.031)	-0.010 (0.048)	**0.528 (0.122)*****	**0.555 (0.127)*****	-0.080 (0.048)	-0.018 (0.067)
Fruiting onset	**-0.691 (0.117)*****	**-0.600 (0.108)*****	-0.052 (0.032)	-0.015 (0.041)	0.165 (0.130)	0.347 (0.152)	0.012 (0.052)	0.053 (0.113)
Fruiting duration	-----	-----	-----	-----	**0.544 (0.104)*****	**0.358 (0.114)***	0.012 (0.051)	0.085 (0.121)
SPAD	**-0.262 (0.087)****	*-0.180 (0.081)*·	-0.002 (0.032)	0.000 (0.034)	**0.293 (0.114)***	0.100 (0.133)	0.006 (0.048)	0.007 (0.074)
F_v/F_m	-0.046 (0.085)	-0.032 (0.076)	-0.030 (0.033)	-0.022 (0.033)	0.213 (0.103)	0.111 (0.098)	-0.021 (0.047)	-0.014 (0.049)
Leaf area	-0.076 (0.096)	0.091 (0.110)	-0.019 (0.031)	-0.013 (0.035)	0.126 (0.110)	0.070 (0.131)	-0.063 (0.046)	-0.060 (0.065)
SLA	0.130 (0.105)	-0.011 (0.161)	0.023 (0.036)	-0.023 (0.077)	-0.203 (0.120)	-0.408 (0.198)	0.058 (0.045)	0.072 (0.129)
LDMC	0.100 (0.093)	0.212 (0.153)	-0.025 (0.034)	-0.010 (0.070)	-0.041 (0.118)	-0.397 (0.194)	0.015 (0.045)	0.082 (0.099)
Leaf thickness	*-0.212 (0.100)*·	-0.026 (0.154)	-0.006 (0.032)	0.015 (0.061)	0.257 (0.122)	0.006 (0.159)	-0.081 (0.045)	0.019 (0.107)
Leaf:stem ratio	**-0.469 (0.098)*****	**-0.260 (0.105)***	**-0.116 (0.030)****	**-0.144 (0.038)****	0.147 (0.111)	0.015 (0.108)	-0.055 (0.050)	-0.063 (0.066)
RGR	*0.268 (0.120)*·	0.080 (0.094)	0.041 (0.037)	0.025 (0.041)	0.097 (0.156)	-0.167 (0.145)	0.074 (0.066)	0.045 (0.080)
Aboveground biomass	-0.030 (0.157)	-0.025 (0.127)	0.004 (0.049)	-0.005 (0.056)	0.225 (0.140)	0.022 (0.126)	0.121 (0.049)	0.117 (0.064)
Genotypic selection analysis								
Flowering onset	**-0.680 (0.095)*****	-0.269 (0.148)	-0.036 (0.038)	0.040 (0.038)	0.023 (0.106)	0.225 (0.287)	0.086 (0.054)	0.226 (0.207)
Flowering duration	**0.319 (0.107)***	-0.115 (0.135)	0.060 (0.038)	0.043 (0.062)	**0.345 (0.092)****	0.383 (0.232)	-0.065 (0.051)	0.084 (0.096)
Fruiting onset	**-0.639 (0.109)*****	**-0.506 (0.109)*****	-0.017 (0.039)	0.075 (0.058)	0.254 (0.105)	0.203 (0.217)	-0.022 (0.054)	-0.117 (0.263)
Fruiting duration	-----	-----	-----	-----	**0.278 (0.098)***	0.113 (0.160)	0.035 (0.053)	0.053 (0.205)
SPAD	-0.065 (0.112)	0.156 (0.099)	0.017 (0.041)	-0.053 (0.074)	0.217 (0.099)	0.047 (0.223)	-0.040 (0.051)	-0.036 (0.113)
F_v/F_m	0.053 (0.108)	0.115 (0.092)	0.023 (0.039)	-0.039 (0.041)	-0.040 (0.099)	-0.009 (0.137)	0.029 (0.052)	0.028 (0.067)
Leaf area	- 0.116 (0.12)	0.056 (0.122)	-0.071 (0.039)	-0.028 (0.046)	0.108 (0.097)	0.168 (0.217)	0.016 (0.052)	0.011 (0.095)
SLA	0.210 (0.114)	0.083 (0.150)	0.075 (0.050)	-0.032 (0.126)	-0.109 (0.099)	-0.372 (0.339)	0.015 (0.051)	0.083 (0.179)
LDMC	-0.126 (0.109)	-0.121 (0.148)	0.012 (0.044)	0.010 (0.101)	-0.040 (0.095)	-0.380 (0.287)	0.018 (0.051)	0.064 (0.135)
Leaf thickness	-0.195 (0.118)	-0.021 (0.188)	-0.051 (0.040)	-0.064 (0.074)	0.189 (0.096)	-0.126 (0.230)	0.001 (0.052)	0.083 (0.144)
Leaf:stem ratio	**-0.509 (0.097)*****	-0.246 (0.127)	**-0.136 (0.033)****	-0.120 (0.049)	0.090 (0.097)	0.134 (0.152)	-0.007 (0.058)	0.005 (0.093)
RGR	**0.493 (0.127)****	0.345 (0.128)	0.063 (0.056)	-0.043 (0.039)	**0.370 (0.124)***	0.072 (0.212)	0.105 (0.064)	0.100 (0.129)
Aboveground biomass	-0.077 (0.137)	0.090 (0.125)	-0.130 (0.059)	0.052 (0.058)	0.017 (0.110)	0.059 (0.148)	0.020 (0.055)	0.060 (0.078)

Supp. 7: Phenotypic and genotypic quadratic selection differentials (*C'*) and gradients (*γ'*), and their standard error (in parentheses) of both species and treatments for total seed number fitness variable. Significant ($p < 0.05$) and marginally significant ($0.05 < p < 0.1$) values after FDR correction are in bold and italic, respectively. Significance levels: · $p < 0.1$; ***** $p < 0.05$; ****** $p < 0.01$.

	***H. squamatum*, drought treatment**		***H. squamatum*, well-watered treatment**		***C. hyssopifolia*, drought treatment**		***C. hyssopifolia*, well-watered treatment**	
Phenotypic selection analysis	*C*' (SE)	*γ*' (SE)	*C*' (SE)	*γ*' (SE)	*C*' (SE)	*γ*' (SE)	*C*' (SE)	*γ*' (SE)
Flowering onset	**-0.360 (0.107) ****	*-0.312 (0.113)* ·	0.033 (0.048)	-0.009 (0.052)	-0.121 (0.206)	0.071 (0.315)	-0.055 (0.060)	0.014 (0.087)
Flowering duration	-0.218 (0.176)	-0.164 (0.145)	0.001 (0.020)	0.067 (0.031)	0.008 (0.188)	-0.249 (0.200)	-0.073 (0.079)	-0.245 (0.114)
Fruiting onset	0.123 (0.131)	0.109 (0.109)	0.058 (0.038)	-----	-0.282 (0.197)	-0.317 (0.238)	-0.066 (0.084)	0.096 (0.196)
Fruiting duration	-----	-----	-----	-----	-0.225 (0.207)	0.182 (0.212)	-0.049 (0.078)	-0.251 (0.188)
SPAD	-0.060 (0.141)	0.052 (0.119)	0.109 (0.050)	0.108 (0.052)	-0.208 (0.201)	0.419 (0.244)	0.021 (0.060)	-0.048 (0.100)
F_v/F_m	0.222 (0.145)	0.174 (0.129)	-0.034 (0.044)	-0.044 (0.043)	0.370 (0.218)	0.241 (0.183)	0.063 (0.076)	0.161 (0.102)
Leaf area	0.117 (0.133)	-0.080 (0.118)	-0.069 (0.040)	-0.055 (0.041)	0.027 (0.148)	*-0.344 (0.147)* ·	0.021 (0.067)	0.052 (0.082)
SLA	-0.075 (0.106)	-0.197 (0.152)	-0.020 (0.052)	0.003 (0.064)	-0.045 (0.139)	*-0.484 (0.177)* ·	0.017 (0.061)	0.103 (0.093)
LDMC	0.193 (0.140)	*0.322 (0.125)* ·	0.027 (0.050)	-0.019 (0.062)	-0.123 (0.134)	*0.392 (0.156)* ·	-0.107 (0.054)	-0.049 (0.088)
Leaf thickness	-0.007 (0.142)	-0.009 (0.150)	-0.022 (0.043)	0.035 (0.053)	-0.115 (0.167)	0.184 (0.161)	0.115 (0.063)	0.090 (0.088)
Leaf:stem ratio	0.225 (0.138)	0.016 (0.134)	-0.082 (0.044)	-0.097 (0.053)	0.089 (0.171)	0.019 (0.141)	-0.037 (0.084)	-0.069 (0.112)
RGR	0.022 (0.116)	0.177 (0.106)	-0.017 (0.040)	-0.013 (0.047)	-0.073 (0.169)	0.085 (0.128)	0.047 (0.063)	-0.030 (0.092)
Aboveground biomass	-0.342 (0.143)	-0.119 (0.140)	**-0.148 (0.049) ***	-0.083 (0.057)	-0.131 (0.218)	-0.097 (0.166)	0.041 (0.071)	0.159 (0.114)
Genotypic selection analysis								
Flowering onset	**-0.379 (0.109) ***	-0.153 (0.135)	-0.011 (0.055)	0.014 (0.096)	*-0.307 (0.140)* ·	0.387 (0.332)	0.153 (0.070)	0.173 (0.307)
Flowering duration	-0.166 (0.166)	-0.136 (0.243)	0.012 (0.050)	-0.094 (0.106)	-0.163 (0.118)	-0.314 (0.275)	0.095 (0.085)	0.126 (0.584)
Fruiting onset	-0.297 (0.174)	-0.364 (0.180)	-0.062 (0.044)	-0.019 (0.064)	**-0.593 (0.167) ***	-0.552 (0.391)	0.002 (0.095)	0.719 (0.690)
Fruiting duration	-----	-----	-----	-----	**-0.576 (0.176) ***	-0.510 (0.380)	-0.047 (0.098)	-1.079 (0.739)
SPAD	0.034 (0.169)	0.150 (0.210)	0.012 (0.057)	-0.057 (0.110)	**-0.480 (0.150) ***	-0.698 (0.312)	0.057 (0.088)	0.107 (0.187)
F_v/F_m	-0.070 (0.153)	0.352 (0.190)	0.013 (0.079)	-0.125 (0.136)	0.065 (0.154)	-0.036 (0.335)	0.004 (0.068)	0.307 (0.247)
Leaf area	-0.040 (0.162)	0.014 (0.168)	-0.032 (0.068)	0.004 (0.105)	-0.090 (0.140)	0.357 (0.384)	0.043 (0.090)	-0.037 (0.195)
SLA	-0.105 (0.152)	-0.090 (0.169)	0.024 (0.080)	0.063 (0.177)	-0.160 (0.177)	-0.361 (0.392)	0.164 (0.081)	0.108 (0.300)
LDMC	-0.002 (0.219)	0.271 (0.216)	0.108 (0.064)	-0.038 (0.109)	0.114 (0.146)	0.230 (0.361)	-0.021 (0.101)	-0.274 (0.284)
Leaf thickness	0.109 (0.179)	0.077 (0.206)	-0.057 (0.054)	0.053 (0.097)	0.124 (0.141)	0.087 (0.324)	0.120 (0.112)	0.319 (0.333)
Leaf:stem ratio	-0.203 (0.150)	0.077 (0.205)	-0.143 (0.054)	-0.188 (0.113)	0.076 (0.179)	-0.189 (0.348)	0.083 (0.092)	-0.052 (0.285)
RGR	-0.070 (0.191)	-0.445 (0.266)	-0.001 (0.065)	0.020 (0.085)	-0.242 (0.173)	-0.271 (0.672)	0.177 (0.083)	0.130 (0.450)
Aboveground biomass	0.345 (0.185)	0.465 (0.178)	-0.056 (0.066)	-0.065 (0.093)	*0.413 (0.176)* ·	0.132 (0.341)	0.147 (0.092)	-0.421 (0.407)

Supp. 8: Genetic variance-covariance matrices (G-matrix) of functional traits for both species and treatments. FlO: flowering onset; FlD: flowering duration; FrO: fruiting onset; FrD: fruiting duration; SPAD: leaf chlorophyll content; FVFM: photochemical efficiency; LA: leaf area; SLA: specific leaf area; LDMC: leaf dry matter content; LT: leaf thickness; L.S: leaf:stem ratio; RGR: relative growth rate; AB: aboveground biomass; Fitness1: Total seed number; Fitness2: Total seed mass.

Helianthemum squamatum, drought treatment

	FlO	FlD	FrO	SPAD	FVFM	LA	SLA	LDMC	LT	L:S	RGR	AB	Fitness1	Fitness2
FlO	0.737	-0.333	0.248	0.121	0.044	0.110	-0.137	0.039	0.135	0.266	-0.213	0.158	-0.417	-0.431
FlD	-0.333	0.749	-0.307	-0.030	-0.022	0.077	0.012	-0.114	0.043	-0.163	0.006	-0.023	0.272	0.284
FrO	0.248	-0.307	0.682	0.044	0.037	0.062	-0.078	0.032	0.042	0.134	-0.080	0.031	-0.256	-0.256
SPAD	0.121	-0.030	0.044	0.536	-0.019	0.016	-0.042	0.041	-0.038	0.082	-0.024	-0.009	-0.113	-0.110
FVFM	0.044	-0.022	0.037	-0.019	0.628	-0.025	0.058	0.040	-0.106	0.046	-0.040	0.065	-0.049	-0.043
LA	0.110	0.077	0.062	0.016	-0.025	0.614	-0.089	-0.173	0.263	0.135	-0.066	0.064	-0.063	-0.050
SLA	-0.137	0.012	-0.078	-0.042	0.058	-0.089	0.637	-0.275	-0.265	-0.129	0.070	-0.034	0.127	0.112
LDMC	0.039	-0.114	0.032	0.041	0.040	-0.173	-0.275	0.584	-0.236	0.101	0.036	-0.037	-0.009	-0.012
LT	0.135	0.043	0.042	-0.038	-0.106	0.263	-0.265	-0.236	0.628	0.067	-0.143	0.133	-0.156	-0.130
L:S	0.266	-0.163	0.134	0.082	0.046	0.135	-0.129	0.101	0.067	0.695	-0.049	-0.030	-0.286	-0.291
RGR	-0.213	0.006	-0.080	-0.024	-0.040	-0.066	0.070	0.036	-0.143	-0.049	0.611	-0.185	0.281	0.274
AB	0.158	-0.023	0.031	-0.009	0.065	0.064	-0.034	-0.037	0.133	-0.030	-0.185	0.575	-0.145	-0.125
Fitness1	-0.417	0.272	-0.256	-0.113	-0.049	-0.063	0.127	-0.009	-0.156	-0.286	0.281	-0.145	0.757	0.709
Fitness2	-0.431	0.284	-0.256	-0.110	-0.043	-0.050	0.112	-0.012	-0.130	-0.291	0.274	-0.125	0.709	0.761

Helianthemum squamatum, well-watered treatment

	FlO	FlD	FrO	SPAD	FVFM	LA	SLA	LDMC	LT	L:S	RGR	AB	Fitness1	Fitness2
FlO	0.646	-0.421	0.064	0.042	0.113	0.091	0.100	-0.012	-0.139	0.106	-0.014	0.014	-0.158	-0.196
FlD	-0.421	0.630	-0.063	0.005	-0.091	-0.106	-0.019	-0.026	0.104	-0.034	0.043	-0.028	0.096	0.114
FrO	0.064	-0.063	0.560	0.071	0.032	-0.010	0.045	0.031	-0.126	-0.057	-0.014	-0.065	-0.055	-0.041
SPAD	0.042	0.005	0.071	0.580	-0.022	0.021	0.084	-0.088	-0.086	-0.051	0.014	-0.098	-0.003	-0.012
FVFM	0.113	-0.091	0.032	-0.022	0.629	0.044	-0.051	0.001	0.093	-0.032	-0.091	0.114	-0.026	0.025
LA	0.091	-0.106	-0.010	0.021	0.044	0.630	-0.026	-0.159	0.130	0.127	-0.020	0.154	-0.110	-0.064
SLA	0.100	-0.019	0.045	0.084	-0.051	-0.026	0.690	-0.429	-0.309	-0.135	0.262	-0.357	0.063	-0.026
LDMC	-0.012	-0.026	0.031	-0.088	0.001	-0.159	-0.429	0.656	-0.088	0.050	-0.130	0.202	-0.024	0.007
LT	-0.139	0.104	-0.126	-0.086	0.093	0.130	-0.309	-0.088	0.619	0.107	-0.164	0.207	-0.036	0.000
L:S	0.106	-0.034	-0.057	-0.051	-0.032	0.127	-0.135	0.050	0.107	0.724	0.053	0.088	-0.284	-0.240
RGR	-0.014	0.043	-0.014	0.014	-0.091	-0.020	0.262	-0.130	-0.164	0.053	0.653	-0.302	0.051	-0.043
AB	0.014	-0.028	-0.065	-0.098	0.114	0.154	-0.357	0.202	0.207	0.088	-0.302	0.669	-0.068	0.040
Fitness1	-0.158	0.096	-0.055	-0.003	-0.026	-0.110	0.063	-0.024	-0.036	-0.284	0.051	-0.068	0.601	0.501
Fitness2	-0.196	0.114	-0.041	-0.012	0.025	-0.064	-0.026	0.007	0.000	-0.240	-0.043	0.040	0.501	0.602

Centaurea hyssopifolia, drought treatment

	FlO	FlD	FrO	FrD	SPAD	FVFM	LA	SLA	LDMC	LT	L:S	RGR	AB	Fitness1	Fitness2
FlO	0.582	-0.223	0.448	0.001	0.034	0.038	-0.040	-0.068	0.028	-0.042	0.142	0.306	-0.149	0.104	0.109
FlD	-0.223	0.591	-0.090	0.246	0.120	-0.016	0.039	-0.112	0.029	0.141	-0.034	-0.029	0.043	0.216	0.323
FrO	0.448	-0.090	0.708	-0.032	0.040	0.036	-0.040	-0.052	-0.025	-0.009	0.098	0.321	-0.110	0.146	0.210
FrD	0.001	0.246	-0.032	0.639	0.152	0.037	0.016	-0.175	0.091	0.058	0.010	0.079	-0.011	0.235	0.295
SPAD	0.034	0.120	0.040	0.152	0.703	-0.047	0.417	-0.193	0.099	0.240	0.115	0.140	0.002	0.153	0.213
FVFM	0.038	-0.016	0.036	0.037	-0.047	0.643	-0.024	0.090	-0.036	-0.045	-0.047	-0.024	-0.048	0.058	0.028
LA	-0.040	0.039	-0.040	0.016	0.417	-0.024	0.701	-0.045	0.001	0.230	0.036	0.100	0.021	0.082	0.125
SLA	-0.068	-0.112	-0.052	-0.175	-0.193	0.090	-0.045	0.635	-0.396	-0.278	-0.063	-0.031	-0.015	-0.109	-0.146
LDMC	0.028	0.029	-0.025	0.091	0.099	-0.036	0.001	-0.396	0.629	-0.058	0.094	-0.049	0.084	-0.030	-0.051
LT	-0.042	0.141	-0.009	0.058	0.240	-0.045	0.230	-0.278	-0.058	0.634	-0.019	0.000	-0.013	0.128	0.205
L:S	0.142	-0.034	0.098	0.010	0.115	-0.047	0.036	-0.063	0.094	-0.019	0.642	0.046	0.068	0.090	0.129
RGR	0.306	-0.029	0.321	0.079	0.140	-0.024	0.100	-0.031	-0.049	0.000	0.046	0.659	-0.200	0.118	0.168
AB	-0.149	0.043	-0.110	-0.011	0.002	-0.048	0.021	-0.015	0.084	-0.013	0.068	-0.200	0.626	0.068	0.073
Fitness1	0.104	0.216	0.146	0.235	0.153	0.058	0.082	-0.109	-0.030	0.128	0.090	0.118	0.068	0.615	0.643
Fitness2	0.109	0.323	0.210	0.295	0.213	0.028	0.125	-0.146	-0.051	0.205	0.129	0.168	0.073	0.643	0.923

Centaurea hyssopifolia, well-watered treatment

	FlO	FlD	FrO	FrD	SPAD	FVFM	LA	SLA	LDMC	LT	L:S	RGR	AB	Fitness1	Fitness2
FlO	0.720	-0.419	0.458	-0.438	-0.027	-0.098	0.061	-0.152	0.084	0.143	0.297	0.380	-0.115	-0.015	0.031
FlD	-0.419	0.694	-0.259	0.274	0.048	0.073	0.004	0.048	-0.049	-0.058	-0.091	-0.249	0.002	-0.046	-0.065
FrO	0.458	-0.259	0.724	-0.568	0.090	-0.098	0.076	-0.259	0.126	0.218	0.342	0.308	-0.091	-0.070	-0.014
FrD	-0.438	0.274	-0.568	0.719	-0.075	0.112	-0.008	0.261	-0.137	-0.203	-0.353	-0.223	0.112	0.108	0.049
SPAD	-0.027	0.048	0.090	-0.075	0.710	-0.045	0.331	-0.163	0.060	0.101	0.020	0.174	0.033	-0.022	0.082
FVFM	-0.098	0.073	-0.098	0.112	-0.045	0.679	-0.054	0.073	0.062	-0.108	-0.136	-0.056	-0.026	0.029	0.006
LA	0.061	0.004	0.076	-0.008	0.331	-0.054	0.722	-0.130	-0.044	0.252	0.057	0.199	0.027	-0.101	0.025
SLA	-0.152	0.048	-0.259	0.261	-0.163	0.073	-0.130	0.719	-0.384	-0.437	-0.314	-0.080	-0.032	0.123	0.067
LDMC	0.084	-0.049	0.126	-0.137	0.060	0.062	-0.044	-0.384	0.697	-0.042	0.190	0.010	0.125	-0.010	0.001
LT	0.143	-0.058	0.218	-0.203	0.101	-0.108	0.252	-0.437	-0.042	0.727	0.236	0.091	-0.060	-0.129	-0.066
L:S	0.297	-0.091	0.342	-0.353	0.020	-0.136	0.057	-0.314	0.190	0.236	0.743	0.194	-0.098	-0.129	-0.099
RGR	0.380	-0.249	0.308	-0.223	0.174	-0.056	0.199	-0.080	0.010	0.091	0.194	0.692	-0.172	0.000	0.057
AB	-0.115	0.002	-0.091	0.112	0.033	-0.026	0.027	-0.032	0.125	-0.060	-0.098	-0.172	0.641	0.108	0.120
Fitness1	-0.015	-0.046	-0.070	0.108	-0.022	0.029	-0.101	0.123	-0.010	-0.129	-0.129	0.000	0.108	0.658	0.491
Fitness2	0.031	-0.065	-0.014	0.049	0.082	0.006	0.025	0.067	0.001	-0.066	-0.099	0.057	0.120	0.491	0.660

Supp. 9: Phenotypic and genotypic directional selection differentials (*S'*) and gradients (*β'*), and their standard error (in parentheses) of both species and treatments for total seed mass fitness variable. Significant ($p < 0.05$) and marginally significant ($0.05 < p < 0.1$) values after FDR correction are in bold and italic, respectively. Significance levels: · $p < 0.1$; ***** $p < 0.05$; ****** $p < 0.01$; ******* $p < 0.001$.

	***H. squamatum*, drought treatment**		***H. squamatum*, well-watered treatment**		***C. hyssopifolia*, drought treatment**		***C. hyssopifolia*, well-watered treatment**	
Phenotypic selection analysis	*S'* (SE)	*β'* (SE)	*S'* (SE)	*β'* (SE)	*S'* (SE)	*β'* (SE)	*S'* (SE)	*β'* (SE)
Flowering onset	**-0.698 (0.101)*****	**-0.442 (0.109)*****	*-0.074 (0.029)·*	-0.069 (0.048)	-0.025 (0.121)	0.059 (0.208)	0.057 (0.058)	0.079 (0.101)
Flowering duration	**0.391 (0.098)*****	**0.229 (0.092)***	*0.074 (0.028)·*	0.019 (0.045)	**0.525 (0.097)*****	**0.609 (0.109)*****	-0.077 (0.052)	0.002 (0.074)
Fruiting onset	**-0.667 (0.118)*****	**-0.573 (0.107)*****	-0.039 (0.029)	-0.019 (0.038)	0.083 (0.128)	0.303 (0.155)	0.026 (0.056)	0.002 (0.124)
Fruiting duration	-----	-----	-----	-----	**0.424 (0.112)****	0.220 (0.106)	-0.006 (0.055)	0.017 (0.133)
SPAD	**-0.282 (0.083)****	**-0.195 (0.079)***	0.011 (0.030)	0.002 (0.032)	0.197 (0.121)	-0.175 (0.121)	0.070 (0.052)	0.046 (0.083)
F_v/F_m	-0.038 (0.083)	-0.021 (0.075)	0.013 (0.030)	0.032 (0.031)	0.058 (0.115)	0.045 (0.090)	-0.036 (0.051)	-0.011 (0.055)
Leaf area	-0.035 (0.097)	0.114 (0.106)	-0.005 (0.029)	-0.004 (0.033)	0.124 (0.115)	0.204 (0.123)	0.005 (0.050)	-0.012 (0.071)
SLA	0.091 (0.101)	0.011 (0.158)	0.006 (0.033)	-0.127 (0.072)	-0.159 (0.109)	-0.307 (0.180)	0.042 (0.048)	0.111 (0.141)
LDMC	0.098 (0.091)	0.232 (0.146)	-0.032 (0.031)	-0.099 (0.066)	-0.068 (0.113)	-0.232 (0.176)	0.010 (0.050)	0.102 (0.109)
Leaf thickness	-0.155 (0.100)	0.007 (0.148)	-0.001 (0.030)	-0.062 (0.057)	**0.352 (0.117)***	0.174 (0.139)	-0.052 (0.049)	0.055 (0.117)
Leaf:stem ratio	**-0.472 (0.098)*****	**-0.293 (0.102)***	**-0.085 (0.029)***	**-0.122 (0.035)****	0.025 (0.118)	0.029 (0.090)	-0.048 (0.055)	-0.065 (0.072)
RGR	**0.283 (0.121)***	0.098 (0.093)	0.031 (0.034)	0.038 (0.039)	0.283 (0.143)	0.110 (0.128)	0.104 (0.071)	0.029 (0.087)
Aboveground biomass	-0.037 (0.155)	0.027 (0.125)	0.043 (0.045)	0.029 (0.052)	0.185 (0.145)	0.002 (0.121)	*0.145 (0.052)·*	0.132 (0.070)
Genotypic selection analysis								
Flowering onset	**-0.680 (0.091)*****	*-0.358 (0.146)·*	-0.059 (0.034)	0.016 (0.055)	-0.070 (0.103)	0.134 (0.266)	0.116 (0.056)	0.307 (0.227)
Flowering duration	**0.346 (0.107)****	-0.056 (0.134)	0.071 (0.034)	0.090 (0.051)	-0.085 (0.114)	0.246 (0.219)	-0.092 (0.053)	0.055 (0.105)
Fruiting onset	**-0.665 (0.099)*****	**-0.465 (0.108)****	-0.021 (0.036)	-0.046 (0.034)	0.253 (0.088)	0.056 (0.201)	0.017 (0.058)	-0.206 (0.288)
Fruiting duration	-----	-----	-----	-----	0.124 (0.109)	0.010 (0.151)	-0.002 (0.057)	-0.006 (0.225)
SPAD	-0.074 (0.109)	0.107 (0.098)	0.022 (0.037)	-0.038 (0.036)	-0.048 (0.113)	-0.166 (0.214)	-0.007 (0.055)	0.019 (0.124)
F_v/F_m	0.093 (0.105)	0.142 (0.091)	0.046 (0.035)	0.065 (0.034)	0.013 (0.11)	-0.129 (0.126)	-0.013 (0.056)	-0.003 (0.073)
Leaf area	-0.041 (0.117)	0.097 (0.121)	-0.046 (0.036)	-0.008 (0.041)	0.028 (0.098)	0.092 (0.202)	0.065 (0.054)	0.052 (0.104)
SLA	0.131 (0.111)	0.062 (0.148)	0.055 (0.047)	-0.117 (0.111)	-0.056 (0.099)	0.088 (0.317)	-0.020 (0.055)	0.017 (0.197)
LDMC	-0.074 (0.106)	-0.031 (0.146)	0.001 (0.040)	-0.059 (0.088)	-0.054 (0.095)	0.013 (0.269)	0.012 (0.055)	-0.027 (0.148)
Leaf thickness	-0.133 (0.117)	0.068 (0.186)	-0.048 (0.037)	-0.127 (0.065)	0.211 (0.093)	0.186 (0.224)	0.033 (0.055)	0.007 (0.158)
Leaf:stem ratio	**-0.362 (0.109)****	-0.146 (0.126)	-0.117 (0.031)	-0.110 (0.043)	-0.008 (0.099)	0.031 (0.139)	0.032 (0.062)	0.054 (0.102)
RGR	**0.483 (0.122)****	*0.349 (0.126)·*	0.022 (0.052)	0.013 (0.051)	0.174 (0.100)	0.041 (0.193)	0.121 (0.069)	0.010 (0.141)
Aboveground biomass	-0.074 (0.133)	0.078 (0.123)	-0.118 (0.054)	-0.043 (0.065)	0.131 (0.135)	0.010 (0.140)	0.032 (0.059)	0.056 (0.085)

Supp. 10: Phenotypic and genotypic quadratic selection differentials (*C'*) and gradients (*γ'*), and their standard error (in parentheses) of both species and treatments for total seed mass fitness variable. Significant ($p < 0.05$) and marginally significant ($0.05 < p < 0.1$) values after FDR correction are in bold and italic, respectively. Significance levels: · $p < 0.1$; ***** $p < 0.05$; ****** $p < 0.01$.

	***H. squamatum*, drought treatment**		***H. squamatum*, well-watered treatment**		***C. hyssopifolia*, drought treatment**		***C. hyssopifolia*, well-watered treatment**	
Phenotypic selection analysis	*C*' (SE)	*γ*' (SE)	*C*' (SE)	*γ*' (SE)	*C*' (SE)	*γ*' (SE)	*C*' (SE)	*γ*' (SE)
Flowering onset	**-0.393 (0.106)****	**-0.321 (0.114)***	0.016 (0.044)	-0.030 (0.048)	-0.151 (0.177)	**-1.055 (0.370)***	-0.093 (0.065)	-0.077 (0.093)
Flowering duration	-0.235 (0.176)	-0.158 (0.139)	-0.005 (0.018)	0.056 (0.029)	0.058 (0.175)	0.040 (0.197)	-0.046 (0.086)	-0.221 (0.121)
Fruiting onset	0.089 (0.129)	0.073 (0.105)	0.037 (0.035)	-----	-0.291 (0.170)	0.231 (0.230)	-0.068 (0.091)	0.157 (0.208)
Fruiting duration	-----	-----	-----	-----	-0.333 (0.222)	0.405 (0.224)	-0.061 (0.085)	-0.222 (0.198)
SPAD	-0.107 (0.127)	0.023 (0.107)	*0.101 (0.046)*·	0.077 (0.049)	-0.073 (0.223)	-0.047 (0.224)	0.053 (0.065)	0.034 (0.111)
F_v/F_m	0.269 (0.145)	0.231 (0.124)	0.022 (0.043)	0.000 (0.043)	0.116 (0.218)	-0.037 (0.150)	0.085 (0.081)	0.208 (0.108)
Leaf area	0.012 (0.134)	-0.110 (0.110)	*-0.080 (0.037)*·	-0.056 (0.038)	0.066 (0.200)	-0.249 (0.153)	-0.036 (0.073)	-0.015 (0.089)
SLA	0.007 (0.108)	-0.173 (0.137)	-0.009 (0.048)	0.001 (0.060)	-0.111 (0.135)	**-0.527 (0.152)****	0.007 (0.066)	0.111 (0.098)
LDMC	0.187 (0.131)	**0.319 (0.120)***	0.015 (0.046)	-0.040 (0.058)	-0.164 (0.124)	**0.429 (0.118)****	-0.126 (0.058)	-0.091 (0.093)
Leaf thickness	-0.086 (0.138)	-0.041 (0.141)	0.015 (0.040)	0.043 (0.050)	-0.010 (0.190)	**0.451 (0.148)***	0.143 (0.069)	0.105 (0.093)
Leaf:stem ratio	0.183 (0.133)	-0.011 (0.130)	*-0.108 (0.041)*·	-0.106 (0.050)	0.095 (0.177)	0.078 (0.134)	-0.035 (0.092)	-0.074 (0.120)
RGR	-0.004 (0.115)	0.160 (0.105)	-0.011 (0.037)	-0.013 (0.047)	-0.132 (0.156)	0.209 (0.122)	0.063 (0.068)	-0.047 (0.096)
Aboveground biomass	-0.313 (0.141)	-0.092 (0.135)	*-0.122 (0.046)*·	-0.083 (0.057)	-0.146 (0.191)	-0.145 (0.149)	0.051 (0.076)	0.265 (0.119)
Genotypic selection analysis								
Flowering onset	**-0.323 (0.106)***	-0.097 (0.127)	-0.008 (0.049)	-0.006 (0.079)	0.071 (0.162)	0.738 (0.212)	0.100 (0.077)	-0.029 (0.249)
Flowering duration	-0.181 (0.166)	-0.221 (0.228)	0.003 (0.045)	-0.053 (0.087)	0.042 (0.125)	0.591 (0.150)	0.080 (0.090)	0.067 (0.472)
Fruiting onset	-0.217 (0.161)	-0.355 (0.169)	-0.074 (0.040)	-0.054 (0.052)	-0.111 (0.195)	0.392 (0.284)	0.014 (0.102)	1.133 (0.559)
Fruiting duration	-----	-----	-----	-----	-0.279 (0.185)	-0.020 (0.194)	-0.030 (0.106)	-1.489 (0.598)
SPAD	0.043 (0.164)	0.116 (0.197)	-0.022 (0.052)	-0.078 (0.090)	0.006 (0.201)	0.624 (0.330)	0.118 (0.093)	0.233 (0.151)
F_v/F_m	-0.110 (0.148)	0.335 (0.178)	0.031 (0.071)	-0.079 (0.112)	0.060 (0.154)	-0.053 (0.132)	0.014 (0.073)	0.320 (0.199)
Leaf area	-0.051 (0.158)	-0.065 (0.157)	-0.078 (0.063)	-0.038 (0.086)	-0.028 (0.138)	0.154 (0.222)	-0.025 (0.095)	-0.141 (0.158)
SLA	0.052 (0.150)	-0.033 (0.159)	-0.024 (0.074)	0.035 (0.146)	-0.069 (0.173)	0.456 (0.229)	0.123 (0.090)	0.013 (0.243)
LDMC	0.176 (0.213)	0.315 (0.203)	0.068 (0.060)	-0.095 (0.090)	0.068 (0.147)	0.416 (0.170)	0.008 (0.108)	-0.309 (0.230)
Leaf thickness	0.031 (0.180)	0.089 (0.193)	-0.022 (0.050)	0.091 (0.080)	-0.028 (0.140)	0.012 (0.134)	0.157 (0.118)	0.404 (0.269)
Leaf:stem ratio	-0.060 (0.169)	0.037 (0.192)	-0.140 (0.051)	-0.166 (0.093)	0.179 (0.169)	0.483 (0.150)	0.129 (0.097)	0.095 (0.231)
RGR	-0.110 (0.182)	-0.450 (0.250)	0.002 (0.061)	0.035 (0.070)	0.015 (0.187)	0.171 (0.268)	0.124 (0.092)	0.190 (0.364)
Aboveground biomass	0.305 (0.179)	0.478 (0.167)	-0.063 (0.060)	-0.081 (0.077)	0.298 (0.179)	0.611 (0.153)	0.184 (0.097)	-0.185 (0.329)

Supp. 11: Results from the linear mixed models used for testing the effect of treatment in the phenotypic expression of individuals of both species (i.e., phenotypic plasticity). *F-statistics*, *P-values*, marginal, and conditional variance (R^2_M and R^2_C, respectively) for each model are shown. Significant ($P < 0.05$) and marginally significant ($0.05 < P < 0.1$) terms are presented in bold and italic, respectively. Degrees of freedom = 1.

	H. squamatum				***C. hyssopifolia***			
	Treatment		**R^2_M**	**R^2_C**	**Treatment**		**R^2_M**	**R^2_C**
	F	*P*			*F*	*P*		
Flowering onset	17.873	**<0.001**	0.066	0.419	1.257	0.271	0.006	0.058
Flowering duration	100.946	**<0.001**	0.279	0.384	181.258	**<0.001**	0.461	0.488
Fruiting onset	93.583	**<0.001**	0.325	0.484	3.872	*0.058*	0.024	0.144
SPAD	38.286	**<0.001**	0.113	0.127	21.458	**<0.001**	0.103	0.283
F_v/F_m	12.172	**0.001**	0.053	0.213	17.378	**<0.001**	0.084	0.127
Leaf area	64.332	**<0.001**	0.195	0.356	45.586	**<0.001**	0.155	0.324
SLA	3.260	*0.078*	0.011	0.264	6.344	**0.017**	0.027	0.109
LDMC	0.493	0.487	0.002	0.225	25.104	**<0.001**	0.103	0.140
Leaf thickness	34.042	**<0.001**	0.090	0.221	112.909	**<0.001**	0.336	0.416
Leaf:stem ratio	5.266	**0.027**	0.022	0.423	0.183	0.672	0.001	0.180
RGR	10.264	**0.003**	0.027	0.226	133.181	**<0.001**	0.400	0.400
Aboveground biomass	27.714	**<0.001**	0.105	0.342	33.330	**<0.001**	0.142	0.261
Total seed number	691.732	**<0.001**	0.751	0.794	240.749	**<0.001**	0.526	0.532
Total seed mass	813.882	**<0.001**	0.753	0.779	340.175	**<0.001**	0.688	0.718

Supp. 12: Genotypic directional selection differentials (*S'*) and their standard error (in brackets) for phenotypic plasticity in both species, using total seed mass in the drought treatment as fitness variable. Significant ($p < 0.05$) values after FDR correction are shown in bold. Significance levels: ***** $p < 0.05$.

	H. squamatum	***C. hyssopifolia***
Genotypic selection for plasticity	*S'* (SE)	*S'* (SE)
Flowering onset	0.205 (0.102)	0.039 (0.093)
Flowering duration	**-0.284 (0.097)***	-0.258 (0.092)
Fruiting onset	0.115 (0.104)	-0.219 (0.094)
Fruiting duration	-----	-0.142 (0.097)
SPAD	**0.260 (0.098)***	-0.113 (0.125)
F_v/F_m	-0.020 (0.105)	0.082 (0.094)
Leaf area	-0.055 (0.111)	-0.033 (0.092)
SLA	**0.257 (0.098)***	-0.037 (0.091)
LDMC	0.128 (0.103)	-0.037 (0.097)
Leaf thickness	0.156 (0.104)	-0.093 (0.091)
Leaf:stem ratio	0.218 (0.100)	-0.094 (0.107)
RGR	-0.172 (0.102)	-0.103 (0.090)
Aboveground biomass	0.024 (0.106)	-0.022 (0.092)

General Discussion:

GENERAL DISCUSSION

Ongoing climate change is threatening plant biodiversity by altering the patterns of temperature and precipitation worldwide. Consequently, understanding how plant populations will respond to such limiting conditions is critical (Franks et al., 2014; Gienapp et al., 2008; Jump and Peñuelas, 2005; Shaw and Etterson, 2012). Future adaptive evolutionary responses to face climate change depend on the evolutionary potential of adaptive traits and phenotypic plasticity, but past neutral and adaptive processes also influence the standing genetic variation both within and among populations (Etterson, 2004; Teplitsky et al., 2014). Therefore, this thesis provides a comprehensive picture of the evolutionary ecology of Mediterranean gypsum endemic plants (i.e., gypsophiles), to gain insight into how gypsophile populations will respond to climate change and the relative role of different factors affecting such responses.

Specifically, in chapter 1, we determined the phylogeographic processes associated with the origin, genetic diversity and structure of the dominant gypsophile *Lepidium subulatum* across its entire distribution range. In chapter 2, we assessed the footprints of selection on functional traits and their plasticity, and the selective pressures driving adaptive population differentiation along the distribution range of *L. subulatum*, which allowed the identification of potentially vulnerable populations to climate change. Chapter 3 evaluated the patterns of selection in natural conditions in *Centaurea hyssopifolia* and *Helianthemum squamatum*, acting through two different fitness components (survival and reproduction) and considering the high spatiotemporal variation of gypsum ecosystems. Finally, in chapter 4, we assessed the potential response to selection of functional traits and their plasticity in *C. hyssopifolia* and *H. squamatum* under ecologically meaningful conditions that simulated the increment of aridity associated with climate change. The results of this thesis provide novel insight to understand how *in situ* evolutionary processes —adaptive evolution and phenotypic plasticity— may alleviate the negative effects of climate change in gypsophile species.

Speciation and neutral evolutionary processes in gypsophile species

Assessing the events associated with the origin of species is crucial to understand the historical factors that have shaped the geographical distribution of different taxa (Avise, 2000, 1998). Furthermore, estimates of neutral genetic diversity and population structure provide valuable information about the role of dispersal ability and other processes in the patterns of genetic and phenotypic variation between populations (Sork, 2016). Since neutral evolutionary processes affect intraspecific phenotypic variation, insights on the neutral genetic diversity and structure of plant populations are needed to fully understand the ecological and demographic processes that may have driven adaptive differentiation between populations and may determine their future evolutionary potential (Leinonen et al., 2006; Merilä and Crnokrak, 2001). Phylogeographic studies are valuable tools to understand several evolutionary processes, since they bridge the gap between the macroevolutionary processes promoting speciation and the microevolutionary processes operating within species (Avise, 2000; Hickerson et al., 2010). Therefore, the first chapter of this thesis assessed the phylogeographical processes associated with the origin and the patterns of neutral genetic diversity and population structure in the dominant gypsophile *Lepidium subulatum*.

Phylogenetic results showed that the two Iberian gypsum species within the *Lepidium* genus (*L. subulatum* and *L. cardamines*) formed a monophyletic clade that was dated ~3.86 Ma (5.96–2.05 Ma). Furthermore, *L. subulatum* diverged from its sister species *L. cardamines* ~3.01 Ma (5.08–1.33Ma). These dates suggest that the ability to tolerate gypsum soils (gypsophily) of gypsum species within the *Lepidium* genus could be related to major geological and paleoclimatic events that occurred around the Plio-Pleistocene in the Mediterranean Region. Geological events in the Plio-Pleistocene related with the Messinian Salinity Crisis (e.g., the tectonic uplift of the Gibraltar Arc) favored both the emergence and precipitation of

gypsum due to evaporitic processes (Escavy et al., 2012; Garcia-Castellanos and Villaseñor, 2011), increasing the colonization rate of gypsum soils and likely promoting the origin of gypsum specialists (Rajakaruna, 2018; Escudero et al., 2015; Moore and Jansen, 2007). Furthermore, the aridification process that started in the Plio-Pleistocene associated to the origin of the Mediterranean climate likely acted as an evolutionary force promoting the evolution of species inhabiting gypsum ecosystems (Escavy et al., 2012; Thompson, 2005). Indeed, the divergence dates for *L. subulatum* in the Plio-Pleistocene matched with those reported in previous studies assessing the origin of other Mediterranean gypsum species such as *Helianthemum squamatum* (L.) Dum. Cours, *Ferula loscosii* (Lange) Willk., and *Nepeta hispanica* Boiss. & Reuter (Aparicio et al., 2017; Pérez-Collazos et al., 2009; Ramos-Gutiérrez et al., 2022). Importantly, the aridification process of the Pio-Pleistocene also occurred in other regions besides the Mediterranean Basin. These climatic changes had significant effects on the speciation of plant species inhabiting Mediterranean-type and semiarid ecosystems around the world (Benítez-Benítez et al., 2018; Coleman et al., 2003; Comes, 2004; Kadereit and Abbott, 2021; Vargas et al., 2009), including edaphic specialist such as gypsophile species from the Chihuahuan Desert (Mandujano et al., 2020; Moore and Jansen, 2007) and granite specialists of South-West Australia (Tapper et al., 2014). These results highlight the importance of geological and paleoclimatic factors as a driver of speciation and diversification of gypsophiles.

The phylogeographic patterns of *L. subulatum* also showed important results related with the origin and expansion of this species, and the eco-evolutionary processes affecting its genetic diversity and population structure. Haplotype analyses suggested the low dispersal ability of the species via seeds. For instance, the great haplotype differentiation observed between closely located regions such as the Tajo and the Ebro Valley suggested that seed flow across regions has been limited during the evolutionary history of *L. subulatum*. Furthermore, North African populations showed only one fixed haplotype, separated by just one mutational

step from the most common haplotype located in the Iberian Peninsula. Due to the isolation between the Iberian Peninsula and North Africa, a higher number of haplotypes or only one, much more divergent haplotype would be expected. Therefore, the observed pattern suggested that the colonization of North Africa by *L. subulatum* was due to a recent, long-distance dispersal event by chance from the Iberian Peninsula instead of past efficient seed dispersal events during the Messinian Salinity Crisis (i.e., when the Mediterranean Sea was desiccated). The limited dispersal ability via seeds of *L. subulatum* was confirmed by differences in genetic structure found between nuclear and chloroplast markers, since effective pollen and seed flow across populations can be estimated by comparing population differentiation between biparentally and maternally inherited markers (Ennos, 1994; Petit et al., 2005; Robledo-Arnuncio, 2011; Schaal et al., 1998; Sork, 2016). The population genetic structure estimated from chloroplast markers was higher than that found in nuclear markers (F_{ST} = 0.461 vs. 0.187, respectively), indicating that gene flow via pollen is higher than via seeds across populations of *L. subulatum*, matching other studies reporting higher effective gene flow via pollen than seeds (Petit et al., 2005; Sork, 2016; and references therein). Higher rates of pollen flow could be favored by the presence of several small populations of *L. subulatum* throughout the Iberian Peninsula, which may allow efficient pollen movement by pollinators across different populations and regions (Matesanz et al., 2019; Santamaría et al., 2018). Furthermore, pollen flow was likely favored due to the advanced flowering phenology of the species, avoiding competition for pollination at this time of the season (Matesanz et al., 2018). These results confirmed the limited seed dispersal ability of *L. subulatum* as has been hypothesized for gypsophile species (Escudero et al., 2015; Moore et al., 2014; Schenk, 2013), constraining the possibility of responding to climate change by migration in this species.

Finally, we observed high overall genetic diversity in both nuclear and chloroplast markers in populations of *L. subulatum*, agreeing with other studies assessing genetic diversity

of both Iberian (Matesanz et al., 2019) and non-Iberian gypsophiles (Aguirre-Liguori et al., 2014). Such high genetic diversity was consistent with large population sizes typical of this species and the lack of evident demographic changes such as bottlenecks across populations. Since severe demographic changes are often associated with a loss of quantitative genetic variation (Aguilar et al., 2008; Young et al., 1996), the lack of demographic changes may also be one of the reasons behind the high evolutionary potential of traits and their plasticity in gypsophile species (see below). Some authors have discussed that evolution in isolated and poor-quality habitats associated to substrate specialization may result in loss of genetic diversity, being the populations of edaphic specialists genetically depauperated and often considered evolutionary dead-end species (Anacker et al., 2011; see Rajakaruna, 2018 for a deeper discussion). However, the results of this thesis highlighted that this is not necessarily the case for Iberian gypsophiles, which showed significant neutral and quantitative genetic variation both at the inter and intrapopulation level, as shown in chapters 1, 2 and 4 of this thesis.

Adaptive evolution: Footprints of natural selection and future adaptation to climate change

Past natural selection and selective pressures driving quantitative population differentiation

Populations from widely-distributed plant species are often distributed along environmental gradients and consequently, experience differential selective pressures imposed by contrasting environmental conditions. Over the evolutionary history of populations, past adaptation to such differences in environmental conditions may drive the genetic and phenotypic differentiation of populations (Blanquart et al., 2013; Kawecki and Ebert, 2004; Siepielski et al., 2013). Nevertheless, phenotypic variation among populations may be also caused by neutral evolutionary processes (Leinonen et al., 2006; Merilä and Crnokrak, 2001). Therefore, in chapter 2, we assessed the importance of past natural selection and neutral evolutionary

processes using $Q_{ST} – F_{ST}$ comparisons, and identified the selective pressures that have likely promoted quantitative population differentiation in *Lepidium subulatum*.

As found in chapter 1, population differentiation in neutral nuclear markers was moderate (F_{ST} = 0.187), finding significant pollen flow across populations and regions. Although gene flow may counteract the effect of natural selection (Franks et al., 2014; Hoffmann and Sgró, 2011; Kawecki and Ebert, 2004; Sork, 2016), Q_{ST} distributions for several ecophysiological traits (i.e., population differentiation in quantitative traits measured under common condition in the experimental common gardens) were significantly higher than F_{ST}, indicating that past natural selection was strong enough to drive quantitative genetic differentiation among populations. These results highlight that past selection events have favored different phenotypes in different populations, i.e., spatially divergent selection, leading to a pattern of adaptive phenotypic differentiation among populations of *L. subulatum*.

Chapter 2 also provided insights on the ecological factors linked to past selection. Gypsum ecosystems are characterized by the presence of several simultaneous stresses that can act as potential selective pressures shaping quantitative population differentiation (Escudero et al., 2015; Rajakaruna, 2018; and references therein). Specifically, the chemical composition of gypsum soils, with high concentrations of calcium and sulfate but low nutrient levels, imposes restrictions for the growth and success of plants in gypsum ecosystems (Escudero et al., 2015; Palacio et al., 2022). Therefore, differences in soil gypsum content and nutrient composition may have acted as a driver of adaptive differentiation among populations. Alternatively, to deal with such restrictive soil chemical composition, natural selection could have favored a uniform optimum phenotype across populations (i.e., a stress resistance syndrome *sensu* Rajakaruna, 2018). Nevertheless, genetically-based phenotypic differences among populations were not associated with gypsum or nutrient content, suggesting that soil chemical composition has not strongly shaped adaptive intraspecific differentiation in *L. subulatum*.

In contrast, we found strong associations between quantitative genetic differentiation and climatic differences among populations of *L. subulatum*, indicating that past divergent selection was likely driven by differences in climatic conditions across the species range. Drought-related environmental conditions have been identified as the main selective pressures for plants inhabiting semiarid and Mediterranean-type ecosystems (Blondel et al., 2010; Ramírez-Valiente et al., 2009; Thompson, 2005). Importantly, our results matched with those that have found past adaptation linked to climatic differences among populations in other non-edaphic specialist species worldwide (e.g., Cooper et al., 2022; Frei et al., 2012; Keller et al., 2011; Ramírez-Valiente et al., 2014; Solé-Medina et al., 2022), indicating the major role of climate in shaping adaptive intraspecific variation. These results, together with those obtained in chapter 1, highlight the importance of climate during the evolutionary history of gypsophiles, being not only associated with their origin but also promoting adaptive evolution across populations.

The climatic differences found among populations were associated to the phenotypic expression of populations. Although previous studies have reported that individuals from climatically hasher populations usually have conservative strategies to cope with drought (Ramírez-Valiente et al., 2009; Solé-Medina et al., 2022), our results matched with studies that showed that adaptive population differentiation was not easily linked to a particular resource-use strategy. For instance, individuals from populations with harsher climatic conditions showed earlier reproductive phenology, agreeing with previous studies that have reported the presence of such acquisitive strategy to escape from summer drought in harsher populations of Mediterranean species (Brouillette et al., 2014; Franks et al., 2007; Matesanz et al., 2020a). However, individuals from drier populations also showed significantly higher water use efficiency under drought conditions, which has been reported as an adaptation associated with a conservative strategy to minimize water loss in drier populations of Mediterranean species

(Lázaro-Nogal et al., 2016; Matesanz and Valladares, 2014; Ramírez-Valiente et al., 2010). Although these results contrasted with the predicted trade-off between high water use efficiency and fast reproduction, recent studies support that populations can show both early phenologies and high WUE due to higher photosynthetic capacity, as an adaptation to lower water availability (Brouillette *et al.*, 2014; Kooyers, 2015; Kooyers *et al.*, 2015; and references therein). These results supported that drought-tolerance and drought-escape strategies are not mutually exclusive in gypsophiles, matching with those found in other species experiencing drought conditions (Brouillette et al., 2014; Kooyers et al., 2015; Ramírez-Valiente et al., 2018, 2011). Natural selection acts on multivariate and complex phenotypes, and in the absence of genetic constraints, phenotypes associated with different stress-response strategies (acquisitive or conservative) may evolve if this syndrome confers a fitness advantage (Phillips and Arnold, 1989; Solé-Medina et al., 2022). The fact that individuals from hasher populations had both conservative and acquisitive phenotypes for certain traits (e.g., higher WUE, lower SLA, and advanced phenology at the same time) highlighted that the evolution of traits related with contrasting resource-use strategies was not genetically constrained in *L. subulatum*, which may at least partly explain the success of gypsophile species in highly stressful habitats such as gypsum ecosystems. In addition, individuals from harsher populations consistently showed higher reproductive output in both watering treatments, indicating that adaptation to a harsher climate has not resulted in a fitness trade-off across conditions (Hereford, 2009; Matesanz et al., 2020b). More importantly, populations from milder climatic conditions showed very low fitness across watering treatments, highlighting the vulnerability of mesic populations in a climate change context.

Adaptive resource-use strategies of dominant gypsophile species in natural conditions

Adaptive phenotypic variation within populations is the substrate for natural selection (Conner and Hartl, 2004; West-Eberhard, 2005), and thus, assessing the patterns of intraspecific trait variation and how it is related to differences in individual fitness (i.e., identifying the traits under selection) is of paramount importance to understand how natural selection may act to face climate change. Mediterranean plants thrive in habitats where several abiotic and biotic stresses operate simultaneously, being drought the main selective pressure for plant development (Blondel et al., 2010; Ramírez-Valiente et al., 2009; Thompson, 2005). Consequently, Mediterranean plants are often assumed to show a drought tolerance strategy associated with conservative resource-use (Blondel et al., 2010; Matesanz and Valladares, 2014). However, the presence of individual variation in traits related to resource-use strategies and, more importantly, whether such intraspecific trait variation is related to fitness has been almost unexplored for most Mediterranean plant species and was to date unknown for gypsophiles. Therefore, chapter 3 of this thesis evaluated the patterns of selection of *Centaurea hyssopifolia* and *Helianthemum squamatum* in natural conditions, and how they vary considering the high spatiotemporal environmental variation of gypsum ecosystems (i.e., accounting for differences between north and south slopes of gypsum hills, and interannual climatic variation).

As expected, we found profound differences between slopes and years. Specifically, we found a higher plant cover and soil water content, but lower biological soil crust cover in north slopes compared to south slopes. Furthermore, soil water content also varied between years, being higher during the second experimental year in both slopes due to the higher rainfall of 2018. In contrast, biotic conditions did not vary between years within the same slope. Despite of the significant differences in the microenvironmental conditions found between north and south slopes, the direction of selection and the identity of adaptive traits did not vary between

slopes. Indeed, patterns of selection were more similar between slopes within years than within slopes between years, suggesting that the climatic conditions of each year had a stronger effect on selection than the microenvironmental conditions of each slope. These results highlight the importance of climate as a strong selective pressure for gypsophile species, as we have found across this thesis.

Interestingly, patterns of selection through reproductive output showed that, in contrast to the conservative strategy predicted for semiarid Mediterranean species, an acquisitive, drought-escape strategy was adaptive for both gypsophile species (especially for *H. squamatum*) even in scenarios where water availability was scarcer, i.e., in south slopes and the dry year. Although plant species inhabiting special substrates often show ecophysiological adaptations to minimize water loss (Damschen et al., 2012; Escudero et al., 2015; Rajakaruna, 2018), selection favored phenotypes associated with an acquisitive strategy at the intraspecific level in gypsophiles, matching with previous studies that have reported the adaptive value of a similar acquisitive syndrome in other Mediterranean and semiarid species (Franks, 2011; Sherrard and Maherali, 2006). Specifically, individuals with earlier phenologies, higher SLA, lower LDMC, lower WUE, and, to a lower extent, higher leaf nitrogen content, showed a higher reproductive output. A phenological advance, associated with a drought escape strategy, has been proved adaptive in other Mediterranean and semiarid species, since it minimizes water loss in later stages of the growing season, when abiotic stress is more pronounced (Franks, 2011; Herrera, 1992; Sherrard and Maherali, 2006). Other morphological, physiological, and leaf chemical composition traits were associated with reproduction in both years and species, although we found stronger selection in *H. squamatum*. In this species, larger and less sclerophyllous leaves (lower LDMC, higher SLA and leaf area) were adaptive in both slopes and years, maximizing photosynthesis and resource assimilation through an acquisitive strategy (Pérez-Ramos et al., 2013; Reich, 2014; Volaire, 2018). Furthermore, individuals with lower

water use efficiency had higher reproductive output. Previous studies have reported the adaptive value of high WUE in species suffering water stress, associated with a conservative and drought-tolerance strategy (Dudley, 1996; Heschel et al., 2002). However, we found that water conservation was not advantageous in natural conditions for both species, suggesting that photosynthetic efficiency is optimized to maximize carbon uptake and advance reproduction (Franks, 2011; McKay et al., 2003; Wu et al., 2010). Overall, natural selection favored an acquisitive, stress-escape strategy in gypsophile species in natural conditions, since it may allow the rapid development and reproduction of individuals before the most limiting conditions imposed by summer drought (Franks, 2011; Heschel and Riginos, 2005; Welles and Funk, 2021).

In addition, due to several experimental individuals of *H. squamatum* died between both years of study, selection analyses were also performed using survival as fitness variable in this species. Selection patterns acting through survival showed that this fitness component was not associated with functional trait variation in *H. squamatum*, highlighting that the same selection pressures may act differently depending on the fitness component evaluated. Previous studies have reported weaker estimates of selection via survival than via reproduction (Kingsolver et al., 2012 and references therein), and a trade-off between survival and reproductive output of individuals (Harshman and Zera, 2007; Obeso, 2002). However, we did not find evidence of a trade-off between both fitness components since survival was not significantly related to the reproductive output of individuals (i.e., the individuals that died were not those with a higher reproductive output). Indeed, in the presence of such a trade-off, opposing selection patterns via both components would be expected (i.e., individuals with acquisitive phenotypes would have had higher reproduction but a lower probability of survival and *vice versa*). Furthermore, survival was neither associated with the microenvironmental conditions experienced by individuals nor with their size (as a proxy for plant age; Connell et al., 2021; Throop and Archer,

2008). Overall, these results highlighted that either the phenotypic expression of individuals, their reproductive output, their size, or the environmental conditions experienced, were not reliable predictors of survival in gypsum ecosystems. Further studies assessing the specific causes related to the survival of gypsophile individuals in natural conditions are needed.

Evolutionary potential of functional traits in a climate change context

To understand the potential for future adaptation to climate change, assessing both the patterns of selection and the presence of genetic variation for adaptive traits in ecologically meaningful environmental conditions is needed. Chapter 4 of this thesis evaluated the patterns of natural selection and the evolutionary potential of functional traits of two dominant gypsophile species, *Helianthemum squamatum* and *Centaurea hyssopifolia*, under experimental conditions that simulated the increment of aridity produced by climate change. We found that, in both species, the number of traits under selection and the magnitude of selection was higher under drought conditions. These results highlight the role of drought as a key selective pressure for gypsophile species, agreeing with the results of previous studies performed with Mediterranean species (Blondel et al., 2010; Lázaro-Nogal et al., 2016; Ramírez-Valiente et al., 2021, 2009; Thompson, 2005), and those obtained in chapters 2 and 3 of this thesis (see Blanco-Sánchez et al., 2022). However, we found profound differences between species in their adaptive strategies and particularly, in the evolutionary potential of traits under selection (i.e., in quantitative genetic variation). Individuals with advanced and longer phenology, higher relative growth rate, and lower leaf chlorophyll content showed higher fitness under drought conditions in *H. squamatum*, a trait syndrome consistent with a drought-escape, acquisitive strategy (Franks, 2011; Volaire, 2018; Welles and Funk, 2021), which was also found to be adaptive in natural conditions (see chapter 3; Blanco-Sánchez et al., 2022). More importantly, we found genetic variation for phenological traits and reproductive fitness in drought conditions in *H.*

squamatum. Since quantitative genetic variation is the substrate for adaptive evolution (Conner and Hartl, 2004; Endler, 1986; Etterson and Shaw, 2001; Kruuk et al., 2003), our results showed the potential of this species to evolve higher reproductive output under drought conditions associated with a phenological advance, which may be crucial to face the harsher climatic conditions imposed by climate change in the Mediterranean region.

In contrast, selection patterns showed an adaptive drought tolerant strategy in *C. hyssopifolia*. Specifically, individuals with thicker leaves and longer flowering and fruiting phenology had a higher reproductive output under dry conditions. Several studies have reported the adaptive value of thicker leaves and longer reproductive phenologies in Mediterranean species, associated with a drought-tolerance strategy and conservative resource use to minimize water loss (Etterson, 2004; Matesanz et al., 2020a; Ramírez-Valiente et al., 2020; Solé-Medina et al., 2022). However, this adaptive strategy contrasted with that found in natural conditions in chapter 3. Shifts in the drought-related adaptive strategies found between chapter 3 and 4 could be related to differences in water availability across studies (Volaire, 2018). In natural conditions, gypsophiles experience severe water stress mostly at the end of the growing season (Escudero et al., 2015), and may rely on different potential water sources throughout their lifecycle (de la Puente et al., 2022), which could minimize the risk of hydraulic failure while expressing an acquisitive strategy. In contrast, in our common garden experiment, individuals were grown under drought conditions during the entire growing season, simulating the predicted increment of aridity. Indeed, in chapter 3, selection on early phenology was weaker in south slopes during the dry year (i.e., the scenario with higher abiotic stress), suggesting that differences in the duration and strength of drought may have underlain the observed differences in selection patterns and resource-use strategies in *C. hyssopifolia* across chapters of this thesis. Nevertheless, functional traits and fitness lacked genetic variation in *C. hyssopifolia*, which will likely limit the possibility of an adaptive evolutionary response to face climate change. It is

worth noting that, despite the lack of genetic variation in the study population, quantitative genetic variation can vary across the species range (Matesanz et al., 2014; Matesanz and Valladares, 2014; Ramírez-Valiente et al., 2011). Therefore, to evaluate the evolutionary potential of functional traits at the species level, future studies should assess the presence of adaptive genetic variation in other populations.

Phenotypic plasticity: a crucial determinant of gypsophile responses to climate change

High plasticity, but similar responses across populations

Phenotypic plasticity has been identified as the main evolutionary process to cope with rapid environmental change, being crucial to guarantee the persistence of plant species in a climate change context (Arnold et al., 2019; Bonamour et al., 2019; Nicotra et al., 2010). Therefore, chapter 2 also evaluated the patterns of phenotypic plasticity across populations of *L. subulatum*. Interestingly, all populations showed similar plastic responses to drought in most traits, and some of these responses may be considered adaptive (e.g., a phenological advance and higher water use efficiency in response to drought), indicating the importance of plasticity to cope with environmental heterogeneity across the entire distribution range of this species. It has been hypothesized that maintaining high levels of plasticity in stressful environments may provide more costs than benefits, and consequently, natural selection may favor less plastic genotypes in stressful environments (Solé-Medina et al., 2022; Stotz et al., 2021; Valladares et al., 2007; Van Kleunen and Fischer, 2005). Therefore, the presence of ample and similar plastic responses across populations suggested that plasticity have been also subjected to past selection in *L. subulatum*. Indeed, the strikingly similar patterns of phenotypic plasticity found suggests that natural selection has favored similar plasticity across populations (i.e., homogenizing selection on plasticity; Pigliucci & Kolodynska, 2002), probably as a consequence of similar environmental heterogeneity across populations (Matesanz et al., 2020a, 2020b; Van Kleunen

and Fischer, 2005). Such high plasticity across populations may have had relevant evolutionary consequences for gysophiles. Plasticity likely allowed gypsophiles to cope with the stressful and variable environments of gypsum habitats while maintaining high genetic variation within populations, favoring in turn the adaptation to different climatic conditions as we found in chapter 2 (Gomez-Mestre and Jovani, 2013; Matesanz et al., 2020b). Importantly, the high differentiation in functional traits but similar plasticity patterns among population indicated that the evolution of traits and plasticities was independent in gypsophile populations (see next section).

Although plastic responses across populations were generally similar, we also found significant P × E interaction in d13C (water use efficiency) and leaf N, traits intimately related with photosynthetic activity (Brouillette et al., 2014; Nicotra and Davidson, 2010; Ramírez-Valiente et al., 2014). Specifically, individuals from populations with harsher climatic conditions showed higher plasticity in water use efficiency and leaf N. These results suggest that populations from harsher sites were more photosynthetically efficient while conserving water under drought conditions, being able to exploit resources when water was abundant. Similar plasticity patterns have been reported adaptive in "water-wise" plant species (see Nicotra and Davidson, 2010 for a deeper discussion), suggesting that individuals from harsher populations also showed more adaptive plastic responses to drought in certain functional traits. Importantly, these differences in plasticity could have also contributed to population differentiation in fitness traits in *L. subulatum*.

High genetic variation for plasticity within populations

The results obtained in chapter 4 also showed significant plasticity to drought in all functional traits and significant genetic variation for plasticity in *C. hyssopifolia* and *H. squamatum*. This, again, contrasts with the prediction that plasticity is reduced in species inhabiting stressful

ecosystems (Stotz et al., 2021; Valladares et al., 2007), and matches the results in chapter 2. Several interplaying factors have been proposed to explain high levels of plasticity and genetic variation for plasticity, including fluctuating selection pressures and large effective population sizes (Kelly, 2019; Saltz et al., 2018). Specifically, the presence of genetic variation for plasticity in all the traits evaluated in both gypsophile species may be related with the presence of fine-grain and coarse-grain environmental heterogeneity in gypsum habitats. This spatiotemporal heterogeneity likely allows the expression of a wide variety of similarly fit phenotypes in response to the variable selective pressures imposed by such stressful and heterogeneous conditions. Furthermore, the presence of genetic variation for plasticity has crucial evolutionary implications for gypsophiles, since it may allow the evolution of adaptive plastic responses by natural selection in response to further environmental change (Matesanz and Valladares, 2014; Nicotra et al., 2010; Van Kleunen and Fischer, 2005). Therefore, high levels of genetic variation for plasticity often provide populations with a better ability to cope with environmental changes such as climate change (Kelly, 2019; Matesanz et al., 2010; Matesanz and Valladares, 2014; Nicotra et al., 2010). This may be especially crucial for *C. hyssopifolia*, since this species lacked genetic variation for functional traits, at least in the studied population. Although previous studies have reported lower genetic variation for phenotypic plasticity than for ecophysiological traits (Lázaro-Nogal et al., 2015; Matesanz and Valladares, 2014; Scheiner, 1993), our results highlight that this may not be the case for Iberian gypsophiles. In addition, the significant differences found between genetic variation of functional traits and their plasticity showed that both trait means and trait plasticity may evolve independently in gypsophile species (Pigliucci, 2005; Weijschedé et al., 2006), matching with the results found in chapter 2 (i.e., similar plasticity patterns across populations, but significant quantitative population differentiation). Overall, particularly in *C. hyssopifolia*, the evolution of adaptive norms of reaction may play a more important role than the evolution of trait means

to cope with climate change, highlighting the importance of phenotypic plasticity for gypsophile species.

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General Conclusions:

GENERAL CONCLUSIONS

1.- Geological and paleoclimatic changes during the Plio-Pleistocene in the Mediterranean Region, i.e., Messinian Salinity Crisis and the origin of the Mediterranean climate, likely promoted the origin of *Lepidium subulatum*.

2.- *Lepidium subulatum* showed limited seed dispersal ability. The lack of effective dispersal mechanisms via seeds, together with the fragmented configuration of gypsum habitats, may constrain migration to suitable habitats to cope with climate change. In contrast, we found significant pollen flow across populations and regions, which likely contributed to maintain high levels of genetic variation throughout the species range.

3.- Despite significant pollen flow across populations and regions that could swamp adaptation, we found significant footprints of past natural selection in *Lepidium subulatum*. Past natural selection favored different phenotypes in different populations, i.e., spatially divergent selection, resulting in adaptive phenotypic differentiation among populations.

4.- Rather than soil chemical composition, climatic differences among populations were the main selective pressure driving adaptive intraspecific variation in *Lepidium subulatum*.

5.- Associated with climatic differences, populations of *L. subulatum* showed significant genetically-based phenotypic differences. Individuals from populations with harsher climatic conditions (drier and warmer) showed higher reproductive output in drought and well-watered conditions, indicating that adaptation to harsher climates has not resulted in a fitness trade-off. In contrast, individuals from populations with milder climatic conditions consistently showed lower fitness across watering conditions, highlighting their vulnerability in a climate change context.

6.- In natural conditions, a drought-escape, acquisitive strategy was linked to higher reproductive fitness in two dominant gypsophiles, being selection patterns generally consistent across species, slope aspects and climatically contrasting years. Such an acquisitive strategy may allow rapid individual development and reproduction before the most limiting climatic conditions of mid-late summer in gypsum ecosystems.

7.- In contrast with patterns of phenotypic selection for reproductive fitness, survival was not associated with the phenotypic expression of individuals, nor with their reproductive output, size, or environmental conditions. Importantly, differences in selection patterns between reproduction and survival highlights that natural selection may act differently in response to the same selective pressures depending on the fitness component evaluated.

8.- Under experimental conditions simulating the increment of aridity produced by climate change, gypsophile species strongly differ in the presence of within-population quantitative genetic variation and consequently, in the evolutionary potential of adaptive traits. Therefore, dominant co-occurring gypsophile species subjected to similar selection pressures may show contrasting evolutionary trajectories in a climate change context, which may alter the composition of gypsum plant communities over time.

9.- Gypsophiles showed adaptive phenotypic plasticity to drought throughout this thesis, indicating the importance of plasticity for these species to cope with environmental changes and face the heterogeneous, stressful conditions of gypsum habitats. *Lepidium subulatum* showed similar plastic responses to drought across populations, suggesting that natural selection favored a common norm of reaction, likely due to similar environmental heterogeneity across its distribution range. Such high plasticity may have favored the maintenance of high quantitative genetic variation within populations, allowing past adaptation to contrasting climatic conditions.

10.- Gypsophile species showed high within-population genetic variation for phenotypic plasticity, reflecting the ability to evolve adaptive plasticity in response to climate change. Importantly, the high phenotypic differentiation but similar phenotypic plasticity among populations, together with the contrasting patterns of genetic variation for quantitative traits and plasticity within populations, indicate that the evolution of adaptive traits and adaptive norms of reactions may occur independently in gypsophiles.

11.- Ecophysiological traits and their plasticity have the potential to evolve in gypsophiles, but the evolutionary potential may vary among species, traits, and environmental conditions. Therefore, phenotypic plasticity and adaptive evolution can interact to shape the adaptive responses of gypsum endemic species to climate change.

12.- Although substrate specialization may result in losses of genetic diversity leading to evolutionary dead-end species, Iberian gypsophiles showed significant neutral and quantitative genetic variation both among and within populations, reflecting their ability to respond to past and future selective pressures. The results obtained throughout this thesis highlight the key role of climate during the evolutionary history of gypsophiles, being associated with their origin, promoting adaptive evolution across populations, and also shaping their future evolutionary responses.