

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS



TESIS DOCTORAL

**Evolutionary implications in an aerial seed bank
of a Mediterranean pine**

**Implicaciones evolutivas en un banco aéreo de semillas
de un pino mediterráneo**

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Marta Callejas Díaz

DIRECTORES

Doctora Delphine Pierrette Grivet y Doctor José María Climent Maldonado

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DOCTORAL THESIS / TESIS DOCTORAL

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2022



A mis padres, Vicky y Chema

El amor y el aprendizaje se parecen en que nunca son un desperdicio

La memoria secreta de las hojas, Hope Jahren



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PARA OBTENER EL TÍTULO DE DOCTOR**

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Evolutionary implications in an aerial seed bank of a Mediterranean pine

Implicaciones evolutivas en un banco aéreo de semillas de un pino mediterráneo

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El camino ha sido largo, ha requerido esfuerzo, tiempo y paciencia, pero ha sido bonito. Como dice Anne Hope Jahren:

<<Una semilla sabe esperar. La mayoría de las semillas esperan un año antes de empezar a crecer; una semilla de cereza puede llegar a esperar hasta cien años sin ninguna dificultad. ¿Y a qué esperan exactamente? Cada semilla aguarda a que suceda algo, y solo ella sabe qué es. Debe darse una combinación única de temperatura, humedad y luz, junto a otros factores adicionales, para convencer a una semilla de que salte al exterior y se decida a cambiar. Para que aproveche su primera y única oportunidad de crecer>>.

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Abstract

Forests provide fundamental ecosystem services, including an important role as carbon sinks and maintaining genetic diversity. However, forest ecosystems are at risk under future climatic scenarios due to extreme climatic events including severe droughts, wildfires and pest and disease outbreaks. Facing these pressures, tree populations can persist through phenotypic plasticity, local adaptation or migration. Understanding the adaptive potential of forest species is of high relevance to predict the evolutionary responses of entire terrestrial ecosystems under a changing climate.

The Mediterranean region is one of the most prominent biodiversity and climate change hotspots. Mediterranean vegetation is well adapted to stress and disturbances, displaying different strategies to cope with environmental changes. Fire is the most important disturbance in the Mediterranean basin and plays a key role in the ecology and evolution of species. To cope with recurrent wildfires, pine species in the Mediterranean region have developed different fire-trait syndromes related to resistance and resilience, targeting individual survival and/or enhancing post-fire recruitment.

One of these fire-related traits is serotiny, which consists in the ability to retain viable seeds stored in aerial seed banks often for long periods of time. Importantly, these stored seeds are produced by the mother plant under varying ontogenetic and physiological conditions, leading to potential maternal effects that may affect offspring performance as demonstrated in many organisms.

Moreover, pollen contribution could change from year to year due to different male and female reproductive phenology and under important events such as wildfires, leading to differences in the offspring genetic variability among years. Therefore, aerial seed banks provide original (i.e. retrospective access to temporal and environmental series) yet challenging (i.e. multiple confounding effects) models for studying the evolutionary implications of genetic and non-genetic transgenerational effects in a temporal scale under a changing environment.

The main objectives of this work were (i) to determine the effect of maternal conditions, linked to different age cohorts, on seed performance in an aerial seed bank of a Mediterranean pine (*Pinus pinaster*); (ii) to determine the genetic changes in offspring quantitative traits across years and to explore whether environmental heterogeneity had an effect on selection; (iii) and, finally, to explore the genetic variability in two

temporarily contrasted cohorts of the aerial seed bank, and see if any link exists with offspring performance.

The main results of this work were the following: (i) We found that seed mass was related to maternal age and growth at the time of seed production. We also confirmed that seed mass had a positive effect on germination success, but beyond differences in seed mass, maternal age had a negative effect while mother tree size (diameter) had a positive effect on germination timing and subsequent survival. (ii) We also found differences in the heritability of seedling traits but no empirical evidence of evolutionary response through time in 12-year sequential cohorts. (iii) Finally, there were significant differences in the genetic variability among trees within the same year but not between a 12 years period. Therefore, it is unlikely that genetic difference across years is responsible for offspring performance differences.

Several outcomes emerged from this thesis. We highlight the importance of maternal conditions (in terms of age, growth and environment) combined with seed mass in shaping seedling establishment and how they can improve our understanding of the mechanisms underlying phenotypic changes and responses of plant offspring. Furthermore, we revealed that genetic variation across offspring exists among mother trees but not between two extreme years. This suggests that fire events did not impact the genetic makeup of offspring across a 12-years span, probably because of the homogenisation of pollen flow. However, pollen flow may be spatially structured leading to genetic variability across progenies from different mother trees that could partially explain the observed phenotypic differences. We observed a strong interaction between mother trees and cohorts, suggesting different interactions and mechanisms at the microscale. There is still a lack of evidence concerning whether those transgenerational effects can affect the evolutionary response. Our findings open new insights in the offspring performance deriving from long-term canopy seed banks, which deserve further attention since they may have high relevance for plant adaptation under climate changes and for adaptive forest management.

Keywords: Aerial seed bank; Fire-prone environment; Genetic variability; Germination; Heritability; Maternal effects; Ontogeny, *Pinus pinaster*; Plasticity; Pollen flow; Seed mass; Seedling size; Selection; Serotiny; Survival; Trans-generational effects; Yearly cohorts.

Resumen

Los bosques brindan múltiples servicios ecosistémicos fundamentales, destacando su papel clave como sumideros de carbono y el mantenimiento de la diversidad genética. Sin embargo, los ecosistemas forestales están en riesgo en los escenarios climáticos futuros debido a los eventos climáticos extremos asociados, tales como sequías severas, incendios forestales y brotes de enfermedades. Frente a estas presiones, las poblaciones de árboles pueden persistir a través de la plasticidad fenotípica, la adaptación local o la migración. Comprender el potencial adaptativo de las especies forestales es de gran importancia para predecir las respuestas evolutivas de los ecosistemas terrestres bajo un clima cambiante.

La región mediterránea es uno de los ‘puntos calientes’ o ‘hotspots’ de biodiversidad y cambio climático más destacados. La vegetación mediterránea está adaptada al estrés y a las perturbaciones, mostrando diferentes estrategias para hacer frente a los cambios ambientales. El fuego es la perturbación más importante de la cuenca mediterránea, desempeñando un papel clave en la ecología y evolución de las especies. Para hacer frente a los incendios forestales recurrentes, los bosques de pinos en la región mediterránea pueden mostrar diferentes síndromes de adaptación al fuego relacionados con la resistencia y la resiliencia, referidos a la supervivencia individual y/o la regeneración post-incendio.

Uno de los rasgos relacionados con la adaptación al fuego es la serotinia, que consiste en la capacidad de retener semillas viables almacenadas en bancos aéreos de semillas a menudo durante largos períodos de tiempo. Es importante destacar que estas semillas almacenadas son producidas por el árbol madre en diversas condiciones ontogénicas y fisiológicas, lo que da lugar a posibles efectos maternos que pueden afectar al comportamiento de la descendencia, como se ha demostrado en muchos organismos.

Además, la contribución paterna (polinizadores) podría cambiar de un año a otro debido a eventos importantes como son los incendios forestales, lo que podría dar lugar a diferencias en la variabilidad genética de la descendencia entre años. Por lo tanto, los bancos aéreos de semillas se muestran como modelos originales (estudio retrospectivo de series temporales y ambientales) pero desafiantes (múltiples factores de confusión) para estudiar las implicaciones evolutivas de los efectos transgeneracionales genéticos y no genéticos en una escala temporal bajo un entorno cambiante.

Los principales objetivos de este trabajo son (i) determinar el efecto de las condiciones maternas, vinculadas a diferentes cohortes de edad, sobre el comportamiento de las semillas en un banco aéreo de semillas de un pino mediterráneo (*Pinus pinaster*); (ii) determinar los cambios genéticos en dos caracteres cuantitativos de la descendencia a lo largo de los años y explorar si la heterogeneidad ambiental tiene un efecto en la respuesta a la selección; (iii) y, finalmente, explorar la variabilidad genética en dos cohortes, contrastadas en el tiempo, del banco aéreo de semillas y ver si existe alguna relación con el comportamiento de la descendencia.

Los resultados principales de este trabajo son los siguientes: (i) Encontramos que el peso de la semilla estaba relacionado con la edad y el crecimiento del árbol madre en el momento en que produjo las semillas. También confirmamos que el peso de la semilla tuvo un efecto positivo en el éxito de germinación, pero más allá de las diferencias en el peso de la semilla, la edad del árbol madre tuvo un efecto negativo sobre el tiempo de germinación y supervivencia, mientras que el tamaño (diámetro) del árbol madre tuvo un efecto positivo. (ii) encontramos diferencias en la heredabilidad de los caracteres cuantitativos de las plántulas, pero no una evidencia empírica de respuesta a la selección a lo largo de las cohortes en los 12 años consecutivos analizados. (iii) Finalmente, encontramos diferencias significativas en la variabilidad genética entre árboles dentro del mismo año, pero no entre las dos cohortes temporales contrastadas. Por lo tanto, es poco probable que la variación genética entre años sea la responsable de las diferencias observadas en el comportamiento de la descendencia.

Diversos resultados surgen de esta tesis. Destacamos la importancia de las condiciones maternas junto con el peso de la semilla en el establecimiento de las plántulas y cómo pueden mejorar nuestra comprensión acerca de los mecanismos subyacentes a los cambios fenotípicos y al desempeño de la descendencia. Además, mostramos que existe variación genética en la descendencia entre árboles madre, pero no entre dos años extremos. Esto sugiere que los incendios no afectaron a la composición genética de la descendencia en un rango de 12 años, probablemente debido a la homogeneización del flujo de polen. Sin embargo, el flujo de polen puede estar estructurado espacialmente dando lugar a una variabilidad genética entre descendientes de diferentes árboles madre que podría explicar parcialmente las diferencias fenotípicas observadas. Observamos también una fuerte interacción entre árboles madre y cohortes, lo que sugiere diferentes interacciones y mecanismos a pequeña escala. Sin embargo, todavía faltan evidencias

acerca de si dichos efectos transgeneracionales pueden afectar la respuesta evolutiva.

Nuestros hallazgos abren nuevas perspectivas sobre el comportamiento de la descendencia procedente de bancos aéreos de semillas, que necesitan ser indagados en mayor medida, ya que pueden tener una gran relevancia para la adaptación de las plantas bajo el cambio climático y para el manejo adaptativo de los bosques naturales.

Palabras clave: Ambiente propenso a incendios; Banco aéreo de semillas; Cohortes anuales; Efectos maternos; Efectos transgeneracionales; Flujo de polen; Germinación; Heredabilidad; Ontogenia, Peso de semilla; *Pinus pinaster*; Plasticidad; Selección; Serotinia; Supervivencia; Tamaño de las plántulas; Variabilidad genética.

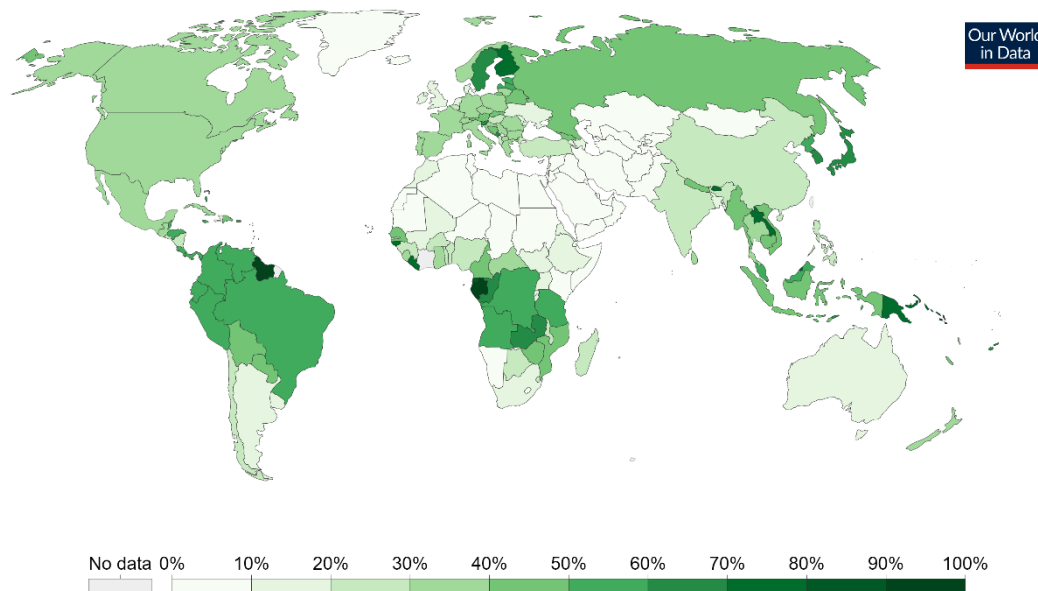




1. Introduction

1.1 Forest ecosystems in a changing environment

Forests cover nearly a third of the Earth's surface, the 31% of the global land area, which corresponds to 4.060 billion hectares, i.e. approximately 0.52 ha of forest per person (Fig. 1; FAO, 2020).



Source: UN Food and Agriculture Organization (FAO) and historical sources.

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Fig. 1. World share of total land area that is covered by forest in 2020 (%). Forest area is land under natural or planted stands of trees of at least 5 meters in situ, whether productive or not, and excludes tree stands in agricultural production systems (Ritchie and Roser, 2021). In Spain, 37.2% of the land area is forested. Source: OurWorldInData.org.

Forests are complex ecosystems that provide habitats for a multitude of plants, animals and microorganisms that coexist, each playing a key role and making the forest biological diversity unique. The world's forests vary broadly in their species composition from temperate and boreal conifer forests with relatively few tree species to tropical forests containing hundreds of them (White *et al.*, 2007).

Forests bring fundamental ecosystem services providing a wide variety of biological, economic and social benefits. They provide services such as soil stabilization and erosion control, water flows regulation in terms of quantity and quality, air and water purification, climate regulation through carbon storage and biological diversity conservation. Forests are usually managed for multiple purposes and supply a variety of products including timber, fruits, leafy vegetables, fodder, chemical substances of pharmaceutical and

cosmetic value, as well as resins. Furthermore, highlighting the positive relationship between nature and health, forests enhance human health and well-being by providing recreational, cultural and aesthetic services (Krieger, 2001; Karjalainen *et al.*, 2010; Nilsson *et al.*, 2010; Loo *et al.*, 2015).

In particular, trees are foundational and keystone species of forest ecosystems (see Fig. 2 for a global map of forest trees) as their architecture and functional and physiological characteristics define forest structure, influencing ecosystem processes such as nutrient cycling, light availability and microclimate (Ellison *et al.*, 2005; Dyderski *et al.*, 2018).

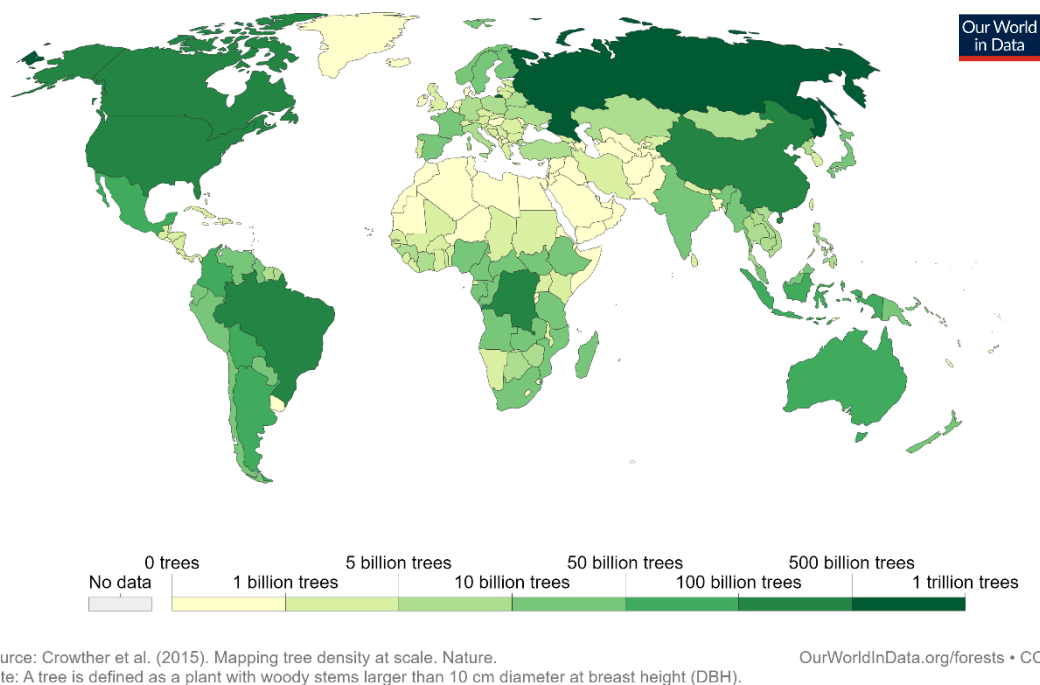


Fig. 2. Global map of forest tree density. Number of trees that exist globally and by country. It is estimated that there are approximately 3.04 trillion trees in the world. In Spain, there are an estimated 11.39 billion trees (Crowther *et al.*, 2015). Source: OurWorldInData.org.

Deforestation, forest management, disturbances and land-use change are currently identified as responsible for a gross loss of approximately 15.3 billion trees each year. The global number of trees has fallen by approximately 45.8% since the onset of human civilization (post-Pleistocene). Although these rates of forest loss are currently highest in tropical regions, the scale and consistency of this negative human effect on a global scale highlights how historical land-use decisions have shaped natural ecosystems (Crowther *et al.*, 2015).

In addition, climate change is causing changes in tree species composition and thus, in the local environment on which a variety of other species depend (Ellison *et al.*, 2005). Therefore, studying the adaptive potential of forest trees is fundamental to predict the evolutionary responses of entire terrestrial ecosystems under a climate change scenario.

1.1.1 Climate Change scenario.

A detailed report on the state of the European climate has confirmed that 2019 was the warmest year on record. The long-term view shows a clear warming trend across the last four decades (ESOTC, 2020). 2020 has been one of the six warmest years on record, with the highest recorded temperature exceeding 38 °C within the Arctic Circle. The global mean temperature for 2020 was 1.2 ± 0.1 °C above the 1850–1900 baseline (WMO, 2021; but see Schurer *et al.*, 2017 to see different pre-industrial baselines).

Annual average land temperatures over Europe are projected to continue increasing by more than the global average temperature. The largest temperature increases are projected over eastern and northern Europe in winter, and over southern Europe in summer. Annual precipitation is generally projected to increase in northern Europe and to decrease in southern Europe, thereby enhancing the differences between currently wet and dry regions (Fig. 3).

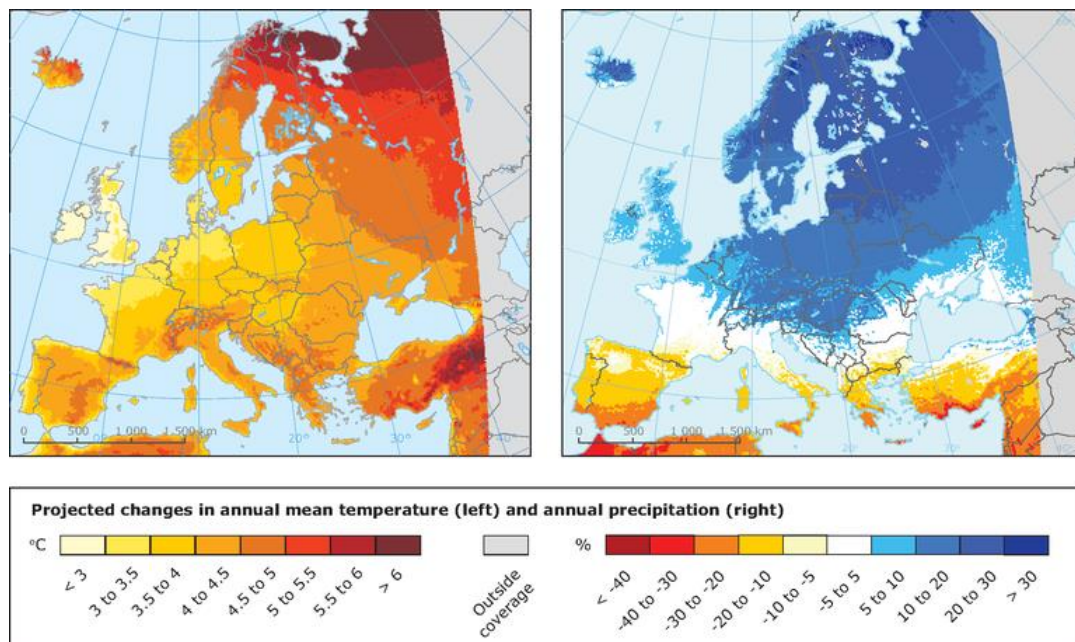


Fig. 3. Projected changes in annual mean temperature (left) and annual precipitation (right), for 2071-2100, compared to 1971-2000, based on the average of a multi-model ensemble forced with the RCP8.5 high emissions scenario. All changes marked with a colour (i.e. not white) are statistically significant. Source: European Environment Agency.

Besides temperature, several Global Climate Indicators describe the changing climate, including greenhouse gases and ozone, changes in land-use, oceans, cryosphere, precipitation, drivers of short-term climate variability like the ‘El Niño–Southern Oscillation’ and high-impact events such as heatwaves, drought and wildfires as seen in 2020, among others (WMO, 2021).

The major drivers of climate change since the mid-20th century have been the increasing levels of greenhouse gases in the atmosphere due to human activities and the changes in land-use (IPCC, 2014; WMO, 2021). The scale of these emissions has been an important component in the designation of a new geological era in which human activity is a primary driver: the Anthropocene (Jorgenson *et al.*, 2019). It is estimated that about 40% of anthropogenic CO₂ emissions have remained in the atmosphere since 1750 and the rest has been removed by sinks and stored in natural carbon cycle reservoirs, i.e. plants, soils and in the ocean (IPCC, 2014). Thus, ecosystems have the potential for large additional climate mitigation (Griscom *et al.*, 2017)

Recently, especially during the 2018-2020 summers, European forests have been affected by severe droughts, windstorms, more severe and widespread wildfires and disease outbreaks together with human disturbances (Stenlid *et al.*, 2011; Seidl *et al.*, 2014). Around two thirds of the total forest biomass in the EU and UK is potentially vulnerable to natural disturbances, 46% is threatened by windthrows, followed by forest fires (29%) and insect outbreaks (25%). (Fig. 4, Feyen *et al.*, 2020).

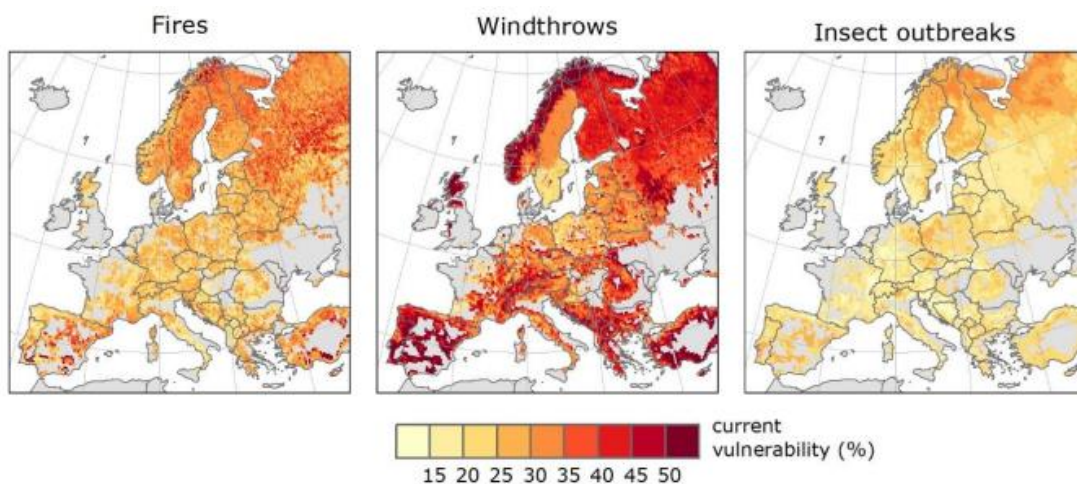


Fig. 4. Current Forest vulnerability to major natural disturbances in Europe, expressed as the share (in %) of biomass that would be lost in case of a disturbance. Areas with forest cover fraction lower than 10% are masked out, in grey (Feyen *et al.*, 2020).

Damage to forests caused by such extreme events and disturbances as well as interactions between them have increased in recent decades and are projected to increase under the climate change scenario. It is therefore clear that this is a major challenge for future forest conservation, which heavily depends on best practices of forest management (Mauser, 2021).

The role of forests in mitigating climate change through carbon sequestration is widely recognised. However, intrinsic aspects of forest biodiversity such as genetic diversity also play a key role in increasing the resilience of natural forests, but it is not always well appreciated (Hamrick, 2004; Whitham *et al.*, 2006; see Geburek and Konrad 2008 for a discussion). Trees can only be part of the solution providing mitigation services if they are well adapted and have the potential to adapt to future changing climates (FAO, 2015).

1.1.2 The case of Mediterranean Forests

The impact of climate change on forests is generally expected to be more severe in hot dry regions, where trees are at their adaptive limits, and in confined areas of moist forest surrounded by drier land (FAO, 2015).

In this context, the Mediterranean region is particularly important because it is a hotspot in terms of both biodiversity and climate change. Firstly, it is the meeting home between three continents: Africa, Asia and Europe and to a great variety of ecosystems and species. On the other hand, the long-standing anthropogenic pressure and the significant current human activity make it one of the world's regions most threatened by current changes (Gauquelin *et al.*, 2018). In fact, following the Regional Climate Change Index (RCCI), the Mediterranean region is one of the most prominent climate change hotspots in terms of vulnerability or climate response (Giorgi, 2006). The Mediterranean climate is characterized by a pronounced bi-seasonality with warm and dry summers and mild wet winters, a large year-to-year variability of total rainfall as well as frequent strong and dry winds that favour the spread of forest fires (Scarascia-Mugnozza *et al.*, 2000).

Potential problems associated with climate change include more frequent fires, intense droughts, break-downs in the synchrony between trees flowering periods and the presence of pollinators, species invasions and more severe pest outbreaks (Seidl *et al.*, 2014; FAO, 2015). Summer drought is a major factor limiting plant distribution,

reproductive success, plant reproductive performance, growth and survival (Benito-Garzón *et al.*, 2011; Bussotti *et al.*, 2015; del Cacho *et al.*, 2013; Andivia *et al.*, 2020). The impact of climate change on the diversity and dynamics of forest communities in the Mediterranean region will depend not only on the expected increase in aridity, but more importantly, on the reduced frequency of wet years (Matías *et al.*, 2011). Indeed, drought-induced growth declines and even tree mortality events have increased in recent decades across the Mediterranean region (Peñuelas *et al.*, 2001; Allen *et al.*, 2010; Dorado-Liñán *et al.*, 2019; Ferriz *et al.*, 2021; Mauser, 2021). Moreover, fire danger intensity is projected to increase, especially in southern European countries, where fires are already more frequent and intense (Feyen *et al.*, 2020).

Therefore, the importance of Mediterranean forest ecosystems and their thriving tree populations is due not only to their location but also to their value in terms of area, biodiversity, goods and services, and ultimately to the key role they are likely to play in the future under a changing climate (FAO, 2019).

1.2 Outcomes in forest tree populations

1.2.1 Adaptive strategies to cope with a changing world

Trees are long-lived organisms characterised by their particular life history, combining long-generation time (allowing divergent strategies at different life stages) and the capacity for long dispersal distances through pollen and seeds (Kremer *et al.*, 2012). During their long lives, they have to cope with changing biotic and abiotic environments. As the environment changes, trees like other living beings can respond through different adaptive strategies: persistence due to phenotypic plasticity, local adaptation, moving to a new area with suitable environmental conditions (migration), a combination of these processes or death (Aitken *et al.*, 2008; Bussotti *et al.*, 2015).

Forest trees possess high levels of genetic variation due to their large population sizes, are capable of long-distance gene flow, and accumulate new mutations due to their longevity and balancing selection (White *et al.*, 2007). Paleoecological evidence indicates that the geographic ranges of tree species have expanded and contracted several times without any apparent loss of genetic diversity. Indeed, much of the genetic variation is found within rather than between populations (Hamrick, 2004). Successful local

adaptation depends on the amount of genetic variability, which is essential for adaptation of species to new conditions through changes in allele frequencies that enhance their ability to survive and reproduce. In addition, phenotypic plasticity, which involves the same genotypes expressing different phenotypes in response to environmental variation, also may allow individuals to enhance their fitness. At the population level, plasticity allows colonization and establishment in different habitats (Matesanz and Valladares, 2014). Adaptation to a new environment may start with plastic phenotypic changes followed by genetic changes, but whether those plastic changes enable or hinder genetic adaptation is under debate (Ho and Zhang, 2018; Perry *et al.*, 2018). Understanding how populations adapt to new conditions is important not only to assess their ecology and evolution but also for predicting their resilience under climate change.

Maladaptive phenotypic plasticity in a changing climate is caused by mismatches between optimal and realised mean phenotypes, in particular when environments become unpredictable (Oostra *et al.*, 2018). Local adaptation will depend on the standing genetic variation, while migration on the capacity of dispersing into new habitats quickly enough and on the ability to compete with already existing species in the new areas (Hamrick, 2004; White *et al.*, 2007; Duputié *et al.*, 2015; Jaramillo-Correa *et al.*, 2015).

Therefore, considering the possible pace of environmental changes, rapid or slow, gradual or sudden, their magnitude and the current landscape fragmentation, the main question that arises is whether tree species would be able to adapt to the new conditions and novel stresses (Hamrick, 2004; Alfaro *et al.*, 2014). Potential increases in tree mortality associated with climate-induced processes are cause of particular concern (Allen *et al.*, 2010) since the rate of evolution of forest tree species may be slower than the rate of present climate change (Davis *et al.* 2005; Alfaro *et al.*, 2014; Trumbore *et al.*, 2015).

1.2.2 Reproductive traits and natural recruitment in fire-prone environments

The Mediterranean vegetation is well adapted to stressful ecological conditions, presenting distinctive trait syndromes that counteract the harmful effects of the environment that we have seen, such as summer xerothermic events and wildfires, ensuring either individual survival and growth and/or successful recruitment (Scarascia-Mugnozza *et al.*, 2000; Grivet *et al.*, 2013; Matesanz and Valladares, 2014).

For example, mechanisms of resistance to fire include high sprouting ability and thick bark in broadleaved species while conifers have early and large seed production, serotinous cones and high ecological plasticity (Scarascia-Mugnozza *et al.*, 2000; Rundel *et al.*, 2018).

In this context, high temperatures and low humidity have been correlated with an increase of wildfires and will affect the post-fire regeneration dynamics if those environmental episodes become more frequent (Doblas-Miranda *et al.*, 2017). Fire frequency and burn severity may increase in the Mediterranean Basin under the warmer and drier climate projected for this region (Fernández-García *et al.*, 2019).

Fire is the most important disturbance in many forest systems and in particular in the Mediterranean basin. For a long time, it has been considered a potential threat to forests and a causal agent of degradation (Pausas *et al.*, 2018). However, fire is an ancient, universal and ecological force that plays a fundamental role in the history of life, shaping plant species distribution and their functional traits (Trabaud, 1994.; Bell, 2001; He and Lamont, 2017). Fire response strategies are basic in nature, and thus common between animals and plants (Pausas, 2019). In particular, pines are among the most evident fire-adapted plant genera, with species presenting diverse fire syndromes under different fire regimes (Keeley, 2012; Pausas, 2015; Badik *et al.*, 2018).

Depending on the fire characteristics and frequency, pine species can display different adaptive syndromes related to either resistance (individual survival) or resilience (post-fire seedling recruitment). Resistance is related to thick bark in many species and postfire resprouting (ability to generate new shoots from dormant buds in fire-scorched stems) in a few species. Resilience is linked to postfire seeding, the ability to generate a fire-resistant seed bank with seeds that germinate profusely after fires (McCune, 1988; de las Heras *et al.*, 2012; Pausas and Keeley, 2014). Some tree traits increase fitness in ecosystems with low-intensity understory fires, like thick basal bark and self-pruning lower branches that eliminates ladder fuels that would lead to stand-replacing crown fires, whereas others enhance fitness in crown-fire ecosystems (the retention of lower branches or the shorter stature favors the fire reaching the canopy, Fig 5.), such as serotiny and early reproduction (Keeley and Zedler, 1998; Pausas, 2015).

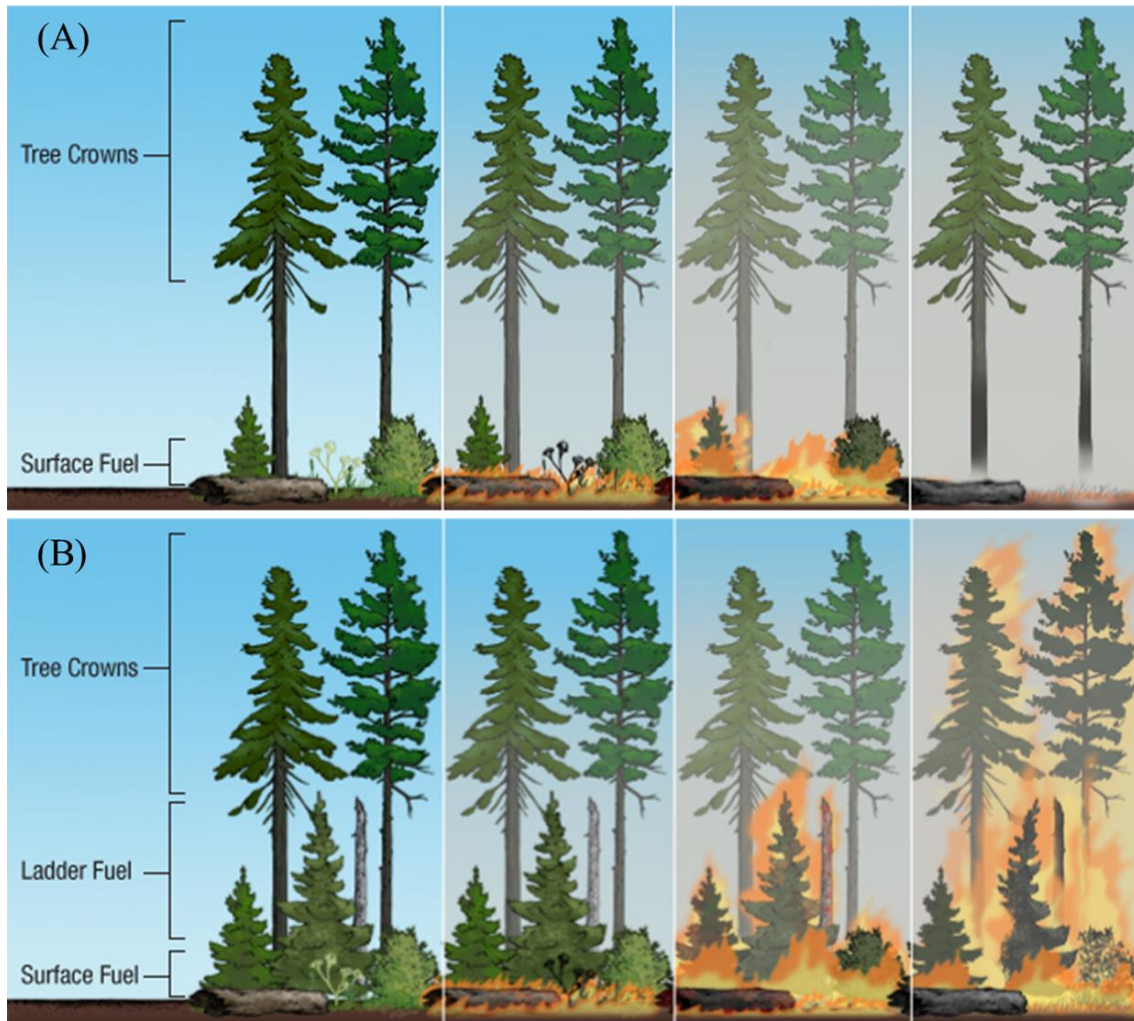


Fig. 5. Representation of forest fires, (A) The greater the distance between surface fuel (grass, logs, twigs, fallen branches, low-lying foliage) and tree crowns, the more difficult it is for fire crown to start, (B) surface fires spread quickly through woody debris and ladder fuels allow the fire to move up toward the forest canopy. Modified from the U.S. Forest Service. Credit: Adam Cole, Nelson Hsu / NPR.

At least three categories of species with fire adaptive syndromes can be recognized: a) *fire-tolerators*, which resist and survive frequent understory fires, characterised by a thick bark and self-pruning of lower branches coupled with epicormic resprouting and recovery, b) *fire-embracers* or *obligate seeders*, with thin bark and old-branch retention coupled with serotiny, representing a fire embracing strategy associated with crown fires and quick post-fire recovery, and c) *fire-avoiders*, which live in environments where fires rarely happen (Pausas, 2015; Lamont *et al.*, 2019). In addition, there are *facultative seeders*, which have both mechanisms for regenerating after fire, that is, they are able to resprout and to germinate after fire via seeds of serotinous cones, and *postfire colonizers* that, as their name indicates, are plants that are established after fire by seeds dispersed from unburned patches (Pausas and Keeley, 2014).

In particular, serotiny is a distinctive trait of the *fire-embracers* or *postfire obligate seeders* (Buma *et al.*, 2013; Pausas and Keeley, 2014), derived from the Latin ‘serotinus’ which means ‘late in occurrence’ (Lamont, 1991; Sheehan, 2020), which consists in the ability to retain seeds in temporarily closed cones (serotinous cones) to form an aerial seed bank that can be released after an environmental trigger, normally fire (Lamont, 1991). This so-called on-plant storage of seeds is a feature of many fire-prone dominant trees and shrubs in North America, Mediterranean Basin, South Africa and Australia (Lamont, 2021). The number of serotinous cones and the extend of the storage period represent a fire-persistence trait, which can be found in species of fire-prone environments in varying degrees (Perry and Lotan, 1979; Keeley *et al.*, 2011; Hernández-Serrano *et al.*, 2013). Besides the strategies of seed storage, another key aspect in plants that rely on seeds for regeneration after fire is the early onset of reproduction that is crucial to build a sufficient aerial seed bank to ensure recruitment in populations exposed to frequent fire regimes (Bradstock and O’Connell, 1988; Tapias *et al.*, 2001).

1.2.3 Long-lasting aerial seed banks: a challenging approach

Natural seed banks represent an adaptive strategy in the life histories of plants growing in disturbance- or stress-prone environments. Seed banks can be divided into two types, soil and canopy seed banks (Lamont, 1991; Su *et al.*, 2019).

Soil seed banks are an essential component of many plant communities since they constitute a reservoir of viable seeds in the soil ready to germinate (transient soil seed banks) or to persist for longer periods (persistent soil seed banks) (Nathan and Muller-Landau, 2000; Thompson, 2000). Soil seed banks promote diversity in plant communities, acting as a temporary buffer against unfavourable conditions that decrease plant survival and seed production (Foronda *et al.*, 2020).

Long-term seed storage in cones within the canopy (serotiny) is considered the main adaptation of *fire-embracers* and is common in families such as *Protaceae*, *Cupressaceae* and *Pinaceae* that dominate the flora of fire-prone areas (Lamont *et al.*, 2020). At least 14 species of *Pinus* in North and Central America, plus three in the Mediterranean Basin (*P. halepensis*, *P. brutia* and *P. pinaster*), one from the Canary Islands (*Pinus*

canariensis), and one from the south-west of China (Su *et al.*, 2019) bear serotinous cones (Richardson, 1998; Tapias *et al.*, 2001).

As we have seen above, wildfires can stimulate thermo-dehiscence of cones which releases a large number of seeds and provides a substantial advantage in terms of regeneration after fire (Lamont, 1991; Vega Hidalgo, 2003). If post-fire conditions are optimal, serotiny has an advantage over soil storage, where only a proportion of seeds will germinate (Lamont and Enright, 2000; Su *et al.*, 2019), as in the case of *Cistus* spp. (Izhaki *et al.*, 2000). Generally, obligate-seeders with soil seed banks can have a staggered period of seed dormancy and release that may last for many months and which might constitute an efficient risk-reducing trait in terms of seedling competition, predation or pathogen attack- and determines plant fitness (Pierce and Cowling, 1991; Nathan and Muller-Landau, 2000; Thompson, 2000; Bastida and Talavera, 2002).

While in recent decades many works have been published about the variation in the degree of serotiny between and within species (like those already cited), the short-term evolutionary implications of canopy seed banks, namely the putatively complex combination of genetic and non-genetic (maternal and epigenetic) factors remain largely unexplored (Feduck *et al.*, 2015; Schulz *et al.*, 2018; Lamont *et al.*, 2020). Maternal effects occur when the environment or physiological state of a mother changes the offspring phenotype without a corresponding change in the genotype (Bock *et al.*, 2019), and epigenetics refer to the modifications in DNA expression that are produced without altering the DNA sequence and that can be inherited (Danchin *et al.*, 2011; Balao *et al.*, 2018).

Long-lasting seed banks are particularly intriguing because they contain viable seeds that were formed across a range of maternal developmental stages and under different environmental conditions. Unlike soil seed banks, aerial seed banks of woody plants in serotinous fruits or cones can be individually dated retrospectively (Tapias *et al.*, 2001, Martín Sanz *et al.*, 2017). Moreover, fruits or cones formed early in the mother plant life coexist with those formed at more developed stages, and this ontogenetic gradient may interact with the environment under which each seed cohort was formed (Leslie and Losada, 2019). In this sense, aerial seed banks provide a valuable model for the study of the causes and consequences of maternal and genetic effects.

1.3 Case study species: *Pinus pinaster* Aiton

Mediterranean pines are large-sized, long-living and iteroparous species that endure a wide variety of changes in their environment during their long lifespan. Although they have evolved under similar environmental conditions, they diversified long ago, ca. 10 Mya, and present distinct biogeographic and demographic histories and may thus have responded quite differently to selective pressures. The evolution of pines should be interpreted in relation to climate (extreme temperatures), geology (oligotrophic soils) and fire (frequency and severity of fire regimes), all constituting environmental stressors since the Mesozoic (Keeley 2012). At the phenotypic level, most life-history traits followed independent evolution pathways in Mediterranean pines, leading to a heterogeneous assembly of species (Grivet *et al.*, 2013). Comparative studies revealed that, compared to other conifers in the Mediterranean and elsewhere in the world, Mediterranean pines present a high level of genetic differentiation and low genetic diversity within populations, along with an increasing level of genetic diversity from west to east and a low level at low elevation. The main factors explaining this pattern are linked to ancestral adaptation to wildfire, reduction of effective population size during the Last Glacial Maximum (LGM) and long-distance dispersal during the Holocene (Grivet and Olsson, 2021). Precisely for this reason and due to their relevance in the Iberian Peninsula, Mediterranean pines have become model organisms to study the evolution of life-history strategies in terms of the investment of resources in growth, reproduction and defences over time (Climent *et al.*, 2008; Grivet *et al.*, 2013; López-Goldar *et al.*, 2020; Vázquez-González *et al.*, 2021).

Pinus pinaster Ait. (Pinaceae), commonly known as maritime pine, is a medium-size coniferous tree (Fig 6.) native to the western Mediterranean basin. It is one of the most important forest species in Spain due to its multiple uses (resin and wood production, recreation and soil protection) and to its ability to grow in very poor soils and under prolonged drought (Alía and Martín, 2003).



Fig. 6. Infographic of *Pinus pinaster* Ait., modified from Ordóñez, J.L. 2019. Source: <http://www.alertaforestal.com>

Maritime pine is widely distributed in the western Mediterranean basin including Southwestern Europe (Iberian Peninsula, France and Italy), and northern Africa (Morocco and Tunisia) (Fig. 7).

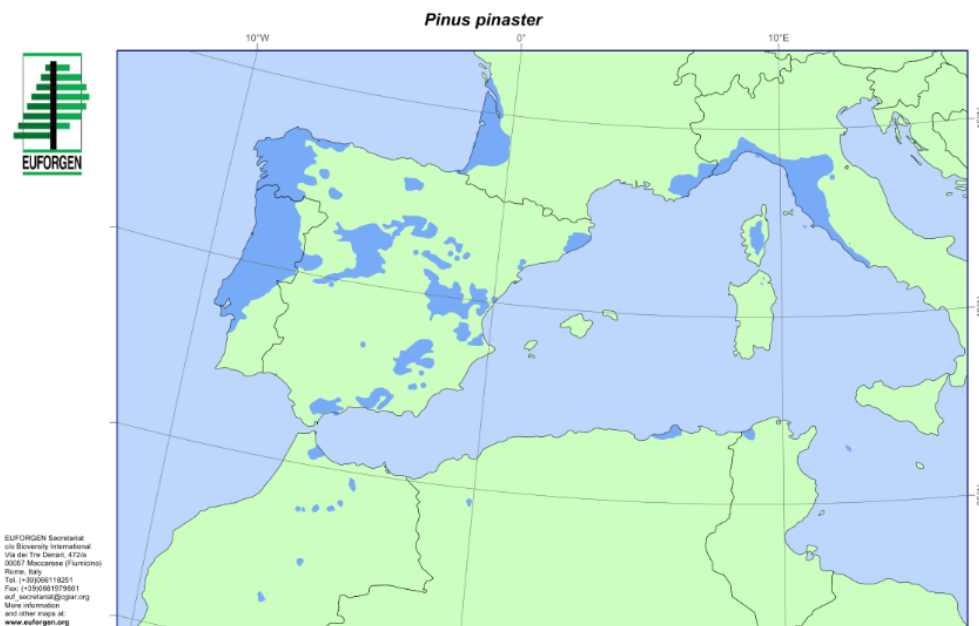


Fig. 7. Distribution map of *Pinus pinaster*, including both natural and naturalized occurrence (EUFORGEN, 2009). Source: www.euforgen.org.

This species can be found in contrasting environments: from acid soils to basic, sandy and poor soils; from sea level to 2100 m of elevation above sea level; and from areas with more than 1400 mm of annual rainfall and no dry season to areas with 350 mm and more than 4 dry months (Alía and Martín, 2003). Therefore, it is found in a broad range of ecosystems including continental as well as Atlantic and Mediterranean coastal forests and woodlands, being more productive and frequent in areas with Atlantic influence compared to continental and Mediterranean climate areas (Alía *et al.*, 1996; Barrio-Anta *et al.*, 2020). Historically, and especially during the mid-20th century, afforestation programmes (i.e. reforestation and afforestation) reshaped its distribution, thus making the differentiation of autochthonous and non-autochthonous stands particularly difficult in many cases (Guiral *et al.*, 2019; Alía and Martín, 2003).

P. pinaster spatial distribution is expected to change under the climate change scenario, especially in its Atlantic north-western area that is foreseen to be drastically reduced (Benito-Garzón *et al.*, 2011). This pine is considered a drought-avoiding tree species that may adapt to drought by physiological responses (e.g., increasing water-use efficiency by rapid stomata closure; Navarro-Cerrillo *et al.*, 2018; Caminero *et al.*, 2018; Andivia *et al.*, 2020). Its growth is mainly constrained by water shortage during the growing season (spring), suggesting that future dry spells and heat waves may be common underlying drivers of dieback and mortality events, particularly in some locations due to large divergence among populations (Chambel *et al.*, 2007).

Several studies have provided evidence of genetic differentiation for adaptive traits among populations of *Pinus pinaster* within its highly fragmented distribution area, suggesting local adaptation to different environments and disturbance regimes (Alía *et al.*, 1997; González-Martínez *et al.*, 2002; Aranda *et al.*, 2009; Santos-del-Blanco *et al.*, 2012; Grivet *et al.* 2017; Sánchez-Salguero *et al.*, 2018; de Miguel *et al.*, 2020). This putative local adaptation justifies the ecological classification of the natural area of distribution into 20 main provenance regions (plus 7 smaller ones) in Spain (Fig. 8). However, direct empirical evidence of higher fitness of local populations compared to non-local ones is still lacking (Ramírez-Valiente *et al.*, 2021).

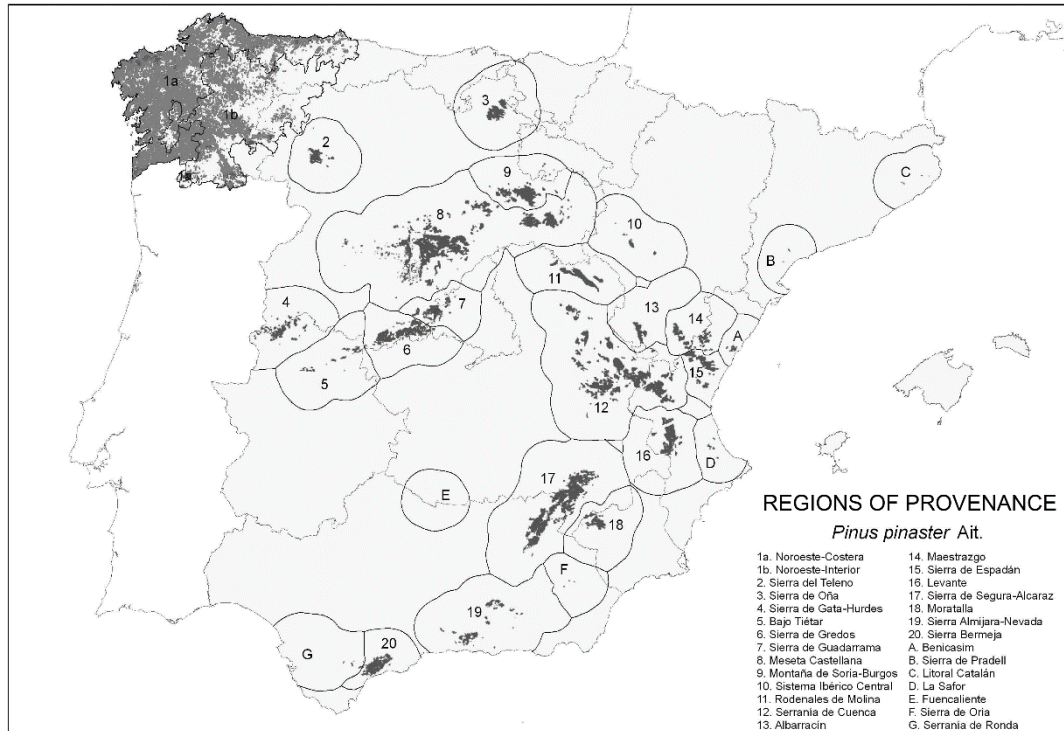


Fig. 8. Distribution of *Pinus pinaster* in Spain represented by dark grey shade and its provenance regions delimited by grey lines (modified from Alía Miranda *et al.*, 2009).

Within the high population differentiation in *P. pinaster*, we can find different combinations of traits pertaining to the fire-adaptation syndromes described in 1.2.2 (Tapias *et al.*, 2004). Particularly, bark thickness, early female reproduction, total cone bearing (i.e. total number of cones per tree, open and closed) and serotiny degree (the average percent of closed cones with respect to the total number, Fig. 9) have been shown to present variation among populations associated with ecotypic meaning (Tapias *et al.*, 2004; de las Heras *et al.*, 2012; Santos-del-Blanco *et al.*, 2012). While in low-productivity continental *P. pinaster* stands (like those in the Duero basin sands), fires are very scarce and of low intensity, in more productive zones the woody understory leads to high intensity crown fires (Tapias *et al.*, 2001; Fernandes and Rigolot, 2007). In these areas with stand-replacing crown fires, female reproduction can begin as early as at the 4 years of age, although the production of fertile seeds normally starts between 10 and 15 years (Tapias *et al.*, 2001; Torres *et al.*, 2006).

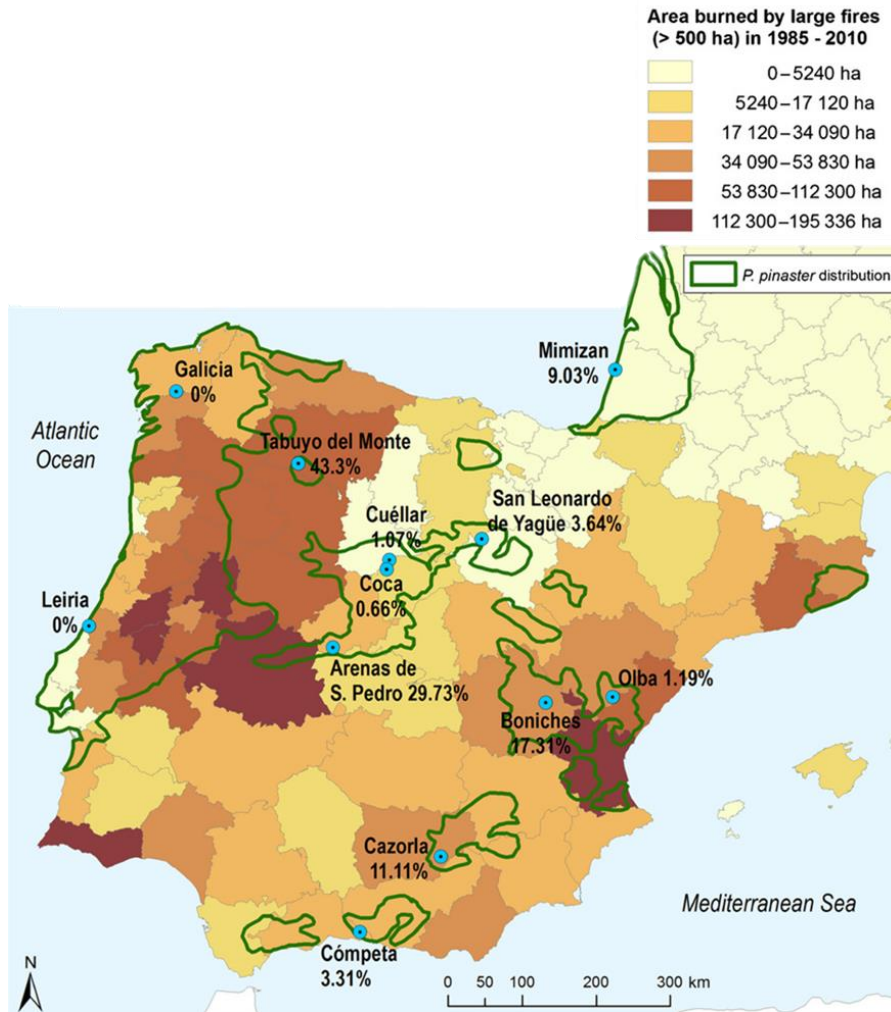


Fig. 9. Serotiny estimates (average % of serotinous cones with respect to total cones) for populations of *Pinus pinaster* (data retrieved from Tapias *et al.*, 2004) superimposed on provincial data of burned area by large fires (> 500 ha, from 1985 to 2010; European Commission, 2010). Maritime pine range in the Iberian Peninsula is shown in green. Modified from Budde *et al.*, 2014.

This relatively early female reproductive onset combined with the high serotiny in some populations makes *P. pinaster* a unique model species to study the evolutionary implications of aerial seed banks.

1.4 Common garden experiments of forest species

Compared with other organisms, trees have long generation times, which make them challenging yet unique models to study ecological and evolutionary questions. When studying the genetic basis of adaptation for forest tree species and populations, common garden experiments are established, by growing individuals in the same environment, as these trials constitute an efficient tool for controlling the effects of phenotypic plasticity

(de Villemeireuil *et al.*, 2016). Additionally, they can be installed in different locations to test for genotype-by-environment interactions and species/population plasticity across biogeographic regions.

Traditional *provenance trials* used germplasm banks to grow individuals for numerous purposes, ranging from the production of wood and non-wood products to the provision of ecosystem services such as the restoration of forests for biodiversity conservation. These trials early revealed that seed origin had a major influence on the performance of planted trees due to the use of local and non-local material (Koskela *et al.*, 2014). Long-term provenance trials are still used and are particularly valuable to provide information of forest dynamics over time and predicting specific responses to climate change (Pretzsch *et al.*, 2019). Meanwhile, new approaches are increasingly being used such as i) short-term common garden experiments in nurseries to gain some insights on the juvenile traits or traits highly correlated to the juvenile and mature phases of the plants, and ii) molecular analyses in laboratories to identify underlying markers involved in populations' adaptation.

On the other hand, multi-site *reciprocal transplant experiments* are commonly used to study and compare the performance of local and non-local genotypes with the aim of testing the adaptation limits (via response functions) of different populations, and demonstrate which seed source/s is/are appropriate for planting in each particular biogeographic area (Lu *et al.*, 2016; Giencke *et al.*, 2018).

Open-pollinated progeny trials with seeds from a single or multiple populations or collected from open-pollinated seed orchards are also widely used in breeding programmes or specific evolutionary studies, such as those focusing on the adaptive potential of populations (Santos del Blanco *et al.*, 2010). In addition, *clonal trials* are created through vegetative propagation and used for multiple purposes (Gaspar *et al.*, 2009). Often the population or provenance level in these common garden experiments is combined with a within-population level comprising identified maternal lineages (half-sibs) or clones.

In recent years, new-paradigm common garden experiments have been deployed in forest tree species, intending to mimic natural or at least semi-natural or 'mesocosms' conditions at germination and early developmental stages, frequently overseen in

traditional experiments using nursery-grown plants (e.g. Alía *et al.*, 2014; Vizcaíno-Palomar *et al.*, 2014; Bachofen *et al.*, 2021).

1.5 Objectives

In this Thesis, we are interested in the short-term evolutionary consequences of aerial seed banks in post-fire recruitment, in particular, in the performance of the progeny of different yearly seed cohorts. To assess germination, survival and growth at early developmental stages, we installed a common garden experiment under semi-natural conditions or “mesocosms”. Our main interest lies in disentangling the factors that affect the offspring performance derived from an aerial seed bank of a Mediterranean pine (*Pinus pinaster*) in the context of the fire-adaptive trait: serotiny.

The objectives and initial hypotheses were the following:

(i) To determine the effect of maternal conditions, linked to different age cohorts, on seed performance in an aerial seed bank of *Pinus pinaster* (Chapter 1). We hypothesized that a) the aerial seed bank will show variation in seed mass between and within mother trees that depends on maternal age and conditions at the time of seed development, b) that maternal age and yearly conditions have an effect on early seedling performance (germination and survival) and c) that these effects on offspring performance are at least partly mediated by variation in seed mass. The specific objectives were:

- To determine whether the aerial seed bank shows variation in seed mass between and within mother trees depending on maternal age (i.e. do older individuals produce lighter or heavier seeds?), growth and environmental conditions at the time of seed development (i.e. do trees produce heavier seeds under favourable conditions?).
- To test whether maternal age, growth and environmental conditions experienced at the time of seed development shape germination success and timing, as well as early survival.
- To assess to which extent these effects on offspring performance are mediated by the variation in seed mass.

(ii) To determine the genetic changes in offspring quantitative traits across years and to explore whether environmental heterogeneity had an effect on selection (Chapter 2). We hypothesized that a) those maternal effects measured in the previous chapter would also help explain seedling performance in terms of growth, and b) heritability estimates decrease when controlling for seed mass and maternal effects. The specific objectives were:

- To test whether different environmental conditions at micro (maternal environment) and macro (general environment) scales among years and mother trees within the same population affect offspring performance in terms of seedling size and ontogeny.
- To determine offspring genetic changes in quantitative traits (size and ontogeny) across years considering potential maternal effects for each particular year.
- To test whether maternal conditions and the environmental heterogeneity had an effect on selection for size and ontogeny, as key traits for natural regeneration.

(iii) To explore the genetic variability in two temporarily contrasted cohorts of the aerial seed bank, which could explain the observed differences in the offspring performance (Chapter 3). Our hypothesis was that within this period of time, recurrent wildfires and/or the mega-wildfire of high-intensity occurred in 2012 could have played a role in shaping the genetic makeup of the population, translating into differences in the offspring performance investigated in this thesis. The specific objective was:

- To understand better the long-term evolutionary consequences of serotinous cones on the genetic variability of a collection of aerial seed banks separated by 12 years, i.e. by comparing the genetic makeup of an old (2003) and a recent (2014) seed cohort of the aerial seed bank.

Table 1. Overview of the structure of the Thesis, including brief details of the three chapters.

	Objectives	Material and Methods				Results
		Sampling site	Field and/or laboratory work	Statistical analyses	Software	
Chapter 1	(i) Effects of maternal conditions, linked to different age cohorts, on seed mass and seedling performance in an aerial seed bank.	Tabuyo del Monte, Sierra del Teleno (León, Spain) Common garden experiment located in Madrid (Spain)	- Mother tree characterisation (basal diameter, tree age and annual ring width) - Canopy seed bank (age of serotinous cones and seed weight) - Climate Data: SPEI ^A - Seed germination and early seedling survival	LMM* GLMM** Survival Analyses: Kaplan-Meier	R version 3.5.3	Callejas <i>et al.</i> , 2021. American Journal of Botany
Chapter 2	(i) Genetic changes in offspring quantitative traits across years. (ii) Effect of environmental heterogeneity on selection.	Common garden experiment located in Madrid (Spain)	- Shoot ontogeny - Seedling height	LMM* Ketata analyses of performance h^2 , CV_A , Response to Selection	R version 4.1.0 ^B	Callejas <i>et al.</i> , in prep
Chapter 3	(i) Genetic variability in two temporal cohorts (age 3 and 14 years) of the aerial seed bank, and test whether temporal genetic heterogeneity is linked to temporal offspring performance.	Common garden experiment located in Madrid (Spain)	- 477 seedlings coming from two contrasted temporal cohorts (C3 y C14) - Genotyping using 8 nuclear microsatellites (nSSR)	Genetic diversity analyses. AMOVA*** Mating system. Gene flow estimation	<ul style="list-style-type: none"> ○ Arlequin 3.5 software^C ○ GeneMapper® version 3.0^D ○ Micro-Checker 2.2.3^E ○ MLTR software^F ○ POLDISP 1.0c^G ○ R version 4.1.0^B 	Callejas <i>et al.</i> , in prep

*Linear Mixed Models. **Generalized Linear Mixed Models. *** Analysis of MOlecular VAriance. // A. Vicente-Serrano *et al.*, 2010. B. R Core Team, 2021. C. Excoffier and Schneider, 2005. D. Applied Biosystems. E. Van Oosterhout *et al.*, 2004. F. Ritland, 2002. G. Robledo-Armuncio *et al.*, 2007.





2. Chapter 1

Maternal effects in shaping offspring performance in an aerial conifer seed bank

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Abstract

Maternal effects have been demonstrated to affect offspring performance in many organisms and, in plants, seeds are important mediators of these effects. Some woody plant species maintain long-lasting canopy seed banks as an adaptation to wildfires. Importantly, these seeds stored in serotinous cones are produced by the mother plant under varying ontogenetic and physiological conditions.

We sampled the canopy seed bank of a highly serotinous *Pinus pinaster* population to test if maternal age and growth, as well as the environmental conditions during each crop year, affected seed mass and ultimately germination and early survival. After determining retrospectively the year of each seed cohort, we followed germination and early survival in a semi-natural common garden.

We found that seed mass was related to maternal age and growth at the time of seed production, i.e. slow growth-older mothers had smaller seeds and fast growth-young mothers had bigger seeds, which could be interpreted either as a proxy of senescence or as a maternal strategy. We also confirmed that seed mass had a positive effect on germination success, but beyond differences in seed mass, maternal age had a negative effect and diameter had a positive effect on germination timing and subsequent survival.

Thereby we highlight the importance of maternal conditions combined with seed mass in shaping seedling establishment. Our findings open new insights in the offspring performance deriving from long-term canopy seed banks, which may have high relevance for plant adaptation.

2.1. Introduction

The phenotypes of all living beings are determined by their genotype, their environment, and the interaction between the two, and there is a growing realisation that an important part of this environment may be provided by the mother during early life stages, such as the embryonic development stage (Mousseau and Fox, 1998; Diggle *et al.*, 2010). As maternal effects have long been studied in a variety of taxa and contexts (Bernardo, 1996; Russell and Lummaa, 2009, Pick *et al.*, 2019), we can find different definitions in the literature (for a discussion of maternal effects in plants, see Roach and Wulff, 1987). Here we use the common (quantitative genetic) definition of a maternal effect as the contribution of the maternal parent to the phenotype of its offspring beyond the equal chromosomal contribution expected from each parent (Roach and Wulff, 1987; Kirkpatrick and Lande, 1989). In other words, maternal effects occur when the environment or physiological state of a mother changes the offspring phenotype without a corresponding change in the genotype (Bock *et al.*, 2019). Importantly, this does not mean that maternal effects can not have a genetic basis (Wolf and Wade, 2016).

Transgenerational maternal effects provide a flexible mechanism by which sedentary or sessile organisms can cope with heterogeneous environments (Galloway, 2007). In plants, seed mass, which is determined by the seed coat, the megagametophyte and the embryo, may be an important mediator of maternal effects (Bischoff and Mueller-Schaerer, 2010). In flowering plants (angiosperms), both the embryo and the endosperm are derived from individual fertilization events, and only the seed coat that encloses these tissues is purely of maternal origin (Westoby and Rice, 1982; Baroux *et al.*, 2002). By contrast, in gymnosperms only the diploid embryo contains genes of both parents, while all other tissues, including the conspicuous megagametophytic tissue surrounding the embryo, are maternal in origin (Linkies *et al.*, 2010). Hence, in gymnosperms there is a potentially wider role for maternal effects mediated by seed provisioning, together with other aspects of the external and internal maternal environment (Herman and Sultan, 2011).

Maternal effects that affect germination and early stages of plant development are among the best-documented examples (Donohue *et al.*, 2005; Bischoff and Mueller-Schaerer, 2010; Cendán *et al.*, 2013). Therefore, seed mass is a cornerstone trait that links the evolutionary ecology of reproduction with seedling establishment and the ecology of

vegetative growth (Shipley *et al.*, 1990; Leishman *et al.*, 2000). In conifers, the haploid megagametophyte is the main storage tissue providing reserves to the embryo for germination, before the needle-like cotyledons start photosynthesising (Burrows *et al.*, 2017). So, it is not surprising that maternal effects related to seed mass and seedling performance (survival and growth) are particularly well-documented in conifers (Sorensen and Campbell, 1993; Zas and Sampedro, 2015).

In addition to seed mass, maternal age has been shown to have a negative effect on offspring performance in other taxa (Lansing, 1954; Priest *et al.*, 2002; Bock *et al.*, 2019). Many organisms exhibit age-related declines in offspring quality and such parental age effects may be due, among others, to age-related changes in parental care or provisioning, or other age-related changes in the environment in which offspring develop (Barks and Laird, 2020).

Given the substantial evolutionary and ecological implications that maternal effects can have, it is essential to investigate the performance of plant offspring derived from heterogeneous maternal conditions, in terms of age, growth and environment. To this end, aerial seed banks provide a privileged experimental system compared to standard germplasm banks (Levin, 1990; Barrett *et al.*, 2005). Long-lasting seed banks are particularly valuable when it comes to assessing the importance of maternal effects because they contain viable seeds that have been formed across a range of maternal developmental stages and environmental conditions. However, this approach has never been previously used in seed ecological research. In addition to providing valuable general insights, serotiny provides a valuable model for the study of the causes and consequences of maternal effects in general.

Considering that seed mass-mediated maternal effects are costly in terms of resources (Martín Sanz *et al.*, 2017), we expect seed mass to vary with respect to the maternal developmental stage and the environmental conditions at time of seed formation, and this variation to mediate variation in offspring performance (i.e. germination success, timing of germination and survival). We retrospectively determined each seed's crop year, as well as the mother tree's age, growth, and the abiotic environment at the time of seed formation, i.e. at each of those crop years. Subsequently, we followed germination and early survival in a semi-natural nursery common garden.

2.2. Material and Methods

2.2.1. Study site and sampling regime

Since we needed a wide enough representation of serotinous cone ages, we chose a maritime pine (*Pinus pinaster* Aiton) population previously characterized as highly serotinous (more than 58% of serotinous trees, Tapias *et al.*, 2004; Calvo *et al.*, 2016). This natural population is located at an altitude of 900 m above sea level in Tabuyo del Monte (latitude 42°18'46"N, longitude 6°12'12"W), northwestern Spain. In November 2017, we randomly sampled 20 trees that were at least twenty meters apart over an area of 55 ha with at least six age cohorts of serotinous cones per branch to enable further retrospective dating (**Fig. 10**). Retrospective cone dating in this species is easier in comparison with other serotinous pines in our area such as *Pinus halepensis*, which usually develop several consecutive growth units per year (high polycyclism) and false xylem growth rings (Buissart *et al.*, 2015). Cones were collected from three branches per tree and stored at 4°C during the lab processing.

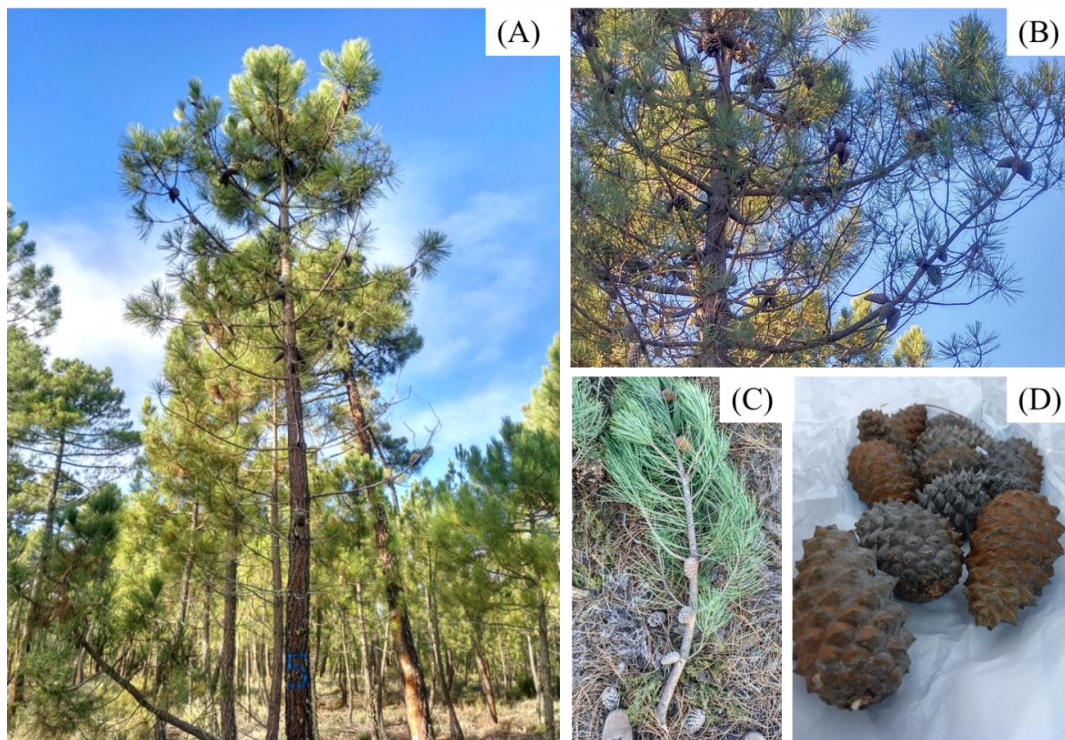


Fig. 10. Representative pictures of the natural population of *Pinus pinaster* Ait. located in Tabuyo del Monte (northwestern Spain): (A) One of the mother trees sampled as an example, (B) an aerial seed bank with serotinous cones from different cohorts, (C) a detail of a branch with serotinous cones (see Appendix 1 to complement figures 1B and 1C) and (D) a subsample of serotinous cones. Pictures by ©Callejas-Díaz, M., 2017.

2.2.2. Cone and seed age characterisation

To infer the year in which a cone was produced in the field, we used the stem node counting method (Lamont, 1985). These estimates were followed-up by counting the number of rings in the branch at the insertion point in the laboratory (Tapias *et al.* 2001, Martín-Sanz *et al.*, 2017). From the estimated cone crop year, we deduced the age of each cone and thereby of the seeds therein (Fig. 11). For example, a cone produced in 2007 contained ten years old seeds in 2017. We discarded four trees due to indistinguishable and/or missing branch wood rings, therefore we kept sixteen mother trees for the next steps of the experiment.

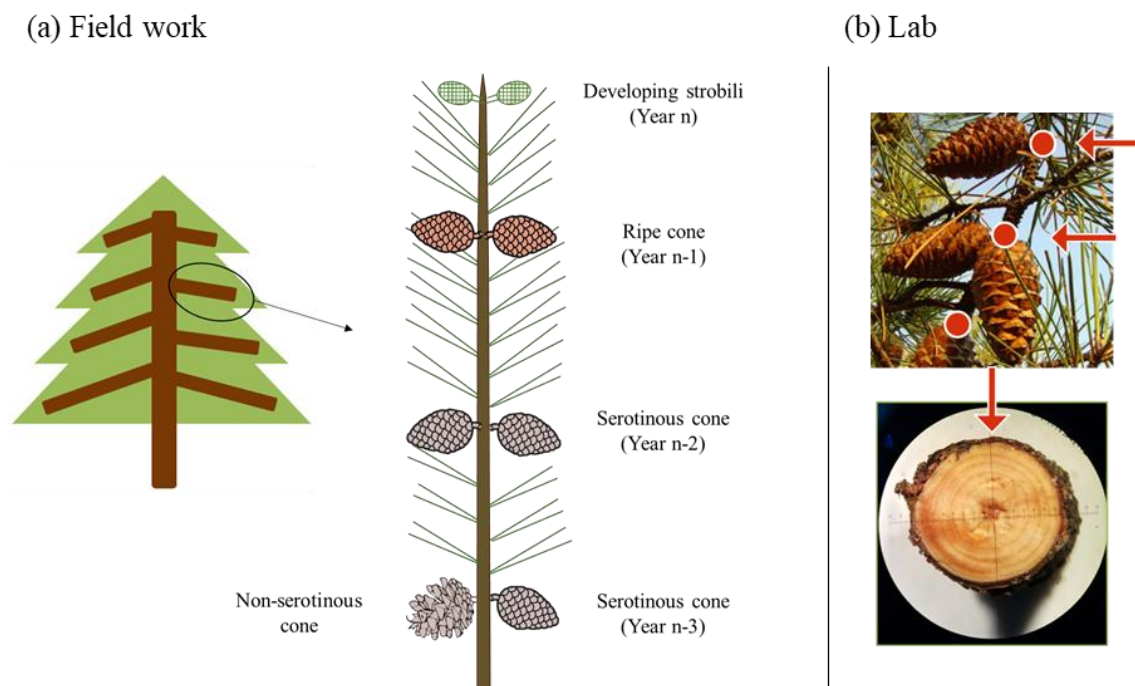


Fig. 11. (a) Schematic representation of the ‘node-counting method’ and (b) the subsequent counting of rings in the laboratory. Diagram modified from Climent, J., by ©Callejas-Díaz, M., 2020. Pine cone design by ©Canalda, J.

2.2.3. Mother tree characterization

We measured the basal diameter of all sampled trees, and we extracted two basal cores with a Pressler increment borer to determine tree age and measure annual radial growth in the laboratory (Stokes and Smiley 1968, Fig. 12).



Fig. 12. Example of core extraction with the help of an increment borer of the Pressler type. (1) Detail of the power drill at the borer insertion point, (2) and (3) a mini-auger is drilled by hand from the bark to the centre (pith) of the tree, and (4) the resulting core sample extracted from the hole, which displays the tree's annual rings at that point in the tree. Pictures by ©Callejas-Díaz, M., 2018.

Each core was mounted and cut with the help of a microtome (Gärtner and Nievergelt, 2010) until tree rings were clearly visible (Fig. 13). Tree ring series were visually crossdated to identify locally absent rings and to check for errors (Fritts, 1976). Crossdating was not statistically verified because most trees were very young and individual time series were shorter than 25 years in several cases. Ring width was measured at 0.01 mm accuracy using the LINTAB system and TSAP-Win (Rinntech, Heidelberg, Germany). If cores failed to reach the centre (Norton *et al.*, 1987) the number of missing rings was estimated by dividing the length of the missing radius by the mean growth rate of the rings adjacent to the largest visible arc on the core following Rozas (2003). To better compare the interannual variability in radial growth among different trees, raw ring-width measures were normalized, i.e. transformed to have a mean of 0 and a standard deviation of 1 before further statistical analysis.

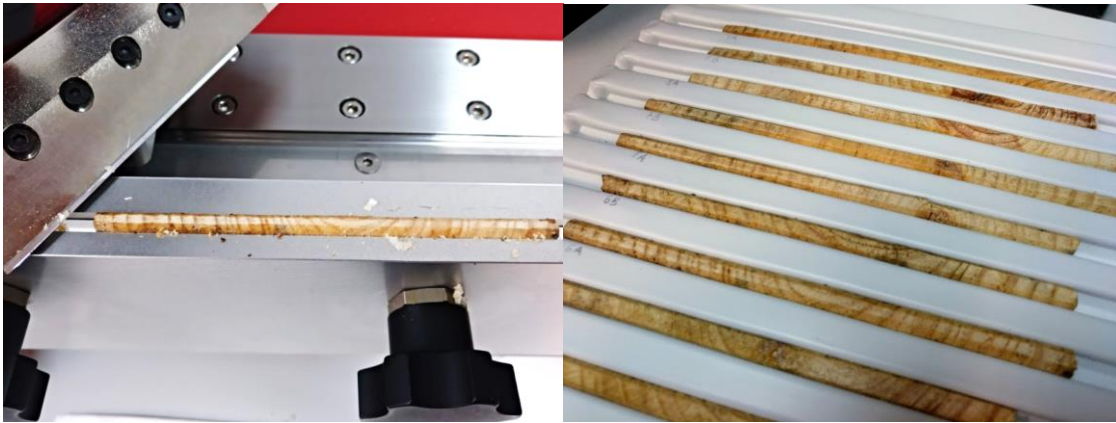


Fig. 13. On the left, core microtome for cutting micro sections of entire increment cores. On the right, processed increment cores with visible tree rings. Pictures by ©Callejas-Díaz, M., 2019.

Based on the basal diameter as measured in the field and the ring-width data collected in the laboratory, we retrospectively inferred the age and diameter of each mother tree in the year a cone was produced.

2.2.4. Climate data

To complement our temporal data on the maternal environment over time, we used the Standardised Precipitation-Evapotranspiration Index (SPEI; <https://spei.csic.es>), a multiscalar drought index based on climatic data that can be compared with other SPEI values over time and space (Vicente-Serrano *et al.*, 2010). The SPEI database offers long-time robust information about drought conditions at the global scale across a range of timescales. We used the SPEI calculated for five months (SPEI5July) corresponding to each embryo-development year. Positive values are associated with above-average wet conditions, and negative values with above-average dry conditions. Here we used SPEI data based on monthly precipitation and potential evapotranspiration as collected by the Climatic Research Unit of the University of East Anglia.

2.2.5. Seed extraction and measurement

Cones were introduced in a chamber at 60°C for 2.5 hours, after which they were opened manually to obtain all seeds (Fig. 14). Further heating could have negative effects

on germination success (Escudero *et al.*, 2002). We weighed the total seed mass from each cone, and after using a float test to remove empty seeds (Serrano Antolín and Calderón Guerrero, 2009) and to ensure the same conditions for all trees and cohorts, ten seeds per cone were selected at random and weighed individually. Note that various tests can be used to ensure that only viable seeds are planted (e.g. float test such as in our case or x-ray), but nevertheless germination cannot be guaranteed (Davis *et al.*, 2004). No dormancy-breakage treatment was applied to our *Pinus pinaster* seeds, since the objective was to mimic the natural germination conditions as much as possible.

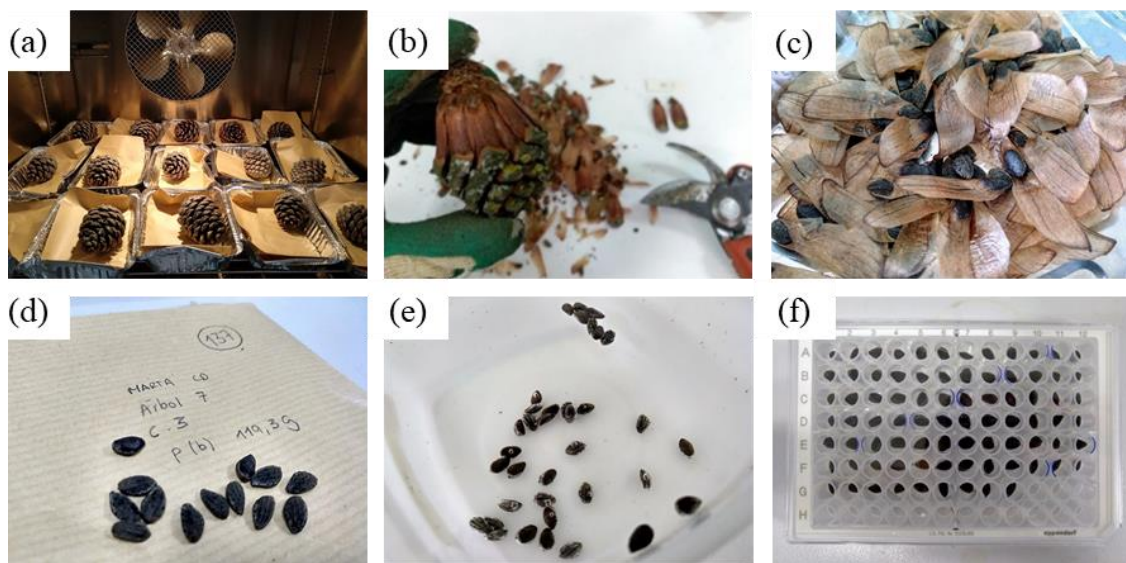


Fig. 14. (a) Individual pine cones already open inside the chamber with controlled conditions, (b) completing the cone opening manually to obtain the seeds (picture by ©Callejas-Díaz, M., 2015), (c) winged seeds of *Pinus pinaster*, (d) detail of a tree cohort seeds without wings, (e) float test to select viable seeds and (f) seeds weighed and individually identified. Pictures by ©Callejas-Díaz, M., 2018.

2.2.6. Common garden experiment

The common garden experiment was conducted under semi-natural conditions in a nursery (latitude 40°27'24.77" N, longitude 3°45'06.32" W, 597 m above sea level) once the lab processing was completed, i.e. six months after the field sampling. Although the environmental conditions at the nursery did not match those at the sampling site, they were similar to the warmer and drier continental range of the species.

We used plastic containers (57 x 37 x 32 cm) filled with natural pine forest soil (*eutric cambisol* after FAO taxonomy) collected in a natural *Pinus pinaster* stand (Fig. 15) located in the Central Range west of Madrid to ensure early seedling mycorrhization (Trappe, 1977; Pera and Álvarez, 1995; Buscardo *et al.*, 2009). A bottom layer of 10 cm of expanded clay was added prior to the natural substrate to ensure water drainage and aeration of the roots.

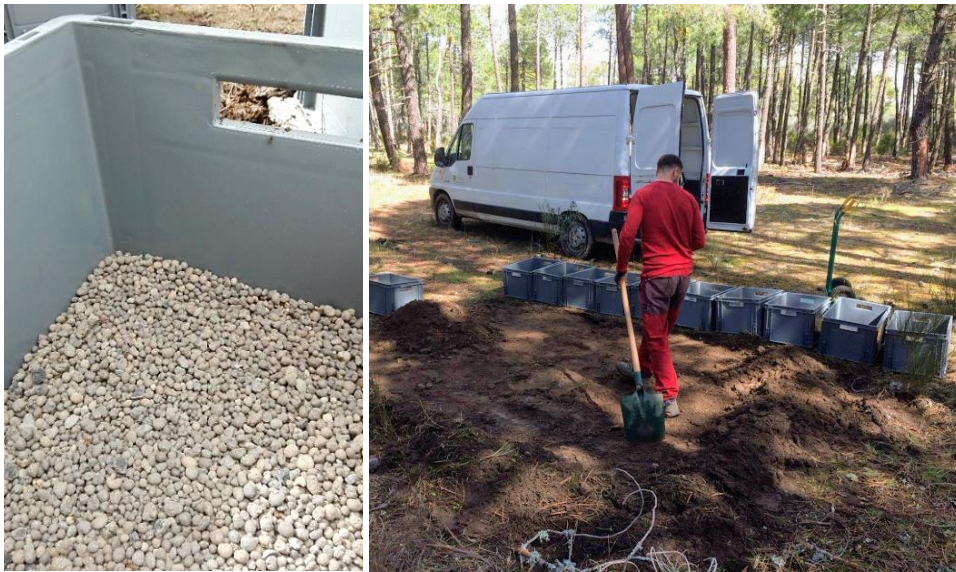


Fig. 15. Detail of the plastic containers with the expanded clay at the bottom, and the natural soil collected in Ávila, west of Madrid. Pictures by ©Climent, J., 2018.

We sowed a total of 4620 seeds (see Supplementary Information, Appendix S1), using a plastic grid to facilitate seed location and further measurements, and also to prevent any confusion with the eventual germination of seeds coming with the soil (although seed soil banks in pines are negligible). In short, with 10 seeds per cone, 3 cones per cohort and tree, and at least 6 cohorts with a maximum of 12 per tree, all for a total of 16 trees. The 30 seeds per cohort per tree were divided into six groups of five seeds each. This resulted in six batches of 770 seeds each. By hand, seeds from each batch were randomly planted across 10 containers (referred to here as a block), resulting in a total of 60 containers (Fig. 16).

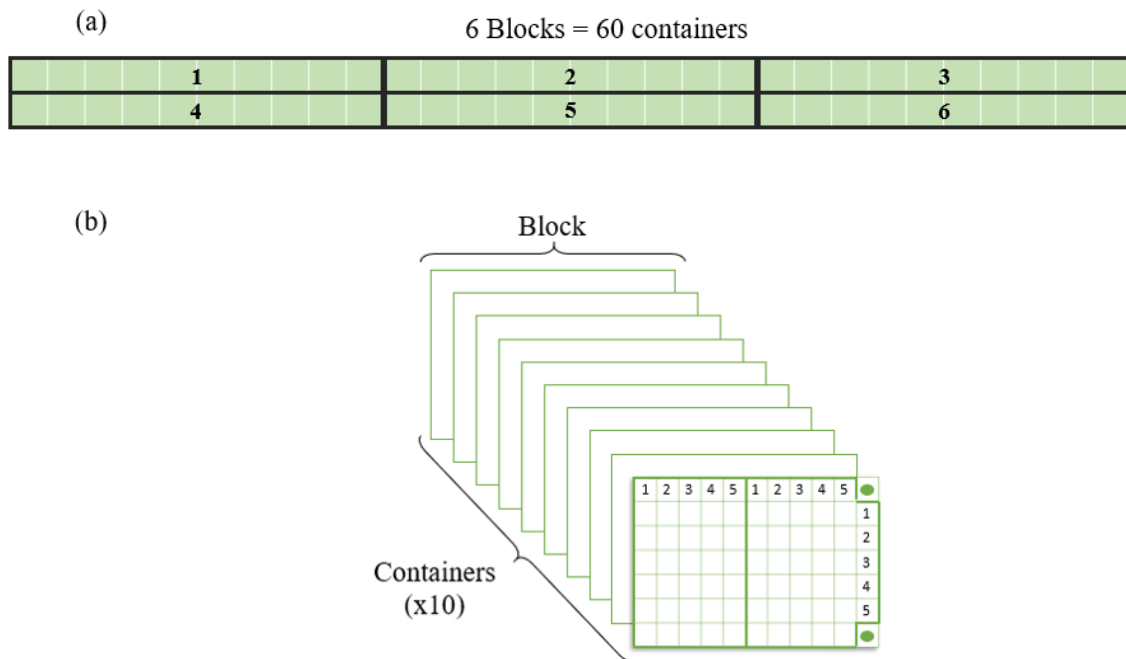


Fig. 16. (a) Schematic representation of the experimental design, and (b) one experimental unit from a total of 6. Each block had ten containers. Each container had 77 seeds (two of them were to fill the gaps: green point), making a total of 4620 seeds.

The location of each seed within a container was recorded to allow individual monitoring (Fig. 17). Containers were covered with a mesh to protect seeds from predation. Pots were kept well-watered to ensure sufficient hydration for germination until the end of July. Germination and survival were recorded every three days from April to October 2018, after which the monitoring frequency was reduced to every 10 days until December. Hence, measurements lasted eight months.



Fig. 17. Representative pictures of the common garden experiment located in Madrid, by ©Callejas-Díaz, M., 2018.

2.2.7. Statistical analyses

First, we used a linear mixed model (LMM) to quantify the percentage of variance in seed mass explained by mother tree ID, cone age cohort and experimental block by fitting each as a random effect. Their statistical significance was assessed using likelihood ratio tests. We subsequently included mother tree age, diameter growth, ring-width and SPEI5July for the year at embryo development/cone production as fixed covariates to quantify their roles in shaping variation in seed mass among mother trees, cohorts and blocks. To account for variation in the effect of age, diameter and ring width among

individuals, all models included a random slope term for these covariates (Schielzeth and Forstmeier 2009). Note that because all seeds were collected in the same year, seed age and cohort were perfectly correlated (i.e. all seeds that are 10 years old were produced in 2007) whatever mother tree age. However, we expected the random cohort effect to mostly capture random variation in the environmental conditions during cone and seed formation, whereas we expected the fixed maternal age covariate to capture systematic age-related changes.

Initially we used within-subject centering (Van de Pol and Wright, 2009) to separate within- versus between-individual effects of maternal age, diameter and ring width. To this end, we aggregated all measurements of each predictor for the same individual into an average value, and subsequently subtracted this individual mean from each measurement within an individual. We then fitted both the mean and the deviation of the mean as predictors in the model. While we acknowledge that statistical power was relatively low for this comparison, for none of these predictors we found a significant difference between within- and among-individual effects (see Results). Hence, we subsequently fitted a similar model, but this time with the original measurements as fixed covariates.

Secondly, we quantified the importance of the maternal condition and seed mass on three aspects of offspring performance: germination probability, timing of germination, and seedling survival. Germination and survival probability were modelled as binary traits (germination/survival after germination until 224 days) with a binomial generalized linear mixed model (GLMM). Germination timing was modelled using a linear mixed model with days needed to germinate (i.e. germination day) as the response variable. All models included mother tree ID, cohort and block as random effects, and seed mass, maternal age, diameter, ring width and SPEI as fixed covariates. These analyses were complemented by time-to-event analyses (also known as survival analyses) to estimate the effect of maternal age on seed germination and survival. We used the Kaplan-Meier method to estimate the probability of germination in a given time (measured in days). All mixed models were fitted using the lme4 package (Bates *et al.*, 2015) in R version 3.5.3 (R Core Team, 2019). Significance was inferred via Satterthwaite's degrees of freedom method as implemented in the lmerTest package (Kuznetsova *et al.*, 2017). Cox mixed-effects models were fitted using the coxme package (Therneau, 2020). Kaplan-Meier survival curves were estimated using the survival package (Therneau, 2015).

2.3 Results

Sampled trees ranged from 20 to 69 years of age with a basal diameter between 16.3 and 34.2 cm. The first seeds germinated 17 days after sowing, and the last seeds recorded germinated as late as the second spring, 55 weeks after sowing.

Mean seed mass \pm standard deviation was 55.2 ± 12.5 mg, and varied significantly among trees (32% of variance explained; $\chi^2_1=1962.8$, $p<0.001$) and cohorts (15% of variance explained; $\chi^2_1=844.54$, $p<0.001$).

By comparing within- and between- mother tree effects prior to fitting the final model, we found a non-significant effect of age within mothers ($t_{14.4}=-0.5$, $p=0.6$), and a negative between- mother tree effect of age ($t_{11.8}=-2.56$, $p=0.025$), i.e. older trees produced smaller seeds, but seed mass did not change with cone age within a tree. Despite a large amount of among-individual variation in within-individual slopes, overall seed mass decreased with maternal age ($t_{13.9}=-3.2$, $p<0.006$, Table 2) (Fig. 18A). The effects of ring width were non-significant both within- ($t_{14.4}=0.3$, $p=0.77$) and between- ($t_{11.8}=0.5$, $p=0.65$) mother trees (Fig. 18B). The effect of tree diameter on seed mass was positive and significant between individuals ($t_{12.1}=2.2$, $p=0.045$), i.e. bigger trees produced heavier seeds (Fig. 18C).

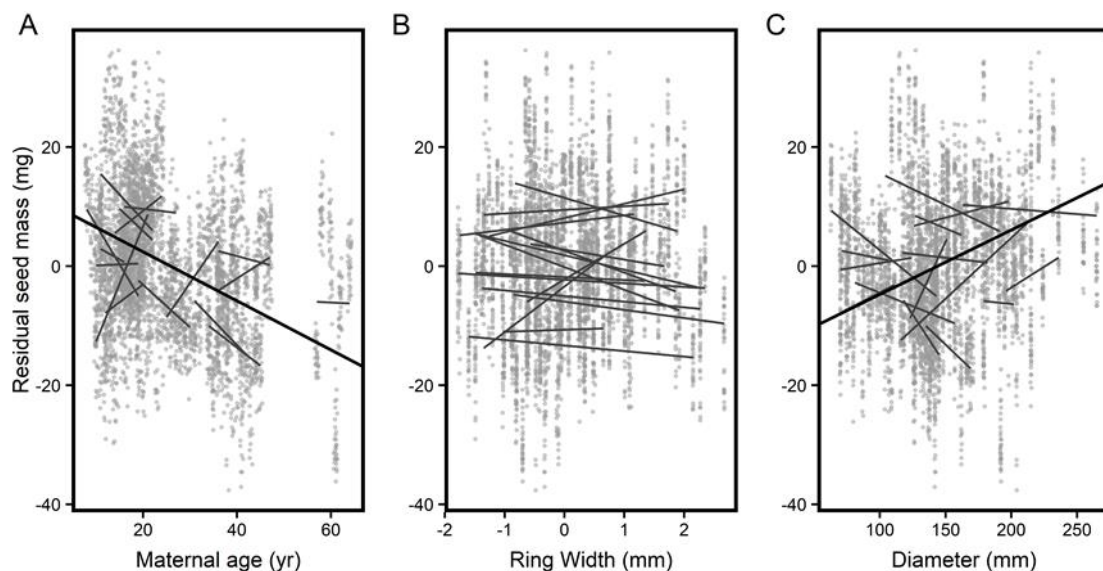


Fig. 18. Within- and between- mother trees effects in seed mass for our sixteen sampled mother trees. The thinner black lines show individual linear regressions for each mother tree. The thicker black line in each panel was drawn from the estimated parameters in the mixed model when that predictor was significant (Table 2). Y-axis represents residual seed mass after accounting for calendar year effects (cohort). In the x-axis, the effect of (A) maternal age; (B) centered ring width and (C) tree diameter.

If we ignore the distinction between within- and among-individual variation, since the differentiation of their effect is not significant, and analyse raw seed mass rather than individual means and deviations from these means (see Methods), we find that although basal diameter increases with age ($t_{10.02}=63.90$, $p<0.001$, Fig. 19), a model including both maternal age and basal diameter again reveals a negative relationship between mother tree age and seed mass (i.e. older trees produce lighter seeds), whereas the effect of basal diameter tends to be positive (i.e. larger trees produce heavier seeds, Fig. 18C). The effects of ring width and SPEI were non-significant. See Table 2 for parameter estimates and statistical details.

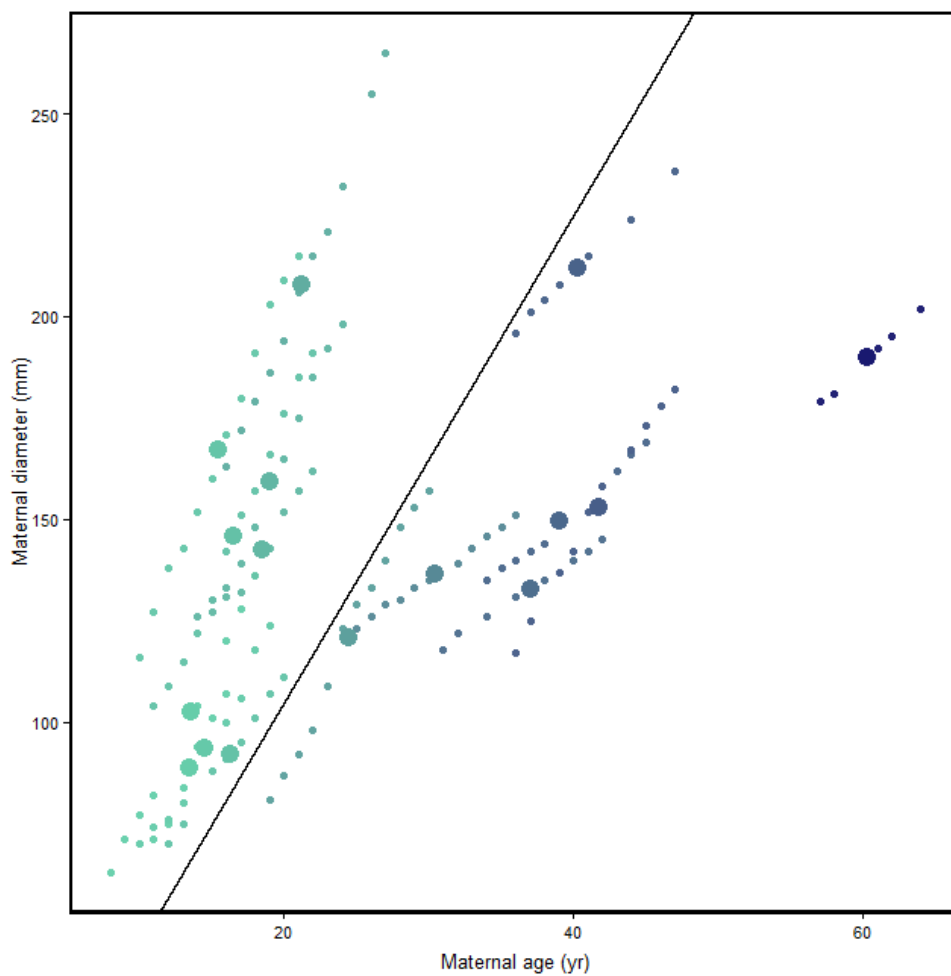


Fig. 19. Relationship between age and diameter of mother trees. Large points correspond to the mean age of each mother tree and small points represent values for each cohort within trees. The black line was drawn from the estimated parameters of the mixed model fitted ($t_{10.02}=63.90$, $p<0.001$).

Table 2. Determinants of seed mass (measured in milligrams). Fixed effect estimates, standard errors, t-values, degrees of freedom using Satterthwaite approximation (as implemented in the lmerTest package, Kuznetsova *et al.*, 2017) and p-values.

	Estimate	Std. Error	t value	df	Pr(> t)
Maternal age	-5.24	1.63	-3.2	13.9	0.006
Tree Diameter	4.37	1.88	2.3	13.1	0.037
Ring width	1.14	2.54	0.5	19.3	0.659
SPEI	-0.18	1.87	-0.1	8.9	0.928

40 days after sowing, 50.8% of the seeds had germinated. Seedling survival was very high over this period, with 98.9% of the seedlings surviving until that date. Up until the onset of winter and the cessation of germination for that year, 224 days after sowing, 89% of the seeds had germinated and 83.3% of all seedlings were still alive. Note that the 11% of seeds that did not germinate until this time point cannot not be considered as failures as they may germinate at a later time point.

Seed mass predicted whether a seed germinated or not as a binary response, with larger seeds being more likely to germinate (Table 3). None of the other predictors were statistically significant.

Table 3. Predictors of germination probability based on a binomial generalised linear mixed model. P-values are based on likelihood ratio tests (LRT). Estimates are on a logit scale.

	Estimate	Std. Error	Chi2	Pr (Chi)
Seed Weight	0.04	0.01	93.23	<0.001
Maternal Age	-0.04	0.14	0.101	0.751
Tree Diameter	-0.01	0.13	0.006	0.937
Ring Width	0.08	0.08	0.976	0.323
SPEI	0.07	0.13	0.065	0.799

The timing of germination on the other hand was unaffected by seed mass, but there was a significantly positive effect of maternal age, with older mothers producing seeds that germinate later, whereas the effect of basal diameter tends to be negative, with larger

mothers producing seeds that germinate earlier (Table 4). The timing of germination was unaffected by ring width and SPEI.

Table 4. Determinants of the timing of germination. Estimated regression parameters, standard errors, *t*-values, degrees of freedom using Satterthwaite approximation (as implemented in the lmerTest package, Kuznetsova *et al.*, 2017) and P-values for the LMM about germination timing.

	Estimate	Std. Error	t value	df	Pr(> t)
Seed weight	-0.07	0.07	-1.1	2451.1	0.286
Maternal age	7.78	2.84	2.7	20.1	0.013
Diameter	-5.51	2.03	-2.7	69.10	0.009
Ring width	0.86	0.86	1.0	255.39	0.314
SPEI	-1.0	1.75	-0.6	9.01	0.580

To illustrate the effect of the mother trees on germination timing, we used the time-to-event analysis method (Fig. 20). The vertical distance between 0 and 1 represents the change in cumulative probability of not germinating as the curve advances. We can see differences between trees.

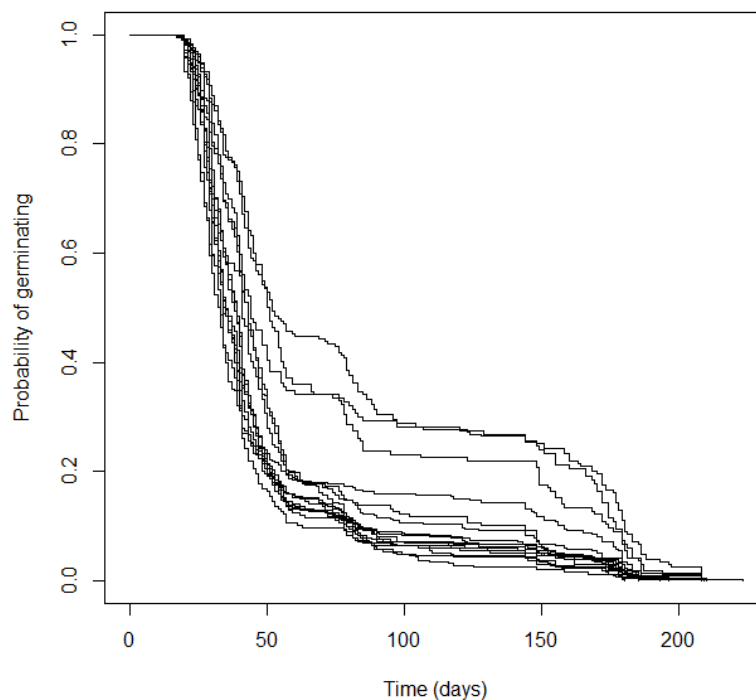


Fig. 20. Kaplan–Meier estimates of germination probability curves for all trees, illustrating the differences in the germination time between individuals. The vertical distance between 0 and 1 represents the change in cumulative probability of germination as the curve advances.

Finally, we found that both seed mass and germination timing showed a significant effect ($\chi^2_1=7.273$, $p<0.01$; $\chi^2_1=127.426$, $p<0.001$ respectively) on mortality, such that higher seed mass significantly reduced seedling mortality risk. Again, we followed up these results using a time-to-event analysis method and we predicted survival rates for representative germination dates (Fig. 21). In this case, the vertical distance between 0 and 1 represents the change in cumulative probability of survival as the curve advances. Seeds that had germinated 22 days after sowing (in blue) survived more than those who had germinated 172 days after sowing (in red). These dates correspond to 5 and 95 percent quantiles, respectively.

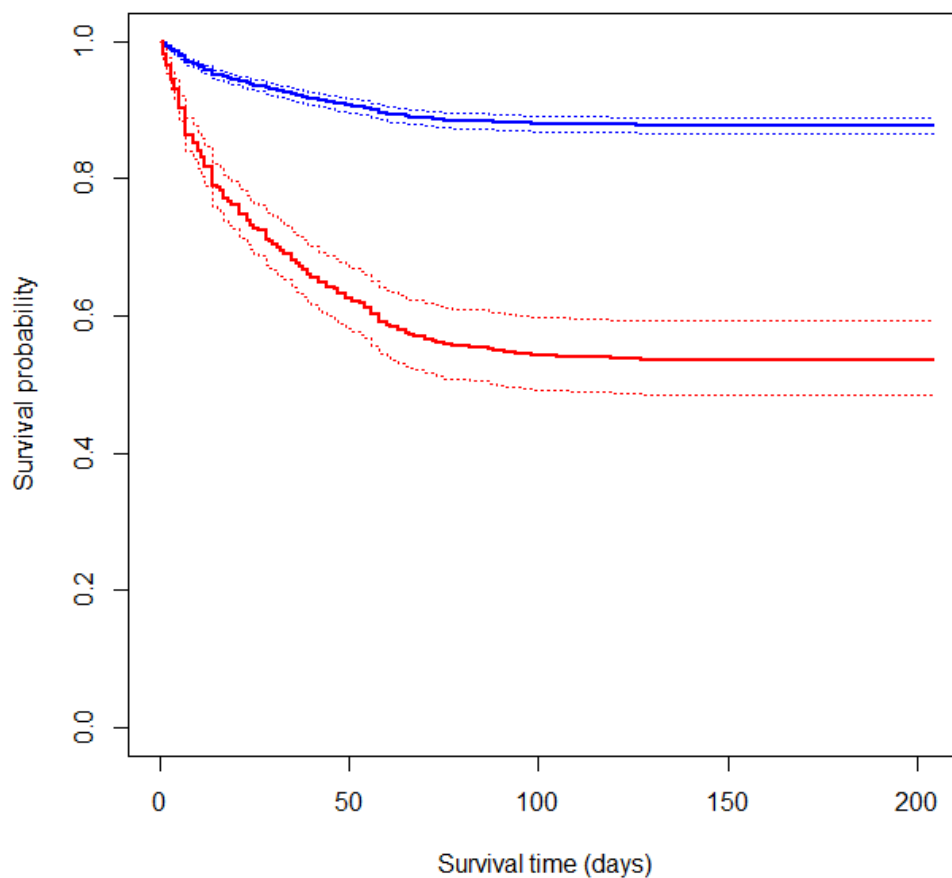


Fig. 21. Kaplan–Meier estimates of survival probability curves for a seed that germinated 22 days after sowing in blue, and for a seed that germinated 172 days after sowing in red (which correspond to 5 and 95 percent quantiles, respectively), illustrating that seeds that germinated earlier had more probability of surviving. To display this plot (see Methods), we used the `coxme` package (Therneau, 2020) to fit a model to the survival object and then, we predicted survival rates for representative germination dates using `survival` package (Therneau, 2020). The vertical distance between 0 and 1 represents the change in cumulative probability of survival as the curve advances. Dotted lines are the confident intervals (2.5% and 97.5%).

2.4 Discussion

In this chapter, we quantified the role of seed mass as a mediator of maternal effects on offspring performance and the importance of maternal age and conditions in shaping these effects. With the seed, the independence of the next generation of plants begins (Bewley, 1997), therefore it is essential to disentangle the factors that will determine its future, including maternal condition and the environmental conditions during seed formation. Maternal age (Cooper *et al.*, 2020) and its sensitivity to the environment can lead to variation in its growth, condition and physiological state (Schmid and Dolt, 1994; Galloway, 2005) that could have important consequences for the next generation, like shown in animals (see, for example Mousseau and Fox, 1998).

We found significant differences in seed mass both between trees and among cohorts of the same mother tree, with 13.6% of the variation in seed mass being attributable to variation among trees versus 9.3% being attributable to cohort effects. This is in line with research on other species, where most variation in seed mass was also observed between individuals (Thompson, 1984; Kołodziejek, 2017; Wang and Ives, 2017). The variation in seed mass, both within and between mother trees, is largely due to effects associated with the maternal tree and environmental conditions during seed development (Bladé and Vallejo, 2008).

The first prediction of the Smith-Fretwell model (1974) is that plants should produce seeds of equal size. However, large seeds are costly (i.e. cost of reproduction) and we therefore expect seed mass to depend on the resource status of the mother plant (Geritz, 1995), which may vary over time and with age (Plaistow *et al.*, 2007). Such variation will cause variation in seed mass between yearly crops of the same individual (Wulff, 1986). In line with this expectation, we found a substantial and statistically significant amount of variation among cohorts within individuals. High within-individual variation is a constant in many organisms, and in particular seed mass typically varies two to fourfold, even within individuals (Michaels *et al.*, 1988). Noteworthy, we found up to five-fold variation between cohorts of some individuals, which is similar to results reported in other species of pine (*Pinus aristata* and *P. flexilis*, Borgman *et al.*, 2014; *P. nigra*, Tiscar and Lucas-Borja, 2010, but see Castro, 1999 in *P. sylvestris*).

We confirmed that the age of the mother tree had a negative effect on seed mass, suggestive of maternal senescence or perhaps of a maternal strategy, resulting in the production of smaller cones containing fewer and heavier seeds at younger age and larger cones with more though smaller seeds at older ages (Cruz *et al.*, 2019). In other studies, maternal age has been shown to have a negative effect on offspring performance either directly or mediated by, for example, an effect of maternal age on seed size (Lembicz *et al.*, 2011) or germination (Alonso-Crespo *et al.*, 2020). At the same time, despite an increase in size with age, mother size (assessed by its diameter), had a significant positive relationship with seed mass; in other words, big young mothers produced larger seeds.

This result confirms that mother size and age have independent effects on seed mass: even when there is a positive correlation between age and diameter, the correlation is less strong than often believed (Fritts 1976; Pederson, 2010) and very often older trees are not the larger ones (Fig. 22), particularly in natural and natural managed forests. In line with this, we found two or even three different growth trajectories across the individual trees (Fig. 19). However, we cannot associate these patterns with differences in the microenvironment, in terms of light or soil conditions. The history of silvicultural practices in this population could help explain these trajectories since they can directly or indirectly influence tree growth (Long *et al.*, 2004).

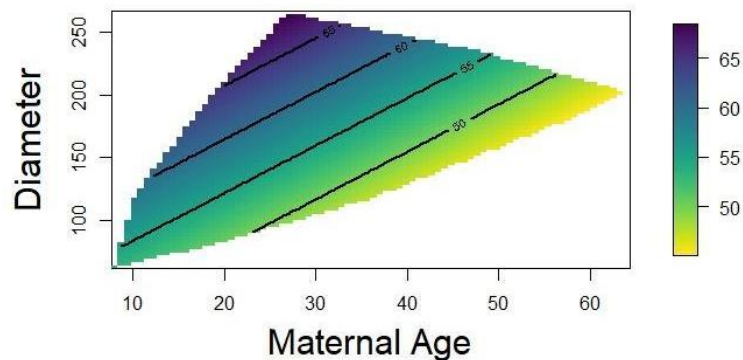


Fig. 22. Contour plot showing the relationship between seed mass, tree age at the embryo development and diameter. Blue colours represent higher seed mass and green colours lower seed mass (mg).

Various studies that have examined the relationship between growth, measured as tree ring width, and reproductive output have provided evidence that reproduction reduces tree performance (Thomas, 2011; Lucas-Borja and Vacchiano, 2018). However, we found that annual ring-width was unrelated with seed mass and hence found no evidence for a trade-off between growth and reproduction. This is in fact in line with other studies

examining maternal effects in other pine species, which found that inter-annual variability in mother twig growth during seed provisioning was not significantly related to differences in seed mass (Borgman *et al.*, 2014). However, seed mass is only one aspect of reproductive investment, and more work using a more comprehensive measure of reproductive investment is needed.

Climatic fluctuations, such as changes in precipitation and temperature patterns associated with climate change, can be an important determinant of reproductive performance (Pérez-Ramos *et al.*, 2010, Basto *et al.*, 2018, Hatzig *et al.*, 2018). However, contrary to other works (Lacey *et al.*, 1997, Murray *et al.*, 2004), we found no effect of the climate experienced by the mothers during embryo development (measured through the SPEI5July, Vicente-Serrano *et al.*, 2010) on seed mass. The fact that our mother trees were of different age and size, and micro-environmental differences in the natural stand could explain a more variable reaction to the same climatic factors at a given year.

We found a significant positive effect of seed mass on germination success in line with a well-supported trend (Castro, 1999, Linkies *et al.*, 2010, Cendán *et al.*, 2013). This could be a result of better-provisioned offspring from higher seed reserves having greater establishment success (Leishman *et al.*, 2000; Herman and Sultan, 2011). However, when accounting for maternal traits, there was no effect of seed mass on the timing of germination. In our study, the age and diameter of the mother were the main effects, such that seeds from older trees (within the age range analysed) had a significant delay in germination, in line with other studies (Leishman *et al.*, 2000; Alvarez *et al.*, 2005), whereas seeds from larger trees germinate earlier. Age effects could be suggestive of senescence or could indicate that seeds from older plants have a greater physical or mechanical dormancy, i.e. seeds are more impermeable or have a harder endosperm (Alvarez *et al.*, 2005). Importantly, germination timing has been postulated to be more determinant to the success of post-fire regeneration than germination percentage *per se* since seedlings will have to compete efficiently for light and water (Cruz *et al.*, 2017). Confirming this assertion, in our experiment early-germinating seeds (before June) had much lower mortality risk (9%) compared to those germinating in midsummer (28% by the end of this season). Early seedling emergence in the growing season confers a greater rate of survival or better growth if early emergence provides advantages with respect to an environmental cue (Verdú and Traveset, 2005; Castro, 2006).

This advantage of early germination is more evident in milder Mediterranean climates where late frosts are less frequent or intense and summer drought is the main source of seedling mortality (Gómez-Aparicio *et al.*, 2005). Drought-related mortality due mainly to midsummer has the potential to act as a filter during early life stages (Warwell and Shaw, 2019). In addition, seed germination timing may influence subsequent seedling phenology and developmental changes by determining the seasonal conditions experienced by seedlings, as studied in annual plants (Donohue, 2009), in which the timing of germination determined whether an annual or biennial life cycle was expressed. However, ensuring a wide range of dormancy due to age effects may be an advantage under an unpredictable Mediterranean climate. Nevertheless, beyond the high influence of germination timing, seed mass had also a positive effect on reducing mortality risk, favouring the survival of the better-provisioned seedlings (Simons and Johnston, 2000).

This work opens a new path in our understanding of the trans-generational plasticity of long-lived plants under a changing environment. This is particularly important as climate change is aggravated and can have implications for the adaptive management of natural forests.





3. Chapter 2

Response to selection in an aerial conifer seed bank

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Abstract

Despite the long-standing interest in the gene-by-environment interaction, potential confounding effects of the temporal scale, such as seed age and different maternal conditions among years have been still poorly investigated.

Seeds obtained from 12 cone cohorts collected from 16 mother trees of *Pinus pinaster* Ait. were planted in a semi-natural common garden located in Madrid, Spain. Seedlings were measured for total height and ontogenetic status during seven months until the winter growth cessation.

We tested whether environmental variation among years, seed mass and maternal conditions, had an effect, as potential sources of variation, on early seedling performance reflecting different responses to selection and heritability estimates.

We found interannual differences in the estimation of heritability that attenuated when we incorporated those potential sources of variation across the temporal scale. We found no empirical evidence of the effect of environmental heterogeneity on selection. These results suggest that these transgenerational effects, even if promoting phenotypic differentiation, could have a secondary role in the short-term evolutionary potential within populations in forest tree species.

3.1. Introduction

Compared to annual species, long-lived forest trees have to face highly variable selection pressures that result from environmental changes over long periods of time (de Miguel *et al.*, 2020; Lamont *et al.*, 2020). To respond to these pressures, tree populations can persist through phenotypic plasticity, local adaptation, migration to a new area with environmental conditions they are adapted to or a combination of these responses (Aitken *et al.*, 2008; Alberto *et al.*, 2013; Bussotti *et al.*, 2015; Gömöry *et al.*, 2020).

However, considering that phenotypic plasticity and migration might be insufficient to cope with these changes and are expected to lag behind the pace of climate change (Shaw and Etterson 2012; Bisbing *et al.*, 2021), successful in situ adaptation will depend on the amount of standing genetic variation and the rate at which this variation arises, is maintained or get to fixation within-populations (Hancock *et al.*, 2011; Shaw and Etterson, 2012; Alía *et al.*, 2014). Therefore, it is critical to continue exploring and measuring the variation amongst individuals or populations across time and space.

The patterns of variation of environmental conditions and resource availability in time and space play a key role in plant performance since species and populations differ in their ability to tolerate those changes and therefore, in their response (Latham, 1992; Sultan, 2000). Unless site conditions are extremely similar, differences in performance are not constant in all environments, and hence across-habitat comparisons can be misleading because environmental differences between stands may hide potential within-habitat effects (Scherer-Lorenzen, *et al.*, 2005). For this reason, numerous studies have focused on studying the responses of tree species to spatial variation through multi-site provenance and progeny trials or experimental environments that are ecologically relevant to the study organism (Sultan, 2000; Santos del Blanco *et al.*, 2010; Alberto *et al.*, 2013). However, seed lots used for these genetic field trials are generally collected in a specific year or even without considering the year of seed production (Sorensen *et al.*, 1977; Sow *et al.*, 2018; Cruz, *et al.*, 2019). Hence, the response to changes in the temporal scale within a tree population have not been studied in such detail and nor in natural conditions, but should not be overlooked since seeds produced in different years may have been exposed to different environmental conditions or may have been produced in extreme years affecting the crossing patterns. In line with this, one major concern is to what extent inter-annual environmental variability can influence the genetic parameters

and thus the response to selection in these long-lived species regarding seedling performance.

A factor that could affect the genetic variation of the produced seeds and the genetic cohort differences per mother tree is the crossability pattern, which is highly influenced by flowering synchronization during the flowering period (Aravanopoulos and Alizoti, 2019). It is also well-known that the environmental conditions experienced in early stages of embryo development or early life can act directly on the developing phenotype and may generate differences between cohorts, i.e. individuals born in the same year under the same environment (Pigeon *et al.*, 2019). This consideration highlights the relevance of maternal effects, i.e. transgenerational plasticity, which occur when the phenotype of an individual is determined not only by its own genotype and the environmental conditions it experiences but also by the year-to-year variation of phenotype or environment of its mother (Mousseau and Fox, 1998; Wang *et al.*, 1994; Kuijper and Hoyle, 2015; Vivas *et al.*, 2020). Maternal effects can be transmitted through diverse mechanisms such, for example, seed provisioning (Roach and Wulff, 1987; Herman and Sultan, 2011; Zas *et al.*, 2013; Vivas *et al.*, 2020). Maternal effects can be confounded with genetic effects (Danchin *et al.*, 2011) in the estimation of heritability since they may cause resemblance between individuals from the same mother tree, overestimating the genetic differences among the progenies of different plants (Sow *et al.*, 2018). Thus, once again, it is confirmed that the study of these effects has profound implications for a better understanding of evolutionary changes and should be considered when forest management decisions are made (Vivas *et al.*, 2020).

Maternal effects can also be transmitted by epigenetic mechanisms that can take place during embryo development (de Vega- Bartol *et al.*, 2013) that, if present, may last stable along tree life through what is called ‘epigenetic memory’ (Yakovlev *et al.*, 2012). More importantly, epigenetics may influence all aspects of the phenotypic variance formula and thus can have evolutionary consequences (see Banta and Richards, 2018 for an extensive review). Therefore, it is essential to investigate the environmental factors linked to annual seed cohorts that can shape the response to selection (McGlothlin and Galloway, 2014).

Ideally, approaching the issue of temporal effects at early stage would be best done in natural or semi-natural conditions as this would allow interacting evolutionary processes to proceed, and thus provide a realistic context for inferences that are relevant to natural populations (Shaw and Etterson, 2012). We propose such an approach by studying a long-lasting aerial seed bank of a conifer species, which allowed us to explore year to year variability within a natural forest tree population. This setting could be considered as ‘multi-year progeny testing’, by treating different annual cohorts as different environments. That way we assessed both the temporal scale approach and the consideration of maternal effects retrospectively.

Our target conifer species, the maritime pine (*Pinus pinaster*) presents a unique model to examine interannual variation. This species presents long-lasting storage of seeds in serotinous cones (i.e. long-closed cones that store seed in the tree crown) that provide access to genetic and environmental variances among yearly cohorts. Year to year variability may have profound implications. Inter-annual environmental variation can explain phenotypic variation (Vizcaíno-Palomar *et al.*, 2020) and can, for example, modify reproductive dynamics (Mutke *et al.*, 2005; Bravo *et al.*, 2017). However, traditional common garden studies usually focus on the spatial (rather than temporal) variability of species and rely on the assumption that variation among populations is due to genetic differentiation (Borgman *et al.*, 2014), overlooking temporal dynamics and maternal effects. Hence, examining year to year variation and the associated maternal effects, by collecting seeds from different years by the same maternal plants, may be a good method for a better interpretation of early plant performance and for investigating possible differences in the response to selection.

For this purpose, we calculated retrospectively each cohort year and we explored some potential sources of variation in early seedling performance considering the environmental variation among years and the role of seed mass and maternal conditions in mediating this (Bischoff and Mueller-Schaerer, 2010; Herman and Sultan, 2011). We measured seedling height at the end of the first growing season and seedling height at the same ontogenetic status as a size-independent index of seedling development (see Methods). Although the relationship between seedling height and developmental status with fitness is not straightforward, these traits are generally assumed to play a key role in early seedling establishment and survival (Verdú and Traveset 2005; Herman and Sultan, 2011; Grossnickle, 2012; de Miguel *et al.*, 2020; Andivia *et al.*, 2021).

In particular, we asked ourselves to what extent we can expect the heritability of seedling height, derived from seeds of an aerial seed bank, to vary among crop years. Our aim is to dissect those factors that affect year to year variation in heritability estimates of seedling height related to interannual variability and to test whether that environmental heterogeneity had an effect on selection.

3.2. Material and Methods

3.2.1 Phenotype measurements and seedling ontogenetic characterisation

Germinating seeds of the experiment described in Chapter 1 (see page 45) were assigned to the values (a) arched hypocotyl emergence, (b) straight hypocotyl uplifting the seed and (c) for the unfolding of cotyledons released from the seed coat as indicated in Figure 23. Shoot ontogeny was further recorded for all seedlings every two weeks until they stopped growing around October, following a categorical scale slightly modified from Chambel *et al.*, (2007, Fig. 23). (0) for the cotyledonary stage, (1) for emergence of the epicotyl rosette, (2) for epicotyl elongation, (3) for the formation of axillary buds and (4) for the development of dwarf shoots with secondary needles, either on the main shoot or on lateral branches. Higher scores reflected a higher developmental (i.e. ontogenetic) stage separately from plant size even when transition from level to level may not be continuous for all plants. Seedling height (in mm) was measured periodically since plants reached stage (2), initially weekly and then every two weeks until they stopped growing around October or the difference between measures was lower than our measuring error. We additionally included in the analyses, seedling height at the ontogenetic stage4 ('H4' hereafter).

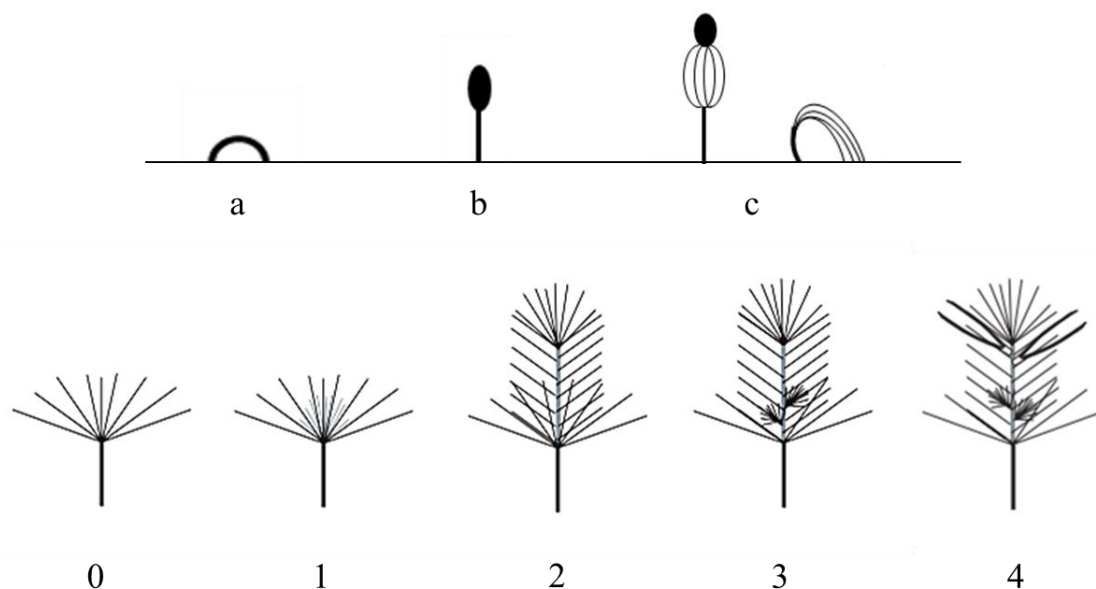


Fig. 23. Ontogenetic scale: (a) arched hypocotyl emergence, (b) straight hypocotyl uplifting the seed and (c) unfolding of cotyledons released from the seed coat, (0) cotyledonary stage, (1) emergence of the epicotyl rosette, (2) epicotyl elongation, (3) formation of axillary buds and (4) dwarf shoots with secondary needles. Modified from Chambel *et al.*, 2007, by ©Callejas-Díaz, M., 2019.

3.2.2 Statistical analyses

We used a linear mixed model (LMM) to assess and quantify the percentage of variance in seedling height explained by mother tree ID, cone age cohort, the interaction between these two and experimental block by fitting each as a random effect (hereafter “General Model”). Their statistical significance was assessed using likelihood ratio tests. Once confirmed the significance of these effects, we obtained Best Linear Unbiased Prediction (BLUP) estimates, which is a technique commonly used in animal and plant breeding studies for estimating random effects, originally developed for ranking and selection in breeding and genetic studies (Henderson, 1963; Robinson, 1991). Best Linear Unbiased Predictors (BLUPs) are weighted means, i.e. a mean where some values contribute more than others, commonly used as point estimates of random effects in mixed models. They predict the expected phenotype of a tree offspring by using phenotypic information collected from relatives (Alía *et al.*, 2014). We plotted BLUP estimates of each mother tree at each cone age cohort for seedling height and H4 against BLUP estimates of cohorts to illustrate Genotype-by-Environment (G×E) interaction patterns. The estimation of random effects allowed us to determine if mother trees were stable across cohorts, to obtain a general view of the interaction patterns, i.e. if they responded in the same way to the annual environment or not.

To further dissect G×E interaction, we selected specific variables of mother trees, i.e. seed mass as a mediator of maternal effects, maternal age and annual ring-width, and on the other hand, a specific variable of cohort, i.e. SPEI (see page 44). Subsequently, we fitted linear mixed models including all these variables as fixed covariates to quantify their roles in shaping variation in seedling height and H4 among mother trees, cohorts, their interaction and blocks. Note that, initially, we expected the random cohort effect to mostly capture random variation in the environmental conditions during cone and seed formation. However, cohort did not show a significant effect by itself, but only its interaction with mother trees (See Results).

The saturated model for the fixed part contained the linear and quadratic terms for each explanatory variable and all the potential pair-wise and three variable linear interactions (i.e. *Seed Mass* × *Maternal Age*; *Seed Mass* × *SPEI*; *Seed Mass* × *Ring Width*; *SPEI* × *Ring Width* and *Seed Mass* × *Ring Width* × *SPEI*).

Then, with the aim of illustrating the overall mother tree ranking in terms of seedling height and H4 performance, we plotted the mean rank across cohorts (environments) against standard deviation of ranks for all mother trees (genotypes). A genotype is considered stable if its rank value is relatively consistent in all the environments. i.e., showing low ranks and having a low standard deviation (Ketata *et al.*, 1989; Flores *et al.*, 1998).

Third, to assess the variation in additive genetic control for seedling height and H4, we used the animal model to estimate global narrow-sense heritability (h^2). The narrow-sense heritability of a trait (h^2) is defined as the proportion of phenotypic variance explained by additive genetic variance, i.e. $h^2 = V_A/V_P$ (Wilson *et al.*, 2010). The additive genetic value (V_A) is the sum of the average effects of the alleles that an individual carries and its variation makes the degree of resemblance between relatives (Falconer and Mackay, 1983). In other words, heritability is a simple measure of the importance of genetic factors in explaining the differences between individuals (Visscher *et al.*, 2008). Note that the offspring within wind-pollinated families collected directly on the mother-plants are considered to be half-sibs and related to each other with a relatedness coefficient $\rho = 0.25$. In these half-sibs family trials, the additive genetic variance is equal to the variance among families (V_F), thus $V_A = 4 \times V_F$ (Gauzere *et al.*, 2013). Thus, we fitted a basic model (“General Model” in Table 9) without including fixed effects as recommended (Wilson, 2008). Subsequently, we did the same for estimating h^2 independently for each cohort. Then, we discounted block effects from each seedling height value and we included fixed covariates step by step, for a better understanding and comparison. “Model 1” included seed mass as a mediator of maternal effects. To account for maternal effects, we included seed mass and maternal age, diameter and ring width (hereafter “Model 2”). Maternal effects can increase similarity among maternal siblings. Consequently, they can look very much like additive genetic effects and if present, but unmodelled, represent a type of ‘common environment effect’ that can - and will- cause upward bias in V_A and so h^2 (Kruuk, 2004; Wilson *et al.*, 2010).

On the other hand, note that SPEI was a cohort-specific covariate that helped to account for environmental changes among years, but could not be added when estimating h^2 independently for each cohort since SPEI was constant within cohorts and thus, it did not help to explain variance differences between individuals of the same age.

In addition, to compare genetic variation, we estimated the coefficient of additive genetic variation (CV_A , also known as coefficient of evolvability), which, unlike h^2 , is a measure of additive genetic variation that is standardized by the trait mean and therefore independent of other sources of variance, making it suitable for comparison among traits and experiments (García-González *et al.*, 2012).

Finally, we calculated the response to selection (R) to determine the predicted evolutionary change for each cohort based on the observed offspring phenotypes. For this, we estimated the selection differential (S), which is the difference in average performance of the cohort group (each cohort sampled) and the selected mother trees (4 best trees in terms of seedling performance), and we scaled the result with the heritability estimate, because the h^2 indicates which proportion of the phenotypic variance can be attributed to genetic variance (Lush, 1937; Falconer and Mackay, 1996; Oldenbroek, and van der Waaij, 2014).

Therefore:

$$R = (\underline{P}_{\text{selected mother trees}} - \underline{P}_{\text{cohort group}}) \times h^2 = S \times h^2$$

All mixed models were fitted using the lme4 package (Bates *et al.*, 2015) in R version 4.1.0 (R Core Team, 2021). Significance was inferred via Satterthwaite's degrees of freedom method as implemented in the lmerTest package (Kuznetsova *et al.*, 2017). The modes of random effects were extracted using ranef function in lme4 package (Bates *et al.*, 2015).

3.3 Results

Mean seedling height \pm standard deviation was 131.4 ± 37.14 mm, and values varied significantly among mother trees (8% of variance explained; $\chi^2_1 = 45.3$, $p < 0.001$), blocks (7,8% of variance explained, $\chi^2_1 = 222.12$, $p < 0.001$), but not among cohorts (0.8% of variance explained, $\chi^2_1 = 1.83$, $p = 0.18$). However, 6% of the variance was explained by the interaction between mother tree and cohort ($\chi^2_1 = 77.93$, $p < 0.001$). With respect to the H4 (See Methods), the mean \pm standard deviation was 186.1 ± 41.28 , and varied among mother trees (8,1%, $\chi^2_1 = 44.22$, $p < 0.001$), and blocks (10,8% of variance explained, $\chi^2_1 = 236.96$, $p < 0.001$). Again, while variation among cohorts was not significant (0% of variance explained, $\chi^2_1 = 0$, $p = 1$), the interaction between mother tree and cohort significantly explained 6,6% of the variance ($\chi^2_1 = 66.78$, $p < 0.001$). See Table 5 for parameter estimates and statistical details of both traits.

Table 5. Likelihood ratio test on random effects of linear mixed effects model analysis to determine Genotype x Environment interaction on seedling height and H4. ANOVA-like table for random-effects with variances, standard deviations, likelihood ratio test, degrees of freedom and p-values of the likelihood ratio test.

Groups	Variance	Std. Dev.	LRT	Df	Pr(>Chisq)
<i>Seedling Height</i>					
Mother Tree	113.96	10.675	45.655	1	<0.001
Cohort	12.28	3.505	1.875	1	0.1709
Mother Tree*Cohort	87.95	9.378	77.892	1	<0.001
Block	111.84	10.575	223.198	1	<0.001
<i>H4</i>					
Mother Tree	141.4	11.89	44.464	1	<0.001
Cohort	0.0	0.0	0.000	1	0.9999
Mother Tree*Cohort	114.6	10.71	66.763	1	<0.001
Block	188.8	13.74	238.335	1	<0.001

BLUP estimates for seedling height by cohorts within mother tree showed a strong, widespread interaction between mother trees and cohorts (Fig. 24). In other words, the responses of the maternal genotypes were different depending on the year when seeds were formed, even when cohort mean values (x axis) for height were similar (e.g. -0.39 and -0.36).

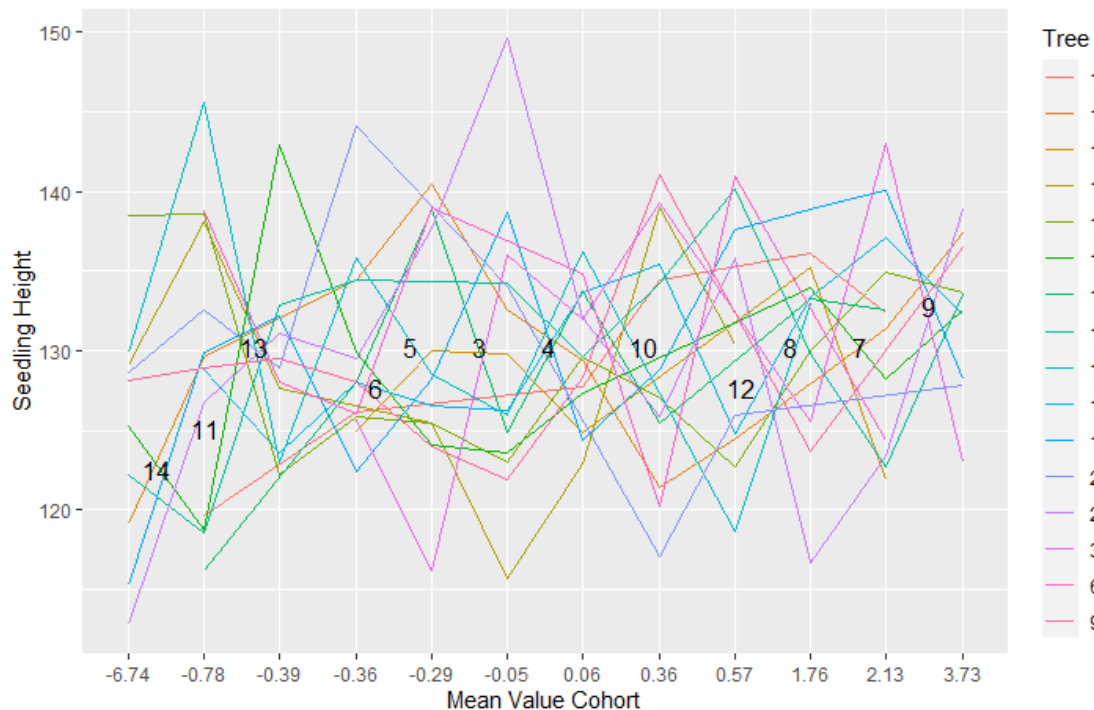


Fig. 24. Genotype x Environment interaction for seedling height. Each coloured line represents the BLUP estimates of Mother Tree x Cohort interaction. Values of the X axis are the Cohort BLUPs, with numbers above indicating each cohort.

We found a similar pattern for the ontogenetic index H4, with a strong interaction between mother tree and cohort (Fig. 25), even considering that cohort did not significantly explain variance by itself.

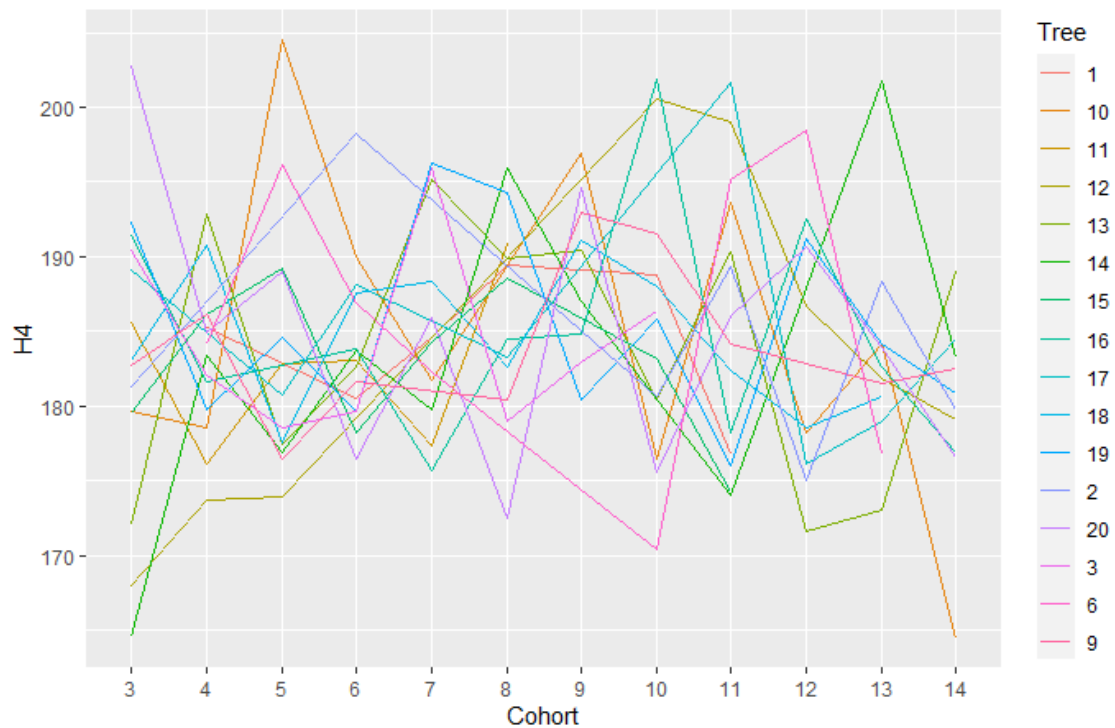


Fig. 25. Genotype x Environment interaction for seedling height at the ontogenetic status 4 (H4). Each coloured line represents the BLUP estimates of Mother Tree x Cohort interaction. In this case, values of the X axis are the cohort IDs since the cohort BLUPs were 0 in all the cases.

We found a moderate overall genetic control for both quantitative traits, i.e. seedling height and H4 with very similar values for narrow-sense heritability and slightly higher coefficient of additive genetic variance (CV_A) for height (Table 6).

Table 6. Genetic control for seedling height in maritime pine under semi-natural conditions. Note that H4 is seedling height at the ontogenetic status 4 (see Methods). These estimates were calculated by fitting a linear mixed model only with random effects, with no fixed covariates. h^2 : narrow-sense heritability, CV_A : coefficient of evolvability, R: response to selection (in %).

Trait	h^2	Percentile	Mean (mm)	CV_A	R (%)
Seedling Height	0.32	0.15 0.36	131.4207	16.25	2.91
H4	0.33	0.16 0.38	186.1097	12.78	2.43

Then, when disentangling the mother by cohort interaction in detail, we found that seed mass and yearly mother tree ring width had a significant positive effect on seedling height, as well as the interaction between SPEI and Ring Width (Table 7). The direction

of these effects indicated that heavier seeds produced taller seedlings (Fig. 26). Moreover, the traits indicative of good maternal conditions during the cohort year, i.e. higher annual ring width and wet yearly conditions, were associated to taller seedlings too (Fig. 27). However, the quadratic term of seed mass showed a negative effect on seedling height, i.e. there is tendency to reduce the growth on heavier seeds. Similarly, the interaction of seed mass with other covariates, such as maternal age, ring width and SPEI, had a negative effect on seedling height.

Table 7. Determinants of seedling height. Fixed effects estimates, standard errors, t-values, degrees of freedom using Satterthwaite approximation and p-values are reported.

	Estimate	Std. Error	t value	df	Pr(> t)
Seed Mass	8.17	1.06	7.7	445.45	< 0.001
Seed Mass ^2	-1.20	0.57	-2.1	936.87	0.0368
Ring Width	3.83	1.30	3.0	131.20	0.0037
Maternal Age	-0.27	1.95	-0.1	28.71	0.8906
SPEI	1.66	1.16	1.4	124.72	0.1559
Seed Mass × MaternalAge	-2.56	0.89	-2.9	413.16	0.0040
Seed Mass × Spei	-2.01	1.07	-1.9	319.58	0.0608
SPEI × RingWidth	3.84	1.37	2.8	133.98	0.0058
Seed Mass × RingWidth	-1.95	1.00	-1.9	243.06	0.0534
Seed Mass × RingWidth × SPEI	-3.14	1.12	-2.8	288.47	0.0052

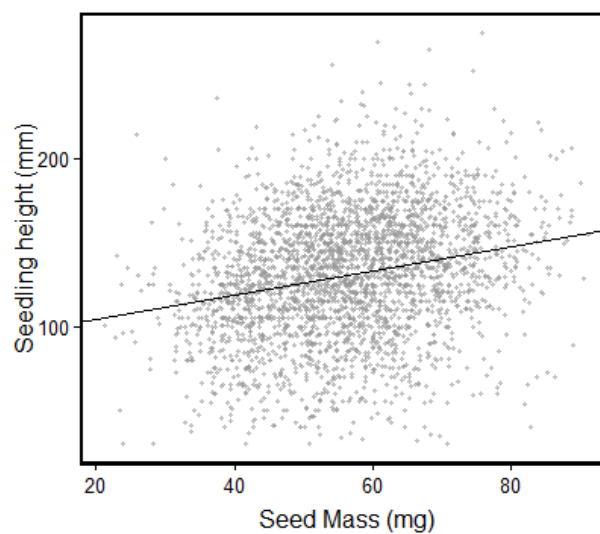


Fig. 26. Relationship between seed mass and seedling height. The black line was drawn from the estimated parameters of the mixed model fitted ($t_{534.83}=9.64$, $p<0.001$).

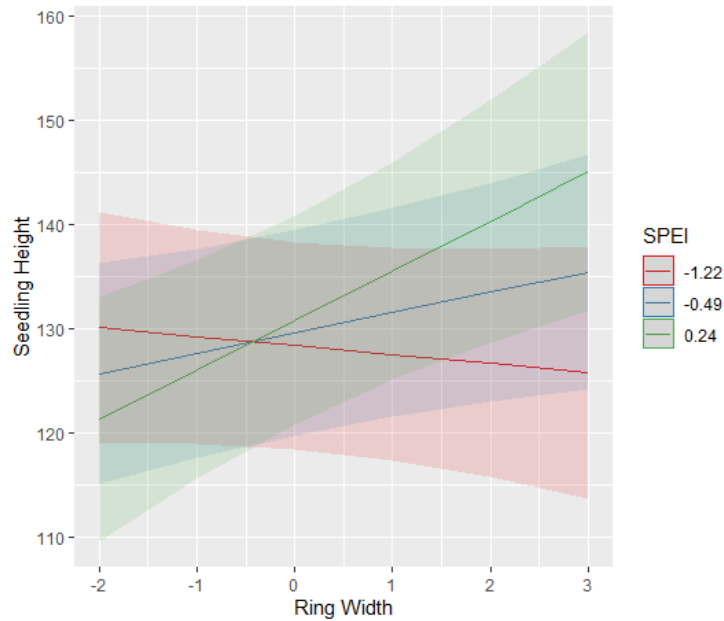


Fig. 27. Two-way interaction plot displaying the predicted values based on the linear mixed model previously fitted (see Table 7). Positive values of SPEI are associated with above average wet conditions, and negative values with above-average dry conditions (Vicente-Serrano *et al.*, 2010).

However, there were sharp differences when looking at the $G \times E$ interaction for H4 as we can see in a simpler model (Table 8). We found that both seed mass and the interaction between Ring Width \times SPEI had significant positive effects on seedling ontogeny.

Table 8. Determinants of seedling height at ontogenetic status 4. Fixed effects estimates, standard errors, t-values, degrees of freedom using Satterthwaite approximation and p-values are reported.

	Estimate	Std. Error	t value	df	Pr(> t)
Seed Mass	8.38	1.14	544.98	7.35	< 0.001
Ring Width	3.02	1.68	140.57	1.80	0.0737
SPEI	1.11	1.50	128.73	0.74	0.4610
Ring Width \times SPEI	3.56	1.72	133.25	2.07	0.0405

To determine the overall performance of each mother tree, we calculated the mean rank for each mother tree across years for both traits (Fig. 28 and Fig. 29). We plotted the mean rank (x axis) of mother trees over cohorts depending on seedling height performance and their standard deviation from the mean (y axis). Low values in the ranking corresponds to a better performance, i.e. mother trees produced offspring with higher seedling height. In line with this, low values in the standard deviation shows

‘stability’, i.e. genotypes with a lower variance for seedling height among cohorts. The plot area was divided in four sections to better illustrate the mother tree stability in terms of seedling performance. ‘Unstable Genotype with Low Performance’ represents high rank mean values and high standard deviation from the mean, i.e. mother trees produced offspring with lower height values and also showed an unstable performance across cohorts, depending on changes in environmental conditions; ‘Stable Genotype with Low Performance’ corresponds to high rank mean values but low standard deviation from the mean, i.e. mother trees produced offspring with lower seedling height values constantly; ‘Unstable Genotype with High Performance’ represents low rank mean values with high deviation from the mean, i.e. mother trees produces in general taller seedlings but varying across cohorts; and ‘Stable Genotype with High Performance’ correspond to low rank mean values and low standard deviation values, i.e. mother trees showed a constant performance irrespective of any change in environmental conditions. The mother trees within the latter section are considered the most stables. For example, in the first case, (Fig. 28) mother tree 6 was the most stable and well adapted across cohorts (environments), and mother tree 9 was the most unstable tree with the low seedling height values.

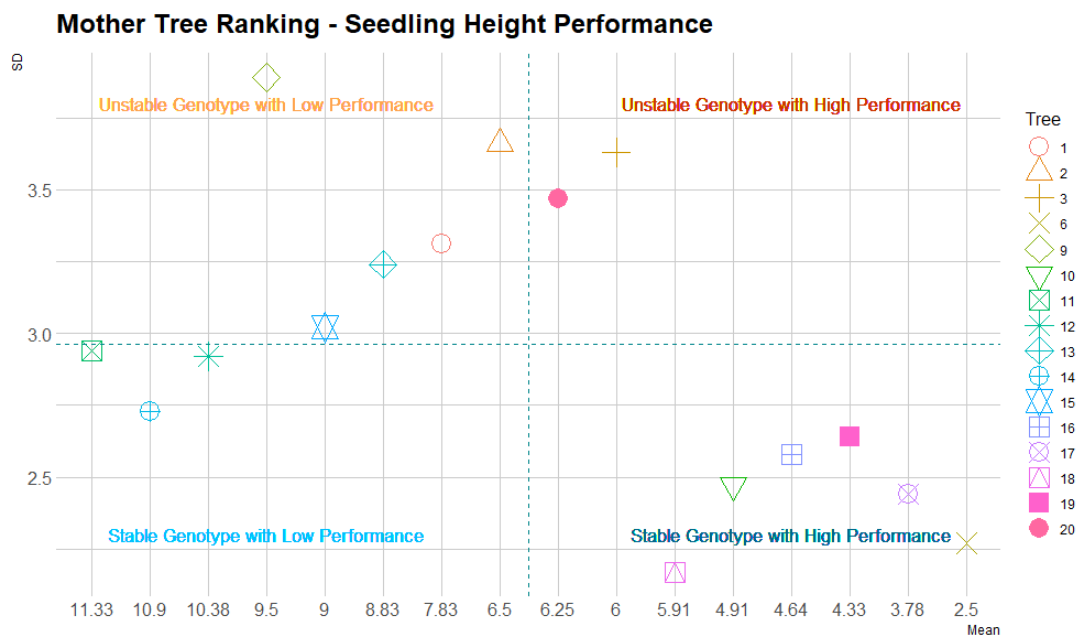


Fig. 28. Mother tree performance stability with respect to seedling height. The x axis indicates the mean rank of a genotype over environments against their standard deviation from the mean. A stable genotype is the one possessing a constant performance irrespective of any changes in environmental conditions, i.e. genotypes with a minimal variance for seedling height over different environments are considered ‘stables’ since they showed no deviation from the mean.

In the case of H4, the mother position over the plot area changed (Fig. 29). We found that mother trees maintained the position with respect to performance (above and below the mean, x axis) except mother trees 2 and 3. However, with respect to stability, we found that standard deviation values are higher in general except for mother tree 17, since the points are higher up in the plot. See for example, mother trees 12 and 14.

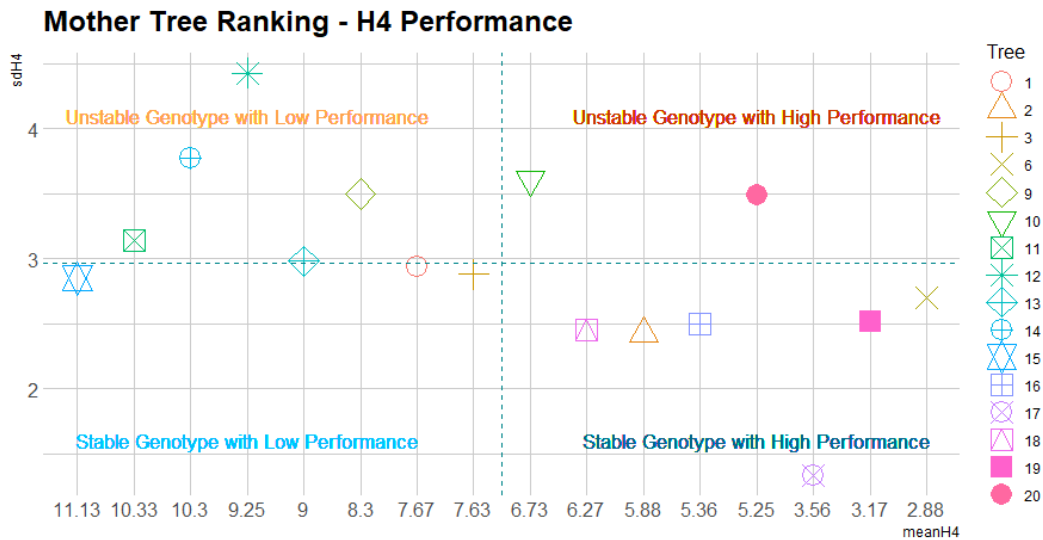


Fig. 29. Mother tree performance stability with respect to H4. The x axis indicates the mean rank of a genotype over environments against their standard deviation from the mean. A stable genotype is the one possessing a constant performance irrespective of any changes in environmental conditions, i.e. genotypes with a minimal variance for seedling height over different environments are considered ‘stables’ since they showed no deviation from the mean.

Finally, when looking at each cohort independently, genetic control on seedling height and H4 varied across cohorts and values were different from zero (Table 5). Again, narrow-sense heritability (h^2) values were high in all cases except for cohort 9 in both traits, which is in accordance with Fig 1, where we can see that there is less variance between trees for this cohort. Note that this h^2 estimation only account for random effects.

However, when discounting maternal effects according to the models, we found a decrease in h^2 values (Table 9). The first model including seed mass showed a general decrease in heritabilities, more notably in some cases, like for example cohort 3. In contrast, when fitting the second model that included all significant predictors (see Table

7 and Table 8), h^2 values decreased but as much as with the previous model. In fact, some values are a bit higher.

The response to selection (R) also varied among cohorts (Table 9), with positive and negative values. But, in general, we did not find high responses, except in the case of cohort 3 of H4, whose value seems to be a bit higher compared to other cases. This means that seedlings at H4 from the selected mother trees grew 7% more than the other seedlings, which in particular corresponds to 13 mm. Therefore, the most informative part of this result is the variation present across cohorts.

Table 9. Quantitative genetic parameters for seedling height and H4 across cohorts in maritime pine under semi-natural conditions, for all models based on Table 5,7 and 8. h^2 : narrow-sense heritability and its percentile, mean and coefficient of variation; CV_A : coefficient of evolvability; R : response to selection (in %); C.V.: Coefficient of variation. General Model: without fixed effects, Model 1: fitting seed mass, Model 2: fitting all predictors (see Methods).

	Cohort	General Model			Model 1			Model 2			Gen Model	Model 1	Model 2				
		h^2	Percentile		CV_A	h^2	Percentile		CV_A	h^2	Percentile		CV_A	R (%)	R (%)	R (%)	
Seedling height	3	0.91	0.33	1.53	30.1	0.36	0.02	0.77	17.4	0.41	0	0.1	18.7	1.88	1.38	1.01	
	4	0.29	0.01	0.67	16.5	0.14	0	0.44	11.2	0.17	0	0.54	12.4	0.32	0.11	0.07	
	5	0.61	0.13	1.15	26.3	0.49	0.05	0.99	23	0.58	0.05	1.22	25.3	2.11	2.67	1.86	
	6	0.48	0.1	0.94	20.4	0.46	0.07	0.9	19.9	0.51	0.05	1.14	21.2	0.05	0.98	0.87	
	7	0.53	0.08	1.12	21.6	0.56	0.06	1.22	22.3	0.75	0.05	1.5	26.4	0.38	-1.85	-2.59	
	8	0.41	0.03	0.89	18	0.3	0	0.72	15	0.44	0	1.09	18.7	1.61	-0.88	-0.91	
	9	0.06	0	0.32	6.8	0.04	0	0.29	5.8	0.02	0	0.38	3.9	0.02	0.21	0.03	
	10	0.1	0	0.36	9.4	0.07	0	0.29	7.5	0.05	0	0.37	6.3	-0.5	0.24	-0.01	
	11	0.68	0.18	1.26	28.6	0.49	0.09	1.01	23.7	0.56	0.06	1.2	25.3	3.49	2.58	1.64	
	12	0.54	0.04	1.15	22.4	0.3	0	0.81	15.8	0.36	0	1.07	17.4	0.11	0.03	-0.13	
	13	0.16	0	0.5	12	0.07	0	0.35	8	0.22	0	0.7	14.2	-0.09	0.00	-0.04	
	14	0.26	0	0.74	18	0	0	0.32	0	0	0	0.56	0	-0.72	0.00	0	
	Mean	0.42				0.27				0.34							
	C.V	60.9				74.0				72.4							
	H4	Cohort	h^2	Percentile		CV_A	h^2	Percentile		CV_A	h^2	Percentile		CV_A	R (%)	R (%)	R (%)
3		1.11	0.38	1.83	27.4	0.57	0.12	1.18	18.2	0.6	0.1	1.18	18.6	7.07	3.63	3.05	
4		0.19	0	0.58	11.7	0.09	0	0.4	7.8	0.11	0	0.43	0.87	0.01	-0.03	0.00	
5		0.76	0.14	1.4	24.5	0.47	0.04	1.04	18.4	0.51	0.03	1.13	19.5	1.63	1.22	0.99	
6		0.35	0	0.81	14.2	0.34	0	0.8	13.8	0.37	0	0.89	14.5	-0.24	0.00	0.02	
7		0.55	0.06	1.17	17.6	0.48	0	1.12	16.3	0.54	0	1.21	17.5	0.75	1.07	0.93	
8		0.28	0	0.76	11.9	0.24	0	0.68	10.9	0.28	0	0.77	11.8	-0.39	-0.25	-0.23	
9		0.05	0	0.33	5.2	0.07	0	0.4	6	0.04	0	0.39	0.46	-0.03	0.04	-0.03	
10		0.14	0	0.46	9.3	0.16	0	0.49	9.9	0.17	0	0.59	10.3	-0.61	-0.63	-0.48	
11		0.52	0.05	1.03	18.8	0.43	0	0.91	17	0.48	0.02	1.07	18	1.10	0.68	0.56	
12		0.75	0.07	1.45	20.8	0.58	0	1.29	17.6	0.45	0	1.12	15.3	2.12	1.54	0.94	
13		0.18	0	0.57	10.2	0.12	0	0.51	8.1	0.12	0	0.56	0.83	-0.21	-0.23	-0.18	
14		0.25	0	0.82	12.9	0	0	0.39	0	0.02	0	0.53	0.36	0.13	0.00	0.02	
Mean		0.43				0.30				0.31							
C.V		74.2				70.1				68.1							

3.4 Discussion

Different alleles provide the capacity to produce a great variety of responses to selection pressures, and therefore, it is essential to study the genetic variation of forest tree traits on which changing climate seems likely to impose selection (Garzón *et al.*, 2011; Sella and Barton, 2019). This is the case of polygenic traits of evolutionary significance, i.e. fitness-related traits such as seedling height, that play a key role in early seedling establishment (Verdú and Traveset 2005; Herman and Sultan, 2011; Grossnickle, 2012; de Miguel *et al.*, 2020). In this work, the question posed is to what extent we can expect the heritability of height and ontogeny of seedlings derived from seeds of an aerial seed bank, to vary among crop years. In addition, whether environmental heterogeneity (environmental variation among years, seed mass and maternal conditions) had an effect on selection. We focused on a single population, which allowed us to study the variability within the time scale with the help of seeds from a range of serotinous cone cohorts formed during different years. With this approach, we considered years as different environments. We determined year to year variability in the estimation of heritability of height and ontogeny considering some potential sources of variation across the temporal scale such as maternal and environmental conditions.

Firstly, when analysing how much variance explained the random effects, we found strong effects of mother tree, experimental block and mother tree \times cohort interaction on seedling height. The estimation of random effects allowed us to determine if mother trees responded in the same way to the annual environment or on the contrary, they differed. As it is shown, mother tree \times cohort interaction varied significantly even when cohort estimates were similar. However, the effect of the annual cohort itself did not show a significant effect, which suggested that, rather than the global environment to which seeds were subjected during development, some potential effects at the microscale could be affecting the trait, i.e. a combination of different conditions linked to each year and mother tree (Zas *et al.*, 2013; Castro, 2021). This interaction could also be the result of different mating patterns among individuals in different years that may keep the overall variation of the population intact. That explains why the genetic variation of post fire regeneration from serotinous cones does not usually deviate from that of the old stand (Lucas-Borja *et al.*, 2016; Aravanopoulos and Alizoti, 2019).

Secondly, we included some fixed predictors to separate some potential maternal effects and the effects of each cohort. As expected, we found a significant effect of seed mass on seedling height, such that heavier seeds grew taller, a result well documented in the literature (Leishman *et al.*, 2000; Suárez-Vidal *et al.*, 2017), which state that seed mass is the largest influence on a seedling's initial reach above and below the ground. In general, heavier seeds may contain more reserves and initially develop into larger seedlings (Bladé and Vallejo, 2008). However, its quadratic term showed a slightly negative effect on growth, indicating that, in some cases, heavier seeds did not necessarily grow more (in terms of height). In line with this, smaller seeds of some species may outgrow seedlings from large seeds, especially when resources are not limited (Paz and Martínez-Ramos, 2003), i.e. microsite may influence the expression of seed mass effects on natural regeneration as a function of resource availability (Parker *et al.*, 2006). We must also keep in mind that we are looking only at the above-ground growth, while seedling biomass partitioning could provide a more complete picture of seedling resource allocation (Mašková and Herben, 2018).

In a previous work (Callejas-Díaz *et al.*, 2022, see Chapter 1), we found that annual ring-width of mother trees was not significantly correlated with differences in seed mass. However, it showed a substantial positive effect on seedling height and H4, as well as the interaction of annual ring-width and SPEI, i.e. mother trees with higher annual growth in combination with wet conditions in that year produced taller seedlings. As seen for this species, abiotic conditions of the maternal environment can influence seedling height, in the sense that progenies derived from mother trees growing in a favourable maternal environment were taller (Vivas *et al.*, 2013).

Seedling growth performance and plasticity is generally assumed to be genetically controlled but also determined by the environment experienced by the mother tree (Bose *et al.*, 2020). Our results suggest that a kind of environmental memory could be operating via maternal effects, e.g. resources inherited through seeds (seed mass-independent effects, Roach and Wulff, 1987), and/or transgenerational epigenetic mechanisms that can induce changes in gene expression (Zas *et al.*, 2013).

Although seed mass had a positive effect on seedling growth, its interaction with maternal age showed a significant negative effect on seedling height, indicating that older trees produced seedlings that grew less, presumably due to older trees producing lighter

seeds as seen in the previous chapter. Moreover, seed germination timing increased with maternal age as seen in the previous chapter, and in other work for this species (Alvarez *et al.*, 2005) and thus could merely imply that seedlings did not reach the height that earlier germinating seeds did.

The negative effect of the three-way interaction of seed mass, SPEI and ring width could be explained as the effect of the combined action of these three predictors is less than the sum of their individual effects for some mother trees, suggesting that their individual positive effect on seedling height is not always straightforward when they interact in some cases.

The results related to mother tree ranking indicate that in cases where seedling performance is high, instability can also be present. This highlights the importance of recognising the role of maternal effects, which can change from one year to another, at least to take into account that the offspring phenotypes can deviate from what breeding programmes are looking for (Vivas *et al.*, 2020).

Genetic control on both seedling height and H4 varied across cohorts within a population and those changes could depend in part on conditions at the micro-scale, indicating different microevolutionary patterns between years. Generally, some significant amount of additive genetic variance can be maintained within natural populations, even for these kinds of characters tightly connected to fitness (Mousseau and Roff, 1987; Sella and Barton, 2019). Variation in fitness and the traits associated with it, in addition to being attributable to many genes, is also related to environmental conditions (Crow, 2008; Hill, 2010).

A common practice when quantifying heritabilities from mixed-effects models, is to omit fixed effects. However, fitting fixed effects allows to finely controlling for confounding effects (De Villemereuil *et al.*, 2018). In line with this, we included seed mass to account for maternal effects and we found that heritability estimates decreased. This confirmed that, although it was not such a big difference, seed provisioning increased resemblance between relatives and, if not modelled, could have been confounded with genetic effects (Danchin *et al.*, 2011).

However, when we fitted the other fixed covariates such as maternal age and ring width, h^2 estimates increased slightly with respect to the model that only included seed

mass (see Methods), revealing that these effects were purely environmental. Their inclusion necessarily reduced the magnitude of V_R and subsequently, h^2 increased in most cases (Byers, 2008; Wilson, 2008).

In any case, the successive inclusion of fixed effects in the models led to a decrease of h^2 in all cohorts with respect to the general model with only random effects, except in cohorts 7 and 9. But, despite being a plastic trait subjected to environmental variation, seedling height is a highly heritable trait in this pine species (Suárez-Vidal *et al.*, 2021).

Because this study was conducted with a subsample of 16 mother trees, it is likely that only a small range of the actual genetic variation of the original population was explored, although some values of h^2 were high. It is therefore likely that the additive genetic variance in seedling height within the whole population could actually be even larger than that reported here. In addition, when accounting for maternal effects we expected the variance explained by the mother tree to go down, as was the case. Moreover, we cannot discard other unmeasured maternal effects that could contribute to the phenotypic variation. As a consequence, our heritability estimates could always be biased. Also, any variation among cohorts could be the result of variation in additive genetic variance, but also of variation in maternal variance.

On the other hand, heritability was estimated assuming that families are true half sibs, but we cannot completely ensure that some of the seedlings from the same maternal tree are, in fact, full siblings. However, due to the mating system in Maritime pine (Gaspar *et al.*, 2009), the associated error in heritability estimates due to the inclusion of full-sibs, when assuming a standard coefficient of relation among open-pollinated sibs of $\frac{1}{4}$ is low and not very likely.

Finally, although we showed that some mother trees produced offspring with better performance, the response to selection was not pronounced. Again, this could be due to the dataset used in this work. But, in addition, maternal effects result in mismatches between genotypes and phenotypes, thereby buffering the influence of selection on allele frequency, which could help explain our results (Yamamichi and Hoso, 2017; Pujol *et al.*, 2018).





4. Chapter 3

Estimating genetic variability between two temporal contrasting cohorts from an aerial seed bank of maritime pine

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Abstract

Wildfires play a key role in the ecology and evolution of plant species, affecting potentially the genetic makeup of populations, by shaping the patterns of spatial and temporal genetic variation in natural populations. In this chapter, we tested whether there were differences in the genetic variability between two contrasting temporal cohorts, separated by 11-year span time, in the highly serotinous population of *Pinus pinaster* of Tabuyo del Monte. Our hypothesis was that within this period of time, recurrent wildfires and/or the mega-wildfire of high-intensity occurred in 2012 could have played a role in shaping the genetic makeup of the population, translating into differences in the offspring performance investigated in the previous chapters.

Using eight nuclear microsatellites, we genotyped 477 seedlings originating from two contrasted temporal cohorts (i.e., cohort 3 with cones formed in 2014, and cohort 14 with cones formed in 2003) collected in 20 adult trees, and grown in the common garden experiment. We analysed the overall within-tree genetic diversity as well as the inter-annual variation of different age cohorts.

We did not find any differences in the genetic variability (in terms of allelic richness, genetic differentiation and variation) between the two pools of offspring (cohorts 3 and 14) analysed. We therefore could not attribute the observed differences in the offspring performance across the two considered cohorts to genetic differences measured in the population under study. However, we revealed a certain level of genetic variation (13%) among pools of offspring across mother trees within a given year. Finally, mating and gene flow analyses showed that paternal clouds within offspring arrays and across offspring arrays of a given year were highly diverse, meaning that offspring did not share the same fathers.

Altogether, our results did not reveal any temporal differences in the genetic makeup of the two cohorts under study, and a marginal spatial heterogeneity of these cohorts across trees within the same year. These outcomes are in agreement with the homogenization effect of pollen flow on genetic diversity.

4.1. Introduction

Forest trees typically possess levels of genetic variation that are among the highest observed in all living organisms, mainly due to their large population size that reduces random genetic drift, their longevity that results in the accumulation of mutations over time, their high levels of outcrossing, capacity for long distance gene flow between populations and balancing selection (White *et al.*, 2007). However, these high levels of genetic variation can be reduced by factors linked to the population history and demography (e.g. bottleneck, genetic drift), human intervention (e.g. thinning) or natural disturbances (e.g. disease, drought, fire or flooding).

Among the latter, wildfires play a key role in the ecology and evolution of plant species in Mediterranean regions (Pausas and Bond, 2018), prompting different plant strategies such as early onset of reproduction that ensures a canopy seed bank (Bradstock and O'connell, 1988; Tapias *et al.*, 2001; see page 24). One of these strategies is the production of serotinous cones, which consist in closed cones that are retained in aerial seed banks often for long periods of time, and usually open with the heat of fire (see more in page 25). The increase of temperatures and drought has been linked to an ongoing increase of fire occurrence, intensity and severity in Mediterranean ecosystems (Doblas-Miranda *et al.*, 2017; Fernández-García *et al.*, 2019). Ecological consequences of fire include alteration in pollen and seed dispersal, mating patterns and gene flow by opening the landscape and reducing the populations' size and density (Shohami and Nathan, 2014).

Depending on their regimes (ranging from frequent, low-severity fires in some ecosystems to infrequent, high-severity fires in others), fires may have a range of consequences on the level and spatial distribution of genetic diversity (Lucas-Borja *et al.*, 2016), through either selective processes (i.e. modifying genetic structure by selecting for particular behavioural or physiological traits) or selectively-neutral demographic processes (modifying genetic structure through either direct effects such as population bottlenecks or redistribution of individuals, or indirect effects such as manipulation of habitat suitability or permeability (Banks *et al.*, 2013). Stand-replacing fires (crown fire) could lead to potential replacement of pollen donors across years (Torres *et al.*, 2016), while these intense fires may open the landscape and favour wind-mediated pollen flow, therefore alleviating the negative effects of a strong reduction in population size on the population genetic layout. This was the case for instance in an Israeli natural stand of

Pinus halepensis experiencing a drastic fire that killed 96% of the pine trees, reducing the nearby pollen donors and leading to a change in the gene flow dynamic (Shohami and Nathan 2014). This drastic reduction of pollen donors led to differences in the amount of genetic variation among years within the aerial seed bank. In this situation, new alleles can arise, be maintained or removed, and/or get to fixation within populations (Hancock *et al.*, 2011). In others cases, fires may have milder consequences on the genetic makeup of the successive generations, as shown in a study with two Iberian populations of *Pinus halepensis* under contrasting fire regimes (high vs. low frequency and intensity of wildfires) (Budde *et al.* 2017). The results showed a preservation of the overall genetic diversity but different spatial genetic structure (SGS) in the progenies of both populations, with stronger SGS and selective pressures in the population under stronger fire regime. The impact of fire on the genetic makeup is thereby very context dependent, and empirical data are lacking to draw general conclusions. In particular, the pattern of accumulation of genetic variation over time in seed banks, such as serotinous cones, is poorly understood (Barrett *et al.*, 2005). Aerial seed banks may act as reservoirs of genetic variation (Barret *et al.*, 2005), giving us the opportunity to compare the pre-disturbance and post-disturbance gene flow within the same population.

In order to understand better the long-term consequences of serotiny on the genetic variability within aerial seed banks, we studied one Spanish population of maritime pine (*Pinus pinaster*) with a subsample of 13 adult trees bearing serotinous cones spanning 12 years (from 2003 to 2014).

In areas such as the Iberian Peninsula, stands of *P. pinaster* have been severely affected by natural disturbances through recurrent forest fires (Derory *et al.*, 2002; Taboada *et al.*, 2021). In particular, Tabuyo del Monte (situated in Sierra del Teleno) is a historically fire-prone area, mainly due to spring dry lightning storms (Santamaría, 2015; Fernandez-Manso *et al.*, 2016). This forest was affected by a mega-wildfire of high-intensity in 2012 (Fernández-García *et al.*, 2019), a year that is included in our seed bank temporal range. Our study focused on the seeds enclosed in serotinous cones of the two extreme years of the series (2003 and 2014) to maximize our chance of finding any genetic footprint of fire events in our population (such as the one from 2012). Our objectives are two-fold, finding out: (i) whether the genetic makeup of a serotinous collection has changed in time due to a reduction or shifting of the pollen contributors. To that aim, we estimated genetic variation, mating parameters and gene flow in the two

cohorts of aerial seed bank across 13 mother trees; and (ii) whether temporally distinct serotinous cohorts explained the performance of seedlings in the common garden described in Chapter 1 and Chapter 2.

4.2. Material and Methods

4.2.1. Study site and field sampling

We collected needle tissue from the same 20 mother trees sampled within the *P. pinaster* population of Tabuyo del Monte previously described (see 2. Chapter 1, 2.2 Material and Methods). We kept a minimum separation distance of 20 m between sampled adults, which ranged from 20 to 69 years of age based on the results of Chapter 1. Seedlings from serotinous cones (spanning 3-14 years) collected from 16 mother trees (see 2.2.2. in page 42) were germinated and collected two years after sowing them in a common garden experiment located in Madrid (see page 45). Seedlings from the two most contrasted temporal cohorts were selected, i.e. cohort 3 (3 years old cones from 2014) and cohort 14 (14 years old cones from 2003) (Fig 30.), from all of the mother trees that had those cohorts represented, i.e. 13 mother trees. The seedling sampling corresponds to a total of 477: 305 samples for cohort 3 and 172 samples for cohort 14, depending on the germination and survival rates (see Table 10). Needles from both 20 the mother trees and the 477 seedlings were dried in silica gel pending DNA extraction.

Table 10. The 477 seedlings sampled in the common garden experiment located in Madrid, from cohorts 3 and 14 subsampled in 13 mother trees, depending on germination and survival. *Data not available (not all the mother trees sampled had cohort 14).

Mother Tree ID	No. Offspring C3	No. Offspring C14	Total no. Offspring
2	26	20	46
3	23	*	23
9	11	8	19
10	25	17	42
12	22	16	38
13	27	23	50
14	23	11	34
15	26	*	26
16	25	17	42
17	22	21	43
18	25	*	25
19	23	21	44
20	27	18	45

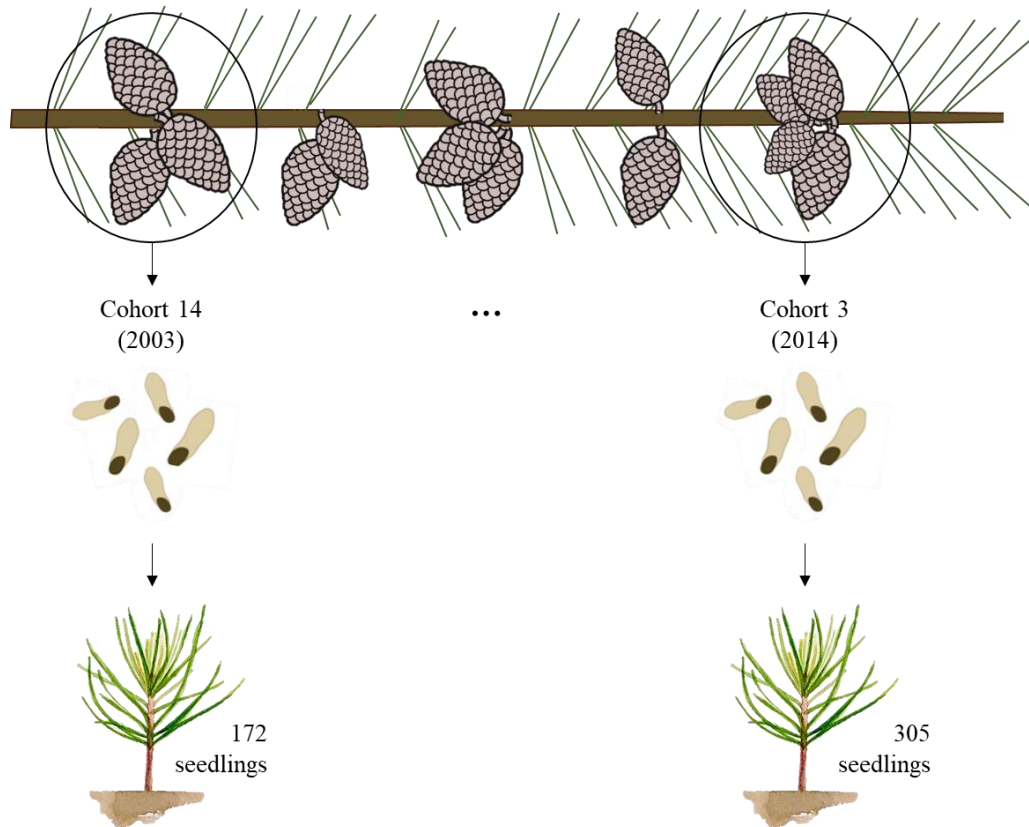


Fig. 30. Schematic representation of serotinous cohorts 3 and 14 in a branch of a mother tree of *Pinus pinaster* (note that between them, were found the other cohorts although not all are represented), ©Callejas-Díaz, M., 2020. Pine cone design by ©Canalda, J.

4.2.2. Genetic characterization

We extracted genomic DNA from 20 mg of dried needle tissue using Invisorb DNA Plant HTS 96 Kit/C (STRATEC Molecular GmbH, Berlin, Germany). DNA quality and quantity were assessed with NanoDrop™ One/OneC (Thermo Fisher Scientific). Microsatellites are powerful tools for characterization of genetic structure and generally have a large number of alleles that allow the differentiation between individuals (Guichoux *et al.*, 2011). We genotyped the 20 mother trees and the 477 seedlings using 12 nuclear microsatellite molecular markers (Simple Sequence Repeats - nSSR) based on the study of Unger *et al.* (2014). Mother trees were genotyped to assess the overall level of genetic diversity at the population level, while genotype comparison between mother tree and seedlings was used to detect and correct genotyping errors (e.g., allele dropout or null alleles).

Amplified fragments were separated using an ABI 3730 genetic analyser (Applied Biosystems, Carlsbad, CA), and fragment sizes were determined using the GeneScan™ 500LIZ® Size Standard (Applied Biosystems) using the software GeneMapper® version 3.0 (Applied Biosystems). The scored loci were then analysed with MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.*, 2004) to test for null alleles and scoring errors. Eight nSSRs were finally retained for subsequent analyses (ctg275, RPtest11, ctg4363, epi3, FRPP94, NZPR1078, gPp14 and pEST2669) based on various criteria, including easiness of scoring, Hardy-Weinberg equilibrium and power to differentiate between individual genotypes. The four discarded nSSRs showed poor, unspecific or inconsistent amplification, and/or resulted in ambiguous scoring (NZPR544, A6F03, NZPR413 and epi5). Note that in the case of NZPR544 and A6F03, we obtained the same fragment sizes for both markers. To avoid redundant information, we selected the one easier to read, which in our case was A6F03, since NZPR544 displayed ambiguous cases with more than two alleles (Fig 31.), even in the repetitions.

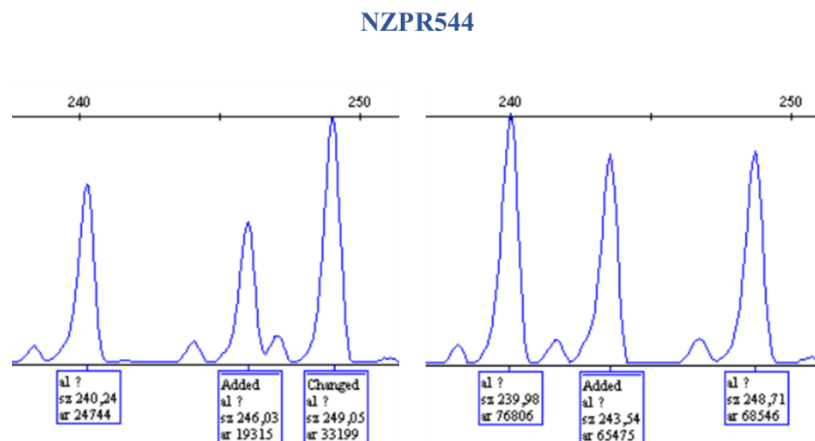


Fig. 31. Example in which more than two alleles appeared, even when doing repetitions. Figure caption obtained from GeneMapper® 3.0 (Applied Biosystems).

However, we found that A6F03 fragment sizes did not meet the assumptions of the Hardy-Weinberg equilibrium, as also occurred with NZPR413. Therefore, both nSSR were discarded too.

Finally, both epi5 and gPp14 also displayed the same alleles (Fig. 32) and we chose to retain gPp14.

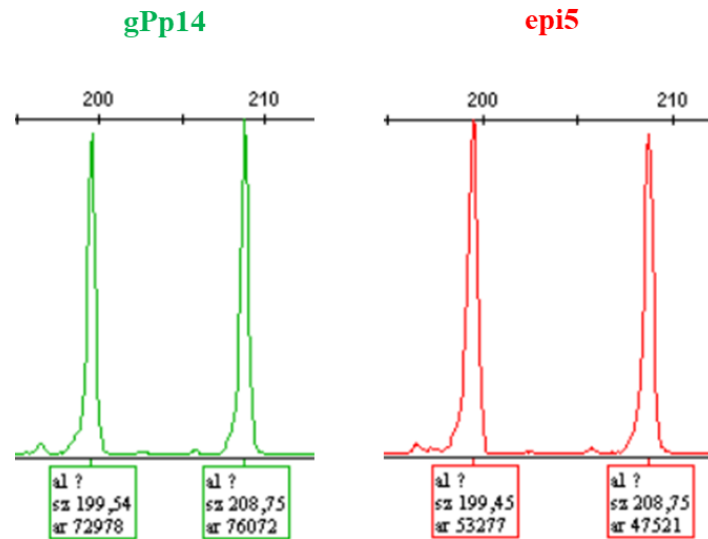


Fig. 32. Example of similar readings of fragment sizes for the nSSR loci gPp14 and epi5, obtained using GeneMapper® 3.0 (Applied Biosystems).

We confirmed that the elimination of any of these markers would not change the results. The eight nSRRs mentioned above were able to genetically discriminate the 20 mother trees.

4.2.3. Analysis of genetic diversity and differentiation

We characterized each retained microsatellite loci for the whole population (i.e. 20 adults) and each cohort (i.e. C3 and C14) by computing observed (H_o) and expected heterozygosity (H_E ; Nei, 1987), allelic richness (Garza and Williamson, 2001), allelic frequencies and the degree of genetic differentiation between cohorts 3 and 14 (F_{ST} , 10.000 permutations, Slatkin, 1995). We also tested for Hardy-Weinberg equilibrium using an exact test based on Markov-chain algorithm (Guo and Thompson, 1992) for each locus. Finally, we carried out the molecular analysis of variance (AMOVA, Excoffier *et al.* 1992) to infer the population genetic structure at different hierarchical levels: between years (cohort C3 versus cohort C14), among mother trees within years, and within mother trees.

All these analyses were performed using Arlequin 3.5 software (Excoffier and Schneider, 2005) and the R packages adegenet (Jombart, 2008), pegas (Paradis, 2010), ade4 (Thioulouse *et al.*, 2018), gstudio (Dyer, 2021) and hierfstat (Jerome, 2021), in R software version 4.1.0 (R Core Team, 2021).

4.2.4. Mating system

Subsequently, we estimated mating system parameters from progeny arrays, such as the difference tm - ts and multilocus correlation of p estimate, using MLTR software (Ritland, 2002). Specifically, the difference between tm (multilocus population outcrossing rate) and ts (singlelocus population outcrossing rate) is the percentage of mating between genetically related parents. In other words, when true selfing is present, the difference between multilocus and single-locus estimates of outcrossing, is often used to characterize the level of biparental inbreeding (i.e., inbreeding due to mating among relatives), although this difference is always an underestimate, as it depends upon the number of loci used (Ritland, 2002). The multilocus correlation of p estimate ($r_{p(m)}$) corresponds to the correlation of outcrossed paternity within offspring arrays (fraction of siblings that share the same father), while its inverse ($1/r_{p(m)}$) can be interpreted as the effective number of fathers (N_{ep}) within offspring arrays (Ritland, 1989).

4.2.5. Gene flow estimation

Contemporary pollen dispersal was estimated using the program POLDISP (Robledo-Arnuncio *et al.*, 2007) that is based on mother-offspring diploid genotypic data and does not require mapping and genotyping potential pollen donors within the study area. The module KINDIST provides estimates of the correlation of paternity within and among maternal sibships, from the genotypes of a sample of seed-plants and their respective maternal progenies. This method was used to estimate the correlated paternity rate within each maternal tree for each cohort (i.e. whether offspring share fathers between years) and the correlated paternity rate of a same cohort between maternal trees (i.e. whether offspring share fathers within the same year). KINDIST computes correlated paternity estimates following the procedure described in Robledo-Arnuncio *et al.* (2006). This procedure is based on the computation of pairwise kinship coefficients (F) between the paternal gametic genotypes of offspring pairs. The expectation of F between two offspring is 0.5 if they have the same (non-inbred) father, and 0 if they have different (unrelated) fathers. Negative values indicate that paternal gametes are less related than in the average population.

Additionally, we estimated the pollen contribution by removing from each offspring the contribution of the maternal contribution, using *gstudio* (Dyer, 2021) in R software version 4.1.0 (R Core Team, 2021). Note that if mother and offspring were both identical heterozygotes, then it made it difficult to know if the dad (pollen donor) gave the first allele and the mother tree gave the second or vice-versa.

Then, knowing the mother and offspring genotypes, and potential fathers, we estimated the paternity exclusion probability. This is the probability of excluding a randomly selected individual from paternity based upon the allele frequencies in the population.

4.3 Results

The eight microsatellites across the 20 mother trees sampled in this single population of *P. pinaster*, showed an average allelic richness of 5.1, ranging from 3 to 11 alleles (Table 11).

Table 11. Standard diversity index: Number of alleles per microsatellite locus among the 20 mother trees sampled in the *P. pinaster* population of Tabuyo del Monte (León, Spain).

nSSR	Allelic Richness: No. of Alleles
ctg275	11
RPtest11	4
ctg4363	4
epi3	5
FRPP94	6
NZPR1078	3
gPp14	4
pEST2669	4
Mean	5.125
s.d.	2.532

The multilocus exclusion probability for the eight microsatellites used in our study was 97%, showing that we captured a reasonable amount of the theoretical maximum exclusion we could expect. The locus *ctg275* had the highest paternity exclusion (Table 12), ranging from 49-77%, consistent with the high number of alleles found for this locus (Table 11 and Table 13).

Table 12. Exclusion probabilities (%) per locus. The probability that one can exclude a randomly selected individual from paternity based upon the allele frequencies in the population. Pexcl: paternity exclusion probabilities (%); PexclMax: theoretical maximum exclusion one could get with a locus where all alleles are at equal frequency (%).

Locus	Pexcl	PexclMax
ctg275	49.23	77.18
ctg4363	41.16	50.39
epi3	38.47	50.39
FRPP94	40.48	65.97
gPp14	7.38	37.04
NZPR1078	34.99	37.04
pEST2669	15.68	37.04
RPtest11	33.51	50.39

The observed heterozygosity (H_O) was 0.54, ranging from 0.15 to 0.80 across loci, while the expected heterozygosity (H_E) was 0.56, ranging from 0.15 to 0.74 across loci. Hardy-Weinberg equilibrium test showed no locus at disequilibrium in terms of allelic frequencies (Table 13). Therefore, all eight loci were included in subsequent analyses.

Table 13. Standard diversity indices for the 20 mother trees sampled: observed (H_O) and expected heterozygosity (H_E), and Hardy-Weinberg equilibrium (HWE) test for each locus.

Locus	H_O	H_E	HWE	
			P-value	s.d.
ctg275	0.80	0.74	0.964	0.00013
RPtest11	0.60	0.57	0.669	0.00046
ctg4363	0.65	0.69	0.870	0.00032
epi3	0.75	0.70	0.896	0.00033
FRPP94	0.65	0.68	0.898	0.00031
NZPR1078	0.55	0.67	0.610	0.00049
gPp14	0.15	0.15	1.000	0.00000
pEST2669	0.20	0.27	0.354	0.00058
Mean	0.54	0.56		
s.d.	0.24	0.22		

The allelic frequencies of mother trees and seedlings (cohorts 3 and 14 together) showed very similar distributions when looking at the alleles they had in common (Fig. 33), with seedlings displaying new alleles compared to mother trees (see for example the locus ctg275). This difference could be attributed to paternal contribution, i.e. pollen gene flow from within or outside the population that brings new alleles.

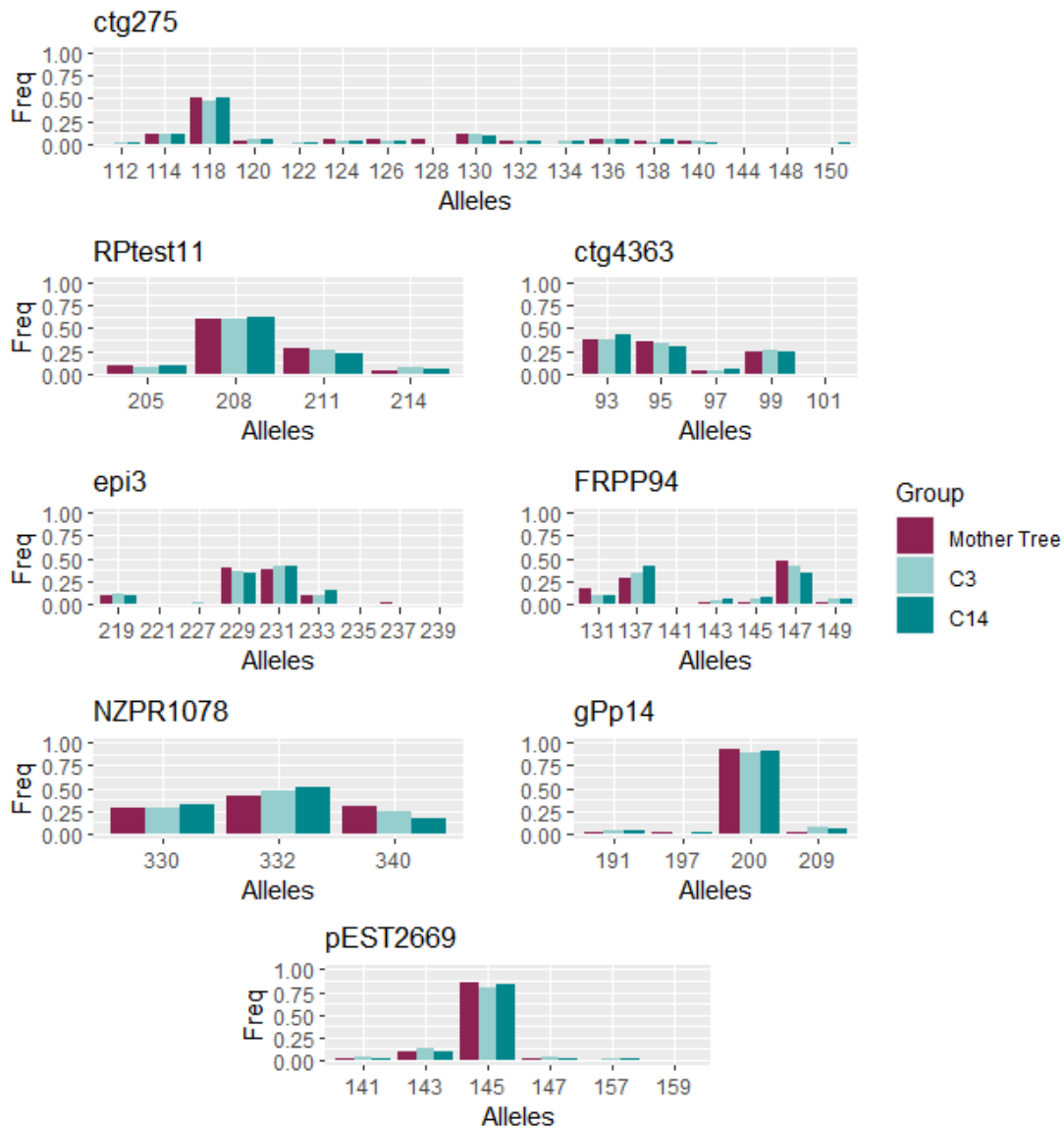


Fig. 33. Allelic frequencies for the eight nSSR loci in the population of *Pinus pinaster* located in Tabuyo del Monte. For each locus, there are displayed alleles that correspond to mother trees (in violet), cohort 3 (light blue) and 14 (turquoise).

Within each of the two temporally contrasted offspring cohorts (C3 and C14) sampled in 13 different mother trees of this population of *P. pinaster*, the eight microsatellites showed a similar level of polymorphism with an average allelic richness of 6.9, ranging from 3 to 17 alleles across the two cohorts (3 to 16 alleles within each cohort, Table 14). Noteworthy mentioning is the higher number of alleles in seedlings compared to adults for four loci (ctg275, epi3, FRPP94, pEST2669; Table 11 and Table 13), a pattern that could be attributed to paternal contribution (i.e. pollen gene flow from within or outside

the population). In particular, ctg275 was the SSR with the highest values of genetic diversity for both mother trees and progeny.

Table 14. Number of alleles per microsatellite locus within offspring cohorts (C3 and C14) from the *Pinus pinaster* population of Tabuyo del Monte (León, Spain).

nSSR	Allelic Richness: No. of Alleles		
	C3	C14	Total
ctg275	16	16	17
RPtest11	4	4	4
ctg4363	4	5	5
epi3	9	7	9
FRPP94	7	6	7
NZPR1078	3	3	3
gPp14	4	4	4
pEST2669	6	6	6
Mean	6.6	6.4	6.9
s.d.	4.3	4.1	4.5

The observed heterozygosity (H_o) was 0.58 for C3 (ranging from 0.20 to 0.79 across loci) and 0.56 for C14 (ranging from 0.21 to 0.75 across locus). Hardy-Weinberg equilibrium test showed no locus at disequilibrium in terms of allelic frequencies (Table 15), except marginally for RPtest11 in C14 ($p=0.046$). Therefore, all eight loci were included in subsequent analyses.

Table 15. Standard diversity indices: observed (H_o) and expected heterozygosity (H_E) for each cohort, and Hardy-Weinberg equilibrium (HWE) test for each locus.

Locus	H_o		H_E		HWE			
	C3	C14	C3	C14	C3		C14	
					P-value	s.d.	P-value	s.d.
ctg275	0.79	0.71	0.75	0.72	0.433	0.0002	0.099	0.0001
RPtest11	0.58	0.55	0.55	0.55	0.645	0.0005	0.046	0.0002
ctg4363	0.70	0.70	0.68	0.68	0.827	0.0003	0.605	0.0005
epi3	0.74	0.70	0.68	0.69	0.604	0.0003	0.344	0.0004
FRPP94	0.66	0.71	0.70	0.70	0.214	0.0004	0.248	0.0004
NZPR1078	0.67	0.63	0.64	0.61	0.273	0.0004	0.965	0.0002
gPp14	0.20	0.18	0.21	0.17	0.505	0.0004	1.000	0.0000
pEST2669	0.33	0.31	0.35	0.29	0.274	0.0005	0.816	0.0004
Mean	0.59	0.56	0.57	0.55				
s.d.	0.21	0.21	0.19	0.21				

The global pairwise F_{ST} comparing the inter-annual genetic structure (i.e. cohort 3 versus cohort 14) over all loci was small and not significantly different between the two years ($F_{ST} = 0.002$, $p = 1$), indicating similar pollen clouds across years.

Similarly, the analysis of molecular variance (AMOVA) revealed that there were no differences in the genetic variability among cohorts 3 and 14, while 13% of the variation was found between mother trees within cohorts (Table 16).

Table 16. Global AMOVA results as a weighted average over 8 loci. We displayed differences between cohorts, between mother trees, between offspring arrays within mother trees and within offspring arrays.

Source of Variation	Df	Sum of Squares	% Variation	P-value
Between Cohorts	1	8.65	-0.95	0.993
Between Mother Trees within Cohorts	21	590.03	12.97	0.001
Between offspring arrays within Mother tree	454	1549.28	-13.74	1.000
Within offspring arrays	477	2230.31	101.71	0.931
Total	953	4378.27	100.00	

When looking at the inbreeding parameters from the progeny array, we found a similar mating percentage between genetically related parents within each of the two cohorts: 19% within cohort 3 and 18.5% within cohort 14 (Table 17). The relatively high t_m - t_s points to the existence of mating between relatives. Average outcrossing rates were high and similar between cohorts, for both multilocus (1.200 ± 0.041 in C3, 1.200 ± 0.000 in C14), and single-locus (1.006 ± 0.016 in C3, 1.015 ± 0.024 in C14) estimates. The multilocus correlation of outcrossed paternity ($r_{p(m)}$) within offspring cohorts was negative in both cases, translating into offspring not sharing the same fathers across mother trees. This translates into an effective number of pollen donors per cohort (N_{ep}), ranging from 10 (C14) to 15 (C3).

Table 17. Inbreeding parameters estimates from the progeny array. Note that tm : the multilocus population outcrossing rate; ts : the single-locus population outcrossing rate; $tm-ts$: biparental inbreeding; *correlation of p estimate*: correlation of outcrossed paternity within offspring arrays, $r_{p(m)}$.

Cohort	<i>tm (s.d.)</i>	<i>ts (s.d.)</i>	<i>tm-ts (s.d.)</i>	<i>Multilocus correlation of p estimate (s.d.)</i>
3	1.20 (0.041)	1.00 (0.016)	0.19 (0.042)	-0.07 (0.034)
14	1.20 (-.000)	1.02 (0.024)	0.18 (0.024)	-0.1 (0.058)

When we analysed the correlated paternity rate within each maternal sibship for contrasting temporal cohorts, we found that estimates were low (Table 18), indicating that offspring arrays from the same year and mother tree have different unrelated fathers.

Table 18. Within-sibship correlated paternity estimates, based on the computation of pairwise kinship coefficients (F) between the paternal gametic genotypes of offspring pairs. The expectation of F between two offspring is 0.5 if they have the same (non-inbred) father.

Mother Tree	Cohort 3	Cohort 14
2	0.06	0.06
3	0.056	NA
9	0.08	0.03
10	0.02	-0.01
12	0.02	0.01
13	-0.00	-0.03
14	0.05	NA
15	0.01	0.08
16	0.01	0.02
17	0.04	-0.03
18	-0.01	NA
19	0.04	0.00
20	0.05	0.12

We found that the among-sibship correlated paternity estimates between mother trees were low too, indicating that offspring from the same year but from different mothers have different unrelated fathers (see Appendix S2).

4.4 Discussion

As explained in the previous chapters, studying an aerial seed bank through a temporal approach can help to disentangle the effect of evolutionary forces in the adaptive responses of plants to environmental change. In this chapter, our aim was to determine whether there were differences in the genetic variability between two contrasting temporal cohorts, separated by 11-year span time, in the highly serotinous population of *Pinus pinaster* of Tabuyo del Monte. Our hypothesis was that within this period of time, recurrent wildfires and/or the mega-wildfire of high-intensity occurred in 2012 (Fernández-García *et al.*, 2019) could have played a role in shaping the pollen flow processes, thereby modifying the genetic makeup of the population, translating into differences in the offspring performance investigated in the previous chapters.

Our results did not point to any significant genetic differences (in terms of allelic richness, genetic differentiation and variation) between the two pools of offspring (cohorts 3 and 14), while they did show a certain level of genetic variation among pools of offspring across mother trees within a given year (13% of the total variation). These results point to a spatial but not temporal heterogeneity of offspring pools in the population under study.

We have to be aware that the observed genetic makeup corresponds to germinated and surviving seedlings in the common garden experiment. Therefore, two points need to be highlighted: (i) It may well be that the genetic makeup in surviving seedlings is different from that of total seeds sowed because selection acted during survival and early growth stages. Rates of survival for cohort 3 and cohort 14 were 73% and 57% respectively, also indicating less representation of C14; (ii) seedlings in the common garden experiment were not subjected to the same microenvironmental variation as generally found in natural conditions for Mediterranean conifers (see references in González-Martínez *et al.* 2006). Therefore, the output found in our study may differ from that in natural conditions.

We also have to keep in mind that we only analysed two years with a limited number of offspring per mother tree and per year, which could limit the power of our analysis. However, by selecting such a long-time span between the two selected years (including an intense fire event in 2012), we optimized the probability of finding any impact of fire

on the genetic makeup of the offspring. Moreover, if we were able to detect significant genetic differences across mother trees with a lower sample size per offspring array, we should have been able to detect some differences at the cohort level, having a much larger sample size. We can therefore pretty safely discard that genetic difference across years is responsible for offspring performance differences.

When focusing at the within cohort level, mating and gene flow analyses provided additional information on the origin of the spatial genetic pattern. Mating outputs showed mainly outcrossing as expected for anemophilous species, and more specifically for pine species (Burczyk *et al.*, 1996; De-Lucas *et al.*, 2008), where an outcrossing rate of 0.9 has been reported (White *et al.*, 2007; tm and $ts \sim 0.96$, de-Lucas *et al.*, 2008). In our study, mating parameters estimates such as tm and ts were similar to those found in a progeny trial study of maritime pine (Gaspar *et al.*, 2009). Biparental inbreeding estimates were high for both cohorts ($tm-ts$ around 19%) in comparison with other work of the same species ($tm-ts=0.040$, De-Lucas *et al.*, 2008), although similar results have been found in other studies (Gaspar *et al.*, 2009, Fernandes *et al.*, 2008). The existence of mating between relatives could translate a mate-selection strategy for conserving characteristics that will allow tree adaptation within microclimates. In the present study, seeds were collected in the same provenance, and the existence of some family relationship among descendants could be expected (Fernandes *et al.*, 2008). Despite the relatively high biparental inbreeding values, we observed an increase of about 6% of the observed heterozygosity in the progeny (0.58 for C3 and 0.56 for C14) as compared with the parental values (0.54).

When focusing on pollen flow, both outcrossing paternity within offspring arrays (Table 6) and correlated paternity within offspring arrays (Table 7) were low, translating into offspring having different unrelated fathers, both within a same array (Table 8) and across arrays from different mother trees. The homogenisation of pollen flow across years is in agreement with what is known about wind-dispersed species such as pines, and more specifically in *P. pinaster* that showed a high immigration rate of pollen in previous studies (González-Martínez *et al.* 2006). Because of the pollen homogenous effect, we would not expect to find any genetic differentiation among offspring arrays, except if the pollen pool was spatially structured (i.e. some fathers would fertilize more often some close-by mother trees).

Our capacity to detect spatial or temporal genetic changes in the offspring arrays highly relied on the exclusion power of our markers. The eight nSSR selected were highly informative since they had enough power to differentiate individuals, with an exclusion probability of 97%. The high level of polymorphism (allelic richness and heterozygosity levels) found in the studied population confirmed results from previous studies done in different populations of *P. pinaster* using the same set of markers (Jaramillo-Correa *et al.*, 2015; Unger *et al.*, 2014 and 2016) or a combination of different markers (Mariette *et al.*, 2001; Derory *et al.*, 2002; González-Martínez *et al.*, 2003; Gaspar *et al.*, 2009).

The present study was carried out to complement the previous chapters, in our attempt to explain the variability in the offspring performance of maritime pine from Tabuyo del Monte provenance. We can conclude that the observed differences in the offspring performance across the two considered cohorts cannot be attributed to genetic differences. In particular, we showed that paternal clouds within offspring arrays and across offspring arrays of the same year are highly diverse, meaning that offspring do not share the same fathers, translating to an homogenisation of pollen flow on the genetic diversity. Beyond the allelic composition and the allele frequency changes through time, other regulatory mechanisms, such as maternal effects and adaptive epigenetic responses, may help to understand tree performance under a rapid climate change. Understanding the interplay between maternal effects, epigenetics and adaptation should enhance the understanding of evolutionary trajectories (Bräutigam *et al.*, 2013).



5. General Discussion

In this thesis, we address the genetic and non-genetic effects in the performance of seeds and progenies deriving from an aerial seed bank of *Pinus pinaster*. We focused on a single population and looked retrospectively in time using seeds from serotinous cone cohorts formed across 12 years (between 2003 and 2014). In a mesocosm-type, semi-natural common garden experiment, we followed seed germination and survival, growth and development of seedlings during the first year after germination. With this approach, we were able to study the impact of time, and yearly maternal conditions and environment on seedling performance.

First, we found that seed mass was related to maternal age and growth at the time of embryo development, i.e. slow growth-older mothers had smaller seeds and fast growth-young mothers had bigger seeds, which could be interpreted either as a result of senescence or as a maternal strategy, resulting in the production of smaller cones containing fewer and heavier seeds at a younger age and larger cones with more though smaller seeds at older ages (Cruz *et al.*, 2019). We also confirmed that seed mass positively affected germination success, a well-described trend in many works (e.g. Herman and Sultan, 2011; Zas *et al.*, 2013). But beyond differences in seed mass, maternal age had a negative effect on germination timing and subsequent survival while diameter had a positive effect. The independent effects of maternal age and diameter indicate that, although there is a positive correlation between age and diameter, the correlation is less strong than often believed (Pederson, 2010). Therefore, the present work reinforces the key role of maternal effects in shaping offspring performance and the role of seed mass as a mediator of these effects (Roach and Wulff, 1987; Bischoff and Mueller-Schaerer, 2010; Vivas *et al.*, 2020).

Second, to further analyse seedling performance, we focused on seedling size and ontogeny, revealing interannual differences in the estimation of heritability. This variability is partially attenuated when incorporating potential sources of variation across the temporal scale, such as maternal and environmental conditions. We found no empirical evidence of the effect of environmental heterogeneity on selection. Since we analysed a subsample of 16 mother trees, it is likely that only a small range of the actual genetic variation of the original population was explored. But alternatively, other sources of variation that we did not measure could bias the estimates of V_A , and consequently the prediction of the response to selection. Namely, maternal effects can produce mismatches

between genotypes and phenotypes. Importantly, responses to selection are often disregarded in quantitative genetic studies of wild populations, despite the presence of genetic variation (Pujol *et al.*, 2018).

Finally, in our attempt to further explain the differences observed in the performance of seedlings from the same mother across years, we explored the genetic variability in two contrasted cohorts (2003 and 2014) of the aerial seed bank. Considering that our population is in a historically fire-prone area, fire events (especially that of 2012; Fernández-García, 2019) could have affected the dynamic of gene flow and the composition of pollen donors between years, implying differences in the genetic makeup of progenies (Shohami and Nathan, 2014). However, we did not find any genetic variation among two contrasting temporal cohorts, suggesting a limited impact of the genetic makeup on the interannual differences of heritabilities.

5.1 Perspectives and implications for future studies

Forest trees are long living sessile organisms that have to cope with changing environments during their long life, adjusting their growth, development and reproduction in order to increase or maintain their own life expectancy and fitness. As the effects caused by abiotic and biotic stresses can last for long periods, favouring different responses and strategies, there is a high probability of interaction with other stresses or disturbances that could lead to more stressful combined events (Amaral *et al.*, 2020). This has strong implications for the resilience of natural forest tree populations under a climate change scenario. In this context and along with genetics, the mechanisms that constitute a link between the genotype and the phenotype, such as maternal effects and epigenetic regulation, need special attention (Pascual *et al.*, 2014; Amaral *et al.*, 2020; Vivas *et al.*, 2020).

In particular, we have seen throughout this thesis that seed banks provide an exciting and challenging model for studying the evolutionary implications of genetic and non-genetic transgenerational effects. Even in long-lived plants like forest trees, maternal effects related to seed resource allocation and epigenetic mechanisms linked to embryogenesis and seed maturation may contribute to the rapid adaptation to

environmental changes (Herman and Sultan, 2011; Yakovlev *et al.*, 2012; Vivas *et al.*, 2013). This thesis provides experimental evidence about the role of the maternal environment and age in shaping the performance of its offspring in a key forest species, complementing past and future studies into genetic effects, transgenerational plasticity - known as epigenetic memory- (Henderson and Jacobsen, 2007; Yakovlev *et al.*, 2012; Correia *et al.*, 2013; Vivas *et al.*, 2013) and into other forms of ecological inheritance such as associations between plants and the microbiome (Vivas *et al.*, 2015).

Maternal effects can enhance the development and resistance, in terms of growth, pests, and stress of offspring plants. This will become increasingly important in the face of rapidly changing environmental conditions (Vivas *et al.*, 2020). Therefore, maternal effects should be considered in tree breeding and conservation in order to reach their goals and to avoid overestimating genetic differences (Räsänen and Kruuk, 2007; Alberto *et al.*, 2013). In our case, including seed mass and maternal conditions allowed a finer control for confounding effects (De Villemereuil *et al.*, 2018). For seedling size at early ages, we have seen that maternal effects were related to an interaction between cohort and mother tree, and not to the main effects, indicative of differences in resource allocation. When correcting for these effects, the degree of genetic control was more stable across years, and we did not observe a significant effect on the differential selection of the progenies among years. This suggests that these transgenerational effects, even if promoting phenotypic differentiation, could have a secondary role in the evolutionary potential within populations in forest tree species.

Future studies are needed to confirm and complement our results, integrating different aspects, for example: measuring to larger extent potential sources of variation, such as maternal temperature (Dewan *et al.*, 2018), microbiota (Fort *et al.*, 2021) or water availability (Vivas *et al.*, 2019), among other effects (see Vivas *et al.*, 2020 for an overview of maternal effects in forest trees); increasing the number of samples to increase the chances of capturing more genetic variation and, including maternal effects in common-garden experiments to account for confounding effects (Borgman *et al.*, 2014). This also must be considered in restoration strategies since the effects of parental environment on offspring phenotype differ depending on the conditions that offspring themselves encounter (Baker *et al.*, 2018; Donelson *et al.*, 2018).

In this thesis, we found a significant genetic variability among mother trees that could partially explain phenotypic differences, meanwhile we did not find any genetic evidence linked to the temporal seedling performance. In addition, we observed a strong interaction between the mother tree and temporal cohorts, suggesting different interactions at the microscale between maternal conditions and the environment. There is still a lack of evidence concerning whether those transgenerational effects can affect the evolutionary response, enhancing plant fitness and adaptation.



6. Conclusions

Conclusiones

1. The studied aerial seed bank of the natural population of *P. pinaster* showed marked differences in seed weight among cohorts and trees. This variation, both within and between mother trees, is largely due to effects associated with maternal tree conditions during seed development.
2. We found a non-significant effect of age within mothers and a negative between-mother tree effect of age, i.e. older trees produced smaller seeds, but seed mass did not change with cone age within a tree. Overall seed mass decreased with maternal age at the time of seed production, i.e. slow growth-older mothers had smaller seeds and fast growth-young mothers had bigger seeds, which could be interpreted either as a proxy of senescence or as a maternal strategy.
3. Despite an increase in size with age, mother size (assessed by its diameter) had a positive effect on seed mass, i.e. larger trees produced heavier seeds.
4. Seed mass had a positive effect on germination success, with larger seeds being more likely to germinate, consistent with a well-described trend in many species. However, seed mass did not affect the timing of germination.
5. Maternal age and diameter were the main effects on germination timing. Seeds from slow growth-older mothers had a significant delay in germination. Age effects could be suggestive of senescence or could indicate that seeds from older plants have a greater physical or mechanical dormancy.
6. We found two or three different growth trajectories across the mother trees. However, with our data, we cannot associate these patterns to microenvironmental differences in the natural stand.
7. Seedling size and ontogeny varied significantly among mother trees and experimental blocks, but not among cohorts. We found a strong and widespread interaction between mother trees and cohorts for these traits, which implies that the effects of the maternal genotypes were different depending on the year when seeds were formed.

8. We found significant effects of maternal and environmental conditions on seedling size and ontogeny (i.e. heavier seeds produced taller seedlings), as well as the interaction between SPEI and Ring Width. These traits indicative of favourable maternal conditions during the cohort year (i.e. higher annual ring width and wet yearly conditions), were associated with taller seedlings too. In addition, we found a combination of effects between seed mass and maternal conditions on seedling size, indicating interactions at the microscale.
9. The narrow-sense heritability for seedling size and ontogeny showed similar moderate values, consistent with the literature. When looking at each cohort independently, heritability estimates on seedling size and ontogeny varied across cohorts. However, when discounting maternal effects, h^2 values were more stable.
10. We did not find empirical evidence of evolutionary response through time in 12-year sequential cohorts. The most informative part of this result is the variation present across cohorts.
11. Our results suggest that a kind of environmental memory could be operating via maternal effects, e.g. resources inherited through seeds, and/or transgenerational epigenetic mechanisms that can induce changes in gene expression.
12. We did not find significant genetic differences (in terms of allelic richness, genetic differentiation and variation) between the two pools of offspring (cohorts 3 and 14), while we detected a certain level of genetic variation among pools of offspring across mother trees within a given year. This indicates a spatial but not temporal heterogeneity of offspring pools in the population under study.
13. We cannot associate genetic differences across years with the offspring performance differences observed in this thesis.
14. Mating results showed mainly outcrossing as expected for anemophilous species, and more specifically for pine species. We also found some amount of biparental inbreeding, as could be expected since seeds were collected in the same provenance.

15. Correlated paternity values were low, meaning that offspring had different unrelated fathers. We showed that paternal clouds within offspring arrays and across offspring arrays of the same year are highly diverse, indicating an homogenisation of pollen flow on the genetic diversity.
16. The eight nSSR selected were highly informative since they had enough power to differentiate individuals, with an exclusion probability of 97%.
17. We can conclude that, beyond the allelic composition and the allele frequency changes through time, other mechanisms, such as maternal effects are involved in the progenies performance. However, we did not find differences in selection between cohorts of the same mothers. Therefore, it is still unclear if the observed marked phenotypic differences could contribute to fast trans-generational adaptation that could be highly relevant under a rapid climate change. Overall, our findings open new insights in the offspring performance deriving from long-term canopy seed banks, which should be completed in the future.

1. El banco aéreo de semillas procedente de la población natural de *P. pinaster* estudiada, mostró marcadas diferencias en el peso de las semillas entre cohortes y árboles. Esta variación, tanto dentro como entre árboles, se debe en gran parte a los efectos asociados a las condiciones del árbol madre.
2. Encontramos un efecto significativo negativo de la edad entre árboles madre. Es decir, los árboles más viejos produjeron semillas más pequeñas, pero el peso de semilla no cambió con la edad de los conos dentro del árbol. El peso de la semilla disminuye con la edad materna en el momento de formación de la semilla, es decir, las madres más viejas de crecimiento lento produjeron semillas más pequeñas y las madres jóvenes de crecimiento rápido produjeron semillas más grandes, lo que podría interpretarse como un indicador de senescencia o como una estrategia materna.
3. A pesar del aumento general del diámetro con la edad, el tamaño de la madre tuvo un efecto positivo en el peso de la semilla. Es decir, los árboles más grandes produjeron semillas más pesadas.
4. El peso de la semilla mostró un efecto positivo sobre el éxito de germinación, confiriendo una mayor probabilidad de germinar, lo que coincide con una tendencia bien descrita en muchas especies. Sin embargo, el peso de semilla no mostró un efecto sobre el tiempo de germinación.
5. La edad materna y el diámetro fueron los efectos principales sobre el tiempo de germinación. Las semillas de árboles más viejos de crecimiento lento, tuvieron un retraso significativo en la germinación. Los efectos de la edad podrían ser un indicador de senescencia o podrían indicar que las semillas de árboles más viejos tienen una mayor latencia o dormición física o mecánica.
6. Observamos al menos dos o tres trayectorias de crecimiento diferentes a través de los árboles madre. Sin embargo, con nuestros datos, no podemos asociar estos patrones a diferencias microambientales en el lugar de origen.

7. Tanto el crecimiento como el desarrollo de las plántulas variaron significativamente entre los árboles madre y los bloques experimentales, pero no entre las cohortes. Encontramos una interacción fuerte y generalizada entre los árboles madre y las cohortes para estos rasgos, lo que implica que los efectos de los genotipos maternos fueron diferentes según el año en que se formaron las semillas.
8. Encontramos efectos significativos de las condiciones maternas y ambientales sobre el tamaño y la ontogenia de las plántulas (semillas más pesadas produjeron plántulas más altas), así como la interacción entre SPEI y el ancho de los anillos. Estos rasgos indicativos de condiciones maternas favorables durante el año de la cohorte (es decir, mayor crecimiento anual y condiciones anuales húmedas), también se asociaron con plántulas más altas. Además, encontramos una combinación de efectos entre la masa de semillas y las condiciones maternas sobre el tamaño de la plántula, lo que indica interacciones a microescala.
9. La heredabilidad en sentido estricto para el crecimiento y desarrollo de las plántulas mostró valores moderados similares, consistentes con la literatura. Cuando analizamos cada cohorte de forma independiente, las estimaciones de heredabilidad para estos mismos rangos variaron entre las cohortes. Sin embargo, al descontar los efectos maternos, los valores de h^2 fueron más estables, lo que sugiere que corregimos con precisión para las variables de confusión.
10. No encontramos evidencia empírica de la respuesta evolutiva a través del tiempo en las 12 cohortes estudiadas. La parte más informativa de este resultado es la variación presente entre cohortes.
11. Nuestros resultados sugieren que una especie de memoria ambiental podría estar operando a través de los efectos maternos, mediante el aprovisionamiento de las semillas y/o mecanismos epigenéticos transgeneracionales que pueden inducir cambios en la expresión génica.

12. No encontramos diferencias genéticas significativas (en términos de riqueza alélica, diferenciación genética y variación) entre los dos grupos de la descendencia (cohortes 3 y 14), aunque sí encontramos variación genética entre árboles madre para un mismo año, lo que indica una heterogeneidad espacial pero no temporal en la descendencia de la población estudiada.
13. No podemos constatar que las diferencias observadas en el comportamiento de la descendencia entre años se deban a una variación genética entre los mismos.
14. Los resultados con respecto al apareamiento, mostraron principalmente cruces externos como se esperaba para las especies anemófilas, y más específicamente para las especies de pino. También encontramos cierta cantidad de endogamia biparental, como era de esperar, ya que las semillas se recolectaron en la misma procedencia.
15. Los valores de correlación en la paternidad eran bajos, lo que significa que la descendencia tenía diferentes padres no emparentados. Demostramos que la nube polínica dentro y entre grupos de descendientes del mismo año es muy diversa, lo que indica una homogeneización del flujo de polen en la diversidad genética.
16. Los ocho marcadores microsatélites nucleares seleccionados fueron altamente informativos ya que mostraron suficiente potencial para diferenciar a los individuos, con una probabilidad de exclusión del 97%.
17. Podemos concluir que, más allá de la composición alélica y los cambios en la frecuencia alélica a través del tiempo, otros mecanismos, como los efectos maternos, están involucrados en el desempeño de la progenie. Sin embargo, no encontramos diferencias en la selección entre cohortes de las mismas madres. Por lo tanto, todavía no está claro si las marcadas diferencias fenotípicas observadas podrían contribuir a una rápida adaptación transgeneracional que podría ser muy relevante bajo un rápido cambio climático. En general, nuestros hallazgos abren nuevas perspectivas sobre el comportamiento de la descendencia procedente de bancos aéreos de semillas, que deberían ser complementadas en el futuro.



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8. Supplementary Information

Appendix S1. Experimental design.

Mother Tree	Number of cohorts/tree	Sampled Cones/Cohort	Sampled seeds/cone	Total seeds
1	6	3	10	180
2	8	3	10	240
3	8	3	10	240
6	8	3	10	240
9	10	3	10	300
10	11	3	10	330
11	6	3	10	180
12	8	3	10	240
13	12	3	10	360
14	10	3	10	300
15	8	3	10	240
16	11	3	10	330
17	9	3	10	270
18	11	3	10	330
19	12	3	10	360
20	12	3	10	360
Seeds for filling the gaps				120
				4.620

Appendix S1a. Description of the study design. Note that the number of cohorts sampled varies among trees.

Appendix S2. Correlated paternity for each cohort among sib-ship.

Appendix S2.1. Among-sibship correlated paternity estimates for Cohort 3. Negative correlated paternity value indicates that the corresponding paternal gametes are less related than the average in the sample.

Cohort 3			Cohort 14		
Mother Tree A	Mother Tree B	Correlated Paternity	Mother Tree A	Mother Tree B	Correlated Paternity
2	3	-0.04869	2	9	-0.02568
2	9	0.05597	2	10	-0.02149
2	10	-0.02808	2	12	-0.03842
2	12	-0.00807	2	13	0.01668
2	13	-0.0167	2	14	-0.02378
2	14	-0.01022	2	16	-0.00666
2	15	-0.00603	2	17	-0.00303
2	16	0.00886	2	19	-0.02949
2	17	-0.03135	2	20	0.06216
2	18	0.02886	9	10	0.03012
2	19	-0.00205	9	12	0.02688
2	20	0.02339	9	13	-0.02264
3	9	-0.08109	9	14	-0.02296
3	10	0.04813	9	16	-0.0192
3	12	0.00073	9	17	0.00618
3	13	-0.00773	9	19	0.02163
3	14	-0.02497	9	20	0.01153
3	15	-0.00384	10	12	0.01175
3	16	0.01876	10	13	0.00933
3	17	-0.00411	10	14	0.0083
3	18	-0.00249	10	16	-0.00356
3	19	0.01631	10	17	0.00487
3	20	0.00402	10	19	0.01938
9	10	0.03422	10	20	-0.03279
9	12	-0.01725	12	13	-0.00028
9	13	-0.00182	12	14	0.05581
9	14	0.02137	12	16	0.02613
9	15	-0.03968	12	17	0.00629
9	16	0.02636	12	19	0.02112
9	17	0.0303	12	20	-0.06218
9	18	0.00438	13	14	-0.01141
9	19	-0.03188	13	16	0.01682
9	20	-0.02696	13	17	0.0138
10	12	-0.01399	13	19	-0.00377
10	13	-0.00789	13	20	0.00852
10	14	0.00415	14	16	-0.00704

10	15	-0.00729	14	17	0.03924
10	16	0.01462	14	19	-0.01995
10	17	0.03487	14	20	-0.04516
10	18	-0.00962	16	17	0.00767
10	19	-0.01969	16	19	0.02936
10	20	-0.02316	16	20	-0.05432
12	13	0.01917	17	19	-0.01007
12	14	-0.01804	17	20	-0.00121
12	15	0.02365	19	20	-0.0109
12	16	0.02154			
12	17	0.01019			
12	18	-0.01612			
12	19	-0.01177			
12	20	-0.00298			
13	14	0.03443			
13	15	0.0186			
13	16	-0.0162			
13	17	-0.00064			
13	18	-0.00108			
13	19	-0.00874			
13	20	-0.00161			
14	15	0.0072			
14	16	-0.02992			
14	17	0.00783			
14	18	-0.00868			
14	19	-0.01546			
14	20	0.01541			
15	16	-0.00971			
15	17	0.01809			
15	18	-0.01174			
15	19	0.00447			
15	20	-0.00883			
16	17	0.02923			
16	18	-0.00191			
16	19	-0.01432			
16	20	-0.02152			
17	18	-0.02249			
17	19	-0.01362			
17	20	-0.0523			
18	19	0.03699			
18	20	0.01862			
19	20	0.00984			

Thank you,



