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TESIS DOCTORAL

*Evaluation of assisted evolution as a conservation
strategy for climate change adaptation*

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RESUMEN

Antecedentes

El actual cambio climático, acelerado por las actividades humanas, está poniendo en peligro la supervivencia de numerosas especies. Ante esta situación, los organismos pueden responder de diferentes maneras. Los movimientos migratorios tanto en latitud como en altitud son una de las respuestas más evidentes y mejor estudiadas. Sin embargo, la capacidad de dispersión de algunos organismos puede estar limitada, como es el caso de las plantas. En estos casos, cobran gran importancia otro tipo de respuestas, como puede ser la plasticidad fenotípica o la adaptación genética. A pesar de que la plasticidad fenotípica podría ofrecer una respuesta con mayor rapidez, los cambios genéticos podrían ser la única solución sostenible a largo plazo. No obstante, dada la rapidez con la que se están produciendo los cambios ambientales, puede que incluso estas respuestas sean insuficientes para asegurar la supervivencia de ciertas poblaciones. En este contexto, se hace necesaria la búsqueda de nuevas herramientas que nos ayuden a la conservación de la biodiversidad. En este sentido, la evolución asistida incluye todas aquellas acciones en las que se realiza una intervención humana sobre alguna de las fuerzas evolutivas, con fines de conservación. Dentro de estas acciones se incluye la migración asistida, la cual consiste en el movimiento intencionado de individuos desde sus poblaciones actuales hacia hábitats más favorables. Puesto que este tipo de actuaciones pueden conllevar numerosos riesgos ecológicos asociados (como puede ser la propagación de enfermedades, o que una especie se vuelva invasora), también se han propuesto otras estrategias que favorezcan la adaptación *in-situ* de los organismos, como son la selección artificial y el flujo genético asistido. Estas dos estrategias se engloban dentro del concepto

de adaptación facilitada, y su objetivo es el de mejorar el potencial adaptativo (capacidad de los organismos de evolucionar y adaptarse a nuevas condiciones ambientales) de las poblaciones en respuesta al cambio climático. Este potencial adaptativo o evolutivo viene determinado por la variabilidad genética que posean las poblaciones. Por tanto, para mejorar dicho potencial, las técnicas de adaptación facilitada lo que buscan es aumentar la presencia de alelos adaptativos que se encuentran en bajas frecuencias en la población diana. Mediante la selección artificial, se seleccionan determinados individuos dentro de una población, que poseen ciertos rasgos de interés, con el objetivo de perpetuar dichos rasgos en las siguientes generaciones. Por su parte, el flujo genético asistido consiste en transferir gametos o individuos de unas poblaciones a otras, con la intención de mejorar su variabilidad genética y, por tanto, su capacidad de adaptación a las condiciones ambientales. No obstante, este tipo de actuaciones también pueden conllevar ciertos riesgos, como puede ser una disminución de la variabilidad genética en el caso de la selección artificial, o la depresión exogámica, en el caso del flujo genético asistido. Además, estas estrategias pueden conllevar respuestas indeseadas en otros rasgos distintos al de interés debido a la existencia de correlaciones entre ellos.

Los cambios en la fenología son una de las respuestas más comunes que presentan los organismos frente al cambio climático. Un aspecto clave en la fenología de las plantas angiospermas es el inicio de floración, el cual se ha descrito que es un carácter de base genética y con una alta heredabilidad. Se trata también de un rasgo poligénico en el que hay implicados cientos de genes, y sobre el que influyen numerosos aspectos genéticos y epigenéticos. Aunque se ha observado que el cambio climático está favoreciendo que las plantas adelanten su floración en zonas templadas, tampoco está claro si van a ser capaces de evolucionar lo suficientemente rápido y las consecuencias que esto puede tener.

Objetivos

El objetivo general de esta tesis es evaluar el uso de la selección artificial y el flujo genético asistido como herramientas para estudiar y, en la medida de lo posible, mejorar, el potencial adaptativo de ciertas poblaciones respecto al avance del inicio de floración para facilitar su adaptación al cambio climático. También pretendemos evaluar los riesgos y beneficios de estas técnicas a la hora de poder utilizarlas en acciones de conservación y restauración. Para llevar a cabo este objetivo, hemos elegido como especie de estudio *Lupinus angustifolius* L., una especie herbácea anual perteneciente a la familia de las leguminosas. El alcance de este objetivo principal se pretende lograr a través de los siguientes cuatro objetivos específicos: i) Utilizar la selección artificial y el flujo genético asistido para adelantar el inicio de floración en cuatro poblaciones de *Lupinus angustifolius*. ii) Evaluar los efectos que la selección artificial y el flujo genético asistido pueden tener sobre otros rasgos reproductivos y vegetativos. iii) Identificar las señales genómicas derivadas de la selección artificial y el flujo genético asistido y que pueden estar asociadas a los fenotipos de floración temprana y el resto de los rasgos evaluados. iv) Testar la progenie derivada de los tratamientos de selección artificial y flujo genético asistido en condiciones naturales para determinar si la aplicación de estas estrategias podría tenerse en cuenta a la hora de realizar acciones de conservación y restauración.

Metodología

Para lograr los objetivos propuestos hemos utilizado una aproximación multidisciplinar en la que se han integrado estudios a nivel fenotípico y también a nivel genómico. Se han seleccionado cuatro poblaciones de *Lupinus angustifolius* localizadas en la península Ibérica, procedentes de dos latitudes y condiciones climáticas contrastadas (las dos poblaciones del norte se encuentran en unas condiciones más frías, mientras que las dos poblaciones del sur se encuentran en unas condiciones más térmicas). Las semillas

procedentes de las cuatro poblaciones se han sembrado en un experimento de jardín común en Móstoles (Madrid). En primer lugar, se han establecido diferentes líneas de selección artificial y flujo genético asistido, creadas tanto por autocruzamiento de los individuos de *L. angustifolius* como por cruzamientos manuales, tanto intrapoblacionales (selección artificial) como interpoblacionales (flujo genético asistido), y dichas líneas se han mantenido en jardín común durante tres generaciones (Capítulos 1 y 3). Se ha evaluado la eficacia de la selección artificial para adelantar el inicio de floración y para caracterizar el potencial evolutivo de las poblaciones de *L. angustifolius*, así como los efectos que esta selección artificial puede tener sobre otros rasgos de las plantas (Capítulo 1). Se ha evaluado el potencial que puede tener el flujo genético asistido para adelantar el inicio de floración en las poblaciones del norte *de L. angustifolius*, utilizando individuos procedentes de las poblaciones del sur para polinizarlas, y también los impactos que se producen sobre el resto de los rasgos medidos (Capítulo 3). De manera complementaria, para ambas estrategias se ha llevado a cabo un estudio de secuenciación dirigida para evaluar los efectos que pueden tener la selección artificial y el flujo genético asistido a nivel genómico (Capítulos 2 y 3). Finalmente, hemos examinado los efectos de la adaptación facilitada en condiciones naturales. Para ello, los descendientes de las líneas de las poblaciones del norte obtenidos en los experimentos de jardín común se han sembrado *in-situ* en sus condiciones ambientales de origen, y se han evaluado los efectos que la selección artificial y el flujo genético asistido han tenido sobre el inicio de floración y también sobre otros rasgos reproductivos y vegetativos (Capítulo 4).

Resultados

Capítulo 1. La utilización de la selección artificial resultó ser una herramienta útil para adelantar el inicio de floración en *Lupinus angustifolius* en condiciones controladas, aunque solo en las poblaciones del norte. Esto indica que estas poblaciones del norte

poseen una mayor diversidad genética y, por tanto, un mayor potencial adaptativo para este rasgo que las poblaciones del sur. Además, la selección artificial produjo modificaciones en otros de los rasgos estudiados (altura, biomasa, crecimiento, área foliar específica o SLA y contenido en materia seca de los foliolos o LDMC), aunque estos cambios dependieron en gran medida de la latitud de la población de origen y del tipo de cruzamiento aplicado. No se observaron cambios en ninguno de los rasgos reproductivos estudiados (número o peso de las semillas producidas), aunque esto podría ser debido a que las plantas del sur no tengan la suficiente presión selectiva en este ambiente de jardín común, al disponer de riego *ad libitum*.

Capítulo 2. A nivel genómico, se confirmó que las poblaciones del norte poseen una mayor diversidad genética que las poblaciones del sur. Además, los individuos procedentes de las poblaciones del sur mostraron un mayor grado de parentesco entre ellos que los individuos de las poblaciones del norte. También se encontró un mayor número de SNPs (*Single Nucleotide Polymorphisms* o polimorfismos de un solo nucleótido) candidatos a estar bajo selección en las poblaciones del norte que en las del sur, y se encontraron algunos SNPs asociados a los rasgos estudiados (inicio de floración, número de semillas producidas, peso de las semillas producidas, peso de las semillas producidas, altura, biomasa, crecimiento, SLA y LDMC). Por otro lado, no se observó una reducción de la diversidad genética debido al proceso de selección artificial.

Capítulo 3. El flujo genético asistido también resultó eficaz para adelantar la floración en *L. angustifolius* en condiciones controladas. Además, este adelanto del inicio de floración también causó que estas plantas tuvieran un menor crecimiento, y que produjeran semillas de mayor peso. Además, se identificaron 36 SNPs que mostraron una diferencia significativa en sus frecuencias alélicas entre las líneas control y de flujo genético. Además, estos 36 SNPs estuvieron asociados a los cambios producidos en el

inicio de floración, el peso de las semillas y el crecimiento de las plantas a nivel fenotípico.

Capítulo 4. En condiciones naturales, las plantas descendientes de las líneas de selección artificial no mostraron ningún cambio significativo ni en el inicio de floración ni en ninguno de los otros rasgos estudiados. Las plantas descendientes de la línea de flujo genético asistido sí mantuvieron los cambios que ya se observaron en condiciones controladas. Es decir, las plantas descendientes de la línea de flujo genético asistido florecieron antes que las plantas procedentes de la línea control y también tuvieron un menor crecimiento. Por tanto, parece que, en este caso, el flujo genético asistido podría ser más eficaz y tener efectos más estables que la selección artificial. No obstante, la falta de efectos observados en las líneas de selección artificial también podría ser debida a que las plantas no sufrieran un gran estrés térmico al encontrarse de nuevo en unas condiciones más frías que las del jardín común.

Conclusiones

1. El uso de la selección artificial ha sido útil para adelantar el inicio de la floración de *Lupinus angustifolius* bajo condiciones controladas de un experimento de jardín común, pero sólo en las poblaciones de latitudes más altas. Por su parte, el tratamiento de flujo genético asistido de poblaciones del norte con polen procedente del sur también ha sido capaz de adelantar el inicio de la floración bajo condiciones controladas de un experimento de jardín común.

2. El adelanto del inicio de la floración ocasionado por las líneas de selección también conllevó la modificación de otros rasgos de la planta (altura, biomasa, crecimiento, SLA y LDMC), aunque no se observó ningún cambio en el éxito reproductivo. En paralelo, el inicio más temprano de la floración del tratamiento

de flujo asistido vino acompañado de un aumento del peso de las semillas y de un menor crecimiento de las plantas.

3. Los estudios genómicos indican que existe una marcada diferenciación genética entre las poblaciones estudiadas del norte y del sur de *Lupinus angustifolius*, presentando las primeras una mayor variabilidad genética.

4. En el experimento de flujo genético asistido se detectó una clara asociación entre los cambios observados a nivel fenotípico y los cambios observados a nivel genómico respecto al inicio de floración, el peso de las semillas producidas y el crecimiento de las plantas.

5. Las plantas descendientes de los tratamientos de selección artificial no conllevaron la modificación de ningún rasgo en el experimento realizado en condiciones naturales, mientras que las plantas procedentes del tratamiento de flujo genético asistido sí mantuvieron los cambios en el inicio de floración y en el crecimiento observados en el experimento bajo condiciones controladas. De este modo, en este caso de estudio, el flujo genético asistido parece ser más eficaz y tener efectos más estables que la selección artificial.

6. Las poblaciones del norte tienen un mayor potencial adaptativo para adelantar el inicio de floración frente al cambio climático, mientras que las poblaciones del sur se encuentran en una situación de mayor vulnerabilidad.

GENERAL INTRODUCTION

Background

The threat of climate change to biodiversity

Biological diversity is essential for human survival. The acceleration of climate change, produced by human activities, is causing that the current rate of biodiversity loss is tens or hundreds of times higher than the average of the last ten million years (IPCC, 2022). In this context, the survival of many species is under threat. Organisms can respond to climate change in many different ways. One of the most common and best-studied responses is the migration of species or populations to higher latitudes or altitudes to occupy areas with environmental characteristics consistent with their tolerance ranges (Forero-Medina et al., 2011; Parmesan & Yohe, 2003; Root et al., 2003; Thomas, 2010). However, it is possible that environmental changes may occur faster than the dispersal capacity of some species. In addition, these movements are not possible for some organisms, such as some plants, which are sessile and have reduced seed dispersal (Berg et al., 2010; Engler et al., 2009). In these cases where migration is not possible or is not sufficient, organisms have to look for alternatives to avoid extinction without moving from the place where they live. These alternatives involve phenotypic plasticity and/or genetic adaptation (Gienapp et al., 2008; Jump & Peñuelas, 2005; Teplitsky & Millien, 2014). Phenotypic plasticity refers to the capacity of the same individual or genotype to express different characteristics depending on the environment in which it is found (Bradshaw, 1965), while genetic adaptation, on the other hand, implies a change in the genetic composition. Although phenotypic plasticity may offer a more rapid response to change than evolutionary adaptation, it may also be insufficient in the long term (Miner et al., 2005). Thus, genetic adaptation may be the only way for some species or populations to survive for a prolonged period of time (Bradshaw, 1965; Gienapp et al.,

2008; Jump & Peñuelas, 2005). However, these responses might not be sufficient to guarantee population survival, given the speed at which the environment is changing (Shaw & Etterson, 2012; Talukder et al., 2022; Urban, 2015).

Conservation strategies to improve species survival

In the face of this situation, new conservation strategies are emerging that can help to conserve biodiversity. Assisted evolution refers to conservation and restoration actions that help organisms to better adapt to new environmental conditions (Jones & Monaco, 2009; van Oppen et al., 2015). Any action that implies a human intervention on any of the evolutionary forces would be encompassed within this term. One of these strategies is assisted migration (Grady et al., 2011; Loss et al., 2011). It consists of intentional movements of individuals and populations to habitats with more suitable environmental conditions (Aitken & Whitlock, 2013; Vitt et al., 2010). However, such actions have also been widely debated among the scientific community, due to the potential risks involved (Aitken & Whitlock, 2013; Laikre et al., 2010; Ricciardi & Simberloff, 2009; Webber & Scott, 2011). It is important to keep in mind that these interventions could cause hybridization of species, unintentional spread of diseases, the disruption of processes such as pollination, or lead to a species becoming invasive (Loss et al., 2011; Traveset & Richardson, 2006; Williams & Dumroese, 2013). Alternative strategies, such as artificial selection or assisted gene flow, have been proposed to solve some of these ecological problems associated to assisted migration (Aitken & Whitlock, 2013; Jones & Monaco, 2009; van Oppen et al., 2015). These strategies are aimed at favoring *in-situ* adaptation processes.

The adaptive or evolutionary potential of a species is defined as its capacity to evolve in response to environmental changes (Funk et al., 2019). It has already been observed that many organisms may have the ability to evolve in response to climate change in a short

period of time (Bradshaw & Holzapfel, 2008). Thus, artificial selection and assisted gene flow aim to improve the adaptive potential of species in response to climate change (Aitken & Whitlock, 2013; Humanes et al., 2021; van Oppen et al., 2015). To this end, they attempt to increase the prevalence of adaptive alleles of the species that are found in low frequencies in the threatened population, by the reinforcement with individuals containing advantageous alleles (van Oppen et al., 2015). Through the process of artificial selection, humans select certain individuals from a population that present particular traits of interest, in order to maintain them in the following generations (Conner, 2016). Moreover, the amount of additive genetic variance for a given trait can be estimated with the help of artificial selection, and therefore it can also be useful in determining how a trait may evolve in response to natural selection (Conner, 2003). On the other hand, gene flow from a source population to a target population increases the genetic variability of the latter and can therefore improve its adaptative potential to environmental modifications, such as climate change (Grummer et al., 2022). Assisted gene flow can be defined as a process in which gametes or individuals are consciously transferred between populations in an attempt to improve the adaptation to current or potential climate conditions (Aitken & Whitlock, 2013). Although artificial selection has been widely used for the domestication of crops and animal species (Dempewolf et al., 2014), and some studies have investigated the potential advantages of gene flow for genetically vulnerable populations (Morente-López et al., 2021; Prieto-Benítez et al., 2021; Sexton et al., 2011), little research has been done on using them for conservation purposes. Based on the above, these two strategies could be a way of both assessing the evolutionary potential of species and increasing their adaptive potential. However, these actions may also entail certain associated risks. Artificial selection could lead to a decrease in the genetic diversity of populations and may also cause undesired responses in traits correlated with

the selected trait (Sheth & Angert, 2016). Assisted gene flow may lead to a decrease in the biological efficiency of individuals (outbreeding depression), as it could lead to the disruption of coadapted gene networks or the introduction of maladapted alleles (Aitken & Whitlock, 2013). Given the great relevance that these interventions can have for biodiversity conservation, and the potential risks and benefits associated, it is essential to develop experimental studies that evaluate these initiatives from both a genetic and ecological point of view.

Flowering onset as a key trait for plant adaptation to climate change

The phenology of many species is strongly influenced by environmental conditions (Cleland et al., 2007; Munguía-Rosas et al., 2011; Tang et al., 2016). For this reason, changes in phenology are one of the main consequences of climate change. Furthermore, there is evidence that many of these phenological changes are regulated by genetic variations more than just phenological plasticity (Bradshaw & Holzapfel, 2009). In plants, the timing of reproduction has a significant impact on their fitness (Forrest & Thomson, 2010; Landa, 1992), making the shift from the vegetative to the reproductive phase a critical step in their life cycle (Blümel et al., 2015). Thus, the phenology of reproduction is likely to be under strong selection (Chuine, 2010; Levin, 2006). In addition, flowering onset has been described as a trait with high heritability (Franks et al., 2007; Franks & Weis, 2008). For all these reasons, flowering onset is an essential aspect of plant adaptation to climate change (Franks & Hoffmann, 2012) and it is a suitable target variable for experimentation with artificial selection and assisted gene flow strategies. However, flowering onset is a polygenic trait in which more than one hundred genes have been identified to be involved in different species (Blümel et al., 2015; Weller & Ortega, 2015). It involves an intricate network of several genetic and epigenetic factors (Blümel et al., 2015), requesting the integration of several both internal and external stimuli

(Putterill et al., 2004). Therefore, it is a complex trait, which can be difficult to characterize and modify in an intentional way.

Objectives and work plan

Taking into account the above considerations, the general objective of this thesis is to evaluate the use of artificial selection and assisted gene flow as tools to assess the adaptive potential of target plant populations and to increase it with the aim of modifying a particular trait of interest to facilitate their adaptation to new environmental conditions derived from climate change. This general objective was applied to selected populations of *Lupinus angustifolius* L. with the aim of advancing their flowering onset. We also aim to evaluate the risks and benefits of these tools in order to potentially use them in conservation and restoration actions. To this end, we identified the following specific objectives: i) To use artificial selection and assisted gene flow techniques to advance flowering onset in four populations of *L. angustifolius*. ii) To evaluate the effects that artificial selection and assisted gene flow may have on reproductive and non-reproductive traits other than flowering onset. iii) To identify genomic signals that may be associated with early flowering phenotypes and other trait changes derived from artificial selection and assisted gene flow. iv) To test the progeny derived from the artificial selection and assisted gene flow treatments in natural conditions to find out if the application of these treatments could be considered in conservation and restoration actions.

This thesis is structured into four interrelated chapters (Figure 1). Each chapter consists of the typical sections of which research articles are composed (*i.e.*, abstract, introduction, materials and methods, results, and discussion), and is intended for publication in international peer-reviewed journals. At the end of the thesis, we present a general discussion addressing the afore-mentioned objectives and the obtained results.
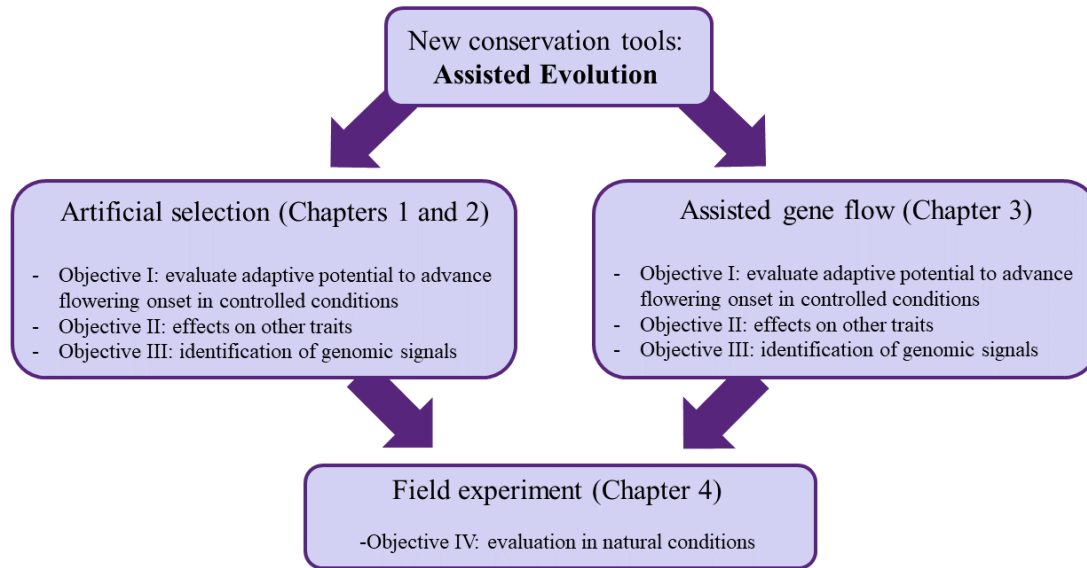
Figure 1. Schematic representation of the organization of the thesis into chapters and the specific objectives addressed in each one of them.

In the **first chapter** we use artificial selection to quantify the evolutionary potential of *L. angustifolius* to advance flowering onset under controlled conditions. By establishing different early flowering lines through both self-crosses and intrapopulation manual crosses, we aim to advance the flowering onset, and then evaluate whether other traits are modified. Thus, we are addressing objectives one and two.

In the **second chapter**, we perform a genomic study using a gene capture approach to identify the possible genomic signals that artificial selection is producing in the lines established in chapter one. In this way, we respond to objective number two. We also used the genomic analysis to assess the genetic diversity and genetic structure of the studied populations.

In the **third chapter**, we address objectives one, two, and three through the gene flow approach performed under controlled conditions. By performing manual interpopulation crosses, we evaluate whether gene flow can be a suitable tool to increase the evolutionary

potential of *L. angustifolius* to advance flowering initiation, the effects that this manipulation can have on other traits, and we also perform a genomic characterization.

Finally, in the **fourth chapter**, we test the progeny derived from artificial selection and assisted gene flow strategies in a field experiment near the northern populations of *L. angustifolius*. We intend to assess whether their effects are in line with those occurring under controlled conditions, and to determine whether they might be suitable tools for use in conservation and restoration actions. Therefore, we achieve objective number four.

Methods

Species used as case study

Lupinus angustifolius L. or blue lupine (Figure 2) is an herbaceous annual plant belonging to the family Fabaceae. It is widely distributed throughout the Mediterranean basin and has also been introduced as a crop in many other parts of the world (Castroviejo & Pascual, 1993). It occurs naturally in well-drained acid or neutral soils, including disturbed areas like roadway medians or abandoned croplands (Rhodes & Maxted, 2016). This species presents symbiosis with nitrogen-fixing bacteria (*Bradyrhizobium sp.*), increasing the availability of total nitrogen in the soil (Jarabo-Lorenzo et al., 2003; Stępkowski et al., 2007). It is an essentially self-pollinating species, whose hermaphrodite flowers self-pollinate before the flower opens (Wolko et al., 2011). It is a plant that can reach up to 100 cm in height, and its inflorescences can have up to 30 blue-purple flowers that produce pods containing 3-7 seeds (Clements et al., 2005). Photoperiod and vernalization control flowering (Rahman & Gladstones, 1974), so flowering onset may occur between March and August depending on climatic conditions (Castroviejo & Pascual, 1993). It is a species whose characteristics make it suitable for experimentation,

since it has a short life cycle, grows in a wide range of humidity and temperature conditions, and its seeds germinate at practically 100 % after mechanical scarification.

Figure 2. Individual of *Lupinus angustifolius* L.
Author of the original photo: S. Sacristán-Bajo

Study populations and experimental design

We selected four populations of *Lupinus angustifolius* L. located in the Iberian Peninsula, distributed by pairs in two climate-contrasting zones (Figure 3, Table 1). Two of them are located in Salamanca (Central Spain, hereafter northern populations), and the other two are located in Badajoz (South Spain, hereafter southern populations). The two regions are approximately 300 km apart, and the two populations in each region are about 20 kilometers apart. These regions receive almost the same amount of precipitation each year, but precipitation is greater in the northern region in the months of May-July when the plants develop their fruits and seeds and the southern region has higher mean, minimum, and maximum annual temperatures and, thus, experience higher water deficits.

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In the summer of 2016, we collected seeds of *L. angustifolius* from each of these populations. We collected seeds from at least 98 mother plants (genotypes) in each population that were at least one meter distant from one another.
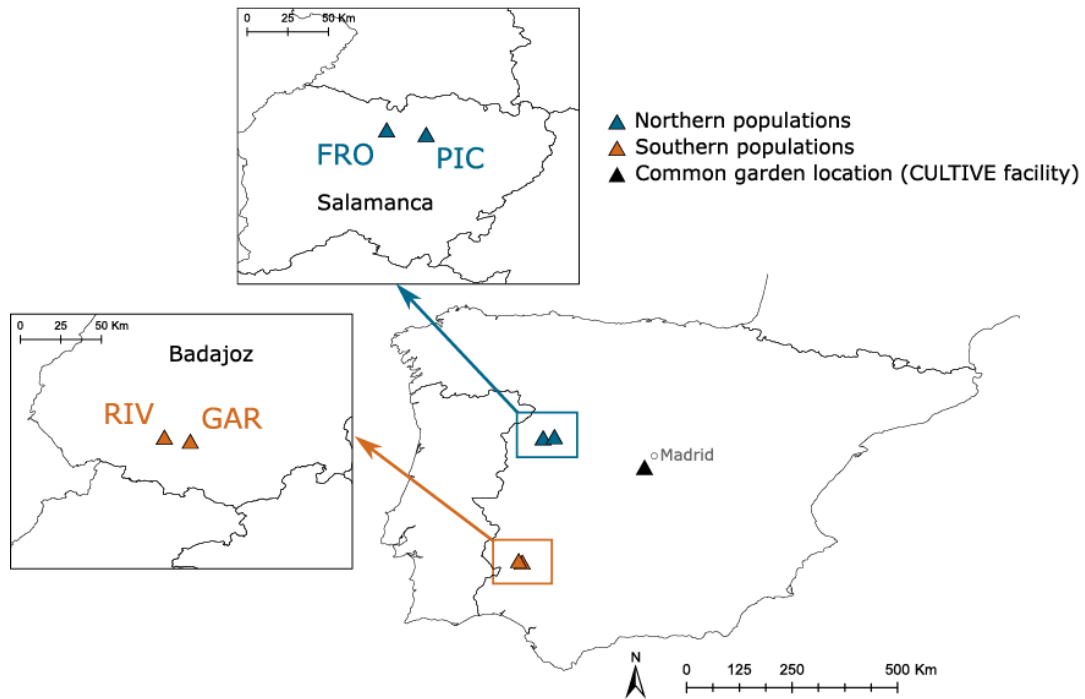
Figure 3. Location of the four populations of *Lupinus angustifolius* L. studied and of the common garden used in this thesis.

Table 1. Climatic data of the four populations of *Lupinus angustifolius* L. and common garden site involved in the thesis. Town, region, geographical coordinates (decimal degrees, WGS84) and climate variables associated to the populations (1985-2015 period) and to the common garden site (2017-2020 period) are shown. The May–July window matches the period when plants are producing fruits and seeds. Climate data were obtained from ClimateEU (Marchi et al., 2020).

Acronym	Town	Region	Latitude	Longitude	Elevation (m.a.s.l.)	Annual mean temperature (ºCelsius)	May-July precipitation (mm)
FRO (N)	Zafrón	Central Spain	41.0241	-6.0281	840	12.4	92
PIC (N)	Zarapicos	Central Spain	41.0043	-5.8130	820	12.6	89
GAR (S)	La Garranchosa	Southern Spain	38.3257	-6.4337	422	16.5	64
RIV (S)	Rivera de la Lanchita	Southern Spain	38.3515	-6.5760	352	16.8	61
-	Common garden (2017-2020)	Central Spain	40.3343	-3.8829	690	14.9	63

In each chapter, we use different experimental designs to achieve the proposed objectives:

Chapter 1. In November of 2016, the seeds of the 98 genotypes were sown in a common garden experiment at the CULTIVE facility (https://urjc-cultive.webnode.es/) at Rey Juan Carlos University (Móstoles, Madrid) (Figure 4a). This facility is located at intermediate conditions between the two groups of populations selected (Figure 3, Table 1). In 2017, we selected approximately the first quartile of individuals that flowered earlier to establish an early flowering selection line (hereafter, EFL). We also randomly selected another 25 % of the individuals among all genotypes to establish a control flowering line (hereafter, CFL). These lines were maintained by self-crossing during the following seasons of the experiment (2017-2018, 2018-2019, and 2019-2020 seasons). In 2018, we manually and randomly crossed the individuals of the early flowering line (Figure 4b), obtaining an outcrossed early flowering line (hereafter, OUT). In 2019, we allowed the OUT line individuals to self-pollinate, creating a segregating F2 line (hereafter, OUTS). We monitored the flowering onset of all individuals daily, and we also measured a number of reproductive and non-reproductive traits of the plants (number and weight of the seeds, height, biomass, shoot growth, specific leaflet area (SLA) and leaflet dry matter content (LDMC).

Chapter 2. In this chapter we carried out the genomic characterization of the lines established in chapter 1. For this purpose, in 2019 we extracted DNA from the leaves of 15 individuals of each of the four populations and the three selection lines corresponding to that year (CFL, EFL and OUT). We conducted a gene capture approach in which we used the *Lupinus angustifolius* annotated genome that is available in GenBank as a reference genome (GenBank accession: PRJNA398717). We determined the biological function of each sequence of the *L. angustifolius* genome, and we selected the Gene Ontology terms related to our traits of interest (*i.e.*, flowering, growth, and abiotic stress).

The sequences obtained through this method were employed as probes to carry out the sequencing process. For each population, we compared the individuals of the artificial selection lines with the control individuals looking for outlier SNP frequencies. We also characterized the genetic diversity and structure of the populations. Finally, we carried out a genome wide association study (GWAS) to look for associations between the phenotypic traits studied and the genomic regions sequenced.

Chapter 3. In November 2017, seeds of the 98 genotypes were sown following the same procedure as in Chapter 1 and in the same common garden. In 2018, we conducted manual interpopulation crosses (Figure 4b) using pollen from plants from southern populations to pollinate plants from northern populations, thus establishing an F1 gene flow line (hereafter, GFL). As a control line, we use the same as in Chapter 1 (CFL). In 2019, the individuals from the CFL of the northern populations were manually crossed with pollen from individuals from the GFL to establish a backcross line (hereafter, BCL). We also let the GFL to self-pollinate, thus establishing an F2 self-pollination line (hereafter, SPL). In this chapter, we carried out a phenotypic characterization, monitoring the flowering onset of the plants and also characterizing a battery of plant traits (in the same way as in Chapter 1). In addition, we performed a genomic characterization of these lines using the same protocol as described in Chapter 1.

Chapter 4. In 2020, all the above-mentioned lines were perpetuated by self-pollination, resulting in a control flowering line (CFL), an early flowering line (EFL), and an F3 generation of the outbred line (hereafter, OUTS2) for the artificial selection experiment, and a control flowering line (CFL) and an F3 generation (hereafter, SPL2) for the gene flow experiment. In November 2020, seeds of all the established lines (*i.e.*, CFL, EFL, OUTS2 and SPL2) of the northern populations were sown to perform a field experiment in Zarapicos (Salamanca, Figure 4c). Figures 4d and 4e show a complete diagram of the

process of creating the different lines through the successive generations. As in Chapters 1 and 3, we carried out a phenotypic characterization of these individuals, monitoring the flowering onset and measuring a series of reproductive and non-reproductive trait.
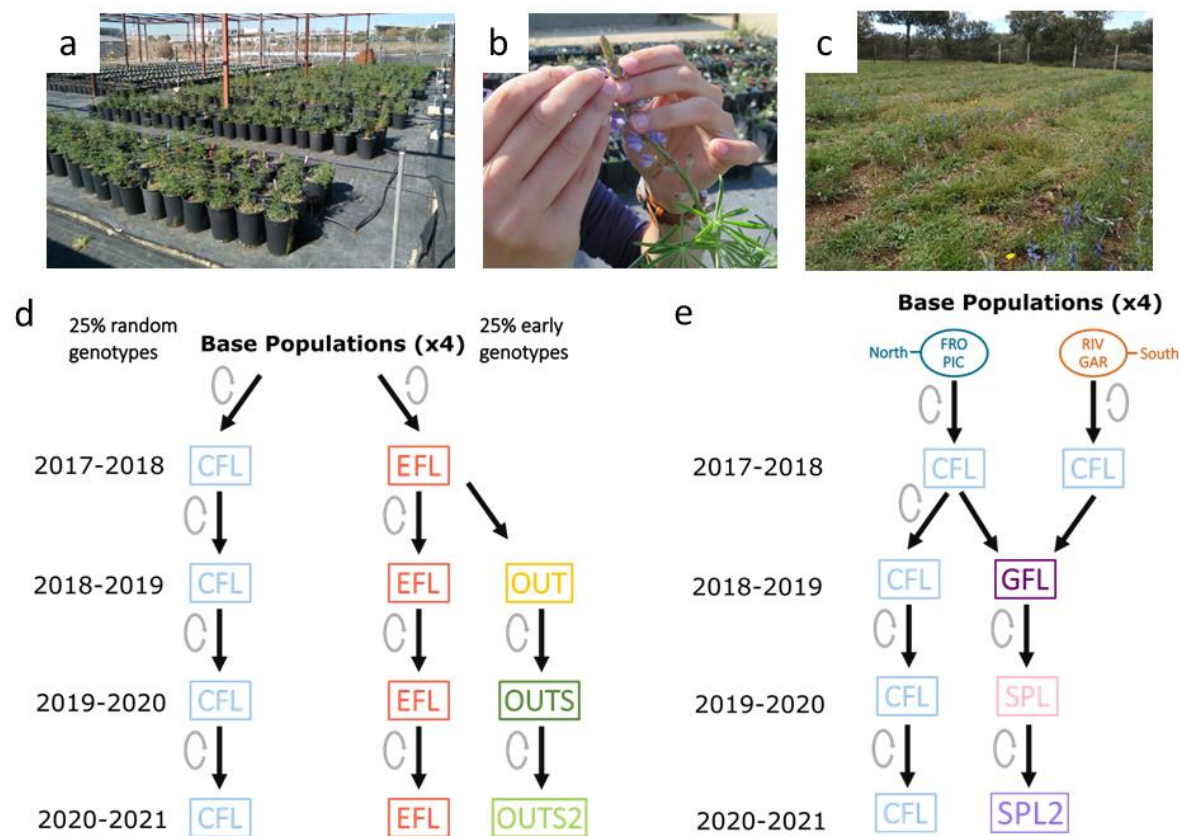
Figure 4. Experimental designs of the experiments carried out with *Lupinus angustifolius*. a) Common garden established at Rey Juan Carlos University, b) Manual pollination of *L. angustifolius*. c) Plot in which the field experiment was carried out in Zarapicos (Salamanca). d) Diagram of the process of establishing the different lines for the artificial selection experiment. e) Diagram of the process of establishing the different lines for the assisted gene flow experiment. Grey circles next to the arrows indicate that the individuals of that line were self-crossed.

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Chapter 1: Population origin determines the adaptive potential for the advancement of flowering onset in *Lupinus angustifolius* L. (Fabaceae)

Abstract

In the present framework of global warming, it is unclear if evolutionary adaptation can happen quick enough to preserve the persistence of many species. Specifically, we lack knowledge about the adaptive potential of the different populations in relation to the various constraints that may hamper particular adaptations. There is evidence indicating that early flowering often provides an adaptive advantage to plants in temperate zones in response to global warming. Thus, the objective of this study was to assess the adaptive potential for advancing flowering onset in *Lupinus angustifolius* L. (Fabaceae). Seeds from four populations from two contrasting latitudes in Spain were collected and sown in a common garden environment. Selecting the 25 % of the individuals that flowered earlier in the first generation, over three generations, three different early flowering selection lines were established, involving both self-crosses and outcrosses. All artificial selection lines advanced their flowering significantly with respect to the control line in the northernmost populations, but not in the southern ones. Selection lines obtained from outcrossing had a greater advancement in flowering than those from self-crossing. No differences were found in the number or weight of the seeds produced between control and artificial selection lines, probably because plants in the common garden were drip irrigated. These results suggest that northern populations may have a greater adaptive potential, and that southern populations may be more vulnerable in the context of climate warming. However, earlier flowering was also associated with changes in other traits (height, biomass, shoot growth, LDMC and SLA), and the effects of these changes varied greatly depending on the latitude of the population and selection line. Assessments of the ability of populations to cope with climate change through this and other approaches are essential to manage species and populations in a more efficient way.

Keywords

Artificial selection, climate change, evolutionary ecology, manual crosses, plant traits, population origin.

Introduction

Species possess different strategies to cope with climate change. One of the best documented responses is that species distributions are shifting towards higher latitudes and/or elevations to occupy areas within their ranges of thermal tolerance (Forero-Medina et al., 2011; Parmesan & Yohe, 2003; Root et al., 2003). However, some species will not be able to migrate fast enough to avoid extinction, especially those that have limited dispersal capacity, such as many plants (Berg et al., 2010). In these cases, phenotypic plasticity and adaptive evolutionary responses are of vital importance (Jump & Peñuelas, 2005; Teplitsky & Millien, 2014). The ability of species to evolve in response to environmental changes constitutes their adaptive or evolutionary potential (Funk et al., 2019). Given the exceptional swiftness of climate change (Shaw & Etterson, 2012), it is unclear for many species if evolutionary adaptation can occur fast enough to ensure population survival. Therefore, characterizing adaptive potential is essential for assessing the resilience and extinction risk of species and populations to climate change. Nevertheless, it can be a very difficult feature to measure and quantify (Funk et al., 2019; Williams et al., 2008).

Artificial selection is a useful way to test a species' evolutionary potential and determine the nature and strength of its evolutionary constraints (Conner, 2003; Hoffmann & Sgró, 2011). It is a process in which humans select individuals of a given species with certain phenotypic traits for breeding, to enhance and perpetuate those traits in future generations (Conner, 2016). It has been used to improve many different traits during the domestication of crops, livestock, and pets, such as changes in size, shape, or color, adaptation to

environmental conditions, or resistance to pests and diseases (Conner, 2016; Dempewolf et al., 2014). However, the use of artificial selection in the fields of conservation biology and adaptive evolution has been so far little explored (Selwood et al., 2015).

Artificial selection can provide estimates of the magnitude of additive genetic variance for a trait and the genetic covariance between the selected trait and other traits. The additive genetic variance of a trait is essential for natural selection to act upon and bring about evolutionary change. On the other hand, genetic covariances with other traits are also important because, through them, natural selection on one trait causes an evolutionary change in a correlated trait, which may or may not be itself under direct selection (Conner, 2003). In the former case, genetic covariances can explain the existence of trade-offs between fitness-related traits (Etterson & Shaw, 2001; Walsh & Blows, 2009; Worley & Barrett, 2000). Therefore, artificial selection can be a sound way to determine how a single trait may evolve under a given strength of natural selection.

Since environmental pressures may vary among populations, a given trait can acquire different values through local adaptation depending on the population origin (Debieu et al., 2013; Milla et al., 2009; Morente-López et al., 2020). Thus, the differences in genetic diversity within and between populations influence the adaptive potential of a species (Funk et al., 2019). Conducting artificial selection experiments on populations originating from different environmental conditions can inform us about the strength and speed with which a trait can evolve in response to environmental changes in each population and which populations have greater adaptive potential for a given trait (Conner, 2003). In this context, they can be used to determine the vulnerability of populations to climate change and to explore how we can act to mitigate their effects.

One of the consequences of global warming is the early arrival of spring and the late arrival of winter in temperate zones, which has prompted the modification of phenological

traits in many species (Bradshaw & Holzapfel, 2009). For instance, in plant species, the transition from the vegetative to the reproductive phase is a crucial step in their life cycle (Blümel et al., 2015) and the timing of reproduction greatly influences reproductive success (Forrest & Thomson, 2010; Landa, 1992; Thomas et al., 2001). There is already considerable evidence that climate change is favoring advanced flowering plants in temperate zones (Büntgen et al., 2022; Fitter & Fitter, 2002; Peñuelas et al., 2002) and that flowering time is a highly heritable character (Franks et al., 2007). Therefore, flowering time is a key trait for plant adaptation to climate change (Franks & Hoffmann, 2012). The proper synchronization of flowering time with ideal environmental conditions is a delicate task that implies the integration of numerous external and internal signals (Putterill et al., 2004). Thus, the control of flowering time entails a complex network of different genetic and epigenetic regulators (Blümel et al., 2015), implying that it is a polygenic trait with more than one hundred implicated genes identified in some species (Blümel et al., 2015; Weller & Ortega, 2015).

The main objective of our study was to use artificial selection to assess the adaptive potential for advancing flowering onset in two sets of populations of contrasting origins of the annual legume *Lupinus angustifolius* L. We hypothesized that, given the polygenic nature of the trait, there would be substantial standing genetic variation to obtain artificially selected subsets whose progeny would flower significantly earlier than the population mean, even in a selfing species such as *L. angustifolius*. Moreover, forcing the outcrossing between the artificially selected individuals might generate greater genetic variation and phenotypes that flower even earlier. Considering that the two sets of populations differ in the temperature regimes of their localities of origin (southern populations have warmer temperature regimes), we expected that flowering time may advance less in southern populations with warmer conditions, because natural selection

may have already acted in these populations to select for early flowering genotypes, thus, reducing available genetic variation. Additionally, there are potential epistatic and pleiotropic interactions between the genes involved in the regulation of flowering time and those involved in the expression of other traits. Thus, we hypothesized that artificial selection to advance the onset of flowering might indirectly affect the expression of other plant traits, which may also contribute to the overall fitness of the individuals. Considering the 'fast-slow' plant economics spectrum that integrates traits through leaves, stems and roots, and results in the existence of trade-offs between traits (Reich, 2014), we would expect that plants that flower earlier would have lower biomass and growth, since they would allocate more resources to reproduction. Using a common garden experiment, we compared early flowering selection lines, obtained through self-crosses or out-crosses, against a control line in four populations of two climatically contrasted regions in the Iberian Peninsula. We recorded flowering onset and measured a suite of other plant traits to answer the following questions: i) Is there adaptive potential for advanced flowering in the study populations of *L. angustifolius*? ii) Can outcrossing of the artificially selected subset advance further flowering onset? iii) Is the intensity of flowering onset advance dependent on the population of origin or the latitude of the material subjected to artificial selection? iv) Which other traits will be affected by advancing flowering onset and how?

Materials and Methods

Study species and collected material

Lupinus angustifolius L. (Fabaceae) is an annual herbaceous plant that occurs in the Mediterranean Region and has been introduced as a cultivated crop all around the world (Castroviejo & Pascual, 1993). The flower is hermaphroditic and mostly self-pollinates before its petals open (Wolko et al., 2011). Natural outcrossing estimates are below 2 %

(Dracup & Thomson, 2000). The inflorescence can have up to 30 violet flowers developing acropetally and produces pods with 3-7 seeds (Clements et al., 2005). Flowering onset is controlled by photoperiod and vernalization, and long days accelerate the start of flowering (Gladstones & Hill, 1969; Rahman & Gladstones, 1974).

In the summer of 2016, we collected seeds from four populations of *L. angustifolius* located in Central and Southern Spain (Figure 1, Table 1). All the populations had a large number of individuals (more than 500). The two populations within each region are located less than 20 km apart, whereas the distance between northern and southern populations is approximately 300 km. In each population, we separately collected seeds from 98 mother plants (genotypes) that were located at least one meter apart from each other.
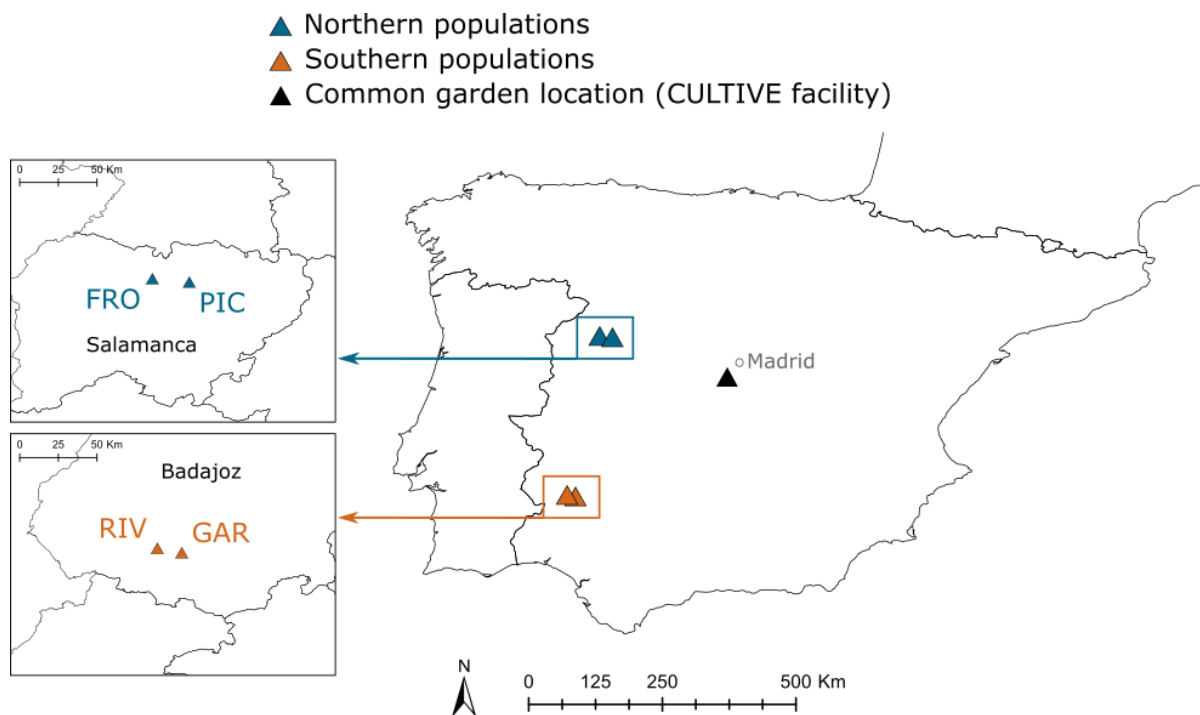
Figure 1. Location of northern (blue) and southern (orange) populations of *Lupinus angustifolius* L. in the Iberian Peninsula. A detail of the populations and the provinces in which they are situated is shown in the squares on the left.

Table 1. Populations of *Lupinus angustifolius* L. and common garden site involved in the study. Town, region, geographical coordinates (decimal degrees, WGS84), and climate variables associated to the populations (1985-2015 period) and to the common garden site (years 2017-2020). May-July period corresponds with the period when the plants are developing fruits and setting seeds. Climate data were obtained from ClimateEU (Marchi et al., 2020).

Acronym	Town	Region	Latitude	Longitude	Elevation (m.a.s.l.)	Annual mean temperature (ºCelsius) and coefficient of variation (in brackets)	May-July precipitation (mm) and coefficient of variation (in brackets)
FRO (N)	Zafrón	Central Spain	41.0241	-6.0281	840	12.4 (3.2)	92 (46)
PIC (N)	Zarapicos	Central Spain	41.0043	-5.8130	820	12.6 (3.1)	89 (45)
GAR (S)	La Garranchosa	Southern Spain	38.3257	-6.4337	422	16.5 (2.4)	64 (64)
RIV (S)	Rivera de la Lanchita	Southern Spain	38.3515	-6.5760	352	16.8 (2.2)	61 (63)
-	Common garden 2017	Central Spain	40.3343	-3.8829	690	15.1	72
-	Common garden 2018	Central Spain	40.3343	-3.8829	690	14.6	95
-	Common garden 2019	Central Spain	40.3343	-3.8829	690	14.8	16
-	Common garden 2020	Central Spain	40.3343	-3.8829	690	15.1	69

Cultivation process and artificial crosses

In the autumn of 2016, seeds from the 98 collected genotypes of each population were sown in a greenhouse at the CULTIVE facility (https://urjc-cultive.webnode.es/) at Rey Juan Carlos University (Móstoles, Madrid). The facility is located in Central Spain, at an intermediate elevation, latitude and temperature regime between the two sets of populations studied (Figure 1, Table 1). The temperature range inside the greenhouse varied between 1 to 25 degrees Celsius, and plants were only exposed to natural light. Seeds were previously scarified by clipping a small fraction of the seed coat to ensure germination and deposited in 6 L pots with a mixture of 50 % sand and 50 % commercial substrate enriched with NPK (Klasmann). For each maternal genotype of each population, two pots were assigned, and three seeds were sown in each, constituting a total of 784 pots. When seeds germinated, one seedling was left in each pot and the rest were clipped. In February 2017, the pots were transferred out of the greenhouse and moved outdoors. Pots were randomly distributed following a block design where populations were evenly represented in each block and regularly watered with drip irrigation to constitute a common garden environment.

In the spring of 2017, the flowering phenology of plants was monitored daily. Flowering onset was calculated as the number of days from the sowing date to the appearance of the first flower of the plant. We considered that each plant had flowered when blue-purple petals of one flower in the main inflorescence could be clearly seen. Artificial selection was implemented one single time as follows: for each population, the plant genotypes that flowered earlier (approximately the first quartile of flowering onset) were tagged and their seeds were separately collected to create an early flowering selection line (hereafter, EFL). For each population, we also generated a control line (hereafter, CFL) by randomly selecting another 25 % among all 98 genotypes. Once again, seeds of each genotype were

separately collected and stored. In the autumn of 2017, the seeds from the chosen genotypes of early flowering selection lines and from the control lines of each population were scarified and sown in pots in the greenhouse as described above. This process was repeated in the same way in subsequent culture cycles in the 2017-2018, 2018-2019 and 2019-2020 seasons. However, in the early flowering selection line, no further selection was implemented. Instead, in the EFL and CFL, seeds of each genotype cultivated in the previous season were separately collected and stored, until use in the subsequent season. In the seasons 2017-2018 and 2018-2019, each genotype was replicated in four pots. In 2019-2020, each genotype was replicated in just two pots.

In the spring of 2018, we manually crossed genotypes from the early flowering selection line between each other to obtain an outcrossed early flowering line (hereafter, OUT). Flowering individuals from the EFL were randomly paired, and manual crosses were carried out in both directions following the protocol described in Supplementary Material, Appendix 1.

In the flowering season of spring 2019, we let the individuals of the OUT line self-pollinate, generating a segregating F2 line (hereafter, OUTS). The OUT line is potentially highly heterozygous and cannot be maintained in a naturally autogamous species. Therefore, it is important to see what happens to the focal trait in subsequent generations when the natural self-pollination process is resumed. Figure 2 shows a diagram of the complete process. The information on the sample sizes per population for each selection line and year at the flowering time is provided in Supplementary Material, Appendix 2 (Table S1).
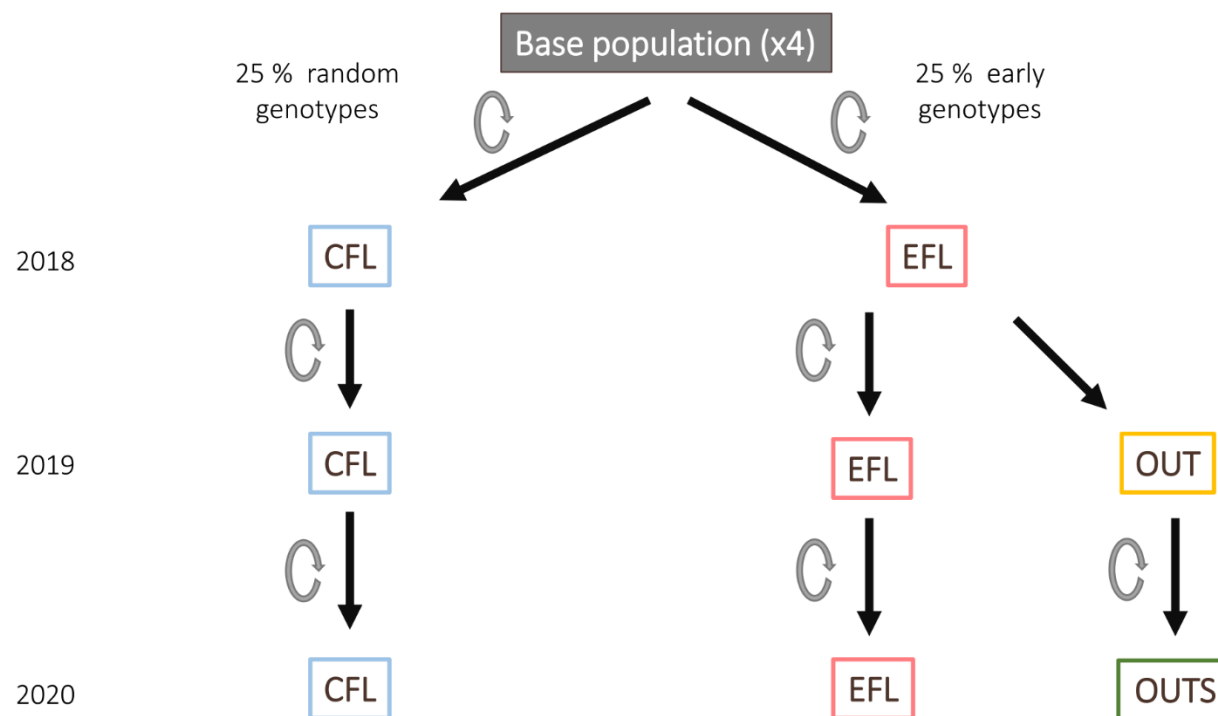
Figure 2. A flowchart depicting the process for generating the different lines. Grey circles next to the arrows indicate that the individuals of that line were self-crossed. CFL: control flowering line; EFL: early flowering line (self-pollinated), OUT: outbred line (cross of different EFL genotypes); OUTS: F2 generation of outbred line resulting from the self-pollination of OUT genotypes.

Trait measurement

Flowering onset was monitored daily for all plants. At the end of each season, the scars that flower peduncles left in the stalks were counted to estimate the number of fruits per plant. The number of seeds per plant was estimated by multiplying the number of fruits of each plant by the average number of seeds per fruit. The average number of seeds per fruit was calculated from the count of the seeds present in up to 15 randomly chosen fruits in each plant. Mean seed weight was estimated from the individual weight of 10 randomly chosen seeds of each plant. Number of seeds per plant and mean seed weight were considered proxies for measuring fitness. Germination rates were not included because they are close to 100 % when seeds are scarified. Similarly, the survival rate of plants under controlled conditions is also close to 100 %.

To measure the specific leaflet area (SLA) and leaflet dry matter content (LDMC), the central leaflets from eight different fully developed leaves belonging to the lateral branches were collected. The fresh leaflets were weighed immediately in a Kern ABJ 120-4M analytical balance (Kern & Sohn GmbH, Albstadt, Germany). After that, leaflets were placed in water-saturated filter paper and stored in plastic bags, and then refrigerated overnight at 4 °C. The next day, we weighed the leaflets again to obtain the saturated weight and measured the foliar area of the leaflets using a foliar scanner Li-3000C (Li-Cor, NE, United States). Finally, leaflets were dried in an oven at 60 °C for at least 72 hours, and then they were weighted to obtain the dry weight. SLA was calculated by dividing the foliar area of a leaflet by its dry weight (Rosbakh et al., 2015). LDMC was calculated by dividing the leaflets dry weight by their saturated weight (Wilson et al., 1999). Total plant height was considered as the distance from the base of the plant to the edge of the main inflorescence at the end of the flowering season. Additionally, we measured the length of the plant from its base to the first flower at the beginning of

flowering onset and at the end of the season, on the same dates for all the plants of the experiment. Consequently, we estimated shoot growth as the difference between these two measurements. We also measured the aboveground biomass of each plant at the end of the season using the balance previously mentioned.

In 2018 and 2019 we measured all the traits described above except shoot growth, which was only measured for year 2019. In the year 2020, due to the pandemic lockdown, we only measured the flowering onset. For this reason, we have characterized the four lines for flowering onset for each population, but only three lines for the remaining traits.

Data analyses

Following Conner (2003), the selection differential was calculated by subtracting the 2017 flowering initiation means of CFL and EFL. Similarly, the response to selection was calculated as the difference between the population means of CFL and EFL in the next generation (2018). The ratio between the response to selection and the selection differential indicates the heritability of the trait in each population. These parameters were calculated to assess how much genetic variation the trait studied has in each population, and how strongly it responded to the selection exerted on them (Conner, 2003).

To assess the differences in flowering onset between populations occurring at the two latitudes prior to selection we calculated the F statistic applying linear models by using the *lm* function implemented in R version 4.1.1. (R Core Team, 2020). To test the effect of artificial selection on flowering onset we calculated the chi-squared statistic of the Type II Wald chi-square tests using generalized linear mixed models (hereafter, GLMMs), and to assess their effect on fitness and morphological traits, we calculated the chi-squared statistic using linear mixed models (hereafter, LMMs). GLMMs and LMMs were fitted using the *glmer* and *lmer* functions included in the R package lme4 version 1.1-27.1 (Bates et al., 2015). Models were run separately for each response variable. Due

to the substantial similarities in response between the populations from the same latitude, we created a new variable called 'latitude' to group the two northern and the two southern populations. We included *selection line* (CFL, EFL, OUT and OUTS), *year* (2018, 2019 and 2020) and *latitude* (North *vs*. South) as fixed effects, and *genotype* and *population* as random effects. Initially, for all the response variables, we tested the interaction between the variables *line* and *year*. Except for SLA and LDMC, this interaction was not significant, so it was excluded from the models. For SLA and LDMC, analyses were performed separately for each year. In all models, we have also included the interaction between *latitude* and *line*. We used a Poisson distribution for the flowering onset variable, and a Gaussian distribution for the rest of the traits. Model residuals were checked graphically for normality and homogeneity of variances using diagnostic plots. R^2 values were calculated using the *summ* function from the package *jtools* version 2.2.0 (Long, 2019). The significance of each fixed effect was quantified using the *Anova* function from R package *car* version 3.0-11 (Fox & Weisberg, 2011). Differences between lines were calculated using Tukey *post hoc* analyses from R package *emmeans* version 1.6.3 (Lenth, 2019). Posterior mean values, standard errors and 95 % credible intervals for the different traits and lines were also calculated with the *emmeans* package. Correlations for the control line in the year 2019 between flowering onset and the rest of the traits were performed using the *corrplot* function from R package *corrplot* version 0.90 (Wei et al., 2017).

Results

Flowering onset

In 2017, the first generation of plants grown in the common garden showed that the onset of flowering of plants from the northern populations (140 ± 12 days) occurred significantly later than that of southern populations (121 ± 10 days) (F = 1006.7, p <

0.001, Df = 1) (Supplementary Material, Appendix 2 Figure S1). The statistical analysis covering the common gardens in the years 2018, 2019, and 2020 also showed a similar pattern ($X^2 = 41.841$, p < 0.001, Df = 1). The artificial selection applied to obtain the EFL revealed that the selection differential, the response to selection, and the heritability of the flowering onset were higher in the northern populations (Table 2, Figure 3).

Table 2. Selection differential, response to selection, and heritability for the flowering onset of the season 2017-2018 for the four studied populations of *Lupinus angustifolius*.

Population	Selection differential	Response to selection	Heritability
FRO	4.481443	1.7420213	0.3887188
PIC	5.371562	2.0188889	0.3758477
GAR	2.905329	0.6503221	0.2238377
RIV	2.924414	0.8248611	0.2820603
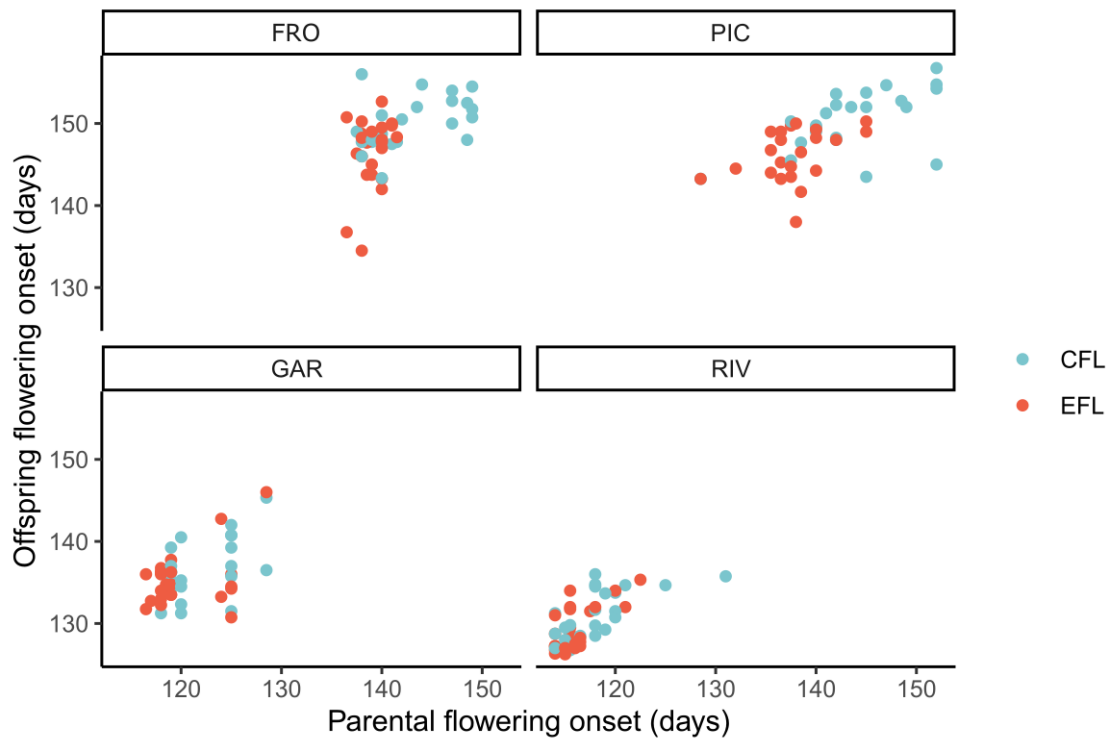
Figure 3. Selection differential for the flowering onset of the season 2017-2018 for the four populations of *Lupinus angustifolius* L. CFL: control flowering line; EFL: early flowering line (self-pollinated). Significant differences (p < 0.05) determined by Tukey test between artificial selection lines and the control line are marked with asterisks (*p < 0.05; **p < 0.01; ***p < 0.001).

The interaction between latitude and line was statistically significant (X^2 = 17.298, p < 0.001, Df = 3), indicating that artificial selection significantly modified flowering onset in northern but not in southern populations (Figure 4, Supplementary Material, Appendix 2, Table S2 and S3). Thus, the EFL, OUT and OUTS lines from the northern populations advanced their flowering time by an average of 6, 16 and 21 days, respectively, compared to the control line (Supplementary Material, Appendix 2, Table S5). Fixed effects explained 59 % of the variation in flowering onset, whereas random effects explained 1.3 % (Supplementary Material, Appendix 2, Table S2). Flowering time for each year, population and line is shown in Supplementary Material, Appendix 2, Figures S2, S3 and S4). Posterior mean values, standard errors, and 95 % confidence intervals for each line are shown in Supplementary Material, Appendix 2, Table S4.
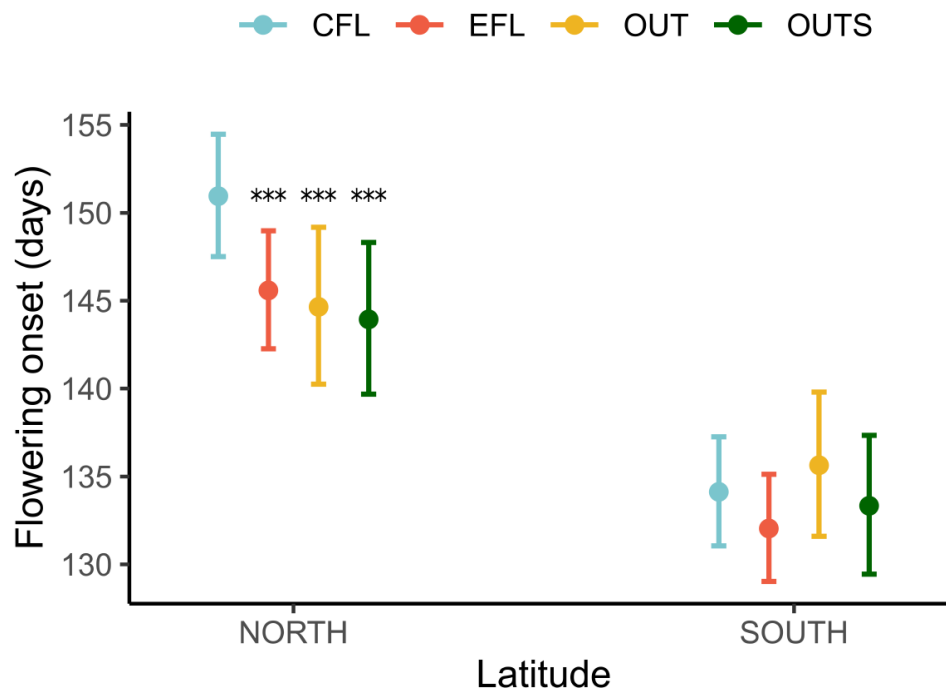
Figure 4. Effect of artificial selection lines (EFL, OUT and OUTS) on advancing flowering onset of *Lupinus angustifolius* L. Populations are grouped by latitude. Years are analyzed together. CFL: control flowering line; EFL: early flowering line (self-pollinated); OUT: outbred line (cross of different EFL genotypes); OUTS: F2 generation of outbred lines resulting from the self-pollination of OUT genotypes. Dots and bars represent the predicted mean from the GLMM model with a Poisson distribution and the 95 % confidence intervals. Significant differences ($p < 0.05$) determined by Tukey test between artificial selection lines and the control line are marked with asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Reproductive success

Artificial selection did not result in differences in reproductive success among lines, *i.e.*, number of seeds ($X^2 = 0.254$, $p = 0.881$, Df = 2) and seed weight ($X^2 = 3.983$, $p = 0.137$, Df = 2) (Supplementary Material, Appendix 2, Figure S5, Tables S2 and S3) in any of the latitudes. However, significant differences were found in the number ($X^2 = 22.800$, $p < 0.001$, Df = 1) and in the weight ($X^2 = 61.890$, $p < 0.001$, Df = 1) of the seeds between latitudes (Supplementary Material, Appendix 2, Tables S2 and S3). Northern population plants produced a greater number of seeds than southern populations, but the weight of their seeds was lower. Fixed effects explained 19 % of the variation in the number of seeds, and 50 % in seed weight, whereas random effects explained 3.4 %, and 13 %,

respectively (Supplementary Material, Appendix 2, Table S2). Posterior mean values, standard errors, and 95 % confidence intervals for the number of seeds and seed weight for each line are shown in Supplementary Material, Appendix 2, Table S4.

Correlated responses to selection for early flowering

The effect of EFL was significantly associated to lower shoot growth in both northern and southern populations, and higher LDMC and lower SLA (year 2019) in southern populations (Figure 5c, e and g). The effect of the OUT line was significantly associated to shorter height and higher SLA (2019) in southern populations and lower biomass and shoot growth in northern populations (Figure 5). A secondary, but relevant, result from the analyses performed on plant height, biomass and shoot growth is that plants from the northern populations were significantly taller and had higher biomass and shoot growth than those from the southern populations (All X^2 test: p < 0.001; Figure 5, Supplementary Material, Appendix 2, Tables S2 and S3). For all studied traits, fixed effects explained between 41 % and 0.7 % of the variance, and random effects, between 29.6 % and 5.1 % (Supplementary Material, Appendix 2, Table S2). Posterior mean values, standard errors, and 95 % confidence intervals for the different plant traits for each line are shown in Supplementary Material, Appendix 2, Table S4.

Several significant correlations were also found between flowering onset and other traits for control flowering line (CFL) and year 2019. In northern populations, plants that flowered earlier showed an increase in their biomass, seed number, and seed weight, and a decrease in shoot growth (Figure 6a). In southern populations, plants that flowered earlier showed a reduction in height, shoot growth and SLA, and an increase in LDMC (Figure 6b).
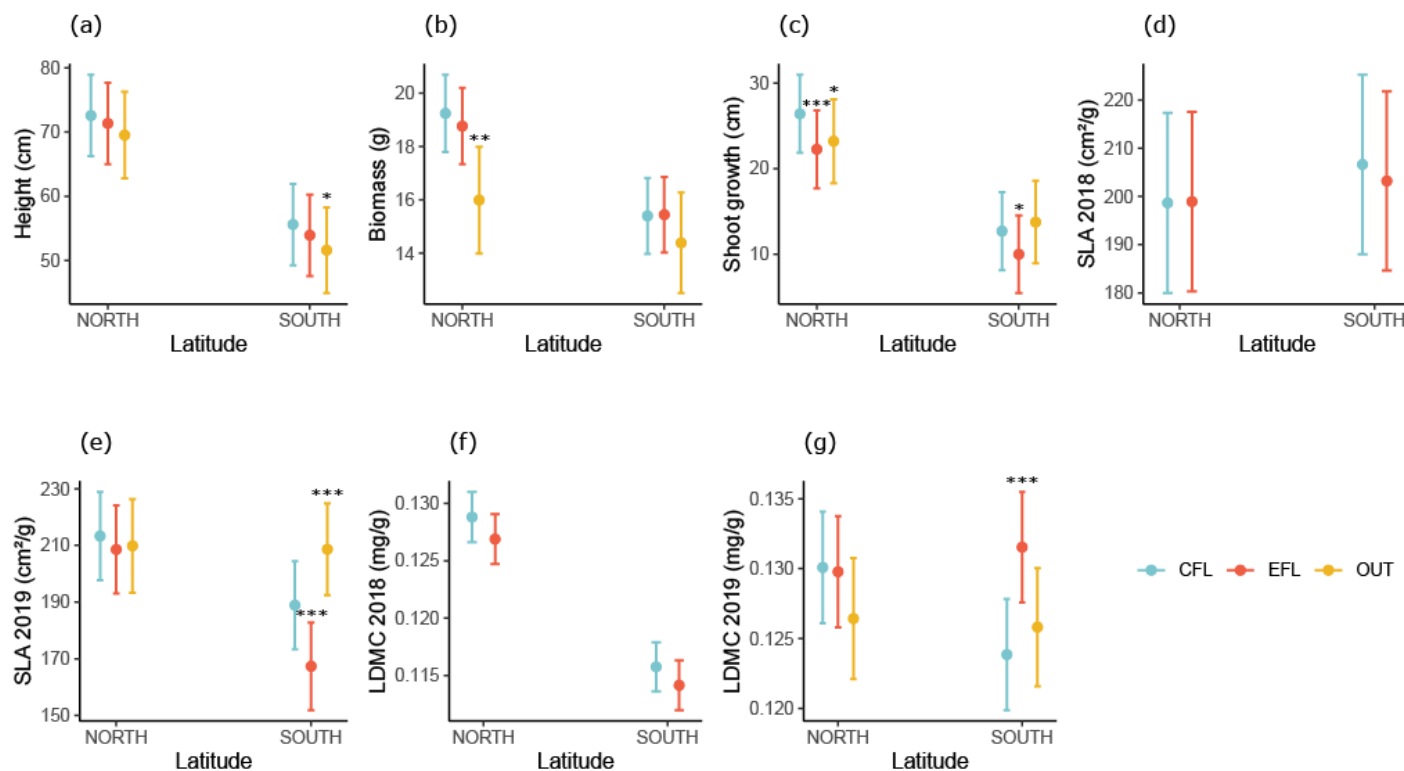
Figure 5. Effect of artificial selection lines (EFL, OUT and OUTS) on other functional traits: (a) plant height, (b) biomass, (c) shoot growth, (d) specific leaflet area (SLA) 2018, (e) specific leaflet area (SLA) 2019, (f) leaflet dry matter content (LDMC) 2018, (g) leaflet dry matter content (LDMC) 2019. Years are analyzed together except for SLA and LDMC. CFL: control flowering line; EFL: early flowering line (self-pollinated); OUT: outbred line (cross of different EFL genotypes). Dots and bars represent the predicted mean from the LMM model with a Gaussian distribution and the 95 % confidence intervals. Significant differences (p < 0.05) determined by Tukey test between artificial selection lines and the control line are marked with asterisks (*p < 0.05; **p < 0.01; ***p < 0.001).
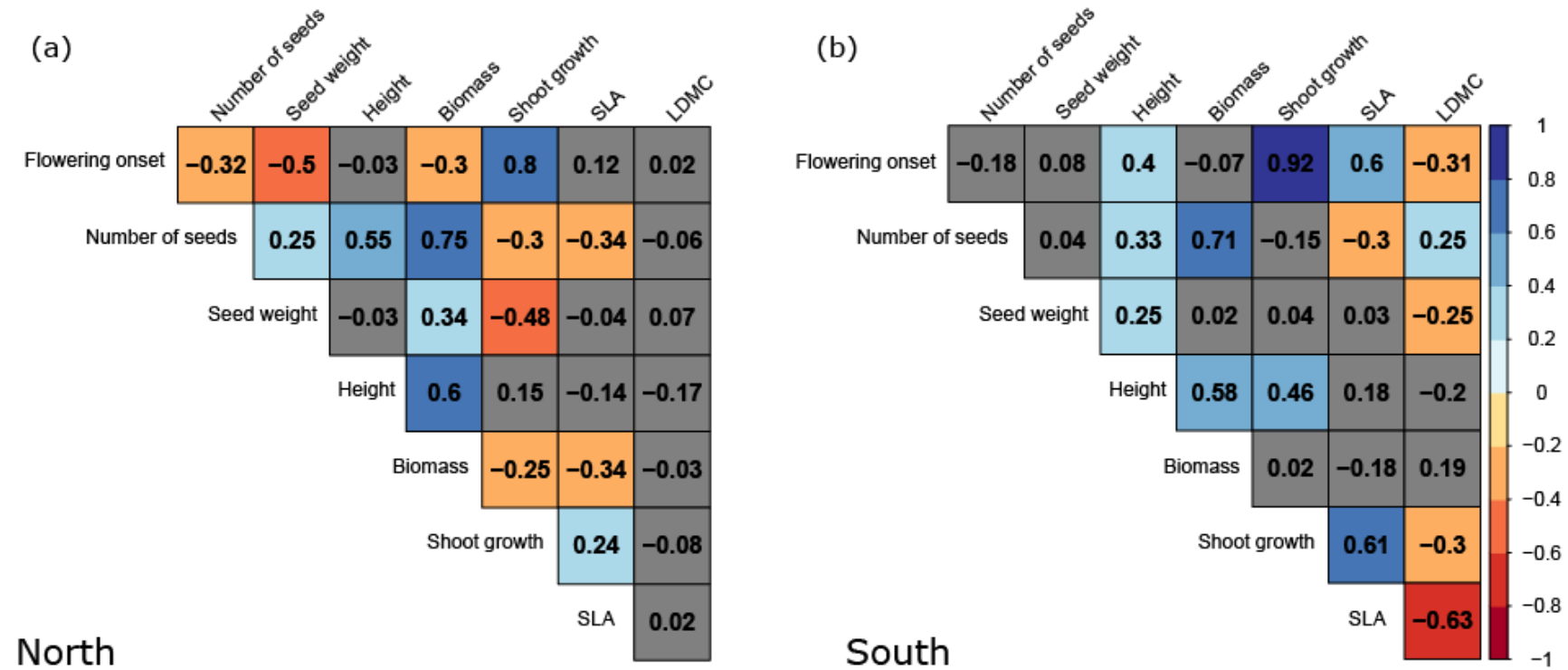
Figure 6. Correlations between flowering onset and other traits for control flowering line (CFL) and year 2019. (a) correlations for northern latitude populations. (b) correlations for southern latitude populations. Positive correlations are represented in cold colours, while negative correlations are represented in warm colours. Non-significant correlations (p > 0.05) are represented in grey.

Discussion

The offspring of artificial selection lines experienced an advance in the flowering onset of *Lupinus angustifolius*. However, the likelihood and magnitude of this change depended largely on the precise environmental conditions experienced by the populations in their native locations and the type of crosses applied (selfing or outcrossing). Thus, flowering onset was significantly modified in the northern populations but not in the southern ones, indicating a greater adaptive potential of the former regarding this trait. The selection lines that were obtained through forced outcrossing between early flowering plants generated plants that flowered even earlier, disclosing the involvement of numerous alleles from multiple genes in the configuration of early flowering phenotypes. Furthermore, advancing flowering onset by artificial selection was associated with changes in other plant traits that varied depending on the latitude of the populations of origin, potentially leading to constraints to adaptation.

Advancement of flowering onset

All artificial selection lines flowered significantly earlier than the control line in the northern populations. However, this did not occur in the southern populations. The outbred line (OUT) and the self-crossed outbred line (OUTS) of the northern populations advanced their flowering onset by a greater number of days (16 and 21 days, respectively) than the self-crossed early flowering line (EFL), which advanced six days with respect to control. The greater advance of OUT and OUTS lines may derive from new allelic combinations that arise from the outcrossing of EFL individuals, which, due to the autogamous nature of the plant, are likely to be homozygous in most loci. This indicates that not only the trait is heritable, as confirmed by the estimates obtained in our experiment, but numerous different alleles from multiple genes are involved in the

configuration of early flowering phenotypes. The range of flowering onset advance obtained is comparable to those obtained in similar studies where artificial selection was used. For example, Burgess et al. (2007), working with *Campanulastrum americanum* (L.) Small, obtained an early flowering line through manual crosses between early flowering plants. After three generations, flowering onset was advanced an average of 13 days compared to the control line. Similarly, Sheth & Angert (2016) obtained an early flowering selection line in *Mimulus cardinalis* Benth that flowered 15 days earlier than the control line after two generations. These two species are xenogamous, whereas *L. angustifolius* is an almost strict autogamous species (Wolko et al., 2011) and thus, one would expect *L. angustifolius* populations to have less standing genetic variation. However, this apparent limitation did not prevent a similar advance in flowering time when compared to the above-mentioned outcrossing species.

In contrast to our results, Sheth & Angert (2016) reported that populations from higher latitudes of *M. cardinalis* flowered earlier than those from lower latitudes, and that the latter presented a response of greater magnitude to artificial selection. The apparently contrasting responses found in *L. angustifolius* and *M. cardinalis* can be explained by the different limiting factors operating in each case. While in the former, hot dry summers force warm populations to flower earlier to complete the reproductive process, in the latter, strong selection on flowering time takes place at the high latitude populations to ensure that plants mature fruits before the growing season ends, in this case, due to the arrival of frosts (Munguía-Rosas et al., 2011; Sheth & Angert, 2016).

The evolutionary potential of some populations to respond to environmental changes may be limited by the lack of genetic variation (Sheth & Angert, 2016). For example, in our experiment, the lack of advancement in flowering time through artificial selection in the southern populations along with their earlier flowering time compared to northern

populations suggests that these populations had already experienced prior natural selection for early flowering due to the higher temperatures and lower interannual variation in temperatures occurring at their locations (Table 1). Consequently, their standing genetic variation for early flowering would be currently lower than that found in the northern populations. Flowering onset may be under weaker selection in northern populations because the growing season is longer due to a later arrival of the drought (Table 1, Matesanz et al., 2020). The lower selection differentials and responses to selection obtained in the southern populations also support this idea (Table 2). These results suggest that northern populations of *L. angustifolius* have a greater adaptive potential and could evolve more rapidly towards earlier flowering phenotypes in response to global warming.

Effect on plant reproductive success

Although early flowering phenotypes are expected to be associated with an increase in reproductive success in temperate zones in response to current global warming (Munguía-Rosas et al., 2011), we did not find an association between selection lines and fitness components (seed number and seed weight) in our study. This is probably explained by the fact that our experiment provided water on demand by drip irrigation throughout the life cycle of the plants and that water availability is the main constraint for plant growth and performance for plants, like *L. angustifolius*, that occur in the Mediterranean region (Blondel et al., 2010; Matesanz et al., 2020; Matesanz & Valladares, 2014). In any case, the effects of early flowering on fitness components may not be straightforward and may involve changes both in seed number and size. For instance, the advance in flowering date might be associated with a reduction in seed number, but increase in seed weight, as we observed in our experiment by the differences in these traits between southern and northern populations (the former flowering earlier than the latter). The trade-off between

number and seed size has been extensively studied (see Lázaro & Larrinaga, 2018). Producing a greater number of seeds can provide a greater number of offspring and greater reproductive success, whereas larger seeds may ensure survival of individuals. Therefore, individuals from stable or resource-rich environments may benefit by producing more seeds, while those from more unstable or resource-poor environments may be more successful by producing fewer but heavier seeds (Leishman et al., 2009; Metz et al., 2010). For instance, Burgess et al. (2007) in their artificial selection greenhouse experiment with *C. americanum*, observed that the early flowering lines produced fewer seeds but yielded greater seed weight than the late flowering lines. However, working with the same populations used in our study, Matesanz et al. (2020) observed that southern populations of *L. angustifolius* (which flower earlier) produced more and heavier seeds than northern populations when plants were subjected to a drought treatment. In view of these results, a different experiment involving the sowing of control and artificial selection lines in natural conditions near the original populations would be needed to thoroughly test the fitness benefits of early flowering plants.

Association with other traits

Artificial selection has the potential to modify the trait of interest within a few generations, but with the peculiarity that it can carry over other traits in the process (Burgess et al., 2007; Sheth & Angert, 2016). Because phenotypes are an integration of different trait values that are closely interrelated, they cannot be interpreted independently (Sobral, 2021). In *L. angustifolius*, the advance of flowering onset also involved a change in some of the studied plant traits. We observed that, depending on the populations of origin, the early flowering lines (EFL and OUT) were associated to lower shoot growth, lower biomass, lower height or lower shoot growth than the control flowering line. These results are in agreement with the hypothesis that we posed related to the resource

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allocation trade-offs (Reich, 2014). In other plant species, it has also been found that plants belonging to the early flowering selection lines not only flower earlier but are also smaller and have fewer branches (Burgess et al., 2007; Munguía-Rosas et al., 2011). Concerning leaf-structure related traits, the EFL had higher LDMC and lower SLA than the CFL, whereas the OUT line had a higher SLA than the CFL. In the first case, the pattern is similar to that found in southern populations with respect to northern populations (Matesanz et al., 2020) and may be related to water-use efficiency (Rao & Wright, 1994; Wright et al., 1994), indicating that individuals that flower earlier and have lower SLA and higher LDMC could acquire an adaptive advantage in drought environments. On the contrary, the positive association between early flowering and higher SLA of the latter was also found in Sheth & Angert (2016) with *M. cardinalis* and is consistent with a rapid-growth life-history strategy (Donovan et al., 2011; Reich, 2014). A possible explanation of some of the differences found between the EFL and the OUT line in some associated traits may rely on the polygenic determination of these traits, the presence of epistatic interactions among genes and the overdominant expression of some loci associated with heterozygous genotypes (Blümel et al., 2015; Bolger, 2021; He et al., 2019; Holland, 2007). The relevance of these trade-offs between flowering time and other traits relies in that they may constrain adaptation when genetic correlations are antagonistic to the direction of selection (Etterson & Shaw, 2001; Walsh & Blows, 2009). The correlations between traits observed in the control line also reveal different adaptive strategies between northern and southern populations, which are reflected in different phenotypic constraints between traits. In northern populations, earlier onset of flowering is correlated with higher biomass, while, in southern populations, earlier flowering is associated with a reduction in plant height and SLA. It is not uncommon for these genetic correlations to vary according to different environmental conditions (Sgrò & Hoffmann,

2004; Sheth & Angert, 2016), because some phenotypic values or combination of traits could have an adaptive advantage in some conditions but not in others (Sobral, 2021). The differences in correlations found between northern and southern populations suggest that northern populations, which have lower water stress, can devote a greater amount of resources to growth, while southern populations must make more efficient use of resources.

Concluding remarks

Artificial selection can allow quantifying genetic variation in traits that are relevant for the adaptation of species to climate change and is useful to determine the adaptive potential of populations. In this study, we experimented with the advancement of flowering time in *L. angustifolius* and found that there are several important aspects to consider. On the one hand, flowering onset of *L. angustifolius* was significantly advanced but only in northern populations. This suggests that northern populations would have a higher capacity for adaptation and that their survival could be higher under the context of climate change, while southern populations would have a higher risk of extinction and would be forced to migrate northward or to higher elevations to track optimal environmental conditions. On the other hand, the advance in flowering onset was found to be associated with changes in other traits, implying that adaptation can be somewhat constrained. These changes were variable depending on the latitude of the populations, implying the existence of different evolutionary constraints to flowering time advancement between northern and southern populations. We did not observe an increase in reproductive success of the early flowering selection lines, probably because these experiments were carried out under controlled conditions without water limitations. We would expect results in natural conditions to provide a fitness advantage to early flowering phenotypes, especially in hot dry years.

In the current times in which climate change is an incipient threat to all organisms, this kind of studies is essential to assess the risks faced by plant populations and help to better manage and conserve them. Given the exceptional swiftness of climate change, it is unclear if adaptive evolution can occur fast enough to ensure the survival of all populations. Thus, the insight derived from these studies also sets the stage for the consideration of novel conservation strategies related to assisted evolution. Assisted evolution refers to all strategies in which there is a human intervention in any of the evolutionary forces to help organisms to adapt to environmental conditions (Humanes et al., 2021; Jones & Monaco, 2009). In this sense, artificial selection could increase the prevalence of adaptive alleles of the species that are found in low frequencies in a threatened population, by the reinforcement with individuals containing advantageous alleles (van Oppen et al., 2015). Although these techniques are being tested on some endangered species, such as corals (van Oppen et al., 2015), their application is still incipient, and more in-depth experimental studies are needed to assess their viability and potential setbacks.

Acknowledgements

Data availability statement

Data associated with this study are made available in the figshare data repository:

https://doi.org/10.6084/m9.figshare.21065602

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Supplementary Material

Appendix 1

Detailed protocol of the manual crosses carried out with *Lupinus angustifolius* L.

- Phase 1 – Emasculation: in the inflorescence, we chose the flowers that had not yet opened and had not been self-pollinated. With the help of tweezers disinfected with alcohol, we separated the petals and removed the anthers, taking care not to damage the rest of the structures. We marked the emasculated flowers with a permanent marker pen, and we covered the inflorescence with an organza bag to avoid pollination by pollinators.

- Phase 2 – Pollination: the day after emasculation the flowers were manually pollinated with mature pollen of opened flowers. We removed the pollen-donors flowers from the inflorescence and holding the flower by the calyx, we pressed it, forcing the pollen come out. Then, we deposited the pollen on the stigma of the previously emasculated recipient flower. Finally, we covered the inflorescence again with the organza bag.

Appendix 2

Table S1. Sample sizes (number of individuals) per population at the flowering time for each selection line and year in which they flowered in the common garden experiments of *Lupinus angustifolius*. CFL: control flowering line; EFL: early flowering line (self-pollinated), OUT: outbred line (cross of different EFL genotypes); OUTS: F2 generation of outbred line resulting from the self-pollination of OUT genotypes.

	2016-2017	2017-2018		2018-2019			2019-2020		
Population	Original line	CFL	EFL	CFL	EFL	OUT	CFL	EFL	OUTS
FRO	193	87	84	63	83	37	46	46	50
PIC	193	82	88	63	86	40	44	44	55
GAR	194	87	93	84	89	47	46	52	60
RIV	191	83	91	84	85	42	44	50	60

Table S2. Effect of selection line, latitude, and year on flowering onset, number of seeds, seed weight, height, biomass, shoot growth, SLA, and LDMC of *Lupinus angustifolius* plants grown in a common garden experiment. Estimates and significance level for fixed effects are shown. Population and Genotype were included as random factors. CFL: control flowering line; EFL: early flowering line (self-pollinated), OUT: outbred line (cross of different EFL genotypes); OUTS: F2 generation of outbred line resulting from the self-pollination of OUT genotypes. Missing factors (CFL = Control line, Latitude North, Year 2018, and interaction CFL: North) are included in the intercept. SLA and LDMC were analyzed separately for each year because they had significant year x line interactions.

Fixed effects	Estimate	Standard error	t value	p value	Pseudo-R^2 (fixed effects)	Pseudo-R^2 (total)
Flowering onset	-	-	-	-	0.589	0.602
Intercept	5.017	0.012	423.927	<0.001	-	-
EFL	-0.036	0.006	-5.695	<0.001	-	-
OUT	-0.043	0.012	-3.594	<0.001	-	-
OUTS	-0.048	0.011	-4.259	<0.001	-	-
South	0.118	0.016	-7.198	<0.001	-	-
2019	-0.179	0.005	-36.544	<0.001	-	-
2020	-0.218	0.006	-36.995	<0.001	-	-
EFL:South	0.021	0.009	2.302	0.021	-	-
OUT:South	0.054	0.016	3.393	0.001	-	-
OUTS:South	0.042	0.014	2.901	0.004	-	-
Number of seeds	-	-	-	-	0.187	0.221
Intercept	1172.085	82.832	14.150	0.001	-	-
EFL	-51.097	48.681	-1.050	0.296	-	-
OUT	-79.296	74.353	-1.066	0.287	-	-
South	-591.653	113.366	-5.219	0.020	-	-
2019	9.486	33.785	0.281	0.779	-	-
EFL:South	130.324	67.573	1.929	0.055	-	-
OUT:South	160.416	99.005	1.620	0.106	-	-
Seed weight	-	-	-	-	0.497	0.627
Intercept	101.711	4.257	243.894	<0.001	-	-
EFL	5.031	2.349	2.142	0.033	-	-
OUT	2.363	4.059	0.582	0.561	-	-
South	46.500	5.917	7.859	0.008	-	-
2019	-16.678	1.261	-13.226	<0.001	-	-
EFL:South	-3.899	3.335	-1.169	0.244	-	-
OUT:South	-5.143	5.504	-0.934	0.351	-	-
Height	-	-	-	-	0.409	0.589

Intercept	72.547	3.236	22.418	0.001	-	-
EFL	-1.217	0.989	-1.232	0.220	-	-
OUT	-3.038	1.587	-1.941	0.057	-	-
South	-16.943	4.552	-3.722	0.059	-	-
2019	3.469	0.577	6.014	<0.001	-	-
EFL:South	-0.456	1.421	-0.321	0.749	-	-
OUT:South	-0.962	2.151	-0.448	0.655	-	-
Biomass	-	-	-	-	0.189	0.298
Intercept	19.239	0.737	26.114	<0.001	-	-
EFL	-0.477	0.571	-0.836	0.405	-	-
OUT	-3.249	0.918	-3.537	<0.001	-	-
South	-3.840	1.008	-3.811	0.029	-	-
2019	-3.496	0.340	-10.296	<0.001	-	-
EFL:South	0.522	0.803	0.651	0.517	-	-
OUT:South	2.238	1.222	1.832	0.068	-	-
Shoot growth	-	-	-	-	0.387	0.683
Intercept	26.387	2.327	11.341	0.005	-	-
EFL	-4.140	0.930	-4.454	<0.001	-	-
OUT	-3.191	1.466	-2.177	0.031	-	-
South	-13.702	3.291	-4.164	0.042	-	-
EFL:South	1.435	1.355	1.059	0.292	-	-
OUT:South	4.243	2.025	2.095	0.037	-	-
SLA 2018	-	-	-	-	0.007	0.194
Intercept	198.673	9.550	20.804	0.001	-	-
EFL	0.276	4.996	0.055	0.956	-	-
South	7.979	13.467	0.593	0.606	-	-
EFL:South	-3.702	7.025	-0.527	0.600	-	-
SLA 2019	-	-	-	-	0.189	0.311
Intercept	213.305	7.952	26.823	<0.001	-	-
EFL	-4.740	4.342	-1.092	0.277	-	-
OUT	-3.453	5.347	-0.646	0.519	-	-
South	-24.390	11.226	-2.172	0.137	-	-
EFL:South	-16.838	6.124	-2.749	0.006	-	-
OUT:South	23.170	7.372	3.143	0.002	-	-
LDMC 2018	-	-	-	-	0.249	0.320
Intercept	0.129	0.001	114.979	<0.001	-	-
EFL	-0.002	0.001	-1.264	0.210	-	-
South	-0.013	0.002	-8.311	<0.001	-	-
EFL:South	0.000	0.002	0.142	0.888	-	-
LDMC 2019	-	-	-	-	0.054	0.105
Intercept	0.130	0.002	63.704	<0.001	-	-
EFL	-0.000	0.001	-0.214	0.831	-	-
OUT	-0.004	0.002	-2.158	0.032	-	-
South	-0.006	0.003	-2.167	0.124	-	-
EFL:South	0.008	0.002	3.950	<0.001	-	-
OUT:South	0.006	0.002	2.404	0.017	-	-

Table S3. Chi-square statistic, degrees of freedom and P-values of the Type II Wald chi-square tests of GLMM and LMM analyses to study the effect of selection line, latitude, and year on flowering onset, number of seeds, seed weight, height, biomass, shoot growth, SLA, and LDMC of *Lupinus angustifolius* plants grown in a common garden experiment. Estimates and significance level for fixed effects are shown. Population and Genotype were included as random factors. CFL: control flowering line; EFL: early flowering line (self-pollinated), OUT: outbred line (cross of different EFL genotypes); OUTS: F2 generation of outbred line resulting from the self-pollination of OUT genotypes. Missing factors (CFL = Control line, Latitude North, Year 2018, and interaction CFL: North) are included in the intercept. SLA and LDMC were analyzed separately for each year because they had significant year x line interactions.

Fixed effects	X^2	Df	Pr $(>|X^2|)$
Flowering onset	-	-	-
Line	35.807	3	<0.001
Year	1987.443	2	<0.001
Latitude	41.841	1	<0.001
Line:latitude	17.298	3	<0.001
Number of seeds	-	-	-
Line	0.254	2	0.881
Year	0.079	1	0.779
Latitude	22.800	1	<0.001
Line:latitude	4.679	2	0.096
Seed weight	-	-	-
Line	3.983	2	0.137
Year	174.921	1	<0.001
Latitude	61.890	1	<0.001
Line:latitude	1.721	2	0.423
Height	-	-	-
Line	11.311	2	0.003
Year	36.166	1	<0.001
Latitude	14.944	1	<0.001
Line:latitude	0.232	2	0.890
Biomass	-	-	-
Line	10.641	2	0.005
Year	106.004	1	<0.001
Latitude	13.090	1	<0.001
Line:latitude	3.358	2	0.187
Shoot growth	-	-	-
Line	27.139	2	<0.001
Latitude	14.572	1	<0.001
Line:latitude	4.510	2	0.105
SLA 2018	-	-	-
Line	0.207	1	0.649
Latitude	0.220	1	0.639

Line:latitude	0.278	1	0.598
SLA 2019	-	-	-
Line	41.084	2	<0.001
Latitude	5.705	1	0.017
Line:latitude	30.247	2	<0.001
LDMC 2018	-	-	-
Line	2.705	1	0.100
Latitude	126.822	1	<0.001
Line:latitude	0.020	1	0.887
LDMC 2019	-	-	-
Line	41.083	2	<0.001
Latitude	5.705	1	0.016
Line:latitude	30.257	2	<0.001

Table S4. Posterior mean values, standard errors, and 95 % confidence intervals for the different traits and lines of *Lupinus angustifolius* plants grown in a common garden experiment. CFL: control flowering line; EFL: early flowering line (self-pollinated), OUT: outbred line (cross of different EFL genotypes); OUTS: F2 generation of outbred line resulting from the self-pollination of OUT genotypes.

	North				South			
	Mean	Std. error	2·5%	97·%	Mean	Std. error	2·5%	97·%
Flowering onset	-	-	-	-	-	-	-	-
CFL	132	1.54	129	135	117	1.37	115	120
EFL	128	1.48	125	130	116	1.34	113	118
OUT	127	1.94	123	131	119	1.78	115	122
OUTS	126	1.84	123	130	117	1.69	114	120
Number of seeds	-	-	-	-	-	-	-	-
CFL	1177	80.80	900	1456	585	79.70	300	871
EFL	1126	79.1	836	1415	664	79.20	375	953
OUT	1098	97.1	855	1340	666	93.90	422	911
Seed weight	-	-	-	-	-	-	-	-
CFL	93.40	4.21	78.60	108	139.90	4.16	124.80	155
EFL	98.40	4.17	83.40	113	141.00	4.15	125.90	156
OUT	95.70	5.11	83.00	108	137.10	4.96	124.30	150
Height	-	-	-	-	-	-	-	-
CFL	74.30	3.22	61.30	87.30	57.30	3.22	44.30	70.40
EFL	73.10	3.21	60.00	86.10	55.70	3.21	42.60	68.80
OUT	71.20	3.41	59.60	82.80	53.30	3.37	41.50	65.20
Biomass	-	-	-	-	-	-	-	-
CFL	17.50	0.72	15.30	19.70	13.70	0.71	11.04	15.90

EFL	17.00	0.71	14.80	19.20	13.70	0.70	11.40	15.90
OUT	14.20	0.98	12.10	16.40	12.60	0.92	10.60	14.70
Shoot growth	-	-	-	-	-	-	-	-
CFL	26.39	2.33	17.47	35.30	12.69	2.33	3.77	21.60
EFL	22.25	2.32	13.28	31.20	9.98	2.31	0.95	19.00
OUT	23.20	2.50	15.29	31.10	13.74	2.46	5.66	21.80
SLA 2018	-	-	-	-	-	-	-	-
CFL	199	9.50	163	235	207	9.50	170	243
EFL	199	9.50	163	235	203	9.50	167	240
SLA 2019	-	-	-	-	-	-	-	-
CFL	213	7.95	185	242	189	7.93	160	218
EFL	209	7.92	180	237	167	7.90	138	196
OUT	210	8.43	184	236	209	8.28	182	235
LDMC 2018	-	-	-	-	-	-	-	-
CFL	0.13	0.00	0.13	0.13	0.12	0.00	0.11	0.12
EFL	0.13	0.00	0.12	0.13	0.11	0.00	0.11	0.12
LDMC 2019	-	-	-	-	-	-	-	-
CFL	0.13	0.00	0.12	0.14	0.12	0.00	0.12	0.13
EFL	0.13	0.00	0.12	0.14	0.13	0.00	0.13	0.14
OUT	0.13	0.00	0.12	0.13	0.13	0.00	0.12	0.13

Table S5. Observed mean ±SD values for the different traits measured and the different lines tested. SLA: specific leaf area, LDMC: leaf dry matter content. CFL: control flowering line; EFL: early flowering line (self-pollinated), OUT: outbred line (cross of different EFL genotypes); OUTS: F2 generation of outbred line resulting from the self-pollination of OUT genotypes.

	Flowering onset (days)				Number of seeds			Seed weight (mg)			Total height (cm)			Shoot growth (cm)			SLA (cm^2/g)			LDMC (mg/g)			Biomass (g)		
	CFL	EFL	OUT	OUTS	CFL	EFL	OUT	CFL	EFL	OUT	CFL	EFL	OUT	CFL	EFL	OUT	CFL	EFL	OUT	CFL	EFL	OUT	CFL	EFL	OUT
North	136.06 ±15.33	130.26 ±15.28	120.92 ±7.54	115.66 ±9.02	1180.99 ±559.19	1123.60 ±693.73	1101.45 ±572.63	92.37 ±23.64	97.71 ±24.70	87.78 ±17.74	74.96 ±10.33	72.90 ±11.35	72.79 ±11.26	27.29 ±9.71	21.14 ±8.05	22.48 8.47	206.14 ±35.44	203.96 ±36.17	209.66 ±44.44	0.13 ±0.01	0.13 ±0.01	0.13 ±0.01	17.63 ±7.30	16.96 ±6.88	12.55 ±4.98
FRO	136.12 ±14.73	130.85 ±14.70	124.32 ±5.73	118.90 ±8.24	1297.12 ±626.99	1208.61 ±720.90	1179.69 ±576.11	96.15 ±22.48	100.45 ±24.86	91.44 ±19.11	74.29 ±9.74	74.89 ±10.56	74.77 ±11.78	26.39 ±8.64	23.05 ±8.06	27.14 ±6.84	205.89 ±39.19	202.58 ±32.47	207.28 ±39.22	0.13 ±0.01	0.13 ±0.01	0.13 ±0.01	17.93 ±7.55	18.52 ±6.88	13.76 ±5.14
PIC	135.99 ±15.96	129.67 ±15.83	117.69 ±7.70	112.72 ±8.75	1056.97 ±446.93	1044.09 ±660.03	1025.39 ±566.86	88.35 ±24.28	95.05 ±24.35	84.54 ±16.01	75.70 ±10.94	71.03 ±11.77	70.92 ±8.93	28.16 ±10.65	19.23 ±7.63	18.08 ±7.51	206.41 ±31.01	205.26 ±39.41	211.86 ±49.17	0.13 ±0.01	0.13 ±0.01	0.12 ±0.02	17.31 ±7.05	15.38 ±6.54	11.34 ±4.57
South	120.02 ±13.13	117.98 ±12.83	113.57 ±6.18	107.23 ±7.54	589.29 ±434.47	665.04 ±429.37	670.70 ±331.97	139.52 ±24.45	140.36 ±26.12	128.28 ±22.15	57.85 ±9.69	55.46 ±9.45	55.45 10.00	12.53 ±7.18	9.64 ±5.09	14.03 ±6.48	197.25 ±39.47	184.18 ±38.05	209.35 ±36.26	0.12 ±0.01	0.12 ±0.01	0.13 ±0.01	13.73 ±5.19	13.36 ±4.23	10.91 ±4.07
GAR	122.52 ±13.29	120.62 ±12.64	116.30 ±4.43	108.37 ±6.82	535.67 ±395.07	574.34 ±409.68	759.19 ±315.38	140.59 ±23.64	147.13 ±28.92	138.88 ±20.73	61.38 ±9.81	59.56 ±8.92	61.99 ±7.63	15.50 ±7.72	12.41 ±4.61	16.95 ±5.81	210.12 ±44.93	195.87 ±39.12	216.08 ±28.70	0.12 ±0.01	0.12 ±0.01	0.13 ±0.01	12.78 ±5.07	13.30 ±4.05	12.08 ±4.06
RIV	117.47 ±12.49	115.24 ±12.48	110.52 ±6.46	106.05 ±8.12	636.41 ±462.73	754.40 ±431.04	582.20 ±328.01	138.37 ±18.34	133.60 ±20.98	117.40 ±18.10	54.18 ±8.10	51.58 ±8.25	47.93 ±6.51	9.41 ±4.97	7.29 ±4.23	10.66 ±5.55	183.97 ±27.29	172.75 ±33.33	201.82 ±36.62	0.12 ±0.01	0.12 ±0.01	0.13 ±0.01	14.70 ±5.14	13.41 ±4.40	9.69 ±3.76
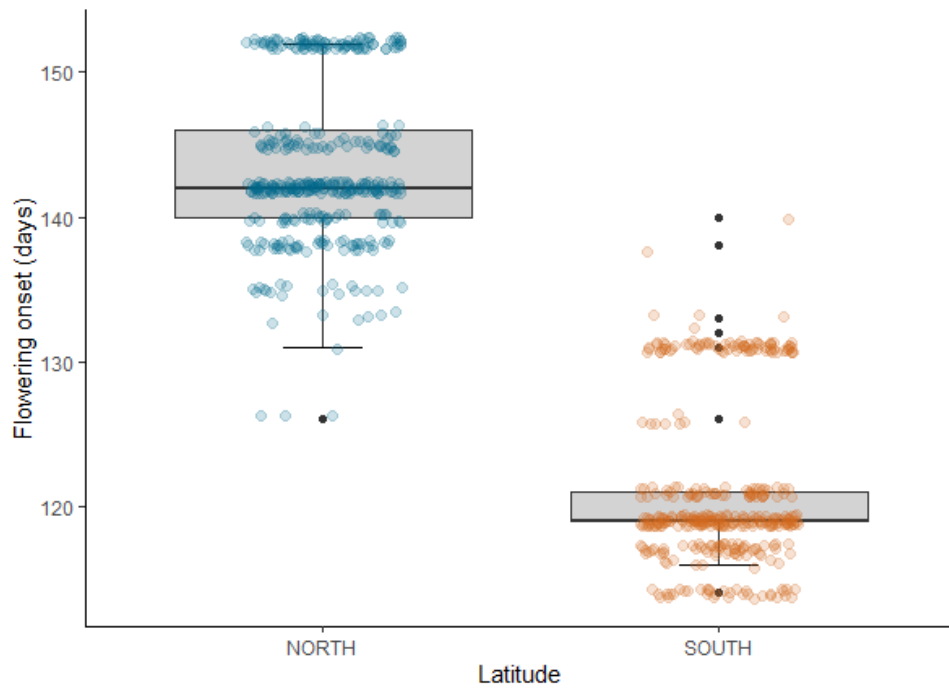
Figure S1. Flowering onset of plants of *Lupinus angustifolius* L. from northern (Zarapicos and Zafrón) and sourthern (La Garranchosa and Rivera de la Lanchita) populations in 2017.
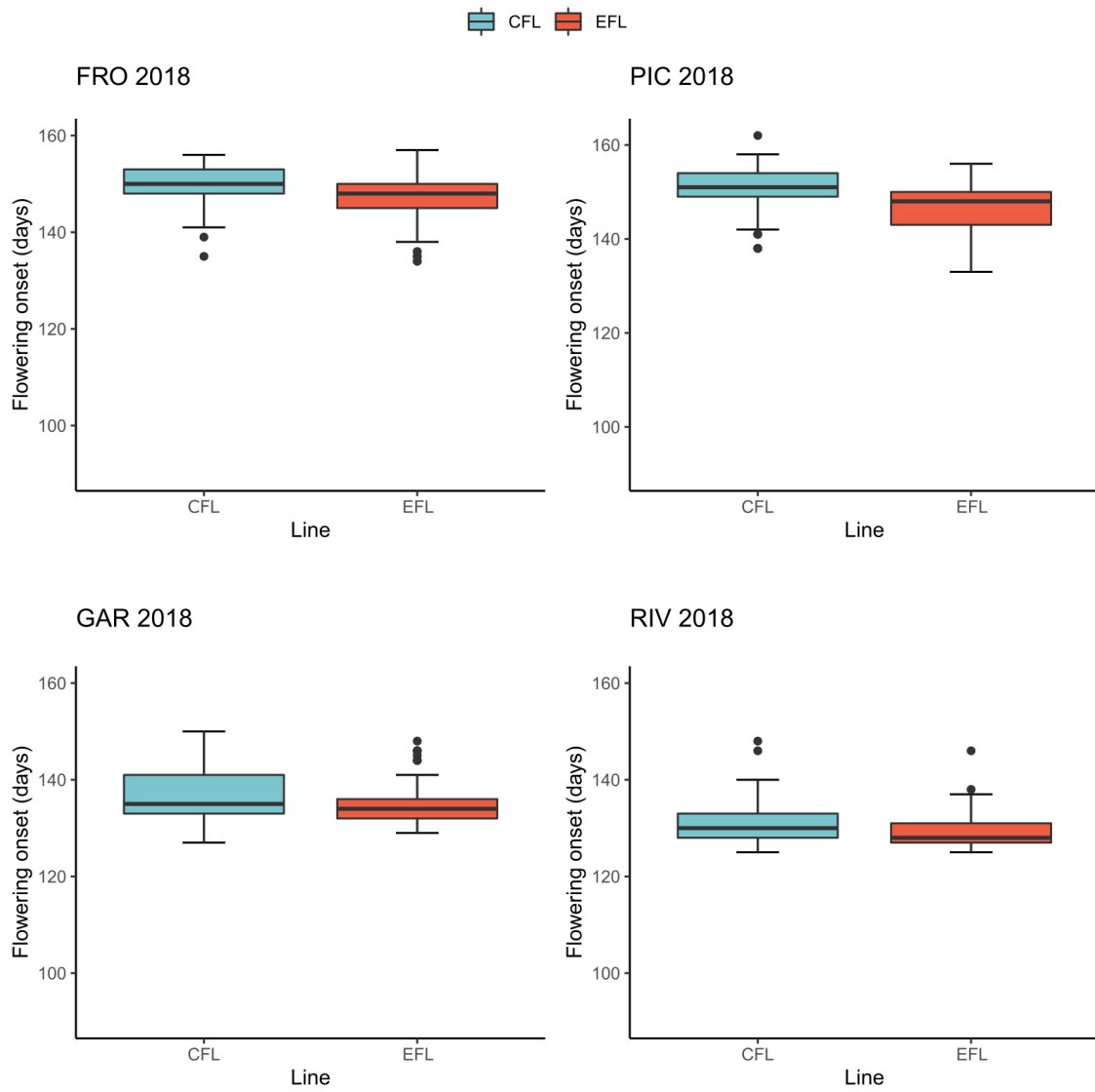
Figure S2. Observed values of flowering onset of *Lupinus angustifolius* for the different populations in the year 2018.
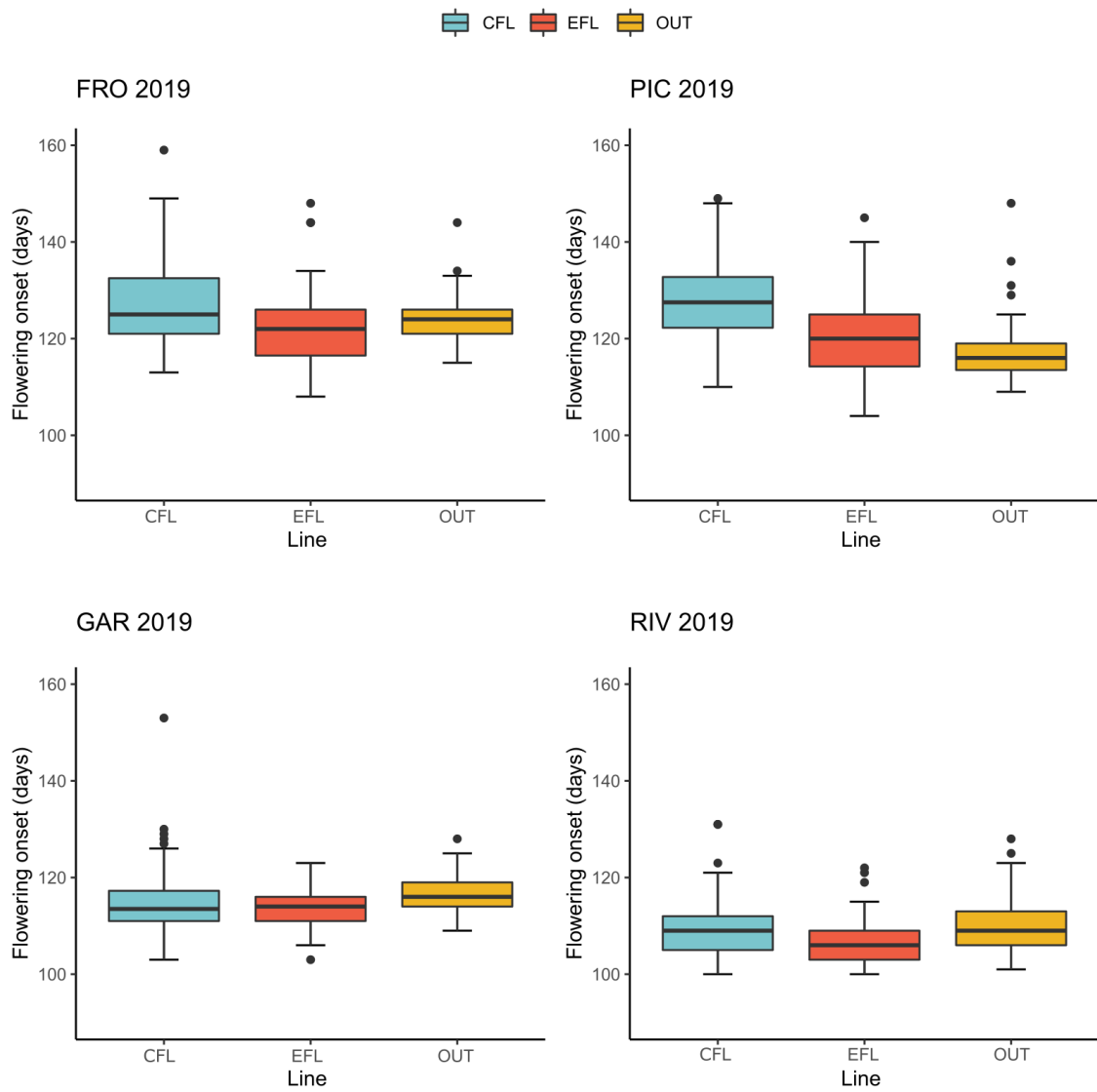
Figure S3. Observed values of flowering onset of *Lupinus angustifolius* for the different populations in the year 2019.
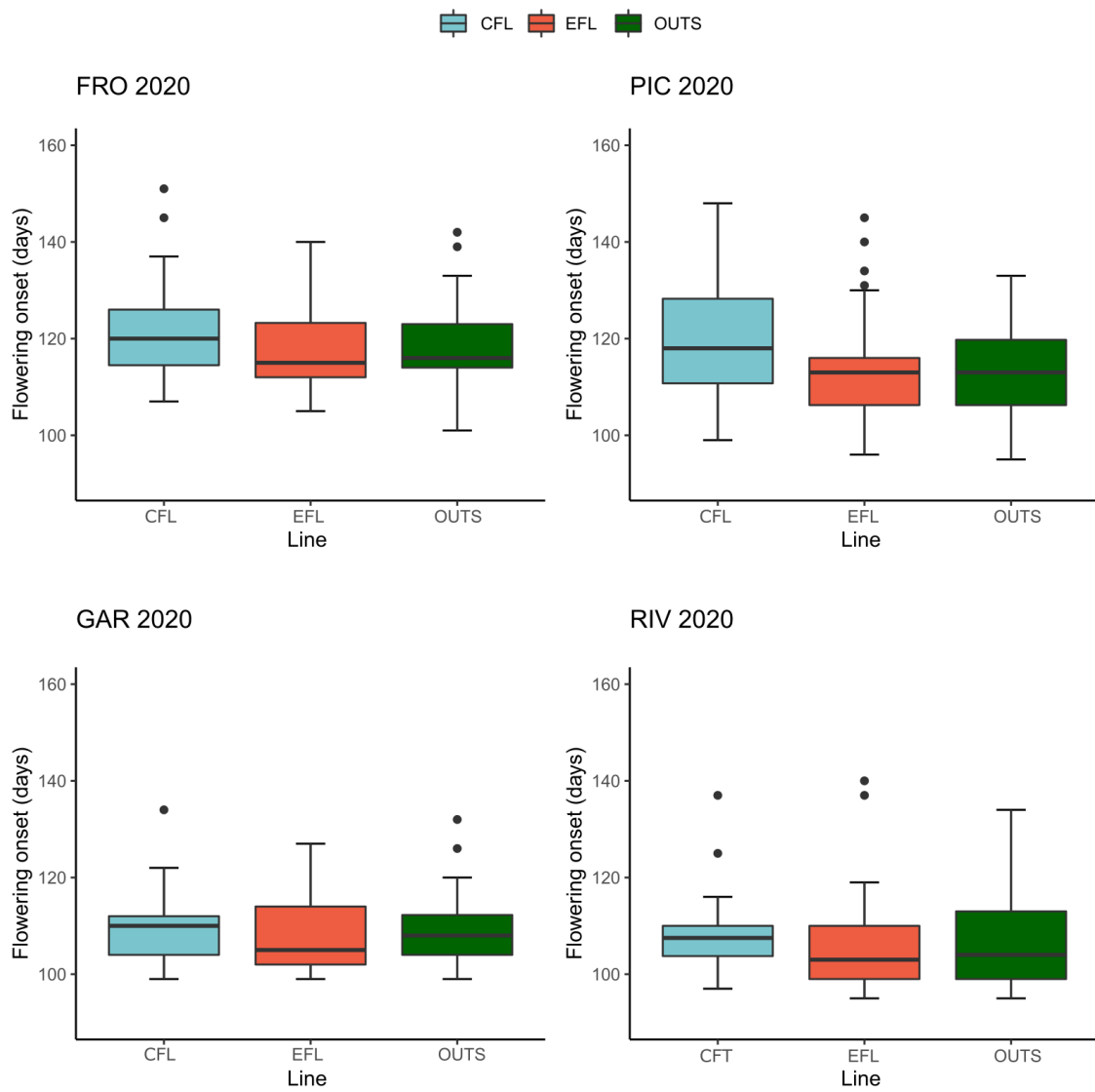
Figure S4. Observed values of flowering onset of *Lupinus angustifolius* for the different populations in the year 2020.
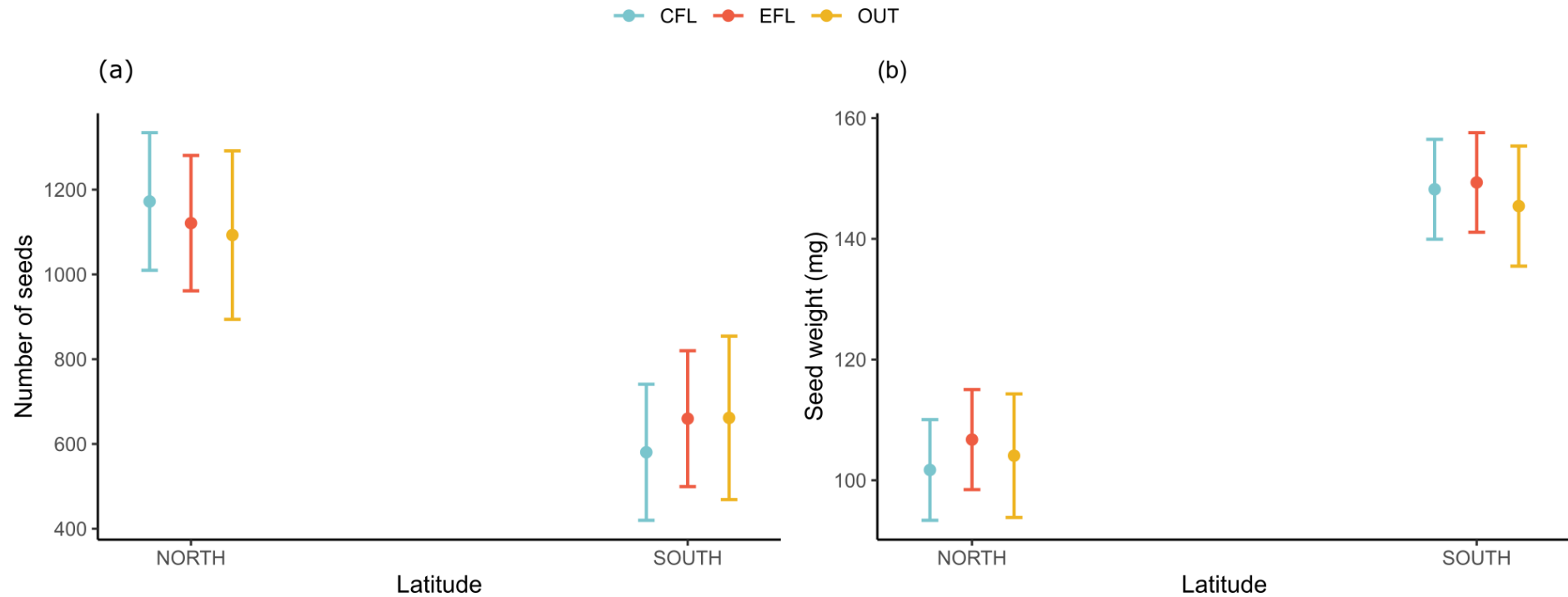
Figure S5. Effect of artificial selection lines (EFL and OUT) on fitness components in northern and southern populations of *Lupinus angustifolius* L.: (a) number of seeds per plant and (b) mean seed weight. Years are analyzed together. CFL: control flowering line; EFL: early flowering line (self-pollinated), OUT: outbred line (cross of different EFL genotypes); OUTS: F2 generation of outbred line resulting from the self-pollination of OUT genotypes. Dots and bars represent the predicted mean from the LMM model with a Gaussian distribution and the 95 % confidence intervals. Differences between the artificial selection lines and the control line were non-significant.

Chapter 2: Artificial selection experiments reveal key genetic diversity for early flowering onset in *Lupinus angustifolius* L. (Fabaceae)

Manuscript in preparation

Abstract

The success of plant populations in response to climate change will depend to a large extent on their capacity for adaptation. Populations with higher genetic diversity are likely to have a greater evolutionary potential to adapt to new conditions. Genomic tools are an excellent resource for identifying this genetic variation and understanding the processes of adaptive evolution. In a previous study, we used an artificial selection approach to characterize the adaptive potential for early flowering onset of four populations of *Lupinus angustifolius* L. (Fabaceae) from two contrasting latitudes. We observed that artificial selection was significantly effective in advancing flowering onset in the northern populations but not in the southern populations, suggesting that the latter would have undergone a previous natural selection for this trait. Thus, northern populations would have greater evolutionary potential in advancing flowering onset in the context of climate change. The main objective of the present study was to find genomic signatures corresponding to the selection lines (lines created by self and outcrossing) established in the four populations in the previous work. We also wanted to assess at the genomic level the differential response to selection obtained between the northern and southern populations. For this purpose, we conducted a gene capture approach aimed at sequencing regions previously known to be related to flowering, reproduction, growth, and abiotic stress. The results obtained support those of the previous study, showing that there is a genetic differentiation between the populations of different latitudes, and that the northern populations have greater genetic diversity and, therefore, greater adaptive potential to cope with climate change. The genomic analyses also revealed a variable number of SNPs significantly different between control and selection lines in each population, and several phenotype-genotype associations. Although a reduction in genetic diversity due to artificial selection would be expected, this was not observed in our study. These results

indicate that our approach provides useful information to assess the genetic vulnerability of these populations and to identify which populations may be in greater need of conservation.

Introduction

Changes in temperature and precipitation regimes caused by climate change inevitably affect species survival. Faced with this situation, populations can respond in different ways: migrate to places more suitable for them, remain in the same place and change their phenotypic expression through adaptive phenotypic plasticity to survive, remain in the same place and optimize the phenotype through genetic adaptation; or become extinct (Forero-Medina et al., 2011; Jump & Peñuelas, 2005; Root et al., 2003; Torres et al., 2023). Organisms with a higher dispersal capacity can easily migrate, while sessile organisms such as plants may have to adopt other strategies in their local habitat (Berg et al., 2010; Engler et al., 2009). Understanding the mechanisms of adaptive evolution of plant species is therefore of great importance for the conservation and management of biodiversity in the current context of global change (Fitzpatrick & Edelsparre, 2018; Hoffmann et al., 2021; Jordan et al., 2017). However, this is a complicated task that requires an examination of the connections between genetic and non-genetic mechanisms, including the role of adaptive plasticity in facilitating adaptation to changing environments (Fitzpatrick & Edelsparre, 2018).

Genetic vulnerability refers to the mismatch between the actual allele frequencies of a population and the allele frequencies estimated necessary to survive under the climatic conditions expected for the future. It is thus a measure of the degree of genetic modification required to cope with climate change through adaptive evolution (Hoffmann et al., 2021; Waldvogel et al., 2020). This is an important concept to consider when making conservation and management decisions. Populations that have a genetic

composition that generates phenotypes that are compatible with those required by forthcoming climatic conditions are expected to survive (Exposito-Alonso et al., 2018; Hoffmann et al., 2021). Moreover, populations that are genetically more diverse are more likely to respond faster to selective pressures, as they are more likely to contain alleles that are necessary for adaptation to new conditions (Hoffmann et al., 2021). In this sense, advances in the world of 'omics' tools, such as High-Troughput Sequencing approaches (hereafter, HTS), can help to better understand these adaptation processes of populations to climate change (Gienapp et al., 2008; Merilä, 2012; Savolainen et al., 2013), uncovering potential genes and pathways involved in climate adaptation (*e.g*. Christmas et al., 2016; Eckert et al., 2013; Steane et al., 2014). Although studies to identify associations between genetic variation and adaptation to climate are currently growing (*e.g*. Ahrens et al., 2019; Housset et al., 2018; Jaramillo-Correa et al., 2015; Rellstab et al., 2020), the feasibility of artificial selection to improve adaptation is still not explored in sufficient depth.

Given the ample evidence that exists for climate change adaptation across different traits, we can use HTS technologies to identify the genetic basis of these adaptive traits (*e.g*. Bradshaw & Holzapfel, 2001; Pulido & Berthold, 2003; Savolainen et al., 2007; Willis et al., 2008). Currently, advances in genomics allow us to sequence hundreds of thousands or millions of Single Nucleotide Polymorphisms (hereafter, SNPs) in a relatively easy and fast way (Hoffmann et al., 2021). Determining the SNPs and genes associated with these traits is very useful for predicting adaptive responses in different populations. However, in most cases, traits are polygenic (*i.e*., trait variation determined by different sets of genes with low effect), making it difficult to identify the multiple loci with which they are associated. In addition, the same gene can affect more than one trait (pleiotropy), there may be interactions between different genes (epistasis), or different sets of genes

may produce the same phenotypic effect (genetic redundancy) (Barghi et al., 2020). These aspects can facilitate or limit adaptation processes and may influence the adaptive potential of populations (Barghi et al., 2020; Hoffmann et al., 2021). One method we can use to measure the evolutionary potential of populations regarding certain traits is artificial selection (Conner, 2003; Hoffmann & Sgró 2011). Through artificial selection, humans select individuals based on their phenotypic characteristics to improve and perpetuate those characteristics in succeeding generations (Conner, 2016). In this way, we can obtain an estimate of a trait's additive genetic variance, which is essential for evolutionary change to take place. Selection experiments combined with genomic tools can therefore be used to predict genetic adaptation rates (Ørsted et al., 2019). By searching for markers (SNPs) with severe levels of differentiation (outliers) between individuals with different phenotypes or between populations, we can find loci that have experienced changes in their allele frequencies and are thus under selection (Stapley et al., 2010). If we can identify these adaptive allele sets and their associations with phenotypes, we will have a better ability to also identify the populations that are more vulnerable and those that may have sufficient variation to cope with climate change (Hoffmann et al., 2015; Hoffmann et al., 2021).

Flowering onset is a highly heritable trait that is crucial in the process of plant adaptation to climate change (Franks et al., 2007; Franks & Hoffmann, 2012). In a previous study (see Sacristán-Bajo et al., 2023, Chapter 1), we evaluated the adaptive potential for earlier flowering onset in *Lupinus angustifolius* L. (Fabaceae). For this purpose, we carried out an artificial selection experiment under common garden conditions in four populations of this species: two at higher latitude and cooler conditions (hereafter, northern populations), and two at lower latitude and warmer conditions (hereafter, southern populations), establishing different selection lines for advanced flowering involving both

intrapopulation manual xenogamous crosses and self-crosses. The progeny of the artificial selection lines flowered earlier than the control line in northern populations but not in southern populations. This suggests that northern populations have more genetic variation on which selection can act. It also indicates that the lower genetic variation in this trait in southern populations may have been generated by past selective pressure towards earlier flowering. In addition, we found that artificial selection for advanced flowering implied changes in other traits such as plant height, biomass, relative growth, Specific Leaflet Area (SLA) and Leaflet Dry Matter Content (LDMC) although we did not find differences in any of the traits associated with reproductive success (seed number or weight). In view of these considerations, the main objective of this study was to identify genomic signals in plants selected for early flowering that could be associated with the early flowering phenotype. Based on the results obtained previously, we hypothesized that the northern populations would have greater genetic diversity than the southern populations, and that the artificial selection carried out would also reduce the genetic diversity of the selected lines. To verify this, we performed a gene capture experiment in which we sequenced genes previously associated with flowering, reproduction, growth and abiotic stress processes. Specifically, we aimed to answer the following questions: i) What are the differences in genetic diversity between northern and southern populations at the genomic level? ii) Does the implemented artificial selection reduce the genetic diversity of the resulting progeny compared to the control treatment or does it modify it qualitatively? iii) Are there loci under selection associated with the flowering onset artificial selection treatments? iv) Can we identify statistically significant associations between flowering onset (or any of the other measured traits) and the allelic variation at the genomic regions analyzed?

Materials and Methods

Study species and cultivation in common garden

Lupinus angustifolius L. or blue lupine ($2n = 40$) is an herbaceous annual legume widely distributed in the Mediterranean basin and cultivated in many other places in the world (Castroviejo & Pascual, 1993). Their hermaphrodite flowers are almost exclusively self-pollinated under natural conditions when the petals are still closed (Wolko et al., 2011), and the species' genetic diversity is thought to be concentrated in the eastern part of the Iberian Peninsula (Mousavi-Derazmahalleh et al., 2018). Flowering time takes place between March and August, depending on weather conditions (Castroviejo & Pascual, 1993). Their racemose inflorescences can have up to 30 purple-blue flowers, which form fruits containing between 3 and 7 seeds (Clements et al., 2005).

To carry out this study, in 2016 we selected four populations located at two different latitudes in the Iberian Peninsula that differ in their climatic conditions (northern populations PIC and FRO and southern populations GAR and RIV, see Sacristán-Bajo et al., 2023, Chapter 1, Table 1, Figure 1). In each population we separately collected seeds from at least 98 mother plants (genotypes). We established a common garden at the CULTIVE facility (https://urjc-cultive.webnode.es/) at Rey Juan Carlos University (Móstoles, Madrid) with a random block design. In November 2016, three seeds from each genotype in each population were scarified and sown in a six L pot inside a greenhouse under the same conditions described in Sacristán-Bajo et al. (2023) (Chapter 1). Inside the greenhouse, the plants received only natural light, the temperature varied between 1 and 25 degrees Celsius and they were sprinkler irrigated on demand. After germination, one seedling was randomly selected to be kept in each pot and the remaining ones were clipped. In February 2017, individuals were moved outside the greenhouse and maintained with drip irrigation until the end of the growing season (June 2017). In spring

2017, we monitored the flowering onset of all individuals. We assumed that the individual had flowered when the blue-purple petals of one flower in the main inflorescence could be easily seen. To create a set of early-flowering individuals, we established an early flowering selection line (hereafter, EFL), consisting of 25 % of the genotypes that first flowered. We also obtained a control line (hereafter CFL), consisting of 25 % randomly selected genotypes from the 98 genotypes available for each population. The individuals of these lines were maintained by self-crossing during the following generations (2017-2018, 2018-2019). In Spring 2018, we created an outcrossed early flowering line (hereafter, OUT) by manually crossing the genotypes from the early flowering selection line between each other. More details on the process carried out to create the lines and the manual pollination protocol can be found in Figure 2 and Supplementary Material 1 of Sacristán-Bajo et al. (2023) (Chapter 1).

At the phenotypic level, we measured a wide range of traits related to phenology, reproductive success (number and weight of seeds), and other vegetative traits (height, biomass, shoot growth, specific leaflet area or SLA and leaflet dry matter content or LDMC). We calculated flowering onset as the number of days between the day of sowing and the day of flowering of the first flower of each individual. We also calculated the shoot growth of each plant as the difference between the height measured at the beginning and at the end of the growing season, measured as the length in centimeters from the base of the plant to the first flower. The number of potential fruits and seeds per plant was estimated, and SLA and LDMC were determined according to the procedures described in Rosbakh et al. (2015) and Wilson et al. (1999). For more details on phenotypic trait data collection, see Sacristán-Bajo et al. (2023) (Chapter 1).

DNA extraction and gene capture experiment

In 2019, we extracted DNA from leaf material corresponding to 15 individuals of each of the three selection lines (CFL, EFL and OUT) for each of the four populations (FRO, PIC, GAR and RIV) resulting in a total of 180 individuals (*i.e.* 15 individuals per treatment x 3 treatments per population x 4 populations). To extract and isolate the DNA, we used the DNeasy Plant minikit (QIAGEN, Valencia, USA).

To select a list of candidate genes that could be associated with flowering onset in *Lupinus angustifolius* we carried out a Blast analysis on its genome (GenBank accession: PRJNA398717) annotated from the National Center for Biotechnology Information (NCBI), which contains all coding sequences (CDs). We determined the gene ontology (GO) terms of the *L. angustifolius* genome using the FullLengtherNext software (Lara et al., 2007). From the full list of GO terms, we selected those related to our traits of interest. In addition to traits related to reproduction, we also included traits related to growth, abiotic stress, nitrogen metabolism and alkaloids, composing a total of 73 gene ontology terms (Table S2). We then created a list with all the gene ontology terms and the sequences related to them using the Go.db package in R software (R Core Team, 2020). The 1716 sequences obtained from this process were used as probes to carry out the targeted sequencing of the gene capture experiment.

The extracted DNA samples were sent to IGATech (Udine, Italy). A Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts) was used to evaluate the quality of the samples. We used the 'SeqCap EZ - HyperPlus' kit from Roche Sequencing Solutions (Pleasanton, CA) to create libraries for target enrichment of approximately 3 Mb of the genomic material of *Lupinus angustifolius*.

Single Nucleotide Polymorphism (SNP) calling

We used Illumina bcl2fastq v2.20 to perform the base calling and sample demultiplexing. Subsequently, we used ERNE v1.4.6 (del Fabbro et al., 2013) and Cutadapt (Martin, 2011) for quality filtering and adapter trimming. Additionally, we used BWA-MEM v0.7.17 (Li & Durbin, 2009) for the alignment to the reference genome. Finally, we used Picard tools (http://broadinstitute.github.io/picard/) to produce on-target alignment statistics and metrics.

We carried out the SNP calling with gatk-4.0 (Depristo et al., 2011) on the total data set. We used VCFtools v0.1.14 (Danecek et al., 2011), and the *vcffilter* function of VCFLIB (Garrison et al., 2021) to filter the raw SNP data. During the filtering process, we kept biallelic SNPs with fewer than 10 % missing data and removed indels from the dataset. We applied additional filters as recommended in the GATK's user guide (https://gatk.broadinstitute.org/) which included the following parameters: Quality depth (QD > 2), symmetric Odds Ratio of 2x2 contingency table to detect strand bias (SOR > 3), phred scaled P-value using Fisher's Exact Test to detect strand bias (FS < 60), z-score from Wilcoxon rank sum test of Alt vs. Ref read mapping qualities (MQRankSum > -12.5), square root of the average of the squares of the mapping qualities (MQ > 40), depth coverage (DP > 10) and u-based z-approximation from the Rank Sum Test for site position within reads (ReadPosRankSum > - 8). Lastly, we used r^2 of 0.6 as the cut-off point to filter SNPs in strong linkage disequilibrium. With this procedure, we moved from the initial identification of ca. 41,419 SNPs to a final dataset of 34,026 SNPs.

Genetic diversity and population structure analyses

Genetic diversity parameters (Pi, Theta, and Tajima's D) were calculated using TASSEL 5.0 (Bradbury et al., 2007). We used a sliding window with a window size of 100 bp and a step size of 10 bp. The population structure was preliminary tested in this study using

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Principal Component Analysis (PCA; Price et al. (2006)), as implemented in TASSEL 5.0 (Bradbury et al., 2007). The kinship analysis was performed using the software KING 2.2.7 (Manichaikul et al., 2010) installed in PLINK 1.9 (Purcell & Chang, 2015). A heat map for the graphical observation of the kinship matrix was created using an automatic correction of IBD coefficients implemented in software R package pheatmap v.1.0.12 (Kolde & Kolde, 2018).

Detecting signatures of selection between populations

We used a script in phyton 2.7. obtained from the hapFLK web page (https://forge-dga.jouy.inra.fr/projects/hapflk) to run the hapFLK statistic implemented in hapFLK v.1.4 (Gautier, 2015) to detect selective sweeps (*i.e.* decrease or elimination of genetic variation in a genomic region where an advantageous mutation has appeared and among nearby nucleotide sequences in a context of directional selection (Harris et al., 2018)). For this analysis, we used only the individuals belonging to the control line of each population (CFL), with the intention of detecting whether there are highly divergent loci between populations as a result of natural selection. The hapFLK test is a haplotype-based approach used to detect selective sweeps based on haplotype frequency differences among populations while considering the hierarchical structure of sampled populations. The hapFLK statistic is an extension of the SNP-based FLK statistic and provides a powerful approach to detect regions of the genome under selection by using a model that incorporates linkage disequilibrium to test for differentiation in haplotype clusters among populations. To account for stratification in the hapFLK statistic, a kinship matrix is required, which is estimated using a neighbour-joining tree and a matrix of Reynold's genetic distances between populations. P-values for hapFLK are computed from a standard normal distribution (Fariello et al. 2013).

For detecting the populations where the selective sweep took place, branch lengths of the population tree were re-estimated for each significant genomic region, using significant SNPs only. For each branch, the *p*-value for the null hypothesis of no difference between the lengths estimated from data in the region and in the whole dataset was computed. This last part was carried out using an R script obtained from the hapFLK web page (https://forge-dga.jouy.inra.fr/projects/hapflk). The calculation of raw *p*-values was based on the null distribution of empirical values after we made sure that these *p*-values were uniformly distributed by plotting them in a histogram (François et al., 2016). Multiple testing correction was done by using a false discovery rate approach (Storey & Tibshirani, 2003).

Detecting signatures of selection between lines

To identify highly divergent regions (*i.e.* outlier loci) between the CFL line and the EFL or OUT line in each population, we applied a sequential approach of Allele Frequency Differences (AFDs) and F_{ST} at the SNP level, using a script in the R statistical environment (R Core Team, 2020). We applied Fishers's exact test on contingency tables (Fisher, 1970) to select those SNPs with significant AFDs between CFL and EFL and, on the other hand, between CFL and OUT. After that, we calculated pairwise F_{ST} values (CFL *vs*. EFL and CFL *vs*. OUT line) for each SNP. To calculate the statistical significance of F_{ST} values we used a chi-square test: $x^2 = 2NF_{ST}(k - 1)$, with $(k - 1)(s - 1)$ degrees of freedom, where N is the total sample size, k is the number of alleles per locus, and s is the number of populations (Workman & Niswander', 1970). For a SNP to be identified as an outlier, it had to be selected by both methods (AFDs and F_{ST} analyses), applying a threshold of $p < 0.001$.

Genome-wide Association Studies (GWAS analyses)

Association analyses were performed via a mixed linear model MLM (Q+Ks). This analysis was performed using all individuals from all populations and lines. This model reduces Type I errors by controlling for genetic relatedness due to both, the population structure (Q) and relationships between individuals (K) (Yu et al., 2006). This analysis was conducted with TASSEL 5.0 (Bradbury et al., 2007), setting the genetic structure (Q) as a fixed factor and a compressed kinship matrix (Ks) as a random factor (K), using the option "compression optimum level". The marker-trait association was tested at the significance level of $p < 0.001$.

In addition, we extracted the additive genetic variance from GWAS analyses to estimate the narrow-sense heritability (the proportion of the total phenotypic variance that can be attributed to additive genetic variance). Estimates of narrow-sense heritability for all traits analyzed in this study were performed by using both the phenotypic variance calculated from the actual phenotypic values and using the residual variances obtained by the GWAS analyses from each locus, this allowed us to obtain two alternative estimates of narrow-sense heritability $h^2 = VG/VP$, where VG is the additive genetic variance and VP was obtained either from actual phenotypic values or from the GWAS residual variances (Zhu & Zhou, 2020).

Results

Genetic diversity

Genetic diversity of the northern populations was greater than that of the southern populations (Table 1). In addition, based on the values of Pi and Theta, within each population, the genetic diversity values between the control and the selection lines were similar (except for PIC population, Table 1), and there was not a consistent pattern of lower genetic diversity in the selection lines. In addition, all Tajima's D values were

negative (which suggests either recent population bottleneck or positive selection), without a clear pattern of lower values in selection or control lines (Table 1). On the other hand, the kinship matrix showed that the individuals of the southern populations (GAR and RIV) had a greater degree of kinship than the individuals of the northern populations (FRO and PIC) (Figure 1).

Table 1. Genetic diversity estimates across populations and lines in the four populations of *Lupinus angustifolius* studied. Nucleotide diversity (Pi), Theta and Tajima's D parameters are shown.

Line	PiPerBP	ThetaPerBP	TajimaD
FRO CFL	0,161045	0,192215	-0,694835
FRO EFL	0,17177	0,186065	-0,28237
FRO OUT	0,19026	0,1999	-0,1905
PIC CFL	0,16589	0,20144	-0,77276
PIC EFL	0,23963	0,26449	-0,360315
PIC OUT	0,20523	0,204515	-0,127355
GAR CFL	0,11462	0,130705	-0,556785
GAR EFL	0,110105	0,12609	-0,56902
GAR OUT	0,095315	0,116865	-0,82258
RIV CFL	0,082155	0,084575	-0,245065
RIV EFL	0,033495	0,044595	-0,98531
RIV OUT	0,04008	0,04613	-0,412185
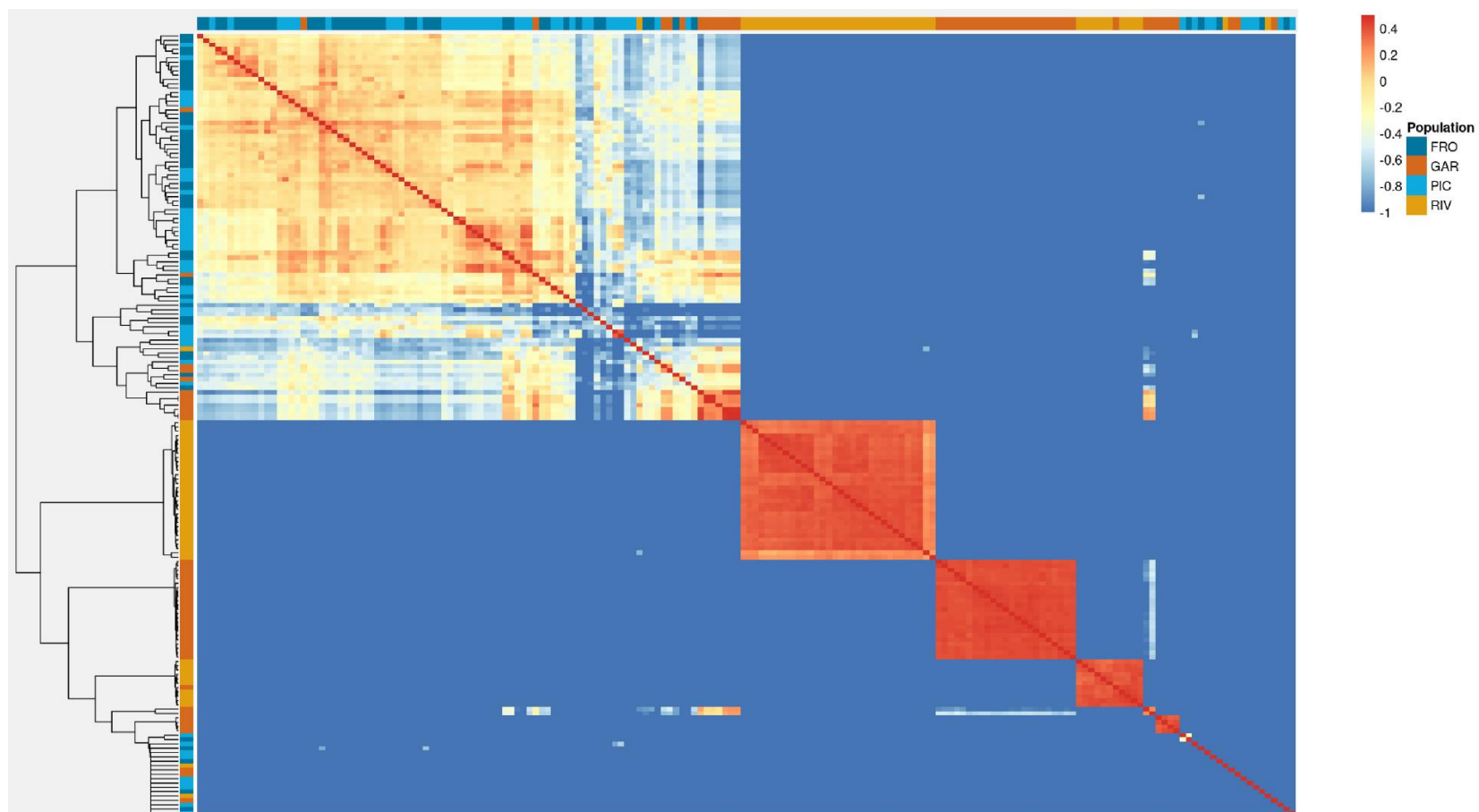
Figure 1. Kinship matrix between the individuals of FRO, PIC, GAR and RIV populations of *Lupinus angustifolius*. Warmer colours depict a higher degree of kinship between each pair of sequenced individuals. The colours shown at the horizontal and vertical axes indicate the population corresponding to each individual according to the legend.

Population structure

The PCA plot revealed that PC1 and PC2 explained 30.2 % and 17.5 % of variance, respectively. FRO and PIC (northern populations) are notably different from GAR and RIV (southern populations) (Figure 2). Moreover, GAR and RIV are also quite different from each other, whereas FRO and PIC showed a weak structure between them (Figure 2). Differences between northern and southern populations range mostly across the first principal component, whereas the second principal component can differentiate between the individuals of the two southern populations (GAR *vs*. RIV). HapFLK analyses detected the same spatial genetic structure (Figure 3), although no significant differences in SNP frequencies were found between populations ($p < 0.001$).
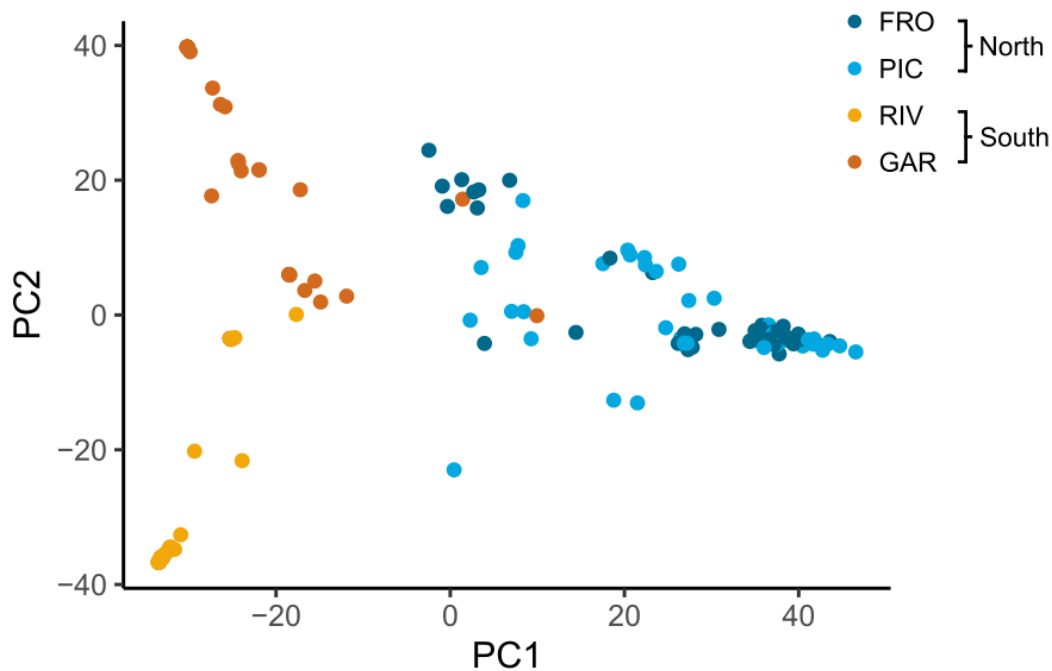
Figure 2. Distribution of individuals of northern (FRO and PIC) and southern (RIV and GAR) populations across the first two principal components of a PCA.
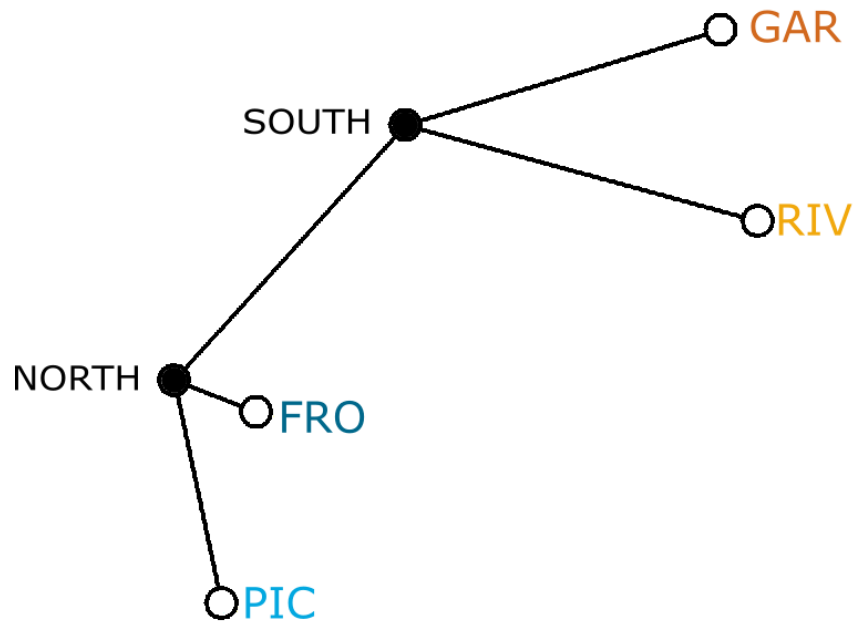
Figure 3. Whole genome tree for the control lines of the four populations of *Lupinus angustifolius* L. obtained from the hapFLK analyses.

Loci under selection

The F_{ST} and AFDs analyses revealed a variable number of SNPs significantly different between control and selection lines (outlier loci) in each population. A summary of the number of loci identified for each population and contrast lines is shown in Table 2, and the lists with the identification of the SNPs involved are shown in the Supplementary Material (Tables S3 to S6). In general, a greater number of SNPs were found to be under selection in the northern populations than in the southern populations. All outlier SNPs were different for each of the populations and line contrasts.

Table 2. Number of SNP outliers identified for each population by both F_{ST} and AFD analyses.

Population	CFL *vs*. EFL	CFL *vs*. OUT
FRO	20	9
PIC	153	665
GAR	0	0
RIV	38	0

Genome-Wide Association Studies (GWAS)

Significant associations were found between the genotype of the individuals and the traits of flowering initiation, number of seeds, seed weight, height, biomass, shoot growth, SLA and LDMC (all at $p < 0.001$). Specifically, we found 42 SNPs associated with flowering onset, 38 SNPs associated with seed number, 16 SNPs associated with seed weight, 31 SNPs associated with plant height, 45 SNP associated with biomass, 28 SNPs associated with shoot growth, 1 SNP associated with SLA, and 9 SNPs associated with LDMC (Figure 5, Table S7). No SNP matched more than one trait.
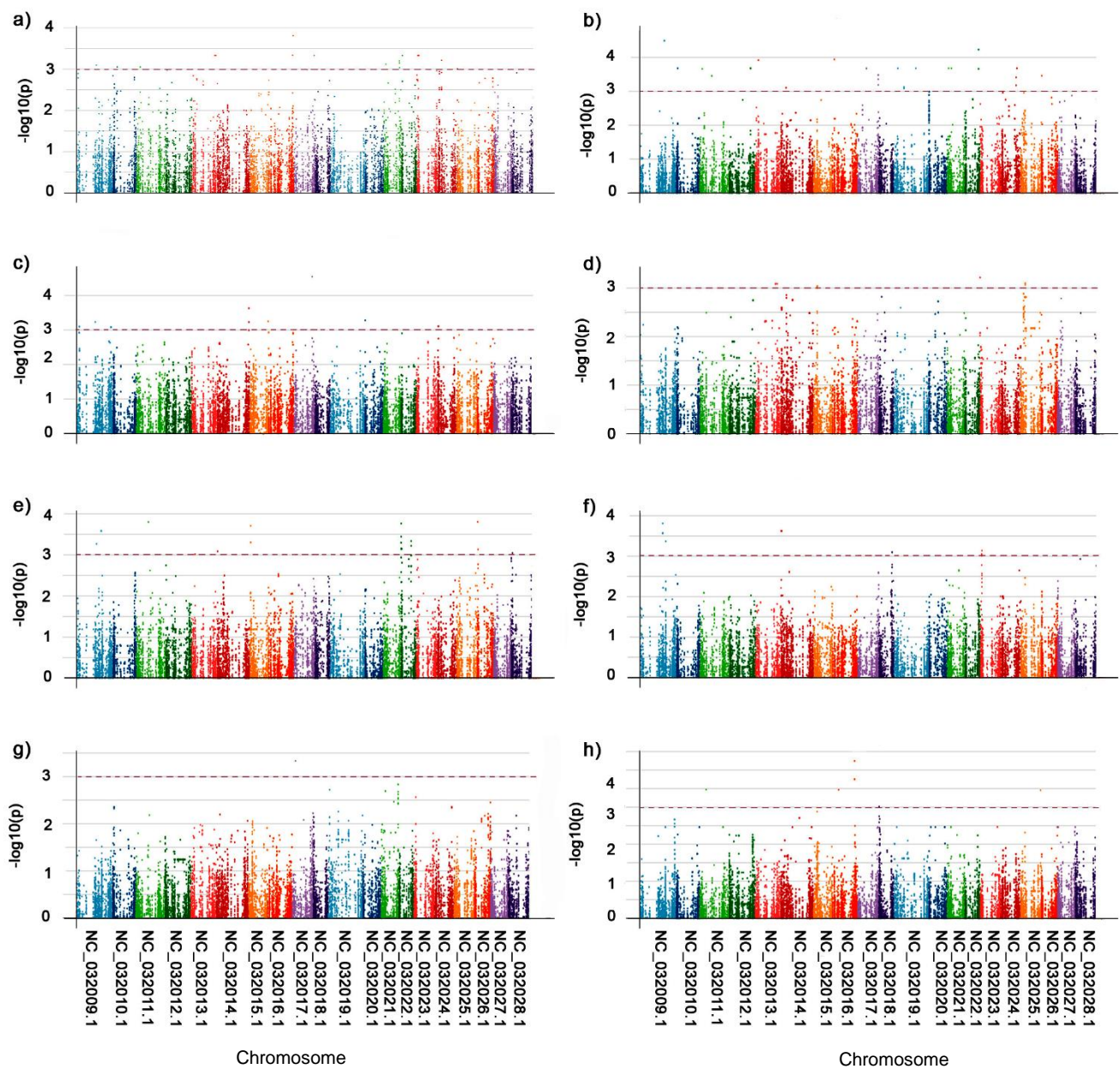
Figure 4. Manhattan plots representing the significant SNPs (p < 0.001) identified in the chromosomes of *L. angustifolius* for the different traits studied. a) flowering onset, b) seed number, c) seed weight, d) height, e) biomass, f) shoot growth, g) SLA, h) LDMC.

In addition, LDMC, shoot growth and number of seeds were found to have high heritability, while SLA and seed weight had low heritability (Table3).

Table 3. Estimates of narrow-sense heritability for all traits analysed in this study. The second column features the values of narrow-sense heritability using the phenotypic variance calculated from the phenotypic data. The third column features the narrow sense heritability using the residual variance.

Trait	Estimated heritability by phenotypic variance	Estimated heritability by residual variance
Flowering onset	0.25	0.5
Number of seeds	0.77	0.6
Seed weight	0.1	0.24
Height	0.3	0.47
Biomass	0.31	0.29
Shoot growth	0.41	0.63
SLA	0.1	0.12

Discussion

The results obtained from the genomic study of the different populations and lines of artificial selection in *Lupinus angustifolius* indicate that there is a genetic differentiation between the northern and southern populations, with the latter showing lower genetic diversity and, at the same time, higher kinship among their individuals. However, no major differences were found between the control lines and the artificial selection lines of any population with respect to genetic diversity parameters. These results, therefore, confirm the ideas that were presented in Chapter 1, confirming that northern populations have greater evolutionary potential regarding the traits associated with the genomic regions studied.

Genetic diversity of populations

In this study, we observed that northern populations have higher genetic diversity than southern populations, for both control and selection lines. In the same line, at the intra-population level, the kinship matrix indicates that individuals from the southern populations have higher similarity to each other than individuals from the northern populations. These results are in line with those obtained in Chapter 1 (Sacristán-Bajo et al., 2023), in which we observed that the response to selection was much greater in the northern populations than in the southern populations. In that chapter, we hypothesized that the southern populations did not experience a significant advance in the onset of flowering because they had already undergone natural selection due to the warmer environmental conditions in their localities. The results obtained in this study support this hypothesis as they confirmed that southern populations have lower genetic diversity in the studied traits compared to northern populations. (Huang et al., 2012a; Izawa, 2007).

The genetic diversity analyses given by nucleotide diversity (pi) and Theta estimator shown in Table 1 did not reveal major changes between the control lines and the artificial selection lines, neither by self-crossing nor by outcrossing, in any of the four populations. In other words, genetic diversity of the artificial selection lines did not significantly differ, in quantitative terms, from the control lines. This lack of significant reduction in genetic diversity is explained by the fact that the selection process was only conducted in one generation. It may also be due to the polygenic character of the flowering onset trait that was the subject of selection, which allows the possibility of obtaining an early flowering onset through multiple allele combinations (Blümel et al., 2015; Grummer et al., 2022), as well as the fact that the selective sweep will only affect the neighboring regions of the genome that are close to the affected genes (Nielsen et al., 2005).

Regarding the Tajima's D statistic (Tajima, 1989), in the northern populations (FRO and PIC), it was observed that the statistic goes from being very negative and closer to -1 in the control line, to being less negative and closer to 0 in the outbreeding line (OUT). Less negative values of Tajima's D are associated to greater average heterozygosity and more scarce rare alleles (Schmidt & Pool, 2002). This could be due to the mating system of the species, since as it is mainly self-pollinated and exogamous crosses are made in the OUT line, we are likely to obtain higher heterozygosity in the latter. This did not take place in the southern populations, probably due to the abundance of rare alleles in the southern populations.

Genetic structure of the populations

The PCA analysis showed a clear differentiation between the northern and southern populations. In addition, the two northern populations are more similar to each other, while the two southern populations are more differentiated, likely due to historical bottlenecks. The hapFLK analysis detected the same pattern although it did not find statistical significance for positive selection on individual genomic regions in any of the studied control populations (CFL). A genetic diversity study of *Lupinus angustifolius* throughout the Mediterranean region found strong genetic differentiation between eastern and western accessions (Mousavi-Derazmahalleh et al., 2018). These two groups differ significantly in their flowering time as a result of adaptation to environmental conditions, since the eastern zone is warmer and drier. As in our case, the southern populations with more thermic conditions flowered earlier (Chapter 1, Sacristán-Bajo et al., 2023, Figure 4).

Genomic signals of artificial selection

In the agronomic context, it has been observed multiple times that artificial selection implemented through multiple generations reduces genetic diversity. Some examples that illustrate this are the greater genetic diversity of wild rice (*Oriza rufipogon*) with regard to cultivated rice (*Oriza sativa*) (Huang et al., 2012; Londo et al., 2006), or the greater genetic diversity of the ancestral varieties of cotton with regard to the more modern varieties (Han et al., 2020), indicating that the artificial selection carried out during the domestication and/or breeding processes reduced their genetic diversity. Therefore, one of the concerns involved in the use of artificial selection in plant conservation is the reduction of genetic diversity (Sheth & Angert, 2016; Whitt et al., 2002).

Although no major reduction in genetic diversity was found in the artificial selection experiment from a quantitative perspective, candidate SNPs (outliers) were found to be under selection in at least one line in three of the four populations, revealing qualitative differences between control and artificial selection lines. F_{ST}-AFDs analyses showed that the number of candidate SNPs under selection was higher in northern populations than in southern populations. It is worth noting the case of PIC (north), in which a particularly high number of candidate SNPs was found, both in the comparisons of the control line with the EFL and the OUT lines. In contrast, with the criteria applied, no SNP was selected as a candidate for any of the comparisons in GAR population (south). These results reinforce the ideas and results obtained in Chapter 1 (Sacristán-Bajo et al., 2023), since it was again observed that the effects of artificial selection are greater in the northern populations than in the southern populations. It is remarkable that there was no SNP overlap among the candidate SNPs identified for the artificial selection on flowering onset in the different populations. This may be explained by the polygenic nature of this trait that allows obtaining similar early flowering phenotypes through different allelic

combinations, but also brings a note of caution due to the possibility of obtaining false positives in spite of the severe restrictions imposed to avoid them (Marigorta et al., 2018; Narum & Hess, 2011).

Phenotype-genotype associations

We found significant associations between several SNPs and different traits. The highest number of associated SNPs were found for biomass (45), flowering onset (42), number of seeds (38), and shoot growth (28), followed by seed weight (16), LDMC (9) and SLA (1). Other studies have identified associations between genotype and flowering onset and reproductive traits in different species (Huang et al., 2012; Jiang et al., 2020; Weller & Ortega, 2015; Zhang et al., 2015), indicating that these traits have a strong genetic component. Additionally, studies in other plant species have found genomic differences related to flowering time which were also associated with geographic variation. For example, studies by Brachi et al. (2010), and Tabas-Madrid et al (2018) found that flowering time in *Arabidopsis thaliana* is a key life-history trait that varies with the environment. Other study found differences in flowering time in rice associated with geographic variation (Izawa, 2007). Slotte et al. (2007) found that variation in flowering time in *Capsella bursa-pastoris* is correlated with latitude, suggesting an adaptation to photoperiod. A study by Burgarella et al. (2016) used a large *Medicago truncatula* core collection to examine the association between nucleotide polymorphisms and flowering time genes involved in the adaptation to environmental heterogeneity. Additionally, Blanco-Pastor et al. (2021) found negative associations between flowering time (heading date) in perennial ryegrass (*Lolium perenne*) and winter temperature. All these studies provide evidence of genomic differences related to flowering time associated with geographic variation as we also found here. Moreover, heritability analyses indicate that flowering onset, number of seeds and shoot growth are traits that have a high heritability,

indicating that they have a strong genetic basis. For this reason, we are likely to find a considerable number of SNPs associated with these traits. In contrast, SLA showed low heritability, and only one SNP was found associated with this trait, indicating that it is probably a trait which is more greatly affected by environment for our study species.

Final considerations

Although several studies have been carried out in different species regarding the genomic signals of artificial selection focused on genetic improvement, the implications for the evolutionary potential of species in the face of climate change have been barely studied. Genomic approaches are powerful tools to complement ecological and evolutionary studies on the adaptation of organisms to climate change. The results obtained in the present study suggest that there is significant genomic differentiation between northern and southern populations in regions related to flowering, reproduction, growth and abiotic stress processes. Since the genetic diversity of southern populations is lower, and fewer candidate SNPs were found to be under selection, southern populations have lower evolutionary potential for the traits studied, and therefore may be more vulnerable to future climate change scenarios. However, since southern populations have a genetic composition that causes them to flower earlier, these genetic resources could be used for example in assisted gene flow actions in northern populations (see Chapter 3). Genomic studies such as this one can be very useful in determining the evolutionary potential of populations, and detecting which populations are at greater risk in the context of climate change, hence requiring more urgent conservation or interventions. In this regard, this study provides a novel approach to the use of genomic data in conservation biology that has scarcely been applied so far (Khan et al., 2016; Shafer et al., 2015), helping us to identify genomic resources that could be used to enhance the adaptation of populations to climate change.

Acknowledgements

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Supplementary Material

Table S1. Ontology Terms selected to design the probes used in the targeted sequencing of individuals of *Lupinus angustifolius* L. and their corresponding biological functions. These data were obtained from the AmiGO resource (Ashburner et al., 2000; Carbon et al., 2009, 2021).

GO term	Biological function
GO:0048653	anther development
GO:0080128	anther septum development
GO:0048657	anther wall tapetum cell differentiation
GO:0010234	anther wall tapetum cell fate specification
GO:0048658	anther wall tapetum development
GO:0048656	anther wall tapetum formation
GO:0048655	anther wall tapetum morphogenesis
GO:0009793	embryo development ending in seed dormancy
GO:0009960	endosperm development
GO:0048559	establishment of floral organ orientation
GO:0010582	floral meristem determinacy
GO:0010451	floral meristem growth
GO:0010227	floral organ abscission
GO:0048437	floral organ development
GO:0048449	floral organ formation
GO:0048444	floral organ morphogenesis
GO:0080187	floral organ senescence
GO:0048438	floral whorl development
GO:0048458	floral whorl formation
GO:0048457	floral whorl morphogenesis
GO:0048459	floral whorl structural organization
GO:0048460	flower formation
GO:0048439	flower morphogenesis
GO:0048461	flower structural organization
GO:0048530	fruit morphogenesis
GO:1990058	fruit replum development
GO:0009835	fruit ripening
GO:0080127	fruit septum development
GO:1990059	fruit valve development
GO:0010450	inflorescence meristem growth
GO:0048281	inflorescence morphogenesis
GO:0010254	nectary development
GO:0048481	plant ovule development
GO:0048482	plant ovule morphogenesis
GO:0048235	pollen sperm cell differentiation
GO:0048868	pollen tube development
GO:0009860	pollen tube growth

GO:0010214	seed coat development
GO:0010162	seed dormancy process
GO:0080112	seed growth
GO:0010431	seed maturation
GO:0048317	seed morphogenesis
GO:0090376	seed trichome differentiation
GO:0090378	seed trichome elongation
GO:0090377	seed trichome initiation
GO:0090380	seed trichome maturation
GO:0080086	stamen filament development
GO:0048480	stigma development
GO:0048479	style development
GO:0010228	vegetative to reproductive phase transition of meristem
GO:0009856	pollination
GO:0009409	response to cold
GO:0009408	response to heat
GO:000941	response to water deprivation
GO:0080186	developmental vegetative growth
GO:0010152	pollen maturation
GO:0022611	dormancy process
GO:0010378	temperature compensation of the circadian clock
GO:0009900	dehiscence
GO:0010212	response to ionizing radiation
GO:0090547	response to low humidity
GO:0019740	nitrogen utilization
GO:0009820	alkaloid metabolic process

Table S2. SNPs identified as signatures of selection between the CFL and EFL lines in FRO population of *Lupinus angustifolius* after being selected by both F_{ST} and AFDs analyses ($p < 0.001$). Information on populations and treatments can be found in Sacristán-Bajo et al., 2023, Table 1, Figures 1 and 2.

SNP	p-value
NC_032013.1_179341	4,26E+07
NC_032013.1_179727	4,26E+07
NC_032013.1_179851	4,26E+07
NC_032013.1_180135	4,26E+07
NC_032013.1_180167	4,26E+07
NC_032013.1_180272	4,26E+07
NC_032013.1_180429	4,26E+07
NC_032013.1_181605	4,26E+07
NC_032013.1_181771	4,26E+07
NC_032013.1_181969	3,04E+08

NC_032013.1_298161	7,87E+08
NC_032013.1_298346	7,87E+08
NC_032013.1_298845	2,12E+07
NC_032013.1_299039	7,87E+08
NC_032013.1_299213	2,12E+07
NC_032013.1_299594	7,87E+08
NC_032013.1_299828	7,87E+08
NC_032016.1_20839680	2,02E+06
NC_032016.1_21438952	4,15E+05
NC_032016.1_21440424	4,37E+07

Table S3. SNPs identified as signatures of selection between the CFL and OUT lines in FRO population of *Lupinus angustifolius* after being selected by both F_{ST} and AFDs analyses (p < 0.001). Information on populations and treatments can be found in (Sacristán-Bajo et al., 2023, Table 1, Figures 1 and 2).

SNP	p-value
NC_032012.1_946480	7,78E+07
NC_032012.1_10631549	7,02E+08
NC_032014.1_366186	1,41E+09
NC_032014.1_8884950	2,03E+08
NC_032014.1_8885261	6,29E+07
NC_032014.1_8885832	5,37E+08
NC_032014.1_8886020	9,92E+08
NC_032014.1_8886033	9,92E+08
NC_032014.1_8886780	3,06E+07

Table S4. SNPs identified as signatures of selection between the CFL and EFL lines in PIC population of *Lupinus angustifolius* after being selected by both F_{ST} and AFDs analyses (p < 0.001). Information on populations and treatments can be found in (Sacristán-Bajo et al., 2023, Table 1, Figures 1 and 2).

SNP	p-value	SNP	p-value
NC_032010.1_24184204	6,76E+07	NC_032018.1_13748699	6,17E+07
NC_032011.1_517278	2,21E+09	NC_032018.1_13751893	8,35E+08
NC_032011.1_520757	2,21E+09	NC_032018.1_13752258	6,17E+07
NC_032012.1_6896743	1,74E+09	NC_032018.1_13752391	6,17E+07
NC_032012.1_21219104	8,29E+08	NC_032018.1_14860489	2,77E+09
NC_032012.1_24784550	8,90E+08	NC_032018.1_14862828	2,77E+09
NC_032012.1_24789659	8,90E+08	NC_032018.1_14863091	2,77E+09
NC_032012.1_24792233	8,90E+08	NC_032019.1_612958	2,53E+08
NC_032012.1_24800510	8,90E+08	NC_032021.1_155931	2,88E+07

NC_032012.1_24801853	2,16E+09	NC_032021.1_156206	7,84E+07
NC_032012.1_24805629	8,90E+08	NC_032021.1_156262	2,44E+07
NC_032012.1_24808032	2,39E+07	NC_032021.1_156407	7,84E+07
NC_032012.1_26086474	1,77E+08	NC_032021.1_156621	1,60E+09
NC_032012.1_26086573	1,77E+08	NC_032021.1_156629	7,16E+07
NC_032012.1_26162630	2,85E+08	NC_032021.1_156812	7,16E+07
NC_032012.1_26162918	4,89E+08	NC_032021.1_156827	2,44E+07
NC_032013.1_513169	1,74E+09	NC_032021.1_1206160	2,21E+09
NC_032013.1_513185	1,74E+09	NC_032021.1_16167811	1,05E+07
NC_032013.1_513658	1,74E+09	NC_032021.1_16723017	8,32E+07
NC_032013.1_514810	1,74E+09	NC_032021.1_16724880	8,32E+07
NC_032013.1_515192	2,74E+09	NC_032021.1_17579312	7,87E+08
NC_032013.1_1119385	2,07E+06	NC_032021.1_17579767	7,02E+08
NC_032013.1_1119460	6,88E+06	NC_032023.1_1781681	1,70E+08
NC_032013.1_1119804	6,88E+06	NC_032023.1_1781975	1,39E+09
NC_032013.1_1119854	6,88E+06	NC_032023.1_17065193	8,90E+08
NC_032013.1_1779261	1,83E+09	NC_032024.1_4526667	7,49E+06
NC_032013.1_1779272	1,83E+09	NC_032024.1_6877955	2,77E+09
NC_032013.1_9881061	2,77E+09	NC_032024.1_7367628	8,29E+08
NC_032013.1_12446168	2,00E+08	NC_032024.1_7367778	8,29E+08
NC_032013.1_12446367	2,00E+08	NC_032024.1_7367941	8,29E+08
NC_032014.1_365590	2,81E+07	NC_032026.1_15054248	1,83E+09
NC_032014.1_580446	7,74E+08	NC_032026.1_15055984	1,83E+09
NC_032014.1_581029	8,29E+08	NC_032026.1_15058377	1,83E+09
NC_032014.1_5880056	7,02E+08	NC_032026.1_15918752	7,02E+08
NC_032014.1_5880075	7,02E+08	NC_032026.1_15920384	7,02E+08
NC_032014.1_5880133	2,07E+08	NC_032026.1_15920648	7,02E+08
NC_032014.1_5880149	2,07E+08	NC_032026.1_15921374	7,02E+08
NC_032014.1_5880168	2,07E+08	NC_032026.1_15921795	7,02E+08
NC_032014.1_5880182	2,07E+08	NC_032026.1_16068098	8,35E+08
NC_032014.1_5880186	2,07E+08	NC_032026.1_16069511	8,35E+08
NC_032014.1_5880245	2,07E+08	NC_032026.1_16069772	8,35E+08
NC_032014.1_6164346	2,21E+09	NC_032026.1_16069859	8,35E+08
NC_032014.1_6165005	2,21E+09	NC_032026.1_16072081	8,35E+08
NC_032014.1_6165388	2,21E+09	NC_032026.1_16073394	8,35E+08
NC_032014.1_6169876	2,21E+09	NC_032026.1_16073957	8,35E+08
NC_032014.1_6178548	8,77E+08	NC_032028.1_334364	1,74E+09
NC_032014.1_6178550	2,21E+09	NC_032028.1_335580	2,88E+07
NC_032014.1_6179308	8,77E+08	NC_032028.1_336188	1,74E+09
NC_032014.1_6181536	1,60E+09	NC_032028.1_336287	1,74E+09
NC_032014.1_6181599	1,60E+09	NC_032028.1_336901	1,74E+09
NC_032014.1_6181768	1,60E+09	NC_032028.1_337347	1,74E+09
NC_032014.1_6181779	1,60E+09	NC_032028.1_337564	1,74E+09
NC_032014.1_6181789	1,60E+09	NC_032028.1_337905	2,88E+07
NC_032014.1_6182217	1,60E+09	NC_032028.1_337961	1,74E+09
NC_032014.1_6182381	1,60E+09	NC_032028.1_338382	2,88E+07
NC_032014.1_32108691	8,29E+08	NC_032028.1_426334	8,29E+08
NC_032014.1_32109629	1,48E+06	NC_032028.1_1359336	2,21E+09
NC_032014.1_32110146	8,32E+07	NC_032028.1_1360019	2,21E+09
NC_032015.1_752833	8,31E+07	NC_032028.1_1360639	2,21E+09
NC_032016.1_364654	9,03E+06	NC_032028.1_1360790	2,21E+09
NC_032016.1_365248	8,29E+08	NC_032028.1_1361012	2,21E+09
NC_032016.1_365345	8,29E+08	NC_032028.1_1362219	1,29E+08
NC_032016.1_365457	9,03E+06	NC_032028.1_1362555	2,21E+09
NC_032016.1_365903	8,29E+08	NC_032028.1_1421164	7,74E+08

NC_032016.1_366869	8,29E+08	NC_032028.1_1421176	5,25E+07
NC_032016.1_367032	9,03E+06	NC_032028.1_1421257	2,21E+09
NC_032016.1_22735388	1,60E+09	NC_032028.1_1452614	3,05E+08
NC_032016.1_22736023	1,60E+09	NC_032028.1_1531216	3,72E+09
NC_032017.1_12306018	1,01E+09	NC_032028.1_1670018	2,20E+09
NC_032017.1_12306095	1,01E+09	NC_032028.1_5359649	5,37E+08
NC_032017.1_12306256	1,01E+09	NC_032028.1_20234830	1,74E+09
NC_032017.1_12306361	1,01E+09	NW_017718123.1_74294	6,09E+08
NC_032017.1_12306436	1,01E+09	NW_017720548.1_62699	2,77E+09
NC_032018.1_3235528	1,74E+09	NW_017723711.1_28799	8,90E+08
NC_032018.1_3239795	1,74E+09	NW_017723711.1_29904	8,90E+08
NC_032018.1_13747233	7,02E+08	NW_017723711.1_29934	8,90E+08
NC_032018.1_13748462	5,92E+08	-	-

Table S5. SNPs identified as signatures of selection between the CFL and OUT lines in PIC population of *Lupinus angustifolius* after being selected by both F_{ST} and AFDs analyses (p<0.001). Information on populations and treatments can be found in (Sacristán-Bajo et al., 2023, Table 1, Figures 1 and 2).

SNP	p-value	SNP	p-value
NC_032009.1_1756378	2,44E+07	NC_032019.1_8162300	1,10E+07
NC_032009.1_20476789	1,41E+09	NC_032019.1_8162598	9,89E+07
NC_032009.1_21358539	1,10E+06	NC_032019.1_8163880	1,10E+07
NC_032009.1_22179944	7,29E+08	NC_032019.1_8814046	7,87E+08
NC_032009.1_22181631	2,66E+08	NC_032019.1_8814149	7,87E+08
NC_032009.1_23308849	1,08E+09	NC_032019.1_8814287	1,88E+08
NC_032009.1_23309370	3,16E+08	NC_032019.1_8814645	1,88E+08
NC_032009.1_23310519	1,08E+09	NC_032019.1_8814888	1,88E+08
NC_032009.1_23310873	3,16E+08	NC_032019.1_8815158	1,88E+08
NC_032009.1_23514794	6,43E+07	NC_032019.1_8815239	1,88E+08
NC_032009.1_23514827	1,88E+08	NC_032019.1_8815523	1,88E+08
NC_032009.1_23515139	1,88E+08	NC_032019.1_8815866	1,88E+08
NC_032009.1_25267356	1,31E+07	NC_032019.1_8816147	1,88E+08
NC_032009.1_26281031	6,96E+08	NC_032019.1_8816371	4,91E+07
NC_032009.1_35506047	7,36E+06	NC_032019.1_8816810	7,87E+08
NC_032010.1_24182989	4,59E+07	NC_032019.1_8817020	1,88E+08
NC_032010.1_24183218	4,59E+07	NC_032019.1_35464272	6,84E+08
NC_032010.1_24183326	4,59E+07	NC_032020.1_316231	1,75E+08
NC_032010.1_24183399	4,59E+07	NC_032020.1_405780	1,89E+07
NC_032010.1_24184204	5,52E+08	NC_032020.1_958922	1,75E+08
NC_032010.1_24476469	2,32E+08	NC_032020.1_964351	1,75E+08
NC_032010.1_24476474	2,32E+08	NC_032020.1_971742	1,75E+08
NC_032010.1_24476505	2,32E+08	NC_032020.1_973654	1,75E+08
NC_032010.1_24476512	2,32E+08	NC_032020.1_974042	1,75E+08
NC_032010.1_24476612	2,32E+08	NC_032020.1_6718172	1,41E+09
NC_032010.1_24477533	2,32E+08	NC_032021.1_155931	2,92E+05
NC_032010.1_24477938	7,31E+07	NC_032021.1_156206	2,92E+05
NC_032011.1_517278	2,46E+07	NC_032021.1_156262	1,97E+04
NC_032011.1_519496	2,53E+08	NC_032021.1_156407	2,92E+05

NC_032011.1_520757	2,46E+07	NC_032021.1_156621	4,48E+04
NC_032011.1_1445585	2,53E+08	NC_032021.1_156629	1,97E+04
NC_032011.1_1445928	2,53E+08	NC_032021.1_156812	1,97E+04
NC_032011.1_1780524	7,84E+07	NC_032021.1_156827	1,97E+04
NC_032011.1_1780652	7,84E+07	NC_032021.1_1018760	7,25E+08
NC_032011.1_24287488	1,76E+09	NC_032021.1_1018864	7,25E+08
NC_032011.1_24287788	1,76E+09	NC_032021.1_1205013	3,61E+07
NC_032011.1_24287835	1,76E+09	NC_032021.1_1206160	5,57E+07
NC_032011.1_24288336	5,52E+08	NC_032021.1_1702612	2,17E+08
NC_032011.1_24288455	5,52E+08	NC_032021.1_1703860	5,95E+06
NC_032011.1_24288475	5,52E+08	NC_032021.1_4021259	1,39E+09
NC_032011.1_24288479	5,52E+08	NC_032021.1_4021752	6,92E+08
NC_032011.1_24288492	5,52E+08	NC_032021.1_4022392	5,00E+06
NC_032011.1_24352426	5,52E+08	NC_032021.1_4475466	6,13E+06
NC_032011.1_24354095	5,52E+08	NC_032021.1_4475697	1,03E+06
NC_032011.1_24768362	5,52E+08	NC_032021.1_4476713	1,47E+05
NC_032011.1_24772698	5,52E+08	NC_032021.1_4477026	6,13E+06
NC_032012.1_949977	2,77E+09	NC_032021.1_4477898	6,13E+06
NC_032012.1_1015435	1,04E+05	NC_032021.1_4478557	6,13E+06
NC_032012.1_1021273	4,91E+07	NC_032021.1_4478737	1,85E+06
NC_032012.1_1286357	1,75E+08	NC_032021.1_4488045	1,47E+05
NC_032012.1_2490203	8,57E+08	NC_032021.1_4646581	4,97E+05
NC_032012.1_6896240	1,27E+06	NC_032021.1_4709260	4,21E+08
NC_032012.1_6896743	1,25E+05	NC_032021.1_4710231	4,21E+08
NC_032012.1_8005277	1,75E+08	NC_032021.1_4711128	4,21E+08
NC_032012.1_21244102	1,77E+08	NC_032021.1_4721289	4,21E+08
NC_032012.1_21615942	1,23E+08	NC_032021.1_4721704	4,21E+08
NC_032012.1_22606527	1,09E+08	NC_032021.1_4721811	4,21E+08
NC_032012.1_22606967	1,09E+08	NC_032021.1_4722139	4,21E+08
NC_032012.1_22607325	1,09E+08	NC_032021.1_4722485	4,99E+08
NC_032012.1_22607661	1,09E+08	NC_032021.1_4772923	4,21E+08
NC_032012.1_22607778	1,09E+08	NC_032021.1_4773164	4,21E+08
NC_032012.1_22608679	4,17E+08	NC_032021.1_4774990	4,21E+08
NC_032012.1_22608808	6,71E+07	NC_032021.1_14971865	1,41E+09
NC_032012.1_22609724	1,09E+08	NC_032021.1_16167811	2,86E+06
NC_032012.1_22609995	7,25E+08	NC_032021.1_16587727	8,90E+08
NC_032012.1_22615844	7,25E+08	NC_032021.1_16588531	1,41E+09
NC_032012.1_22618151	1,09E+08	NC_032021.1_16588792	1,41E+09
NC_032012.1_22618798	1,59E+08	NC_032021.1_16723017	2,13E+06
NC_032012.1_22622004	1,09E+08	NC_032021.1_16723200	5,52E+08
NC_032012.1_22628974	1,09E+08	NC_032021.1_16723719	9,92E+05
NC_032012.1_22638538	2,03E+08	NC_032021.1_16724880	2,13E+06
NC_032012.1_22638714	1,09E+08	NC_032021.1_16724883	9,92E+05
NC_032012.1_22638783	1,09E+08	NC_032021.1_16724973	1,46E+08
NC_032012.1_22638850	1,09E+08	NC_032021.1_16725039	1,46E+08
NC_032012.1_24784152	4,98E+07	NC_032021.1_17579767	4,99E+08
NC_032012.1_24784550	1,10E+06	NC_032022.1_885796	4,99E+08
NC_032012.1_24789570	4,98E+07	NC_032022.1_891065	1,39E+09
NC_032012.1_24789659	1,10E+06	NC_032022.1_892689	1,39E+09
NC_032012.1_24792233	1,10E+06	NC_032022.1_895682	1,39E+09
NC_032012.1_24795489	1,78E+08	NC_032022.1_904281	1,51E+08
NC_032012.1_24800510	1,10E+06	NC_032022.1_904722	1,51E+08
NC_032012.1_24801612	4,98E+07	NC_032022.1_905979	1,39E+09
NC_032012.1_24801853	9,06E+06	NC_032022.1_1559167	3,05E+08
NC_032012.1_24805629	1,10E+06	NC_032022.1_1573890	4,17E+08

NC_032012.1_24808032	1,10E+06		NC_032022.1_4398316	3,38E+07
NC_032012.1_24816362	5,66E+07		NC_032022.1_4398337	3,38E+07
NC_032012.1_25135035	1,74E+09		NC_032022.1_4399199	2,32E+08
NC_032012.1_25241747	1,01E+08		NC_032022.1_8552929	1,75E+08
NC_032012.1_25242891	1,01E+08		NC_032022.1_8743618	5,52E+08
NC_032012.1_25242954	1,01E+08		NC_032022.1_8744767	1,57E+08
NC_032012.1_25244289	3,77E+08		NC_032022.1_11061127	1,75E+08
NC_032012.1_25244311	9,92E+08		NC_032022.1_11064184	1,72E+09
NC_032012.1_25244795	2,44E+08		NC_032022.1_11081761	1,75E+08
NC_032012.1_25246787	1,01E+08		NC_032022.1_14498407	1,14E+08
NC_032012.1_25248050	1,01E+08		NC_032022.1_14498748	1,14E+08
NC_032012.1_25249316	1,01E+08		NC_032022.1_14499374	1,14E+08
NC_032012.1_25250068	1,01E+08		NC_032022.1_14500437	1,14E+08
NC_032012.1_25251513	1,01E+08		NC_032022.1_14502178	3,67E+08
NC_032012.1_25252579	3,00E+07		NC_032022.1_14525786	6,18E+07
NC_032012.1_25252797	3,00E+07		NC_032022.1_14525918	6,09E+08
NC_032012.1_25253119	3,58E+08		NC_032023.1_1985614	1,32E+09
NC_032012.1_25253344	3,77E+08		NC_032023.1_2097673	1,26E+08
NC_032012.1_26086474	1,77E+08		NC_032023.1_2099726	1,96E+09
NC_032012.1_26086573	1,77E+08		NC_032024.1_898836	1,77E+08
NC_032012.1_26162918	4,29E+08		NC_032024.1_2505841	8,29E+08
NC_032013.1_297181	2,46E+08		NC_032024.1_2508967	8,29E+08
NC_032013.1_297568	8,09E+08		NC_032024.1_2511508	8,29E+08
NC_032013.1_1119385	6,29E+07		NC_032024.1_5007384	1,76E+09
NC_032013.1_1119460	6,29E+07		NC_032024.1_5158078	1,41E+09
NC_032013.1_1119804	6,29E+07		NC_032024.1_5158699	5,57E+07
NC_032013.1_1776369	1,08E+09		NC_032024.1_5159041	1,41E+09
NC_032013.1_1776561	1,08E+09		NC_032024.1_5180449	1,41E+09
NC_032013.1_1776646	1,08E+09		NC_032024.1_5181950	1,41E+09
NC_032013.1_1777165	1,06E+09		NC_032024.1_6877955	1,87E+08
NC_032013.1_1777177	1,06E+09		NC_032024.1_6878678	2,44E+08
NC_032013.1_1777190	1,06E+09		NC_032024.1_7262034	1,08E+09
NC_032013.1_1777224	1,26E+08		NC_032024.1_7262204	1,08E+09
NC_032013.1_1777253	1,08E+09		NC_032024.1_7262265	2,65E+07
NC_032013.1_1777664	1,08E+09		NC_032024.1_7264524	1,23E+08
NC_032013.1_1777834	1,08E+09		NC_032024.1_7264621	1,08E+09
NC_032013.1_1777888	1,08E+09		NC_032024.1_7265038	2,82E+08
NC_032013.1_1779220	4,29E+08		NC_032024.1_7265749	3,31E+08
NC_032013.1_1779261	3,22E+08		NC_032024.1_7266092	1,08E+09
NC_032013.1_1779272	8,84E+07		NC_032024.1_7266139	1,08E+09
NC_032013.1_3093000	1,75E+08		NC_032024.1_7266213	1,08E+09
NC_032013.1_3093274	1,76E+09		NC_032024.1_7266310	1,41E+09
NC_032013.1_3093541	2,86E+06		NC_032024.1_7268131	6,97E+07
NC_032013.1_3403251	1,76E+09		NC_032024.1_7367628	4,70E+07
NC_032013.1_3403255	2,07E+06		NC_032024.1_7367778	4,70E+07
NC_032013.1_3403293	2,51E+06		NC_032024.1_7367941	4,70E+07
NC_032013.1_3403978	5,87E+06		NC_032024.1_7803417	1,45E+08
NC_032013.1_3405457	1,75E+08		NC_032024.1_7804887	1,22E+09
NC_032013.1_3642167	1,75E+08		NC_032024.1_16723820	7,25E+08
NC_032013.1_5289773	3,77E+08		NC_032024.1_16724888	5,08E+08
NC_032013.1_5294640	1,39E+09		NC_032024.1_18975653	1,01E+09
NC_032013.1_8875192	3,80E+07		NC_032024.1_18977012	1,01E+09
NC_032013.1_8875850	7,74E+08		NC_032024.1_18979571	1,01E+09
NC_032013.1_8876338	3,80E+07		NC_032024.1_18981222	1,01E+09
NC_032013.1_8877848	1,75E+08		NC_032024.1_19287477	1,45E+08

NC_032013.1_8878022	2,44E+05	NC_032025.1_1224839	6,76E+07
NC_032013.1_9881061	1,75E+08	NC_032025.1_1225148	6,76E+07
NC_032014.1_365590	4,59E+07	NC_032025.1_1225840	6,76E+07
NC_032014.1_580021	1,77E+08	NC_032025.1_1225911	6,76E+07
NC_032014.1_580446	1,70E+08	NC_032025.1_1225978	6,76E+07
NC_032014.1_581019	8,25E+08	NC_032025.1_1225993	6,76E+07
NC_032014.1_581029	4,50E+08	NC_032025.1_1226068	6,76E+07
NC_032014.1_6165388	1,76E+09	NC_032025.1_1226095	6,76E+07
NC_032014.1_6169876	1,76E+09	NC_032025.1_1226100	6,76E+07
NC_032014.1_6178550	1,76E+09	NC_032025.1_1226382	6,76E+07
NC_032014.1_7227175	1,76E+09	NC_032025.1_1295081	6,76E+07
NC_032014.1_19048462	7,25E+08	NC_032025.1_1295238	6,76E+07
NC_032014.1_19056556	7,25E+08	NC_032025.1_1297885	6,76E+07
NC_032014.1_22401781	2,43E+08	NC_032025.1_20811496	8,04E+05
NC_032014.1_32108691	9,03E+06	NC_032025.1_21099318	1,10E+06
NC_032014.1_32109629	3,29E+06	NC_032025.1_21099342	1,10E+06
NC_032014.1_32110146	2,44E+05	NC_032025.1_21099486	3,67E+08
NC_032015.1_752833	2,00E+07	NC_032025.1_21099506	1,07E+08
NC_032015.1_753548	1,87E+08	NC_032025.1_21099926	1,02E+07
NC_032015.1_753556	1,18E+08	NC_032025.1_21101358	3,09E+07
NC_032015.1_753635	4,95E+08	NC_032025.1_21101528	3,09E+07
NC_032015.1_753644	4,60E+07	NC_032025.1_21102659	3,09E+07
NC_032015.1_753737	4,58E+08	NC_032025.1_21103774	3,09E+07
NC_032015.1_753746	1,78E+09	NC_032025.1_21103893	3,09E+07
NC_032015.1_753754	4,58E+08	NC_032025.1_21103909	7,82E+06
NC_032015.1_753784	4,58E+08	NC_032025.1_21104565	3,09E+07
NC_032015.1_753805	4,58E+08	NC_032025.1_21104687	3,09E+07
NC_032015.1_754120	4,58E+08	NC_032025.1_21104892	1,02E+07
NC_032015.1_756437	4,95E+08	NC_032025.1_21105338	1,57E+06
NC_032015.1_756560	4,60E+07	NC_032025.1_21105518	6,97E+06
NC_032015.1_13801452	1,76E+09	NC_032026.1_13723147	1,22E+09
NC_032015.1_13801500	1,76E+09	NC_032026.1_13723509	1,22E+09
NC_032015.1_13801508	1,76E+09	NC_032026.1_13724134	1,22E+09
NC_032016.1_364654	1,76E+09	NC_032026.1_13724167	1,22E+09
NC_032016.1_365248	4,50E+08	NC_032026.1_13727301	1,69E+07
NC_032016.1_365345	4,50E+08	NC_032026.1_14515475	2,12E+07
NC_032016.1_365457	5,57E+07	NC_032026.1_14518051	4,18E+06
NC_032016.1_365903	4,50E+08	NC_032026.1_14518144	4,18E+06
NC_032016.1_366869	1,75E+08	NC_032026.1_14518240	1,35E+08
NC_032016.1_367032	5,57E+07	NC_032026.1_14518687	5,25E+07
NC_032016.1_4790261	7,02E+08	NC_032026.1_14521133	4,89E+08
NC_032016.1_8838558	3,96E+07	NC_032026.1_14521633	3,25E+09
NC_032016.1_9876063	1,13E+09	NC_032026.1_14522226	6,96E+08
NC_032016.1_10288832	6,29E+07	NC_032026.1_14970004	1,57E+08
NC_032016.1_24002013	5,37E+08	NC_032026.1_14970361	3,31E+08
NC_032016.1_24421286	1,13E+09	NC_032026.1_14970790	1,57E+08
NC_032017.1_18778563	1,08E+09	NC_032026.1_14970811	1,57E+08
NC_032017.1_18778809	1,08E+09	NC_032026.1_14970850	1,57E+08
NC_032017.1_19927737	6,46E+05	NC_032026.1_14971051	1,57E+08
NC_032017.1_19927910	6,46E+05	NC_032026.1_14971114	6,09E+08
NC_032017.1_19929119	2,12E+07	NC_032026.1_14971316	1,56E+09
NC_032017.1_19929505	2,12E+07	NC_032026.1_14972027	3,31E+08
NC_032017.1_19929666	1,10E+07	NC_032026.1_15054248	5,25E+07
NC_032017.1_19929771	1,85E+06	NC_032026.1_15055984	5,25E+07
NC_032017.1_20263175	4,73E+08	NC_032026.1_15058377	5,25E+07

NC_032017.1_20581931	6,76E+07	NC_032026.1_15073415	3,67E+08
NC_032017.1_20582403	6,76E+07	NC_032026.1_15073516	3,31E+08
NC_032017.1_20750106	7,79E+06	NC_032026.1_15073971	1,26E+08
NC_032017.1_21525726	2,32E+08	NC_032026.1_15074023	1,22E+09
NC_032017.1_21526042	2,02E+06	NC_032026.1_15074209	3,31E+08
NC_032017.1_21526109	1,01E+08	NC_032026.1_15074350	3,31E+08
NC_032017.1_21526247	6,72E+08	NC_032026.1_15074446	3,67E+08
NC_032017.1_21672520	2,07E+08	NC_032026.1_15075846	6,84E+08
NC_032018.1_1227266	3,16E+08	NC_032026.1_15225382	2,85E+08
NC_032018.1_2376410	3,96E+07	NC_032026.1_15918975	5,25E+07
NC_032018.1_2376650	1,39E+08	NC_032026.1_15919767	2,55E+07
NC_032018.1_2378777	3,96E+07	NC_032026.1_16071796	1,60E+09
NC_032018.1_3049657	3,01E+04	NC_032028.1_335580	3,93E+07
NC_032018.1_3235528	5,26E+05	NC_032028.1_337905	2,88E+07
NC_032018.1_3238616	2,37E+06	NC_032028.1_337961	1,74E+09
NC_032018.1_3239795	6,19E+05	NC_032028.1_338382	2,88E+07
NC_032018.1_3240416	7,97E+06	NC_032028.1_426334	8,29E+08
NC_032018.1_3240444	7,97E+06	NC_032028.1_459979	1,41E+09
NC_032018.1_3299836	5,64E+06	NC_032028.1_460163	1,41E+09
NC_032018.1_3301019	2,17E+08	NC_032028.1_460389	2,20E+08
NC_032018.1_3302425	2,17E+08	NC_032028.1_712604	2,20E+09
NC_032018.1_3302921	2,17E+08	NC_032028.1_1357518	9,10E+05
NC_032018.1_3303053	2,17E+08	NC_032028.1_1357583	9,10E+05
NC_032018.1_3303087	2,17E+08	NC_032028.1_1359336	2,10E+06
NC_032018.1_4016698	5,37E+08	NC_032028.1_1360019	2,10E+06
NC_032018.1_5431885	1,85E+07	NC_032028.1_1360639	2,10E+06
NC_032018.1_5432435	1,85E+07	NC_032028.1_1360790	2,10E+06
NC_032018.1_5432604	1,85E+07	NC_032028.1_1361012	2,10E+06
NC_032018.1_5432991	1,85E+07	NC_032028.1_1362219	7,11E+05
NC_032018.1_5432997	1,85E+07	NC_032028.1_1362555	2,10E+06
NC_032018.1_13747233	5,57E+06	NC_032028.1_1421164	1,79E+04
NC_032018.1_13747419	1,61E+07	NC_032028.1_1421176	6,19E+05
NC_032018.1_13748462	5,57E+06	NC_032028.1_1421257	2,10E+06
NC_032018.1_13748500	9,32E+08	NC_032028.1_1421554	2,70E+06
NC_032018.1_13748699	5,57E+06	NC_032028.1_1422929	1,04E+05
NC_032018.1_13748991	1,61E+07	NC_032028.1_1451417	3,38E+07
NC_032018.1_13749124	1,61E+07	NC_032028.1_1451515	3,38E+07
NC_032018.1_13749333	1,61E+07	NC_032028.1_1451761	6,15E+08
NC_032018.1_13750066	1,61E+07	NC_032028.1_1451903	1,67E+08
NC_032018.1_13751430	1,61E+07	NC_032028.1_1451906	6,15E+08
NC_032018.1_13751893	2,03E+08	NC_032028.1_1452539	6,72E+06
NC_032018.1_13751935	6,29E+07	NC_032028.1_1452614	2,49E+06
NC_032018.1_13752258	5,57E+06	NC_032028.1_1669427	1,11E+09
NC_032018.1_13752391	5,57E+06	NC_032028.1_1670018	1,89E+09
NC_032018.1_13752755	1,61E+07	NC_032028.1_2124211	7,02E+08
NC_032018.1_13821672	4,91E+07	NC_032028.1_2124296	2,30E+08
NC_032018.1_13821819	6,72E+06	NC_032028.1_2126464	7,02E+08
NC_032018.1_13824193	6,72E+06	NC_032028.1_2136356	6,14E+08
NC_032018.1_14737323	1,51E+08	NC_032028.1_5201062	2,85E+08
NC_032018.1_14737601	1,51E+08	NC_032028.1_5359649	4,73E+08
NC_032018.1_14738509	5,32E+07	NC_032028.1_20232432	1,13E+09
NC_032018.1_14740371	3,77E+08	NC_032028.1_20232583	4,97E+08
NC_032018.1_14740684	1,39E+08	NC_032028.1_20232606	1,79E+08
NC_032018.1_14765029	1,51E+08	NC_032028.1_20234830	5,40E+08
NC_032018.1_14765630	1,51E+08	NC_032028.1_20236695	1,13E+09

NC_032018.1_14765875	1,51E+08	NC_032028.1_20238313	1,13E+09
NC_032018.1_14766213	1,51E+08	NC_032028.1_20575392	2,39E+07
NC_032018.1_14766614	1,51E+08	NC_032028.1_20575537	9,32E+08
NC_032018.1_14766850	1,51E+08	NC_032028.1_20576492	7,79E+06
NC_032018.1_14767699	1,51E+08	NC_032028.1_20576832	2,86E+06
NC_032018.1_14767766	1,51E+08	NC_032028.1_20578090	1,06E+08
NC_032018.1_14768242	1,51E+08	NC_032028.1_20578885	7,79E+06
NC_032018.1_14770177	1,51E+08	NC_032028.1_20579046	6,27E+06
NC_032018.1_14770426	1,51E+08	NC_032028.1_20579612	1,65E+06
NC_032018.1_14770435	1,51E+08	NC_032028.1_20579670	7,79E+06
NC_032018.1_14770445	1,51E+08	NC_032028.1_20579693	7,79E+06
NC_032018.1_14770503	1,51E+08	NC_032028.1_20579823	7,79E+06
NC_032018.1_14771253	1,51E+08	NC_032028.1_20579836	7,90E+05
NC_032018.1_14771356	1,51E+08	NC_032028.1_20579843	7,79E+06
NC_032018.1_14772066	1,51E+08	NC_032028.1_20579945	7,79E+06
NC_032018.1_14772425	1,51E+08	NC_032028.1_21221016	2,86E+06
NC_032018.1_14772461	1,51E+08	NC_032028.1_21223343	9,10E+05
NC_032018.1_14860308	7,82E+06	NC_032028.1_21230379	2,14E+06
NC_032018.1_14860311	7,82E+06	NW_017715973.1_4694	1,75E+08
NC_032018.1_14860404	1,79E+07	NW_017717327.1_31751	1,70E+08
NC_032018.1_14860489	1,79E+07	NW_017717327.1_32714	3,85E+07
NC_032018.1_14860509	1,79E+07	NW_017718123.1_74294	5,37E+08
NC_032018.1_14860517	1,79E+07	NW_017719659.1_17627	9,92E+08
NC_032018.1_14860592	5,57E+07	NW_017720548.1_3964	1,85E+07
NC_032018.1_14861923	1,79E+07	NW_017720548.1_4542	4,42E+08
NC_032018.1_14861998	1,79E+07	NW_017720548.1_5506	1,27E+06
NC_032018.1_14862090	1,79E+07	NW_017720548.1_5620	2,14E+08
NC_032018.1_14862100	1,79E+07	NW_017720548.1_5736	2,14E+08
NC_032018.1_14862492	1,79E+07	NW_017720548.1_62098	5,57E+06
NC_032018.1_14862535	1,79E+07	NW_017720548.1_62699	1,75E+08
NC_032018.1_14862590	1,79E+07	NW_017720548.1_64253	6,46E+05
NC_032018.1_14862798	1,79E+07	NW_017720548.1_64368	6,46E+05
NC_032018.1_14862828	5,57E+07	NW_017720548.1_64375	5,57E+06
NC_032018.1_14862829	5,57E+07	NW_017720558.1_9869	1,85E+07
NC_032018.1_14863004	1,79E+07	NW_017720558.1_10422	4,00E+06
NC_032018.1_14863091	1,79E+07	NW_017720558.1_11280	3,48E+04
NC_032018.1_14864131	1,79E+07	NW_017720558.1_11454	2,12E+07
NC_032018.1_14864230	1,79E+07	NW_017720558.1_29059	5,57E+06
NC_032018.1_14864451	1,79E+07	NW_017720558.1_30806	4,00E+06
NC_032018.1_14865101	1,79E+07	NW_017721386.1_41754	1,45E+08
NC_032018.1_14865114	7,82E+06	NW_017721386.1_48758	1,45E+08
NC_032018.1_14865198	1,79E+07	NW_017721386.1_48945	1,45E+08
NC_032018.1_14865401	1,79E+07	NW_017721994.1_9127	3,89E+08
NC_032018.1_14865420	1,79E+07	NW_017722795.1_48488	2,39E+07
NC_032018.1_14865616	1,79E+07	NW_017722795.1_49210	2,39E+07
NC_032019.1_612958	1,76E+09	NW_017722795.1_50107	2,39E+07
NC_032019.1_1158639	1,37E+08	NW_017722795.1_50348	2,39E+07
NC_032019.1_3945642	7,74E+08	NW_017722795.1_51923	2,39E+07
NC_032019.1_3945653	7,74E+08	NW_017722795.1_52048	2,39E+07
NC_032019.1_3945712	7,74E+08	NW_017722795.1_52531	2,39E+07
NC_032019.1_3945775	7,74E+08	NW_017722795.1_53022	5,87E+06
NC_032019.1_3945851	3,34E+08	NW_017722813.1_46423	8,90E+08
NC_032019.1_3946032	1,78E+09	NW_017722813.1_46796	4,89E+08
NC_032019.1_3946242	1,78E+09	NW_017723711.1_28312	8,90E+08
NC_032019.1_3946284	1,78E+09	NW_017723711.1_28685	3,04E+08

NC_032019.1_4141037	8,31E+07	NW_017723711.1_28799	8,90E+08
NC_032019.1_4331486	8,31E+07	NW_017723711.1_29904	8,90E+08
NC_032019.1_4331699	3,04E+08	NW_017723711.1_29934	8,90E+08
NC_032019.1_4334043	4,29E+08	NW_017723711.1_30009	9,63E+08
NC_032019.1_4374216	4,29E+08	NW_017725414.1_16551	3,71E+06
NC_032019.1_4376297	4,29E+08	NW_017725541.1_54625	1,75E+08
NC_032019.1_4377398	4,29E+08	NW_017725541.1_54879	1,45E+08
NC_032019.1_4377473	4,29E+08	NW_017725541.1_55680	4,50E+08
NC_032019.1_4378051	4,29E+08	NW_017725591.1_2684	7,25E+08
NC_032019.1_4378562	4,29E+08	NW_017725720.1_79622	7,25E+08
NC_032019.1_4379329	4,29E+08	NW_017725720.1_79720	7,25E+08
NC_032019.1_4380461	4,89E+08	NW_017725720.1_80993	7,25E+08
NC_032019.1_4380690	4,29E+08	NW_017725720.1_82983	7,25E+08
NC_032019.1_4381127	4,29E+08	NW_017725927.1_17314	2,16E+06
NC_032019.1_4381727	4,29E+08	NW_017726866.1_98887	9,32E+08
NC_032019.1_4382095	4,29E+08	NW_017726866.1_108814	1,62E+08
NC_032019.1_4382151	3,05E+08	NW_017726866.1_111138	1,62E+08
NC_032019.1_4618005	3,38E+07	NW_017727752.1_254615	1,74E+09
NC_032019.1_4618260	3,38E+07	NW_017727831.1_12535	2,32E+08
NC_032019.1_4620049	3,38E+07	NW_017727831.1_13422	2,32E+08
NC_032019.1_4620210	3,38E+07	NW_017727831.1_17505	7,25E+08
NC_032019.1_4620396	3,38E+07	NW_017727831.1_17817	7,25E+08
NC_032019.1_6351809	1,22E+09	NW_017727843.1_190728	7,25E+08
NC_032019.1_7252969	1,39E+08	NW_017727843.1_191152	7,31E+07
NC_032019.1_7253966	5,37E+08	NW_017727843.1_191596	7,25E+08
NC_032019.1_7254591	6,04E+08	NW_017727843.1_193100	1,07E+08
NC_032019.1_8156012	4,45E+07	NW_017728885.1_488952	1,25E+08
NC_032019.1_8156146	1,10E+07	NW_017728885.1_501231	8,25E+08
NC_032019.1_8161536	1,10E+07	-	-

Table S6. SNPs identified as signatures of selection between the CFL and EFL lines in RIV population of *Lupinus angustifolius* after being selected by both F_{ST} and AFDs analyses (p < 0.001). Information on populations and treatments can be found in (Sacristán-Bajo et al., 2023, Table 1, Figures 1 and 2).

SNP	p-value
NC_032010.1_1600034	2,07E+09
NC_032010.1_1659647	1,20E+09
NC_032010.1_1688261	4,01E+08
NC_032010.1_1690216	2,41E+09
NC_032014.1_5880542	4,55E+09
NC_032015.1_1719363	2,11E+09
NC_032015.1_1719376	1,23E+09
NC_032015.1_1719391	4,55E+09
NC_032015.1_4720102	2,41E+09
NC_032015.1_9481856	6,76E+08
NC_032016.1_9605982	2,11E+09
NC_032017.1_20299850	2,11E+09
NC_032018.1_708422	4,55E+09

NC_032018.1_13748462	4,55E+09
NC_032018.1_14346250	6,76E+08
NC_032018.1_14346260	6,76E+08
NC_032019.1_34061875	2,41E+09
NC_032021.1_577636	4,55E+09
NC_032022.1_964984	1,23E+09
NC_032022.1_1565613	6,76E+08
NC_032022.1_15645423	4,55E+09
NC_032025.1_74141	2,41E+09
NC_032025.1_1221183	2,41E+09
NC_032025.1_1222246	8,83E+07
NC_032025.1_2328680	1,20E+09
NC_032025.1_10591289	4,55E+09
NC_032027.1_2135985	4,01E+08
NC_032027.1_16546489	2,41E+09
NC_032027.1_17259201	2,41E+09
NC_032027.1_17259244	2,41E+09
NC_032027.1_17259338	2,41E+09
NC_032028.1_1359874	4,55E+09
NC_032028.1_18358070	4,55E+09
NW_017720548.1_4507	2,56E+09
NW_017720548.1_4542	2,56E+09
NW_017725414.1_16519	4,55E+09
NW_017728885.1_466465	2,41E+09
NW_017728885.1_475655	2,07E+09

Table S7. Significant SNP (p < 0.001) identified in the genome-wide association study (GWAS) related to the traits flowering onset, seed number, seed weight, height, biomass, shoot growth, SLA and LDMC in the four populations of *Lupinus angustifolius* studied.

Trait	SNP	p-value
Flowering onset	SNC_032023.1_1418306	4,70E+10
Flowering onset	SNC_032023.1_2095333	4,70E+10
Flowering onset	SNC_032009.1_20476789	7,99E+10
Flowering onset	SNC_032011.1_4073570	8,87E+10
Flowering onset	SNC_032011.1_4459654	8,87E+10
Flowering onset	SNC_032024.1_20743522	9,83E+10
Flowering onset	SNC_032024.1_20743995	9,83E+10
Flowering onset	SNC_032024.1_20744064	9,83E+10
Flowering onset	SNC_032016.1_24686878	1,56E+11
Flowering onset	SNC_032016.1_24687025	1,56E+11
Flowering onset	SNC_032013.1_24313465	4,62E+11
Flowering onset	SNC_032023.1_1778383	4,63E+11
Flowering onset	SNC_032024.1_4526705	6,11E+11
Flowering onset	SNC_032021.1_16043477	6,29E+11
Flowering onset	SNC_032021.1_16042439	6,29E+11
Flowering onset	SNW_017725541.1_55329	6,74E+11
Flowering onset	SNW_017725541.1_54232	6,74E+11
Flowering onset	SNW_017725541.1_56083	6,74E+11
Flowering onset	SNC_032021.1_16042591	6.,94E+11

Flowering onset	SNC_032021.1_16042587	7,12E+11
Flowering onset	SNC_032021.1_2401151	7,57E+11
Flowering onset	SNC_032010.1_5274244	8,85E+11
Flowering onset	SNC_032011.1_4071280	8,87E+11
Flowering onset	SNC_032011.1_4073686	8,87E+11
Flowering onset	SNC_032021.1_16168355	9,19E+11
Flowering onset	SNC_032021.1_16168369	9,19E+11
Flowering onset	SNC_032024.1_20743842	9,82E+11
Flowering onset	SNC_032016.1_24220039	4,61E+12
Flowering onset	SNC_032023.1_2096423	4,62E+12
Flowering onset	SNC_032023.1_2096446	4,62E+12
Flowering onset	SNC_032013.1_23446522	4,67E+12
Flowering onset	SNC_032013.1_24313447	4,67E+12
Flowering onset	SNC_032013.1_24660400	4,67E+12
Flowering onset	SNC_032013.1_24669375	4,67E+12
Flowering onset	SNC_032017.1_20754713	4,67E+12
Flowering onset	SNC_032017.1_20754714	4,67E+12
Flowering onset	SNC_032022.1_1571296	4,67E+12
Flowering onset	SNC_032023.1_924128	4,67E+12
Flowering onset	SNC_032023.1_924218	4,67E+12
Flowering onset	SNC_032023.1_2096463	4,67E+12
Flowering onset	SNC_032023.1_2096653	4,67E+12
Flowering onset	SNC_032023.1_2097883	4,67E+12
Seed number	SNC_032022.1_14525650	5,87E+09
Seed number	SNC_032013.1_3403142	1,21E+10
Seed number	SNC_032013.1_3403768	1,21E+10
Seed number	SNC_032017.1_21526052	4,39E+10
Seed number	SNC_032009.1_25275208	3,22E+11
Seed number	SNC_032009.1_25275211	3,22E+11
Seed number	SNC_032009.1_25275214	3,22E+11
Seed number	SNC_032017.1_21525957	3,30E+11
Seed number	SNC_032017.1_21525670	3,31E+11
Seed number	SNC_032026.1_218275	3,48E+11
Seed number	SNC_032011.1_12586404	3,52E+11
Seed number	SNC_032011.1_12586407	3,52E+11
Seed number	SNC_032011.1_12586409	3,52E+11
Seed number	SNC_032011.1_12586411	3,52E+11
Seed number	SNC_032011.1_12586414	3,52E+11
Seed number	SNC_032024.1_15767875	3,88E+11
Seed number	SNC_032017.1_21526181	6,46E+11
Seed number	SNC_032024.1_15767882	6,65E+11
Seed number	SNC_032019.1_10574110	7,48E+11
Seed number	SNC_032014.1_5433948	7,84E+11
Seed number	SNC_032019.1_10574083	8,28E+11
Seed number	SNC_032016.1_2153487	1,14E+12
Seed number	SNC_032013.1_3403161	1,21E+12
Seed number	SNW_017717327.1_33273	1,87E+12
Seed number	SNW_017717827.1_2640	2,03E+12
Seed number	SNC_032010.1_2465349	2,09E+12
Seed number	SNC_032012.1_22624088	2,09E+12
Seed number	SNC_032012.1_22624089	2,09E+12
Seed number	SNC_032019.1_22634121	2,09E+12
Seed number	SNC_032021.1_1663733	2,09E+12
Seed number	SNC_032021.1_4490171	2,09E+12
Seed number	SNC_032024.1_16607911	2,09E+12

Seed number	SNC_032019.1_4380532	2,10E+12
Seed number	SNC_032017.1_9425961	2,10E+12
Seed number	SNC_032017.1_9425959	2,11E+12
Seed number	SNC_032017.1_9425962	2,11E+12
Seed number	SNC_032011.1_2873929	2,17E+12
Seed number	SNC_032022.1_14525671	2,18E+12
Seed number	SNC_032017.1_21525726	3,30E+12
Seed weight	SNC_032014.1_32384330	2,35E+11
Seed weight	SNC_032017.1_19161676	2,85E+11
Seed weight	SNC_032020.1_316575	5,26E+11
Seed weight	SNC_032015.1_19229978	5,63E+11
Seed weight	SNC_032015.1_19229979	5,63E+11
Seed weight	SNC_032009.1_19131816	5,86E+11
Seed weight	SNC_032014.1_32381526	5,88E+11
Seed weight	SNC_032024.1_1867115	7,81E+11
Seed weight	SNC_032024.1_1867169	7,81E+11
Seed weight	SNC_032009.1_2907377	7,98E+11
Seed weight	SNC_032009.1_2907005	7,98E+11
Seed weight	SNC_032009.1_35254194	8,28E+11
Seed weight	SNC_032009.1_35259347	8,28E+11
Seed weight	SNC_032009.1_35261823	8,28E+11
Seed weight	SNC_032009.1_35260731	8,28E+11
Seed weight	SNC_032009.1_35262814	8,28E+11
Height	SNC_032025.1_4688793	7,98E+10
Height	SNC_032023.1_149183	6,07E+11
Height	SNC_032023.1_148995	6,07E+11
Height	SNC_032023.1_149063	6,07E+11
Height	SNC_032023.1_149076	6,07E+11
Height	SNC_032023.1_149654	6,07E+11
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Height	SNC_032025.1_4688783	8,01E+11
Height	SNC_032025.1_4688786	8,01E+11
Height	SNC_032013.1_20836897	8,17E+11
Height	SNC_032013.1_20837146	8,17E+11
Height	SNC_032013.1_20837236	8,17E+11
Height	SNC_032013.1_20837242	8,17E+11
Height	SNC_032013.1_20837261	8,17E+11
Height	SNC_032013.1_20837466	8,17E+11
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Height	SNC_032013.1_20838011	8,17E+11
Height	SNC_032013.1_22533687	8,17E+11
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Height	SNC_032015.1_4338234	9,13E+11
Height	SNC_032015.1_4338236	9,13E+11
Height	SNC_032015.1_4338250	9,13E+11
Height	SNC_032015.1_4338255	9,13E+11
Height	SNC_032015.1_4338296	9,52E+11
Height	SNC_032015.1_4338783	9,95E+11
Height	SNC_032015.1_4338943	9,95E+11
Biomass	SNC_032022.1_889068	7,11E+10

Biomass	SNC_032022.1_892717	7,11E+10
Biomass	SNC_032022.1_892921	7,11E+10
Biomass	SNC_032022.1_895553	7,11E+10
Biomass	SNC_032022.1_897474	7,11E+10
Biomass	SNC_032022.1_1556893	7,12E+10
Biomass	SNC_032026.1_218275	1,57E+11
Biomass	SNC_032015.1_1344234	1,95E+11
Biomass	SNC_032009.1_25275208	2,62E+11
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Biomass	SNC_032009.1_25275214	2,62E+11
Biomass	SNC_032022.1_11061008	4,60E+11
Biomass	SNC_032015.1_1345936	4,97E+11
Biomass	SNC_032015.1_1346121	4,97E+11
Biomass	SNC_032015.1_1346569	4,97E+11
Biomass	SNC_032015.1_1346970	4,97E+11
Biomass	SNC_032022.1_802757	5,05E+11
Biomass	SNC_032022.1_802736	5,07E+11
Biomass	SNC_032022.1_802749	5,07E+11
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Biomass	SNC_032022.1_893526	7,11E+11
Biomass	SNC_032022.1_893543	7,11E+11
Biomass	SNC_032022.1_893588	7,11E+11
Biomass	SNC_032022.1_897420	7,11E+11
Biomass	SNC_032022.1_897426	7,11E+11
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Biomass	SNC_032013.1_3403768	9,63E+11
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Biomass	SNC_032011.1_12586407	1,58E+12
Biomass	SNC_032011.1_12586409	1,58E+12
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Biomass	SNC_032011.1_12586414	1,58E+12
Biomass	SNC_032022.1_803208	1,72E+12
Biomass	SNC_032022.1_802782	3,58E+12
Shoot growth	SNC_032023.1_1416779	9,78E+10
Shoot growth	SNC_032014.1_362075	2,39E+11
Shoot growth	SNC_032014.1_362104	2,39E+11
Shoot growth	SNC_032014.1_362223	2,39E+11
Shoot growth	SNC_032014.1_363358	2,39E+11
Shoot growth	SNC_032009.1_23207296	2,70E+11
Shoot growth	SNC_032009.1_23207871	2,70E+11
Shoot growth	SNC_032009.1_23208201	2,70E+11
Shoot growth	SNC_032009.1_23208381	2,70E+11
Shoot growth	SNC_032009.1_23208973	2,70E+11
Shoot growth	SNC_032009.1_26286336	4,29E+11

Shoot growth	SNC_032023.1_1686384	7,28E+11
Shoot growth	SNC_032018.1_13749811	8,00E+11
Shoot growth	SNC_032018.1_13749914	8,00E+11
Shoot growth	SNC_032018.1_13750062	8,00E+11
Shoot growth	SNC_032018.1_13750345	8,00E+11
Shoot growth	SNC_032023.1_1416768	8,95E+11
Shoot growth	SNC_032023.1_1416769	8,95E+11
Shoot growth	SNC_032023.1_1416790	8,95E+11
Shoot growth	SNC_032023.1_1416791	8,95E+11
Shoot growth	SNC_032009.1_23209611	1,54E+12
Shoot growth	SNC_032014.1_361825	2,39E+12
Shoot growth	SNC_032014.1_362071	2,39E+12
Shoot growth	SNC_032014.1_362176	2,39E+12
Shoot growth	SNC_032014.1_367868	2,39E+12
Shoot growth	SNC_032014.1_369459	2,39E+12
Shoot growth	SNC_032009.1_23207126	2,70E+12
Shoot growth	SNC_032009.1_23209275	2,70E+12
SLA	SNC_032017.1_3568920	4,63E+12
LDMC	SNC_032016.1_22733733	5,67E+10
LDMC	SNC_032018.1_708032	9,71E+10
LDMC	SNC_032018.1_708240	9,71E+10
LDMC	SNW_017722654.1_3517	3,30E+11
LDMC	SNC_032016.1_6413474	3,39E+11
LDMC	SNC_032016.1_22735388	1,76E+12
LDMC	SNC_032016.1_22736023	1,76E+12
LDMC	SNC_032011.1_6808829	3,35E+12
LDMC	SNC_032025.1_20062444	3,50E+12

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Chapter 3: Effects of assisted gene flow on the flowering onset of the annual legume *Lupinus angustifolius* L.: from phenotype to genotype.

Manuscript in preparation

Abstract

Current climate change may impede species to evolutionary adapt quickly enough to environmental changes, threatening their survival. In keystone populations, to overcome this challenge, it may be necessary to consider the introduction of adaptive alleles through assisted gene flow. Considering that flowering time is a crucial trait in plant response to global warming, the objective of our study was to test the potential benefits and limitations of assisted gene flow for enhancing the evolutionary potential of *Lupinus angustifolius* L. (Fabaceae) populations through the advancement of flowering time. Previous studies have shown that southern populations of *L. angustifolius* flower earlier than northern populations. We collected seeds from four populations in Spain from two different latitudes, and we established them in a common garden environment. To advance the flowering onset of northern populations, we used pollen from southern individuals to pollinate plants from northern populations, creating an F1 gene flow line. In the following season, we allowed the plants belonging to the F1 gene flow line to self-pollinate to create an F2 self-pollination line. In parallel, we created a backcross line by pollinating individuals from the F1 gene flow line with pollen from northern plants. We also included a control line resulting from a random selection of individuals in each population in the first generation and their descendants from self-crosses in the second generation. We measured flowering onset, reproductive success and other plant traits in all individuals resulting from these lines. To characterize the effects of the assisted gene flow line at the genomic level, we carried out a gene capture analysis to sequence genes related to reproduction, growth, abiotic stress, nitrogen metabolism, and alkaloids in individuals from the F1 gene flow line and the control line in the first generation. All gene flow-derived lines flowered significantly earlier than the control line. Furthermore, plants from the F1 gene flow line produced heavier seeds and had a lower shoot growth than the

control line. Genomic analyses identified 36 SNPs outliers that were associated to flowering onset, seed weight, and shoot growth. These results highlight that assisted gene flow can increase the evolutionary potential of populations by modifying the values of a specific trait. However, the modification of one trait may affect the values of other plant traits. The intrinsic characteristics of the populations will have a fundamental effect on the results of assisted gene flow. Therefore, the selection of the donor population is a critical step to consider in assisted gene flow.

Keywords

Adaptive potential, assisted gene flow, climate change, evolutionary changes, experimental evolution, genomics

Introduction

The current climate change, accelerated by human activity, is compromising the ability of organisms to survive (IPCC, 2022). Species can react to climate change through different kinds of responses, such as migration to more favorable areas, phenotypic plasticity, or evolutionary adaptation (Jump & Peñuelas, 2005; Parmesan & Yohe, 2003). Whereas migration ability can be limited for some organisms, evolutionary adaptation depends on the genetic variation, demography, and historical processes of populations (Sheth & Angert, 2016). Since, in some cases, the environment is changing faster than the rate at which species can adapt or migrate, a mismatch may occur between climate and the adaptations of organisms (Aitken & Whitlock, 2013). Therefore, it is necessary to propose strategies that can reduce the risk of population extinction in the context of climate change by moving populations to more suitable areas or by improving their adaptive potential.

Assisted migration is one of the strategies that has been proposed in this line (Grady et al., 2011; Loss et al., 2011). It consists of the physical translocation of populations to habitats, outside the natural range of the species, that are expected to be more favorable for the species according to future climate predictions (Aitken & Whitlock, 2013; Vitt et al., 2010). However, assisted migration proposals have been widely debated due to the biological risks they can entail. It has been argued that this kind of intervention can cause major impacts on biotic communities, alter nutrient cycles, or disrupt ecological processes such as pollination or seed dispersal (Mack et al., 2000; Traveset & Richardson, 2006). Hybridization with other species could also occur, in addition to the possibility of the species becoming invasive or the involuntary transfer of pathogens (Loss et al., 2011; Williams & Dumroese, 2013). Furthermore, the impacts of these introductions may not be appreciable in the short term and may vary greatly over space and time (Ricciardi & Simberloff, 2009).

Assisted gene flow is an alternative proposal that could help to solve some of these issues (Aitken & Whitlock, 2013; Prieto-Benítez et al., 2021; Wadgymar et al., 2015). It is defined as the movement of gametes or individuals between existing populations to facilitate adaptation (Aitken & Whitlock, 2013; Whiteley et al., 2015). It is known that gene flow between populations can improve genetic variability, allowing them to develop adaptive responses to new scenarios such as climate change (Grummer et al., 2022). To facilitate gene flow, the creation of corridors has also been proposed (Beier, 2012; Heller & Zavaleta, 2009). However, these connections are not always possible due, for example, to habitat fragmentation (Heller & Zavaleta, 2009) or natural barriers. In addition, natural gene flow is limited for some species, such as those plants that are strictly autogamous. There are some studies that have explored the possible benefits that gene flow can have on genetically impoverished populations (Morente-López et al., 2021; Prieto-Benítez et

al., 2021; Sexton et al., 2011). However, the benefit of using directed gene flow to improve the adaptive potential of populations in a context of climate change has been little studied. In contrast to assisted migration, assisted gene flow only implies the movement of genes or individuals between natural populations of the species already present in the ecosystem, with less risks from an ecological point of view (Aitken & Whitlock, 2013). An advantage of assisted gene flow compared to natural gene flow resides in its wide geographical potential, as the movement of gametes can take place over very long distances. Furthermore, when done in a directional manner, the alleles that are introduced are more likely to be pre-adapted to actual or potential environmental circumstances, while natural gene flow can occur in any direction and lead to possible maladaptation (Aitken & Whitlock, 2013). However, assisted gene flow can also involve some genetic risks associated with outbreeding depression, resulting in a loss of local adaptation as some alleles fail to adapt to the new conditions, leading to fitness loss (Aitken & Whitlock, 2013; Byrne et al., 2011; Edmands, 2007; Frankham et al., 2011; Grummer et al., 2022). Then, deepening our understanding of the risks and benefits of using assisted gene flow can help us to understand and improve the evolutionary capacity of populations (Frankham et al., 2017).

Within the same species, many traits of great ecological importance can vary along environmental gradients (De Frenne et al., 2013; Milla et al., 2009). For example, phenological traits and climate conditions are usually closely related, so organisms are constantly trying to match their phenologies with the most favourable environmental circumstances (Pau et al., 2011). Phenological shifts are therefore among the most prominent impacts of climate change (Bradshaw & Holzapfel, 2009; Parmesan & Yohe, 2003), and flowering onset is a critical aspect in the adaptation of plants to climate change (Franks & Hoffmann, 2012). It has been shown that populations present differences in

their flowering onset depending on the latitude, with lower latitude populations flowering earlier in most cases (Lévesque et al., 2005). Several studies have confirmed that flowering onset is a genetically controlled trait with high heritability (Riihimäki & Savolainen, 2004; Franks et al., 2007; Méndez-Vigo et al., 2013). Moreover, it has been sown that flowering onset is a polygenic trait in which a large number of genes are involved and has a complex regulation (Blümel et al., 2015; Fagny & Austerlitz, 2021). It is important to consider that the timing of flowering onset is often correlated with other traits of great importance for plant survival and adaptation, which may constrain its evolution (Etterson & Shaw, 2001; Sacristán-Bajo et al., 2023; Walsh & Blows, 2009). Therefore, a better understanding of the genomic basis of flowering onset and the possible genetic constraints due to correlations between traits may help to design more accurately potential future assisted gene flow actions.

Given the scarce evidence available on this subject, the main objective of this study was to test the potential of assisted gene flow to advance flowering onset in plant populations, assessing the risks and benefits that it can entail. In this context, we set up an experimental study with *Lupinus angustifolius* L. (Fabaceae), that involved manual crosses between four populations from two climatically different areas of the Iberian Peninsula, and the phenotypic and genotypic characterization of the progeny in a common garden environment. In previous studies carried out with these same populations (Sacristán-Bajo et al., 2023, Chapter 1), it has been observed that southernmost populations (which have warmer climate patterns) flower earlier than northernmost populations; therefore, we expect that the gene flow from southern populations to northern populations will produce an advance in the onset of flowering in the offspring with respect to the average individuals of the northern populations. Since the southern populations used as sources for the gene flow are genetically distinct in many traits from the recipient populations

(Sacristan-Bajo et al., 2023, Chapter 1 and Chapter 2), we hypothesize that the introduction of new alleles originating from the south in populations from the north will also generate changes in other traits. We expect that the first generation of gene flow will produce hybrids with intermediate phenotypes. The second generation, produced by self-fertilization of the hybrids, will produce segregation of the traits, giving rise to very different phenotypes. On the other hand, the backcross line, obtained by the subsequent pollination of the hybrids with individuals from the northern populations, may also generate segregation of traits, although they are expected to be more similar to those originally found in the northern populations. We also hypothesize that it will be possible to find a genomic signature associated to the expected changes in the phenotypes. To test these hypotheses, we recorded the timing of flowering onset and other plant traits (plant height, inflorescence length, fruit number, seed per fruit, seed weight, specific leaflet area or SLA and leaflet dry matter content or LDMC), and we sequenced a set of genes related to the flowering process and abiotic stress. We, then, compared the results of gene flow lines against those of the control line to answer the following questions: i) Is it possible to advance the flowering of northern populations through assisted gene flow from the southern populations? ii) If so, are there other traits that are also modified with the application of the gene flow treatment? iii) What is the genomic signature of the phenotypic changes brought about by the gene flow lines?

Materials and Methods

Study species and source populations

The blue lupine (*Lupinus angustifolius* L.) is an annual legume distributed throughout the Mediterranean basin, which has been domesticated as a crop and is grown in many places around the world (Castroviejo & Pascual, 1993). This plant can reach up to more than 100 cm in height and has characteristic palmate leaves divided in 5-9 leaflets. Its flowers

are hermaphrodite, with an inflorescence that can have up to 30 flowers. The fruit is a dehiscent legume with 3-7 seeds (Clements et al., 2005). The species mainly self-pollinates before its petals open (Wolko et al., 2011), with outcrossing estimates below 2 % (Dracup & Thomson, 2000). Depending on latitude and environmental conditions, flowering occurs between March and August (Castroviejo & Pascual, 1993), since its flowering onset is influenced by photoperiod and temperature (Rahman & Gladstones, 1974).

For our study, we selected four populations distributed by pairs in two regions of contrasting climatic conditions, the northern one located in Salamanca (Central Spain), and the southern one located in Badajoz (South Spain) (Sacristán-Bajo et al, 2023, Chapter 1, Figure 1, Table 1). The distance between the two regions is around 300 km, and the populations within each region are less than 20 km apart. Both regions have similar annual precipitation, but the southern one has higher mean, minimum and maximum temperatures and, consequently, experiences higher water deficits. We collected seeds in each population from at least 98 genotypes (mother plants) located at least one meter apart from each other.

Common garden and gene flow experiment

The common garden experiment was carried out in the CULTIVE facility (https://urjc-cultive.webnode.es/) at Rey Juan Carlos University (Móstoles, Madrid). In November 2016, 12 seeds from each of 22 randomly selected maternal genotypes per population were scarified to ensure germination and sown, in groups of three, in four 6 L pots, following the same protocol described in Sacristán-Bajo et al. (2023) (Chapter 1). Inside the greenhouse, the temperature varied between 1 to 25 degrees Celsius, and plants only received natural light. In spring 2017, the pots were transferred outside of the greenhouse to the CULTIVE experimental field and were distributed in a randomized block design

where the plants from the different populations were evenly represented in each block. The substrate in the pots was kept at field capacity with a system of drip irrigation. The temperature conditions at this site are intermediate between those found at the northern and southern regions of origin (Table 1). Before the plants flowered, their inflorescences in their main shoot were bagged to obtain seeds derived from self-pollination. This first growing season was only used to eliminate maternal effects.

In November 2017, seeds separately collected from each individual were sown in the same way and with the same conditions as described above, and the resulting plants were transferred to the CULTIVE experimental field in February 2018. During the 2018 flowering season, manual between-population crosses were carried out to create an 'F1 gene flow line' (hereafter GFL, Figure 1). Plants from the northern region were pollinated using pollen from plants from the southern region, matching the RIV population with the PIC population and the GAR population with the FRO population. All possible crosses between these two pairs of populations (considered as replicates) were performed constrained by the need of having overlapped flowering periods between their individuals. The procedure to carry out manual crosses was the same as that described in the Supplementary Material of Sacristán-Bajo et al. (2023) (Chapter 1), based on the emasculation of individuals of the northern region and their subsequent pollination with pollen from individuals from the southern region. The success of the manual crossings performed was low (around 7 %). As a result, 21 mother plants from both populations were successfully crossed to obtain eight different genotypic crosses in FRO population and 13 different genotypic crosses in PIC population.

Seeds produced were separately collected for each mother plant. In parallel, for each northern population, the genotypes were naturally self-crossed, and their seeds were separately collected to generate the 'control line' (hereafter CFL, Figure 1). In the 2018-

2019 season, seeds were sown, and seedlings were cultured and transferred outdoors in the same way as described above, containing, for each population, individuals from the CFL (64 and 65 from FRO and PIC, respectively) and GFL (33 and 43 from FRO and PIC, respectively) treatments. In the 2019 flowering season, the CFL individuals of the corresponding northern populations were manually pollinated using GFL individuals as pollen donors, creating a 'backcross line' (hereafter BCL, Figure 1). Additionally, an 'F2 self-pollination line' (hereafter SPL, Figure 1) from the GFL was generated by self-pollination. The seeds of these lines, as well as those derived from the self-pollination of CFL, were again separately collected for each mother plant. In the 2019-2020 season, the seeds from these lines were sown and the resulting seedlings were grown and transferred outdoors as indicated above. To summarize, a diagram of the complete process is shown in Figure 1. Results of the backcross line (BCL) are only shown for FRO population, since it was not possible to obtain seeds through these manual crosses for PIC population.
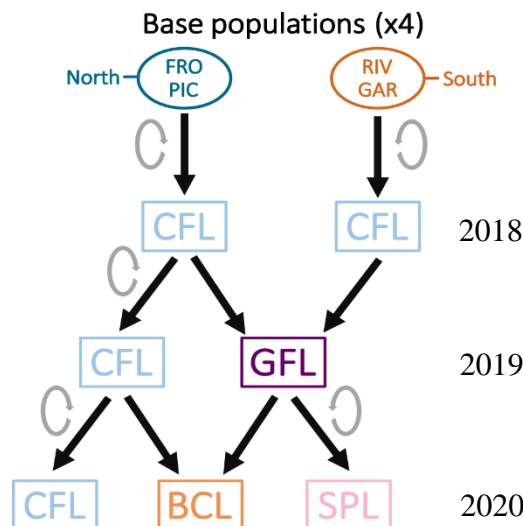

Figure 1. Flowchart describing the different lines performed with individuals of four populations of *Lupinus angustifolius* through time. CFL: control line; GFL: F1 gene flow line; BCL: F2 backcross line; SPL: F2 self-pollination line. Grey circled arrows indicate that the individuals of that line were self-crossed. Black arrows denote the transmission of gametes for the next generation. The years indicated on the left correspond to the flowering season in which adult plants of the indicated lines had been grown and the crosses performed in that season are represented below with the arrows.

Traits measurement

The day of flowering initiation was recorded for each plant as the day when the first purple flower of the main inflorescence was clearly visible. Thus, we defined the flowering onset variable as the number of days between the date of sowing and the flowering start date. We estimated the number of fruits per plant based on the total number of floral scars at the end of the season. The average number of seeds per fruit was determined by counting the seeds in 15 different fruits per plant. The number of seeds per plant was calculated by multiplying the number of fruits per plant by the average number of seeds per fruit. The individual weights of 10 random seeds from each plant were used to calculate the mean seed weight. The mean seed weight and the number of seeds per plant were used as proxies for determining plant fitness.

We also calculated the height of the plants (cm) at the flowering peak (when most plants had bloomed) by measuring the distance from the ground level to the base of the main inflorescence. At the start of flowering and at the end of the culture cycle, we measured the length of the plant (cm) from its base to the first flower. The difference between these two values was used to estimate the shoot growth (cm) of each individual. We also measured the aboveground biomass (g) of each plant at the end of the culture cycle. The central leaflet from eight fully developed leaves belonging to the lateral branches was gathered to determine the specific leaflet area (SLA) and dry matter content (LDMC). The fresh leaflets were weighed immediately in a Kern ABJ 120-4M analytical balance (Kern & Sohn GmbH, Albstadt, Germany). The leaflets were then placed in water-soaked filter paper and stored in plastic bags before being refrigerated overnight at 4 °C. We weighed the leaflets again the next day to get the turgid weight and used a foliar scanner Li-3000C (Li-Cor, NE, United States) to measure the area of the leaflets. Finally, the leaflets were dried for at least 72 hours in a 60 °C oven before being weighed again to

determine their dry weight. SLA was calculated by dividing the area of a leaflet by its dry weight (Rosbakh et al., 2015). LDMC was determined by dividing the leaflets dry weight by its saturated weight (Wilson et al., 1999).

The flowering onset was measured for the years 2019 and 2020, but due to the mobility restrictions of the pandemic lockdown, the rest of the traits were only measured for the year 2019.

Phenotypic analyses

We used the R statistical environment version 4.1.1 to conduct all the statistical analyses mentioned below (R Core Team, 2020). We applied linear and generalized linear mixed models (hereafter LMMs and GLMMs) to analyze the effect of the F1 gene flow line as well as the F2 self-pollination line and the backcross line on flowering onset and the rest of the traits, using the CFL as controls. Therefore, for each trait, we included *line* (CFL and GFL for the year 2019, and CFL, SPL and BCL for the year 2020) and *population* (FRO and PIC) as fixed effects, and *genotype* (mother plant) as a random effect. Since the flowering onset variable holds count data, we used a Poisson error distribution (GLMMs). For the rest of the variables, we used a Gaussian error distribution (LMMs). Diagnostic plots were used to visually inspect the model residuals for normality and variance homogeneity. We tested the interaction between the variables *line* and *population*. As the interaction was not significant, we decided not to include it in the models. The *glmer* and *lmer* functions from the *lme4* package version 1.1-27.1 were used to fit the GLMMs and LMMs (Bates et al., 2015). The *Anova* function from the *car* package version 3.0-11 was used to determine the significance of each fixed effect (Fox & Weisberg, 2011). If necessary (as for the flowering onset in 2020), Tukey *post hoc* analysis from the *emmeans* function from the *emmeans* package version 1.6.3 was used to calculate differences between lines (Lenth, 2019). R^2 values were calculated using the

summ function from the *jtools* package version 2.2.0 (Long, 2019). The *corrplot* function version 0.90 from the *corrplot* package was used to plot correlations between flowering onset and the other traits (Wei et al., 2017).

Genomic analyses

- DNA extraction and selection of candidate genes

Leaf material was collected for DNA extraction in 2019 from individuals of CFL and GFL lines that were also phenotyped. Leaves from a total of 60 individuals were collected, 30 of each line (CFL and GFL), and 15 of each population (FRO and PIC) within each line. We used DNeasy Plant minikit (QIAGEN, Valencia, USA) to extract and isolate DNA.

We designed a gene capture experiment, taking the annotated *L. angustifolius* genome from the National Center for Biotechnology Information (NCBI) as starting point (GenBank accession: PRJNA398717). This genome contains all coding sequences (CDs) belonging to the *L. angustifolius* genome. FullLengtherNext software (Lara et al., 2007) and the *L. angustifolius* genome were used to perform a Blast analysis and obtain the biological function (*i.e.*, gene ontology terms) for each sequence in *L. angustifolius*. We selected 73 gene ontology terms related to reproduction, growth, abiotic stress, nitrogen metabolism, and alkaloids. After that, we used Go.db R package to create a list with all the gene ontology terms superior (broader) and inferior (more specific) (*i.e.* Gene Ontology Terms parent and children according to the convention used for describing relationships between GO Terms) of these gene ontology terms. Finally, we filtered the file which contains all coding sequences to obtain the candidate genes of interest, based on the list of gene ontology terms. These sequences were used as probes to carry out the targeted sequencing.

- Sequencing and Single Nucleotide Polymorphism (SNP) calling

The extracted DNA was sent to IGATech (Udine, Italy). The quality of the genomic DNA was checked using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). Libraries for target enrichment of ~3 Mb of *L. angustifolius* genomic material were produced using the Roche Sequencing Solutions' 'SeqCap EZ – HyperPlus' kit (Roche Sequencing Solutions, Pleasanton, CA) with 200 ng/L of input DNA.

After that, base calling and demultiplexing was carried out with Illumina bcl2fastq v2.20. ERNE v1.4.6 (Del Fabbro et al., 2013) and Cutadapt (Martin, 2011) software was used for quality and adapter trimming, BWA-MEM v0.7.17 (Li & Durbin, 2009) for the alignment to the reference genome, and Picard tools (http://broadinstitute.github.io/picard/) to produce on-target alignment statistics and metrics.

SNP calling was performed on the entire sample simultaneously with gatk-4.0 (Depristo et al., 2011). This step allowed the initial identification of ca. 41,419 SNPs. Raw SNP data were filtered using VCFtools v0.1.14 (Danecek et al., 2011), and the *vcffilter* function of VCFLIB (Garrison et al., 2021). Only biallelic SNPs with fewer than 10 % missing data were kept. Indels were also removed from the dataset. SNPs were then filtered following the hard filtering suggested by GATK's user guide (https://gatk.broadinstitute.org/). Hence, SNPs were filtered based on their quality depth (QD > 2), Phred scaled P-value using Fisher's Exact Test to detect strand bias (FS < 60), Symmetric Odds Ratio of 2x2 contingency table to detect strand bias (SOR < 3), Square root of the average of the squares of the mapping qualities (MQ > 40), Z-score from Wilcoxon rank sum test of Alt vs. Ref read mapping qualities (MQRankSum > -12.5), u-based z-approximation from the Rank Sum Test for site position within reads

(ReadPosRankSum > - 8) and depth coverage (DP >10). This stringent filtering reduced the SNP dataset to 34,026 SNPs. Finally, SNPs in high linkage disequilibrium were filtered using r^2 of 0.6 as the cut-off point, which generated a final dataset of 22,802 SNPs.

- Detecting signatures of selection

We applied a sequential strategy to identify highly divergent loci between the CFL and the GFL lines. We first calculated allele frequency differences (AFDs) between the CFL and the GFL at the individual SNP level and selected those SNPs that had experienced an allele frequency change in the same direction in both populations (FRO and PIC). We then selected those SNPs with significant AFDs by applying a Fishers's exact test (Fisher, 1970). Secondly, pairwise F_{ST} values (CFL *vs*. GFL) were calculated for each SNP. Statistical significance of F_{ST} values was tested for each locus by the chi-square test, $x^2 = 2NF_{ST}(k - 1)$, with $(k - 1)(s - 1)$ degrees of freedom, where N is the total sample size, k is the number of alleles per locus, and s is the number of populations (Workman & Niswander', 1970). We only considered that an SNP showed divergent patterns of differentiation when it was selected as an outlier by both F_{ST} analyses and at the same time it showed consistent AFDs in the two pairs of CFL vs. GFL comparisons. Lastly, these highly divergent loci underwent an individual genotype-phenotype validation (Chen et al., 2022). For this purpose, a linear mixed model with random family effects was fitted using the above-mentioned traits as dependent variables, the genotype of each SNP as a three-level explanatory factor (homozygous for the minor allele, homozygous for the major allele and heterozygous), individual as a random factor and a kinship matrix as a random genetic effect to control for kinship effects. This validation allowed us to detect those SNPs with a large effect on the phenotype. F_{ST} values and allele frequencies were calculated using VCFtools v0.1.14. Kinship matrix was calculated using the centered-IBS method implemented in TASSEL v5.2.81 (Bradbury et al., 2007). Linear mixed models

were fitted using the *lmekin* function implemented in *coxme* R package (Therneau, 2020). The visual representation of the location of these SNPs in the chromosomes of *L. angustifolius* was performed using MG2C version 2.1 software (Jiangtao et al., 2015).

Results

Flowering onset

Significant differences in flowering onset were found between the GFL and the CFL lines in 2019 (Figure 2). In 2020 significant differences were also found between SPL and the CFL lines for both populations, and between BCL and CFL for FRO population (Figure 2). In 2019, plants from the GFL flowered an average of 7 days earlier than control plants in FRO population, and an average of 8 days earlier in PIC population ($X^2 = 20.21$, p < 0.001, Df = 1) (Figure 2a, Supplementary Material Table S1, S2 and S3). In 2020 the SPL flowered 6 days earlier than the CFL in both populations, and the BCL flowered 12 days earlier than the CFL in FRO population ($X^2 = 13.00$, p = 0.001, Df = 2) (Figure 2b, Supplementary Material Tables S1 to S3). The values of R^2 indicated that the fixed effects explained 10 % of the variation, and the random effects explained 2.2 % of the variation in the year 2019. In the year 2020, fixed effects explained 25.3 % of the variation, whereas the random effects explained 16.4 % (Supplementary Material Table S2). Posterior mean values, standard errors, and 95 % confidence intervals for each line are shown in Supplementary Material Table S4.
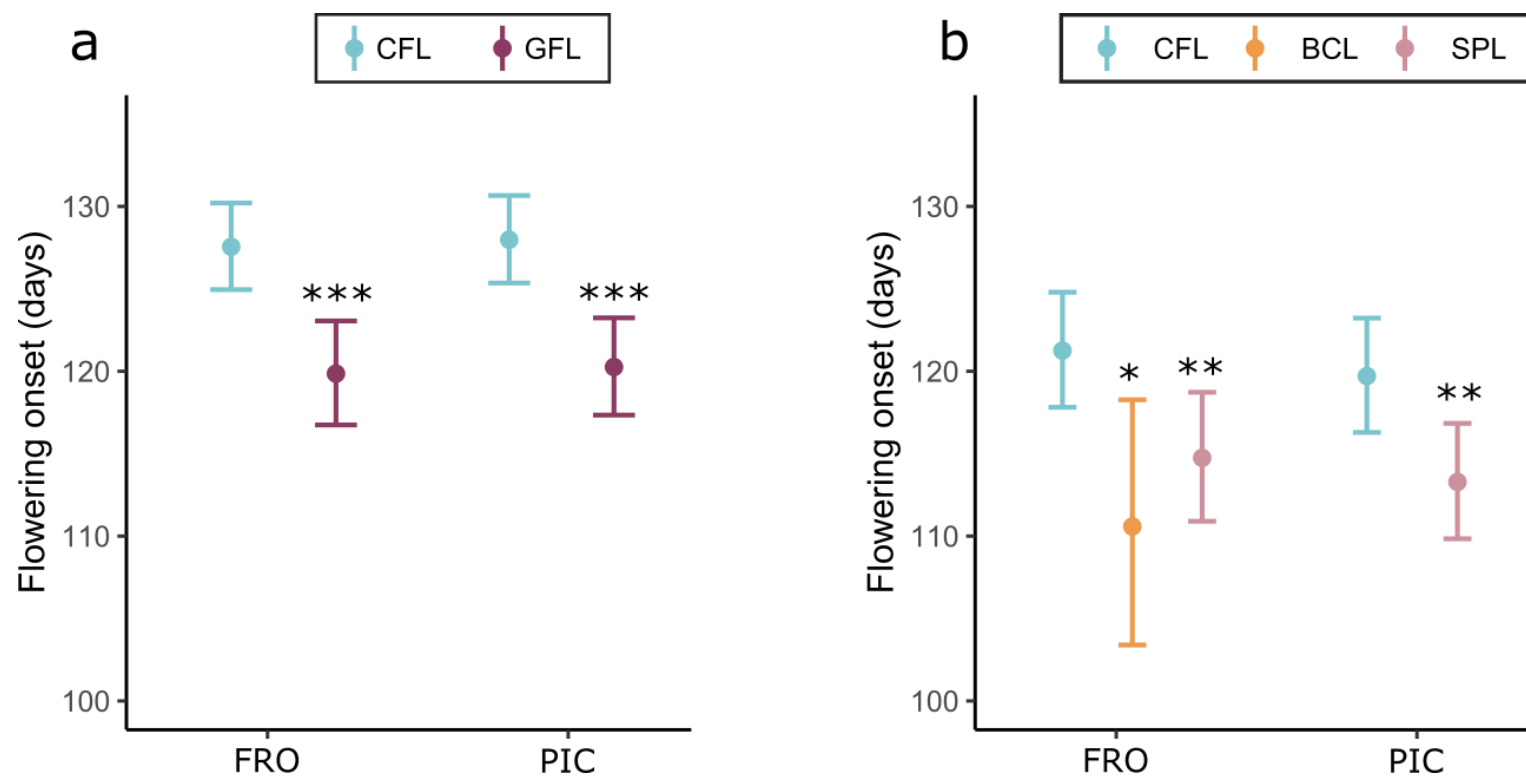
Figure 2. Effects of the F1 gene flow line, backcross line, and self-pollination line on the advancement of flowering onset of *Lupinus angustifolius*. CFL: control line; GFL: F1 gene flow line; BCL: F2 backcross line; SPL: F2 self-pollination line. The values for flowering onset correspond to the estimates obtained from the GLMM model. Dots and bars represent the predicted mean from the GLMM model with a Poisson distribution and 95 % confidence intervals. Significant differences ($p < 0.05$) determined by Tukey test between the generated lines and the control line are marked with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

Reproductive success

No significant differences were found between the GFL and the CFL for seed number (X^2 = 1.38, p = 0.24, Df = 1) (Figure S1a, Supplementary Material Table S3), but significant differences were obtained for seed weight (X^2 = 29.96, p < 0.001, Df = 1). Seeds from GFL were heavier than those from the CFL (Figure 3a, Supplementary Material Tables S1, S2 and S3). In addition, significant differences were found between the two populations both for seed number (X^2 = 4.64, p = 0.03, Df = 1) (Figure S1a, Supplementary Material Table S3) and seed weight (X^2 = 8.20, p = 0.004 Df = 1) (Figure 3a, Supplementary Material Table S3). For seed number, fixed effects explained 12.5 % of the variation, whereas random effects explained 8.6 %. For seed weight, fixed effects explained 38.8 % of the variation, and random effects explained 16.3 % (Supplementary Material Table S2). Posterior mean values, standard errors, and 95 % confidence intervals for each line are shown in Supplementary Material Table S4.

Non reproductive related traits

With respect to height, biomass, SLA and LDMC, no significant differences were found between the control line and any of the established lines (Supplementary Material Table S2, Figure S1). Significant differences were only found between the GFL and the CFL for shoot growth (X^2 = 4.11, p = 0.04, Df = 1). Plants from the GFL had lower shoot growth than those from the CFL (Figure 3b, Supplementary Material Table S1, S2 and S3). Depending on the studied traits, R^2 values indicated that fixed effects explained between 0.3 % and 6.1 % of the variation, and random effects, between 0 % and 46.8 % (Supplementary Material Table S2). Posterior mean values, standard errors, and 95 % confidence intervals for each line are shown in Supplementary Material Table S4.
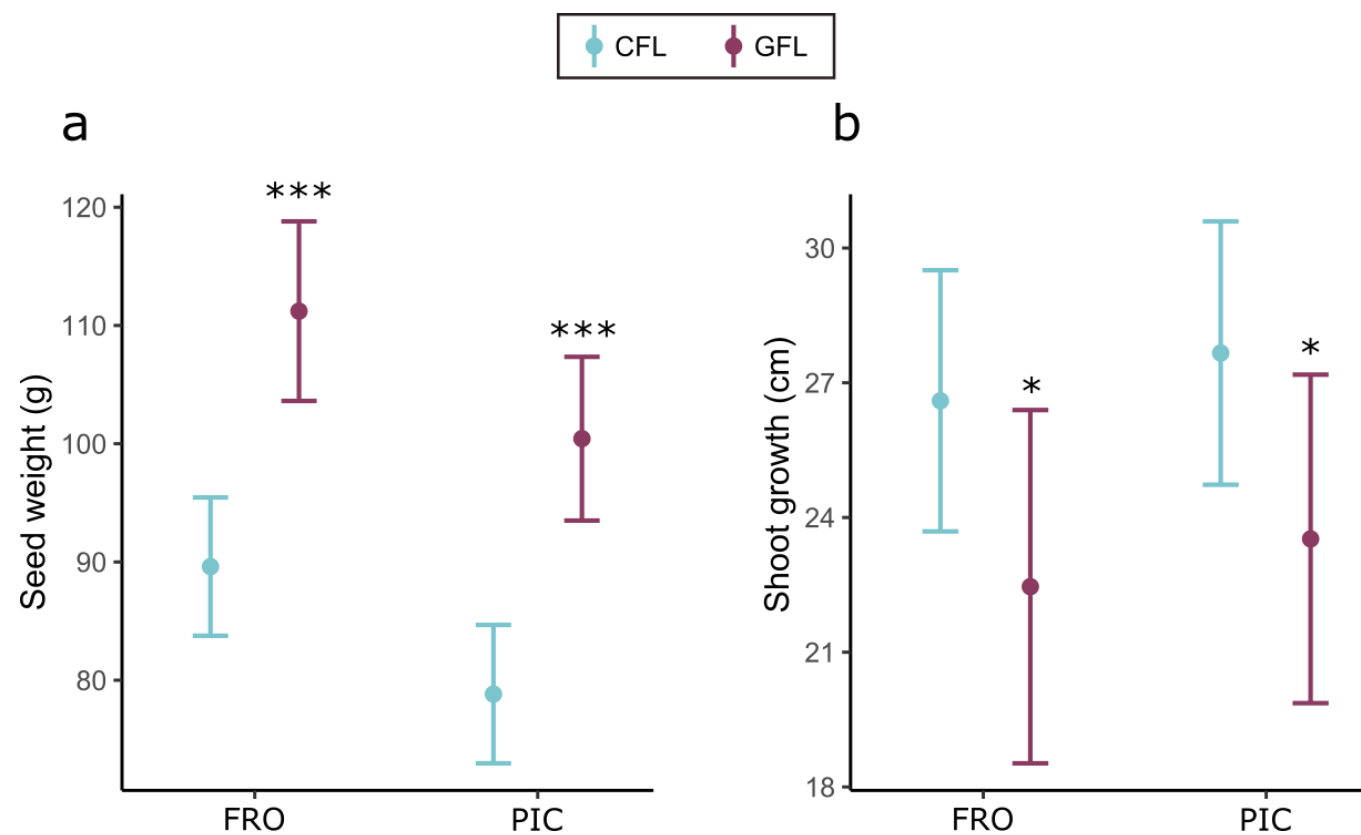
Figure 3. Effect of the gene flow line on the seed weight and shoot growth of *Lupinus angustifolius*. CFL: control line; GFL: F1 gene flow line. Dots and bars represent the predicted mean from the LMM model with a Gaussian distribution and 95 % confidence intervals. Significant differences (p < 0.05) determined by Tukey test between the gene flow line and the control line are marked with asterisks (*p < 0.05; **p < 0.01; ***p < 0.001)

Flowering onset correlations

Different correlations were found between flowering onset and other plant traits for CFL and year 2019 (Supplementary Material Figure S2). It is noteworthy that the correlations between traits varied depending on the population. For the FRO population, plants that flowered earlier showed an increase in their height ($r = -0.33$), biomass ($r = -0.39$), and seed weight ($r = -0.41$), and a decrease in their shoot growth ($r = 0.62$) (Supplementary Material Figure S2a). For the PIC population, plants that flowered earlier showed an increase in seed number ($r = -0.35$) and seed weight ($r = -0.57$), and a reduction in height ($r = 0.33$) and shoot growth ($r = 0.92$) (Supplementary Material Figure S2b).

Loci under selection

We identified 36 SNPs that revealed divergent patterns of differentiation because they were identified as outliers both by F_{ST} analyses and exhibited consistent AFDs in the two groups of CFL versus GFL comparisons (Supplementary Material Table S5). In addition, these 36 SNPs had a significant effect on flowering onset, seed weight, and shoot growth (Supplementary Material Figures S3, S4 and S5) and a great change in the allele frequencies was observed between the CFL and the GFL (Supplementary Material Figure S6). These SNPs were distributed on 11 of the 20 chromosomes of *Lupinus angustifolius* (Figure 4). The functional annotation revealed that the loci to which SNPs identified as outliers are related to different biological processes. Six of them were related to reproduction, another six of them were related to growth, nine of them were related to abiotic stresses, and 13 of them were related to flowering (See Supplementary Material Table S6).
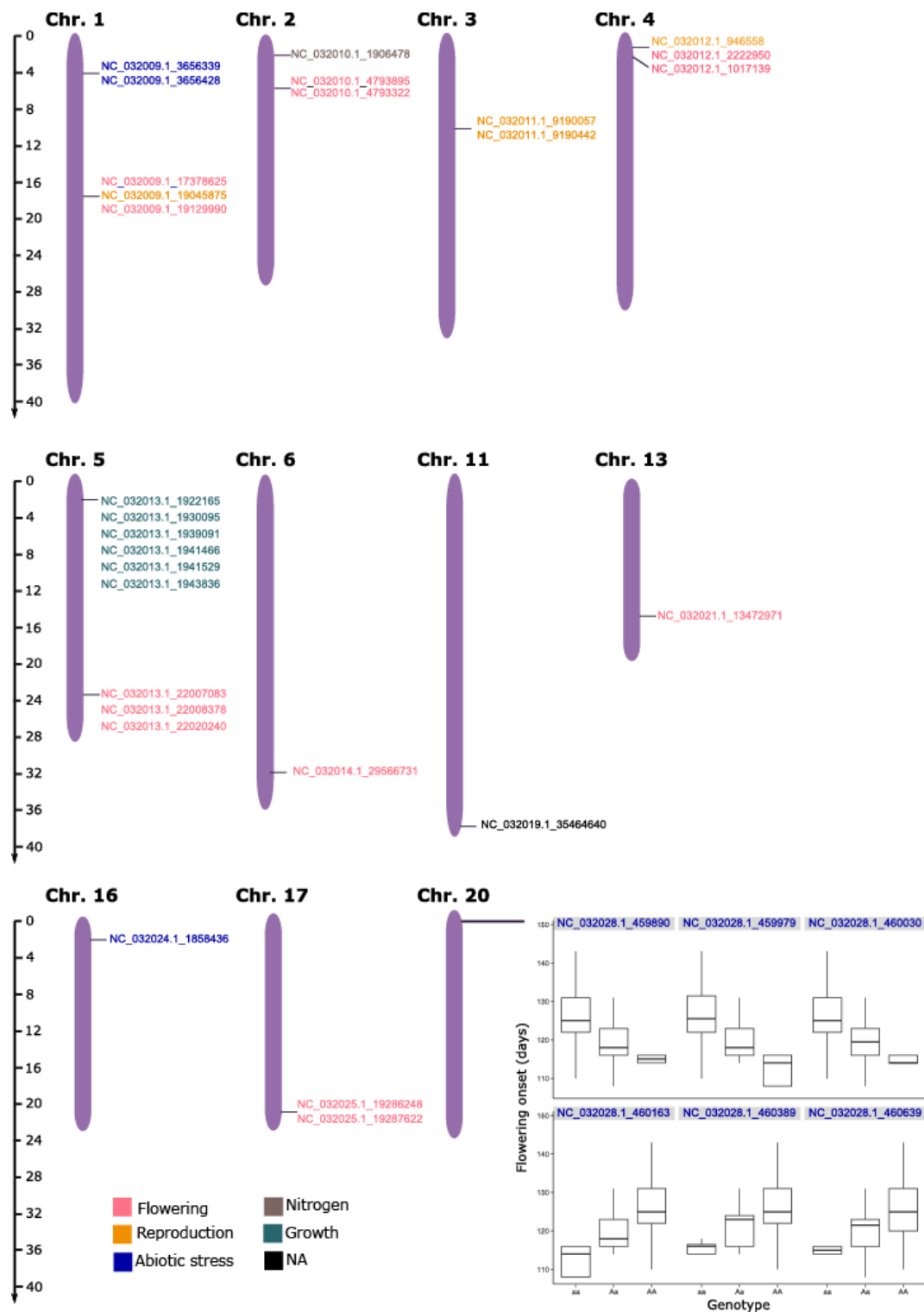
Figure 4. Location on the chromosomes of *Lupinus angustifolius* of the SNPs identified to be under selection and with a significant effect on flowering onset, seed weight, and shoot growth. Detail of the SNPs localized in chromosome 20 as an example. The name of the NCBI Reference Sequence and the position of the SNP in number of base pairs are indicated separated by a low bar.

Discussion

The different lines derived from assisted gene flow produced a significant flowering advance in both populations of *Lupinus angustifolius*. The assisted gene flow also caused modifications in seed weight and shoot growth. The genomic analysis identified 36 SNPs with contrasting frequency differences between the GFL and CFL in both populations. We found that these 36 SNPs significantly explained variation in flowering onset, seed weight and shoot growth, supporting the genetic basis of flowering advancement and the presence of genetic correlations between flowering onset, seed weight and shoot growth detected at the phenotypic level. The present observations highlight the importance of evaluating the effects of gene flow from an integrative perspective.

Effects of artificial gene flow on plant phenology, reproductive success, and non-reproductive traits

In Sacristán-Bajo et al. (2023) (Chapter 1), we observed that southern populations flower earlier than northern populations when grown together in a common garden. Therefore, it is reasonable to expect that hybrids from artificial crosses of northern mother plants with pollen from southern populations (GFL) will flower earlier than their respective northern population controls. It has already been suggested that natural gene flow may influence genetic changes related to climate change in plants (van Dijk & Hautekèete, 2014). These authors compared changes in flowering onset in a common garden environment in populations of *Beta vulgaris* from different latitudes and two collection years (20 years apart). They found evidence of genetic changes in response to climate change, as lower latitude populations delayed flowering, while higher latitude populations advanced flowering. Similarly to our observations, Bontrager & Angert (2019) found that gene flow from historically warmer populations enhanced the adaptive responses of colder populations in a context of rising temperatures. Prieto-Benítez et al. (2021) also

conducted artificial gene flow experiments with the objective of modifying flowering onset in *Silene ciliata* Pourr. (Caryophyllaceae)*,* a perennial mountain plant. Contrary to our results, they observed that gene flow from populations that flowered earlier did not advance the onset of flowering in the recipient populations, but rather delayed it. This shows that the effects of assisted gene flow on flowering onset can be complex and species or context dependent. Outbreeding depression or epistatic effects between genes (Blümel et al., 2015; He et al., 2019; Prieto-Benítez et al., 2021) could make gene flow difficult to predict or ineffective. This could be the cause the backcross line (BCL) produced a higher flowering advance than the F1 gene flow line, as the adverse effects that gene flow can cause in the first generation might have been mitigated with the addition of greater genome representation from the original populations (northern populations). This opens the possibility of carrying out successive generations of backcrossing with the population of origin while selecting for the progeny with early flowering, followed by the reintroduction of these individuals into their populations of origin.

Gene flow may not only affect the trait of interest to be shifted (in this case, flowering onset), but this modification may lead to changes in other traits, including traits related to reproductive success (Aitken & Whitlock, 2013; Morente-López et al., 2021; Prieto-Benítez et al., 2021). In congruence with the fact that the southern populations produce fewer but heavier seeds than the northern populations (Matesanz et al., 2020; Sacristán-Bajo et al., 2023), the GFL individuals produced heavier seeds than the CFL individuals, making the hybrids more similar to the individuals of the southern populations with regard to these traits. In our study, the GFL individuals showed lower shoot growth and also a tendency to lower SLA than the CFL individuals. Correlation analyses between the studied traits also support the existence of these same associations between flowering

onset, seed weight and shoot growth. For both populations, flowering onset correlated in the same direction for these same traits (negatively with seed weight and positively with shoot growth). This indicates again that early flowering plants have higher seed weight and lower shoot growth. In addition, these changes have also been observed at the genomic level (see next section). Several other studies have shown that gene flow leads to changes in different plant traits. For example, Chacón-Sánchez et al., (2021) reviewed the effects of gene flow between cultivated and wild types of several species of the genus *Phaseolus* (Leguminosae). Morphological, seed and other traits were influenced by gene flow events and with important consequences for the species performance. In a context of climate change, shifts towards traits more similar to plants from the southern areas may be an adaptive advantage. In the same direction as our findings, Matesanz et al. (2020) observed that populations in southern areas and those subjected to drought treatment had higher seed weight, lower growth rate and thicker leaves. Plant resources are limited, and when destined for one purpose, they cannot be used for others (Reich, 2014). Therefore, the production of heavier seeds could ensure their survival in more unfavorable environments, such as the southern sites with drier conditions (Leishman et al., 2009; Metz et al., 2010), whereas lower shoot growth and lower SLA could indicate a more efficient investment of resources (Wright et al., 1994). These results obtained in our study and in other studies reinforce the idea that the modification of certain target traits through gene flow will also come associated to changes in other traits, because biological characteristics of organisms are intricately linked, and they must be interpreted as a whole (Sobral, 2021).

Genomic effects of artificial gene flow

The combination of genome-wide studies with phenotypic characterization is essential to identify regions related to adaptive variation (Evans et al., 2014). In our study, we

identified 36 highly divergent SNPs between control and gene flow line, both by F_{ST} and AFDs analyses, indicating that these SNPs have undergone a process of change at the genomic level due to assisted gene flow. Furthermore, we determined that flowering onset, shoot growth and seed weight are partially explained by these same SNPs. This reinforces the idea that assisted gene flow has had an impact on these traits at the genetic level, already observed in the phenotypic study carried out in the common garden experiment.

Some of these 36 loci have also been identified in other studies and have been related to growth, flowering, or abiotic stresses. For example, the axial regulator YABBY 1-like has been related to flower development (Kumaran et al., 2002; Siegfried et al., 1999), similarly to our findings. The xyloglucan endotransglucosylase/hydrolase protein that we had associated to the stamen development through the Blast analysis, has also been associated to root growth (Maris et al., 2020) and abiotic stresses such as drought, salinity and cold temperatures (Keun et al., 2006). Similarly, the 3-oxoacyl protein that we had associated to cold response, has also been associated to drought conditions Nazari et al., (2020). The protein Flowering Locus T (FT) has been identified in *Arabidopsis* and other species, including legumes, as one of the main components that promotes the onset of flowering (*e.g*. Kardailsky et al., 1999; Pin & Nilsson, 2012; Weller & Ortega, 2015). As in our case, this protein is known to play a role in different pathways, such as photoperiod (among others). Related to this, the protein EBS regulates chromatin expression, controlling the expression of genes such as FT (López-González et al., 2014). It is also involved in other processes, such as organ development or seed dormancy (Gómez-Mena et al., 2001; Piñeiro et al., 2003). SNPs related to the FT gene and EBS protein showed a shift in their allele frequencies between the control line and the gene flow line (Supplementary Material Figure S6). Thus, we found that changes produced at the

phenotypic level through gene flow (such as earlier flowering) are also explained at the genomic level.

This is one of the first studies that has combined the evaluation of the use of assisted gene flow with a genomic approach. Our findings demonstrate that including genomic analyses in assisted gene flow studies provides much more accurate information about changes occurring at the genome level. In addition, the identification of these genes opens the door to the development of specific markers to identify early flowering genotypes in the species that may also allow the production of heavier seeds.

Final conclusions

Assisted gene flow through human actions can contribute to the adaptation of populations to climate change by providing suitable genetic variation (Grummer et al., 2022). With our experiments, we have observed that assisted gene flow has made possible to alter characteristics that are crucial for the adaptation of species to climate change, such as flowering onset, which could imply an enhancement of its adaptive potential in a dryer and warmer environment. In our case, the modification of flowering onset was also associated with higher seed weight produced by plants in the F1 gene flow line and lower shoot growth. In addition, genomic analyses confirmed that these changes in phenotype due to gene flow are also observed at the genomic level. Although this could be interpreted as a better adaptation to climate change, since these characteristics are more similar to those existing in southern populations, there are still many aspects to consider. First, unexpected effects may occur in different traits. Moreover, the impacts of assisted gene flow will greatly depend on the characteristics of donor and recipient populations, so this strategy will only make sense if the source populations are previously adapted to the environmental conditions now being experienced by the target population (Aitken & Whitlock, 2013; Prieto-Benítez et al., 2021). Ultimately, although the feasibility of these

techniques depends on numerous factors, this proof of concept using assisted gene flow suggests that these strategies should not be underestimated, as they can be of great use in conserving biodiversity in the current context of climate change. In addition, it demonstrates the potential of including genomic analyses to identify the regions being targeted and the real impact on the genome of the populations.

Acknowledgements

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Supplementary Material

Table S1. Observed mean ± SD values for the different traits measured and the different lines tested. CFL: control line; GFL: F1 gene flow line; SPL: F2 self-pollination line; BCL: backcross line. SLA: Specific leaflet area; LDMC: leaflet dry matter content.

Line	Flowering onset 2019 (days)		Flowering onset 2020 (days)			Number of seeds		Seed weigh (mg)		Height (cm)		Biomass (g)		Shoot growth (cm)		SLA (cm²/g)		LDMC (mg/g)	
	CFL	GFL	CFL	BCL	SPL	CFL	GFL	CFL	GFL	CFL	GFL	CFL	GFL	CFL	GFL	CFL	GFL	CFL	GFL
FRO	127,38 ±9,39	120,22 ±6,84	121,27 ±9,52	109,44 ±7,91	115,03 ±8,81	1239,46 ±679,73	1059,49 ±607,55	87,95 ±19,59	114,97 ±10,75	73,57 ±10,75	68,72 ±7,98	13,81 ±6,85	13,43 ±5,91	26,39 ±8,64	22,84 ±7,85	211,69 ±33,22	201,03 ±48,78	0,13 ±0,01	0,13 ±0,02
PIC	128,18 ±9,65	120,02 ±6,98	119,98 ±13,12	-	113,24 ±8,22	1003,01 ±481,72	954,48 ±405,46	80,79 ±23,92	97,48 ±17,06	74,11 ±11,47	72,56 ±9,31	13,10 ±5,20	14,15 ±4,29	28,16 ±10,65	23,58 ±8,41	215,03 ±29,83	201,46 ±46,38	0,13 ±0,01	0,12 ±0,02

Table S2. Effect of the different lines and population on flowering onset, number of seeds, seed weight, height, biomass, shoot growth, SLA and LDMC of *Lupinus angustifolius* plants in the common garden experiment. Estimates and significance level for fixed effects are shown. Genotype was included as a random factor. CFL: control line; GFL: F1 gene flow line; SPL: F2 self-pollination line; BCL: backcross line. Missing factors (CFL = Control line and population FRO) are included in the intercept.

Fixed effects	Parameter value	Standard error	t value	p value	Pseudo-R^2 (fixed effects)	Pseudo-R^2 (total)
Flowering onset 2019	-	-	-	-	0.100	0.122
Intercept	4.849	0.011	461.128	<0.001	-	-
GFL	-0.062	0.014	-4.495	<0.001	-	-
PIC	0.003	0.013	0.251	0.802	-	-
Flowering onset 2020	-	-	-	-	0.089	0.253
Intercept	4.798	0.015	326.774	<0.001	-	-
BCL	-0.092	0.037	-2.467	0.013	-	-
SPL	-0.055	0.018	-3.031	0.002	-	-
PIC	-0.013	0.018	-0.714	0.475	-	-
Number of seeds	-	-	-	-	0.039	0.125
Intercept	1217.632	71.250	17.089	<0.001	-	-
GFL	-107.615	91.740	-1.173	0.247	-	-
PIC	-190.473	88.460	-2.153	0.036	-	-
Seed weight	-	-	-	-	0.225	0.388
Intercept	89.608	2.985	30.016	<0.001	-	-
GFL	21.602	3.947	5.743	<0.001	-	-
PIC	-10.784	3.765	-2.864	0.006	-	-
Height	-	-	-	-	0.024	0.201
Intercept	72.992	1.390	52.494	<0.001	-	-
GFL	-2.994	1.839	-1.628	0.110	-	-
PIC	1.606	1.748	0.918	0.363	-	-
Biomass	-	-	-	-	0.003	0.195
Intercept	13.538	0.823	16.448	<0.001	-	-
GFL	0.582	1.094	0.532	0.598	-	-
PIC	-0.275	1.040	-0.264	0.793	-	-
Shoot growth	-	-	-	-	0.047	0.515
Intercept	26.599	1.484	17.927		-	-
GFL	-4.139	2.042	-2.027		-	-
PIC	1.064	1.902	0.559		-	-
SLA	-	-	-	-	0.025	0.164
Intercept	212.419	4.907	43.289	<0.001	-	-
GFL	-12.522	6.537	-1.915	0.061	-	-
PIC	1.833	6.208	0.295	0.769	-	-

LDMC	-	-	-	-	0.061	0.061
Intercept	0.134	0.002	79.366	<0.001	-	-
GFL	-0.001	0.002	-0.496	0.622	-	-
PIC	-0.007	0.002	-3.499	0.001	-	-

Table S3. Chi-square statistic, degrees of freedom and p-values of the Type II Wald chi-square tests of GLMM and LMM analyses to study the effect of selection line, population, and year on flowering onset, number of seeds, seed weight, height, biomass, shoot growth, SLA and LDMC of *Lupinus angustifolius* plants grown in the common garden experiment. Estimates and significance level for fixed effects are shown. Genotype was included as a random factor.

Fixed effects	X^2	**Df**	**Pr (>$|X^2|$)**
Flowering onset 2019	-	-	-
Line	20.207	1	<0.001
Population	0.063	1	0.802
Flowering onset 2020	-	-	-
Line	12.998	2	<0.001
Population	0.510	1	0.475
Number of seeds	-	-	-
Line	1.376	1	0.241
Population	4.636	1	0.031
Seed weight	-	-	-
Line	29.959	1	<0.001
Population	8.204	1	0.004
Height	-	-	-
Line	2.651	1	0.104
Population	0.843	1	0.359
Biomass	-	-	-
Line	0.283	1	0.595
Population	0.070	1	0.792
Shoot growth	-	-	-
Line	4.110	1	0.043
Population	0.313	1	0.576
SLA	-	-	-
Line	3.669	1	0.055
Population	0.0872	1	0.768
LDMC	-	-	-
Line	0.246	1	0.620
Population	12.243	1	<0.001

Table S4. Posterior mean values, standard errors and 95 % confidence intervals for the different traits and lines of *Lupinus angustifolius* plants grown in a common garden experiment. CFL: control line; GFL: F1 gene flow line; SPL: F2 self-pollination line; BCL: backcross line.

	FRO				PIC			
	Mean	Std. error	2·5%	97·%	Mean	Std. error	2·5%	97·%
Flowering onset 2019	-	-	-	-	-	-	-	-
CFL	128	1.34	125	130	128	1.35	125	131
GFL	120	1.61	117	123	120	1.50	117	123
Flowering onset 2020	-	-	-	-	-	-	-	-
CFL	121	1.78	118	125	120	1.77	116	123
BCL	111	3.79	103	118	109	4.23	101	118
SPL	115	2.00	111	119	113	1.79	110	117
Number of seeds	-	-	-	-	-	-	-	-
CFL	1218	71.4	1075	1360	1027	70.3	887	1168
GFL	1110	89.1	931	1289	920	82.2	754	1085
Seed weight	-	-	-	-	-	-	-	-
CFL	89.60	2.99	83.60	95.60	78.80	2.99	72.80	84.80
GFL	111.20	3.88	103.40	119.00	100.40	3.54	93.30	107.50
Height	-	-	-	-	-	-	-	-
CFL	73.00	1.39	70.20	75.80	74.60	1.38	71.80	77.40
GFL	70.00	1.79	66.40	73.60	71.60	1.67	68.30	75.00
Biomass	-	-	-	-	-	-	-	-
CFL	13.50	0.83	11.90	15.20	13.30	0.83	11.60	14.90
GFL	14.40	1.07	12.00	16.30	13.80	0.99	11.90	15.80
Shoot growth	-	-	-	-	-	-	-	-
CFL	26.60	1.48	23.60	29.60	27.70	1.50	24.70	30.70
GFL	22.50	2.01	18.40	26.50	23.50	1.87	19.80	27.30
SLA	-	-	-	-	-	-	-	-
CFL	212	4.91	203	222	214	4.93	204	224
GFL	200	6.36	187	213	202	5.94	190	214
LDMC	-	-	-	-	-	-	-	-
CFL	0.13	0.00	0.13	0.14	0.13	0.00	0.12	0.13
GFL	0.13	0.00	0.13	0.14	0.13	0.00	0.12	0.13

Table S5. Minimum allele frequency (MAF), False Discovery Rate (FDR), F_{ST} statistic and F_{ST}-FDR values for each SNP identified as outlier in the genomic analyses.

Population	SNP	Gene	Protein	FDR	F_{ST}	F_{ST}-FDR	CFL MAF	GFL MAF
FRO	NC_032009.1_17378625	LOC109350320	XP_019447099.1	0.010	0.313	<0.001	0.067	0.467
FRO	NC_032009.1_19045875	LOC109351227	XP_019448173.1	0.006	0.348	<0.001	0.067	0.500
FRO	NC_032009.1_19129990	LOC109351285	XP_019448262.1	0.006	0.348	<0.001	0.067	0.500
FRO	NC_032009.1_3656339	LOC109347866	XP_019443510.1	0.010	0.313	<0.001	0.067	0.467
FRO	NC_032009.1_3656428	LOC109347866	XP_019443510.1	0.010	0.313	<0.001	0.067	0.467
FRO	NC_032010.1_1906478	LOC109331801	XP_019422070.1	0.023	0.266	0.001	0.100	0.467
FRO	NC_032010.1_4793322	LOC109328838	XP_019417994.1	0.045	0.212	0.003	0.133	0.464
FRO	NC_032010.1_4793895	LOC109328838	XP_019417994.1	0.048	0.214	0.003	0.133	0.467
FRO	NC_032011.1_9190057	LOC109343327	XP_019437115.1	0.033	0.247	0.001	0.033	0.333
FRO	NC_032011.1_9190442	LOC109343327	XP_019437115.1	0.033	0.247	0.001	0.033	0.333
FRO	NC_032012.1_1017139	LOC109345004	XP_019439298.1	0.009	0.309	<0.001	0.250	0.700
FRO	NC_032012.1_2222950	LOC109345069	XP_019439392.1	0.001	0.489	<0.001	0.067	0.607
FRO	NC_032012.1_946558	LOC109344999	XP_019439290.1	0.002	0.410	<0.001	0.033	0.500
FRO	NC_032013.1_1922165	LOC109347401	XP_019442778.1	0.023	0.271	0.001	0.100	0.467
FRO	NC_032013.1_1930095	LOC109347401	XP_019442778.1	0.023	0.271	0.001	0.100	0.467
FRO	NC_032013.1_1939091	LOC109347400	XP_019442777.1	0.048	0.219	0.002	0.133	0.467
FRO	NC_032013.1_1941466	LOC109347400	XP_019442777.1	0.048	0.219	0.002	0.133	0.467
FRO	NC_032013.1_1941529	LOC109347400	XP_019442777.1	0.048	0.219	0.002	0.133	0.467
FRO	NC_032013.1_1943836	LOC109347400	XP_019442777.1	0.023	0.271	0.001	0.100	0.467
FRO	NC_032013.1_22007083	LOC109348120	XP_019443898.1	0.029	0.238	0.001	0.033	0.321
FRO	NC_032013.1_22008378	LOC109348120	XP_019443898.1	0.033	0.240	0.001	0.033	0.333
FRO	NC_032013.1_22020240	LOC109348121	XP_019443899.1	0.033	0.247	0.001	0.033	0.333

FRO	NC_032014.1_29566731	LOC109350790	XP_019447641.1	0.035	0.229	0.002	0.467	0.833
FRO	NC_032019.1_35464640	LOC109360702	XP_019461304.1	0.048	0.209	0.003	0.133	0.467
FRO	NC_032021.1_13472971	LOC109325863	XP_019414003.1	0.040	0.227	0.002	0.433	0.100
FRO	NC_032024.1_1858436	LOC109329933	XP_019419387.1	0.008	0.326	<0.001	0.100	0.533
FRO	NC_032025.1_19286248	LOC109331033	XP_019420861.1	0.002	0.457	<0.001	0.133	0.679
FRO	NC_032025.1_19287622	LOC109331033	XP_019420861.1	0.035	0.243	0.001	0.167	0.533
FRO	NC_032028.1_459890	LOC109335596	XP_019427286.1	0.01	0.323	<0.001	0.067	0.467
FRO	NC_032028.1_459979	LOC109335596	XP_019427286.1	0.023	0.266	0.001	0.100	0.467
FRO	NC_032028.1_460030	LOC109335596	XP_019427286.1	0.006	0.357	<0.001	0.067	0.500
FRO	NC_032028.1_460163	LOC109335596	XP_019427286.1	0.021	0.265	0.001	0.100	0.464
FRO	NC_032028.1_460389	LOC109335596	XP_019427286.1	0.048	0.204	0.003	0.133	0.467
FRO	NC_032028.1_460639	LOC109335596	XP_019427286.1	0.003	0.392	<0.001	0.067	0.533
FRO	NW_017722081.1_3838	LOC109338905	XP_019431800.1	0.023	0.276	0.001	0.100	0.467
FRO	NW_017722081.1_4164	LOC109338905	XP_019431800.1	0.013	0.314	<0.001	0.100	0.500
PIC	NC_032009.1_17378625	LOC109350320	XP_019447099.1	0.045	0.315	0.001	0.033	0.400
PIC	NC_032009.1_19045875	LOC109351227	XP_019448173.1	0.045	0.315	0.001	0.033	0.400
PIC	NC_032009.1_19129990	LOC109351285	XP_019448262.1	0.045	0.315	0.001	0.033	0.400
PIC	NC_032009.1_3656339	LOC109347866	XP_019443510.1	0.040	0.328	0.001	0.067	0.467
PIC	NC_032009.1_3656428	LOC109347866	XP_019443510.1	0.040	0.328	0.001	0.067	0.467
PIC	NC_032010.1_1906478	LOC109331801	XP_019422070.1	0.029	0.358	<0.001	0.033	0.433
PIC	NC_032010.1_4793322	LOC109328838	XP_019417994.1	0.029	0.358	<0.001	0.033	0.433
PIC	NC_032010.1_4793895	LOC109328838	XP_019417994.1	0.029	0.358	<0.001	0.033	0.433
PIC	NC_032011.1_9190057	LOC109343327	XP_019437115.1	0.040	0.321	0.001	<0.001	0.333
PIC	NC_032011.1_9190442	LOC109343327	XP_019437115.1	0.040	0.321	0.001	<0.001	0.333
PIC	NC_032012.1_1017139	LOC109345004	XP_019439298.1	0.040	0.315	0.001	0.433	0.867
PIC	NC_032012.1_2222950	LOC109345069	XP_019439392.1	0.032	0.349	<0.001	0.367	0.833
PIC	NC_032012.1_946558	LOC109344999	XP_019439290.1	0.029	0.358	<0.001	0.033	0.433
PIC	NC_032013.1_1922165	LOC109347401	XP_019442778.1	0.040	0.328	0.001	0.067	0.467

PIC	NC_032013.1_1930095	LOC109347401	XP_019442778.1	0.040	0.328	0.001	0.067	0.467
PIC	NC_032013.1_1939091	LOC109347400	XP_019442777.1	0.040	0.328	0.001	0.067	0.467
PIC	NC_032013.1_1941466	LOC109347400	XP_019442777.1	0.040	0.328	0.001	0.067	0.467
PIC	NC_032013.1_1941529	LOC109347400	XP_019442777.1	0.040	0.328	0.001	0.067	0.467
PIC	NC_032013.1_1943836	LOC109347400	XP_019442777.1	0.040	0.328	0.001	0.067	0.467
PIC	NC_032013.1_22007083	LOC109348120	XP_019443898.1	0.040	0.321	0.001	<0.001	0.333
PIC	NC_032013.1_22008378	LOC109348120	XP_019443898.1	0.040	0.314	0.001	<0.001	0.333
PIC	NC_032013.1_22020240	LOC109348121	XP_019443899.1	0.040	0.321	0.001	<0.001	0.333
PIC	NC_032014.1_29566731	LOC109350790	XP_019447641.1	0.040	0.300	0.001	0.667	1
PIC	NC_032019.1_35464640	LOC109360702	XP_019461304.1	0.040	0.328	0.001	0.067	0.467
PIC	NC_032021.1_13472971	LOC109325863	XP_019414003.1	0.029	0.385	<0.001	0.900	0.433
PIC	NC_032024.1_1858436	LOC109329933	XP_019419387.1	0.040	0.318	0.001	0.067	0.467
PIC	NC_032025.1_19286248	LOC109331033	XP_019420861.1	0.040	0.324	0.001	0.107	0.533
PIC	NC_032025.1_19287622	LOC109331033	XP_019420861.1	0.040	0.328	0.001	0.067	0.467
PIC	NC_032028.1_459890	LOC109335596	XP_019427286.1	0.009	0.429	<0.001	<0.001	0.433
PIC	NC_032028.1_459979	LOC109335596	XP_019427286.1	0.009	0.459	<0.001	<0.001	0.467
PIC	NC_032028.1_460030	LOC109335596	XP_019427286.1	0.009	0.429	<0.001	<0.001	0.433
PIC	NC_032028.1_460163	LOC109335596	XP_019427286.1	0.009	0.423	<0.001	<0.001	0.433
PIC	NC_032028.1_460389	LOC109335596	XP_019427286.1	0.005	0.555	<0.001	<0.001	0.567
PIC	NC_032028.1_460639	LOC109335596	XP_019427286.1	0.021	0.393	<0.001	<0.001	0.400
PIC	NW_017722081.1_3838	LOC109338905	XP_019431800.1	0.029	0.358	<0.001	0.033	0.433
PIC	NW_017722081.1_4164	LOC109338905	XP_019431800.1	0.029	0.358	<0.001	0.033	0.433

Table S6. Functional annotation of the 36 SNPs identified as outliers in the genomic analyses.

Name	Protein name	Protein ID	GO biological process
NC_032009.1_17378625	axial regulator YABBY 1-like	XP_019447099.1	Flower development (flowering)
NC_032009.1_19045875	uncharacterized protein	XP_019448173.1	Meiotic nuclear division (reproduction)
NC_032009.1_19129990	xyloglucan endotransglucosylase/hydrolase protein 28	XP_019448262	Stamen filament development (flowering)
NC_032009.1_3656339	3-oxoacyl-[acyl-carrier-protein] synthase II, chloroplastic-like	XP_019443510	Response to cold (response to abiotic stress)
NC_032009.1_3656428	3-oxoacyl-[acyl-carrier-protein] synthase II, chloroplastic-like	XP_019443510.1	Response to cold (response to abiotic stress)
NC_032010.1_1906478	nitrogen regulatory protein P-II homolog	XP_019422070.1	Regulation of nitrogen utilization (nitrogen)
NC_032010.1_4793322	rop guanine nucleotide exchange factor 12-like	XP_019417994.1	Pollen tube growth (flowering)
NC_032010.1_4793895	rop guanine nucleotide exchange factor 12-like	XP_019417994.1	Pollen tube growth (flowering)
NC_032011.1_9190057	chaperone protein dnaJ GFA2, mitochondrial-like isoform X3	XP_019437115.1	Pollination (reproduction)
NC_032011.1_9190442	chaperone protein dnaJ GFA2, mitochondrial-like isoform X3	XP_019437115.1	Pollination (reproduction)
NC_032012.1_1017139	isoleucine--tRNA ligase, chloroplastic/mitochondrial	XP_019439298.1	Ovule development (flowering)
NC_032012.1_2222950	cytochrome P450 90A1-like	XP_019439392.1	Anther differentiation (flowering)

NC_032012.1_946558	protein pleiotropic regulatory locus 1-like	XP_019439290.1	Cotyledon development (reproduction)
NC_032013.1_1922165	cell division cycle protein 27 homolog B-like isoform X1	XP_019442778.1	Root meristem specification (growth)
NC_032013.1_1930095	cell division cycle protein 27 homolog B-like isoform X1	XP_019442778.1	Root meristem specification (growth)
NC_032013.1_1939091	cell division cycle protein 27 homolog B-like	XP_019442777.1	Root meristem specification (growth)
NC_032013.1_1941466	cell division cycle protein 27 homolog B-like	XP_019442777.1	Root meristem specification (growth)
NC_032013.1_1941529	cell division cycle protein 27 homolog B-like	XP_019442777.1	Root meristem specification (growth)
NC_032013.1_1943836	cell division cycle protein 27 homolog B-like	XP_019442777.1	Root meristem specification (growth)
NC_032013.1_22007083	chromatin remodeling protein EBS-like	XP_019443898.1	Regulation of long-day photoperiodism (flowering)
NC_032013.1_22008378	chromatin remodeling protein EBS-like	XP_019443898.1	Regulation of long-day photoperiodism (flowering)
NC_032013.1_22020240	chromatin remodeling protein EBS-like	XP_019443899.1	Regulation of long-day photoperiodism (flowering)
NC_032014.1_29566731	Cyclic nucleotide-binding domain-containing protein; putative cyclic nucleotide-gated ion channel 8 isoform X2	OIW09331.1; XP_019447642.1	Pollen tube growth (flowering)
NC_032019.1_35464640	ubiquitin protein ligase	OIW01082.1	Chromatin organization (NA)
NC_032021.1_13472971	armadillo repeat-containing protein LFR	XP_019414003.1	Anther development (flowering)

NC_032024.1_1858436	nuclear export mediator factor NEMF	XP_019419388.1	Cold acclimation (response to abiotic stress)
NC_032025.1_19286248	protein FLOWERING LOCUS T-like	OIV93971 1; XP_019420861	Response to short-day photoperiodism (flowering)
NC_032025.1_19287622	protein FLOWERING LOCUS T-like	XP_019420861.1	Response to short-day photoperiodism (flowering)
NC_032028.1_459890	transcription factor RF2b-like	OIV91432.1; XP_019427286.1	Response to sulfate (response to abiotic stress)
NC_032028.1_459979	transcription factor RF2b-like	OIV91432.1; XP_019427286.2	Response to sulfate (response to abiotic stress)
NC_032028.1_460030	transcription factor RF2b-like	OIV91432.1; XP_019427286.3	Response to sulfate (response to abiotic stress)
NC_032028.1_460163	transcription factor RF2b-like	OIV91432.1; XP_019427286.4	Response to sulfate (response to abiotic stress)
NC_032028.1_460389	transcription factor RF2b-like	OIV91432.1; XP_019427286.5	Response to sulfate (response to abiotic stress)
NC_032028.1_460639	transcription factor RF2b-like	XP_019427286.1	Response to sulfate (response to abiotic stress)
NW_017722081.1_3838	ER lumen protein-retaining receptor	XP_019431801.1_1	Meiotic nuclear division (reproduction)
NW_017722081.1_4164	ER lumen protein-retaining receptor	XP_019431801.1_1	Meiotic nuclear division (reproduction)
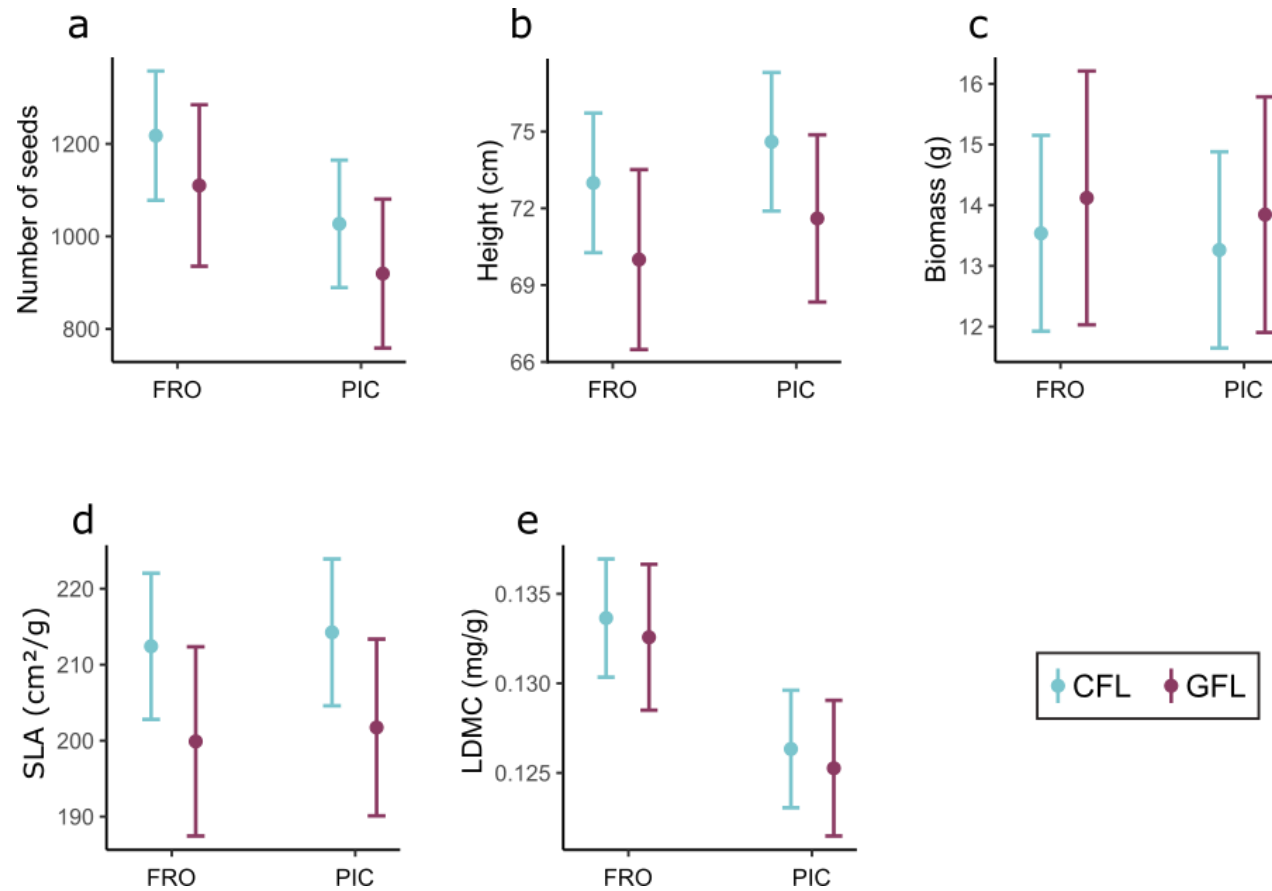
Figure S1. Effect of the gene flow line (GFL) on the different traits of *Lupinus angustifolius* L.: a) number of seeds, b) height, c) biomass, d) SLA, e) LDMC. Dots and bars represent the predicted mean from the LMM model with a Gaussian distribution and 95 % confidence intervals. Differences between the gene flow line and the control line were non-significant.
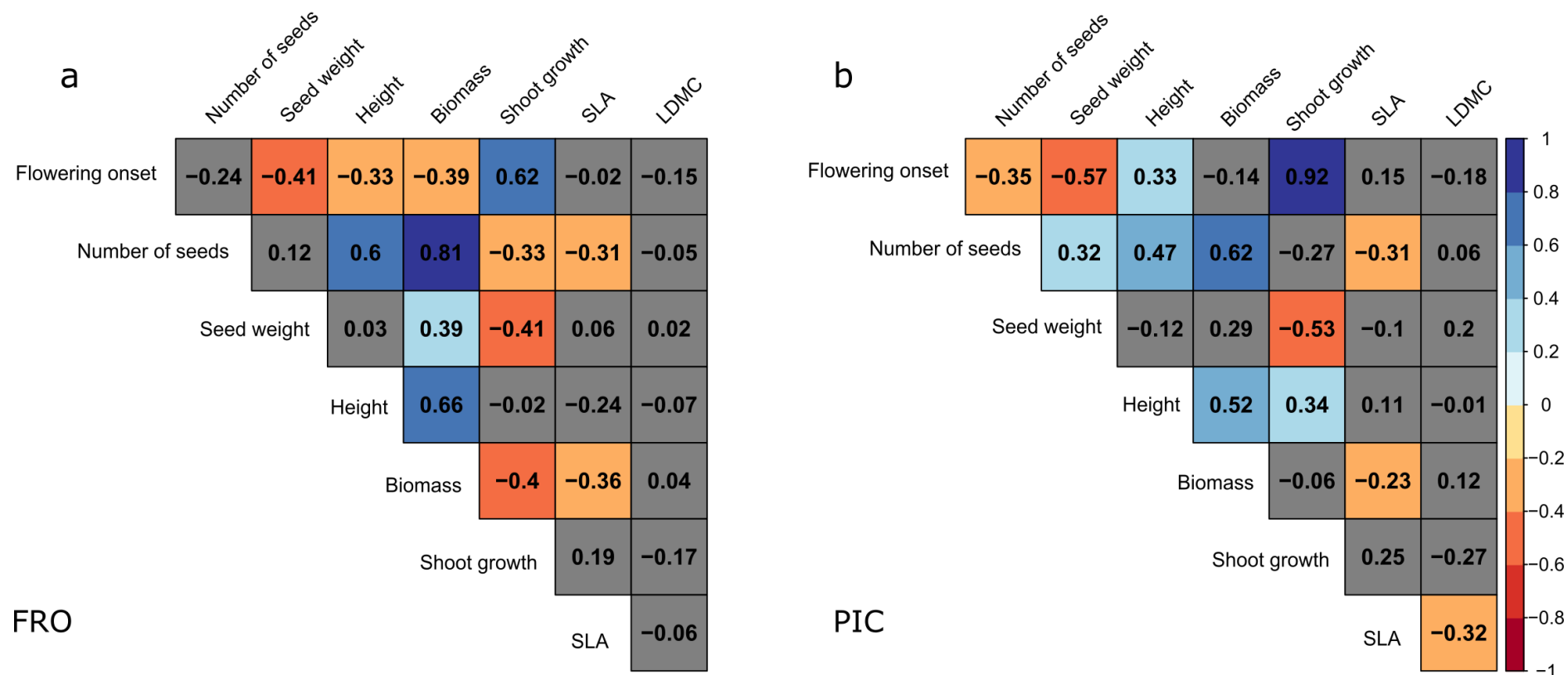
Figure S2. Correlations between flowering onset and other plant traits for control line and year 2019. a) correlations for FRO population. b) correlations for PIC population. Positive correlations are represented in cold colors, while negative correlations are represented in warm colors. Non-significant correlations ($p > 0.05$) are represented in grey.
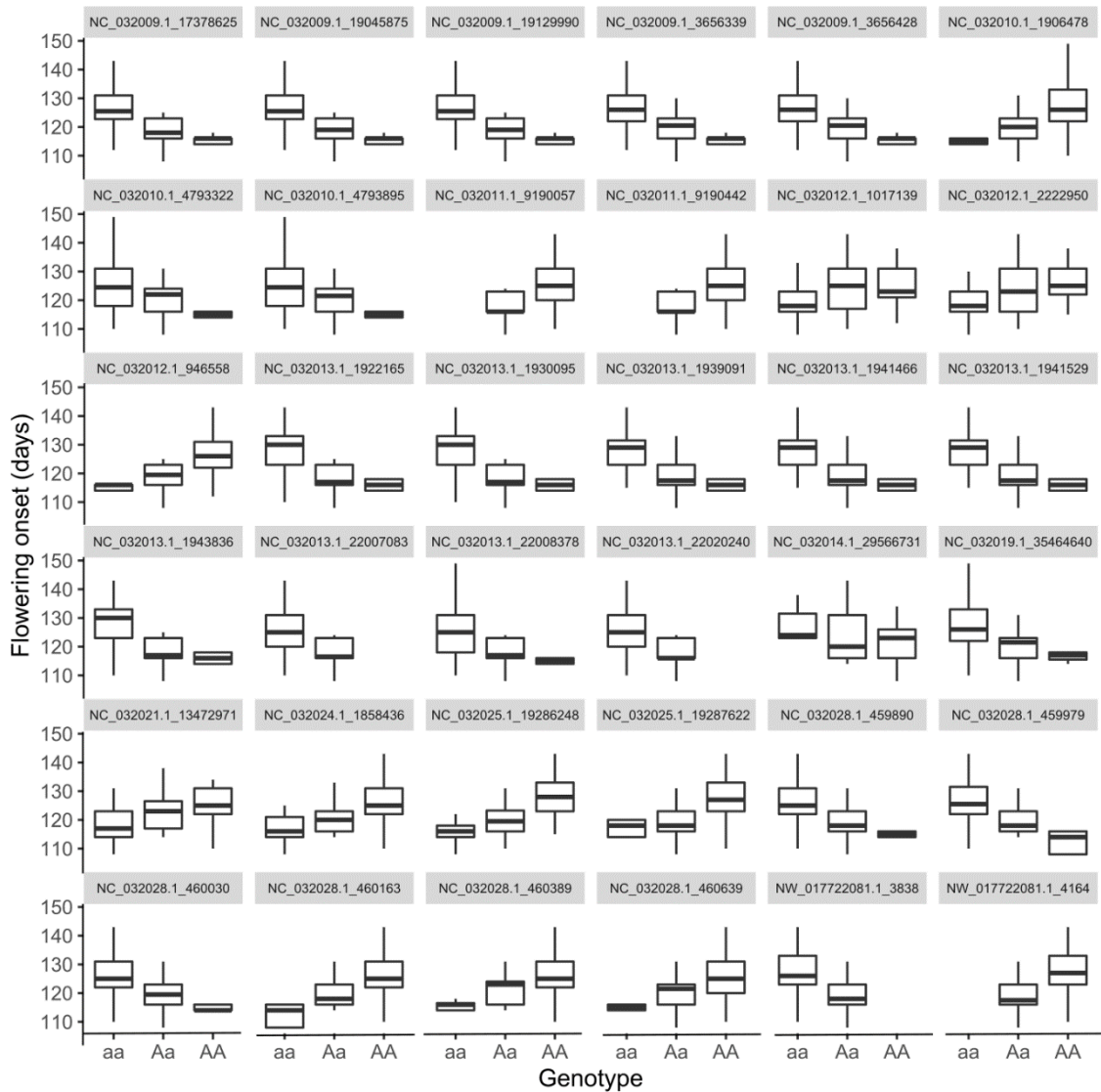
Figure S3. Distribution of flowering onset (days) according to the genotypes (homozygous dominant, recessive and heterozygous) for the 36 significant SNPs detected.
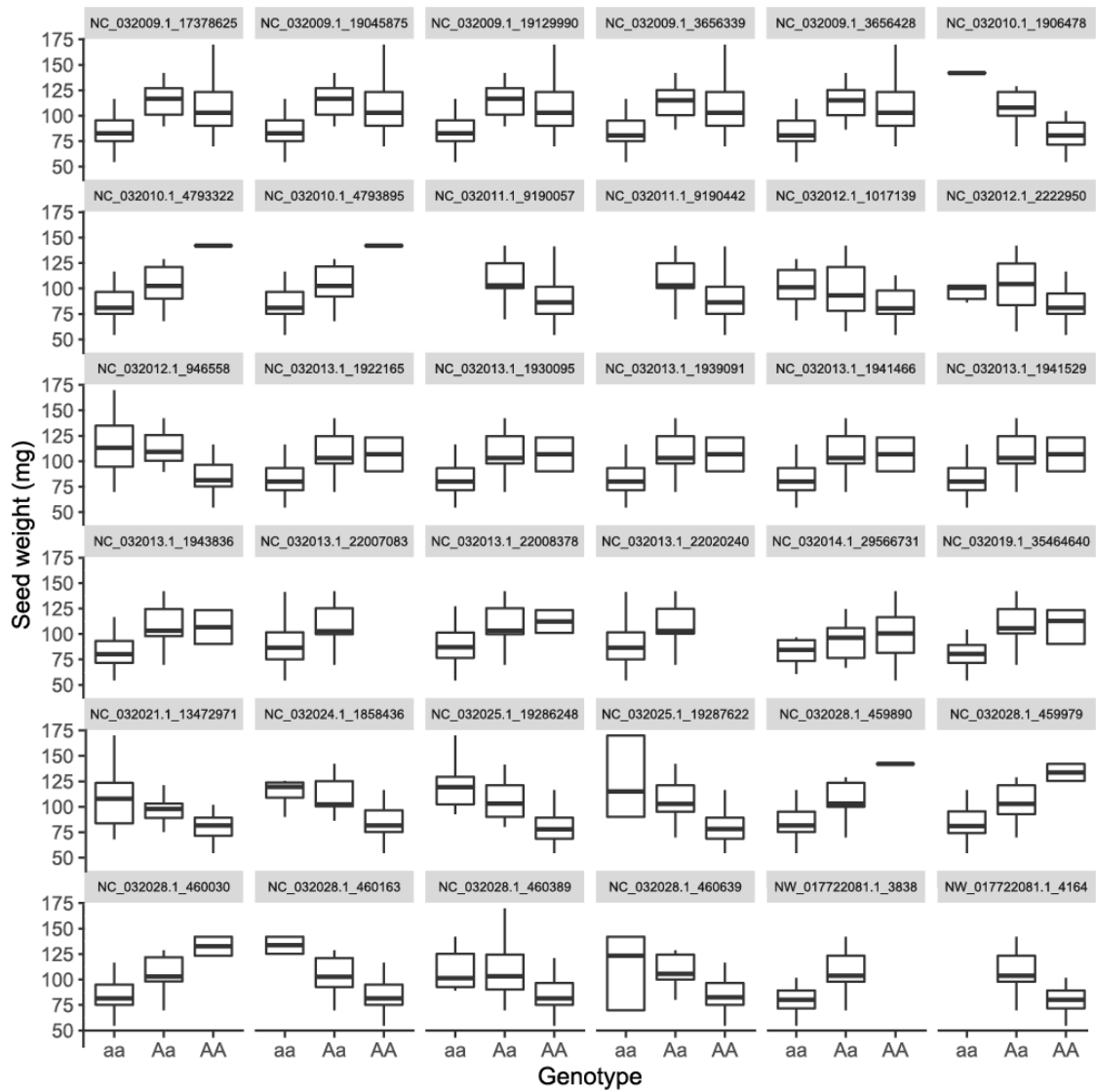
Figure S4. Distribution of seed weight (mg) according to the genotypes (homozygous dominant, recessive and heterozygous) for the 36 significant SNPs detected.
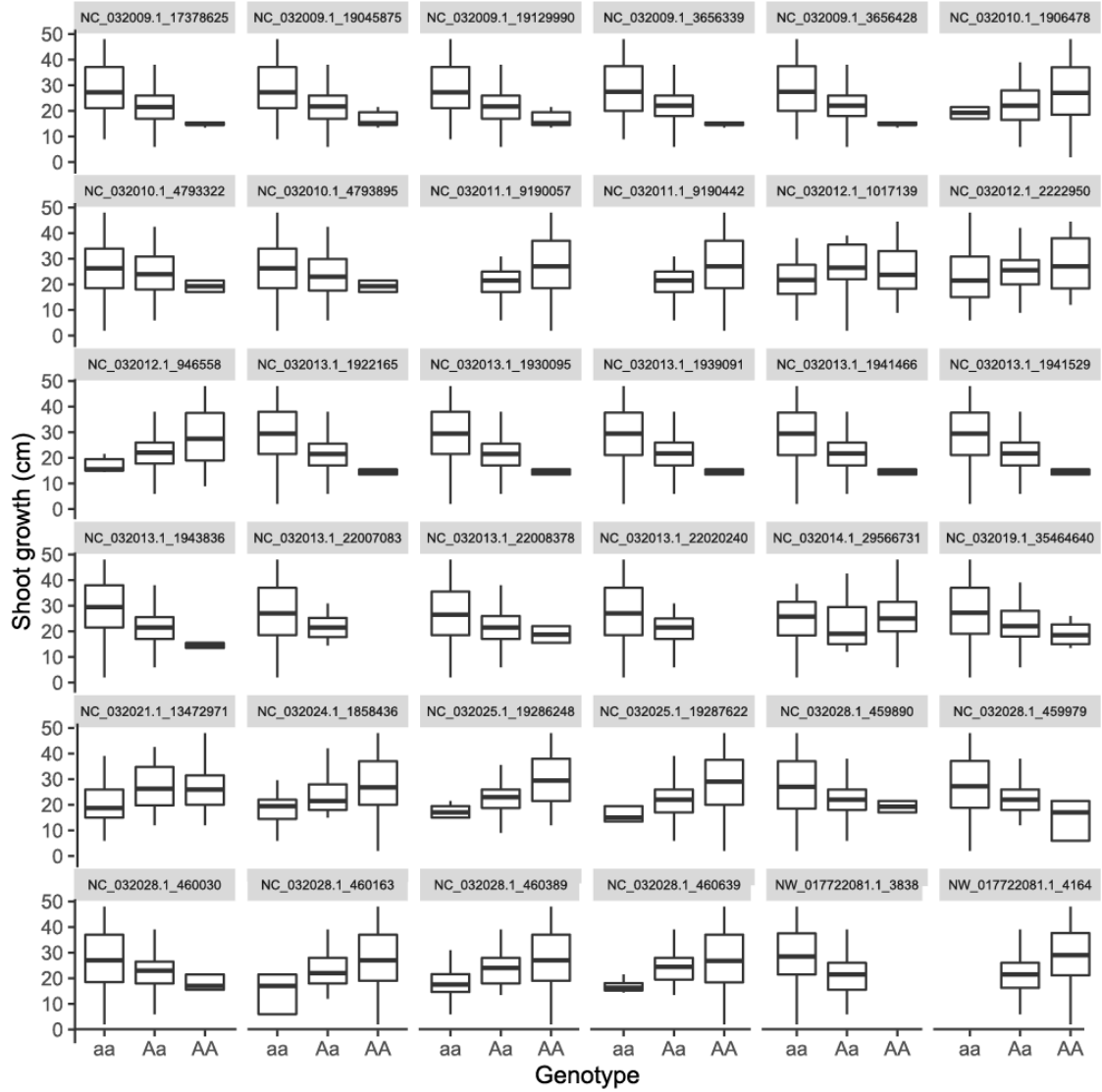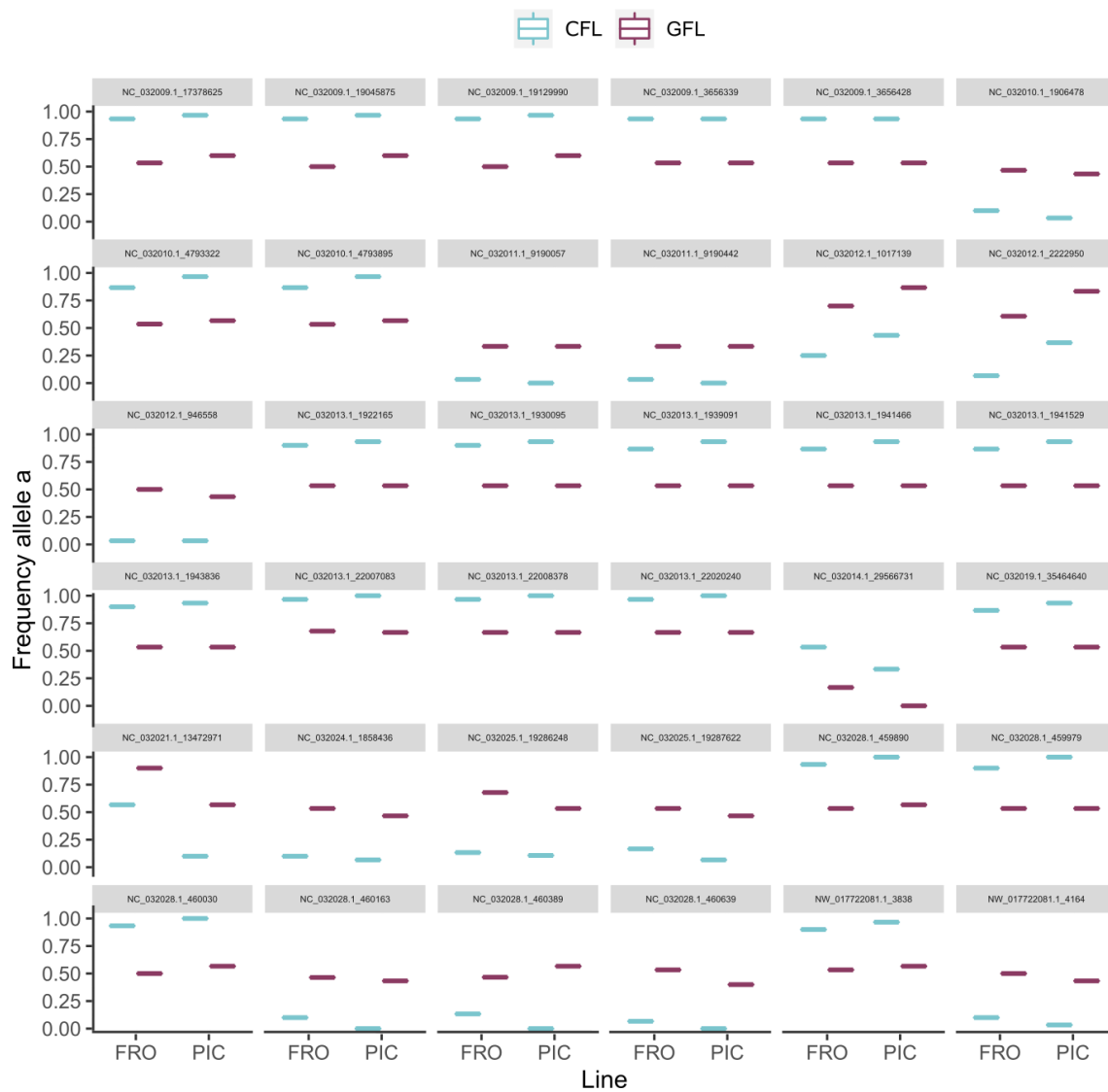
Figure S5. Distribution of shoot growth (cm) according to the genotypes (homozygous dominant, recessive and heterozygous) for the 36 significant SNPs detected.

Figure S6. Changes in allele frequencies between the control treatment (blue) and the gene flow treatment (purple) for both populations (FRO and PIC) for the 36 significant SNPs detected.

Chapter 4: *In-situ* evaluation of artificial selection and assisted gene flow for the advancement of flowering onset in *Lupinus angustifolius* L. (Fabaceae)

<u>Manuscript in preparation</u>

Abstract

Artificial selection and assisted gene flow have recently arisen as useful tools to better manage and protect biodiversity and to increase the adaptive potential of species, in the face of current rapid climate change. The aim of this study was to test the implementation of artificial selection and assisted gene flow by assessing the behaviour of the progeny, resulting from these treatments in a natural setting, in a study case concerning the advance of flowering onset in *Lupinus angustifolius* L. (Fabaceae). Seeds from four wild populations and two contrasting latitudes in Spain were collected and two experiments targeting each treatment settled, for three years. For the artificial selection experiment, two distinct early flowering selection lines were created over three generations for each of the populations, using both self-crosses and outcrosses. For the assisted gene flow experiment, we established an F1 generation by pollinating plants from northern populations with pollen from southern individuals. The F1 was self-pollinated to produce an F2 self-pollination line in the following season. All generated lines were maintained through self-pollination for a third season. In autumn 2020, seeds obtained (F3) from the two groups of treatments from the northern populations were sown in a common garden under natural conditions, close to the original wild populations' locations. In spring 2021, we measured the flowering onset and different vegetative and reproductive plant traits. Plants derived from the artificial selection experiment did not significantly differ from control individuals in any of the measured traits, contrary to the results obtained in previous works under controlled conditions. On the other hand, plants derived from the assisted gene flow lines, flowered significantly earlier and showed lower shoot growth than the control line, in line with results obtained under controlled conditions in previous works. These results show that assisted gene flow was more efficient than artificial selection in modifying flowering initiation under natural conditions.

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Keywords

Adaptive potential, *biodiversity conservation*, *climate change*, *facilitated adaptation*, *natural conditions*, *restoration*.

Introduction

Many organisms are moving in latitude or altitude in response to climate change (Forero-Medina et al., 2011; Walther et al., 2002). However, these migratory movements may not be possible for some plant species without mobility and short dispersal (Engler et al., 2009). Other strategies that organisms may use are those related to *in-situ* adaptation, such as phenotypic plasticity or genetic adaptation (Jump & Peñuelas, 2005). Even so, the intensification of current climate change may cause these responses to be insufficient to prevent the extinction of some populations and biodiversity loss (Talukder et al., 2022; Urban, 2015). In this context, for improving species management, it is necessary to determine populations adaptive capacity and to find out if it is possible to enhance their evolutionary potential.

Plant populations threatened by climate change can improve their situation if they count on genetic variability to increase the fitness of individuals to new environmental conditions (Gomulkiewicz & Shaw, 2013). Artificial selection and assisted gene flow are two approaches that can help us to quantify and even increase the adaptive potential of a given population (Aitken & Whitlock, 2013; Conner, 2003; Hoffmann & Sgró, 2011). On the one hand, through artificial selection, we can select individuals of a population that possess certain traits of interest in order to preserve them for future generations (Conner, 2003, 2016). On the other hand, assisted gene flow consists of the intentional movement of alleles, gametes or individuals between different populations to promote adaptation to present or future environmental conditions (Aitken & Whitlock, 2013; Whiteley et al., 2015). Both strategies fall in the concept of facilitated adaptation, which involves

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intervening in one or more evolutionary forces – by the introduction of beneficial alleles – to help organisms to adapt to new environmental conditions or pressures, such as those derived from climate change (Humanes et al., 2021; Jones & Monaco, 2009; Torres et al., 2023; van Oppen et al., 2015). Nonetheless, it is noteworthy that the implementation of these actions can entail certain risks, including loss of genetic integrity, local adaptations, and technical difficulties (Torres et al., 2023), just to mention some. Particularly, artificial selection may be associated with a decrease in genetic diversity which may impair evolutionary resilience (Chen et al., 2017; Sheth & Angert, 2016; Whitt et al., 2002). On the other hand, assisted gene flow may break linkage disequilibrium, with the introduction of maladapted alleles or the disruption of coadapted gene networks, resulting in a decrease in the fitness of individuals (*i.e*. maladaptation, outbreeding depression) (Aitken & Whitlock, 2013). In addition, both strategies can lead to undesired correlated responses in other functional traits, constraining adaptation due to trade-offs (Sacristán-Bajo et al., 2023; Sheth & Angert, 2016).

Artificial selection and assisted gene flow have been experimentally assessed in their potential effects in populations recently (see Chapters 1 and 3, Sacristán-Bajo et al., 2023, and van Oppen et al., 2015,). However, these approaches have been made under partially controlled conditions or as proofs of concept. In the face of the new challenges posed by climate change, it is, necessary to test these approaches also in natural conditions to be able to evaluate whether these tools could help us conserve and manage biodiversity in a better way. Field experiments have great potential to replicate evolutionary events that are difficult to observe under controlled conditions (Garland & Kelly, 2006). For example, under controlled conditions, trade-offs may be absent or attenuated, whereas in nature these trade-offs may be a major constraint to the occurrence of evolutionary changes. However, we must be aware that field experiments entail some difficulties, such

as the existence of many uncontrolled factors that can make it difficult to distinguish between cause and effect (Garland & Rose, 2009). To predict and interpret evolutionary responses, it is crucial to understand these restrictions (Garland & Rose, 2009).

The identification of traits of high relevance for adaptation to climate change, on which we can take action, is an essential point in the use of these methods. Flowering onset is one of the most important events for the reproductive success of plants (Blümel et al., 2015; Forrest & Thomson, 2010; Thomas et al., 2001), which is known to have a genetic basis (Franks et al., 2007; Franks & Hoffmann, 2012). Climate change has led to shift flowering periods in temperate zones (Büntgen et al., 2022; Fitter & Fitter, 2002), in the same way as many organisms adjust their phenology to these environmental changes (Bradshaw & Holzapfel, 2008; Cohen et al., 2018). Therefore, flowering onset is a key trait for plant adaptation to climate change and may be a good target trait on which artificial selection and assisted gene flow strategies could be tested.

The aim of this study was to test management tools to enhance species adaptation. In this way, we tested in a field experiment under natural conditions the progeny of artificial selection and assisted gene flow treatments to advance flowering onset, to evaluate whether they could be applied in conservation and restoration actions. The test was based on a previous common garden experiment that took place under controlled *ex-situ* conditions, involving two pairs of populations from two contrasting climatic zones of the annual plant *Lupinus angustifolius* L. (Fabaceae) (hereafter, northern and southern populations), where the artificial selection and assisted gene flow treatments had been implemented (see Sacristán-Bajo et al., 2023; Chapters 1 and 3). The artificial selection experiment under controlled *ex-situ* conditions showed that selection lines derived from self and out-crosses produced a significant advancement of flowering onset compared to the control line in northern populations, but not in southern ones. Artificial selection lines

did not affect fitness-related traits but did produce significant changes in some vegetative traits (Sacristán-Bajo et al., 2023; Chapter 1). In the assisted gene flow experiment under controlled *ex-situ* conditions, lines resulting from crosses of northern populations with pollen from southern populations also produced a significant advancement of flowering onset. Furthermore, the gene flow line also produced an increase in seed weight compared to the control line, and affected other vegetative traits (see Chapter 3). With the field approach, we intend two things: first, to validate that the trait modified under controlled conditions still maintains the modification in the field, and second, to assess whether the modification has a positive effect on the fitness of individuals.

Taking the previous results into consideration, the offspring obtained from the experiments carried out under controlled conditions was then used to establish a common garden experiment under natural conditions, in a location near one of the northern populations. We then hypothesized that, under natural conditions, the lines derived from the artificial selection treatments and those from the assisted gene flow treatment would also produce plants that flower earlier than those from the control line. In addition, since selective pressures are stronger under natural conditions, we also hypothesized that both artificial selection and assisted gene flow lines would experience changes in fitness-related traits, and probably in other vegetative traits as well with regard to the control treatment plants. Finally, since the gene flow treatment had shown greater change in flowering advance than the selection treatments under controlled environmental conditions, we hypothesized that under natural conditions the gene flow treatment would also manifest greater flowering advance than the artificial selection treatment.

Materials and Methods

Study species and experimental design

Lupinus angustifolius L. is an annual legume that is extensively prevalent throughout the Mediterranean basin and has been introduced and grown as a crop in many other parts of the world (Castroviejo & Pascual, 1993). In natural conditions, it inhabits in well-drained acid or neutral soils, including disturbed environments such as roadsides or abandoned fields (Rhodes & Maxted, 2016). The species germinates in autumn and blooms between March and August depending on environmental conditions (Castroviejo & Pascual, 1993). Its blue-purple flowers are zygomorphic and hermaphroditic, and mainly self-pollinate before the petals open (Wolko et al., 2011).

For this study, in 2016 we collected seeds from four populations located in two contrasting climatic zones in Central and Southern Spain (Figure 1, Table S1). We collected seeds from at least 98 mother plants (hereafter, genotypes) in each population, which were sown in a common garden environment in the CULTIVE facility (https://urjc-cultive.webnode.es/) at Rey Juan Carlos University (Móstoles, Madrid). An artificial selection experiment and a geneflow experiment were conducted under common garden conditions. For the artificial selection experiment, in the 2017 season, an early flowering line was established by selecting the first quartile of individuals that flowered the earliest (hereafter, EFL). In parallel, a control line was applied by selecting another 25 % of randomly selected individuals (hereafter, CFL). In the spring of 2018, we obtained an outcrossed early flowering line (hereafter, OUT), by manually crossing genotypes from the early flowering selection line between each other. In the spring season of 2019, the individuals of the OUT line were self-crossed to generate a segregating F2 line (hereafter, OUTS). In the next season of 2020, the individuals of the OUTS line were perpetuated by self-pollination, generating an F3 generation of the outbred line (hereafter, OUTS2).

The EFL and CFL lines were also perpetuated by self-pollination generating F3 generations in 2020. For the gene flow experiment, in the 2018 season, we applied an F1 gene flow line (hereafter, GFL) by manually pollinating plants from the northern populations using pollen from the southern populations. The control line (CFL) was the same as the one used for the artificial selection experiment. In the flowering season of 2019, we started an F2 self-pollination line by the self-pollination of the F1 gene flow line (hereafter, SPL). The individuals of the SPL were self-pollinated, creating a F3 generation (hereafter, SPL2) in the season of 2020. A more detailed description of the complete process, the growing conditions and the hand-pollination process is provided in Sacristán-Bajo et al., 2022 (Chapters 1 and 3). Figure 2 shows a diagram of the entire procedure. In conclusion, in the 2020 season we collected F3 generation seeds of the CFL, EFL, OUTS2 and SPL2 lines, which were used in the field experiment.
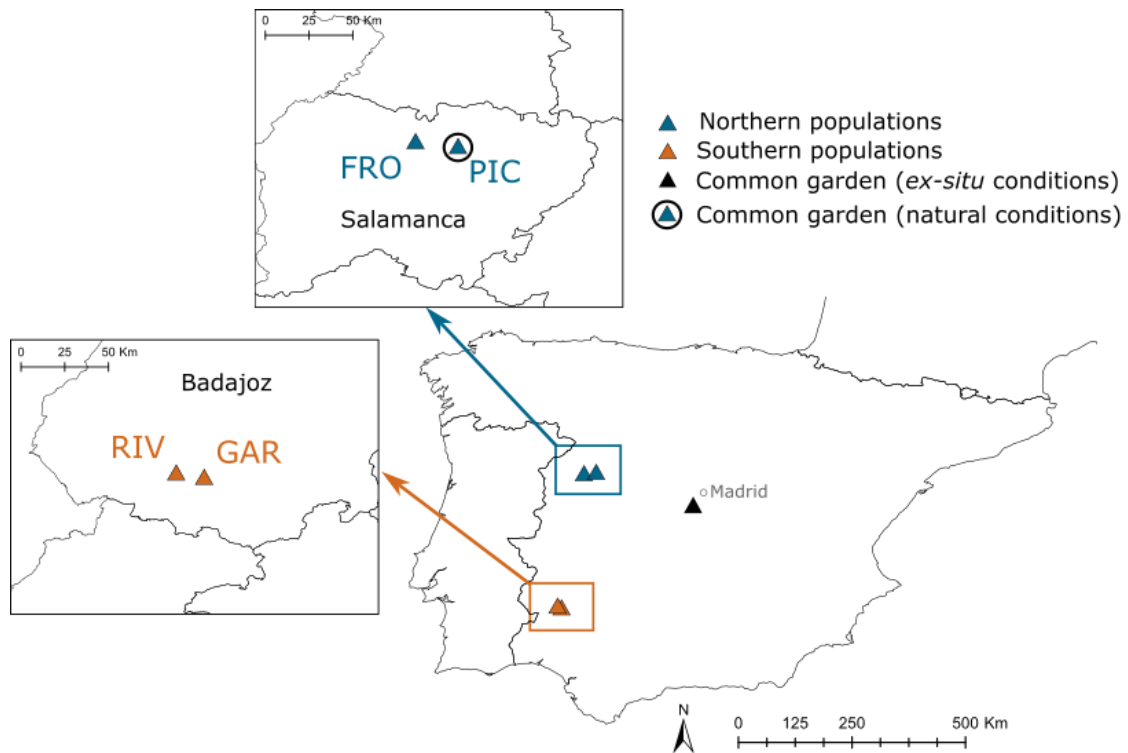

Figure 1. Location of the original populations of *Lupinus angustifolius* L., the site where the *ex-situ* common garden was implemented, and the plot used in the field experiment (common garden, natural conditions).
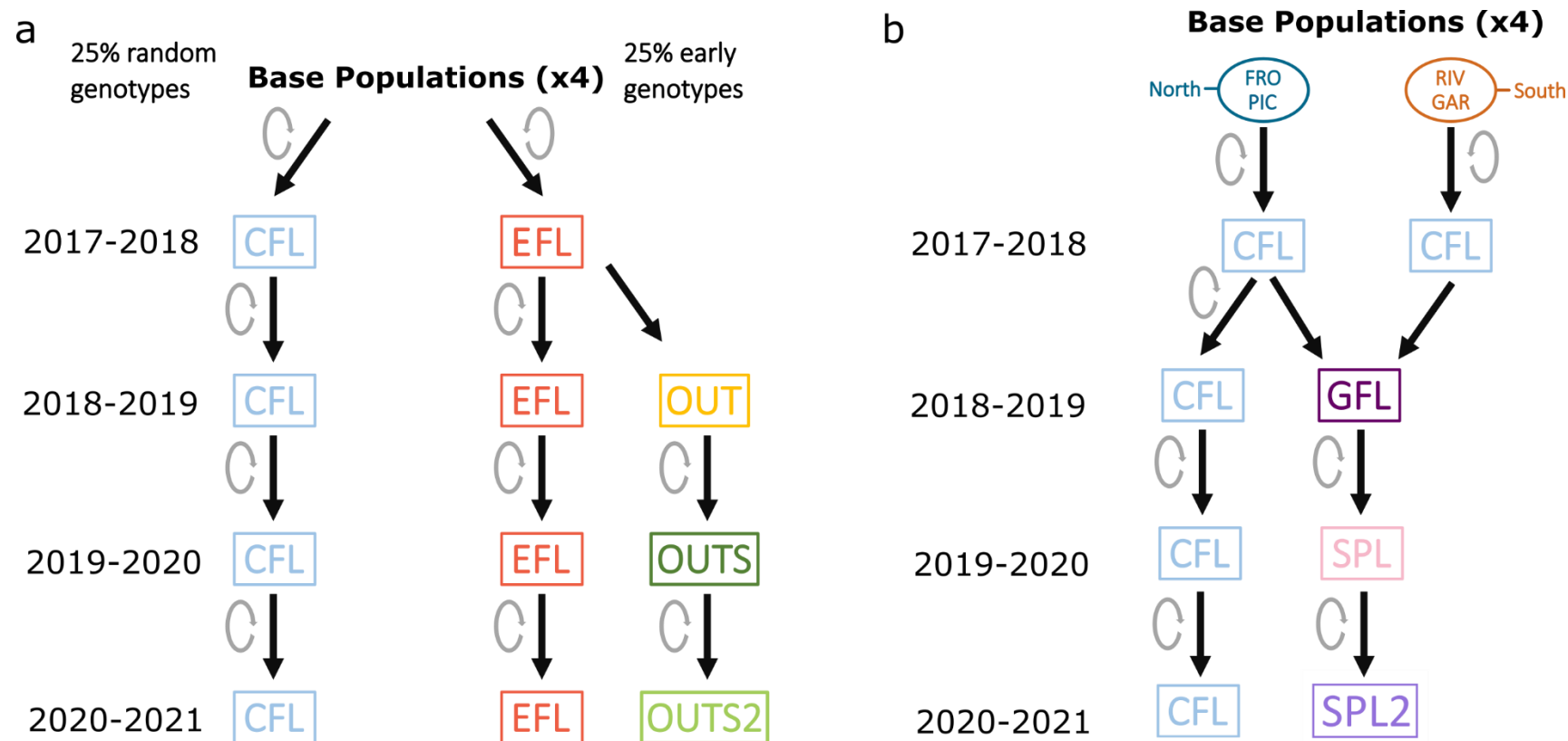
Figure 2. Process describing the different lines obtained through time in four populations of *Lupinus angustifolius*. Grey circles next to the arrows indicate that the individuals of that line were self-crossed. CFL: control flowering line; EFL: early flowering line (selfed), OUT: outbred line (cross of different EFL genotypes); OUTS: F2 generation of outbred line resulting from the self-pollination of OUT genotypes. OUTS2: F3 generation of outbred line resulting from the self-pollination of OUTS genotypes. GFL: F1 gene flow line (manually crossed). SPL: F2 self-pollination line of the F1 gene flow line. SPL2: F3 generation resulting from the self-pollination of the SPL genotypes.

In November 2020, seeds from each of the lines of the northern populations were sown under natural conditions in a grassland located in Zarapicos (Salamanca, Figure 1 and Figure 3) extensively grazed by cattle. We applied a randomized block design formed by four blocks, including seeds of both populations and all lines of the artificial selection and assisted gene flow experiments in each block (Figure 3a). Once each block was delimited, we established 234 sowing points in each block, separated 20 cm from each other and arranged in two lines separated 20 cm from each other. The blocks were separated from each other by a one-meter corridor. The sowing points were briefly prepared with a hoe, and we sowed three seeds of the same genotype at each sowing point, in a triangle-shaped arrangement (Figure 3b and Figure 3c). We sowed a total of 2808 seeds: 36 seeds for each genotype, 10 genotypes per treatment (except for the SPL2 treatment in FRO population, in which we used 8 genotypes), and four treatments per population for the two populations. The seeds were previously weighed and scarified, and once sown, no additional treatment or irrigation was added, since the purpose was to conduct an experiment that closely mimicked the natural conditions experienced by their natural populations of origin, which occur within a radius of 20 km from the location of the experiment.

Figure 3. Experimental plot under natural conditions. a) In each block the sowing points of *Lupinus angustifolius* were arranged in two parallel lines that were 20 cm apart. b) Seedlings of *Lupinus angustifolius*. c) Triangular arrangement of three seedlings of a genotype at a sowing point.

Traits measurement

We monitored and recorded flowering onset, which was considered the date of opening of the first flower, for all plants. The sampling of flowering data was carried out three days a week. Flowering onset on the previous and posterior day of the visit were inferred, based on the developmental stage of the flower. The days to flowering onset were calculated as the number of days between the date of sowing and the date of flowering onset of each plant. We also measured plant height at two different times. Plant height was measured as the distance from the ground level to the top end of the vegetative section of the stem. A first measurement was taken on March 17th 2021, at the beginning of the flowering period, and a second measurement was taken between May 14th and 18th 2021, at the end of the flowering period. The difference between the two allowed us to calculate the shoot growth. In addition, once the reproductive season was over (from early June, when there were no flowers left and they had all turned into fruits), we collected the plants

and counted the number of seeds produced by each plant, which was considered as a proxy for measuring plant fitness. At the end of the season, we also weighed the total dry biomass of the plant.

Statistical analyses

The artificial selection and the assisted gene flow treatments were separately analyzed against the same control treatment in two sets of analyses. All the statistical analyses were carried out using the R statistical environment version 4.1.1 (R Core Team, 2020). We considered flowering onset, shoot growth, biomass, and number of seeds as response variables. To test the effect of artificial selection and assisted gene flow on flowering onset we used generalized linear mixed models (hereafter, GLMMs), and to assess their effect on the rest of the traits, we used linear mixed models (hereafter, LMMs). For each response variable, we ran separate models, using the *glmmTMB* R package version 1.1.3 (Brooks et al., 2017). For flowering onset, we used a Poisson distribution, and for the rest of the traits, we used a Gaussian distribution. For each variable, we included *line* and *population* as fixed effects, and *genotype* (mother plant), as a random effect in all fitted models. Germinated seed weight was included as a covariate in the models. Diagnostic plots were used to visually examine the model residuals. The significance of each fixed effect was evaluated using the *Anova* function from the R package version 3.0-11 (Fox & Weisberg, 2011). The *r.squaredGLMM* function from the package *MuMIn* version 1.47.1 (Nakagawa & Schielzeth, 2013) was used to calculate the R^2 values. Posterior mean values, standard errors and 95 % confidence intervals for the different traits and lines were calculated using the *emmeans* package version 1.6.3 (Lenth, 2019). Correlations between the flowering onset and the other variables (control line) were plotted using the *corrplot* function from the package *corrplot* version 0.90 (Wei et al., 2017).

Results

Artificial selection experiment

No significant differences were found in flowering onset between the EFL and the CFL or between the OUTS2 line and the CFL for any of the populations ($X^2 = 3.512$, p $= 0.173$, Df $= 2$) (Figure 4a, Supplementary Material Tables S2 and S3). There were also no significant differences in any of the other traits measured (number of seeds, biomass, shoot growth) between any of the artificial selection lines and the control line ($X^2_{\text{seed number}}$ $= 5.35$, p $_{\text{seed number}} = 0.07$, Df $_{\text{seed number}} = 2$; $X^2_{\text{biomass}} = 0.49$, p $_{\text{biomass}} = 0.78$, Df $_{\text{biomass}} = 2$; $X^2_{\text{shoot growth}} = 3.98$, p $_{\text{shoot growth}} = 0.14$, Df $_{\text{shoot growth}} = 2$) (Figure 4b, 4c and 4d, Supplementary Material Tables S2 and S3). However, significant differences were found between FRO and PIC populations for the number of seeds and the biomass ($X^2_{\text{seed number}}$ $= 22.87$, p $_{\text{seed number}} < 0.001$, Df $_{\text{seed number}} = 1$; $X^2_{\text{biomass}} = 22.48$, p $_{\text{biomass}} < 0.001$, Df $_{\text{biomass}} = 1$) (Figure 4b, and 4c Supplementary Material Tables S2 and S3). Plants from PIC population had a greater number of seeds and higher biomass than those from FRO population (Figure 4b and 4c, Supplementary Material Table S4). Posterior mean values, standard errors, and 95 % confidence intervals for each line and variable are shown in Supplementary Material Table S5.
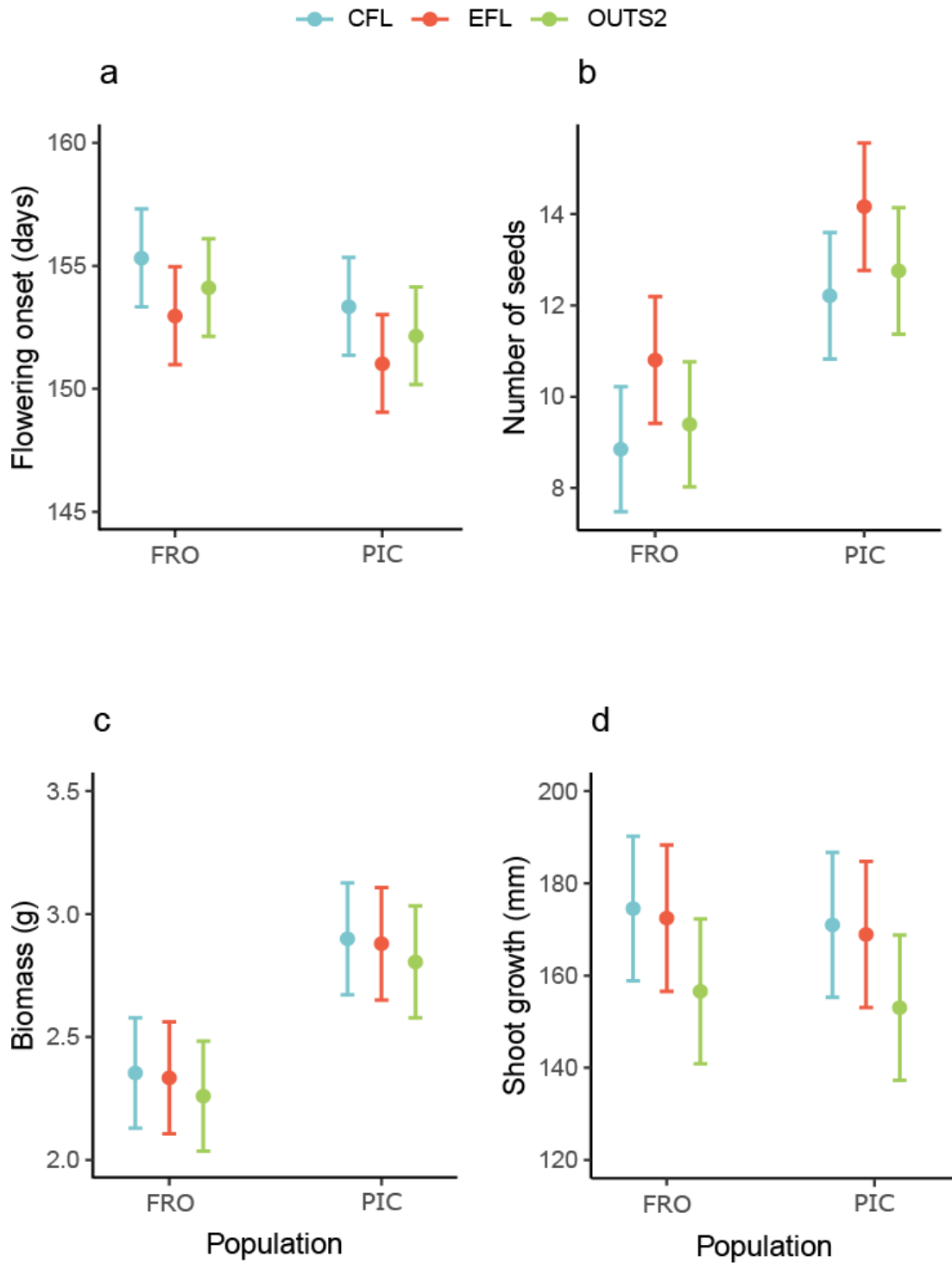
Figure 4. Effect of the artificial selection lines (EFL and OUTS2) on the flowering onset and other traits of *L. angustifolius* L. a) flowering onset, b) number of seeds, c) biomass, d) shoot growth. CFL: control flowering line; EFL: early flowering line (selfed); OUTS2: F3 generation of outbred line (OUT) resulting from two generations of self-pollination.

Gene flow experiment

Significant differences were found in flowering onset between the SPL2 and the CFL. SPL2 individuals flowered an average of 10 days earlier than those from the CFL in FRO population, and 4 days earlier in PIC population ($X^2 = 17.12$, p < 0.001, Df = 1) (Figure 5a, Supplementary Material Tables S4, S6 and S7). Significant differences were also found between lines in shoot growth. Individuals from the SPL2 grew an average of 25 mm less than those from the CFL in FRO population. In PIC population, they grew 21 mm less on average than individuals from the CFL ($X^2 = 8.26$, p = 0.004, Df = 1) (Figure 5b, Supplementary Material Tables S4, S6 and S7). According to the R^2 values, fixed effects explained 10.2 % of the variation for the flowering onset, and 11.6 % for shoot growth, whereas random effects explained 5 % and 39.6 % of the variation, respectively (Supplementary Material Table S6). No significant differences were found between lines for the number of seeds or biomass ($X^2_{\text{seed number}} = 2.16$, $p_{\text{seed number}} = 0.14$, $Df_{\text{seed number}} = 1$; $X^2_{\text{biomass}} = 0.003$, $p_{\text{biomass}} = 0.96$, $Df_{\text{biomass}} = 1$), but significant differences were found for these traits between populations ($X^2_{\text{seed number}} = 8.75$, $p_{\text{seed number}} = 0.003$, $Df_{\text{seed number}} = 1$; $X^2_{\text{biomass}} = 21.79$, $p_{\text{biomass}} < 0.001$, $Df_{\text{biomass}} = 1$) (Figure 5b and 5c, Supplementary Material Tables S6 and S7). In the same way as in the artificial selection experiment, individuals from PIC population had a greater number of seeds and higher biomass than plants from FRO population (Figure 5b and 5c, Supplementary Material Table S4). Posterior mean values, standard errors, and 95 % confidence intervals for each line and variable are shown in Supplementary Material Table S8.
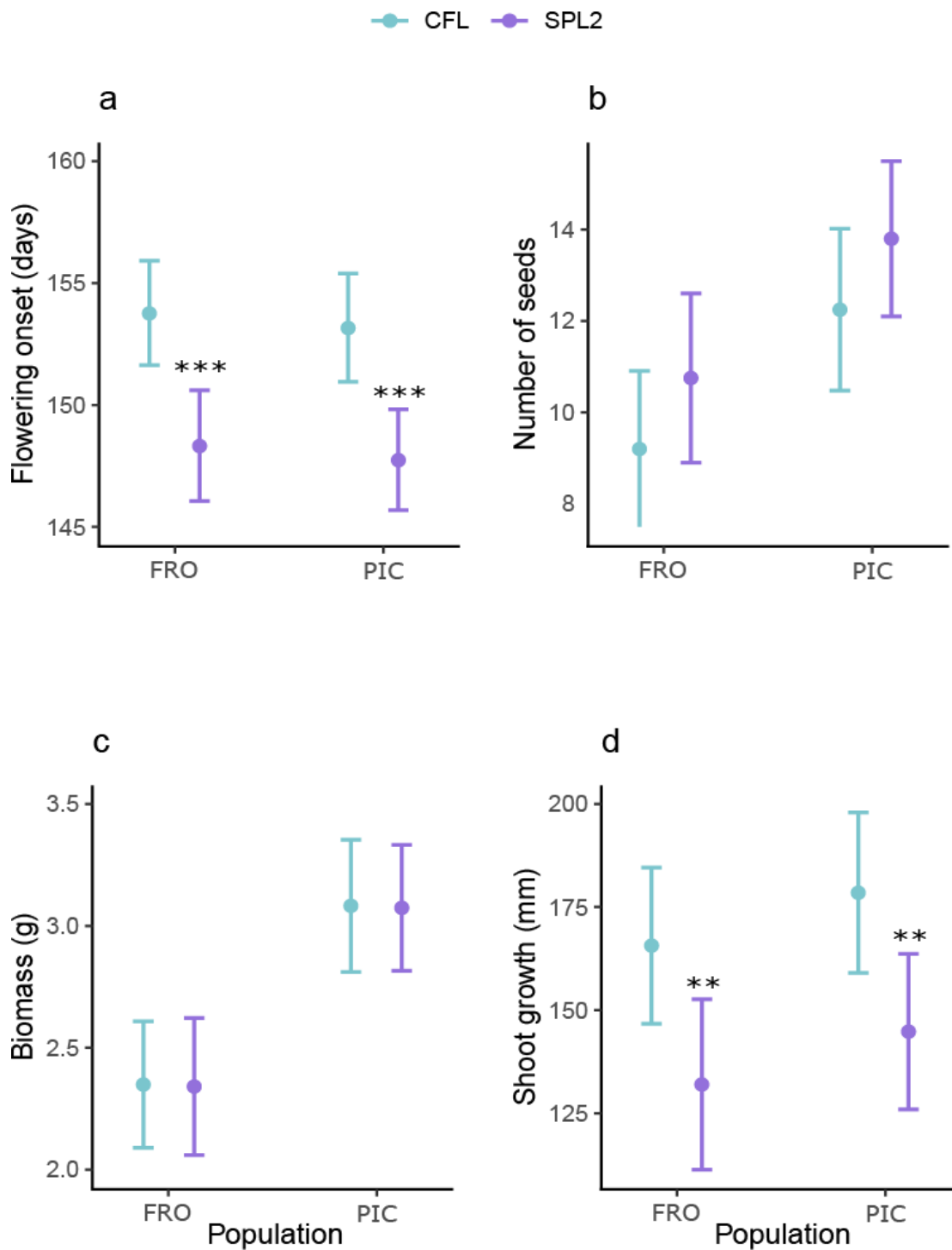
Figure 5. Effect of the assisted gene flow line (SPL2) on the flowering onset an other traits of *L. angustifolius* L. a) flowering onset b) number of seeds, c) biomass, d) shoot growth. CFL: control flowering line; SPL2: F3 generation resulting from two generations of self-pollination of the gene flow line (GFL).

Correlations between traits

Flowering onset was correlated with number of seeds, biomass and shoot growth (Supplementary Material Figure S1). In both FRO and PIC populations, plants that flowered earlier also had a greater number of seeds ($r_{FRO} = -0.49$, $r_{PIC} = -0.36$), and higher biomass ($r_{FRO} = -0.34$, $r_{PIC} = -0.50$) (Supplementary Material Figure S1a,b). On the contrary, shoot growth had a negative correlation with flowering onset in FRO population ($r = -0.15$) (Supplementary Material Figure S1a) but positive in PIC population ($r = 0.18$) (Supplementary Material Figure S1b).

Discussion

According to our results, artificial selection and assisted gene flow differed in their effectiveness in advancing flowering under natural conditions. Lines from the artificial selection experiment did not change their flowering onset or any of the other traits studied with regard to control. On the other hand, the line from the assisted gene flow experiment significantly advanced flowering in both populations, while at the same time their shoot growth was reduced. The results obtained under natural and controlled conditions for the assisted gene flow experiment were similar (see Chapter 3), while the modifications in flowering onset and other traits observed in the artificial selection experiment under controlled conditions were not observed in the field. These results point to the existence of a genotype-by-environment interaction in the case of artificial selection, and also indicate that the mechanisms operating on the expression of flowering onset may be different in each case. Thus, these results underline the need for field studies and research in different environmental conditions to assess whether the implementation of the different facilitated adaptation strategies could be beneficial in conservation or restoration actions.

Effects of artificial selection

In this case, none of the artificial selection lines (EFL and OUTS2) showed a significant advance in flowering onset, compared to the control line, even though the year in which the *in-situ* experiment was conducted was drier than usual (Table S1). All plants from the different lines flowered on average 154-155 days after sowing. This contrasts with the results obtained under controlled conditions, in which the selection lines did significantly advance flowering compared to the control line in northern populations, and plants belonging to the different lines flowered on average between 115 and 136 days (Sacristán-Bajo et al., 2023, Chapter 1). Plants need to accumulate certain degrees of heat to start flowering (Galán et al., 2001). Since the common garden located in the field presents a colder temperature than the one established in controlled conditions, it is not surprising that flowering is generally delayed in the latter. These aspects would explain why plants flower later in the experiment under natural conditions than under controlled conditions, but they do not explain why under controlled conditions we found differences between the control and selection lines and in the field experiment we did not. Since the mortality and germination rate was homogeneous for all lines and populations, the differences found in the results between both experiments point to a strong genotype-by-environment (G x E) interaction. This G x E interaction refers to those cases in which phenotypic plasticity varies between genotypes (crossing reaction norms) (Clausen et al., 1940; Núñez-Farfán & Schlichting, 2001). This can occur when the degree of expression of the genes that control the trait varies between different environments (Basford & Cooper, 1998). G x E interactions have important implications, since predictions about the responses of selected populations or lines under certain environmental conditions may be altered if these conditions change (Matesanz & Ramírez-Valiente, 2019). The fact that Sacristán-Bajo et al., 2023 (Chapter 1) found that the selection differential and the

response to selection were greater in northern than in southern populations supports the idea that northern populations in their natural conditions do not have a selective pressure to flower earlier as that experienced by populations in the south. It should also be noted that the individuals belonging to the OUTS2 line sown in the field are genetically different from those coming from the OUT and OUTS lines sown under controlled conditions. It is expected that the hybrids and their F1 descendants (OUT and OUTS respectively) tested under controlled conditions have higher heterozygosity than the field-tested F2 (OUTS), which have already gone through two generations of self-pollination.

There are also other factors that are not being considered and could be influencing these results, such as vernalization, the composition of the substrate (Ausín & Alonso-Blanco, 2005) or the biotic relationships that may be established under different conditions. For example, vernalization is a process by which the flowering time of certain plants is promoted by their exposure to low temperatures (Adhikari et al., 2012). The flowering onset of different lupin species is controlled by vernalization, but some cultivars of *Lupinus angustifolius* have been selected so as not to require this process (Gladstones & Hill, 1969; Rahman & Gladstones, 1974). It could have happened that by selecting early flowering lines under controlled conditions with reduced exposure to low temperatures we are selected vernalization-independent genotypes (*i.e.* that do not require low temperatures to flower), and that the control line has both vernalization-dependent and independent genotypes. Thus, under natural conditions in which plants experience a period of low temperatures, all vernalization dependent and independent genotypes would have synchronized their flowering, and therefore no differences have been observed between the control and the artificial selection lines.

Effects of assisted gene flow

In the case of assisted gene flow, the changes between lines observed in the experiment carried out in the *ex-situ*-controlled conditions were maintained in the experiment performed under natural conditions. Interestingly, even after two generations of self-crossing following manual crosses between the two populations, and with genotypes less likely to be heterozygous, the differences between lines are still maintained. This reinforces and confirms the idea presented in Chapter 3, indicating that the changes made through assisted gene flow have a strong genetic basis, in some cases involving the incorporation of novel alleles. In this way, assisted gene flow can provide the genetic variability required to allow an appropriate adaptive response to future climate change scenarios (Aitken & Whitlock, 2013). Although the effects of assisted gene flow to improve the evolutionary potential of certain populations under natural conditions have been little studied, some approaches have recently been made using simulations to evaluate the use of assisted gene flow in different scenarios (Grummer et al., 2022). These authors concluded that assisted gene flow could be harmful in the short term (10-20 generations) due to the outbreeding depression. When the target trait is polygenic (*i.e.* regulated by numerous loci with small effect), it may take up much longer for assisted gene flow to produce favourable effects (Grummer et al., 2022). However, and even though flowering onset is a polygenic trait, in our case the results have shown no detrimental effects of assisted gene flow even in the first generations. On the other hand, and as explained in Chapter 3, it is expected that gene flow and modification of flowering initiation will lead to changes in other traits. Due to resource limitations and also for purely mechanistic reasons, the traits of organisms are closely related to each other, implying trade-offs (Reich, 2014; Sobral, 2021). In our case, plants belonging to the SPL2 had a lower shoot growth, but they did not show any change in reproductive success or

biomass. The reduction in growth is in line with what was observed in the experiment carried out in the *ex-situ* common garden, where this reduction was also observed. In terms of reproductive success, the *ex-situ* common garden experiment also showed no change in the number of seeds, although these seeds had a greater weight (Chapter 3). However, in the case of the experiment under natural conditions, it was not possible to measure the weight of the seeds, so we do not know whether this component of reproductive success would have been affected. Although no changes in reproductive success or biomass were observed in the SPL2 line, the correlation analysis carried out with the CFL did show that plants that flower earlier produce greater number of seeds and higher biomass. As mentioned in Chapter 1, it is common for these genetic correlations to vary depending on the environment, since combinations of traits that are adaptively advantageous in certain environmental conditions may not be advantageous in others (Sheth & Angert, 2016; Sobral, 2021).

Artificial selection vs. *assisted gene flow*

In the light of the observed results, we can conclude that assisted gene flow was a more effective tool than artificial selection to advance the flowering onset of *L. angustifolius* in natural conditions. Although artificial selection under controlled conditions advanced flowering onset by a greater number of days than assisted gene flow in the northern populations (see Chapters 1 and 3), under natural conditions this advance of artificial selection was not reflected, while the changes were maintained in the case of assisted gene flow. It is therefore likely that in artificial selection and assisted gene flow experiments we are modifying the onset of flowering by different mechanisms. Through assisted gene flow we are modifying at the genetic level the onset of flowering by introducing early flowering alleles from southern populations into northern populations, as was also observed in Chapter 2 through genomic analyses. In the case of artificial

selection, on the other hand, the modifications observed in the onset of flowering under controlled conditions are probably more likely to be due to regulatory processes, such as epigenetic mechanisms or changes in regulatory genes that are more affected by environmental conditions (Ausín & Alonso-Blanco, 2005; Mylne et al., 2004). Plants belonging to the SPL2 line of gene flow contain in average 50 % of the genome from northern populations and 50 % of the genome from southern populations. This fact, in addition to bringing forward flowering, decreases their northern identity and perhaps also their ability to adapt to other environmental factors that we do not know or are not taking into account. It is likely that these unfavourable aspects (maladaptation) will be eliminated in later generations (Aitken & Whitlock; Grummer et al., 2022), but the possibility of performing several generations of backcrossing with the populations of origin should also be explored, so that the genome of the resulting individuals recovers the genetic identity of the original populations while retaining the desired trait, as indicated in Chapter 3.

In the end, flowering is a very complex aspect that involves the interaction of numerous genes and different genetic and epigenetic mechanisms (Ausín & Alonso-Blanco, 2005; Blümel et al., 2015), and therefore its interpretation and prediction cannot be done in a simple way.

Final remarks

With our experiments, we have observed that assisted gene flow had greater effect than artificial selection, and that the effect is more permanent in varying environmental conditions. This outlines the importance of carrying out experiments in several common gardens under different environmental conditions. In this case, since the experiment under natural conditions was carried out in a colder area than the common garden under

controlled conditions, it would be desirable to implement additional field experiments in natural conditions at warmer temperatures to simulate the effects of global warming. Climate change is a challenge for biodiversity conservation and ecological restoration (Hancock et al., 2011). For this reason, studies such as this one are necessary to help us determine the adaptive potential of populations to climate change, and to find the best strategies to enhance the probability of their survival.

Acknowledgements

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Supplementary Material

Table S1. Populations of *Lupinus angustifolius* L. and common garden site involved in the study. Town, region, geographical coordinates (decimal degrees, WGS84) and climate variables associated to the populations (1985-2015 period) and to the common garden site are shown. March-June period corresponds with the period when plants are producing fruits and seeds. Climate data were obtained from ClimateEU (Marchi et al., 2020), and from the climatic station closest to the plot where the *in-situ* experiment was carried out (Salamanca).

Acronym	Town	Region	Latitude	Longitude	Elevation (m.a.s.l.)	Annual mean temperature (º Celsius)	March-June precipitation (mm)
PIC (1985-2015)	Zarapicos	Central Spain	41.0043	-5.8130	820	12.6	89
PIC (2021)	Zarapicos	Central Spain	41.0043	-5.8130	820	14.1	62
FRO (1985-2015)	Zafrón	Central Spain	41.0241	-6.0281	840	12.4	92

Table S2. Effect of the different lines and population on flowering onset, number of seeds, biomass and shoot growth of *Lupinus angustifolius* plants in the artificial selection experiment. Estimates and significance level for fixed effects are shown. Genotype was included as a random factor. EFL: early flowering line; OUTS2: F3 generation of outbred line resulting from two generations of self-pollination of OUT genotypes. Missing factors (CFL = control line and population FRO) are included in the intercept.

Fixed effects	Parameter value	Standard error	z value	p value	R^2_m	R^2_c
Flowering onset	-	-	-	-	0.065	0.115
Intercept	5.180	0.023	224.220	<0.001	-	-
EFL	-0.008	0.008	-0.970	0.334	-	-
OUTS2	-0.015	0.008	-1.870	0.061	-	-
PIC	-0.013	0.007	-1.940	0.053	-	-
Germinated seed weight	-0.001	<0.001	-6.100	<0.001	-	-
Number of seeds	-	-	-	-	0.063	0.117
Intercept	0.155	2.418	0.064	0.949	-	-
EFL	0.545	0.859	0.634	0.025	-	-
OUTS2	1.953	0.868	2.249	0.526		
PIC	3.359	0.702	4.783	<0.001	-	-
Germinated seed weight	0.081	0.021	3.763	<0.001	-	-
Biomass	-	-	-	-	0.082	0.127
Intercept	0.101	0.396	0.254	0.799	-	-
EFL	-0.094	0.141	-0.668	0.889	-	-
OUTS2	-0.020	0.142	-0.139	0.504		
PIC	0.546	0.115	4.741	<0.001	-	-
Germinated seed weight	0.021	0.004	5.949	<0.001	-	-
Shoot growth	-	-	-	-	0.029	0.386
Intercept	183.808	27.428	0.064	0.949	-	-
EFL	-17.950	9.814	0.634	0.526		
OUTS2	-2.063	9.885	2.249	0.025	-	-
PIC	-3.547	8.014	4.783	<0.001	-	-
Germinated seed weight	-0.086	0.244	3.763	<0.001	-	-

Table S3. Chi-square statistic, degrees of freedom and P-values of the Type II Wald chi-square tests of GLMM and LMM analyses to study the effect of selection line, population, and year on flowering onset, number of seeds, biomass and shoot growth of *Lupinus angustifolius* plants in the artificial selection experiment. Estimates and significance level for fixed effects are shown. Genotype was included as a random factor. EFL: early flowering line; OUTS2: F3 generation of outbred line resulting from two generations of self-pollination of OUT genotypes. Missing factors (CFL = control line and population FRO) are included in the intercept.

Fixed effects	X^2	Df	Pr $(>|X^2|)$
Flowering onset	-	-	-
Line	3.512	2	0.173
Population	3.755	1	0.053
Germinated seed weight	37.213	1	<0.001
Number of seeds	-	-	-
Line	5.350	2	0.069
Population	22.874	1	<0.001
Germinated seed weight	14.164	1	<0.001
Biomass	-	-	-
Line	0.493	2	0.781
Population	22.476	1	<0.001
Germinated seed weight	35.389	1	<0.001
Shoot growth	-	-	-
Line	3.978	2	0.137
Population	0.196	1	0.658
Germinated seed weight	0.125	1	0.723

Table S4. Observed mean ±SD values for the different traits measured and the different lines tested in both populations.

	Flowering onset (days)				Number of seeds				Biomass (g)				Shoot growth (mm)			
	CFL	EFL	OUTS2	SPL2	CFL	EFL	OUTS2	SPL2	CFL	EFL	OUTS2	SPL2	CFL	EFL	OUTS2	SPL2
FRO	155.45 ±7.07	152.933 ±8.06	154.51 ±6.36	145.76 ±7.04	9.50 ±8.71	9.37 ±6.66	10.27 ±8.98	10.59 ±6.36	2.37 ±1.49	2.29 ±1.30	2.29 ±1.32	2.38 ±1.31	147.53 ±48.10	152.24 ±45.74	181.10 ±45.23	122.12 ±43.08
PIC	152.64 ±8.16	152.23 ±8.23	151.22 ±8.58	148.79 ±7.14	12.03 ±10.05	13.51 ±9.27	13.92 ±8.91	14.22 ±9.49	2.97 ±1.82	2.94 ±1.69	2.75 ±1.53	3.16 ±1.88	173.66 ±58.64	159.60 ±41.13	161.58 ±55.56	152.96 ±49.23

Table S5. Posterior mean values, standard errors and 95 % confidence intervals for the different traits and lines of *Lupinus angustifolius* plants grown in the artificial selection experiment. CFL: control line; EFL: early flowering line; OUTS2: F3 generation of outbred line resulting from two generations of self-pollination of OUT genotypes.

	FRO				PIC			
	Mean	**Std. error**	**2·5%**	**97·%**	**Mean**	**Std. error**	**2·5%**	**97·%**
Flowering onset 2020	-	-	-	-	-	-	-	-
CFL	5.045	0.007	5.033	5.058	5.033	0.007	5.020	5.046
EFL	5.038	0.007	5.025	5.050	5.025	0.007	5.012	5.038
OUTS2	5.030	0.007	5.017	5.043	5.017	0.007	5.004	5.031
Number of seeds	-	-	-	-	-	-	-	-
CFL	8.850	0.698	7.480	10.200	12.210	0.707	10.820	13.600
EFL	9.390	0.697	8.030	10.800	12.750	0.708	11.360	14.100
OUTS2	10.800	0.707	9.410	12.200	14.160	0.712	12.770	15.600
Biomass	-	-	-	-	-	-	-	-
CFL	2.350	0.114	2.130	2.580	2.900	0.116	2.670	3.130
EFL	2.260	0.114	2.040	2.480	2.810	0.116	2.580	3.030
OUTS2	2.330	0.116	2.110	2.560	2.880	0.117	2.650	3.110
Shoot growth	-	-	-	-	-	-	-	-
CFL	175	8.000	159	190	171	8.020	155	187
EFL	157	8.010	141	172	153	8.040	137	169
OUTS2	172	8.090	157	188	169	8.080	153	185

Table S6. Effect of the different lines and population on flowering onset, number of seeds, biomass and shoot growth of *Lupinus angustifolius* plants in the assisted gene flow experiment. Estimates and significance level for fixed effects are shown. Genotype was included as a random factor. SPL2: F3 generation resulting from two selfed generations after the crossing of the northern populations with pollen from the southern populations. Missing factors (CFL = control line and population FRO) are included in the intercept.

Fixed effects	Parameter value	Standard error	z value	p value	R^2_m	R^2_c
Flowering onset	-	-	-	-	0.102	0.152
Intercept	5.151	0.029	176.430	<0.001	-	-
SPL2	-0.036	0.009	-4.140	<0.001	-	-
PIC	-0.004	0.008	-0.460	0.644	-	-
Germinated seed weight	-0.001	0.000	-3.990	<0.001	-	-
Number of seeds	-	-	-	-	0.044	0.130
Intercept	5.057	3.486	1.451	0.147	-	-
SPL2	1.555	1.058	1.469	0.142	-	-
PIC	3.051	1.031	2.958	0.003	-	-
Germinated seed weight	0.037	0.031	1.198	0.231	-	-
Biomass	-	-	-	-	0.063	0.107
Intercept	0.652	0.531	1.229	0.219	-	-
SPL2	-0.008	0.161	-0.050	0.960	-	-
PIC	0.733	0.157	4.668	<0.001	-	-
Germinated seed weight	0.015	0.005	3.231	0.001	-	-
Shoot growth	-	-	-	-	0.116	0.512
Intercept	180.377	38.608	4.672	<0.001	-	-
SPL2	-33.663	11.711	-2.875	0.004		
PIC	12.820	11.414	1.123	0.261	-	-
Germinated seed weight	-0.130	0.339	-0.385	0.701	-	-

Table S7. Chi-square statistic, degrees of freedom and P-values of the Type II Wald chi-square tests of GLMM and LMM analyses to study the effect of selection line, population, and year on flowering onset, number of seeds, biomass and shoot growth of *Lupinus angustifolius* plants in the assisted gene flow experiment. Estimates and significance level for fixed effects are shown. Genotype was included as a random factor. SPL2: F3 generation resulting from two selfed generations after the crossing of the northern populations with pollen from the southern populations. Missing factors (CFL = control line and population FRO) are included in the intercept.

Fixed effects	X^2	Df	Pr $(>|X^2|)$
Flowering onset	-	-	-
Line	17.122	1	<0.001
Population	0.214	1	0.643
Germinated seed weight	15.982	1	<0.001
Number of seeds	-	-	-
Line	2.159	1	0.142
Population	8.750	1	0.003
Germinated seed weight	1.435	1	0.231
Biomass	-	-	-
Line	0.003	1	0.960
Population	21.790	1	<0.001
Germinated seed weight	10.441	1	0.001
Shoot growth	-	-	-
Line	8.263	1	0.004
Population	1.261	1	0.261
Germinated seed weight	0.148	1	0.701

Table S8. Posterior mean values, standard errors and 95 % confidence intervals for the different traits and lines of *Lupinus angustifolius* plants grown the assisted gene flow experiment. CFL: control line; SPL2: F3 generation resulting from two selfed generations after the crossing of the northern populations with pollen from the southern populations.

|	FRO				PIC			
	Mean	**Std. error**	**2·5%**	**97·%**	**Mean**	**Std. error**	**2·5%**	**97·%**
Flowering onset 2020	-	-	-	-	-	-	-	-
CFL	5.035	0.007	5.021	5.049	5.031	0.007	5.017	5.046
SPL2	4.999	0.008	4.984	5.015	4.995	0.007	4.981	5.009
Number of seeds	-	-	-	-	-	-	-	-
CFL	9.200	0.871	7.490	10.900	12.200	0.905	10.470	14.000
SPL2	10.800	0.947	8.890	12.600	13.800	0.867	12.100	15.500
Biomass	-	-	-	-	-	-	-	-
CFL	2.350	0.132	2.090	2.610	3.080	0.138	2.81	3.35
SPL2	2.340	0.144	2.060	2.620	3.070	0.132	2.82	3.33
Shoot growth	-	-	-	-	-	-	-	-
CFL	166	9.650	147	185	178	9.930	159	198
SPL2	132	10.530	111	153	145	9.620	126	164
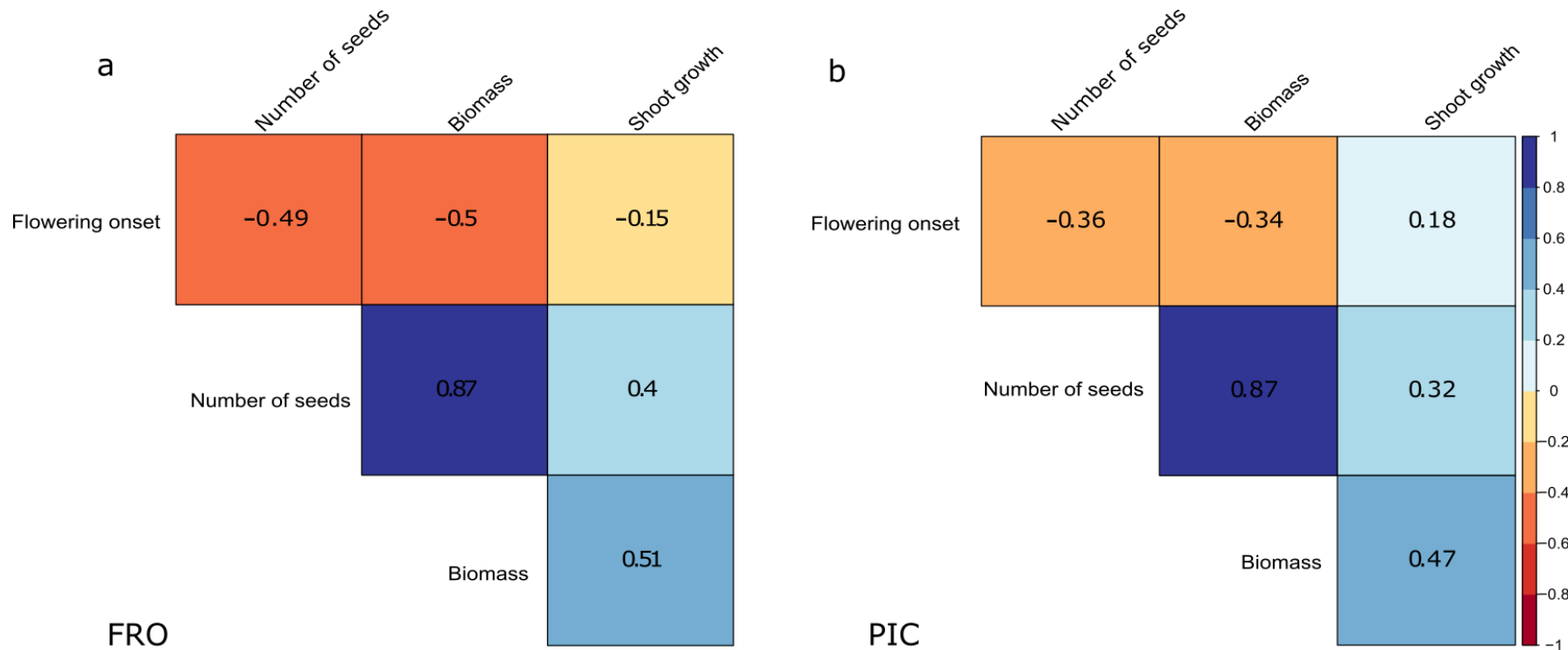
Figure S1. Correlations between flowering onset and other plant traits for the control line (CFL). a) correlations for FRO population. b) correlations for PIC population. Positive correlations are represented in cold colors and negative correlations are represented in warm colors. Non-significant correlations (p > 0.05) are represented in grey.

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GENERAL DISCUSSION

Overview

Adaptive responses at the genetic level may be the only way that certain species or populations can survive the environmental conditions imposed by climate change (Catullo et al., 2019; Gienapp et al., 2008; Jump & Peñuelas, 2005). In this sense, the main objective of this thesis has been to evaluate the potential risks and benefits of applying facilitated adaptation techniques (artificial selection and assisted gene flow) to modify a key trait for plant adaptation to climate change, using the legume *Lupinus angustifolius* L. as study species. For this purpose, we carried out a proof of concept of these novel strategies, by conducting artificial selection and assisted gene flow experiments under both controlled and natural conditions and evaluating the effects of these techniques at both phenotypic and genotypic levels. The following sections discuss: i) the potential effects that facilitated adaptation techniques had on the trait of interest, the flowering onset, ii) the indirect and unintended effects that modification of flowering onset can have, and iii) the implications that the use of these facilitated adaptation tools has in terms of biodiversity conservation.

Efficiency of artificial selection and assisted gene flow techniques

The effectiveness of artificial selection in modifying traits of interest depended to a large extent on the background of the populations on which the selection was applied, and also, on the environmental conditions where the experiments were carried out. Under controlled conditions, our results showed that artificial selection was effective in advancing the flowering date in the northern populations, but not in the southern ones (Chapter 1, Figure 4; Sacristán-Bajo et al., 2023). This is probably related to the fact that the northern populations were genetically more diverse than the southern populations with regard to the genes studied linked to flowering and abiotic stress tolerance (Chapter 2, Table 1). These results suggest that the southern populations may have already been

subjected to natural selection to advance flowering time due to the warmer conditions to which they are exposed, and that this reduction in genetic diversity has made artificial selection less effective in them, given that genetic diversity is the raw material on which selection can operate (Lande, 1976). However, when the comparative experiment was performed in natural conditions, near the original site of Zarapicos population, no such differences were observed between control and selection lines.

On the other hand, assisted gene flow was an effective tool to advance flowering under both controlled and field conditions (Chapter 3, Figure 3; Chapter 4, Figure 5). These findings indicate that the gene flow approach was more effective and stable for advancing the flowering of *Lupinus angustifolius* than the artificial selection tool. This could be due to several reasons. First, the mechanisms that are acting to control the onset of flowering may be different. Flowering onset is a complex trait controlled by both genetic and epigenetic factors, and in which numerous genes are involved (Blümel et al., 2015; Weller & Ortega, 2015). Thus, in the case of artificial selection, we could be acting through genes that affect epigenetic or regulatory processes, and, where these effects are not similarly maintained among genotypes when environmental conditions change. In other words, there would be a genotype-by-environment (G x E) interaction where gene expression varies differently among genotypes between environments (Basford & Cooper, 1998). In the case of gene flow, the introduction of early flowering alleles from southern populations into northern populations may be associated to more structural genes whose expression is less dependent on the environmental conditions.

In any case, this and other studies confirm that these tools can be used to modify important traits for adaptation to climate change (*e.g*. Prieto-Benítez et al., 2021; Sheth & Angert, 2016), although there are many other additional aspects that need to be considered.

Side effects of artificial selection and assisted gene flow

It is impossible to interpret the attributes of organisms separately from one another, since the resources that plants allocate to a given function cannot be designated to another, resulting in trade-offs (Reich, 2014; Sobral, 2021). Then, as expected, the modification of the flowering onset has also been associated with changes in other traits, and these changes also depended on the source populations, the environmental conditions and the type of crossbreeding applied. In the case of artificial selection, the early flowering line obtained by self-crossing (EFL), showed higher LDMC and lower SLA than the control line in southern populations, but not in northern ones. It also exhibited lower shoot growth both in northern and southern populations (Chapter 1, Figure 5). However, the effect of the outcrossing (OUT) line was an increase in SLA and a decrease of height in southern populations, and lower biomass and shoot growth (Chapter 1, Figure 5). These results are related to a more efficient use of water under conditions of higher water stress (Rao & Wright, 1994; Wright et al., 1994) and with the existing trade-off between growth and flowering time (Reich, 2014). Although early flowering phenotypes have sometimes been associated with greater reproductive success (Munguía-Rosas et al., 2011), in our study artificial selection did not affect seed number or weight (Chapter 1, Supplementary Material Figure 5). The abundant water availability to which the plants were subjected under controlled conditions may have inactivated the selective pressures that could have triggered a differential response. In the case of the artificial selection experiment under natural conditions, in addition to the lack of significant changes in flowering onset, no changes in other traits were observed either (Chapter 4, Figure 4). This variety of results indicates that the effects on other traits resulting from artificial selection can be highly variable and, in many cases, difficult to predict.

One of the major concerns with the use of artificial selection is that it may lead to a reduction in genetic diversity, which in turn constraints the evolutionary potential of populations (Sheth & Angert, 2016; Whitt et al., 2002). Nevertheless, we did not observe this loss of genetic diversity in our case in the regions related to the traits of interest (Chapter 2, Table 1). This could be related to the fact that we have only performed one selection cycle, or maybe that the group of selected individuals harbor a wide genetic diversity in other genomic regions.

In terms of gene flow, plants from the flow line (GFL) under controlled conditions produced heavier seeds and showed lower shoot growth than plants from the control line (Chapter 3, Figure 4). Moreover, these differences were maintained in their offspring when the experiment was performed under field conditions (Chapter 4, Figure 5). In addition, in Chapter 3 it was also shown that the SNPs that had a significant change in allele frequencies between the GFL and the CFL also had a significant effect on flowering onset, seed weight and shoot growth (Chapter 3, Supplementary Material Figures S3 to S6). These results again indicate that the assisted gene flow treatments had a greater effect than the artificial selection treatments. Gene flow has also been associated with a possible risk of inducing a reduction in the reproductive success of individuals (Aitken & Whitlock, 2013; Frankham et al., 2011); however, we did not observe this in our experiments either. One aspect to take into account in this regard is that the plants belonging to the assisted gene flow line have 50 % of their genome belonging to another population, so it would be necessary to perform different cycles of backcrossing with the populations of origin, until the plants recover almost all of their native genome while maintaining the desired phenotype of early flowering. We have performed one backcross cycle, but up to six additional backcross cycles would be necessary to achieve this result.

Additionally, different correlations were also found between flowering onset and the other traits measured, both in the gene flow experiment and in the artificial selection experiment (Chapter 1, Figure 6; Chapter 3, Supplementary Material Figure S2; Chapter 4, Supplementary Material Figure S1). These correlations were different in some cases between northern and southern populations or depending on the populations, probably because certain trait combinations may result in some adaptive advantages in some situations but not in others (Sheth & Angert, 2016; Sobral, 2021). For example, northern populations, which are subject to less stressful conditions, can allocate more resources to growth. Southern populations, which are in a more stressful and unstable environment, must ensure the survival of their offspring, and for this reason are likely to produce fewer but larger seeds, while northern populations produce more seeds of smaller size. Due to these changes associated with the modification in the trait of interest, it is of vital importance to know in detail the gene networks that control the target trait, in order to be able to better predict the effects that facilitated adaptation techniques can produce.

Implications for conservation

According to our results, *Lupinus angustifolius* populations at higher latitudes have greater genetic diversity and adaptive potential for earlier flowering onset than southern populations. Thus, they could evolve faster to generate early flowering phenotypes. Meanwhile, southern populations may be at greater risk of extinction due to climate change. In this sense, facilitated adaptation approaches can help identify vulnerable populations that need conservation efforts. Furthermore, artificial selection and assisted gene flow can improve the evolutionary potential of certain populations and identify suitable donors and recipients for assisted gene flow actions. Our study was conducted as a proof-of-concept, but if *L. angustifolius* were endangered and its subsistence depended on the advancement of flowering onset, facilitated adaptation tools could improve the

adaptive potential of northern populations under climate change conditions. These tools also identified southern populations as more vulnerable but containing the earliest flowering alleles necessary for assisted gene flow actions in northern populations.

Our experiments showed that using assisted evolution techniques through artificial selection and assisted gene flow is promising in theory, but complex when it comes to implementation due to the polygenic nature of most traits, different trade-offs and correlations between traits, and genotype-by-environment interactions (G x E). At the methodological level, we also encountered some difficulties. Since we used an almost strictly autogamous species, manual crosses had low efficiency and it was difficult to obtain a large number of seeds. In addition, crosses can have an added difficulty in assisted gene flow if the flowering times of different populations do not overlap (Wadgymar et al., 2015). Therefore, the success of these techniques partly depends on the nature of the species used. However, with the appropriate knowledge and tools, this efficiency could be improved, and limitations could be overcome.

The concepts behind facilitated adaptation approaches, such as artificial selection and gene flow, are not new. Artificial selection has been a tool widely used throughout human history to improve some traits of interest in organisms, especially in species used for crop and livestock production (Conner, 2016; Dempewolf et al., 2014), and gene flow has been proposed as a way to improve the fitness of small inbred or maladapted populations (Frankham, 2015; Morente-López et al., 2021; Prieto-Benítez et al., 2021; Sexton et al., 2011). However, their direct application in conservation is still in its infancy, probably due to concerns about their use (Aitken & Whitlock, 2013; Torres et al., 2023; Wadgymar et al., 2015). Despite the reticence among conservationists about using these assisted evolution tools, some interventions on highly threatened populations have had positive results (Hedrick, 1995; van Oppen et al., 2015). In short, facilitated adaptation tools

require further studies before use, but the results may be promising. Studies such as those presented in this thesis provide information on the present and future situation of populations in the face of climate change and open the way for further research into new conservation tools to improve species survival.

As a final thought, it is important to note that while using techniques that significantly alter the course of natural evolution may seem concerning, human activities (agriculture, infrastructures, pollution, climate change, etc.…) are already deeply affecting the evolution of organisms, in most cases without any assessment about the potential consequences in such depth. Therefore, if a population is in an extremely critical situation, why not try this alternative approach if it is a feasible option?

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GENERAL CONCLUSIONS

1. The use of artificial selection has been useful in advancing the flowering onset of *Lupinus angustifolius* under controlled conditions of a common garden experiment, but only in populations from higher latitudes. On the other hand, assisted gene flow treatment of northern populations with pollen from the south has also been able to advance the flowering onset under controlled conditions of a common garden experiment.

2. The earlier flowering onset caused by the selection lines also led to changes in other plant traits (height, biomass, growth, SLA and LDMC), although no change in reproductive success was observed. In parallel, the earlier flowering onset of the assisted gene flow treatment was accompanied by an increase in seed weight and lower plant shoot growth.

3. Genomic studies indicate that there is a marked genetic differentiation between the northern and southern populations of *Lupinus angustifolius*, with the former showing greater genetic variability.

4. In the assisted gene flow experiment, a clear association was detected between the changes observed at the phenotypic level and the changes observed at the genomic level with respect to flowering onset, seed weight and shoot growth.

5. The plants derived from the artificial selection treatments did not lead to the modification of any trait in the experiment under natural conditions, while the plants from the assisted gene flow treatment did maintain the changes in flowering onset and shoot growth observed in the experiment under controlled conditions. Thus, in this

case study, assisted gene flow appears to be more effective and have more stable effects than artificial selection.

6. Northern populations have greater adaptive potential to bring forward the flowering onset in the face of climate change, while southern populations are in a situation of greater vulnerability.