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DEPARTAMENTO DE

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Pollen flow in fragmented populations of Myrtus communis and Pistacia lentiscus: the importance of mating, pollination systems and the landscape context

Flujo polínico en poblaciones fragmentadas de Myrtus communis y Pistacia lentiscus: el papel de los sistemas de apareamiento, polinización y de la estructura del paisaje

TESIS DOCTORAL

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Sofia Vilaça Nora, Licenciada en Biología y Máster en Ecología, Biodiversidad y Gestión de Ecosistemas por la Universidad de Aveiro, Portugal, presenta esta memoria para optar al grado de Doctora por la Universidad de Sevilla a 29 de septiembre de 2017.

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INFORMAN:

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

Y para que así conste, firman el presente documento en Sevilla a 29 de septiembre de 2017.

Fdo: Abelardo Aparicio

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Do chão sabemos que se levantam as searas e as árvores, levantam-se os animais que correm os campos ou voam por cima deles, levantam-se os homens e as suas esperanças.

Também do chão pode levantar-se um livro, como uma espiga de trigo ou uma flor brava.

José Saramago

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General Abstract

Over the last century, habitat loss and fragmentation have been the main components of current anthropogenic global change and the greatest threats to the global biodiversity. The scientific community has shown a growing interest in understanding the consequences of the anthropogenic disturbance of the biosphere and habitat fragmentation studies have been one of the scientific fields that most flourishing in the last decades. The detrimental effects of habitat fragmentation on plant reproductive success and mating system patterns have been widely documented and there is a general agreement that these effects can ultimately compromise plant persistence. However, species responses to habitat loss and fragmentation are often variable, emphasizing the need to general knowledge on the complex and variable mechanisms of species responses to changes in habitat configuration and size.

The main purpose of this Doctoral Thesis is to evaluate how habitat fragmentation (fragment size and connectivity) affects and determines both historical and contemporary gene flow and mating systems patterns, as well as the reproductive success of populations of the two common Mediterranean shrubs, *Myrtus communis* and *Pistacia lentiscus*. By comparing populations of these two species with contrasting mating and pollination systems, co-occurring in a diverse regional mosaic of Mediterranean forest patches in the Guadalquivir River Valley, this thesis highlights on the complexities surrounding fragmented landscapes and the influence of fragmentation on ecological interactions. Chapter 1 infers patterns of historical gene flow of *Myrtus* and *Pistacia* populations under different landscape contexts. The influence of mating and pollination systems characteristics and population-specific response in both study species is perceived in shaping species' genetic patterns. Both species revealed weak detrimental effects of habitat fragmentation, such as low values of effective population size and evidence for recent genetic bottlenecks, although results were more pronounced in *Myrtus* than in *Pistacia* populations. Both Chapter 2 and Chapter 3 are comparative studies that assessed the role of habitat fragmentation in conditioning plant's reproductive success and contemporary patterns of pollen flow of *Myrtus* and *Pistacia* populations. In these chapters we evaluated the variation in plant reproductive success and mating system estimates and also the main ecological factors responsible for the variation at both the individual and population levels. Rather than a direct

effect of habitat fragmentation, ecological components are more important on species' reproductive success and mating system patterns but, interestingly, their influence vary along the fragmentation gradient. Finally, Chapter 4 analyzes how correlated paternity shapes *Myrtus* and *Pistacia* early progeny performance both in a greenhouse environment and under natural (field) conditions. This study represents one of the few existing empirical examples that evaluate the role of the correlated paternity in shaping the early performance of maternal progenies.

This Doctoral Thesis also demonstrates that the impacts of habitat loss and fragmentation, a part of being most of the times context and species dependent, they are often also more nuanced than simple and easy observed losses of genetic diversity and increase differentiation among populations. Some key consequences of forest fragmentation have now been identified as increased inbreeding depression in progeny sired in a fragmented landscape and decreased progeny fitness due to low numbers of effective pollen donors. It also confirms that variation in mating and pollination systems is an important factor in determining the type and magnitude of species response to habitat fragmentation.

Resumen General

Durante el último siglo, la pérdida de hábitat y la fragmentación han sido los principales componentes del actual cambio antropogénico y las mayores amenazas para la biodiversidad mundial. La comunidad científica ha mostrado un creciente interés por comprender las consecuencias de la perturbación antropogénica de la biosfera y los estudios de fragmentación de hábitats han sido uno de los campos científicos más florecientes en las últimas décadas. Los efectos perjudiciales de la fragmentación del hábitat sobre el éxito reproductivo de las plantas y los patrones del sistema de apareamiento han sido ampliamente documentados y hay un acuerdo general de que estos efectos pueden comprometer la persistencia de las plantas. Sin embargo, las respuestas de las especies a la pérdida y fragmentación del hábitat son a menudo variables, haciendo hincapié en la necesidad de conocimientos generales sobre los mecanismos complejos y variables de las respuestas de las especies a los cambios en la configuración y tamaño del hábitat.

El objetivo principal de esta tesis doctoral es evaluar cómo la fragmentación del hábitat (tamaño y conectividad de los fragmentos) afecta y determina tanto el patrón histórico y contemporáneo de flujo génico como a los patrones de apareamiento, así como el éxito reproductivo de las poblaciones de dos arbustos comunes en el Mediterráneo, *Myrtus communis* y *Pistacia lentiscus*. Comparando las poblaciones de estas dos especies con contrastantes sistemas de apareamiento y polinización, coincidiendo en un mosaico regional diverso de parches de bosque que es el Valle del Guadalquivir, esta tesis destaca las complejidades que rodean los paisajes fragmentados y la influencia de la fragmentación en las interacciones ecológicas. El Capítulo 1 infiere patrones de flujo genético histórico de las poblaciones de *Myrtus* y *Pistacia* bajo diferentes contextos paisajísticos. La influencia de las características de los sistemas de apareamiento y polinización y la respuesta específica de la población en ambas especies de estudio se percibe en los patrones genéticos de las especies. Ambas especies revelaron efectos perjudiciales débiles a la fragmentación del hábitat, tales como, por ejemplo, valores bajos de tamaño efectivo de población y evidencia de cuellos de botella genéticos recientes, aunque los resultados fueron más pronunciados en *Myrtus* que en poblaciones de *Pistacia*. Tanto el Capítulo 2 como el Capítulo 3 son estudios comparativos que

evalúan el papel de la fragmentación del hábitat en el éxito reproductivo de las plantas y los patrones contemporáneos del flujo polínico de las poblaciones de *Myrtus* y *Pistacia.* En estos capítulos se evaluó la variación en el éxito reproductivo de la planta y en las estimas del los patrones del sistema de apareamiento, pero también cuales son los principales factores ecológicos responsables de esta variabilidad tanto al nivel individual como al de la población. En lugar de un efecto directo de la fragmentación del hábitat, los componentes ecológicos son más determinantes en el éxito reproductivo de las especies y en los patrones del sistema de apareamiento y, curiosamente, su influencia varía a lo largo del gradiente de fragmentación. Finalmente, el capítulo 4 analiza cómo la paternidad correlacionada modela el fitness temprano de la progenie de *Myrtus* y *Pistacia* tanto en invernadero como en condiciones naturales. Este estudio representa uno de los pocos ejemplos empíricos existentes que evalúan el papel de la paternidad correlacionada en la determinación del fitness temprano de las progenies.

Esta tesis doctoral demuestra que los impactos de la pérdida y fragmentación del hábitat, una parte de la mayoría de las veces es dependiente del contexto y las especies en cuestión, a menudo los efectos también son más sutiles que simples pérdidas observadas de diversidad genética y incremento de la diferenciación entre poblaciones. Se han identificado como algunas de las consecuencias clave de la fragmentación el un aumento de la depresión de endogamia en la progenie producida en un paisaje fragmentado y disminución del fitness de la progenie debido al bajo número de donantes efectivos de polen. También confirma que la variabilidad en los sistemas de apareamiento y polinización es un factor importante para determinar el tipo y magnitud de la respuesta de las especies a la fragmentación del hábitat.

General Introduction

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GENERAL INTRODUCTION

Habitat fragmentation

For decades the scientific community has shown a growing interest in understanding the consequences of the anthropogenic disturbance of the biosphere, originating a new independent conceptual and theoretical framework of the Biology of Conservation (Figure 1). In fact, habitat fragmentation studies have been one of the scientific fields that most flourishing in the last decades, empowered not only by the effective need to address one of the greatest threats to the global biodiversity (Sala *et al.* 2000) but also by the recent availability of highly polymorphic genetic markers that enabled the development of refined analysis techniques (Ashley 2010).

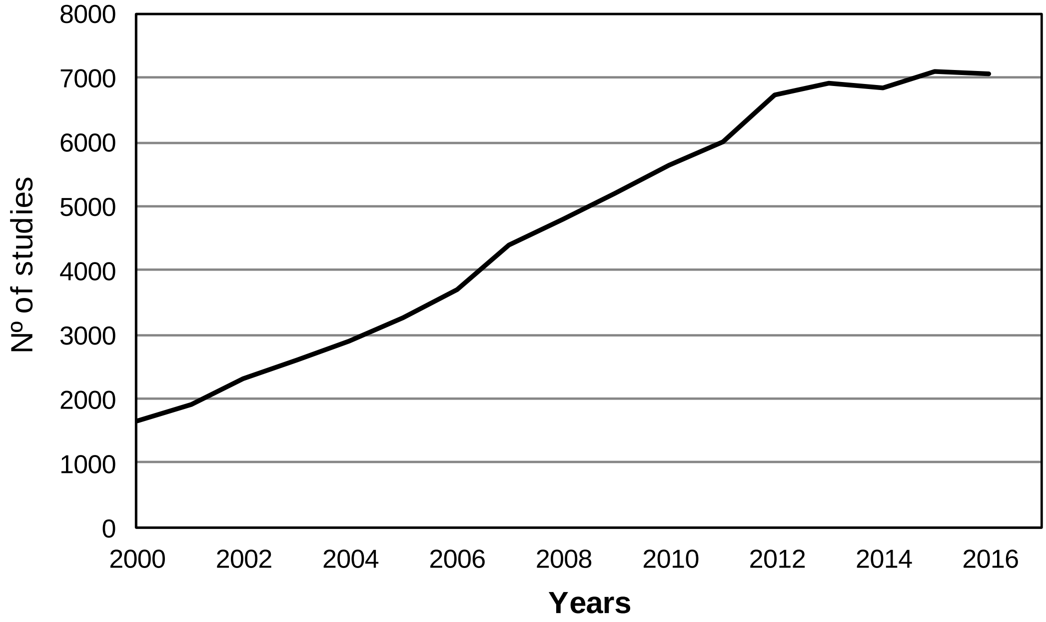


Figure 1. Number of studies including a reference to 'Habitat Fragmentation' from 2000 to 2016. Source: *Google scholar*.

Habitat fragmentation is the process by which an original habitat is reduced and broken into a constellation of habitat remnants that differ in size, shape and connectedness (Fahrig 2003). The detrimental effects of habitat fragmentation have been widely documented, revealing important taxonomic and genetic losses at different scales. Loss of habitat quality in remnant fragments, edge effects and isolation of populations seem to lead to an inevitable decline of all biodiversity parameters and can ultimately compromise plant persistence (McIntyre & Hobbs 1999; Fahrig 2003; Ewers & Didham 2006). From a genetic point of view, the population genetics theory predicts that the decrease of the effective population size and the increase of isolation will make fragmented populations more susceptible to genetic drift and inbreeding, decreasing the success and evolutionary potential of species and increasing population's risk of extinction (Ellstrand & Elam 1993; Young *et al.* 1996). However, empirical evidence does not always support the theory, species responses to habitat loss and fragmentation are often variable and not so straightforward and predictable (e.g. Aizen & Feinsinger 1994; Ewers & Didham 2006; Kramer *et al.* 2008), creating a scientific paradigm. For instance, Ouborg *et al*. (2006) provide an intellectual and analytical challenge to this paradigm by arguing that population size decline and isolation are not interchangeable parameters, the existence of inter-specific relationships in a community may also influence the success or failure of a particular species and that species have a particular idiosyncrasy (rare, common, invasive, threatened, etc.) that can greatly alter the genetic consequences of fragmentation. Kramer *et al.* (2008) argue that, in the case of plants, the genetic paradigm may lead to false interpretations when the genetic reality does not coincide with the ecological reality of the populations. That is, when the limit of a small forest fragment does not delimit the real population in which the genetic exchange is taking place via pollen or seeds or when the long life of species postpones over time observable evidence of genetic drift or inbred depression. In their own words "genetic degradation may not be as important as ecological degradation until many decades after habitat fragmentation is present". Therefore, we should consider the idiosyncrasy of the different systems of study (e. g. species with very different life histories traits) and the inherent difficulty of making generalizations. The same process of fragmentation can have very different consequences for different species since the interactions of vital characters with the environment and intra- and inter-specific competition relationships are very complex and sometimes unpredictable (Leimu *et al.* 2006; Honnay & Jacquemyn 2007; Aguilar *et al.* 2008; Fortuna *et al.* 2009; Aparicio *et al.* 2012).

Gene flow in fragmented populations

Although plants are sessile in their vegetative state, they have a great capacity of movement in some phases of their life cycle: as gametophytes (haploids) inside the pollen grains and as embryos (diploids) within fruits and seeds. It is this mobility that determines the actual degree of populations' isolation and their genetic fate when fragmented. Effective medium- and long-distance dispersal mechanisms can allow relatively high levels of gene flow in fragmented systems, while restricted or failed dispersal systems over time and/or space can cause disruption of genetic connectivity even in continuous populations (White *et al.* 1999; Lowe *et al.* 2005). Yet, the amount of genetic flow (via seeds or pollen) does not in itself prevent the negative effects of a possible genetic isolation (i.e. inbreeding depression, lack of diversity). Gene flow quality is determined by the number of sources (individuals or populations) from which the genes originate, as well as their genetic variance and differentiation from the recipient population. Therefore, the effective characterization of the quantity and genetic diversity associated with the movement of propagules is fundamental to understand the dynamics of fragmentation and its consequences, and to assist in the effective design of strategies for conservation.

In pollen flow studies, there are direct methods of genetic assignment to individuals (paternity analysis) and indirect methods based on the genetic structure of the pollen cloud (Smouse & Sork 2004). While direct methods of paternity analyzes allow a relatively accurate identification of pollen sources (Sork *et al.* 2005), they are logistically limited due to the requirement of an exhaustive sampling and genotyping of all the breeding individuals of the study population, something very difficult to obtain in natural dense populations. Therefore, for situations where it is not feasible to genotype all individuals in the population or when the spatial scale of the study is very broad, indirect methods have been developed that are less logistically demanding since they do not require an exhaustive sampling. With the geographical position and genotype of a feasible number of maternal plants and a fraction of their respective progeny, these methods provide, among other data, estimations of the composition of the pollen cloud (Φ_{FT}), the effective number of pollen donors (N_{ep}), the effective pollination neighbourhood area (A_{ep}) and the average distance of pollen dispersion (δ) (Smouse *et al.* 2001; Hardy *et al.* 2004;

Robledo-Arnuncio *et al.* 2007). On an even larger spatial scale, methods based on the accurate genetic assignment of individuals to their original populations offer a more adequate tool than the aforementioned methods for studying the genetic flow between fragments or populations, although these methods require a certain level of genetic differentiation among populations (Manel *et al.* 2005; Robledo-Arnuncio 2011). Regardless of the used method, a common result of many of these studies is that the rates of immigrant pollen in fragmented populations can be very high and even attributed to populations in the range of hundreds or even thousands of meters. For example, Bacles & Ennos (2008) have estimated pollen migration rates of 43-68% in individuals of the anemophilous species *Fraxinus excelsior*, and Byrne *et al.* (2008) values up to 55.3% in the entomophilous species *Eucalyptus wandoo*. As for the average distance of pollen dispersion, in anemophilous species it can vary between 10-15 m estimated for *Juglans mandshurica* (Bai *et al*. 2007) and 7.6 km estimated in *Populus trichocarpa* (Slavov *et al*. 2009). In insect-pollinated species, there have been estimated distances ranging from 21 m in *Centaurea corymbosa* (Hardy *et al.* 2004) up to the impressive 88.6 km in the fig *Ficus sycomorus* (Ahmed *et al.* 2009) (whose pollination is conducted by the small wasp *Ceratosolen arabicus* which is dispersed by the wind over long distances). Thus, more evidence suggests that many physically fragmented populations are not genetically isolated since fragment boundaries may not represent physical barriers to effective long-distance dispersal (Sork & Smouse 2006; Kamm *et al.* 2009; Lander *et al.* 2010; Robledo-Arnuncio 2011).

Mating systems in fragmented populations

The mating system is a fundamental micro-evolutionary process that can determine the amount and distribution of genetic variation within and among populations of plant species and their effective population size (Coates *et al.* 2007; Duminil *et al.* 2007; Barret & Harder 2017). Mating system standard estimates are usually the proportion of selfed (selfing rate, t_s) or outcrossed (outcrossing rate, t_m) offspring, biparental inbreeding (outcrossed mating among genetically related individuals, t_m-t_s) and the proportion of full-sibs within maternal progeny arrays (correlated paternity, r_p). Mating strategies can vary from obligate outcrossing (~ 50% of angiosperms and 40% of conifers; Igic & Kohn 2006; Leslie *et al.* 2013) to predominantly selffertilized (5-6% of angiosperms, Igic & Kohn 2006). Between these extremes, there is panoply of mixtures of selfed and outcrossed offspring (mixed-mating). Each reproductive strategy has its benefits and drawbacks (Goodwillie *et al.* 2005; Wright *et al.* 2013). Outcrossing provides genetic diversity of offspring genotypes and, probably, enhanced offspring performance (Morran *et al.* 2009). However, promoting outcrossing can have its costs directly through the dispended of energy necessary for attracting pollen vectors (e. g. the production of massive floral displays or floral rewards) and indirectly due to the idiosyncrasy of pollen dispersal (Richards *et al.* 2009) pollen germination and growth (Harder *et al.* 2016). Under unpredictable pollination services or mate availability, such as the case of population undergoing the effects of habitat fragmentation and anthropogenic disturbances, selfing can bear some advantages, a reproductive assurance mechanism (Charlesworth 2006). However, selfing costs arise after fertilization, with increasing of homozygozity, allowing expression of deleterious recessive alleles and causing inbreeding depression (Charlesworth & Willis 2009). Less understood are the mechanisms evolving mixed-mating systems, even though it is estimated that 42% of angiosperms have this mating system strategy (Goodwillie *et al.* 2005). However, plants with this type of mating are considered to be in advantage over plants with pure mating strategies, because they combine the possibility to cross their genes, while guaranteeing reproduction in the absence of pollinators or potential mates (Goodwillie *et al.* 2005).

Mating system in plants is not constant for an individual or population, on reverse it depends on local abiotic and biotic environmental conditions that create overall mating opportunities (Barret & Harder 2017). However, apart from the outcrossing - selfing paradigm that has dominated research on plant mating systems (Barret & Harder 2017), the specific ecological mechanisms (e. g. local influences) causing variation in mating systems patterns are often poorly understood. Correlated paternity received much less attention than the other estimates. Even though reported frequently, correlated paternity has received limited analysis. Among the factors that cause correlated paternity in plants are the correlated pollen dispersion (which may affect fruits with numerous seeds and non-independent pollen transport mechanisms) and the limited availability of potential parents. In turn, this availability depends on parent’s effective density, flowering heterogeneity and synchrony, and the existence of incompatibility systems (Hardy *et al.* 2004). On the other hand, correlated paternity values are also affected by the pollination systems in a significant and intrinsic way. This may lead to anemophilous and entomophilous species being subjected to different levels of gene drift in fragmented landscapes. Several studies have documented significantly higher values of correlated paternity in species pollinated by animals than in wind-pollinated species (Smouse & Sork 2004). This may be because in entomophilous species the degree of genetic connectivity of populations is dependent on the distribution, abundance and behavior of their pollination vectors, which can be also affected by fragmentation (Aguilar *et al.* 2008, Kramer *et al.* 2008, although see Hamrick 2004).

An increase in correlated paternity can have important ecological and evolutionary consequences, since progenies composed for the most part of full siblings could theoretically have a lower capacity to exploit heterogeneous environments compared to unrelated seed cohorts (Karron & Marshal 1990; Rousset & Billiard 2000). This fact may be crucial in extreme fragmented habitats where spatial connectivity is very low and the availability of microhabitats for establishment of seedlings is much lower than in a continuous habitat. In addition, the increase of local genetic structure resulting from the dispersion of maternal progenies with a high level of parental correlation may, after several generations, contribute to processes of inbreeding depression at level local. Empirical studies testing the impact of correlated paternity on progeny performance are virtually absent, but support for this idea can be found in some studies that put in evidence that plants in isolated fragments produce seeds with lower germination rates and seedlings with less genetic diversity and vigor compared to those produced in continuous forests (Nason & Hamrick 1997; Rocha & Aguilar 2001; Fernández-M & Sork 2005).

Study species

In this thesis work we focused our studies in two plant species that are abundant and dominant sclerophyllous shrubs of the Mediterranean woodlands and share important life-history traits (e.g. both produce endozoochorus fleshy fruits), but have contrasted inbreeding and pollen dispersal systems:

Myrtus communis L.

Myrtus is a hermaphroditic, self-compatible shrub with a density-dependent mixed mating system (González-Varo *et al.* 2009) that can live for decades. It blooms massively in early summer (from mid-June to early July). Its showy flowers are white with one style and multiple stamens and its pollen is dispersed by hymenopterans and dipterans (González-Varo *et al.* 2009). Its fruits consist of small multi-seeded berries that begin to be dispersed in mid-October by small-to-medium-sized birds, either resident species or wintering migrants (mostly Sylviidae and Turdidae; see González-Varo 2010).

Figure 2. *Myrtus communis* flower being pollinated by *Megachille* sp. Credit: RG Albaladejo.

Pistacia lentiscus L.

Pistacia is a dioecious shrub (rarely a small tree) and therefore an obligate outcrosser which can live for decades. It is wind-pollinated and blooms massively between mid-March and late April (Jordano 1988). Male flowers are apetalous with 8-10 stamens grouped in dense inflorescences of 8-10 flowers. Female flowers are also apetalous and constituted by a small unilocular ovary and grouped in inflorescences of 4-36 flowers. Fruit development in *Pistacia* is delayed until late summer. This long period of fruit development is common within the genus *Pistacia* and is caused by a delay in the singamy (Grundwag 1976). During this time, *Pistacia* fruits change color from white at the initial stage to red (intermediate stage) and black when they are fully matured (Jordano 1988). Moreover, black matured fruits might be seedless or with empty seeds as a result of ontogenetic processes that lead to parthenocarpy and abortion (Gillaspy *et al.* 1993). The parthenocarpic and aborted fruits in *Pistacia* have been widely studied and the causes of these deceptive fruits are mainly attributed to resource competition in a limited environment (Jordano 1988), or to reduce seed predation rates by chalcidoid wasps (Verdú & García-Fayos 1998) and by granivorous birds (Verdú & García-Fayos 2001). In the study area, *Pistacia* fruits are dispersed from September to December by virtually the same avian dispersal-guild as *Myrtus* (Jordano 1989).

Figure 3. Male (left) and female (right) flowers and inflorescences of *Pistacia lentiscus*.

Study area

The Guadalquivir River Valley depression is an extensive region (21,000 km^2) located between the natural boundaries of Sierra Morena and the Baetic Mountains of Western Andalusia (south Spain) (Figure 2). The topography of the area is flat with altitude ranging between sea level and 200 m. Climate is typically Mediterranean, with warm dry summers and mild humid winters. The mean annual precipitation in the region is *c.* 550 mm (500-598 mm) and January and July temperatures average are 10ºC (9.2–11.6 ºC) and 26ºC (25.5-27.2 ºC), respectively (AEMET; http://www.aemet.es).

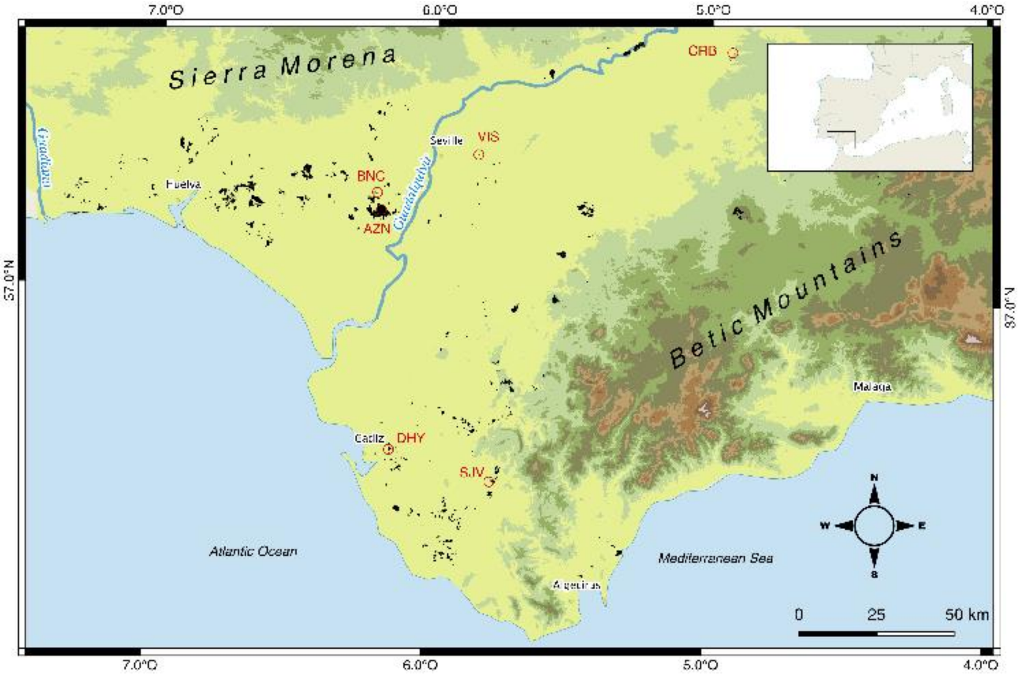


Figure 4. Study area, showing the location of the fragmented forest in Aparicio (2008) (black polygons. Studied populations in red with population codes as in Figure 5.

Originally, the dominant vegetation in this area was thermophilic Mediterranean shrubs associated with cork oak (*Quercus suber* L.) and holm oak (*Quercus ilex* subsp. *ballota* (Desf.) Samp.) forests, accompanied by *Quercus faginea* Lam. in wetter conditions (Carrión & Fernández 2009). However, the region has suffered a long history of management, initiated in the Neolithic period, about 7,000 years ago (Aparicio 2008; Martín *et al.* 2013). The favorable conditions in the Guadalquivir River Valley convert it in one of the main zones of settlement of different civilizations, which translates into a quick development of cereal and olives cultures, vineyards, as well as pasture and mine extraction. During the Roman period, the advance in technology contributed to an increase in rhythm of deforestation. It is estimated that at the end of this period, around 50% of the Iberian Peninsula was already devoid of forest. This proportion could be higher in many parts of Andalusia where the endless landscapes of olive trees were already present (Martín *et al.* 2013). This process extended until nowadays, with the introduction of industrial agriculture that left almost the entire region dominated by intensive monocultures (e.g. olives, cereals, sunflower, cotton, vineyards and diverse orchards).

Therefore, the secular alteration of this fertile region has relegated their Mediterranean forests to a type of landscape that can be considered as relictual (*sensu* McIntyre & Hobbs 1999). Nowadays, the remnant woodland patches cover less than 1% of their potential area. This natural or semi-natural forest vegetation consists of a constellation of approximately 500 fragments with scarce physical connectivity and low degree of habitat quality and conservation (legally protected areas excluded; Aparicio 2008). Almost 70% of the patches are private and managed to harvest pine nuts, cork and firewood, for coal production, cattle breeding and, to a lesser extent, beekeeping and agriculture. Public patches (the remaining 30%) are mainly used for recreation.

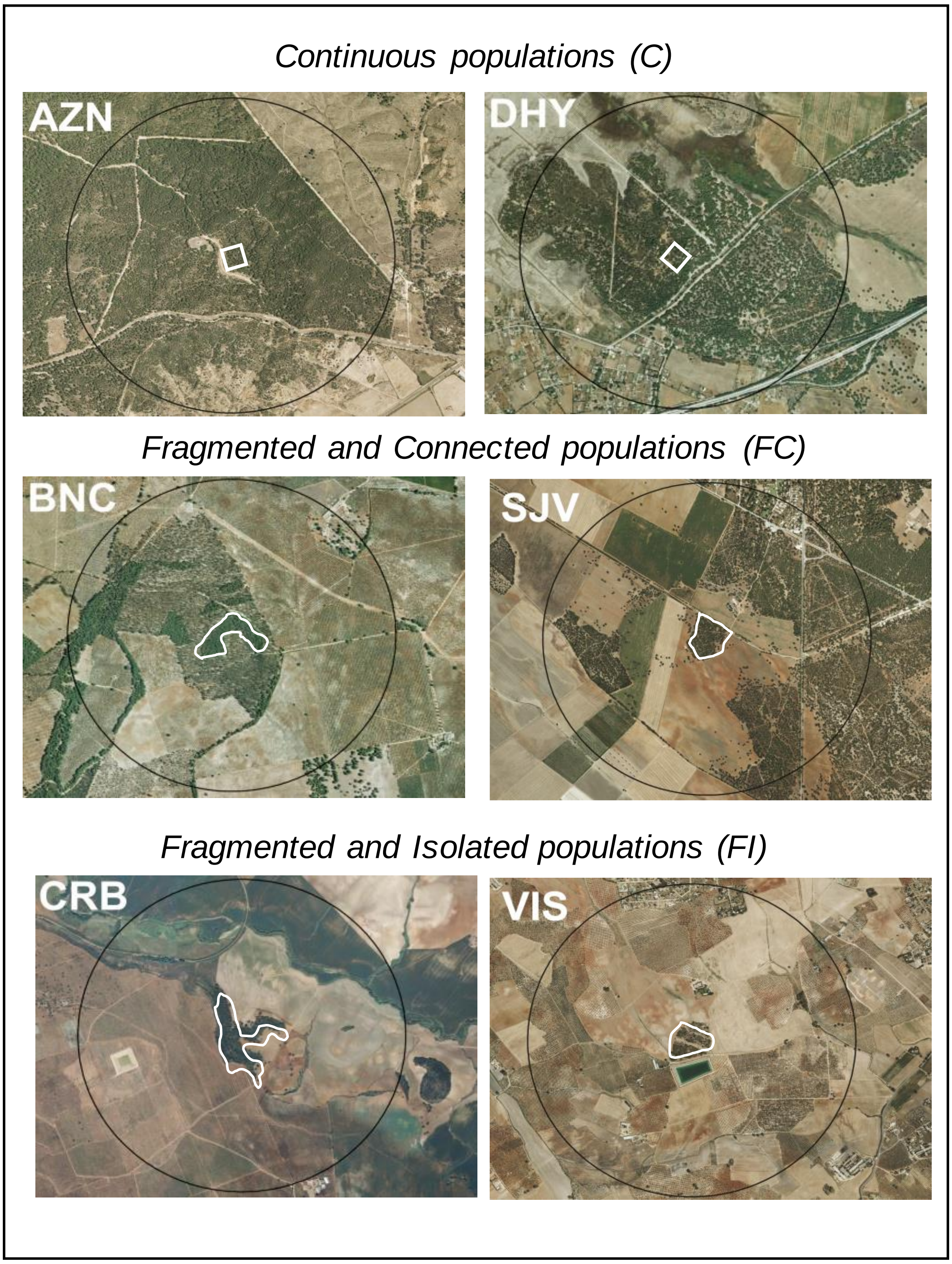
Both natural old (with more than 300 – 400 years of existence) and semi-natural forest patches, even with human modifications, maintain a good part of their natural characteristics. The tree cover (*c*. 8 – 12 m height) is mainly represented by stone pines (*Pinus pinea* L.), holm oaks (*Quercus ilex* subsp. *ballota* (Desf.) Samp.) and cork oaks (*Quercus suber* L.). Although, the presence of stones pines is associated in most of the cases to human plantations, dating back to the eighteenth century, due to the economical importance of this species (Carrión & Fernández 2009). The shrub layer is composed by a mixture of early successional shrubs (< 1 m height), mainly belonging to Cistaceae (e. g. *Cistus salviifolius* L., *Cistus crispus* L., *Cistus ladanifer* L., *Cistus monspeliensis* L. and *Halimium halimifolium* (L.) Willk.), Labiatae (e. g. *Lavandula stoechas* L., *Rosmarinus officinalis* L. and *Teucrium fruticans* L.) and Leguminosae (e. g. *Genista hirsuta* Vahl and *Ulex australis* Clemente) and late successional sclerophyllous shrubs (> 1 m height), such as *Myrtus communis* L. (Myrtaceae), *Pistacia lentiscus* L. (Anacardiaceae), *Olea europaea* var. *sylvestris* (Mill.) Lehr (Oleaceae), *Phillyrea angustifolia* L.

(Oleaceae), *Daphne gnidium* L. (Thymelaeaceae), *Rhamnus lycioides* L. (Rhamnaceae) and *Quercus coccifera* L. (Fagaceae) (Aparicio 2008).

Regardless of their origin, ownership or degree of transformations, these forest patches have an enormous value and importance within the ecosystem they integrate. They play a prominent role in the conservation of biodiversity and connectivity of both plant and animal populations. By sometimes being the only element of natural vegetation within a radius of kilometers, they provide shelter for many species to rest, breed and feed (Martín *et al.* 2013). They also represent a valuable biodiversity reservoir: more than 1,000 plant species have been catalogued, 39 of which have proved to be relevant chorological novelties and 70 are included in the red list of threatened species of Andalusia, even a new species for science has been described, *Limonium silvestrei* Aparicio (Aparicio 2008).

Study populations

From a previous exhaustive floristic and ecological survey conducted in the region (see Aparicio 2008), we detected 114 woodland patches where the two study species co-exist. Out of these fragments, we selected four small patches (Table 1) that were then classified either as 'fragmented-connected' (*FC*) or 'fragmented-isolated' (*FI*) according to the amount of suitable forest area contained in 1-km radius buffer around the centroid of the patch. This threshold was established based on evidence on the study species or similar species that suggest pollen dispersal occurs mostly below this distance (for details see Chapter 1). We also selected two continuous populations (*C*) by setting two rectangular plots of 5 ha within two large stone pine woodlands (above 100 ha) where the two species were very common. We thus established a gradient of habitat fragmentation: *C* < *FC* < *FI* (Figure 5).



Continuous populations (C)

Fragmented and Connected populations (FC)

Fragmented and Isolated populations (FI)

Figure 5. Aerial images of the six study populations categorized into three landscape contexts: continuous [Aznalcázar (AZN) and Dehesa de las Yéguas (DHY)], fragmented-connected [Benacazón (BNC) and San José del Valle (SJV)] and fragmented-isolated (Córdoba (CRB) and El Viso (VIS)]. Buffers of 1-km radius from the centroid of the focal population are also drawn.

Thesis objectives

The main goal of this Doctoral Thesis is to evaluate how habitat fragmentation (fragment size and connectivity) affects and determines both historical and contemporary gene flow and mating systems patterns, as well as the reproductive success of plant populations of the two common Mediterranean shrubs, *Myrtus communis* and *Pistacia lentiscus*. By conducting comparative studies with two distinct species that share important life-history traits (e. g. both produce endozoochorus fleshy fruits), but have contrasted mating and pollen dispersal systems, this thesis aims to analyze the complexities surrounding fragmented systems and the influence of fragmentation on multiple ecological interactions. In order to accomplish this goal, it was fundamental the use of several different methodologies such as field ecology, population genetics (molecular techniques using nuclear microsatellites), experimentation under controlled conditions and different statistical techniques (e. g. general and generalized linear mixed models, model selection and structural equation models among others).

Given the scarcity of fine-scale population genetic information from non-tropical plants, this thesis aspires to respond to several specific questions in this system of Mediterranean forest fragments: Do fragmented populations exhibit lower levels of genetic diversity and higher levels of genetic differentiation and spatial genetic structure than continuous populations? Is it possible to observe a decline of the effective population sizes in the recent past and evidence of genetic bottlenecks as consequence of habitat fragmentation and isolation? How many pollen donors are currently fertilizing seeds? What are the rates of endogamy, correlated paternity, and biparental mating? These rates will have any consequences to the future progeny fitness and survival? Which ecological components are more determinant to plant's reproductive success and mating system patterns? More importantly, do these relationships change under different fragmentation contexts? And are habitat fragmentation effects consistent across species with contrasted breeding and pollen dispersal systems?

Thesis structure

This Doctoral Thesis consists of four chapters. The content and purpose of each of the chapters, which correspond to published scientific articles or in the process of being published, are detailed below.

In **Chapter 1** (*Genetic variation and structure in different landscape contexts*) was conducted standard genetic analyses (genetic diversity and structure, effective population size, detection for potential bottlenecks and spatial genetic structure) on adult individuals in order to infer patterns of historical gene flow of *Myrtus* and *Pistacia* populations under different landscape contexts (*Fragmented-Isolated*; *Fragmented-connected* and *Continuous* populations).

Chapter 2 (*Habitat fragmentation effects on plant reproductive success*) is a comparative study of *Myrtus* and *Pistacia* that assess the role of habitat fragmentation in conditioning plant's reproductive success, either by disturbing directly plant's ability to reproduce and/or by changing plant's relationships with biotic and abiotic ecological components that are determinant to their reproductive success.

In **Chapter 3** (*Ecological determinants of mating patterns in fragmented populations*) the contemporary patterns of pollen flow of *Myrtus* and *Pistacia* populations under three different contexts of fragmentation are studied. In this chapter we evaluated the variation in mating system estimates in both species and also the main ecological factors responsible (e. g. distance to the patch edge, flowering synchrony or canopy neighborhood density) for this variation at both the individual and population levels.

Chapter 4 (*Correlated paternity effects on progeny performance*) analyzes how correlated paternity shapes *Myrtus* and *Pistacia* early progeny performance (seedling emergence, seedling emergence time, seedling growth, biomass, lifetime and survival). The consistency of the observed relationships is tested both in a greenhouse environment and under natural (field) conditions where the effects could be potentially masked by other factors such as the response to environmental heterogeneity.

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1

Genetic variation and structure in different landscape contexts

Nora S, Albaladejo RG, Aparicio A. 2015. Genetic variation and structure in the Mediterranean shrubs *Myrtus communis* and *Pistacia lentiscus* in different landscape contexts.

Plant Biology, 17: 311-319.

Variación y estructura genética en distintos contextos paisajísticos

Resumen

Los estudios que implican diferentes configuraciones del hábitat arrojan información sobre las interacciones complejas que existen entre los rasgos funcionales de las especies y su entorno, y así pueden ayudar a predecir patrones relacionados con la variación genética de sus poblaciones. En este estudio comparamos los patrones de variación genética en dos especies del matorral Mediterráneo (*Myrtus communis* y *Pistacia lentiscus*) que conviven en las poblaciones seleccionadas y que representan tres paisajes con diferente grado de fragmentación: poblaciones continuas, poblaciones fragmentadas-conectadas y poblaciones fragmentadas-aisladas. A través del análisis de la variabilidad de loci de microsatélites nuestros resultados revelaron respuestas sutiles a los diferentes contextos de fragmentación. En su lugar, encontramos fuertes respuestas específicas de la población en las dos especies. Sin embargo, a pesar de que las dos especies mostraron niveles similares de diversidad genética, *Myrtus* se caracterizó por poseer unos niveles más altos de homocigosidad y diferenciación genética poblacional, patrones más acusados de estructuración genética espacial dentro de las poblaciones, valores más bajos de número efectivo de las poblaciones y evidencias más claras sobre la existencia de cuellos de botella genéticos recientes que *Pistacia*. Estos resultados destacan la influencia que sucesos pasados (p.e. conectividad histórica, fluctuaciones del tamaño poblacional) y factores locales (p.e. disponibilidad de microhábitat adecuados para el reclutamiento, calidad del hábitat, densidad de plantas, fauna nativa) tienen a la hora de determinar los patrones genéticos observables en las especies, ya que la influencia de la configuración del paisaje per se (p.e. tamaño y/o aislamiento de los fragmentos) puede ser limitada.

Palabras clave: Especies anemófilas; especies entomófilas; fragmentación del hábitat; genética poblacional; paisaje Mediterránea; sistemas de apareamiento.

Abstract

Studies concerning different habitat configurations can provide insights into the complex interactions between species' life-history traits and the environment and can help to predict patterns in population genetics. In this study, we compared patterns of genetic variation in two Mediterranean shrub species (*Myrtus communis* and *Pistacia lentiscus*) that co-occur in populations within three contrasting landscape contexts: continuous, fragmented-connected and fragmented-isolated populations. Analyzing variation at microsatellites loci, our results revealed weak responses to the landscape contexts. We rather found a population-specific response in both study species. However, despite both study species sharing similar levels of genetic diversity, *Myrtus* displayed higher levels of homozygosity and genetic differentiation among populations, stronger patterns of within-population spatial genetic structure, lower values of mutation- scaled effective population size and stronger evidence for recent genetic bottlenecks than *Pistacia*. This result highlights the influence of past events (e.g. historical connectivity, fluctuations in population size) and local factors (e.g. microhabitat availability for recruitment, habitat quality, plant density, native fauna) and that the landscape configuration per se (i.e. fragment size and/or isolation) might not completely determine the species' genetic patterns.

Keywords: Habitat fragmentation; insect-pollinated species; mating systems; Mediterranean landscapes; population genetics; wind-pollinated species.

INTRODUCTION

Fragmentation modifies the original configuration of natural habitats by reducing habitat extension and quality, and can eventually compromise the persistence of even common and naturally abundant species (Fahrig 2003; Honnay & Jacquemyn 2007). Aside from demographic and ecological effects (Leimu *et al.* 2010; Meirmans *et al.* 2011), fragmentation can also shape patterns of genetic diversity, both within and among populations, and influence the long-term survival of species in anthropogenic habitats (Sork & Smouse 2006). In this context, the 'paradigm of small population genetics' (Bacles & Jump 2011) predicts that a reduction in population size will ultimately lead to a decrease in genetic diversity, an increase in inbreeding (either complete inbreeding or biparental inbreeding) and individual homozygosity and, consequently, a reduction in fitness (Ellstrand & Elam 1993; Young *et al.* 1996).

Nevertheless, under the perspective of population genetics, the consequences of habitat fragmentation have to be examined within the interaction between species' life-history traits (e.g. growth form, perenniality, seed and pollen dispersal, mating and breeding systems, vegetative reproduction, geographic range and longevity) that directly or indirectly affect their ability to spread their genes, and the landscape context (habitat size, landscape-scale connectivity and matrix characteristics). In fragmentation studies it is therefore essential to consider all the complex mechanisms and contexts that either enhance or block gene flow (Bacles & Jump 2011).

For example, the mating system, i.e. the ability to self, has been shown to be one of the drivers behind species' vulnerability to fragmentation (Duminil *et al.* 2007; Aguilar *et al.* 2008). Indeed, self-compatible and self-incompatible species diverge in the way they retain genetic diversity, since self-compatible species tend to contain less genetic diversity within and more genetic differentiation between populations than self-incompatible species (Ellstrand & Elam 1993; Hamrick & Godt 1996). Moreover, pollen dispersal modes are also responsible for how species react to human management and disturbance. Animal-pollinated species tend to be more prone to the effects of genetic drift in fragmented habitats, despite their inherent potential for the long-distance dispersal of pollen grains (Smouse & Sork 2004; Hughes et al. 2007). Finally, it is important to stress the role of historical events such as fluctuations in population size and past bottlenecks in shaping how genetic diversity is currently structured at different landscape scales (Vekemans & Hardy 2004; Bacles & Jump 2011).

In contrast, although difficult to disentangle from species' functional attributes, the landscape context also influences the effective population size and connectivity of populations, as well as their long-term genetic structure (Manel *et al.* 2003; Storfer *et al.* 2010). In fact, it has been stated that whenever populations are constrained to small remnants of habitat, they have high probability of becoming extinct due to environmental or demographic stochasticity, natural catastrophes or reduced genetic diversity (Ewers & Didham 2006). Thus, currently, habitat loss is considered to be one of the major causes of species extinctions (Sala *et al.* 2000). Additionally, the degree of connectivity of populations should be species dependent as different species are likely to have different degrees of susceptibility to the effects of isolation (Ewers & Didham 2006; Holderegger & Wagner 2008). In fact, the disruption of connectivity should only occur when the potential for dispersal does not exceed the degree of isolation of populations, making it difficult to overcome fragmentation (Kramer *et al.* 2008).

In this study, we evaluated the patterns of population genetic diversity and structure of two widespread Mediterranean shrub species, *Myrtus communis* L. and *Pistacia lentiscus* L., in a regional mosaic of forest fragments in southwest Spain. Within this area we selected woodlands representing three contrasting landscape contexts (continuous, fragmented-connected and fragmented-isolated populations); where the two study species co-exist, to ensure the same local history of management and disturbance. We hypothesized that, as a consequence of a long history of deforestation, fragmented populations (especially the fragmented and isolated populations) should exhibit lower levels of genetic diversity and higher levels of genetic differentiation and spatial genetic structure than continuous populations. Moreover, we expected the consequences of fragmentation and isolation to be reflected in a decline of the effective population sizes in the recent past, leading to evidence of genetic bottlenecks. However, important factors in shaping species' genetic variability, like species' life-history traits (e.g. mating system, pollen and seed dispersal mechanism), could mask those effects (Aparicio *et al.* 2012). Thus, we expected the insect-pollinated and self-compatible species, *M. communis*, to show stronger consequences of habitat fragmentation than the wind-pollinated and outcrossing species, *P. lentiscus*.

MATERIAL AND METHODS

Study species, study sites and sampling design

The studied species were *M. communis* L. and *P. lentiscus* L. (*Myrtus* and *Pistacia* hereafter). Both species are common shrubs in the sclerophyllous Mediterranean maquis. *Myrtus* is a hermaphroditic, self-compatible shrub with a density-dependent mixed mating system (González-Varo *et al.* 2009) that can live for decades (González-Varo pers. comm.). It blooms massively in early summer (from mid-June to early July), its showy flowers are visited and its pollen is dispersed by hymenopterans and dipterans (González-Varo *et al.* 2009). Its fruits consist of small berries that begin to be dispersed in mid-October by small-to-medium-sized birds, either resident species or wintering migrants (mostly Sylviidae and Turdidae; see González-Varo 2010).

Pistacia is a dioecious shrub (rarely small tree) and therefore an obligate outcrosser, which can live for decades. Its minute flowers bloom from mid-March to late April, and extensive pollen dispersal by wind takes place (Albaladejo *et al.* 2009). Its fruits consist of small black drupes that ripen and are dispersed from September to December in the study area by virtually the same avian dispersal guild as Myrtus (Jordano 1989).

We conducted this study in the lower catchment area of the Guadalquivir River in western Andalusia (S Spain) (Fig. 1A). This region has a long history of management, initiated in the Neolithic period,with further periods of strong deforestation in Roman times and the Middle Ages (Carrión *et al.* 2001; Valbuena-Carabaña *et al.* 2010). Nowadays, this fertile region is dominated by intensive monocultures (e.g. olives, cereals, sunflower, cotton, vineyards and diverse orchards). This landscape is considered relictual (sensu McIntyre & Hobbs 1999), where the remnant woodland patches cover <1% of their potential area (Aparicio 2008).

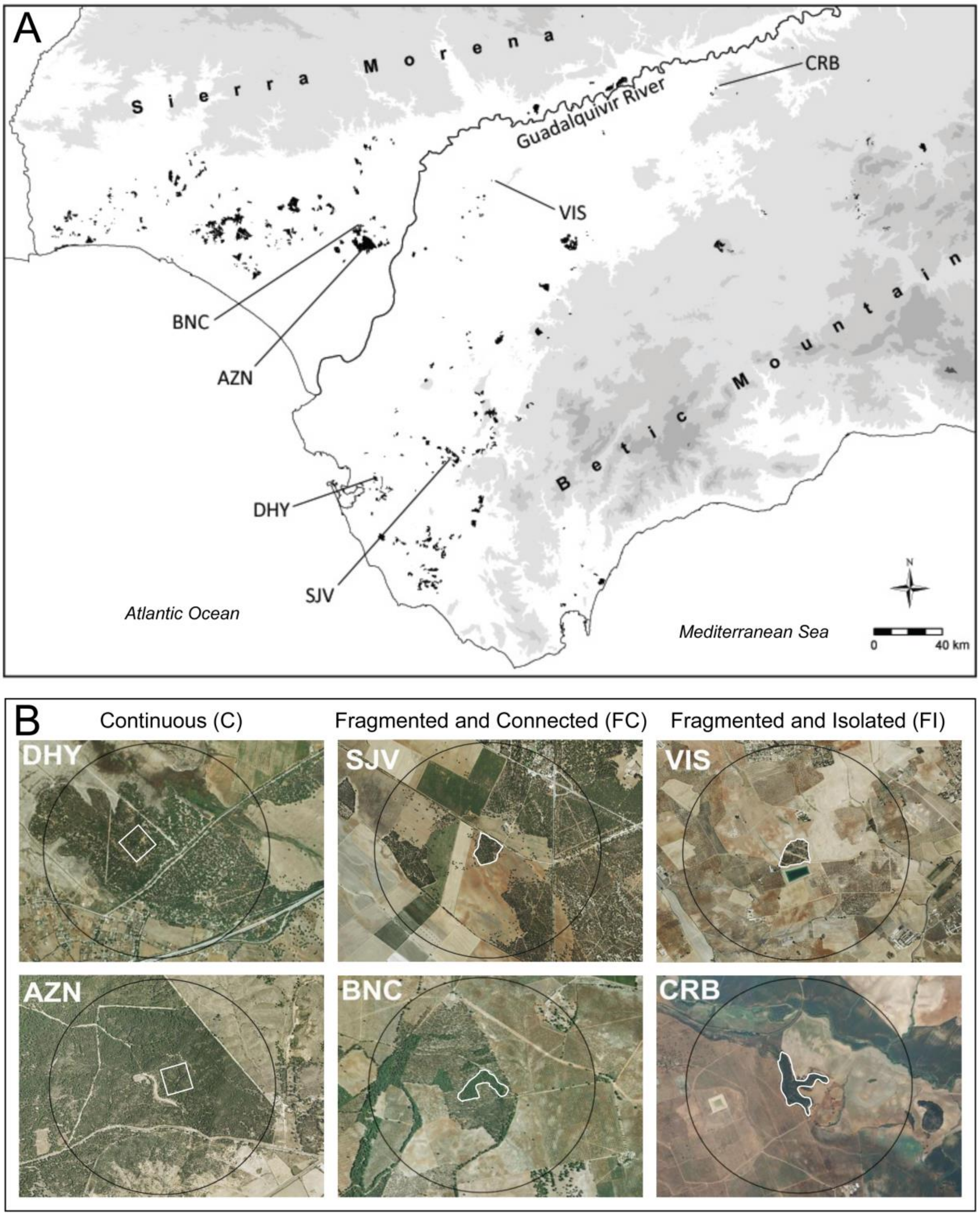

Figure 1. Study area showing the location of the six studied populations in the region (A) and aerial images of the six study populations categorized into three landscape contexts: continuous (AZN and DHY), fragmented-connected (BNC and SJV) and fragmented-isolated (CRB and VIS) (B). Buffers of 1-km radius from the centroid of the focal population are also drawn. Population codes as in Table 1.

1. Genetic variation and structure in different landscape contexts

From a previous exhaustive floristic and ecological survey conducted in the region (see Aparicio 2008), we detected 114 woodland patches where the two study species co-exist. Out of these fragments, we selected four small patches (Table 1) that were then classified either as ‘fragmented-connected’ (*FC*) or ‘fragmented-isolated’ (*FI*) according to the amount of suitable forest area contained in 1-km radius buffer around the centroid of the patch. This threshold was established based on evidence on the study species or similar species that suggest pollen dispersal occurs mostly below this distance. Thus, pollen dispersal kernels and paternity analyses conducted in *Pistacia* revealed average pollination distances below 500 m (Albaladejo *et al.* 2012). And, although no such detailed information is available for *Myrtus*, studies on similar insect-pollinated shrubs can provide useful information. For example, in a population of *Prunus mahaleb* (an hermaphroditic shrub, with a similar guild of pollen dispersers) in southern Spain, García *et al.* (2007) detected pollen dispersal distances of up to 600 m, and average pollen distances below 100 m. Further, studies on pollinator foraging skills (e.g. Gathmann & Tscharntke 2002) have revealed maximum foraging distances of 150–600 m in solitary bees like *Megachile* spp., a genus with species frequently found pollinating *Myrtus* flowers (González-Varo *et al.* 2009). Finally, we also selected two continuous populations (*C*) by setting two rectangular plots of 5 ha within two large stone pine woodlands (above 100 ha) where the two species were very common. We thus established a gradient of habitat fragmentation: FI > FC > C (Table 1; Fig. 1B).

Within each of the six fragments, we collected young leaves from 30 *Myrtus* and 40 *Pistacia* (20 female and 20 male) adult plants (without taking samples from the same ramets) and stored them in abundant silica gel (overall 420 plants were sampled; 180 *Myrtus* and 240 *Pistacia* plants). We recorded the coordinates of each sampled plant with a global positioning system (GPS) unit (Garmin GPSmap 60CSx).

Table 1. Characteristics of the studied populations of *Myrtus communis* and *Pistacia lentiscus*. Connectivity is the woodland area contained in 1 km-radius buffer around the centroid of the focal patch. Population types are: continuous (*C*), fragmented-connected (*FC*) and fragmented-isolated (*FI*) populations.

Population	Coordinates (N,W)	Type	Area (ha)	Connectivity(ha)	*Myrtus communis*			*Pistacia lentiscus*			
					Pop. size†	Density (ind/ha)	Nearest pop. (km)	Pop. size	Density (ind/ha)	Nearest pop. (km)	Sex-ratio (F/M)
AZN	37º14'04'' 6º10'03''	*C*	5	219	245	49	0	610	122	0	1.38^{ns}
DHY	36º33'15'' 6º08'08''	*C*	5	125	189	37.8	0	1475	295	0	2.05*
BNC	37º17'37'' 6º12'29''	*FC*	5.2	21	390	75	0.1	157	30.2	0.1	2.07^{ns}
SJV	36º37'17'' 5º51'42''	*FC*	5.3	96	120	22.6	0.1	1054	198.9	0.2	1.41^{ns}
CRB	37º43'36'' 4º55'25''	*FI*	9.8	0	70	7.14	20	1816	185.3	1	2.47*
VIS	37º26'20'' 5º44'27''	*FI*	5.1	0	200	39.2	10	521	102.2	10	0.63^{ns}

[†] Area for the continuous population type refers to the 5 ha plots.

^{ns} Not significant, *P < 0.05.

DNA isolation and microsatellite analyses

We extracted total DNA from ca. 50 mg dry leaf material using the Invisorb DNA Plant HTS 96 Kit (Invitek, Berlin, Germany) according to the manufacturer's protocol, and amplified seven and ten polymorphic nuclear microsatellites specifically developed for *Pistacia* and *Myrtus* (Albaladejo *et al.* 2008, 2010), respectively. Approximately 30 ng of template DNA were amplified with 1 U fast MyTaqTM Red DNA Polymerase (Bioline, London, UK) in 5 9 reaction buffer to a final volume of 10 ll. Amplification reactions were conducted following the three-primers protocol of Schuelke (2000), with 0.2 lM of the reverse and the M13 universal primer (the latter labelled with FAM, NED, VIC or PET to the 50-end), and 0.07 lM of the modified forward primer with the M13 primer sequence added at its 50-end. PCR cycle conditions basically consisted in a denaturation step at 95 °C for 1 min followed by ten touchdown cycles at 95 °C (15 s), 60 °C (15 s; 0.5 °C cycle 1 or 1 °C cycle 1, depending on the microsatellite), 72 °C (10 s), followed by 30 cycles at 95 °C (15 s), 50 °C or 55 °C (15 s), 72 °C (10 s), and a final extension step at 72 °C for 1 min. Further details can be found in Albaladejo *et al.* (2008, 2010).

We analyzed labelled PCR products with an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, USA) at the Unidad de Genomica (UCM, Madrid, Spain). We scored fragment sizes automatically with GeneMapper 3.7 (Applied Biosystems) and then checked manually when necessary. For genetic analyses, we only retained genotypes successfully scored for at least five loci in *Pistacia* and eight loci in *Myrtus*. To confirm the reliability and consistency of results, we randomly select 20 samples of each species and compared the profiles side-by-side with their replicates, yielding an overall estimate of scoring repeatability of 99%.

Genetic diversity and structure

We checked for the independence among pairs of amplified loci through linkage disequilibrium (LD) tests, applying Bonferroni's correction for multiple tests. We characterized levels of genetic diversity using the following parameters: number of alleles per locus (*A*), effective number of alleles per locus (A_e) and Nei's gene diversity (H_e). We estimated unbiased inbreeding coefficients (F_{IS}) simultaneously with null allele frequencies following the individual inbreeding model as implemented with Inest (Chybicki & Burczyk 2009). With the estimated allele frequencies accounting for the presence of null alleles, we recomputed the calculation of expected heterozygosity (H_{e_null}). We assessed individual genetic diversity levels via the homozygosity by loci (*HL*) index, a calculation that improves raw heterozygosity estimates by weighting the contribution of each locus to the individual homozygosity value in terms of their allelic variability (Aparicio *et al.* 2006). We calculated the *HL* index with the R script Genhet (Coulon 2010).

We estimated the level of among-population genetic differentiation (F_{ST}) with Freena (Chapuis & Estoup 2007), which provides unbiased estimates accounting for null allele presence. For each species, first, we calculated the global F_{ST} among all populations, and second, we calculated F_{ST} values for each population by averaging F_{ST} values among population pairs (for analyses at population level). We assessed the significance of F_{ST} values by constructing 95% confidence intervals using permutation procedures (1000 replicates). We analysed differences between species for H_{e_null}, *HL*, F_{IS} and F_{ST} values using Wilcoxon's paired tests but avoided comparing measures of allelic diversity (*A* and A_e) since they directly depend on the battery of the markers used, which are necessarily different for the two study species.

We estimated selfing rates ($\hat{s}$) at population level with the software Rmes (David *et al.* 2007), which provides two unbiased estimators that account for the occurrence of null alleles in microsatellite loci. We ran the maximum likelihood (*ML*) multilocus heterozygosity method (precision set to 0.001 and $K_{max} = 10$), as well as the method based on the estimation of the two-locus heterozygosity disequilibrium ($\hat{g}_2$; with 1000 interactions). It is important to note that for dioecious species such as *Pistacia* the $\hat{s}$ values only account for selfing in the populations acquired through biparental inbreeding (David *et al.* 2007).

Effective population size and detection of genetic bottlenecks

The effective population size (N_e) is a key parameter in evolution and conservation biology (Allendorf & Luikart 2007) as it is useful for predicting the fate of small populations and for the prompt detection of population fragmentation (England *et al.* 2010) and decline (Antao *et al.* 2011). Nevertheless, the effective population size calculation depends highly on the mutation rate used that can vary in several orders of magnitude across loci and generations (Weber & Wong 1993), making any estimate prone to very large variation. For this reason, in this study we made no attempt to estimate the current effective population size with this method, we rather estimated the mutation-scaled effective population size, theta (Θ). In diploid organisms, Θ is equal to $4N_e\mu$ (with N_e being the effective population size and μ the mutation rate for the microsatellite data set). We obtained Θ estimates and the associated 95% confidence intervals with the program Migrate-N 3.2.16 (Beerli 2009) using the maximum likelihood inference, each run consisting of ten short chains (sampling 1000 trees) and three long chains (sampling 5000 trees), with a burn-in set to 10000 trees. We performed three independent replicates and ran each search with a static heating scheme of four chains at different temperatures (1000000, 3.0, 1.5 and 1.0).

We used the program Bottleneck 1.2.02 (Cornuet & Luikart 1997) to detect potential reductions in N_e in the recent past of the study populations. We employed Wilcoxon's tests to detect excess heterozygosity in comparison to simulated values under mutation-drift equilibrium with three mutational models of microsatellite variation: the infinite allele model (IAM), the step-wise mutational model (SMM) and the intermediate two-phase mutational model (TPM), the latter allowing for 10% of single-step changes (Cristescu *et al.* 2010). The variation rate was set to 12, as recommended by Piry *et al.* (1999) for microsatellite markers.

Spatial genetic structure

We tested for detectable structuring in the gene pools of *Myrtus* and *Pistacia* and their spatial delimitation using the Bayesian clustering algorithm of Structure 2.3.1 (Pritchard *et al.* 2000). For each species we conducted five independent runs for each preselected K (number of genetic clusters), from K = 1 to 6. We conducted all runs following the admixture model and correlated allele frequencies among populations. Due to the overall low values of genetic differentiation among populations (especially for *Pistacia*, see Results), the sample group information was allowed to help in the clustering process following Hubisz *et al.* (2009). We set the number of MCMC iterations to 106, plus an initial burn-in of 105 iterations. We assessed the optimal number of genetic clusters with the log-likelihood values of each run based on the guidelines given by these authors (Pritchard *et al.* 2000; Hubisz *et al.* 2009), and implemented the procedure used by Evanno *et al.* (2005) with the help of the website program Structure Harvester (Earl & vonHoldt 2012). We summarized the results of the independent replicate runs for the optimal K with Clumpp 1.1.2 (Jakobsson & Rosenberg 2007), and visualized them with Distruct 1.1 (Rosenberg 2004).

We characterized the within-population spatial genetic structure for the two species in each population type by constructing average correlograms and plotting average kinship coefficients (*Fij*; Loiselle *et al.* 1995) among individual pairs (only within populations) against distance (represented by seven distance classes designed to contain the same number of observations). We assessed significance of the within-population spatial genetic structure by comparing the observed slope of the regression against the slope obtained by randomly permuting (1000 times) individual locations. We conducted all analyses with the software Spagedi 1.4 (Hardy & Vekemans 2002). To quantify the strength of the observed patterns of within-population spatial genetic structure, *i.e.* the strength of the correlation of the genetic relatedness and spatial linear distance, we computed the S_p statistic (Vekemans & Hardy 2004), which is a valuable parameter because it allows direct quantitative comparisons among populations and species (Hardy et al. 2006).

RESULTS

Genetic diversity and structure

The summarized values of the genetic diversity parameters are given in Table 2. Microsatellite markers were highly polymorphic in both species, with 101 and 85 alleles for *Myrtus* and *Pistacia*, respectively (the missing data rate in our data sets were 3.5% and 1.9%, respectively). Linkage disequilibrium tests showed that all pairs of loci in the two species were independent. Null alleles were detected only in a few loci and at low frequencies in both species (Table S1).

Neither *Myrtus* nor *Pistacia* showed higher levels of diversity in the continuous populations than in the fragmented ones. For instance, in *Myrtus* the lowest allelic richness (A and A_e) and highest levels of homozygosity (*HL*) occurred in the *C* population AZN, while the lowest gene diversity (H_{e_null}) occurred in the *FI* population VIS (see Table 2). In *Pistacia*, the *FI* population VIS consistently showed the lowest values for all measurements of diversity (except for *HL*), while the highest values occurred in the *FC* populations BNC (for A) and SJV (for H_{e_null}), and in the *C* population AZN (for A_e). We found no significant inbreeding in any type of population or species, thereby indicating a situation of virtual panmixia (Table 2). Accordingly, estimates of selfing rates ($\hat{s}$) were not significantly different from zero in any of the studied populations of the two species, regardless of the method (*ML* and $\hat{g}_2$) used for calculation (results not shown).

At the species level, *Pistacia* displayed neither significantly higher gene diversity (average H_{e_null} = 0.706 versus 0.706) nor less inbreeding (F_{IS} = 0.014 versus 0.011) than *Myrtus* populations (Table 2). Nevertheless, Myrtus showed significantly higher levels of individual homozygosity (*HL* = 0.428 versus 0.296) and genetic differentiation (F_{ST} = 0.044 versus 0.013) among populations than *Pistacia*. Also, averaged pairwise F_{ST} values were significant in all *Myrtus* populations, while in *Pistacia*, population genetic differentiation increased progressively as expected, from *C* to *FI* populations, although only values for the *FI* populations were statistically significant.

Table 2. Standard genetic diversity measures for *Myrtus communis* and *Pistacia lentiscus* in continuous (*C*), fragmented-connected (*FC*) and fragmented-isolated (*FI*) populations.

	N	A	A_e	H_{e_null}	HL	F_{IS}	F_{ST}
Myrtus communis							
C							
AZN	30	5.60 (± 0.60)	3.110 (± 0.386)	0.694 (± 0.119)	0.483 (± 0.174)	0.016 (± 0.018)	**0.050** (0.029 - 0.077)
DHY	30	6.90 (± 0.77)	3.415 (± 0.363)	0.725 (± 0.080)	0.408 (± 0.157)	0.008 (± 0.010)	**0.048** (0.025 - 0.080)
FC							
BNC	30	6.00 (± 0.54)	3.340 (± 0.336)	0.720 (± 0.089)	0.426 (± 0.110)	0.008 (± 0.010)	**0.039** (0.017 - 0.065)
SJV	29	6.80 (± 1.00)	3.891 (± 0.552)	0.717 (± 0.165)	0.431 (± 0.139)	0.008 (± 0.012)	**0.033** (0.015 - 0.055)
FI							
CRB	28	6.30 (± 0.92)	3.624 (± 0.593)	0.692 (± 0.196)	0.376 (± 0.172)	0.011 (± 0.013)	**0.045** (0.018 - 0.082)
VIS	29	5.80 (± 0.76)	3.192 (± 0.359)	0.685 (± 0.187)	0.446 (± 0.172)	0.014 (± 0.017)	**0.050** (0.024 - 0.081)
Average	29.3 (± 0.8)	6.23 (± 0.53)	3.429 (± 0.289)	0.706 (± 0.017)	0.428 (± 0.036)	0.011 (± 0.003)	**0.044** (0.031 – 0.062)
Pistacia lentiscus							
C							
AZN	38	8.57 (± 2.01)	4.538 (± 1.214)	0.721 (± 0.174)	0.321 (± 0.202)	0.021 (± 0.021)	0.010 (-0.004 - 0.025)
DHY	40	7.86 (± 1.64)	4.173 (± 0.873)	0.715 (± 0.169)	0.289 (± 0.173)	0.012 (± 0.012)	0.006 (-0.005 - 0.021)
FC							
BNC	36	8.71 (± 1.99)	4.263 (± 1.012)	0.699 (± 0.210)	0.332 (± 0.165)	0.011 (± 0.015)	0.014 (-0.0001 - 0.035)
SJV	36	7.71 (± 1.30)	4.033 (± 0.741)	0.732 (± 0.145)	0.340 (± 0.174)	0.023 (± 0.020)	0.010 (-0.002 - 0.023)
FI							
CRB	40	8.14 (± 1.70)	4.140 (± 0.789)	0.704 (± 0.193)	0.252 (± 0.146)	0.007 (± 0.009)	**0.018** (0.001 - 0.038)
VIS	40	7.43 (± 1.74)	3.836 (± 0.947)	0.667 (± 0.202)	0.243 (± 0.151)	0.008 (± 0.009)	**0.017** (0.001 - 0.037)
Average	38.3 (± 2.0)	8.07 (± 0.50)	4.164 (± 0.235)	0.706 (± 0.023)	0.296 (± 0.042)	0.014 (± 0.007)	**0.013** (0.005 – 0.021)
Wilcoxon's test results for difference between species		-	-	Z = 0.105 *P* = 0.917	Z = 2.201 *P* = 0.028	Z = 0.524 *P* = 0.600	Z = 2.201 *P* = 0.028

Number of sampled individuals (*N*), mean number of alleles per locus (*A*), effective number of alleles per locus (A_e), gene diversity corrected for the presence of null alleles (H_{e_null}), homozygosity by loci index (*HL*), inbreeding coefficient (F_{IS}) and average values of pairwise genetic differentiation (F_{ST}). Values in parentheses are ± 1 SD except for F_{ST} where they show 95% confidence intervals. Significant F_{ST} values in bold when the 95% confidence intervals do not overlap zero.

Effective population size and genetic bottlenecks

Overall, the average *Θ* values (±SD) provided by Migrate-N were 1.64 ± 0.33 for *Pistacia* and 1.46 ± 0.23 for *Myrtus*. Interestingly, the *FI* populations in the two species had the lowest values of *Θ*, although their 95% confidence intervals overlapped with those of other populations (Fig. 2).

The program Bottleneck detected significant reductions in effective population size in all *FI* and *FC* populations of *Myrtus* under the IAM and TPM models of microsatellite evolution (Table S2). The two *FI* populations in *Pistacia* displayed signs of genetic bottleneck but only under the IAM model. As expected, we detected no signs of genetic bottleneck in any *C* population of the study species. The strict SMM model provided no evidence of genetic bottlenecks in any population of our study.

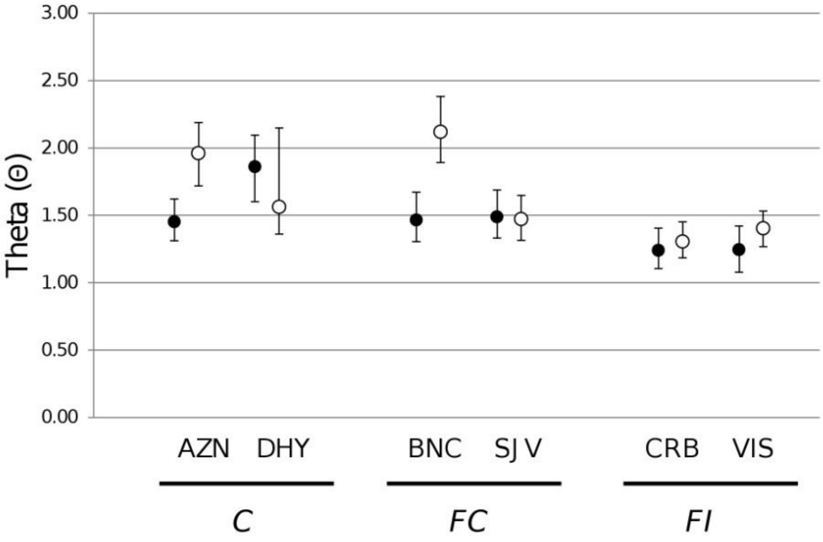

Figure 2. Mutation-scaled effective population size (*Θ*) estimates (and the associated 95% confidence intervals) inferred by Migrate-N for *Pistacia lentiscus* and *Myrtus communis* in the six study populations.

Spatial genetic structure

The Bayesian clustering algorithm of Structure gave contrasting results for the two study species. For *Pistacia* the software consistently showed that K = 1 was the optimal solution (Fig. S1a), and revealed no signs of genetic structuring in the populations. In contrast, for *Myrtus* the optimal solution was K = 2 (Fig. S1b, c), with individuals from the same population showing a large proportion of corresponding membership (from 79% in population CRB to 95% in DHY; Fig. 3). A geographical pattern was also evident in *Myrtus*, with the spatially close populations AZN and BNC (see Fig. 1) belonging to the same genetic cluster. A subtle genetic structure was also evident at K = 4 (Fig. 3, Fig. S1b, c), with individuals from the populations VIS and DHY appearing as single distinct genetic units, and those from SJV and CRB clustering together as another genetic unit.

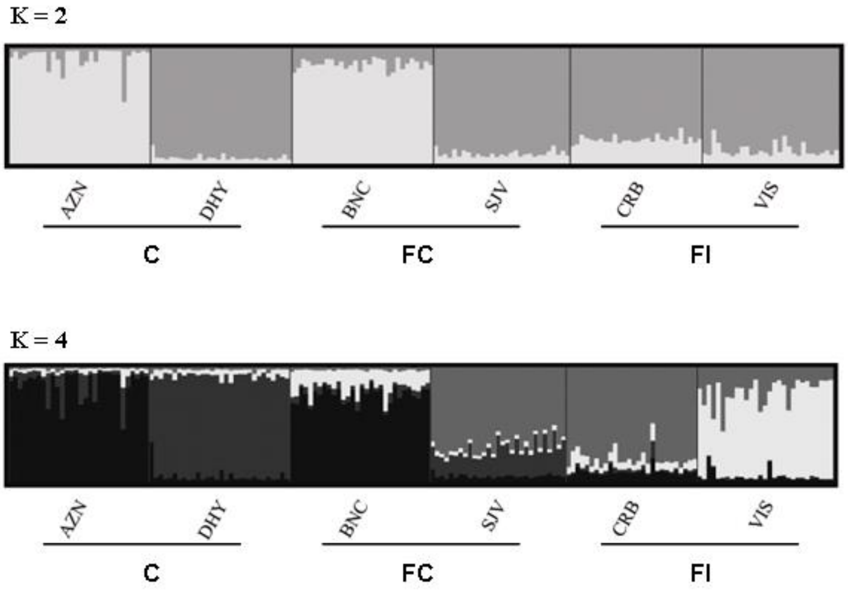

Figure 3. Genetic structure of *Myrtus communis* populations inferred by Structure. Each vertical bar represents an individual and each shade a distinct gene pool in each of the K-inferred clusters. Population codes as in Table 1.

Overall, correlogram analyses revealed low or absent spatial genetic structure within populations in the two study species in the three landscape contexts. In both species, when comparing the three types of populations, within-population spatial genetic structure was unexpectedly higher in *C* populations than in *FC* and *FI* populations (Table 3). In *Myrtus*, the regression slope of the kinship coefficients against the (log) distance was significant in the *C* populations (b_{log} = 0.014; P = 0.007) and only marginally significant in the *FC* populations (b_{log} = 0.010; P = 0.065). In *Pistacia*, the regression slope was only marginally significant in *C* populations (b_{log} = 0.009; P = 0.059) and not significant in the others. Although in general the values were low, we found higher S_p values in *Myrtus* (mean value of the three landscape contexts ± SD = 0.010 ± 0.004) than in *Pistacia* (0.005 ± 0.004), indicating slightly stronger patterns of within-population spatial genetic structure in *Myrtus* populations (Table 3, Fig. S2).

Table 3. Estimates of within-population spatial genetic structure for continuous (*C*), fragmented-connected (*FC*) and fragmented-isolated (*FI*) populations in *Myrtus communis* and *Pistacia lentiscus*.

Genetic structure parameters	*Myrtus communis*			*Pistacia lentiscus*		
	C	*FC*	*FI*	*C*	*FC*	*FI*
b_{log}	-0.0141*	-0.0101†	-0.0059	-0.0088†	-0.0057	-0.0001
$F_{(1)}$	0.0195*	0.0061	0.0102	0.0048	0.0121	-0.0032
S_p	0.0144	0.0102	0.0060	0.0088	0.0058	0.0001

b_{log} is the slope of the regression of kinship coefficients on the (log) spatial distance between individuals; $F_{(1)}$ are the pair-wise kinship estimates between individuals for the first distance interval, and S_p is the intensity statistic from Vekemans & Hardy (2004).

$^{\dagger}P < 0.1$, $^{*}P < 0.05$.

DISCUSSION

Comparative studies evaluating disparate spatial habitat configurations could provide insights on how fragmentation drives species genetic variation and structure, and are, therefore, relevant in assessing the consequences of the anthropogenic disturbance of natural habitats (Aparicio *et al.* 2012). Our study of two species within three contrasting landscape contexts analyzed the interplay between landscape fragmentation and species' response driving genetic variation.

Unexpectedly, we observed relatively weak effects of the landscape context on the population genetic diversity and structure of both study species. Conversely, our data pointed to a notable population-specific local response. For instance, while in the fragmented-isolated population VIS (a small and intensively managed stone pine woodland used for horse riding that suffered occasional fires) we found a negative genetic impact on both species, in the other fragmented-isolated population CRB (a small holm oak stand devoted only to small game hunting) both species had diversity levels that were comparable to the physically connected woodlands (*C* and *FC* population types). Our data suggest that species' genetic patterns depend far more on local factors affecting demographic processes than on the landscape configuration itself (Kramer *et al.* 2008).

Overall, we found that *Myrtus* and *Pistacia* populations differed in some parameters of genetic diversity, and at least some of these results could be due to differences of species' life-history traits. Both species have contrasted pollen dispersal model and mating system, which are functional traits intimately linked to genetic variation (Duminil *et al.* 2007). And, although in our design it is not possible to separate the effects of the two traits (the pollen dispersal mode and the ability to self), our results were consistent with theoretical expectations regarding the mating systems of the study species (Duminil *et al.* 2009; Vranckx *et al.* 2012). When assessing genetic diversity at the individual level (*HL*), we found that *Myrtus* had a significantly larger proportion of homozygous genotypes in all population types than *Pistacia*. This is due to the fact that in the calculation of *HL*, the loci with the stronger contribution to the homozygosity index are those with more alleles and more evenly frequent, thus penalizing those loci carrying rare, infrequent alleles (Aparicio *et al*. 2006). The analyzed loci in *Myrtus* had fewer alleles and were less evenly frequent than those of *Pistacia* (see A and A_e values in Table 2). A higher ability for among-population pollen dispersal in *Pistacia* might homogenize better the allele frequencies and dilute the observable effects of genetic drift at the adult stage.

In contrast, population type did not determine standard measures of inbreeding coefficient or selfing (F_{IS}, $\hat{s}$), which were very low and not significantly different from zero in all populations of both species. Although counter-intuitive, especially for the self-compatible *Myrtus*, this result was not unexpected because recent meta-analyses have shown that inbreeding coefficients in adult plant populations do not respond directly to either plant mating system or habitat fragmentation (Aguilar *et al.* 2008; Vranckx *et al.* 2012). The gradual purging of the most homozygous genotypes during the earliest life stages (David *et al.* 2007; Dornier & Cheptou 2012) from populations guarantees that the inbreeding coefficient in the adult stage is not significantly different from zero. This argument is relevant because selfing rates obtained for progeny in *Myrtus* populations in the same study area ranged from 0.28 to 0.87 (data recalculated from González-Varo *et al.* 2010). Indeed, high rates of inbred progeny in *Myrtus* are known to reflect significantly lower fitness (in terms of germination rates and seedling survival; González-Varo *et al.* 2010, 2012).

Myrtus populations were apparently more susceptible to the effect of genetic drift since all the populations were genetically more differentiated (F_{ST}) and displayed a stronger pattern of spatial genetic structure, as assessed through clustering analysis. This pattern is in accordance with previous studies at larger spatial scales (Aparicio *et al.* 2012). Interestingly, even in *Pistacia*, a species that harbors a set of functional traits rendering it more resilient to the effects of fragmentation (Hamrick 2004), the two fragmented-isolated populations displayed higher and significant values of genetic differentiation than populations in the other landscape contexts.

The effects of fragmentation can also be reflected in parameters related to the effective population size. The lowest estimates of mutation-scaled effective population size for both species were found in the fragmented-isolated populations. According to this result, the *FI* populations of both species seemed to have undergone a genetic bottleneck in the recent past (generations), at least when considering an infinite allele model of microsatellite evolution. The fact that the battery of microsatellite loci used in this study consisted mostly in dinucleotide repeats, interrupted and compound microsatellite loci (see Albaladejo *et al.* 2008, 2010) makes them more prone to follow a multiple-step mutation pattern (like the IAM) rather than a single-step mutation model (see Cristescu *et al.* 2010) as is frequently found in the literature (*e.g.* Dubreuil *et al.* 2010).

It is worth noting that while the *FC* populations of *Myrtus* also displayed signs of being bottlenecked (assuming either the IAM or the TPM models), those of *Pistacia* did not. Both species share the same guild of seed dispersers and have similar long life spans; therefore these differences could be mostly attributable to their respective mating and pollen-dispersal systems (Aguilar *et al.* 2006). González-Varo *et al.* (2010, 2012) have recently shown that selfing in wild *Myrtus* populations is frequent, ensuring seed production for the next season at the cost of reducing genetic diversity in progeny and the negative consequences for fitness (discussed above). Furthermore, the progeny composition of *Myrtus* plants highly depends on the local pollinator environment, rather than merely on habitat fragmentation (González-Varo *et al.* 2009). In contrast, Albaladejo *et al.* (2012) detected extensive pollen flow in *Pistacia*, which enhances resilience to the genetic consequences of physical isolation.

Overall, patterns of within-population genetic structure were low in the two species and similar to those reported for animal-dispersed species (*e.g.* S_p = 0.0088, compiled in Vekemans & Hardy 2004). However, some patterns could be observed that deserve explanation. Contrary to expectations, both species displayed stronger patterns of within-population spatial genetic structure in the continuous than in the fragmented populations. Spatial genetic structure in plant populations is shaped by many factors, including seed dispersal (Hardy *et al.* 2006; Jordano 2010). For instance, some studies have documented that habitat fragmentation affects the composition and behaviour of the frugivorous bird community (Cordeiro & Howe 2003; Rey & Alcántara 2014) leading to the creation of local patterns of spatial genetic autocorrelation (Voigt *et al*. 2009). However, in our study area, small woodlands retain a dispersal guild of almost the same abundance and composition as in large forest patches (Gonzalez-Varo *et al.* 2010). Thus, the observed patterns of within-population spatial genetic structure seem to depend more on other factors that also condition or enhance these patterns, such as topographic heterogeneity or plant density, particularly if seed dispersal is limited (Dubreuil *et al.* 2010).

Albeit low, within-population spatial genetic structure in *Myrtus* was higher than in *Pistacia*, which could be explained by the mating and pollination systems (Vekemans & Hardy 2004;

Hardy *et al.* 2006). In self-incompatible species, both seeds and pollen contributed to the overall spatial gene dispersal, while in self-compatible species a fraction of the pollen is retained in the same plant where it originated. Also, insect-pollinated plants are frequently associated with higher S_p values than wind-pollinated plants (0.0171 versus 0.0064, averaged by Vekemans & Hardy 2004). However, the fact that we dealt with two species displaying a combination of those traits (self-compatible and insect-pollinated versus dioecious and wind-pollinated) did not allow us to discriminate between these non-mutually exclusive factors.

Conclusions

This study highlights how plant species genetic variability is affected differently to habitat fragmentation, and reveals that the landscape configuration (fragment size and/or isolation) per se might not determine the species' genetic patterns. In fact, our results can be added to a growing body of literature (e.g. González-Varo *et al.* 2011; Thornton *et al.* 2012) suggesting that other factors besides the landscape configuration (e.g. historical connectivity, habitat quality for recruitment, plant density, native fauna) are determinant in assessing the consequences of fragmentation. Further, although the effects are variable, our study also shows that some consequences of extreme habitat fragmentation might be detectable even in adult populations of plant species that have a set of life-history traits well-suited to buffer the negative impacts of fragmentation (Hamrick 2004). Overall, despite the inherent difficulty, we emphasize the importance of controlling the spatial scales at which processes such as gene flow are effective when assessing how global threats affect biodiversity (Kramer *et al.* 2008; Bacles & Jump 2011). Studies like this should allow us to contextualize more detailed conservation action plans.

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SUPPLEMENTARY INFORMATION

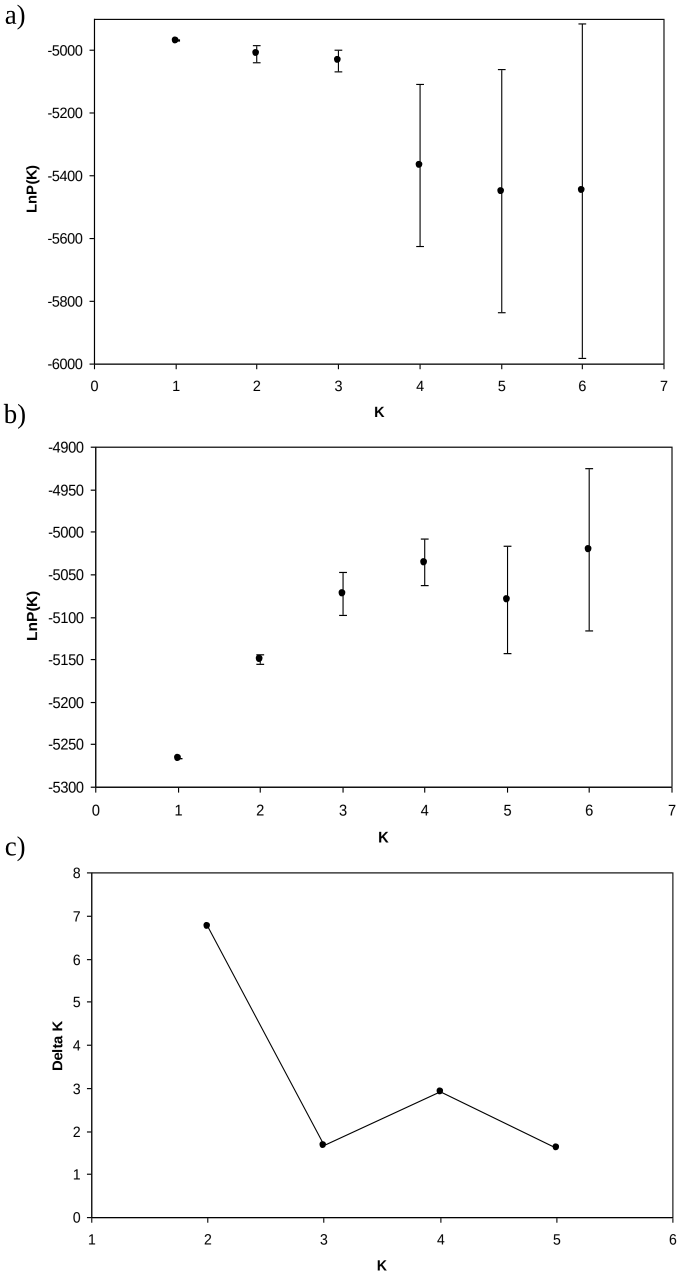

Figure S1. a) Mean value of the loglikelihood [LnP(*K*)] (±SD) over 5 runs for each *K* (nº. of genetic clusters) for *Pistacia lentiscus* data. b) Mean value of LnP(*K*) (±SD) over 5 runs for each *K* for *Myrtus communis* data. c) Delta *K* values (following Evanno *et al*. 2005) as a function of *K* for *Myrtus communis*.

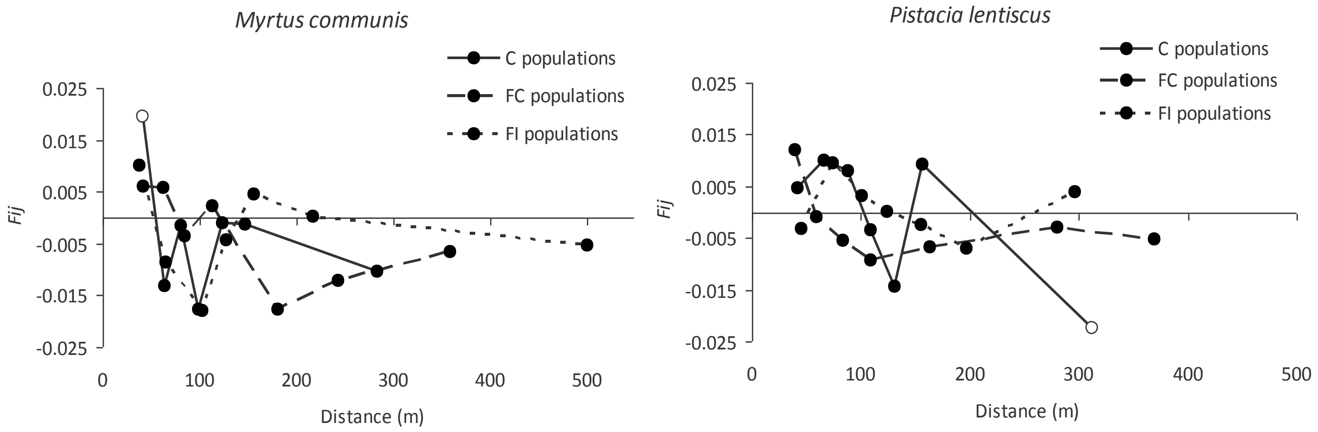

Figure S2. Spatial correlograms for *Myrtus communis* and *Pistacia lentiscus* populations. Empty symbols are kinship coefficients significantly ($P < 0.05$) different from the null hypothesis of random spatial structure based on permutations tests (see text for details).

Table S1. Average null allele frequencies of the amplified loci for *Myrtus communis* and *Pistacia lentiscus.*

Species/Locus	Average null allele frequency
Myrtus communis	
Myrcom2	0.273
Myrcom3	0.098
Myrcom4	0.190
Myrcom5	0.044
Myrcom6	0.082
Myrcom7	0.034
Myrcom8	0.141
Myrcom9	0.062
Myrcom10	0.172
Myrcom11	0.054
Pistacia lentiscus	
Pislen21	0.082
Pislen114	0.038
Pislen333	0.048
Pislen501	0.062
Pislen510	0.038
Pislen526	0.068
PislenR05	0.142

Table S2. Probability values for a significant excess of heterozygosity compared to simulated values expected under mutation-drift equilibrium with three models of microsatellite evolution. Significant values in bold. Population types: *C* (continuous), *FC* (fragmented-connected), and *FI* (fragmented-isolated) populations. IAM, infinite allele model; TPM, two phase mutation model; SMM, stepwise mutation model.

Population	*Myrtus communis*			*Pistacia lentiscus*		
	IAM	TPM	SMM	IAM	TPM	SMM
C						
AZN	0.080	0.161	0.862	0.234	0.469	0.766
DHY	0.065	0.461	0.998	0.148	0.406	0.945
FC						
BNC	**0.042**	**0.042**	0.991	0.531	0.656	0.945
SJV	**0.001**	**0.042**	0.947	0.148	0.289	0.98
FI						
CRB	**0.005**	**0.024**	0.976	**0.039**	0.188	0.980
VIS	**0.002**	**0.019**	0.986	**0.027**	0.234	0.980

2

Habitat fragmentation effects on plant reproductive success

Nora S, Aparicio A, Albaladejo RG. Habitat fragmentation effects on plant reproductive success: The response of two common shrub species (*Myrtus communis* L. and *Pistacia lentiscus* L.).

Unpublished Manuscript.

Efectos de la fragmentación del hábitat en éxito reproductivo de las plantas

Resumen

Globalmente, la fragmentación del hábitat tiene un efecto general negativo sobre la polinización y la reproducción de las plantas. Aún así, las respuestas de las especies a la pérdida y fragmentación del hábitat son a menudo variables y sigue existiendo un debate sobre las causas inmediatas de la disminución de las especies en los paisajes fragmentados. En este estudio comparamos el éxito reproductivo de dos especies comunes de arbustos mediterráneos (*Myrtus communis* y *Pistacia lentiscus*) que conviven en poblaciones las seleccionadas y que representan tres paisajes con diferente grado de fragmentación. Utilizando modelos de ecuaciones estructurales evaluamos cómo la fragmentación del hábitat afecta a múltiples factores ecológicos y genéticos que de un modo directo o indirecto determinan el éxito reproductivo de estas especies. En *Myrtus*, detectamos que la influencia de los componentes ecológicos son determinantes para el éxito reproductivo a lo largo del gradiente de fragmentación. Se encontró un efecto positivo de la sincronía floral en la tasa de fructificación en poblaciones fragmentadas y aisladas, y un efecto negativo de la distancia al borde del fragmento sobre tasa de producción de semillas en poblaciones fragmentadas y conectadas. Además, las tasas de visita de los polinizadores en *Myrtus* fueron más elevadas en poblaciones fragmentadas y están positivamente relacionadas con las tasas de fructificación y producción de semillas. En *Pistacia*, encontramos un efecto negativo directo de la fragmentación del hábitat en la proporción de semillas viables. La densidad de plantas conspecificas alrededor de las plantas maternas estuvo positivamente relacionada con su tasa de fructificación en las poblaciones fragmentadas y conectadas. Este estudio pone de relieve el complejo escenario que gira en torno a la fragmentación del hábitat donde no hay factores únicos involucrados sino la interacción entre muchos factores que son los responsables finales del éxito reproductivo de las plantas.

Palabras clave: Éxito reproductivo; modelo de ecuaciones estructurales; *Myrtus communis*; *Pistacia lentiscus*.

Abstract

Worldwide habitat loss and fragmentation has an overall large and negative effect on pollination and plant reproduction. Still, species responses to habitat loss and fragmentation are often variable and a discussion still persists on the immediate causes of specie declines on fragmented landscapes. In this study we compared the reproductive success of two common Mediterranean shrub species (*Myrtus communis* and *Pistacia lentiscus*) with contrasting breeding and pollen dispersal systems that co-occur in populations within three contrasting landscapes contexts: continuous, fragmented-connected and fragmented-isolated populations. Using the integrating approach SEM, we also evaluated how habitat fragmentation affects and alters multiple ecological and genetic factors that both direct and indirectly determine plant reproductive success of *Myrtus* and *Pistacia*. Rather than a direct effect on *Myrtus*' reproductive success, we detected the distinct influence of ecological components are more determinant to the reproductive success along the fragmentation gradient. We found a positive effect of flowering synchrony on plant's fruit set on fragmented and isolated populations and a negative effect of the distance to the fragment edge on seed set on fragmented and connected populations. Moreover, pollinators' richness and visitation rates were higher in fragmented populations, being the latter also positively related to fruit and seed set. Within this pattern, the only exception was the fragmented-isolated population CRB that had the lowest richness of pollinators due to a massive abundance of honeybees (*Apis mellifera*) and the presence of honey hives within their immediate vicinity. In *Pistacia*, we found a negative effect of habitat fragmentation on seed viability, as fragmented-isolated population presented significantly the lowest percentages. Density of conspecific plants around maternal plants is positively related to fruit set, i.e. maternal plants with more males in their surroundings will produce more fruits in Pistacia fragmented-connected populations. This study highlights the complex scenario that revolve around habitat fragmentation where there are no single factors involved but the interplay between many factors is responsible of the reproductive success of plants.

Keywords: *Myrtus communis*; *Pistacia lentiscus*; reproductive success; SEM analyses.

INTRODUCTION

Pollination and plant reproduction are fundamental processes in the ecology, conservation and management of flowering plant populations (Richards 1997; Leimu *et al*. 2010). A successful pollination and the subsequent reproductive output define plant’s maximum recruitment potential for the next generation in that reproductive event (Wilcock & Neiland 2002). Due to the importance of these processes for long-term persistence of populations, it is essential to understand their promoters and susceptibilities for natural plant populations.

Nowadays, habitat loss and fragmentation represents the major threat to biodiversity of terrestrial ecosystems (Fahrig 2003; Didham *et al.* 2012; Haddad *et al.* 2015). Worldwide most plant populations have been exposed to some degree of habitat loss and fragmentation through agricultural intensification and urban expansion (Saunders *et al.* 1991). Habitat fragmentation can affect plant reproductive success (Aguilar *et al.* 2006; Jacquemyn *et al.* 2012). Reproductive disruption or alteration due to habitat fragmentation can be caused by directly affecting individual plant performance (Saunders *et al.* 1991). For example, changes in environmental conditions (e.g. light, water and nutrients availability) can compromise plant resources availability to successfully conduct flower production, seed development or fruit maturation (Saunders *et al.* 1991). Additionally, habitat fragmentation can compromise plant reproductive output indirectly (Valdés & García 2011) by affecting plant ability for successful and effective pollination through both insufficient pollen quantity and/or quality (self vs. cross-pollination) deposited on stigmas, leading to a low ovule fertilization rate, seed production or less vigorous offspring (Aizen & Feinsinger 1994a; Knight *et al.* 2005; Aizen & Harder 2007). In fragmented habitats, inefficient pollen dispersal could occur due to changes in the population size or the spatial distribution of individuals (Wilcock & Neiland 2002) that may limit the number of pollen donors and compatible pollen (Wolf & Harrison 2001; Aizen & Harder 2007).

Animal-pollinated species are also susceptible of pollination failure due to alterations in pollinator abundance and diversity (Aizen & Feinsinger 2003; Winfree *et al.* 2009, 2011). Many factors can affect pollinator populations in fragmented habitats, such as the reduced attractiveness of forest fragments due to scarce floral rewards (Steffan-Dewenter & Tscharntke 1999; Cheptou & Avendano 2006), lack of suitable habitats for nesting (Steffan-Dewenter *et al.*

2006), diffused pesticides from the matrix into the fragments or the invasion of competitors or predators (Rathcke & Jules 1993; Aizen & Feinsinger 1994b). Habitat fragmentation may even restrict the movements of pollinators (Tewksbury *et al.* 2002; Öckinger *et al.* 2009) or change their behaviour because of differing foraging contexts (Aguilar *et al.* 2006; Kremen *et al.* 2007; Winfree *et al.* 2011), ultimately causing changes in their patterns of visitation.

The detrimental effects of habitat fragmentation on plant reproductive success have been widely documented and there is a general agreement that these effects can ultimately compromise plant persistence (reviewed in Aguilar *et al.* 2006). However, species responses to habitat loss and fragmentation are often variable (e.g. Aizen & Feinsinger 1994a; Ewers & Didham 2006; Kramer *et al.* 2008) and a core discussion persists about the immediate causes of species declines on fragmented landscapes (Fischer & Lindenmayer 2007; Didham *et al.* 2012; Fahrig 2013; Haddad *et al.* 2015). For instance, controversy often arises whether species responses to fragmentation are ruled by general patterns or are purely idiosyncratic (Haila 2002; Didham *et al.* 2012). Unquestionably, efforts to understand how ecological systems are affected by habitat loss and fragmentation are a challenging assignment.

In this study, we used an integrative approach to evaluate how habitat fragmentation affects the reproductive success of *Myrtus communis* L. and *Pistacia lentiscus* L. (*Myrtus* and *Pistacia*, hereafter) populations. These two species are abundant and dominant sclerophyllous shrubs of the Mediterranean woodlands and share important life-history traits (e.g. both produce endozoochorus fleshy fruits), but have contrasted inbreeding and pollen dispersal systems. *Myrtus* is a hermaphroditic, self-compatible and insect-pollinated shrub, while *Pistacia* is a wind-pollinated, dioecious shrub and therefore an obligate outcrosser. Within a regional mosaic of forest fragments in southwest Spain we selected three contrasting landscape contexts (continuous, fragmented-connected and fragmented-isolated populations) and we closely monitored selected *Myrtus* and *Pistacia* plants from the flowering period to fructification to evaluate their reproductive output (here measured as fruit and seed set, number of produced seeds and seed viability). With this comparative study approach we attempted to minimize the differences in spatial distribution and management history of the sampled populations (Aparicio *et al.* 2012). We also characterized each individual plant through a set of ecological and genetic variables with recognized influence into the reproductive output to assess whether different life history traits are reflected on species' reproductive success or common patterns can be detected across species regardless of their mutual differences. Specifically, we aimed to address three core questions: i) Which ecological components are more determinant to the reproductive success of *Myrtus* and *Pistacia*?; ii) Do these relationships change under different fragmentation contexts?; and iii) Are habitat fragmentation effects consistent across species with contrasted breeding and pollen dispersal systems? We expected the anemophilous and dioecious species, *Pistacia*, to be more resilent to the detrimental effects of habitat fragmentation. Moreover, we also expected that both flowering synchrony and conspecif neighbourhood density to play an important role in shaping reproductive success of both species. By addressing these questions we expected to contribute to a better knowledge on habitat fragmentation effects on plant's reproductive success.

MATERIAL AND METHODS

Study species

Myrtus communis L. is a self-compatible shrub with a density-dependent mixed-mating system (González-Varo *et al.* 2009a). This species is the sole representative of the Myrtaceae family in the flora of the Mediterranean Basin. Its hermaphrodite flowers are white, with one style and multiple stamens, and open in the early summer (from mid-June to early July). In the study region, pollination is mostly conducted by hymenopterans (74-93% of visits; González-Varo *et al*. 2009b) and dipterans. Fruits are a multi-seeded berries (mean ± SD = 4.6 ± 2.8 seeds; González-Varo *et al.* 2012), dark-blue when mature. The dispersal season begins at mid-October or early November ending between March and early April (González-Varo 2010). Their main dispersers are passerine birds (mostly Sylviidae and Turdidae) and granivorous passerines (mainly *Carduelis chloris*) are known to be the main seed predators in the study area (González-Varo 2010). *Myrtus* seeds are non-dormant (Baskin & Baskin 1998), and germination occurs few weeks after dispersal.

Pistacia lentiscus L. is a common Mediterranean evergreen, sclerophyllous, dioecious shrub (Verdú & García-Fayos 1998). This species is one of the few representatives of the Anacardiaceae family in the flora of the Mediterranean Basin. This wind-pollinated species blooms massively between mid-March to late April (Jordano 1989), showing an extensive pollen dispersal ability (Albaladejo *et al.* 2009, 2012). Male flowers are grouped in inflorescences of 8-10 flowers and female flowers grouped in inflorescences from 4 to 21 flowers. After pollination during spring, fruit (one-seeded drupes; Verdú & García-Fayos 1998) development in *Pistacia* is delayed until late summer. This long period of fruit development is common within the genus *Pistacia* and is caused by a delay in the singamy (Grundwag 1976). During this time, *Pistacia* fruits change color from white at the initial stage to red (intermediate stage) and black when they are fully matured (Jordano 1988). Moreover, black matured fruits might be seedless or with empty seeds as a result of ontogenetic processes that lead to parthenocarpy and abortion (Gillaspy *et al.* 1993). The parthenocarpic and aborted fruits in *Pistacia* have been widely studied and the causes of these deceptive fruits are mainly attributed to resource competition in a limited environment (Jordano 1988), or to reduce seed predation rates by chalcidoid wasps (Verdú & García-Fayos 1998) and by granivorous birds (Verdú & García-Fayos 2001). After pollination, the zygote remains dormant during several weeks (Grundwag 1976) and fruit final size is only reached in late summer. Fruit colour is strongly associated with seed viability: black fruits may contain viable seeds, whereas white and red fruits contain nonviable seeds (owing to abortion or parthenocarpy; see Jordano 1988, 1989). In the study region, seed dispersal is conducted mostly by Sylviidae and Turdidae (blackcaps, thrushes and robins) from September to March (Herrera 1984; Jordano 1989; Parejo-Farnés, personal communication). A temporary seed bank is formed and germination occurs within a year (García-Fayos & Verdú 1998).

Study area and selected patches

Our study was conducted in the lower catchment area of the Guadalquivir River in western Andalusia (S Spain). This fertile region, currently dominated by intensive monocultures (e.g. olives, cereals, sunflower, cotton, vineyards and diverse orchards), bears a long history of anthropogenic management and deforestation (Martín *et al.* 2013) and currently remnants of woodland forests cover less than 1% of their potential area (Aparicio 2008).

We conducted this study in six patches out of the 114 forest patches where the two study species coexist in the study area (for a detailed description of the study system selection see Nora *et al.* 2015). Four out of these fragments were classified either as ‘fragmented and connected’ (*FC*) or ‘fragmented and isolated’ (*FI*) according to the amount of suitable forest area contained in 1 km-radius buffer around the centroid of the patch. This threshold was established based on evidences of pollen dispersal on the study species or similar ones, occurring mostly below this distance, and was also supported by detectable signs of genetic bottlenecks in the adult populations of *Myrtus* and *Pistacia* within the *FI* patches (Nora *et al.* 2015). The two remaining study patches were continuous populations (*C*) serving as a control situation which consisted in 5 ha rectangular plots within two large pine-stone woodlands (above 100 ha) where the two study species were very common. We thus established the following gradient of habitat fragmentation: *C* < *FC* < *FI*.

Sampling design and maternal plant reproductive output

Within each forest patch, we selected and marked 30 *Myrtus* adult plants and 40 *Pistacia* plants (20 male and 20 female plants) (n_{total} = 420 plants). Individual plants were selected *a priori* in order to capture all the local heterogeneity available in each forest patch (mainly conspecific density and distance to the patch edge) which may have a reflection in mating system parameters at individual plant level (Franceschinelli & Bawa 2000; de Lucas *et al.* 2008; Chapter 3). Locations of all plants were recorded with a GPS unit. Previous to the flowering season (March and June for *Pistacia* and *Myrtus*, respectively) we marked five branches in each selected plant, each branch harbouring 10-15 flowers (mean ± SD = 11.86 ± 3.65 flowers in *Myrtus*) or inflorescences (12.66 ± 3.55 and 12.93 ± 4.50 female and male inflorescences in *Pistacia*). We monitored each study plant weekly during the flowering period, which we considered to be defined as the period between the onset of anthesis of the first and the senescence of the last flower. We considered a *Pistacia* female flower as senesced when the stigmas dried out and in *Myrtus* when the flowers shed the petals. We monitored *Pistacia* male plants until the end of the flowering period (n = 7756 male inflorescences) and *Myrtus* and *Pistacia* maternal plants until fruit maturation ($n_{Pistacia}$ = 7848 female inflorescences and n_{Myrtus} = 10674 flowers).

We assessed reproductive output by estimating 'fruit set' as the ratio of the final number of mature fruits related to the initial number of marked flowers. In *Pistacia*, the initial number of flowers was estimated as the number of final fruits plus the number of scars in the inflorescence axis left by both unpollinated flowers and fruits fallen by abscission (Verdú & García-Fayos 1998). For *Pistacia* we also evaluated 'seed viability' in each maternal plant by collecting 257.9 (± 139.9) fully mature black fruits per plant and checking whether they contained a viable seed inside through the flotation/sink method (Albaladejo *et al.* 2009). Apart from fruit set, in *Myrtus* we also estimated the 'number of seeds per fruit' and the 'seed set'. The number of seeds per fruit was averaged after dissecting 20 fruits per plant. Seed set (number of mature seeds per fruit/number of initially ovules per flower) was estimated by dissecting five fruits per plant under magnifying glasses and recording the number of ovules, aborted and mature seeds. The total number of unfertilized ovules plus the aborted seeds and the mature seeds found within a fruit was assumed to be equal to the total number of ovules initially present in the ovary.

Myrtus pollinators' observations

Habitat fragmentation can cause an alteration in pollinator abundance and diversity with important consequences to pollination success in animal-pollinated species (Aizen & Feinsinger 2003; Winfree *et al.* 2009, 2011). Therefore, in this study it was essential to survey the *Myrtus* pollinator's assemblage composition and the frequency of visits to *Myrtus* plants in the same flowering season. We conducted five minutes observation censuses over random individual plants at their flowering peak. All observations were made in good weather on warm days from 10:00 to 14:00 h, the maximum pollinator activity period. In the field, we identified insect visitors as morpho-species, which included insects of similar morphology and behaviour in the flowers (see González-Varo *et al.* 2009b). Insects were identified to the species or genus level. We performed censuses 1 m apart from the shrub canopy and at least in two different days in each population. The average time spent during the censuses was 6.08 h (± 2.27) per population. Due to the high number of flowers contacted by some visitors, visitation rates were recorded at plant level. We estimated the 'visitation rate' to individual plants (V/H; number of visits/5 min.) and the 'relative frequency of visits' by each insect morpho-species (percentage of visits).

Maternal plant predictor variables

As our main goal in this study was to evaluate in an integrative approach the influence of habitat fragmentation on multiple ecological interactions and plant reproduction of *Myrtus* and *Pistacia*, we selected a set of ecological and genetic factors that we anticipated to take part into the dynamics of these plants reproductive process:

Distance to patch edge

The habitat that is under the influence of the patch's boundary (i.e. patch edge, Murcia 1995) is a transition zone where the forest habitat and the surrounding matrix interact. Plants under the influence of edges can have primary responses (e.g. physical damage, microclimatic and/or geochemical alterations) and secondary (e.g. vegetative growth, mortality alterations) responses to fragmentation that could lead to changes in their reproduction output (Harper *et al.* 2005; Hagen *et al*. 2012). We computed the nearest distance of each plant to the edge of the forest patch ('distance to edge', hereafter) with ArcGis 9.0, for each maternal plant, using the geographic location of each maternal plant and the digital cartography of the study area.

Conspecific neighbourhood density

The density of conspecifics around a focal maternal plant, characterized by the spacing between conspecific individuals (Kunin 1997), may also influence plant reproduction success (Feinsinger *et al.* 1991; Kunin 1997; Wilcock & Neiland 2002). For example, studies with animal-pollinated species have observed that pollinators may increase inter-plant visits in high plant density areas (Feinsinger *et al.* 1991, Kunin 1993), whereas in isolated areas (plants with low conspecific neighbourhood density) pollinators may spend more time in the same plant promoting more within-plant visits and, therefore, selfing events (either by autogamy or geitonogamy; Karron *et al*. 2009). Moreover, pollination of wind-pollinated species can also be affected by alterations in the conspecific density promoted by habitat fragmentation since conspecific density around maternal plants may act as a barrier for pollen dispersal for these species (Dyer & Sork 2001; Bacles *et al.* 2005). To characterize the conspecific neighbourhood density of each maternal plant ('conspecific neighbourhood density', hereafter) we calculated a proximity index, a dimensionless metric (adapted from the Proxi index by McGarigal *et al.* [2002]) which takes into account the number and size of conspecific neighbours (potential pollen donors) within a 10 m buffer radius around the focal maternal plant weighted by the squared distance to the focal maternal plant. Only male plants were considered for the dioecious *Pistacia*.

Flowering synchrony

Flowering phenology and synchrony are fundamental factors with significant impact on pollination success (Primack 1985; Rathcke & Lacey 1985; Elzinga *et al*. 2007). Profound pollen limitation may occur if no synchrony takes place between maternal plant stigma's receptivity and pollen release by pollen donors in both animal and wind-pollinated species. From our phenological records we estimated the 'flowering synchrony' of each maternal plant relative to the population, according to the Augspurger's (1983) index. This index calculates the degree to which the plant’s blooming period overlapped with the blooming periods of each other possible pollen donor in the same population. Augspurger's index ranges from 0 when there is no synchrony, to 1 when the flowering overlap is complete.

Individual genetic diversity

In plants, many studies have revealed positive correlations between multilocus heterozygosity at neutral markers and fitness components (e.g. Oostermeijer *et al*. 1995; Breed *et al*. 2012). These heterozygosity-fitness correlations (HFC) have been the subject of review since the mid 80’s (Mitton & Grant 1984; Britten 1996; David 1998; Szulkin *et al*. 2010). In fact, a recent study have reported the occurrence of a positive relationship between heterozygosity and *Myrtus* seedling growth rate, an important and integrative life history trait (González-Varo *et al*. 2012). Interestingly, the authors found the relationship to be associated with habitat fragmentation, because it was absent in small and probably bottlenecked populations, where inbreeding and other different genetic processes (e.g. genetic drift) could have overridden this effect. Maternal genotypes of the studied plants here are available and have been published in a companion study (Nora *et al.* 2015). Multilocus genotypes were characterized through seven and eight polymorphic nuclear microsatellites in *Pistacia* and *Myrtus*, respectively. Details on DNA isolation, amplification conditions and scoring can be found in Nora *et al*. (2015). Maternal genetic diversity was assessed via the homozygosity by loci index (*HL*), a calculation that improves raw observed heterozygosity values (H_o) by weighting the contribution of each locus to the individual homozygosity value in terms of their allelic variability (Aparicio *et al.* 2006).

Statistical analyses

For each species, we tested differences in reproductive success parameters using general and generalized linear mixed models in R 3.4.1 (R Developmental Core Team 2017). We conducted all analyses considering population type and population identity (nested within type) as fixed factors, and the plant identity as a random factor. We used a Gaussian distribution for modelling the response variables ‘fruit set’, ‘number of seeds per fruit’ and ‘percentage of viable seeds’, since they were normal or log-normal distributed. We performed the analyses with the package *nlme* v. 3.1-111 (Pinheiro *et al.* 2013). For the response variable ‘seed set’, we run models using a beta binomial distribution with the package *lme4* (Bates *et al.* 2014) and *car* (Fox & Weisberg 2011).

Pollinators’ abundance differences among *Myrtus* populations were evaluated with GLMMs in R 3.4.1 (R Developmental Core Team 2017) using V/H as response variable. We considered both the population type and population identity (nested within type) as fixed factors, and each individual five-minute census as a random-effect factor since consecutive censuses may display some degree of temporal correlation. We used a negative binomial distribution in the models after checking the absence of patterns in the residuals. We also conducted a Spearman correlation test to assess relationships of the ‘visitation rate’ and the *Myrtus* reproductive output estimates ‘fruit set’ and ‘seed set’.

To evaluate the effects of habitat fragmentation on the interactions between maternal traits and plant reproductive success in an integrative way we used structural equation models (SEM). Unlike general linear models (GLMs) or additive models (GAMs), SEM are used to unravel the structure linking traits that are correlated in a multivariate way (Shipley 2016) and bears the capacity to evaluate complex systems composed of non-independent variables (Grace 2006). For this reason, SEM is recognized as an important tool in ecology (Iriondo *et al.* 2003; Valdés & García 2011; Cacho *et al.* 2013; Brudvig *et al.* 2015) and more specifically has been proved to be useful disentangling the complex effects of habitat loss and fragmentation (e.g. Didham *et al.* 2012). We first started by building a path scheme in which we represent all the hypothesized causal relationships between the individually measured traits (predictors variables) and the reproductive success variables (response), based on previous knowledge of the ecological system (Fig. 1). We expected response variables to be non-independent between them. Thus, in *Myrtus*, we expected ‘fruit set’ to exert a direct effect on ‘seed set’, and the latter to influence ‘the number of seeds per fruit’, while in *Pistacia*, we expected ‘fruit set’ to exert a direct effect on ‘seed viability’. Further, in the *Myrtus* model we hypothesized that all predictors (flowering synchrony, conspecific neighbourhood, distance to patch edge and maternal homozygosity) have a direct effect on the response variables (fruit set, seed set and number of seeds/fruit). In the *Pistacia* model, we hypothesized that all predictor variables (flowering synchrony, conspecific neighbourhood, distance to patch edge and maternal homozygosity) have a direct effect on ‘fruit set’ and on ‘seed viability’.

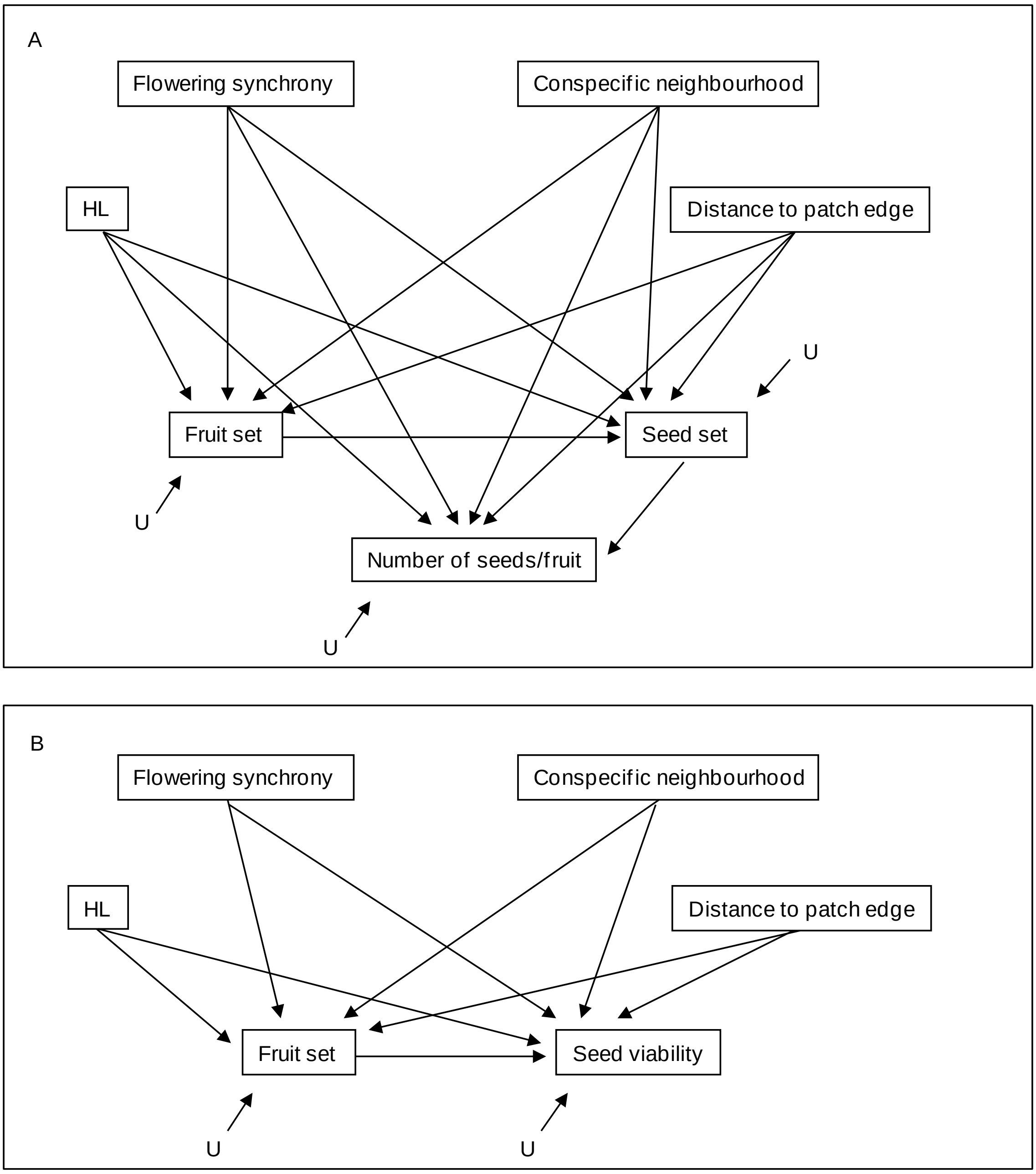

Figure 1. Hypothetical structural equation models of the relationships among maternal plant traits and reproductive success response variables for *Myrtus* (A), and *Pistacia* populations (B). U represents the unexplained variance of the response variables.

As our main goal in performing SEM analyses was to explore whether there is a variation on the influence of maternal plant traits on the reproductive success along our fragmentation gradient (*C* < *FC* < *FI*), we ran a multigroup SEM using population type as grouping factor (Grace 2006; Shipley 2016) instead of constructing a different SEM for each population type. The advantage of multigroup SEM is the capacity to compare statistically between groups and determine which parameters differ (or are the same) in each group (Shipley 2016). We built a constrained model in which all free parameters were forced to be equal across the three types of populations, with the exception of the variables ‘flowering synchrony’, ‘conspecific density’ and ‘distance to the forest edge’, because we expected the effects of these variables to be highly dependent of the population type. Subsequently, we developed a series of nested models where equality constraints were removed one by one at a time to detect which one would improve the model, providing a lower AIC value (Shipley 2016). We added in these models the population of origin (not shown in Fig. 1) as a categorical factor to control for the potential effect that unmeasured traits at population-level may exert on the response variables. The final model goodness of fit was evaluated with Bollen-Stine bootstraps. A significant value indicates that the model is a poor description of the data, while a nonsignificant value indicates that the predicted pattern of the model is not distinguishable from the observed (Shipley 2016). We also tested the fit of the model to the data through the Comparative Fit Index (CFI). Values of CFI greater than 0.9 indicate an acceptable fit of the model to the data (Grace 2006). Variables were transformed if necessary to achieve normality. We carried out all SEM analyses with the package *lavaan* v 0.5-12 (Rosseel 2012) in R 3.4.1 (R Developmental Core Team 2017).

RESULTS

Reproductive output under distinct landscape contexts at population level

The results of fruit and seed production for both *Myrtus* and *Pistacia* populations are summarized in Table 1. In *Myrtus*, approximately half the flowers converted successfully into fruits (overall mean ± SE = 0.54 ± 0.02). 'Seed set' values were very low (0.07 ± 0.002) and the 'number of seeds per fruit' varied from one to 17 in the studied populations (4.19 ± 0.13). In *Myrtus*, we did not detected significant differences in the measured fitness parameters neither among population types nor between populations nested within types (Table 2).

Table 1. Mean values (± SE) of reproductive parameters for *Myrtus* and *Pistacia* populations according to the gradient of habitat fragmentation: continuous populations (*C*), fragmented-connected populations (*FC*), and fragmented-isolated populations (*FI*).

	Myrtus communis			*Pistacia lentiscus*	
	Fruit set	Nº seeds/fruit	Seed set	Fruit set	Seed viability
***C* populations**					
AZN	0.47 ± 0.04	3.90 ± 0.32	0.06 ± 0.01	0.29 ± 0.02	0.41 ± 0.05
DHY	0.52 ± 0.04	3.91 ± 0.33	0.06 ± 0.01	0.16 ± 0.03	0.77 ± 0.05
***FC* populations**					
BNC	0.56 ± 0.04	4.88 ± 0.35	0.08 ± 0.01	0.17 ± 0.02	0.19 ± 0.05
SJV	0.52 ± 0.04	4.16 ± 0.34	0.07 ± 0.01	0.16 ± 0.02	0.85 ± 0.05
***FI* populations**					
CRB	0.64 ± 0.04	4.14 ± 0.32	0.08 ± 0.01	0.26 ± 0.02	0.10 ± 0.05
VIS	0.54 ± 0.04	4.23 ± 0.32	0.06 ± 0.01	0.21 ± 0.03	0.06 ± 0.05
Overall	0.54 ± 0.02	4.19 ± 0.13	0.07 ± 0.02	0.21 ± 0.01	0.40 ± 0.03

Fruit set of black mature fruits in *Pistacia* populations was low (mean ± SE = 0.21 ± 0.01). 'Seed viability' (0.40 ± 0.03) varied largely among populations, ranging from 0.06 to 0.85. We found strong significant differences between population types (*C*, *FC* and *FI*) in the 'fruit set' (Table 2) with both *FC* populations (BNC and SJV) displaying the lowest values of 'fruit set'.

However, we also detected significant differences between populations (nested within type), mostly attributable to the contrasted values of ‘fruit set’ from the *C* populations (0.29 in AZN vs. 0.16 in DHY). Regarding ‘seed viability’, we found significant differences between population types and also between populations within types (Table 2). While both *FI* populations (CRB, VIS) showed the lowest values (0.10 and 0.06, respectively) of ‘seed viability’, *C* and *FC* populations displayed highly contrasted values (high values in DHY and SJV and moderate values in AZN and BNC). Moreover, it is worth noting that populations with the higher values of ‘fruit set’ did not showed the higher proportion of viable seeds.

Table 2. Results of the general and generalized linear mixed models to test for differences among population types and among populations within types in the observed reproductive parameters for *Myrtus communis* and *Pistacia lentiscus*.

	Type		Population (within type)	
	χ^2	*P*	χ^2	*P*
Myrtus communis				
Fruit set	4.86	0.088	4.13	0.248
Nº seeds/fruit	0.92	0.631	0.30	0.960
Seed set	1.93	0.380	5.79	0.122
Pistacia lentiscus				
Fruit set	9.47	0.009	7.86	0.049
Seed viability	55.87	< 0.001	117.77	< 0.001

Pollinator assemblage and visitation rates in Myrtus populations

A total of 1147 insects belonging to at least 19 distinct morph-species were observed visiting *Myrtus* flowers during our study (Table 3). Hymenoptera accounted for 82-99% of visits in each population, the remaining of the visits were accounted by Dipterans (Fig. 2). Honeybees were consistently more frequent than other Hymenoptera, especially in CRB, where we found the presence of domestic hives in the proximity. We detected differences in the insect visitation rates (Fig. 2) between population types (Likelihood ratio test LRT χ^2 = 194.06, $P < 0.001$) and also between populations within type (LRT χ^2 = 163.36, *P* < 0.001), having CRB the highest and *C* populations (AZN and DHY) the lowest visitation rates. Insect visitation rate was significantly associated with seed set (Spearman's *r* = 0.83, *P* = 0.039, *n* = 6; Fig. 3) and marginally with fruit set (Spearman's *r* = 0.673, *P* = 0.148, *n* = 6; Fig. 3) but not related with number of seeds / fruit (Spearman's *r* = 0.43, *P* = 0.397, *n* = 6; Fig. 3).

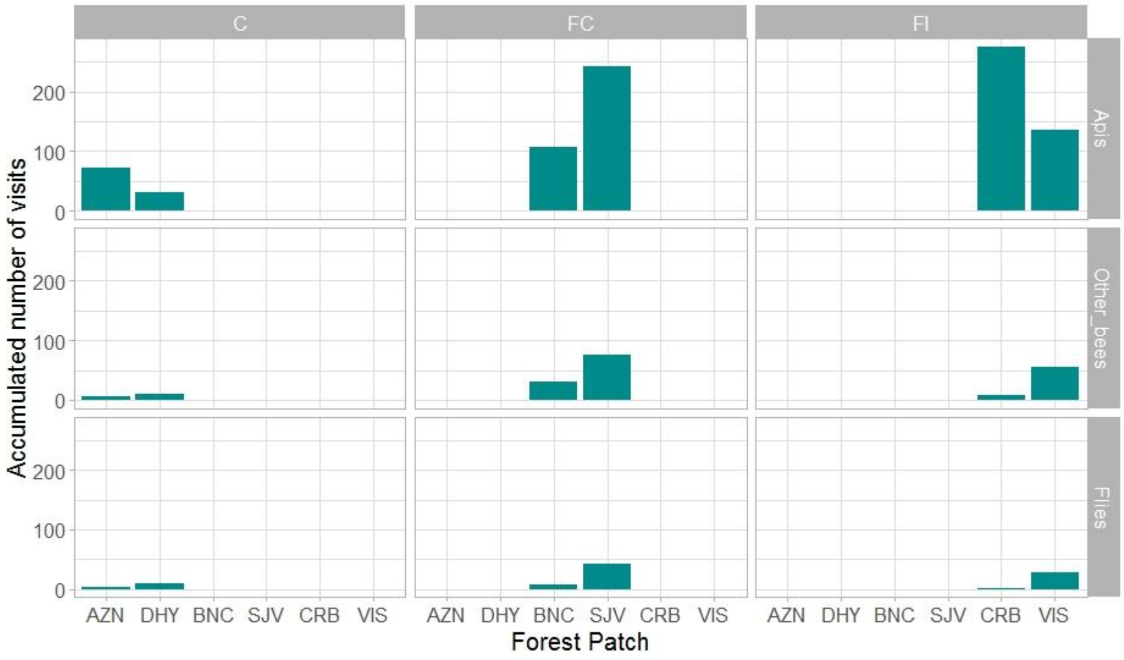

Figure 2. Accumulated number of visits of visits to *Myrtus* flowers of honeybees (*Apis mellifera*), other bee species (other hymenopterans) and flies (dipterans) in the study populations.

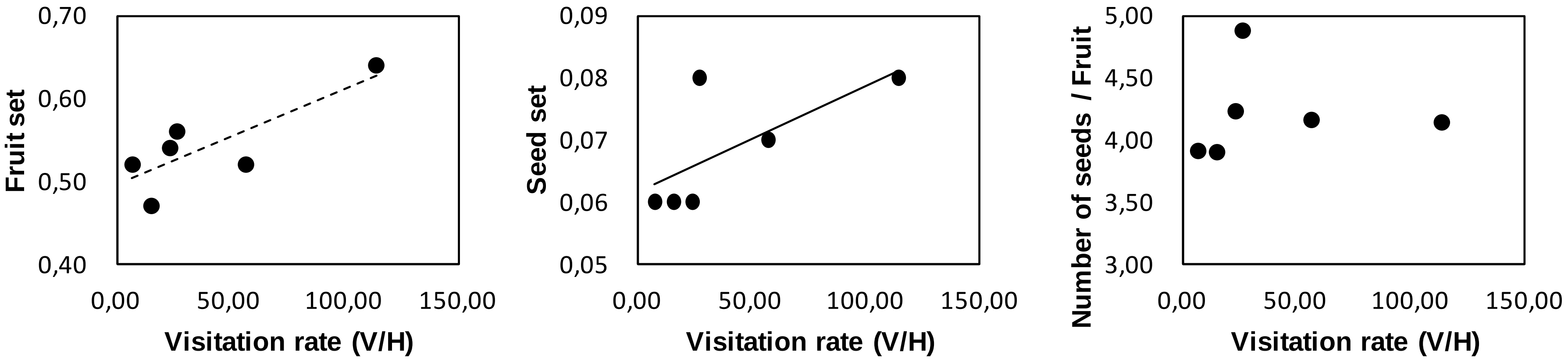

Figure 3. Relationship between reproductive output (fruit set, seed set and number of fruits / seed) and the insect visitation rates in *Myrtus* populations.

Table 3. *Myrtus* pollinators: V/H = visits/hour; % = percentage of visits (relative to the total number of visits) attributable to a given morpho-species for each population. * = observed outside the census time. n = number of 5 minutes observation periods.

	C populations				*FC* populations				*FI* populations			
FLORAL VISITORS	AZN (n=65)		DHY (n=90)		BNC (n=65)		SJV (n=77)		CRB (n=30)		VIS (n=111)	
	V/H	%	V/H	%	V/H	%	V/H	%	V/H	%	V/H	%
Himenoptera	**14.40**	**96.30**	**5.47**	**82.00**	**25.29**	**95.14**	**49.71**	**88.12**	**112.80**	**99.30**	**20.22**	**87.38**
Apis mellifera (Apidae)	13.29	88.89	4.27	64.00	19.75	74.31	37.87	67.13	110.00	96.83	14.70	63.55
Amegilla quadrifasciata (Anthophoridae)	0.37	2.47			0.18	0.69	0.16	0.28			0.22	0.93
Bombus terrestris (Apidae)												
Ceratina cucurbitina (Anthophoridae)	0.37	2.47	1.07	16.00	0.55	2.08	5.61	9.94	2.80	2.46	0.97	4.21
Colletes fodiens (Colletidae)					2.22	8.33	0.16	0.28			0.11	0.47
Lasioglossum sp. (Halictidae)							0.31	0.55				
Megachile ligniseca (Megachilidae)			*								*	
Megachile pilidens (Megachilidae)	*				1.48	5.56	1.71	3.04			2.49	10.75
Pseudapis bispinosa (Halictidae)					0.37	1.39	1.25	2.21			1.51	6.54
Xylocopa violacea (Anthophoridae)			0.13	2.00			0.16	0.28				
Hymenoptera indet.	0.37	2.47			0.74	2.78	2.49	4.42			0.22	0.93
Diptera	**0.55**	**3.70**	**1.20**	**18.00**	**0.92**	**3.47**	**6.70**	**11.88**	**0.80**	**0.70**	**2.92**	**12.62**
Anthrax spp. (Bombyliidae)			0.13	2.00							0.22	0.93
Eristalinus taeniops (Syrphidae)											0.11	0.47
Eristalis tenax (Syrphidae)			*						0.40	0.35	0.22	0.93
Stomorhina lunata (Calliphoridae)			0.40	6.00							1.30	5.61
Syritta pipiens (Syrphidae)							0.47	0.83				
Villa spp. (Bombyliidae)	0.18	1.23					0.31	0.55			0.11	0.47
Xanthogramma marginale (Syrphidae)							0.16	0.28				
Diptera indet.	0.37	2.47	0.67	10.00	0.92	3.47	5.77	10.22	0.40	0.35	0.97	4.21
Total of V/H	**14.95**		**6.67**		**26.22**		**56.42**		**113.60**		**23.14**	
Number of hymenopterans species	3		3		6		8		2		6	
Number of dipterans species	2		3		1		4		2		6	
Total of species	5		6		7		12		4		12	

Influence of plant traits and landscape contexts on reproductive output at plant level

The results of *Myrtus* SEM analyses are shown in Figure 4A. Multigroup SEM fit the data well and all three models explained a large amount of the variation for 'seed set' and 'number of seeds per fruit': from 24% to 34% in 'seed set' and from 47% to 72% in 'number of seeds per fruit'. These values were lower in 'fruit set', explaining 27% of variation in the model only for *FI* populations and only a 9% and 3% in the models of *C* and *FC* populations, respectively. Overall, the models showed how 'fruit set' had an important positive and direct effect on 'seed set' (unstandardized coefficient ± SE = 0.12 ± 0.03, $P < 0.001$) and an indirect effect on the 'number of seeds per fruit' as 'seed set' exerted a positive direct effect to the 'number of seeds per fruit' (5.02 ± 0.56, $P < 0.001$). When analyzing the differences among groups (i.e. types of populations), we found that plant's flowering synchrony showed a strong and significant positive effect on 'fruit set' (0.72 ± 0.21, $P < 0.001$) only in *FI* populations. The distance to the patch edge negatively affected (although marginally) seed set on *FC* populations (-0.03 ± 0.02, $P = 0.053$), indicating that in these populations, plants closer to the patch edge produce a slightly higher seed set. We found no signals of significant heterozygosity-fitness correlations nor effects due to conspecific density in the measured reproductive variables.

In the *Pistacia* analysis, multigroup SEM also had a good overall goodness of fit of the data (Fig. 4B). Predictors explained from 11 to 49% of the variation in the fruit set and from 19 to 79% in the observed seed set. *Pistacia* multigroup SEM revealed only a few significant coefficient paths. We found that the conspecific neighbourhood density and the distance to patch edge significantly affected 'fruit set', although only in *FC* populations (unstandardized coefficients ± SE = 0.13 ± 0.05, $P = 0.017$ and 0.27 ± 0.06, $P < 0.001$, respectively). No influence was found of fruit set on seed viability and none of the predictor variables influenced 'seed viability' in any type of population. Also, we did not detect effects of either flowering synchrony or HL on the *Pistacia* reproductive success.

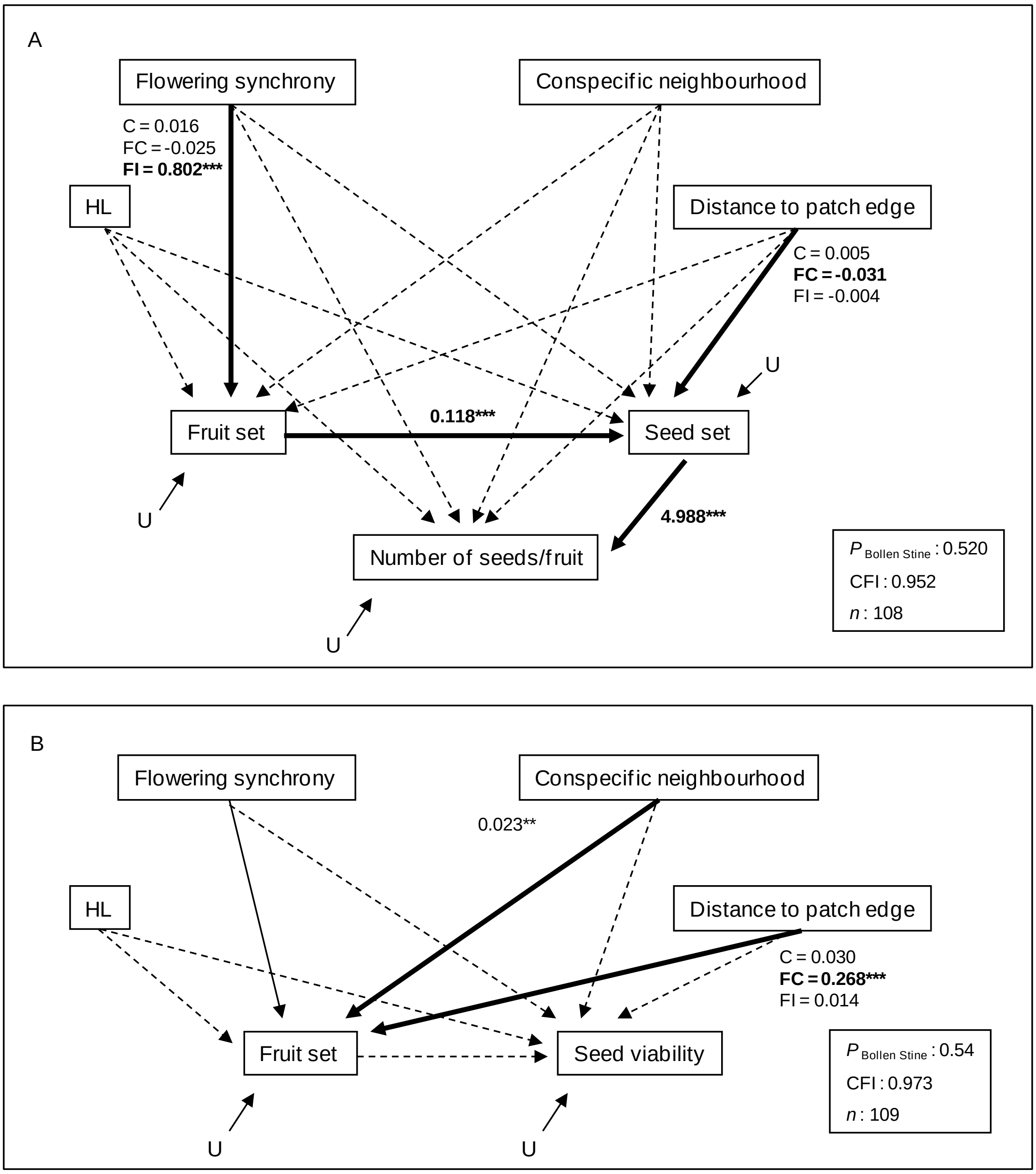

Figure 4. Fitted structural equation models (SEM) for *Myrtus* (A) and *Pistacia* (B) continuous (*C*), fragmented and connected (*FC*) and fragmented and isolated (*FI*) populations using a multi-group approach. The unstandardized coefficients are given for significant paths: * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$. For simplicity, non-significant paths are represented by dashed arrows.

DISCUSSION

Reproductive output under distinct landscape contexts at population level

Habitat fragmentation has an overall large and negative effect on pollination and plant reproduction (Aguilar *et al.* 2006) and also alters ecological interactions (Jacquemyn *et al.* 2012) that influence movement and the outcome of pollen. In this study we analyzed the complexities of plant reproduction in fragmented systems (Aizen & Feinsinger 1994a) by evaluating the influence of habitat fragmentation on multiple ecological interactions and plant reproduction of two species with distinct pollination strategies and mating systems (*Myrtus* and *Pistacia*).

Myrtus *reproductive success*

We found no significant direct effect of habitat fragmentation on *Myrtus* reproductive output (fruit set, number of seeds/fruit or seed set). Although contrary to our expectations, this lack of effects was also observed by González-Varo *et al*. (2009b, 2012) in the same region on the fruit set between small and large patches of *Myrtus*. Similar to previous studies, *Myrtus* reproductive output appears to depend more on local pollinator environment than on fragmentation (González-Varo *et al.* 2009b). Being a self-compatible species with a mixed-mating system, *Myrtus* reproductive output (except for the number of seeds/fruit) is expected to be more constrained by pollen quantity than by pollen quality (i.e. self vs. outcross pollen) (González-Varo *et al.* 2009b, 2010).

In this study, pollen quantity (here regarded as the insect visitation rate) is positively related to populations' fruit and seed set, although only statistically significant for seed set (Fig. S1). Visitation rates varied greatly among populations and types of populations (Table S1). In fact, the population CRB with the highest visitation rates, showed a number of visits 2-fold higher than the second more visited population (SJV). This difference is highly caused by *Apis mellifera* (honeybees) and the presence of honey hives in the immediate vicinity (<100 m) of the patch. CRB is mainly monopolized by this species that represents 96.83% of the total number of visits on an individual plant. In the Mediterranean region, *Apis mellifera* occurs in highly densities due to the extensive presence of domestic hives (Goulson 2003). Only in Andalusia, approximately a million hives were legally established in 2005 and around half of them were located within our study area, the Guadalquivir River Valley (Anon 2005). *Apis mellifera* is also the most abundant species in all other study populations with frequency rates varying from 88.89 to 63.55%. This species has a high tolerance to anthropogenic disturbances (Aizen & Feinsinger 2002; Steffan-Dewenter *et al.* 2002; Goulson 2003; Winfree *et al.* 2009) and is reported to provide a 'rescue-effect' on pollination and female plant fecundity in fragmented systems, in the absence or decrease of native pollinators, especially for pollinator-dependent plants (Aizen & Feinsinger 1994b; Dick 2001; Goulson 2003; Winfree *et al.* 2009). However, it has been demonstrated that they are poor pollinators to native plants (Aizen & Feinsinger 1994a, 1994b) due to their distinct foraging behavior. *Apis mellifera* have a tendency to spent longer periods in the same plant and contact more plants per visit than other insects (Lázaro & Traveset 2005), promoting selfing events instead of favoring the transfer and deposition of outcross pollen (Aguilar *et al.* 2006).

We found fragmented populations (*FC* and *FI*) to display higher visitations rates than continuous populations. In small patches pollinators tend to spend proportionally more time in the plants along their foraging routes (Goulson 2000). In our case, insect visitation rates were significantly associated with seed set and marginally with fruit set, indicating that higher visitations rates in fragmented patches may have helped fruit and seed production and probably contributed to diminish the negative effects of habitat fragmentation to be noticeable. Even though non-significant, the mean fruit set and mean seed set were slightly higher in fragmented than in continuous populations. This pattern of received pollen limitation was also found in large populations of *Myrtus* in the same region (González-Varo *et al.* 2009b). As pointed out by these authors, the mixed-mating system of *Myrtus* and specially their ability to produce self progeny difficult the detection of inbreeding depression effects in fruit and seed production (Husband & Schemske 1996). An ongoing research will determine whether these visitation rates are translated in more outcrossing events in detriment of autogamy or geitonogamy and, therefore, a more vigorous progeny. Nevertheless, our results must be interpreted with caution, we are aware that we only have monitored a single pollination season 'snapshot' and consequently, this study does not considers temporal variability. Insect pollinator populations often fluctuate between years (Williams *et al.* 2001) and plant populations are susceptible to qualitatively and/or quantitatively distinct pollinator environments through time, especially perennial plants (Herrera 1988; Price *et al.* 2005; Aizen & Harder 2007).

Among the most important species' life history traits that may condition plant's vulnerability to habitat loss and fragmentation are the mating system and the degree of pollinator specialization (Rusterholz & Baur 2010). Being self-compatible and having a generalized pollination system, species like *Myrtus* may show some resilience in a disturbed and changing environment (Johnson & Steiner 2000; Aguilar *et al.* 2006). When pollinators' abundance and richness are unpredictable, many self-compatible plants compensate a shortage of outcross pollen by self-fertilization as a mechanism of reproductive assurance (Eckert *et al.* 2006; Opedal *et al.* 2016). Therefore, the effects of habitat fragmentation might be underestimated when only analyzing plant's reproductive output (fruit and seed set), because effective consequences could only be detectable in further steps of the regeneration cycle such as seed and/or seedling fitness.

Pistacia *reproductive success*

Fruit set values found in this study (21%) were accordingly to those found in literature that could vary from 10% in Italy (Martínez-Palle & Aronne 2000) to 52.9% in Pujol, Spain (Verdú & García-Fayos 1998). No distinct pattern of the effect of habitat fragmentation was observed in the fruit set as both *FC* populations (BNC and SJV) had the lowest values, together with the continuous population DHY. Moreover, fruit set was not related seed viability, as reported in other studies (Jordano 1988; Verdú & García-Fayos 2001).

A negative effect of habitat fragmentation was detected in 'seed viability' as the *FI* populations showed the lowest values. This decline in seed viability could be explained by inbreeding depression in fragmented and isolated populations. In fact, the others study populations showed disparate results, with DHY and SJV bearing much higher percentages of seed viability. These two populations are geographically close and are the most southern populations in our study area, indicating that possibly similar climatologic conditions (mainly wind strength and direction and precipitation rates) may be related to the observed values. Wind-pollinated species, such as *Pistacia*, greatly depend on the quantity of pollen availability and precipitation during flowering has been reported to create unfavorable conditions for their pollination (Whitehead 1969). In general *Pistacia* had very low values of seed viability, which is a common characteristic of anemophilous species. Plants with unspecialized pollination syndromes tend to produce much more flowers than fully mature fruits (Jordano 1988). For example, Feret and collaborators (1982) studied the acorn production of four *Quercus alba* trees during two years and they reported a failure of 70% of the flowers to produce mature acorns. Moreover, our percentage (40%) is very similar to the one found by Martínez-Palle and Aronne (2000) in Italy (50%), and by Jordano (1988) in Doñana, Spain (19.9%). Previous studies conducted on this species or the same genus have reported that seed viability is mainly affected by climatic conditions, especially water resources (Jordano 1988; Verdú 1994) and maternal plant life history traits (Verdú & García-Fayos 2002). The influence of flowering phenological synchrony and the distance to the nearest male plant on *Pistacia* seed viability was also detected by Jordano (1988).

Wind-pollination has been frequently viewed as a disadvantage and a less evolved process when compared to animal pollination, mainly due to its passivity, dependence on abiotic traits such as wind intensity and direction, humidity, rainfall and temperature (Whitehead 1983; Culley *et al.* 2002) and less efficiency regarding pollen deposition on conspecific stigmas. However, wind-pollinated species are regarded as more predisposed to persist in fragmented landscapes, due to pollinators' constant unreliability that make anemophily more effective than entomophily (Whitehead 1969, Regal 1982). However, contrary to these predictions, recent evidence suggests that pollen limitation in wind-pollinated plants may be as common as it is in animal-pollinated species (Friedman & Barrett 2009; Barrett 2010).

Influence of maternal plant traits and landscape context on reproductive success

SEM analyses conducted on *Myrtus* populations have detected some ecological components that are determinant to the reproductive success regarding the interplay with the fragmentation context. Distance to patch edge showed a negative effect on seed set, a variable more affected by pollen quality (discussed above), but only in *FC* populations. It is likely that in fragmented patches, plants closer to the edge are pollinated by insects that arrived from neighbour patches that brought pollen with a more different pool of alleles. In fact, fruit set was not affected by this predictor variable, which could validate the importance of pollen quality of the external pollen leading to a lower ratio of seed abortion and a higher seed set. Recent studies have reported a positive relation between edges and forests gaps on flower and fruit production (e.g. Burgess *et al.* 2006; Athayde & Morellato 2014), though is far from being a general trend (e.g. Harper *et al.* 2005; Hagen *et al*. 2012), and the pollinator guild of the target plant species is undoubtedly a capital factor. The fact that distance to patch edge seemed not to affect *FI* populations indicates that the physically isolated patches we selected may be located too far from other potential pollen sources to receive a significant proportion of visits by pollinators carrying external pollen. Plants in *FI* populations might rely mainly on local pollen sources and thus are more prone to be affected by variables affecting within patch pollen availability such as flowering synchrony with other plants within the patch. Our SEM analyses seems to support this hypothesis since we found flowering synchrony to exert a strong direct effect to the fruit set in these populations which further indirectly affects the seed set.

When analyzing the SEM results in *Pistacia*, we detected the influence of the conspecific neighborhood and the distance to patch edge on the fruit set of plants located in *FC* populations (Fig.6). Density of conspecific plants around maternal plants is positively related to fruit set, i.e. maternal plants with more males in their surroundings will produce more fruits in this type of populations. This result could indicate that maternal plants are receiving most of their pollen from neighbourhood plants. In the literature has been frequently documented in wind-pollinated species their preference for mating with neighbouring individuals (Burczyk *et al.* 1996; Dow & Ashley 1998). However this pattern could lead to an increase of biparental inbreeding (Stacy *et al.* 1996; Hodgins & Barrett 2006). Former studies in *Pistacia* found that, even though, *Pistacia* pollen can travel long distances, a large fraction of the produced pollen doe not move very far from their paternal plant (Albaladejo *et al.* 2009b, 2012). Therefore, maternal plants with higher flowering male density had higher levels of biparental inbreeding. Though counter-intuitive, the positive relation of distance to patch edge with fruit set may be an artifact of the conspecifc neighbourhood density effect. These two predictor variables, although globally uncorrelated (Spearman's r = -0.14, P = 0.147, n = 109), showed a positive and significant correlation in *FC* populations (Spearman's r = 0.55, P < 0.001, n = 38). This means that the more distant to the patch edge, the more aggregate male plants are in these populations. Regarding the influence of ecological and genetic variables on the percentage of seed viability across distinct types of populations, we detected no effects in the SEM analyses.

Conclusions

With this study we highlight the importance of comparative studies with species with distinct life history traits in assessing habitat fragmentation effects not only on plant's reproductive success and also encourage other studies to incorporate, in an integrative approach, both direct and indirect effects of habitat fragmentation on the influence of ecological and genetic components that are determinant to their reproductive output. As observed, habitat fragmentation promotes a quite complex scenario where there are no single factors involved but the interplay between many factors is responsible of the reproductive success of plants. Moreover, our study stress how biotic ecological variables (e.g. pollinator composition, flowering synchrony), combined with the spatial architecture of the landscape, shapes species reaction to habitat fragmentation and the idiosyncratic nature of the species response to habitat disturbance.

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Ecological determinants of mating patterns in fragmented populations

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Determinantes ecológicos de los patrones de apareamiento en poblaciones fragmentadas

Resumen

Un buen conocimiento de los sistemas de apareamiento en plantas es fundamental para comprender las consecuencias genéticas de la fragmentación del hábitat. En este estudio se evaluaron los patrones de apareamiento y sus factores determinantes en dos arbustos mediterráneos, *Myrtus communis* y *Pistacia lentiscus*. Para las dos especies, se estimaron utilizando marcadores genéticos de microsatélites, los parámetros del sistema de apareamiento en seis poblaciones seleccionadas y que representan tres paisajes con diferente grado de fragmentación. También se consideró el impacto del ambiente local sobre los patrones de apareamiento midiendo la distancia al borde del bosque, la cobertura arbustiva y la densidad de vecinos conspecíficos. Las poblaciones de la especie entomófila y autocompatible (*Myrtus*) revelaron tasas de xenogamia relativamente bajas (t_m = 0,61) y tasas de endogamia biparental (t_m-t_s = 0,21) y paternidad correlacionada (r_p = 0,51) elevadas. Sin embargo, la especie anemófila y dioica (*Pistacia*) presentó valores muy bajos de paternidad correlacionada (r_p = 0,10), mientras que todavía conserva valores relativamente altos de endogamia biparental (t_m-t_s = 0,21). Además, los patrones de apareamiento también fueron influenciados por diferentes factores paisajísticos y ecológicos. La paternidad correlacionada en *Myrtus* reveló efectos de la densidad de vecinos conspecíficos y de la fenología de floración. En *Pistacia*, se detectó la influencia de la fragmentación del hábitat y del aislamiento, efectos de borde y también de la sincronía de la floración. En conclusión, nuestro estudio confirmó la complejidad y la alta dependencia de las especies y el contexto de los patrones de apareamiento, destacando la importancia de los estudios multi-escala y de múltiples especies para entender la variación encontrada en los sistemas de apareamiento.

Palabras clave: Especies anemófilas; especies entomófilas; fragmentación del hábitat; genética de poblaciones; paisajes mediterráneos; sistemas de apareamiento.

Abstract

Good knowledge of plant mating system is fundamental to understand the genetic consequences of habitat fragmentation. In this study, we evaluated mating system patterns and its determinants on two Mediterranean shrubs, *Myrtus communis* L. and *Pistacia lentiscus* L., with distinct life history traits. For each species, we estimated mating system parameters in six landscapes with contrasted levels of habitat fragmentation and isolation, using microsatellite markers. We also considered the impact of the local environment on mating patterns by measuring distance to the forest edge, canopy cover and conspecific neighborhood density. Populations of the insect-pollinated and self-compatible species, *Myrtus*, displayed low rates of outcrossing (t_m = 0.61) and high rates of biparental inbreeding (t_m-t_s = 0.21) and correlated paternity (r_p = 0.51). Yet, the wind-pollinated and dioecious species, *Pistacia*, presented low values of correlated paternity (r_p = 0.10) while still retaining relatively high values of biparental inbreeding (t_m-t_s = 0.21). Moreover, mating system patterns were also influenced by distinct landscape and ecological factors. Correlated paternity in *Myrtus* was influenced by conspecific neighbourhood density and flowering phenology. In *Pistacia*, we detected the influence of the habitat fragmentation and isolation and edge effects and, as in the case of *Myrtus*, also flowering phenology. Conclusively, our study confirmed the complexity and the highly species and context dependency of mating patterns, highlighting the importance of multi-scale and multi-species studies to understand mating system variation.

Keywords: Habitat fragmentation; insect-pollinated species; mating systems; Mediterranean landscapes; population genetics; wind-pollinated species.

INTRODUCTION

Fragmentation has become a major concern in ecology and conservation biology (Fahrig 2003; Hanski & Gaggiotti 2004; Eckert *et al.* 2009). Many populations that were once continuous now exist only as fragments, transformed into scattered smaller populations (Ghazoul 2005) with altered size, degrees of connectivity and under the influence of edge effects (Magrach *et al.* 2014). Nowadays, anthropogenic habitat deterioration has become so persistent that it is threatening an array of life-history processes that have a direct influence on the genetic connectivity and the reproductive success of populations, and thus, compromising the long-term persistence of many plant species (Sork & Smouse 2006; Aguilar *et al*. 2008; Eckert *et al.* 2009). The mating system is one of these processes, a fundamental micro-evolutionary process that can determine the amount and distribution of genetic variation within and among populations of plant species (Coates *et al.* 2007; Duminil *et al.* 2007). Mating system patterns are investigated by characterizing parameters such as outcrossing rates, biparental inbreeding (mating among genetically related individuals) and correlated paternity (proportion of full-sibs within maternal progeny arrays). By far, the latter has received much less attention than the other parameters (Barrett & Harder 2017). However, its importance has been enhanced by recent empirical studies showing that correlated paternity has an impact on progeny fitness and recruitment (Breed *et al.* 2012, 2014; Nora *et al.* 2016). Theory predicts that maternal plants that receive high pollen diversity (i.e. low correlated paternity) might display progeny with increased fitness through female selection of more compatible pollen (Yasui 1998; Skogsmyr & Lankinen 2002; Pannell & Labouche 2013). Further, correlated paternity may be important not only for an individual progeny but for the overall female fitness, since higher pollen diversity received by maternal plants may conduct to more genetically diverse progeny that would exploit more efficiently an ecologically heterogeneous environment (Young 1981; Karron & Marshall 1990).

Numerous studies have examined the impact of anthropogenic habitat fragmentation on plant mating systems, including forest-dealing species. Even though the detrimental effects of habitat fragmentation have been widely documented, a large number of plant species can ameliorate the population genetic pressures created by habitat fragmentation (Sork *et al*. 2002; Mimura *et al*. 2009; Côrtes *et al*. 2013), through an array of mechanisms (Kramer *et al*. 2008;

Lowe *et al.* 2015). Some species can compensate detrimental effects of habitat fragmentation and degradation through extensive gene flow via pollen and/or seed (Bacles *et al.* 2005; Breed *et al.* 2011; Lander *et al.* 2011). The long-lived nature of some plant species and overlap of generations within one single populations ca act to delay the loss of genetic diversity (e.g. Lowe *et al.* 2005; Petit & Hampe 2006; Bacles & Jump 2010; Davies *et al.* 2010). Moreover, some species adapted to a flexible mating system that reassure reproductive success through selfed progeny (Charlesworth 2006).

Further, it is aknwoledged the importance of variation in mating and breeding systems in determining the type and magnitude of species' response to habitat fragmentation (Lowe et al. 2015). Also, the large variability in mating system patterns that is often observed in maternal plants within the same population (e.g. Guazere *et al.* 2013; Saro *et al.* 2014) suggests that these patterns are highly context-dependent and several ecological factors, others than the breeding system (Neal & Anderson 2005), are responsible for this high variability, such as landscape heterogeneity, vegetation structure or the local neighbourhood of conspecific plants, including its density (Franceschinelli & Bawa 2000; García *et al*. 2005; de Lucas *et al.* 2008).

Conspecific neighbourhood density is often considered as a main factor determining plant mating system patterns, affecting both the outcrossing rates (Robledo-Arnuncio *et al.* 2004) and correlated paternity (Dick *et al*. 2003; Fuchs *et al.* 2003). A number of studies found that in dense clusters, the pollen received by maternal plants might be monopolized by only a few pollen donors and hence, less diverse than pollen pools of maternal plants inhabiting less dense stands (Dyer & Sork 2001). However, this trend is not always observed. For instance, in insect-pollinated species, highly isolated plants may receive fewer and longer pollinator visits promoting selfing (either by autogamy or geitonogamy; Karron *et al.* 2009) and decreasing the provision of different pollen sources (i.e. increasing correlated paternity, Breed *et al.* 2013). The effects of conspecific density would also be intrinsically related with the heterogeneity in flowering phenology among plants. Even in a dense neighbourhood of potential pollen donors, male availability may be limited if no synchrony takes place between maternal plant stigma's receptivity and pollen release in both insect-pollinated (Hardy *et al.* 2004) and wind-pollinated species (Valbuena-Carabaña *et al*. 2005). Besides, canopy structure may cause effects on mating patterns (Bacles *et al.* 2005; Tomita *et al.* 2008). This may be especially important in wind-pollinated species, as the canopy around maternal plants acts as a barrier for pollen dispersal (Dyer & Sork 2001; Bacles *et al.* 2005). Likewise, the alteration of native forests through habitat fragmentation may also affect species' mating system patterns by creating, for example, edge effects. The habitat that is under the influence of the patch's boundary (i.e. patch edge) suffers from both direct (e.g. physical damage, microclimatic and/or geochemical alterations) and indirect responses to fragmentation. For instance, among the several indirect responses, plants may suffer alterations in vegetative growth and reproduction output (i.e. fruit set, seed set) (e.g. Harper *et al.* 2005; Chapter 2).

In this study, we investigated the variation in mating system (i.e. outcrossing rates, biparental inbreeding and correlated paternity) of two common Mediterranean shrub species, *Myrtus communis* L. and *Pistacia lentiscus* L. (*Myrtus* and *Pistacia*, hereafter) that share important life-history traits (bird-dispersed, long-lived shrubs or small trees), but contrast in breeding and pollen dispersal systems (hermaphroditic self-compatible insect-pollinated vs. dioecious wind-dispersed, respectively). To control the idiosyncrasy of the results (Bacles & Jump 2011; Aparicio *et al.* 2012), we restrictively selected populations where both study species coexisted in three different categorical contexts of fragmentation: continuous populations, fragmented-connected populations and fragmented-isolated populations. More importantly, we also explored which ecological factors are responsible for mating system variation at both the individual and population levels; specifically, we considered the effects of local maternal neighbourhood (such as canopy cover and conspecific density), the flowering phenological synchronicity and patch metrics on individual mating system. A previous study (Nora *et al.* 2015) on the genetic variation has shown that *Myrtus* adult population has higher among-population genetic differentiation, higher homozygosity levels and stronger evidences for past bottlenecks than *Pistacia* in a common historical context; thus evidencing stronger negative effects of habitat fragmentation in the former species. Here in this study we aim to understad how shifts in contemporary mating patterns caused by landscape heterogeneity (habitat fragmentation and isolation) and the local environment (within population) impact the genetic diversity of naturally produced progeny. Regarding these two study species we expect a stronger genetic impact in progeny of *Myrtus* than in *Pistacia.*

MATERIAL AND METHODS

Study species

Myrtus communis and *Pistacia lentiscus* are common shrubs in Mediterranean forest dominated by helm oaks (*Quercus ilex*), cork oaks (*Q. suber*) and pines (mainly *Pinus pinea* and *P. halepensis*). *Myrtus* is a hermaphroditic and self-compatible species characterized by a density-dependent mixed mating system (González-Varo *et al.* 2009a). Its flowers are white with one style and multiple stamens that in early summer (from mid-June to early July) are pollinated by hymenopterans and dipterans (González-Varo *et al.* 2009b; Chapter 2). Flowers are arranged in lax inflorescences. The fruit is a multi-seeded berry (4.6 ± 2.8 seeds; Chapter 2), which become dark-blue when mature, that begins to be dispersed in mid-October by small and medium-sized birds, either resident species or wintering migrants (mostly Sylviidae and Turdidae, see González-Varo 2010).

Pistacia is a dioecious and wind pollinated species. Male flowers are apetalous with 8-10 stamens grouped in dense inflorescences of 8-10 flowers (Jordano 1989). Female flowers are also apetalous and constituted by a small unilocular ovary and grouped in inflorescences of 4-36 flowers (Chapter 2). *Pistacia* fruits are small one-seeded drupes that are white or red before maturation and only become dark if a viable seed has developed inside. In the study area, *Pistacia* fruits are dispersed from September to December by virtually the same avian dispersal-guild as *Myrtu*s (Jordano 1989).

Study area

The study was conducted in the lower catchment area of the Guadalquivir River in western Andalusia (S Spain) (Fig. 1). This region has a long history of human alteration and management (Martín *et al*. 2013). Nowadays, this fertile region is dominated by intensive monocultures (e.g. olives, cereals, sunflower, cotton, vineyards and diverse orchards), and the remnant woodland patches cover less than 1% of their potential area (Aparicio 2008).

Within this area, we selected four small forest patches where *Myrtus* and *Pistacia* coexist (Nora *et al.* 2015). The patches were then classified either as 'fragmented and connected' (*FC*) or 'fragmented and isolated' (*FI*) according to the amount of suitable forest area contained in 1 km-radius buffer around the centroid of the patch. This threshold distance was based on previous evidence suggesting pollen dispersal to occur mostly below this distance (see Nora *et al*. 2015). Evidence for genetic isolation in the *FI* patches was also supported by detectable signs of genetic bottlenecks (Nora *et al.* 2015). Additionally, we also selected two continuous populations (*C*) to serve as controls by setting two rectangular plots of 5 hectares within two large pine-stone woodlands (above 100 hectares) where the two species are very common. We thus established a gradient of habitat fragmentation: *C* < *FC* < *FI* (Fig. 1).

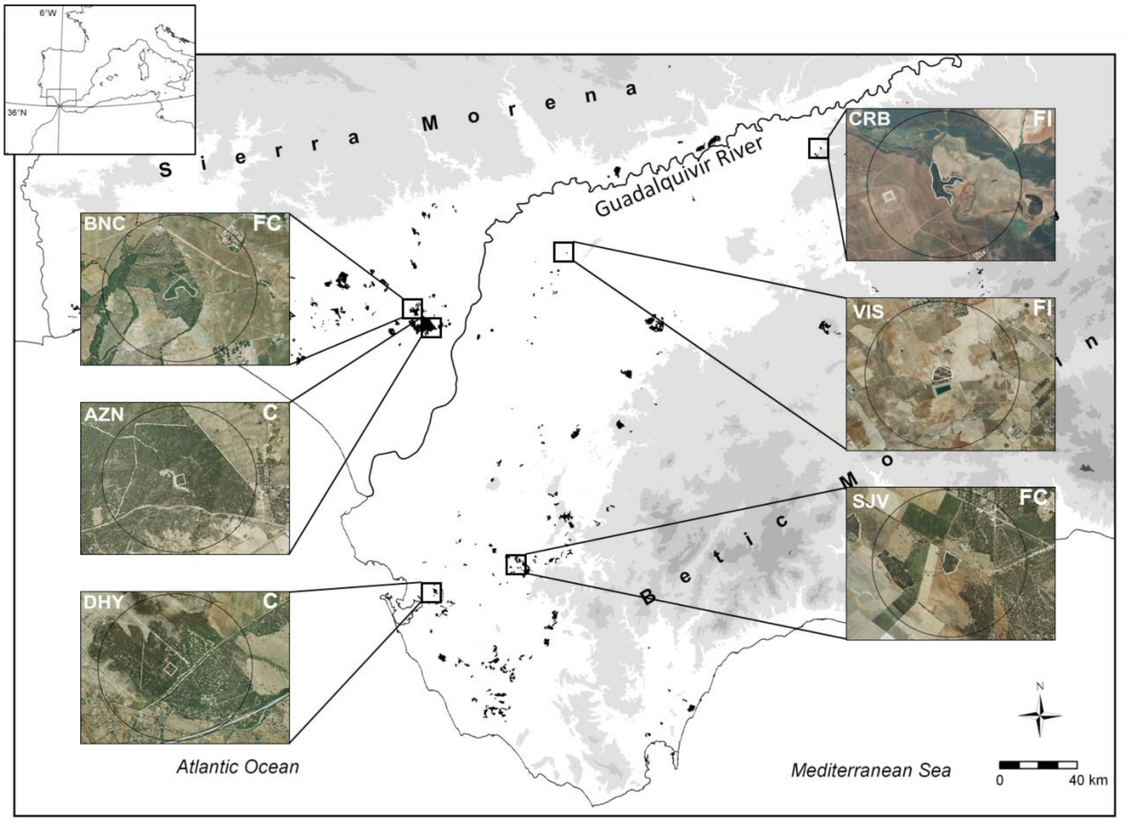

Figure 1. Map of the Guadalquivir river Valley (southwestern Spain) and aerial images showing the location of the six study populations categorized into three landscape contexts: continuous (AZN and DHY), fragmented-connected (BNC and SJV), and fragmented-isolated (CRB and VIS). Buffers of 1-km radius from the centroid of the focal population are also drawn.

Sampling design, DNA extraction and microsatellite genotyping

In each population, we selected 20 *Myrtus* adult plants, and 20 female and 20 male *Pistacia* plants to conduct mating pattern and contemporary pollen flow analyses. The locations of maternal plants were recorded with a GPS unit. The within-population distance between pairs of maternal plants ranged from 2.23 to 249.79 m in *Myrtus*, (average ± SD = 118.65 ± 74.07 m), and from 8.48 to 368.95 m in *Pistacia* (average ± SD = 121.65 ± 76.48 m).

During October and November of 2010, fully mature fruits were collected directly from maternal plants. For DNA isolation we used *Myrtus* seedlings with the two cotyledons fully developed and *Pistacia* embryos. Twenty *Myrtus* seeds from each of the maternal plant (one seed per fruit to avoid the effects of correlated pollination in a single pollination event) were germinated in separated Petri dishes containing filter paper saturated with distilled water (*Myrtus* germination rates are above 90%, González-Varo *et al.* 2009a; Nora *et al.* 2016). In *Pistacia*, no prior germination was necessary because it is possible to separate the embryo tissue from the maternal endocarp and the endosperm.

DNA was extracted with the Invisorb DNA Plant HTS 96 Kit (Invitek, Germany) according to the manufacturer's protocol, and we amplified seven and eight polymorphic nuclear microsatellites in *Pistacia* and *Myrtus*, respectively. Further details about the amplification reactions and PCR cycle conditions can be found in Albaladejo *et al.* (2008, 2010) and Nora *et al*. (2015). We analysed labelled (with 6-FAM, NED, VIC and PET dyes) PCR products with an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, US) at the 'Unidad de Genómica' (UCM, Madrid, Spain). Fragment sizes were scored with GeneMapper 3.7 (Applied Biosystems, Foster City, US) and corrected manually. We only retained multilocus genotypes successfully scored with at least four loci. Maternal genotypes were already available from Nora *et al.* (2015). We checked for genotyping errors by the detection of mother-offspring mismatches and obtained an overall mismatching estimate of 2.01% for *Myrtu*s and 1.72% for *Pistacia*. Overall, 1,888 offspring of *Myrtus* (mean ± SD = 15.70 ± 0.70 seeds per maternal plant) and 1,720 of *Pistacia* (14.70 ± 3.00 seeds per maternal plant) were available for mating system analyses.

Genetic variation and mating patterns

Genetic variation analysis

We evaluated the genetic diversity of the maternal plants and their offspring at population level by estimating the mean number of alleles per locus (A), the effective number of alleles per locus (A_e), the unbiased expected heterozygosity (H_e), the observed heterozygosity (H_o) and the inbreeding coefficient (F_{IS}). Confidence intervals at 95% level (95% CIs) for adults were constructed from standard errors. To avoid the effect of family structure on estimating these parameters in the progeny, we randomly selected one seedling per maternal plant to create a data set consisting of 20 offspring from different mothers per population. This procedure was repeated 1,000 times to assess 95% CIs. We considered that differences between adults and offspring were significant when 95% CIs did not overlap. These analyses were made with the package *gstudio* (Dyer 2014) and customized scripts in R 3.4.1 (R Core Team 2017).

Mating pattern

To assess mating system, we calculated the multilocus (t_m) and single locus (t_s) outcrossing rates, both at population and family levels with the software Mltr 3.4 (Ritland 2002). We ran the analyses using the Newton-Raphson algorithm as missing data were infrequent in our data set (less than 5%). Pollen and ovules were constrained to have the same gene frequencies. At the population level, standard errors and confidence intervals were assessed by bootstrap (1,000 replicates) using families as the resampling unit. At the family level, families were considered as populations, using individuals within families as the resampling unit. For *Pistacia* (as this species is dioecious), we used only the parameters t_m and t_s to estimate the biparental inbreeding (t_m-t_s, i.e. mating between genetically related individuals).

We also estimated the within-mother correlated paternity (r_p) following the method by Hardy *et al.* (2004) with the software Poldisp (Robledo-Arnuncio *et al.* 2007). The effective number of pollen donors contributing to the progeny per maternal plant (N_{ep}) was calculated as $1/r_p$ and the effective pollination neighbourhood area (A_{ep}) as N_{ep}/d (Smouse *et al*. 2001), where *d* is the census density of male reproductive plants (ind/ha). We calculated these estimates at family level and then averaged population level values.

We estimated the global genetic differentiation (Φ_{FT}) among maternal plants pollen clouds within populations with Twogener (Smouse *et al.* 2001). Note that Φ_{FT} and r_p estimate virtually the same phenomenon as theoretically we expect $\Phi_{FT} = r_p/2$ in the absence of inbreeding (Hardy *et al*. 2004). The standard errors for global Φ_{FT} were calculated by jackknifing over loci, and the randomized A&W test (Sork *et al.* 2005) was applied to compare Φ_{FT} values among populations. These analyses were conducted with the R code developed by A. S. Hirao (Hirao *et al.* 2006).

In *Myrtus*, calculation of mating system parameters (r_p, Φ_{FT} and N_{ep}) were also conducted after removing selfed progeny (determined visually by the presence of only maternal alleles) to test whether variation in among-mothers selfing rates was conditioning the levels of correlated paternity (Robledo-Arnuncio *et al.* 2007). Wilcoxon paired tests were used to compare mating system parameters considering either the total progeny or only the outcrossed progeny.

Maternal plant ecological metrics

For each of the 120 *Myrtus* and 117 *Pistacia* maternal plants, we characterized the following set of variables at the plant level (Table S1):

Distance to patch edge

The nearest distance of each plant to the edge of the forest patch ('distance to edge', hereafter) was computed with ArcGis 9.0, using the geographic location of each maternal plant and digital cartography of the study area (see Aparicio 2008).

Ecological maternal neighbourhood

We characterized the neighbourhood composition and structure around each maternal plant ('canopy neighbourhood density') by measuring the percentage of shrub and tree cover on soil projection weighted by height, along four 10-m line-transects placed in N, E, S and W directions from the focal plant. Bare soil percentage was strongly negatively correlated to the woody cover (Spearman's $r = -0.953$, $P < 0.001$) and thus was not considered.

Further, the conspecific neighbourhood of each maternal plant (‘conspecific neighbourhood density’) was characterized by a proximity index, a dimensionless metric (adapted from McGarigal *et al.* 2002) which takes into account the number and size of conspecific neighbours (potential pollen donors) within a 10 m buffer radius weighted by their distance to the focal maternal plant. Only male plants were considered for dioecious *Pistacia*.

Maternal plant flowering phenology

In March and July of 2010, just before the flowering season of each species, we marked five branches with 10-15 flowers/inflorescences (mean ± SD = 11.95 ± 3.62 flowers per branch in *Myrtus*, 12.66 ± 3.55 inflorescences per branch in *Pistacia* female plants and 12.93 ± 4.50 inflorescences in *Pistacia* male plants) in each of the selected plants, and then monitored them weekly. From these phenological records, we estimated 'flowering synchrony' of each maternal plant relative to the population, according to the Augspurger's index (Augspurger 1983). This index calculates the degree to which the plant’s blooming period overlapped with the blooming periods of each other possible pollen donor in the same population. Augspurger's index ranges from 0, when there is no synchrony, to 1, when the flowering overlap is complete. Likewise, the 'maximum flowering synchrony' was also estimated by taking into account only the plant’s maximum bloom period, i.e. when more than 50% of flowers were open.

Model construction and selection

At the individual level, relationships between mating system parameters (t_m, t_m-t_s, r_p) and the explanatory variables ('population type', 'distance to edge', 'canopy neighbourhood density', 'conspecific neighbourhood density', 'flowering synchrony' and 'maximum flowering synchrony') were evaluated through generalized linear mixed models (GLMMs) by fitting error distribution to a binomial distribution through a logit-link function. The population identity was added as a random effect to control for the non-independence of maternal plants occurring in the same population and thus sharing the same management history. Then, we tested population identity significance with likelihood ratio tests between two competing models, one including the random factor and the other with fixed factors only. Prior to model construction, collinearity among predictor variables was checked through Pearson's correlations (r), and we excluded variables with $r > |0.5|$. Pearson's correlations showed no collinearity among variables except for *Myrtus,* the variables 'canopy neighbourhood density' and 'distance to the edge' ($r = 0.98$, $P < 0.001$), but none of them appeared to significantly impact mating system when used alternatively in models (data not shown). We followed a model construction and selection approach with the following steps. Firstly, we tested full models including all two-way interaction terms between explanatory variables. Non-significant interaction terms were then omitted in further models. Secondly, we constructed models that included all possible main factors plus the significant interaction terms and ranked the resulting models following the Akaike's information criterion (AIC) and their Akaike's weight (w_i) (Burnham & Anderson 2002). The Akaike's weight is the probability of each model to be considered the best model over all competing models (Jonhson & Omland 2004). We only considered models with either the lowest AIC or with ΔAIC (the difference in AIC between each model and the model with the lowest AIC) ≤ 2 (Jonhson & Omland 2004). The selected models were validated by inspecting the residuals (see Zuur *et al.* 2009). The relative importance of each explanatory fixed factor was also estimated as the sum of the w_i over all selected models in which the variable was present (Burnham & Anderson 2002). Models were run using the package *lme4* (Bates *et al.* 2014) in R 3.4.1 (R Core Team 2017).

RESULTS

Genetic variation

Significant differences in genetic variation between adults and offspring were found in *Myrtus* (Table 1). In general, *Myrtus* offspring presented higher values of F_{IS} and lower values of diversity (A, A_e, H_e and H_o) than adults. Indeed, in this species we observed significant differences among life stages in the mean number of alleles per locus (A) and the effective number of alleles per locus (A_e) in the population DHY, the expected heterozygosity (H_e) in both *C* populations (AZN and DHY) and VIS, and the observed heterozygosity (H_o) in the *C* populations, BNC and VIS. The decrease in H_o in the offspring of *C* populations, BNC and VIS was also accompanied by a significant increase in F_{IS} (not observed in VIS). In *Pistacia*, we observed the same trend, although with slightly lower magnitude rendering most comparisons not significant. In fact, only the observed number of alleles (A) was significantly lower in offspring than in adults, in all populations.

Mating patterns

Over the six populations, the mean (± SE) multilocus outcrossing rate (t_m) in *Myrtus* was 0.61 ± 0.02 and ranged from 0.45 in the population AZN to 0.70 in SJV (Table 2). *Myrtus* displayed high rates of biparental inbreeding (t_m-t_s = 0.21 ± 0.01), correlated paternity (r_p = 0.51 ± 0.02) and global differentiation among pollen gene pools (Φ_{FT} = 0.30 ± 0.02). When considering only outcrossing events, correlated paternity decreased significantly (Wilcoxon's test Z = 2.20, P = 0.03), although still remained relatively high with a mean of 0.30 (± 0.02). These results translated into a mean number of pollen donors (N_{ep}) of 3.27 ± 0.56 and average effective pollination neighbourhood area (A_{ep}) of 0.09 ± 0.04 ha, which corresponds to a radius around the maternal plants from 7.95-29.48 m. Accordling, when considering only outcrossed seeds (see Table 3), the mean N_{ep} values increased to 7.06 ± 1.43 and average of A_{ep} to 0.26 ± 0.06 ha.

Table 1. Mean values of genetic variation parameters for adult plants (± SE) and progenies (with 95% CI) of *Myrtus communis* and *Pistacia lentiscus*: A, mean number of alleles per locus; A_e, number of effective alleles per locus; H_e, expected heterozygosity; H_o, observed heterozygosity and F_{IS}, inbreeding coefficient. Values in bold indicate progeny parameters significantly different from the adult ones.

	Adults (±SE)						Progeny (95%CI)					
	C		*FC*		*FI*		*C*		*FC*		*FI*	
	AZN	DHY	BNC	SJV	CRB	VIS	AZN	DHY	BNC	SJV	CRB	VIS
Myrtus communis												
n	20	20	20	20	20	20	316	314	312	318	312	316
A	5.30±1.42	6.70±2.16	5.70±1.77	6.70±3.06	5.60±2.41	5.60±2.17	5.11(4.50-5.63)	**5.65(5.00-6.25)**	5.39(5.00-5.88)	6.21(5.63-6.88)	6.50(5.60-7.25)	5.24(4.75-5.75)
A_e	3.22±1.17	3.68±1.42	3.39±1.04	4.08±1.88	3.56±1.67	3.24±1.14	2.93(2.64-3.23)	**3.08(2.74-3.49)**	3.26(2.99-3.55)	3.78(3.33-4.26)	4.10(3.50-4.59)	3.05(2.75-3.37)
H_e	0.64±0.16	0.69±0.11	0.67±0.15	0.68±0.19	0.63±0.25	0.63±0.23	**0.59(0.54-0.63)**	**0.64(0.60-0.68)**	0.64(0.61-0.67)	0.68(0.64-0.72)	0.66(0.63-0.69)	**0.59(0.55-0.62)**
H_o	0.53±0.26	0.58±0.22	0.54±0.29	0.55±0.22	0.53±0.27	0.53±0.23	**0.40(0.35-0.46)**	**0.40(0.34-0.46)**	**0.44(0.37-0.50)**	0.49(0.41-0.57)	0.48(0.42-0.55)	**0.44(0.38-0.51)**
F_{IS}	0.17±0.34	0.17±0.28	0.19±0.34	0.16±0.29	0.16±0.22	0.15±0.19	**0.33(0.20-0.45)**	**0.40(0.30-0.50)**	**0.34(0.23-0.45)**	0.28(0.16-0.40)	0.31(0.17-0.43)	0.24(0.14-0.37)
Pistacia lentiscus												
n	20	20	20	20	19	18	290	314	261	315	282	255
A	8.57±5.32	7.86±4.34	8.71±5.25	7.71±3.45	8.14±4.49	7.43±4.61	**6.45(5.86-7.00)**	**6.11(5.57-6.71)**	**6.63(5.86-7.29)**	**6.57(5.86-7.14)**	**6.36(5.57-7.00)**	**6.15(5.43-6.86)**
A_e	4.54±3.21	4.17±2.31	4.26±2.68	4.03±1.96	4.14±2.09	3.84±2.51	4.13(3.65-4.59)	3.91(3.50-4.32)	4.17(3.71-4.59)	4.02(3.50-4.51)	3.91(3.40-4.43)	3.92(3.44-4.34)
H_e	0.67±0.23	0.67±0.21	0.63±0.28	0.67±0.21	0.66±0.24	0.62±0.25	0.66(0.63-0.69)	0.65(0.62-0.68)	0.64(0.61-0.66)	0.65(0.61-0.68)	0.65(0.62-0.68)	0.64(0.61-0.67)
H_o	0.60±0.25	0.64±0.23	0.58±0.30	0.61±0.18	0.68±0.28	0.68±0.30	0.65(0.59-0.72)	0.63(0.56-0.69)	0.63(0.57-0.69)	0.61(0.54-0.68)	0.64(0.57-0.71)	0.64(0.57-0.71)
F_{IS}	0.14±0.20	0.07±0.13	0.15±0.22	0.09±0.13	0.00±0.30	0.00±0.30	0.03(-0.08-0.14)	0.06(-0.06-0.19)	0.02(-0.09-0.16)	0.08(-0.05-0.21)	0.04(-0.09-0.18)	0.02(-0.12-0.16)

Table 2 Mating system parameters in *Myrtus communis* and *Pistacia lentiscus* populations: t_m, multilocus outcrossing; t_m-t_s, biparental inbreeding; r_p, correlated paternity; $r_{p\_without\_self}$, correlated paternity of outcrossed seeds; Φ_{FT}, pollen cloud genetic differentiation and N_{ep}, effective number of pollen donors. Mean values (±SE) are reported.

	N_{adults}	$n_{progeny}$	t_m	t_m-t_s	r_p	$r_{p\_without\_self}$	Φ_{FT}	N_{ep}
Myrtus communis								
C								
AZN	20	316	0.45±0.05	0.14±0.03	0.58±0.06	0.24±0.04	0.34±0.05	2.30±1.36
DHY	20	314	0.69±0.04	0.26±0.03	0.49±0.06	0.32±0.04	0.27±0.03	2.63±1.37
FC								
BNC	20	312	0.60±0.05	0.19±0.03	0.56±0.06	0.35±0.04	0.34±0.04	3.15±1.36
SJV	20	318	0.70±0.05	0.23±0.03	0.39±0.06	0.22±0.04	0.22±0.01	6.72±1.36
FI								
CRB	20	312	0.59±0.05	0.23±0.03	0.51±0.06	0.35±0.05	0.30±0.02	2.45±1.39
VIS	20	316	0.62±0.05	0.25±0.03	0.51±0.05	0.32±0.04	0.31±0.04	2.32±1.36
Total	120	1888	0.61±0.02	0.21±0.01	0.51±0.02	0.30±0.02	0.30±0.02	3.27±0.56
Pistacia lentiscus								
C								
AZN	20	290	1.20±0.00	0.22±0.03	0.09±0.03	-	0.05±0.01	11.23±121.00
DHY	20	314	1.00±0.05	0.23±0.03	0.04±0.03	-	0.02±0.01	54.34±117.78
FC								
BNC	20	261	0.99±0.07	0.17±0.03	0.12±0.03	-	0.07±0.01	47.37±117.78
SJV	20	315	1.03±0.07	0.25±0.03	0.14±0.03	-	0.08±0.01	272.95±114.80
FI								
CRB	19	283	1.02±0.08	0.18±0.03	0.10±0.03	-	0.06±0.01	42.84±121.01
VIS	18	254	1.01±0.09	0.19±0.03	0.11±0.04	-	0.05±0.01	28.32±121.01
Total	117	1717	1.04±0.03	0.21±0.01	0.10±0.01	-	0.06±0.008	91.11±48.19

As expected in a dioecious species, estimated t_m in *Pistacia* was very close to one (Table 2). This species displayed low correlated paternity ($r_p = 0.10 \pm 0.01$) and global differentiation among pollen gene pools ($\Phi_{FT} = 0.06 \pm 0.008$) but relatively high biparental inbreeding (t_m-$t_s = 0.21 \pm 0.01$). Accordingly, the average N_{ep} was high 91.11 ± 48.19 and the effective pollination neighbourhood area (A_{ep}) low (0.38 ± 0.10 ha), corresponding to a radius around the maternal plants of 17.95-47.10 m.

At family level, mating system showed high variation in both *Myrtus* and *Pistacia*. For example, correlated paternity ranged in *Myrtus* populations from minimum values such as 0.01 (SJV) and maximum values of 0.16 (DHY) to 1.00. Likewise, in *Pistacia*, we found correlated paternity values ranging from minimum values as null (AZN, BNC, CRB, DHY, VIS) to 0.002

(SJV) and maximum values 0.13 (DHY) to 0.72 (VIS). *Myrtus* outcrossing rates also varied highly within populations, from minimum values of null (AZN and SJV) to 0.09 (BNC) and maximum values of 0.69 (AZN) to 1 (BNC).

Model construction and selection

Generalized linear mixed models (GLMMs) revealed the importance of patch features and ecological factors on mating system patterns in both species. However, we found local environmental variables to affect mating system differently in *Myrtus* and *Pistacia*.

Table 3. Results from generalized mixed effects models (GLMMs) examining the patch and ecological characteristics influence on mating system parameters of *Myrtus communis*. Best model is presented for each response variable, with respective Akaike's information criteria (AIC) and Akaike weight (w_i); the relative importance (%) of each explanatory variable is also given. Significant *P*-values in bold.

	Correlated paternity (r_p)			Outcrossing rate (t_m)		
	t/X²-value	*P*	Rel. imp. (%)	*t/X²-value*	*P*	Rel. imp. (%)
Intercept	**2.53**	**0.013**		**4.41**	**<0.001**	
Population type			14			15
Canopy neighbourhood density			29			36
Conspecific neighbourhood density	**-2.88**	**<0.001**	96	**2.46**	**0.016**	87
Flowering synchrony	0.37	0.715	96	-0.78	0.434	94
Flow. Synchrony x Consp. density	**2.65**	**0.009**	85	**-2.25**	**0.027**	68
Population identity[1]	0.21	0.650		2.99	0.080	
df		6			6	
AIC		8.31			-19.28	
w_i		0.53			0.37	

[1] Population identity significance was derived using likelihood ratio tests (see text for details).

In *Myrtus*, the best-fitting GLMM revealed the same set of explanatory variables for t_m and r_p (Table 3). Population identity, modelled as a random factor in GLMMs, was not significant in the models (*P* > 0.05, Table 3), indicating that maternal plants from the same population showed no correlated values of r_p or t_m. Conspecific neighbourhood density had a significant effect in *Myrtus* mating system (with a relative importance of 96% and 87% among all the models considered for r_p and t_m, respectively). This variable had a negative effect on r_p and positive effect on t_m. However, the effect of conspecific neighbourhood density was dependent on the flowering synchrony as it is showed by the significant interaction between these two variables (Table 3, Fig. 2). This interaction (relative importance of 85% and 68%) revealed a significantly negative influence of the conspecific neighbourhood on the correlated paternity but only in poorly synchronized maternal plants. Highly synchronized maternal plants showed no influence of conspecific neighbourhood density on r_p values. We observed the same trend on the response variable r_p after excluding the selfing events (Table 4), although with marginal significance only (conspecific neighbourhood density: t = -1.87, P = 0.06 and interaction: t = 1.72, P = 0.09). No significant effects of any measured ecological variables on biparental inbreeding rates were found in *Myrtus* (results not shown).

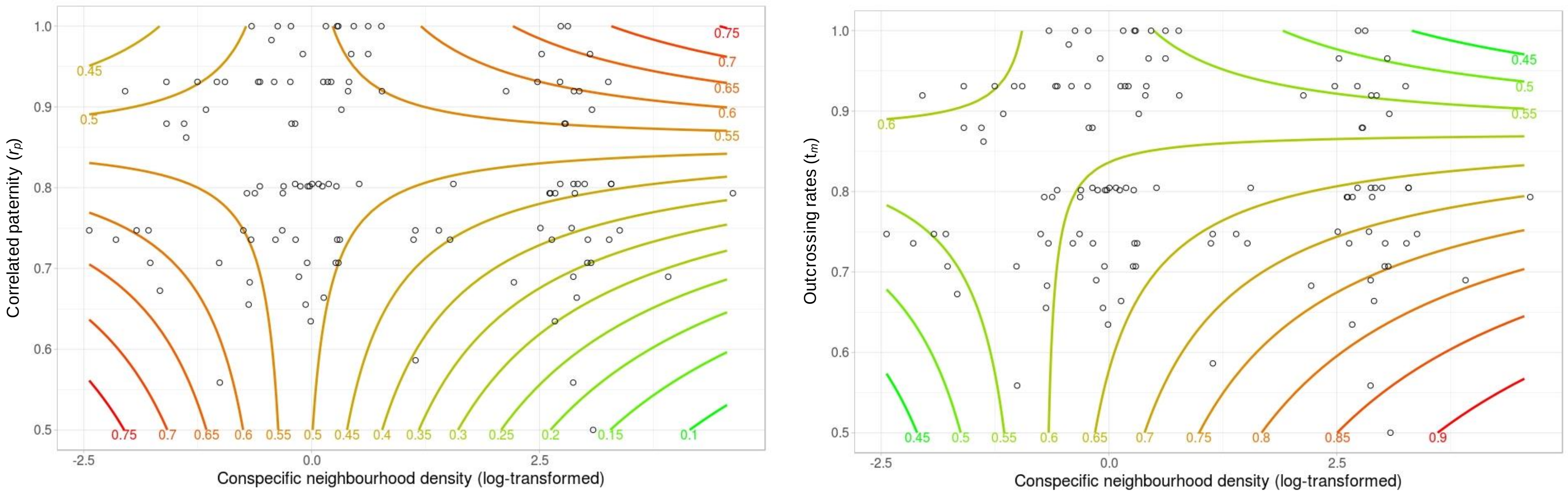

Figure 2. Relationships of conspecific neighbourhood density with correlated paternity (r_p) and outcrossing rates (t_m) in *Myrtus communis*.

Table 4. Results from generalized mixed effects models (GLMMs) examining the patch and ecological characteristics influence on correlated paternity excluding selfing events of *Myrtus communis.* Best model is presented, with respective Akaike's information criteria (AIC) and Akaike weight (w_i); the relative importance (%) of each explanatory variable is also given. *P* -values in bold.

	t / X^2	P	Rel. imp. (%)
Intercept	2.96	**0.004**	
Population type			31
Canopy neighbourhood density			36
Conspecific neighbourhood density	-1.87	**0.064**	65
Flowering synchrony	-0.91	0.366	48
Flow. Synchrony x Consp. density	1.72	**0.089**	20
Population identity[1]			
df		6	
AIC		-39.17	
wi		0.11	

[1] Population identity significance was derived using likelihood ratio tests (see text for details).

In *Pistacia*, GLMMs evidenced a strong influence of local environment on mating system (Table 5, Fig. 3). Population identity was not significant in explaining levels of variation in r_p or t_m-t_s (P > 0.05, Table 5). 'Maximum flowering synchrony' showed a significant and negative effect in r_p (relative importance of 100%), meaning that correlated paternity decreases when flowering synchrony increases. However, it is important to emphasize that the model selection approach revealed significant effects of only 'maximum flowering synchrony' and not of 'total flowering synchrony'. *Pistacia* correlated paternity was also conditioned by population type and the distance of the maternal plant to the patch edge (relative importance of 66% and 61%, respectively). While *FC* populations were not significantly different in correlated paternity from both *C* and *FI* populations (t = 2.12, P = 0.12 and t = 0.46, P = 0.66), *C* populations had marginally lower correlated paternity than *FI* populations (t = 2.89, P = 0.06). The distance of the maternal plant to the forest edge affected positively and significantly correlated paternity, maternal plants closer to the border of the forest patch displaying lower values. Regarding biparental inbreeding, we observed that both population type (relative importance of 69%) and canopy density around the maternal plant (relative importance of 92%) influenced *Pistacia* mating with relatives (Table 6; Fig. 3). Moreover, the canopy density around the maternal plant affected negatively levels of *Pistacia* biparental inbreeding, indicating that under higher densities mating between related plants is reduced.

Table 5. Results from generalized mixed effects models (GLMMs) examining the patch and ecological characteristics influence on mating system parameters of *Pistacia lentiscus.* Best model is presented for each response variable, with respective Akaike's information criteria (AIC) and Akaike weight (w_i); the relative importance (%) of each explanatory variable is also given. Significant *P*-values in bold.

	Correlated paternity (r_p)			Biparental inbreeding (t_m-t_s)		
	t/X²-value	*P*	Rel. imp. (%)	*t/X²-value*	*P*	Rel. imp. (%)
Intercept	0.67	0.51		**12.52**	**<0.001**	
Population type	**8.37**	**0.02**	66	**6.89**	**0.03**	69
Distance to patch edge	**2.11**	**0.04**	61			69
Canopy neighbourhood density			32	**-3.20**	**0.002**	92
Conspecific neighbourhood density			44			46
Maximum flowering synchrony	**-4.14**	**<0.001**	100			49
Population identity[1]	4.95	0.99		3.64	1.00	
df		8			6	
AIC		-204.77			-163.85	
w_i		0.22			0.13	

[1] Population identity significance was derived using likelihood ratio tests (see text for details).

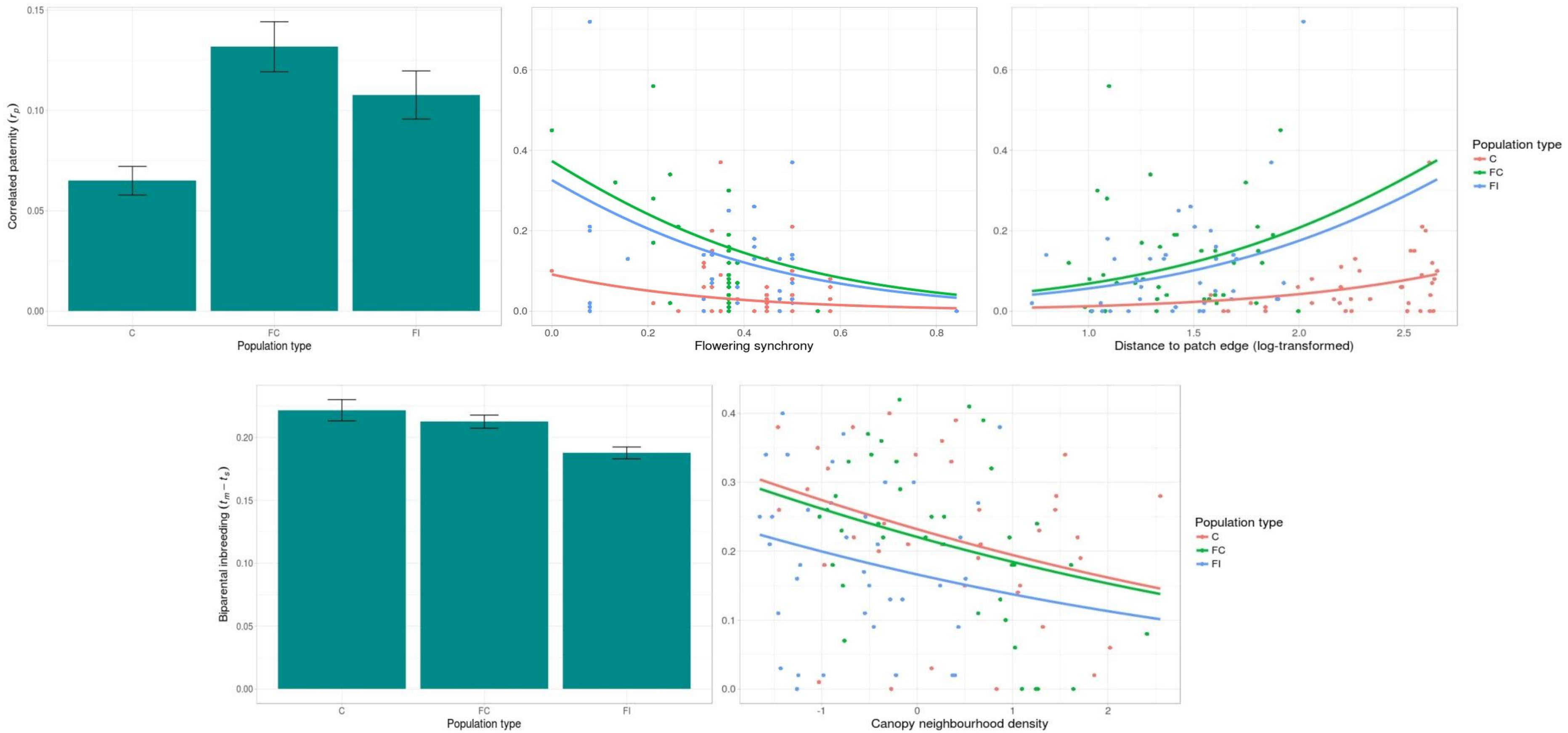

Figure 3. Relationships between patch (population type and distance to patch edge) and ecological (floral synchrony and canopy neighbourhood density) characteristics and mating system parameters (correlated paternity and biparental inbreeding) in *Pistacia lentiscus*. .

DISCUSSION

Mating system studies in fragmented habitats are fundamental to understand the genetic consequences of habitat fragmentation and its impact on populations' genetic diversity and compromising populations' persistence (Loveless & Hamrick 1984). Our study provides new insights about the mating system patterns and its determinants on two Mediterranean shrubs with similar life history traits, demonstrating that mating system patterns are complex and highly variable among species and populations. Here, we also demonstrate that local ecological heterogeneity is an outstanding factor underlying such variation.

Genetic variation and mating system

The analyses of genetic variation across cohorts revealed a trend towards a decrease of genetic diversity in offspring when compared to adults in both species. These results suggest that the amount of genetic variation that will be available for the next generation is lower than the one the current adult population harbors and are in agreement with previous studies showing a decline of genetic diversity in offspring within fragmented populations (Aguilar *et al*. 2008; Sork & Smouse 2006; Eckert *et al.* 2009; Vranckx *et al.* 2011). However, these results should be interpreted with caution because in this study we only analysed a sample of progenies from a single year (which could not represent the whole fruiting period, because seed sampling was concentrated in a short period and not all seeds ripe at the same time).

In our study, we found disparate mating system patterns between the two study species, despite their similar life-history traits, probably due to differences in their breeding and dispersal systems. Moreover, the insect-pollinated and self-compatible *Myrtus* presented higher rates of correlated paternity, biparental inbreeding and global differentiation among pollen gene pools, even when considering only outcrossing events. The observed values of N_{ep} on *Myrtus* populations were low, indicating that the majority of seeds are sired by only a few pollen donors and only few progeny has different fathers. These results seem consistent with values reported for other insect-pollinated species (Smouse & Sork 2004). In general, animal-pollinated species have inherently lower N_{ep} values than wind-pollinated ones (Smouse & Sork 2004), even though some pollinators are capable to move considerable distances (e.g. *Ficus sycomorus* pollinators, Ahmed *et al.* 2009). Accordingly, *Pistacia* populations displayed lower values of correlated paternity and a higher effective number of pollen donors (N_{ep} = 11.78), similar to other wind-pollinated species (N_{ep} ~ 10 in *Quercus semiserrata*, Pakkad *et al.* 2008 and *Pinus echinata*, Dyer & Sork 2001). Interestingly, both species displayed similar and relatively high levels of biparental inbreeding (see below).

Factors influencing mating patterns

Pollen dispersal idiosyncrasy is influenced by landscape context and ecological variables (Sork *et al.* 1999). The high level of variability detected among maternal plants in our study in both species confirms this fact. In *Myrtus* we found local ecological factors played a fundamental role in shaping mating system. Patterns of pollen-mediated gene flow are significantly influenced by the spatial arrangement of individuals on the landscape (Meagher & Vassiliadis 2003), which could explain that the conspecific neighbourhood density were the most determinant factor in our study of *Myrtus* mating system (negative effect on r_p and positive effect on t_m). A previous study on *Myrtus* in the same region found a similar trend towards a positive correlation between conspecific aggregation and outcrossing rates (González-Varo *et al*. 2009). In this study, we have also detected a significant interaction between conspecific neighbourhood density and the flowering synchrony of the maternal plant with respect to the population, which is another important factor shaping mating system patterns (Hardy *et al.* 2004). This interaction revealed that the conspecific neighbourhood only had a significantly negative influence on the correlated paternity in poorly synchronized maternal plants. In fact, we consider that these two effects (conspecific neighbourhood density and flowering phenological synchronization) are intrinsically related because pollinators are expected to alter their foraging strategies to match the distribution of floral resources (Pyke 1984). In isolated plants or when few flowering plants are available, insect pollinators will concentrate all the effort where most of the floral rewards are. Consequently, accordingly to the theory of optimal foraging, pollinators will spend more time in the same plant and will be less likely to shift from one plant to another due to their inherent increased cost (Heinrich & Raven 1972; Charnov 1976; Pyke 1984; Ottewell *et al*. 2009). This is especially true for honeybees (*Apis mellifera*), the most frequent pollinator in the species (González-Varo et al. 2009; Chapter 2), which prefer foraging for long periods of time in the same plant and tend to move among neighbouring plants (Monzon *et al.* 2004). This reduction in the pollination neighbourhoods (Breed *et al.* 2013) will eventually lead to the increase of selfing (Karron *et al*. 2009) and also the decrease of provision of different pollen sources (i.e. increasing correlated paternity; Bianchi & Cunningham 2012), as confirmed in this study. Besides, even though all *Myrtus* populations presented high values of flowering synchrony (*C*: 0.86 ± 0.02; *FC*: 0.81 ± 0.02; *FI*: 0.79 ± 0.02, S. Nora unpublished results), our analyses indicated that maternal plants from continuous populations displayed higher flowering synchrony than maternal plants from fragmented-connected (t = -3.07; P = 0.003) and fragmented-isolated (t = -2.30; P = 0.02) populations. Even though, we must be cautious with the interpretation of these results because other effects such as those derived from inbreeding, which we cannot tear apart in this study, might have been also influencing the studied relationships. Our results highlight the indirect effects of habitat fragmentation which will eventually drive to an increment of selfing events and correlated paternity and, consequently, to the impoverishment of genetic diversity available for the next generation.

Unlike *Myrtus*, *Pistacia* mating system patterns were mostly influenced by habitat patch characteristics, involving population type and distance of the maternal plant to the forest edge. Fragmentation influenced positively *Pistacia* correlated paternity. Maternal plants from continuous populations displayed lower correlated paternity values than maternal plants from fragmented-connected and fragmented-isolated populations. Further, the distance of the maternal plant to the forest patch edge negatively affected correlated paternity, leading to lower correlated paternity values in maternal plants closer to the patch edge. The negative effects of habitat fragmentation in *Pistacia* are in agreement with some meta-analyses that have revealed a trend towards an increase of selfing and correlated paternity with habitat fragmentation (Breed *et al.* 2003; Aguilar *et al.* 2008). These results also supported recent studies that acknowledge that even wind-pollinated species might be vulnerable and susceptible of genetic erosion as insect-pollinated species are, in contrast to what was previously assumed (Sork & Smouse 2006). The fact that maternal plants near the patch edges had lower values of correlated paternity suggests that these plants could be the recipient of pollen from other forest patches.

This incoming pollen could potentially be buffering the negative effects of habitat fragmentation (Dick *et al.* 2003; Byrne et al. 2008). In fact, long distance dispersal events have been frequently reported for wind–pollinated species (Bacles *et al.* 2005; Robledo-Arnuncio & Gil 2005; Buschbom *et al.* 2011). Specifically, long distance pollen movement was detected in *Pistacia* in a highly fragmented landscape (Albaladejo *et al.* 2012). However, these maternal plants, even if experiencing lower levels of correlated paternity, still have to bare large microclimatic changes associated to the edge of the forest patch that may affect further key stages of the life cycle such as fructification, seed dispersal or seedling recruitment (Tomimatsu & Ohara 2004). We have observed relatively high levels of biparental inbreeding in *Pistacia*, indicating that although some maternal plants could be receiving a non-negligible amount of pollen flow from outside the population, an important fraction of the pollen-mediated gene flow is due to effective pollination events from related individuals. Curiously, no relation was found between mating system parameters and the conspecific neighbourhood density of maternal plants at the 10 m radius buffer we established. The selected radius in this study could have been insufficient for this species. In fact, the estimates of effective pollination neighbourhood area in *Pistacia* populations were all well over the 10 m radius. The fact that continuous populations presented the highest levels of biparental inbreeding also points in that direction. Biparental inbreeding was also affected negatively in *Pistacia* populations by the canopy density around the maternal plants. This result is contradictory with the current literature where canopy density is often regarded as a barrier for pollen mediated gene flow (Dyer & Sork 2001; Guazere *et al.* 2013). Surely other factors, not accounted for in this study, are affecting biparental inbreeding levels and could help to explain this result.

Divergent flowering times are expected to promote mating system variation (Fuchs *et al.* 2003; Robledo-Arnuncio *et al.* 2004). This flowering lag can have a strong effect in mating system patterns, not only in animal-pollinated species, but also in wind-pollinated species as we have found in *Pistacia*, where flowering synchrony showed a significant negative effect in the levels of correlated paternity. Low availability of males at the time of maternal plant stigma's receptivity will conduct to a low diversity of pollen pools received by maternal plants and thus to an increase of correlated paternity. Curiously, because *Pistacia* is wind-pollinated, this trend was only observed during the maximum flowering period (when more than 50% of the flowers from the same plant are flowering). This result contrasted with the one obtained in *Myrtus* (where the trend was observed all over the flowering period), apparently because *Myrtus* is entomophilous and, therefore, has a more effective pollen transfer system than *Pistacia* (anemophilous).

Conclusions

The main aim of this study evaluate shifts in contemporary mating patterns caused by landscape heterogeneity (habitat fragmentation and isolation) and the local environment (within population) impact the genetic diversity of naturally produced progeny of two species with some similar life-history traits. Our results highlight that habitat fragmentation and within patch ecological variation have important consequences in shifting mating system patterns, depending mainly on species' pollen dispersal attributes, reinforcing the need for more multispecies studies. In accordance with recent meta-analyses of the effects of habitat fragmentation on plant mating patterns (Aguilar *et al.* 2008; Breed *et al.* 2013), we observed both direct and indirect negative effects of habitat fragmentation, through an increase of selfing rates and correlated paternity, but only in the wind-pollinated species (*Pistacia*). Moreover, specially derived from results in *Pistacia*, we detected the importance of small patches of vegetation within the landscape (as long as they are not highly isolated), stressing their conservation value in maintaining genetic connectivity at the landscape level. However, our study only evaluates the genetic variability left available for the next generation, and the effective quantity and quality of the pollen pool transmitted successfully to successive generations still needs further investigation. Therefore, further long-term assessment is needed to understand the wide effects of habitat fragmentation.

This study highlights the need for more studies with common species, which are fundamental components of their ecosystems and, as shown in this study, are as equally susceptible to habitat fragmentation. It also highlights the importance of incorporate observations of local ecological characteristics that indirectly could affect species mating systems on fragmentation studies. Not forgetting the importance that both the ecological characteristics and heterogeneity of landscape and within-patch have in making effective conservation planning and management decisions.

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SUPPLEMENTARY INFORMATION

Table S1. Ecological characteristics of the studied populations of *Myrtus communis* and *Pistacia lentiscus*. Population types are: continuous (*C*), fragmented-connected (*FC*) and fragmented-isolated (*FI*) populations.

	C populations		FC populations		FI populations	
	AZN	DHY	BNC	SJV	CRB	VIS
Myrtus communis						
Mean distance to nearest patch edge (m)	364.76±6.05	124.82±6.05	22.94±6.05	53.83±6.05	31.95±6.05	43.76±6.05
Mean flowering synchrony	0.87±0.07	0.86±0.12	0.82±0.15	0.75±0.11	0.77±0.11	0.81±0.11
(max. period)	0.65±0.19	0.40±0.07	0.81±0.18	0.58±0.14	0.56±0.08	0.53±0.19
Mean distance to nearest conspecific (m)	3.69±1.12	2.56±1.15	1.64±1.12	1.39±1.12	8.99±1.15	1.81±1.12
Mean nº conspecific neighbours	0.40±0.35	2.37±0.36	0.50±0.35	1.60±0.35	1.84±0.36	0.45±0.35
Mean canopy density neighbouhood (%)	14.17±1.09	12.99±1.09	17.42±1.14	14.38±1.04	14.31±1.20	10.54±1.20
Pistacia lentiscus						
Mean distance to nearest patch edge (m)	384.99±8.24	123.42±7.82	17.27±8.02	48.27±8.24	33.34±5.75	44.94±8.13
Mean flowering synchrony	0.76±0.06	0.69±0.13	0.50±0.11	0.62±0.12	0.72±0.13	0.70±0.12
(max. period)	0.39±0.09	0.44±0.13	0.36±0.07	0.32±0.11	0.37±0.18	0.38±0.16
Mean distance to nearest male conspecific	4.52±1.30	3.16±1.30	7.98±1.30	2.42±1.37	5.48±1.30	2.47±1.30
Mean nº conspecific neighbours	1.85±0.42	3.10±0.42	1.20±0.42	3.83±0.45	2.20±0.42	4.15±0.42
Mean canopy density neighbouhood (%)	15.48±1.14	15.40±1.14	15.56±1.08	15.03±1.21	17.09±1.03	13.32±1.03

4

Correlated paternity effects on progeny performance

Nora S, Aparicio A, Albaladejo RG. 2016. High correlated paternity leads to negative effects on progeny performance in two Mediterranean shrub species.

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Efectos de la fragmentación del hábitat en la polinización y en la ecología vegetal reproductiva

Resumen

El deterioro antrópico del hábitat puede promover cambios en los sistemas de apareamiento de las plantas que posteriormente pueden afectar a la viabilidad de la progenie, condicionando así el reclutamiento de plantas para la próxima generación. Sin embargo, la mayoría de los estudios se centra en el parámetro tasa de cruzamiento, mientras que otros parámetros como la paternidad correlacionada han sido raramente estudiados. En este estudio, se investigó la relación entre la paternidad correlacionada y la viabilidad de la progenie de *Myrtus communis* y *Pistacia lentiscus*. Para ello, estimamos los parámetros del sistema de apareamiento utilizando marcadores genéticos de microsatélites y realizamos experimentos de siembra en el invernadero y en el campo. Nuestros resultados mostraron que algunos componentes de la viabilidad de la progenie fueron afectados negativamente por la alta paternidad correlacionada de las plantas maternas. En *Myrtus*, la alta paternidad correlacionada tuvo un efecto negativo sobre la proporción y el tiempo de emergencia de las plántulas en el campo y en el invernadero, respectivamente. En *Pistacia*, el tiempo de emergencia de las plántulas en el campo también fue influenciado negativamente por la alta paternidad correlacionada y un análisis de supervivencia de la progenie en el campo demostró una mayor mortalidad de plántulas de plantas maternas con alta paternidad correlacionada. En general, encontramos efectos de la paternidad correlacionada en la viabilidad de la progenie de *Myrtus*, una especie autocompatible. Además, también se detectaron efectos de la paternidad correlacionada en el tiempo de emergencia y la supervivencia de la progenie en *Pistacia*, una especie auto-incompatible. Este estudio representa uno de los pocos ejemplos empíricos existentes que destacan la influencia que la paternidad correlacionada puede ejercer sobre la viabilidad de la progenie en múltiples etapas durante el crecimiento temprano de las plántulas.

Palabras clave: Experimento de campo; experimento en invernadero; fitness de la progenie; paternidad correlacionada; sistemas de apareamiento; tasas de cruzamiento.

Abstract

Anthropogenic habitat deterioration can promote changes in plant mating systems that subsequently may affect progeny performance, thereby conditioning plant recruitment for the next generation. However, very few studies yet tested mating system parameters other than outcrossing rates; and the direct effects of the genetic diversity of the pollen received by maternal plants (i.e. correlated paternity) has often been overlooked. In this study, we investigated the relation between correlated paternity and progeny performance in two common Mediterranean shrubs, *Myrtus communis* and *Pistacia lentiscus*. To do so, we collected open-pollinated progeny from selected maternal plants, calculated mating system parameters using microsatellite genotyping and conducted sowing experiments under greenhouse and field conditions. Our results showed that some progeny fitness components were negatively affected by the high correlated paternity of maternal plants. In *Myrtus,* high correlated paternity had a negative effect on the proportion and timing of seedling emergence in the natural field conditions and in the greenhouse sowing experiment, respectively. In *Pistacia*, seedling emergence time under field conditions was also negatively influenced by high correlated paternity and a progeny survival analysis in the field experiment showed greater mortality of seedlings from maternal plants with high correlated paternity. Overall, we found effects of correlated paternity on the progeny performance of *Myrtus*, a self-compatible species. Further, we also detected effects of correlated paternity on the progeny emergence time and survival in *Pistacia*, an obligate outcrossed species. This study represents one of the few existing empirical examples which highlight the influence that correlated paternity may exert on progeny performance in multiple stages during early seedling growth.

Keywords: Correlated paternity, field experiment, greenhouse experiment, mating system, outcrossing rates, progeny fitness.

INTRODUCTION

Mating patterns are fundamental for determining the amount and distribution of genetic variation within and between populations of plant species (Hamrick *et al*. 1996; Duminil *et al.* 2007). Anthropogenic habitat deterioration may promote changes in mating patterns (Coates *et al.* 2007; Eckert *et al.* 2009), which can directly influence the genetic variation in progeny and, even more importantly, may be reflected in fitness costs that will compromise plant recruitment in the forthcoming generation (Lowe *et al.* 2005; Eckert *et al.* 2009). Mating system can be described by parameters such as selfing, outcrossing rates and correlated paternity. Numerous studies have found high variability in mating parameters such as outcrossing rates and correlated paternity levels among species(Eckert *et al.* 2009), among populations within species (Rocha *et al.* 2001; Mimura *et al.* 2009) and even among maternal plants (e.g. García *et al.* 2005; Oddou-Muratorio *et al*. 2006; Byrne *et al.* 2007; de Lucas *et al.* 2008; González-Varo *et al.* 2009a). This variation is highly context-dependent and reflects the influence of diverse ecological factors such as landscape heterogeneity, vegetation structure and the local neighbourhood of conspecific plants (Franceschinelli & Bawa 2000; García *et al.* 2005; de Lucas *et al*. 2008). Nevertheless, mating system parameters are mainly controlled by plant reproductive traits that permit or prevent self-fertilization (i.e. the breeding system; see Neal & Anderson 2005).

However, although numerous studies aimed at evaluating variation in mating systems within species (Ghazoul 2005; Lowe *et al.* 2005), only a few have ever focused on their influence on progeny performance (Breed *et al.* 2014, 2015). Furthermore, most current studies consider no other mating system parameter than selfing rates and overlook the direct effects of the genetic diversity of the pollen received by the maternal plants (but see Breed *et al*. 2012a, 2012b; Llorens *et al.* 2013). Correlated paternity (the proportion of full-sibs within maternal progeny arrays) is an important parameter that can provide deep insights into the pollination biology of plant species (Hardy *et al.* 2004; Verdú *et al.* 2006). Pollen diversity received by the maternal plants may be advantageous for progeny fitness because it can promote pollen competition, thereby allowing post-pollination selection for those males with the fastest-growing pollen tubes (Yasui 1998; Pannel & Labouche 2013), and/or may increase female choice by post-pollination mechanisms occurring both before ovule fertilization and during seed development (as cryptic female choice) and enable them to select between progeny that differ in quality and/or compatibility (Yasui 1998; Pannel & Labouche 2013).

In this study, we analysed how correlated paternity affects progeny performance in two common shrub species, *Pistacia lentiscus* L. (Anacardiaceae) and *Myrtus communis* L. (Myrtaceae) (*Pistacia* and *Myrtus*, hereafter). Even though their life-history traits are similar in a number of ways (e.g. both are long-lived and their seeds are dispersed mainly by birds), they differ in their breeding and pollen dispersal systems. *Myrtus* has a mixed-mating system with hermaphroditic flowers pollinated by insects, while *Pistacia* is a wind-pollinated dioecious species (and thus an obligate outcrosser). The mating systems of both species have been extensively studied. For instance, Albaladejo *et al.* (2009, 2012) found a wide range for correlated paternity values in *Pistacia* linked to significant spatiotemporal variation (from 0.03 to 0.23), which, to some extent, could be attributed to inherent individual factors (such as phenological synchronisation) or the local neighbourhood of conspecific plants. Additionally, *Pistacia* is capable of extensive pollen movement and high rates of pollen flow (Albaladejo *et al.* 2012). Regarding *Myrtus* pollen dispersal, less information is known. *Myrtus* populations often show very high levels of correlated paternity (in the range 0.40–0.61; (González-Varo *et al.* 2009a, S. Nora, unpublished results). Moreover, variation in outcrossing rates in *Myrtus* populations (from 0.13 to 0.62) has been shown to significantly affect progeny performance, with higher outcrossing rates being associated with higher seedling emergence and survival under greenhouse conditions (González-Varo *et al.* 2010). However, to date no attempts have been made to test the impact of correlated paternity variation on progeny performance in these two species.

Here, we combined mating system analysis with data on progeny performance measurements based on both greenhouse and natural field sowing experiments. Specifically, we aimed (1) to assess the influence of correlated paternity on early progeny performance (here evaluated as seedling emergence, seedling emergence time, seedling growth, biomass, lifetime and survival) in *Myrtus communis* and *Pistacia lentiscus*, and (2) to verify the consistency of the observed relationships both in a greenhouse environment and under natural (field) conditions, where they could be potentially masked by other factors such as the response to environmental heterogeneity.

MATERIAL AND METHODS

Study species and system

Myrtus communis and *Pistacia lentiscus* are widespread and abundant shrubs in sclerophyllous Mediterranean woodlands. In the study area, *Myrtus* grows up to 4 m in height and blooms massively in early summer (from mid-June to early July) and its white flowers are hermaphroditic and pollinated mostly by hymenopterans and dipterans (González-Varo *et al.* 2009b). *Myrtus* fruit are berries (mean ± SD = 5.2 ± 2.7 seeds; González-Varo *et al.* 2010) that turn dark blue when mature. Fruits mature from mid-October to late November and seeds are dispersed by passerine birds, mostly Sylviidae and Turdidae (González-Varo 2010). *Pistacia* is a dioecious shrub up to 4 m in height. It is wind-pollinated and blooms massively between mid-March and late April (Jordano 1988). Its fruits are small black one-seeded drupes and mature in September–December and are consumed by a similar guild of dispersers as those of *Myrtus* (Jordano 1989).

This study was conducted in the Guadalquivir river valley (southwestern Spain), a large (21,000 km^2), fertile and intensively cultivated Mediterranean lowland. This region has a long history of human intervention and management (Valbuena-Carabaña *et al.* 2010) and remnant woodland patches cover less than 1% of their potential area (Aparicio 2008). The study area, Dehesa de las Yeguas (36º33'15''N, 6º08'08''W), is ca. 100 hectares of semi-natural stone pine (*Pinus pinea* L.) forest (Fig 1). In the study area, shrub cover represents 63.82% of the total study area and is mainly composed of small (<1 m) dry-fruited shrubs (Cistaceae, Lamiaceae and Leguminosae) and tall (>1 m) fleshly fruited shrubs, amongst which *Pistacia* and *Myrtus* are the dominant species. The climate is typically Mediterranean, with a mean annual precipitation of ca. 650 mm and an intense summer drought (approximately 10 mm rainfall in July and August), and a mean monthly temperature of 19ºC (ranging from 9ºC in February to 25ºC in August) (data from *subsistema CLIMA* available at http://www.juntadeandalucia.es/medioambiente).

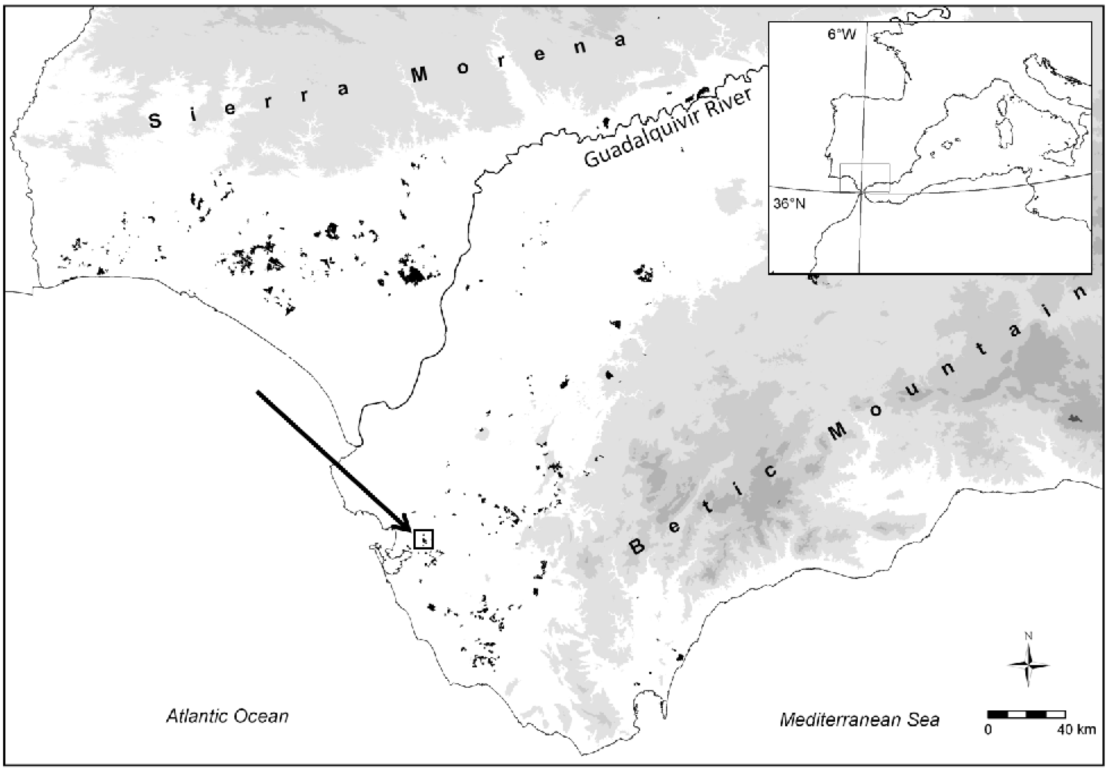

Figure 1. Map of the Guadalquivir River Valley (southwestern Spain), the location of the stone pine forest selected as a study area (Dehesa de las Yeguas). The unshaded area represents lowland areas (< 200 m above sea level), the remaining woodland patches are shaded in black and higher elevations and legally protected areas are in light and dark grey, respectively. The arrow indicates the location of the study area.

In November 2010, we collected naturally produced mature fruits from throughout the canopy of 18 *Myrtus* and 18 *Pistacia* maternal plants. Study plants were selected to cover the range of natural heterogeneity in the local environment (mainly plant density, from clumped to isolated plants). Therefore, local variation was chosen *a priori* to try to capture the highest variability in mating system parameters at individual plant level (Yasui 1998; Franceschinelli & Bawa 2000; García *et al*. 2005; de Lucas *et al.* 2008).

Microsatellite genotyping and individual mating system analyses

For genetic analysis we randomly selected 16 seeds per maternal plant. *Pistacia* embryos were easily excised from the maternal endocarp and endosperm, so they did not require germination. In *Myrtus* only one seed per fruit was used to avoid the effects of correlated pollination in a single pollination event. Seeds from each maternal plant were germinated in separate Petri dishes containing filter paper saturated with distilled water. Previous studies indicate that germination rates in *Myrtus* are very high (González-Varo *et al.* 2009a) and in this study almost all seeds germinated within a few days (mean germination rates ± SD = 95.0% ± 6.9%). For DNA isolation, *Myrtus* seedlings were harvest once both cotyledons were fully developed.

We extracted DNA from *Myrtus* seedlings and *Pistacia* embryos with the Invisorb DNA Plant HTS 96 Kit (Invitek, Germany) following the manufacturer's protocol. We amplified seven and eight polymorphic nuclear microsatellites in *Pistacia* and *Myrtus*, respectively. Further details regarding the amplification reactions and PCR cycle conditions can be found in Albaladejo *et al.* (2008, 2010) and Nora *et al*. (2015). We analysed fluorescently labelled (with 6-FAM, NED, VIC and PET dyes) PCR products with an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, US) at the *Unidad de Genómica* (UCM, Madrid, Spain). Fragment sizes were automatically scored with GeneMapper 3.7 (Applied Biosystems, Foster City, US) and corrected manually when necessary. We only retained multilocus genotypes that successfully scored with at least four loci. Maternal genotypes had already been analysed for a companion study (Nora *et al.* 2015). We checked for genotyping errors by identifying mother-offspring mismatches and obtained an overall mismatching estimate of 1.90% for *Myrtus* and 2.14% for *Pistacia*.

Independence among pairs of loci of maternal genotypes was checked through linkage disequilibrium (LD) tests using the Genepop web server (http://genepop.curtin.edu.au) and tested for null alleles with Micro-Checker (Oosterhout *et al*. 2004). We calculated maternal genetic diversity levels via the homozygosity by loci (*HL*) index, a calculation that improves raw heterozygosity estimates by weighting the contribution of each locus to the individual homozygosity value in terms of their allelic variability (Aparicio *et al.* 2006). We calculated *HL* with the R script Genhet (Coulon 2010). We estimated the multilocus (t_m) and single locus (t_s) outcrossing rates and the correlated paternity (r_p) for maternal plants with the software Mltr 3.4 (Ritland 2002). Pollen and ovules were constrained to have the same gene frequencies and standard errors, and confidence intervals were assessed by bootstrap (1000 replicates) with individuals within families as the resampling unit. For *Pistacia* (as this species is dioecious), we used only the parameters t_m and t_s to estimate the biparental inbreeding (t_m-t_s, i.e. mating between genetically related individuals).

Progeny performance under greenhouse conditions

In April 2011, we individually sowed a total of 720 seeds (20 seeds from each maternal plant of both species) in trays of 60 pots (5 x 5 cm and 17 cm in depth). We filled each pot with horticultural blend (5:1 peat and perlite) and sowed the seeds at a depth of c. 0.5 cm. We watered the trays twice a week and randomly changed their position every 2–3 weeks to ensure homogeneous conditions inside the greenhouse throughout the 424 days of the experiment. We regularly monitored ‘seedling emergence’ (every 2–3 days for two months after sowing) and ‘seedling mortality’ until the end of the experiment. A seedling was regarded to have emerged when it extended cotyledons. For each emerged seedling we also recorded the following data: (1) ‘seedling emergence time’ as the number of days between sowing and seedling emergence (monitored every 2–3 days for two months after sowing), (2) ‘seedling growth’, measured as seedling height at four dates distributed regularly throughout the experiment (days 36, 113, 190 and 267) and including a final measurement at the end (day 424), and (3) ‘seedling biomass’ measured as the dry weight at the end of the experiment. To measure the biomass, after harvesting we dried the surviving plants at 60º C for 72 h and, in order to obtain accurate measurements of the biomass allocation, we separately measured the shoot and root dry weight of each plant.

Progeny performance under field conditions

We performed a sowing experiment using 30 seeds from each maternal plant (totalling 1080 seeds) to assess seedling performance under natural conditions in Dehesa de las Yeguas

(Spain). To avoid bias between seedling emergency of sowed seeds of our experiment and seedlings from natural dispersed seeds, we started this experiment in early March 2011, the end of the natural dispersion period (cf. González-Varo *et al.* 2011). We sowed seeds in three distinct microhabitats: (1) under conspecific shrubs (*Myrtus* seeds under *Myrtus* shrubs and *Pistacia* seeds under *Pistacia* shrubs), (2) under heterospecific shrubs (*Myrtus* seeds under *Pistacia* shrubs and *Pistacia* seeds under *Myrtus* shrubs), and (3) in open ground under tree cover. We focused our experiment on these microhabitats because they are traditionally considered suitable places for birds to drop seeds and are consequently favourable places for seedling recruitment (e.g. Izhaki *et al.* 1991; Herrrera *et al.* 1994; Rey *et al.* 2000; González-Varo *et al.* 2011). We established 10 sowing plots per microhabitat (30 plots in total). In each plot, we removed any naturally dispersed seedlings and sowed uniformly one seed from each of the selected maternal plants (i.e. 18 seeds of each species) at a depth of 0.5–1 cm. To match natural conditions as close as possible, we then added a thin layer of litter on top of the seed after sowing. Each plot was protected by a wire mesh cage (with an upper surface grid area of 15 × 10 cm and 10 cm in height) to prevent predation by rodents.

We conducted this experiment and monitored each emerged seedling until September 2011 (i.e. after the first summer). We monitored ‘seedling emergence’ and ‘seedling mortality’ weekly for three months and fortnightly thereafter until the end of the experiment. Like in the greenhouse, in this field experiment, seedlings were regarded to have emerged when they fully extended cotyledons. For each seedling we also recorded the following variables: (1) ‘seedling emergence time’, as the number of days between sowing and seedling emergence and (2) ‘seedling lifetime’, as the total number of days a seedling lived from emergence until death or until the end of the experiment (for those seedlings that survived). To control soil moisture, we used a time domain reflectometer (TDR, Campbell Scientific Inc., Logan, UT, USA) with 12-cm depth rods to measure soil volumetric water content (VWC; %). This measure is fundamental because the water deficit, especially during the summer, is a critical factor for seedling survival in many Mediterranean plant species (e.g. Herrera *et al.* 1994; Rey *et al.* 2000). Measurements were taken at an adjacent point (~20 cm) to each sowing plot every two weeks in the first three months and monthly thereafter.

Statistical analyses

For each species, we compared seedling performance between experiments (greenhouse vs. field) using the non-parametric Wilcoxon paired test. Correlations between seedling height and seedling total dry biomass, as well as between shoot and root dry biomass were made using Spearman's correlations. We reported data as means ± SE.

We evaluated the relationships between the correlated paternity (r_p) and progeny performance measurements using generalized linear models (GLMs) in R 3.0.2 (R Developmental Core Team 2016). We conducted all analyses at maternal plant level and used a gamma distribution with a log link function for modelling the response variables. To check whether r_p was correlated with other mating system variables we performed Spearman's correlation tests and observed no significant correlations between correlated paternity and any other genetic parameter (HL, t_m-t_s or t_m) neither in *Myrtus* nor in *Pistacia* (S1 Table). We accounted for the effects of seed mass on the response variables 'proportion of seedling emergence', 'seedling growth' and 'seedling lifetime', and the effects of 'seedling emergence time' on the variables 'seedling growth', 'seedling biomass' and 'seedling lifetime' by including these variables as covariates in the models. To obtain the mean seed mass for each maternal plant we randomly sampled 20 seeds per maternal plant (one seed per *Myrtus* fruit) and weighed it to the nearest 0.1 mg.

We evaluated seedling survival rates of *Pistacia* in the field sowing experiment with survival analyses (Kleinbaum 1996) and we tested the effects of correlated paternity using Cox's proportional hazard regression (Cox 1972). This analysis was performed with the software Statistica v.6 (StatSoft 2001).We considered seedlings that at the end of the study were still alive as censored data. No *Myrtus* seedlings survived the first summer in any of the microhabitats (see Results) and so this analysis could not be performed for this species.

RESULTS

Genetic diversity and mating parameters

We genotyped open-pollinated progeny from 18 maternal plants of *Myrtus* (n = 266) and *Pistacia* (n = 283). Microsatellite markers were highly polymorphic in both species with a total of 57 and 40 different alleles identified across all maternal plants for *Myrtus* and *Pistacia*, respectively. Linkage disequilibrium tests showed that all pairs of loci in the two species were independent and null allele presence was not significant at any loci within any species.

Overall, *Myrtus* maternal plants had high levels of homozygosity (mean HL = 0.386 ± 0.035) and correlated paternity (mean r_p = 0.395 ± 0.045) (S2 Table). On the other hand, *Pistacia* maternal plants had relatively low levels of both homozygosity (mean HL = 0.271 ± 0.034) and correlated paternity (mean r_p = 0.060 ± 0.006). Both parameters presented a large variation among *Myrtus* maternal plants: HL ranged from 0.18 to 0.69 and r_p from 0.10 to 0.87. In *Pistacia* maternal plants, we also found high variability in HL levels, ranging from 0.00 to 0.49 but r_p values were more stable among maternal plants, ranging from 0.03 to 0.11. As expected in a dioecious species, estimated outcrossing rate (t_m) in *Pistacia* was very close to one (S2 Table). The mean value of t_m in *Myrtus* maternal plants was 0.704 ± 0.044, ranging from 0.25 to 0.98 (S2 Table). *Myrtus* displayed relatively higher rates of biparental inbreeding than *Pistacia* (t_m-t_s = 0.264 ± 0.027 vs. 0.065 ± 0.005).

Progeny performance under greenhouse conditions

The mean percentage of seedling emergence was 53.33% (± 2.26) in *Myrtus* and 49.17% (± 1.73) in *Pistacia*. *Myrtus* seedlings took on average 20.04 (± 0.40) days to emerge (emergence time was 14–36 days after sowing), while *Pistacia* seedlings took 18.90 (± 0.40) days for emergence (range of 14–44 days) (S3 Table). Seedling mortality was low throughout the experiment in the greenhouse (15.03% in *Myrtus* and 16.38% in *Pistacia*). Approximately one month after the start of the experiment, *Pistacia* seedlings had a mean height of 26.9 (± 0.8) mm and *Myrtus* seedlings a mean of 16.7 (± 0.8) mm (S3 Table). At the end of the experiment, the mean heights were 391.6 (± 9.8) mm for *Pistacia* seedlings and 257.5 (± 9.3) mm for *Myrtus* seedlings. *Pistacia* seedlings had a final mean dry biomass of 2.87 (± 0.04) g and *Myrtus* seedlings 2.39 (± 0.04) g (S3 Table). In both species, seedling height at the end of the experiment was highly correlated with seedling total dry biomass (Spearman's $r_{Myrtus} = 0.87$, $P < 0.001$, and $r_{Pistacia} = 0.77$, $P < 0.001$). Shoot and root dry biomass were also highly correlated in both species (Spearman's $r_{Myrtus} = 0.92$, $P < 0.001$ and $r_{Pistacia} = 0.71$, $P < 0.001$).

The influence of correlated paternity on seedling performance in the greenhouse environment is summarized in Table 1. Correlated paternity had no effect on *Myrtus* 'proportion of seedling emergence', 'seedling growth' or 'seedling biomass' but had a significant positive effect on 'seedling emergence time' (Fig 2). Correlated paternity had no significant effect on *Pistacia* seedling performance measurements ('proportion of seedling emergence', 'seedling emergence time', 'seedling growth' and 'seedling biomass') (Table 1).

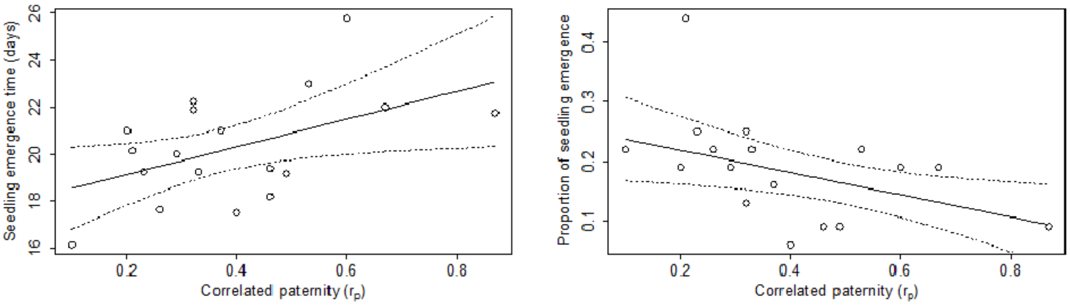

Figure 2. Relationships between correlated paternity and seedling performance variables for *Myrtus communis* derived from GLMs in the greenhouse (left) and field (right) experiment.

The covariate 'seed mass' had a significant positive effect on 'proportion of seedling emergence' in *Myrtus* and in the first measurement of seedling height ('seedling height after 36 days') in both species (Table 1). 'Seedling emergence time' only had a significant effect on seedling height in *Pistacia.*

Progeny performance under field conditions

Pistacia had similar seedling emergence rates in field and greenhouse environments (Wilcoxon's test; $Z = 0.15$, $P = 0.88$). However, field conditions had a strongly significant negative effect on *Myrtus* seedling emergence (Wilcoxon’s test; $Z = 3.59$, $P < 0.001$). Both *Myrtus* and *Pistacia* seedlings took longer to emerge in the field than in the greenhouse (Wilcoxon’s test; $Z = 3.72$, $P < 0.001$ and $Z = 3.72$, $P < 0.001$, respectively) (S3 Table). *Myrtus* seedlings in the field took on average 39.19 (± 0.95) days to emerge, whereas *Pistacia* seedlings emerged slightly earlier, 34.24 (± 0.60) days.

We did not conduct GLMs on seedling survival due to low seedling survival in *Pistacia* (only 40 seedlings survived to the end of the experiment) and due to the lack of surviving *Myrtus* seedlings. *Myrtus* seedling mortality rate rose dramatically from 12.38% at day 29 to approximately 100% at day 78 and was clearly associated with the measured volumetric water content in the ground (see Fig 3). For this reason, the potential of seedling survival for this species was assessed indirectly by using seedlings’ lifetimes as a response variable (see Table 2).

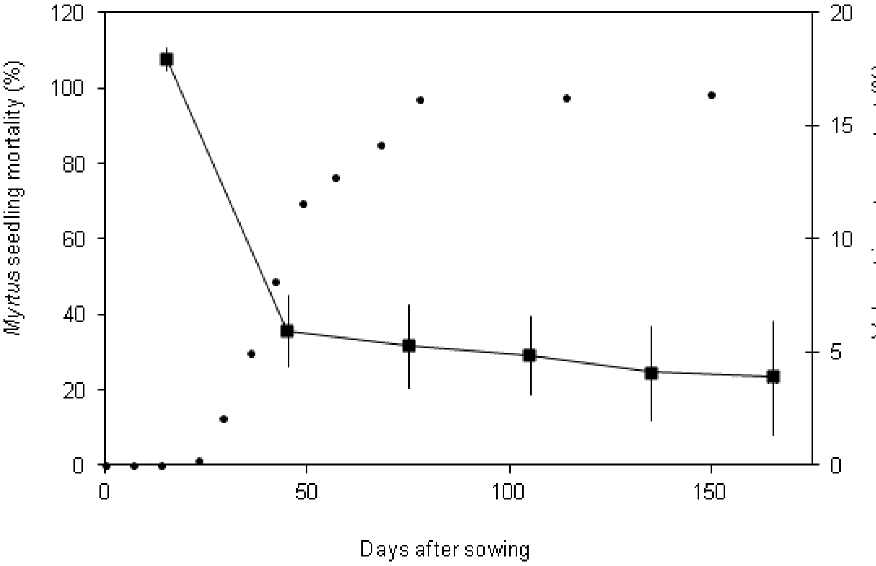

Figure 3. Cumulative percentage of *Myrtus communis* seedling mortality in field environment (dots) and temporal dynamics in mean soil volumetric water content (%) in the plot where sowing experiments were carried out (thin line).

Table 1. Results from generalized linear models (GLMs) examining the influence of correlated paternity on fitness measurements in seedlings of *Myrtus communis* and *Pistacia lentiscus* grown under greenhouse conditions.

Fitness measurements	Correlated paternity (r_p)					Other covariates
	Estimate	±	SE	*t*	*P*	
Myrtus communis						
Emergence (proportion)	-0.480	±	1.012	-0.474	0.642	**Sm***
Emergence time (days)	**-0.013**	**±**	**0.006**	**-2.190**	**0.045***	Sm
Seedling height (mm)						
After 36 days	0.053	±	0.352	0.150	0.883	**Sm***, Et
After 113 days	-0.039	±	0.041	-0.964	0.351	Sm, Et
After 190 days	-0.029	±	0.024	-1.208	0.247	Sm, Et
After 267 days	-0.027	±	0.023	-1.179	0.258	Sm, Et
After 424 days	-0.026	±	0.019	-1.376	0.190	Sm, Et
Dry biomass (g)						
Total	-0.009	±	0.013	-0.732	0.476	Sm, Et
Shoot	-0.021	±	0.032	-0.656	0.522	Sm, Et
Root	-0.015	±	0.018	-0.870	0.399	Sm, Et
Pistacia lentiscus						
Emergence (proportion)	8.423	±	8.440	0.998	0.334	Sm
Emergence time (days)	-0.079	±	0.069	-1.156	0.266	Sm
Seedling height (mm)						
After 36 days	-0.639	±	0.719	-0.889	0.389	**Sm***, **Et****
After 113 days	-0.008	±	0.130	-0.065	0.949	Sm, Et
After 190 days	0.005	±	0.082	0.058	0.954	Sm, Et
After 267 days	-0.013	±	0.080	-0.161	0.875	Sm, Et
After 424 days	0.007	±	0.072	0.103	0.919	Sm, Et
Dry biomass (g)						
Total	0.033	±	0.050	0.649	0.527	Sm, Et
Shoot	0.066	±	0.132	0.502	0.623	Sm, Et
Root	0.001	±	0.001	0.889	0.389	Sm, Et

Covariates: seed mass (Sm) and emergence time (Et). Significant *P*-values in bold; * < 0.05; ** < 0.01.

4. Correlated paternity effects on progeny performance

The influence of correlated paternity on seedling performance in the field experiment is summarized in Table 2. Contrary to the effects detected in the greenhouse experiment, we found no significant trend towards the influence of correlated paternity on ‘seedling emergence time’ and ‘seedling lifetime’ in *Myrtus* under field conditions. For this species we found a significant negative relationship of correlated paternity on ‘proportion of seedling emergence’ (Fig 2). Correlated paternity had no association with *Pistacia* seedling performance measurements ‘seedling emergence’ and ‘seedling lifetime’. However, we found a significant relationship between correlated paternity and ‘seedling emergence time’ in *Pistacia* under field conditions. The covariate ‘emergence time’ had a significant effect on ‘seedling lifetime’ in *Myrtus*.

Table 2. Results of the generalized linear models (GLMs) examining the influence of correlated paternity on fitness measurements in seedlings of *Myrtus communis* and *Pistacia lentiscus* grown under natural field conditions.

Fitness measurements	Correlated paternity (r_p)					Other covariates
	Estimate	±	SE	t	P	
Myrtus communis						
Emergence (proportion)	7.660	±	3.492	2.193	**0.045***	Sm
Emergence time (days)	0.004	±	0.005	0.779	0.448	Sm
Lifetime (days)	-0.003	±	0.009	-0.335	0.743	Sm, **Et***
Pistacia lentiscus						
Emergence (proportion)	-0.364	±	4.722	-0.077	0.940	Sm
Emergence time (days)	-0.043	±	0.020	-2.196	**0.044***	Sm
Lifetime (days)	0.051	±	0.048	1.061	0.307	Sm, Et

Covariates: seed mass (Sm) and emergence time (Et). Significant *P*-values in bold; * < 0.05.

Survival analysis regression test detected the influence of correlated paternity in *Pistacia* seedling survival. When running the analyses we found significant negative effect of correlated paternity (Chi^2 = 4.09, df = 1, P = 0.043; Fig 4), indicating that progeny from maternal plants with high correlated paternity had lower survival rates than progeny from maternal plants with low correlated paternity.

DISCUSSION

Plant recruitment is an essential stage of plant population dynamics and the long-term persistence of plant populations. In order to understand plant recruitment, it is fundamental to determinate which factors are affecting the demographic processes involved in early seedling performance (e.g. seedling emergence, growth and first-year survival). Our study is one of the few to date that has investigated whether the pollen diversity naturally received by maternal plants –here assessed by the correlated paternity– has an influence on the subsequent progeny performance. To accomplish our goal, we integrated mating system analysis with data on progeny performance both in greenhouse and field-sowing experiments.

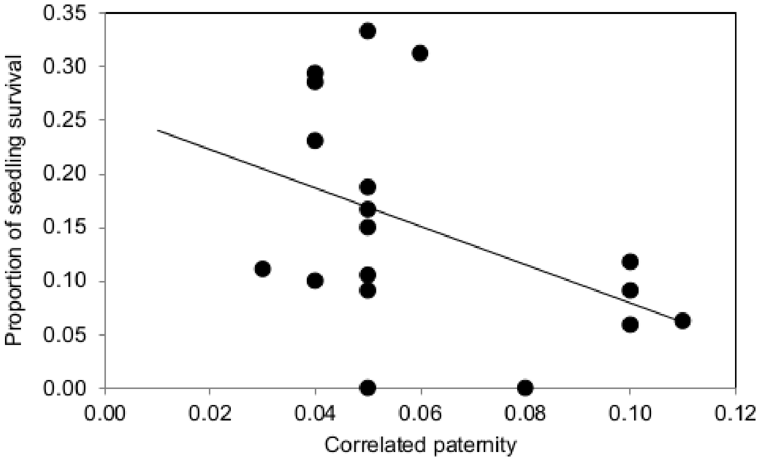

Figure 4. *Pistacia lentiscus* seedlings survival as a function of correlated paternity in the field environment.

The effects of correlated paternity on progeny performance were more evident in *Myrtus*. Under greenhouse conditions, we observed that high correlated paternity was associated to a delay in seedling emergence. In Mediterranean environments early emergence is of critical importance in determining subsequent seedling growth (Verdú *et al.* 2005), as was also observed in our study, in which emergence time influenced seedling fitness. Furthermore, the negative influence of high correlated paternity on the percentage of seedling emergence was also evident in the field environment, i.e. under more stressful conditions. These results fit the hypothesis that maternal plants that receive high pollen diversity (i.e. have low correlated paternity) generate progeny with increased fitness. As hypothesized in the literature, this relation could be due to female selection for more compatible pollen (Yasui 1998; Skogsmyr & Lankinen 2002), a process that could occur both before fertilization (e.g. by pollen germination and pollen-tube growth) or after (González-Varo & Traveset 2010; Pannel & Labouche 2013). In addition, our results concur with the few other empirical studies in which the effects of correlated paternity on fitness progeny have been tested. For instance, Llorens *et al.* (2013) reported both direct and indirect (through the increase of seed size) relationships of paternal diversity on seed germination, seedling weight and survival in *Banksia sphaerocarpa*. Also, Breed *et al*. (2012a, 2012b) reported negative effects of high correlated paternity on sapling growth in *Swietenia macrophylla* and *Eucalyptus socialis*.

Contrary to the results obtained under greenhouse conditions, seedling emergence time in the field was not significantly affected by correlated paternity, even though progeny from maternal plants with high correlated paternity had greater emergence times that progeny from maternal plants with low correlated paternity. We probably only observed this relationship in the greenhouse because under field conditions the influence of correlated paternity was masked by other factors that may condition this fitness variable more critically. Several studies documented the particularly importance of abiotic factors (such as water, light, temperature, microhabitats and disturbances) on seedling establishment under the harsh summer conditions of the Mediterranean climate (e.g. Rey & Alcántara 2000; González-Varo *et al.* 2011).Still, even under this type of natural conditions, we were able to detect the influence of correlated paternity in the percentage of seedling emergence in *Myrtus*.

It has been suggested that higher pollen diversity moderates the effects of inbreeding depression on progeny performance by lessening the likelihood that pollen with expressed deleterious alleles is involved in reproduction (Armbruster & Rogers 2004). In our study, although it is difficult to completely disentangle the effects of correlated paternity from the effects of inbreeding, in some cases it was possible to detect a trend indicating that correlated paternity could have an effect on progeny fitness independently of inbreeding (whenever an effect of r_p independently of t_m or *HL* results is observed), which agrees with findings in other studies (e.g. Breed *et al*. 2012b). Nevertheless, this subject still needs further research.

4. Correlated paternity effects on progeny performance

In *Pistacia*, no significant impact of correlated paternity on progeny performance was evident in the greenhouse conditions. *Pistacia* is an obligate outcrossed species so it is not surprising that it has low levels of correlated paternity. Thus, the inherent pollination biology of *Pistacia*, (leading to inherent low levels of correlated paternity) is diluting the potential trends that we found in *Myrtus*.

However, under field conditions we were able to detect the influence of correlated paternity on *Pistacia* seedling performance. For instance, and even though abiotic factors (especially drought) may be a major cause of the low seedling establishment in the Mediterranean (Herrera *et al.* 1994; García-Fayos & Verdú 1998; Rey & Alcántara 2000), even more than genetic factors (González-Varo *et al.* 2011), our survival analyses did find some evidence of the positive influence of low correlated paternity in a very important stage of the progeny fitness: survival after the first summer. Surviving the first summer in the harsh Mediterranean conditions is critical and this period is the most likely to act as a bottleneck for plant recruitment (Rey & Alcántara 2000; Traveset *et al.* 2003; Gómez-Aparicio 2008; González-Varo *et al.* 2011).

Under field conditions, we also observed the influence of correlated paternity in delaying *Pistacia* emergence time, a fundamental characteristic in the Mediterranean, as mentioned before. In fact, several *Pistacia* characteristics such as early seedling emergence make this species competent and able to survive efficiently in the Mediterranean climate (Verdú & García-Fayos 2001). Early emergence leads to benefits for seedling growth and fecundity, especially important in a Mediterranean environment in which species have to grow sufficiently during rainy spring to survive the summer drought (Verdú & Traveset 2005). The timing of emergence will in fact determine the seedling's fate as a plant. In our study, it is likely that the early emergence of *Pistacia* seedlings (as compared to *Myrtus*) in the field coincided with more benign conditions that resembled those in the greenhouse, which may have led to the similar percentage of emergence observed in the two sowing experiments for this plant. After the first summer, only 8% of *Pistacia* seedlings survived in the field experiment, a low survival rate but higher than that of *Myrtus* (in which no seedlings survived to the end of the experiment). Summer water deficit is the main factor of stress and mortality for seedlings in Mediterranean woody plants (Herrera *et al.* 1994; García-Fayos & Verdú 1998; Rey & Alcántara 2000; González-Varo *et al.* 2011). Nearly all dead seedlings in our field experiment were found to be desiccated (S. Nora, personal observation) and so seedling mortality is probably mostly attributable to drought. In fact, *Myrtus* seedling mortality was already 48.57% after just two months of the experiment (approximately at the end of April), by which time the measured volumetric water content in the ground had fallen dramatically. Plant species that do not form persistent soil seed banks (such as *Myrtus* or *Pistacia*) depend on a short temporal window after dispersal or on rare rainfall events to be able to recruit. Bearing in mind these conditions, the probability of natural seedling establishment in these two species – and especially in *Myrtus* – is extremely limited (González-Varo *et al.* 2011).

In this study, seedlings assessed for progeny fitness were not genotyped, which have been ideally the best approach in order to obtain direct fitness – mating system associations. However, we rather used family mating system estimates to detect associations between correlated paternity and progeny fitness. We considered this approach in order to avoid bias in the estimation of the mating system parameters, because genotyping only emerged seedlings could have biased upwardly the mating system estimates (considering that the percentage of emerged seeds in the greenhouse was ~ 50% for both species and in the field experiment the percentage was even lower). Bearing this in mind, it is fundamental to reckon that in this study the detected effects between mating system parameters and the fitness variables are indirect.

Our results on progeny fitness-correlated paternity associations found multiple non-significant effects and thus, we recognize that our results might lack significance once a multiple correction is applied. However, we considered that our study present novel results which could promote the forthcoming of new researches on the effects correlated paternity in other plant species and future meta-analysis studies.

Conclusions

We found that the correlated paternity has an impact on different aspects of progeny performance. In this study, we report a strong influence of correlated paternity on the progeny performance of *Myrtus*, a self-compatible species. Even so, in *Pistacia* (outcrossed species) the negative consequences of high correlated paternity on progeny emergence time and survival under field conditions were also detected. We conclude that our findings, in combination with a handful of recent empirical studies, suggest that mating pattern parameters – other than outcrossing rates – influence progeny performance in multiple stages during early seedling growth.

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SUPPLEMENTARY INFORMATION

Table S1. Correlations among maternal plant-level mating system estimates: maternal homozygosity by loci (HL), biparental inbreeding (tm-ts), outcrossing rate (tm) and correlated paternity (rp). Pearson's correlation coefficient with significant P-values in bold (* < 0.05).

	HL	t_m-t_s	t_m
Myrtus communis			
t_m-t_s	-0.21^{ns}		
t_m	0.13^{ns}	**0.49***	
r_p	-0.02^{ns}	0.38^{ns}	-0.27^{ns}
Pistacia lentiscus			
t_m-t_s	0.09^{ns}		
r_p	0.13^{ns}	0.19^{ns}	

Table S2. Individual (SE) genetic parameters (maternal homozygosity by loci and mating system parameters) computed for the selected maternal plants.

	Maternal homozygosity by loci (*HL*)	Mating system parameters		
		Outcrossing rate (t_m)	Biparental inbreeding (t_m-t_s)	Correlated paternity (r_p)
Myrtus communis				
M01	0.18	0.46 (0.03)	0.17 (0.02)	0.46 (0.09)
M02	0.24	0.68 (0.03)	0.38 (0.02)	0.33 (0.06)
M03	0.25	0.82 (0.03)	0.34 (0.02)	0.46 (0.07)
M04	0.25	0.69 (0.03)	0.32 (0.02)	0.32 (0.09)
M05	0.26	0.83 (0.03)	0.35 (0.02)	0.21 (0.06)
M06	0.26	0.82 (0.03)	0.22 (0.02)	0.26 (0.04)
M07	0.31	0.38 (0.03)	0.18 (0.02)	0.29 (0.14)
M08	0.32	0.82 (0.03)	0.15 (0.02)	0.32 (0.05)
M09	0.36	0.90 (0.03)	0.41 (0.02)	0.40 (0.04)
M10	0.38	0.25 (0.03)	0.08 (0.01)	0.53 (0.16)
M11	0.40	0.73 (0.03)	0.35 (0.02)	0.60 (0.05)
M12	0.40	0.98 (0.03)	0.29 (0.04)	0.20 (0.07)
M13	0.40	0.78 (0.03)	0.46 (0.03)	0.87 (0.03)
M14	0.45	0.63 (0.03)	0.14 (0.02)	0.37 (0.05)
M15	0.57	0.58 (0.03)	0.26 (0.02)	0.67 (0.06)
M16	0.59	0.76 (0.03)	0.27 (0.02)	0.23 (0.06)
M17	0.63	0.77 (0.03)	0.31 (0.02)	0.49 (0.05)
M18	0.69	0.82 (0.03)	0.07 (0.02)	0.10 (0.11)
Pistacia lentiscus				
P01	0.00	0.98 (0.00)	0.06 (0.00)	0.05 (0.00)
P02	0.32	0.94 (0.01)	0.03 (0.01)	0.05 (0.00)
P03	0.37	0.98 (0.00)	0.06 (0.00)	0.04 (0.00)
P04	0.05	0.94 (0.01)	0.05 (0.01)	0.05 (0.00)
P05	0.15	0.96 (0.00)	0.08 (0.01)	0.06 (0.01)
P06	0.15	0.99 (0.00)	0.06 (0.00)	0.03 (0.00)
P07	0.29	0.99 (0.00)	0.06 (0.00)	0.08 (0.01)
P08	0.26	0.99 (0.00)	0.06 (0.00)	0.04 (0.00)
P09	0.37	0.99 (0.00)	0.08 (0.00)	0.04 (0.00)
P10	0.34	0.99 (0.00)	0.10 (0.01)	0.10 (0.01)
P11	0.42	0.99 (0.00)	0.07 (0.00)	0.04 (0.00)
P12	0.22	0.99 (0.00)	0.07 (0.00)	0.05 (0.00)
P13	0.46	0.93 (0.01)	0.03 (0.01)	0.05 (0.00)
P14	0.39	0.99 (0.00)	0.11 (0.01)	0.10 (0.01)
P15	0.32	0.98 (0.00)	0.06 (0.00)	0.10 (0.02)
P16	0.49	0.99 (0.00)	0.10 (0.01)	0.05 (0.00)
P17	0.23	0.92 (0.02)	0.02 (0.01)	0.11 (0.01)
P18	0.05	0.99 (0.00)	0.08 (0.00)	0.05 (0.00)

Table S3. Progeny performance of *Myrtus communis* and *Pistacia lentiscus* under greenhouse and field conditions. Data are given as means ± SE.

Fitness measurements	*Myrtus communis*	*Pistacia lentiscus*
Greenhouse experiment		
Emergence (%)	53.33 ± 2.26	49.17 ± 1.73
Emergence time (days)	20.04 ± 0.40	18.90 ± 0.40
Seedling height (mm)		
After 36 days	16.70 ± 0.75	26.89 ± 0.78
After 113 days	136.56 ± 5.15	212.90 ± 5.41
After 190 days	220.00 ± 8.60	354.44 ± 9.02
After 267 days	230.39 ± 8.90	368.37 ± 9.35
After 424 days	257.50 ± 9.30	391.60 ± 9.80
Dry biomass (g)		
Total	2.39 ± 0.04	2.87 ± 0.04
Shoot	1.28 ± 0.03	1.58 ± 0.03
Root	1.11 ± 0.01	1.30 ± 0.01
Field experiment		
Emergence (%)	18.22 ± 1.86	49.07 ± 1.90
under *Myrtus*	24.24 ± 2.73	47.22 ± 3.73
under *Pistacia*	14.44 ± 2.87	46.11 ± 3.73
open ground	15.66 ± 2.73	53.89 ± 3.73
Emergence time (days)	39.19 ± 0.95	34.24 ± 0.60
under *Myrtus*	39.94 ± 1.70	35.52 ± 0.93
under *Pistacia*	34.50 ± 2.31	36.35 ± 0.91
open ground	41.97 ± 2.11	31.31 ± 0.86
Lifetime (days)	23.44 ± 0.11	94.50 ± 6.38
under *Myrtus*	18.92 ± 3.78	98.02 ± 12.41
under *Pistacia*	25.23 ± 5.14	38.28 ± 12.64
open ground	28.97 ± 4.70	138.00 ± 11.62
Survival (%)	-	15.15 ± 2.21
under *Myrtus*	-	15.29 ± 3.77
under *Pistacia*	-	2.44 ± 3.84
open ground	-	25.77 ± 3.53

General Discussion

GENERAL DISCUSSION

Pollen flow in fragmented populations

Over the last century, habitat loss and fragmentation have been the main components of current anthropogenic global change and the main drivers of biodiversity change in terrestrial ecosystems (Sala *et al.* 2000; Newbold *et al.* 2015). Accordingly, this is a fundamental research topic in Ecology and Conservation Biology (Tscharntke *et al.* 2012). Even if a dramatic progress has been made in the last decades, it is still very difficult to identify and quantify general patterns and processes on the effects of habitat fragmentation (Fardila *et al.* 2017). The island biogeography theory was among the earliest theories to predict the effects of fragmentation (MacArthur & Wilson 1967). In this theory, habitat fragments are regarded as habitat islands, surrounded by a matrix of human land use and unsuitable habitat for natural species. This theory has been regarded ever since as the bases of most empirical studies of habitat fragmentation. However, several authors recently discussed the critical differences that exist between habitat fragments and islands (Fahrig 2013; Mendenhall *et al.* 2014; Matthews 2015). For instance, the surrounding matrix in which some fragments are immersed may be potentially permeable to organisms' movement (Tischendorf & Fahrig 2000) and, therefore, influence the demography and survival of local populations (Brudvig *et al.* 2017). While the number of empirical studies on habitat fragmentation has increased exponentially in the last two decades, the need for new conceptual knowledge and the development of a theoretical framework specifically fitting the habitat fragmentation context has been currently requested (Fahrig 2013; Mendenhall *et al.* 2014; Matthews 2015). General knowledge on the complex and variable mechanisms of species responses (e.g. Aizen & Feinsinger 1994; Ewers & Didham 2006; Kramer *et al.* 2008) to changes in habitat configuration and size is needed as empirical evidence does not always support the current theory, creating a scientific paradigm (see Kramer *et al.* 2008; Lowe *et al.* 2015).

Important questions to understand the impacts of population fragmentation are related to the genetic connectivity of populations and the key influence of pollen dispersal of different species. Both population genetic theoretical models and empirical studies support that effective dispersal mechanisms may allow relatively high levels of gene flow even in fragmented systems, maintaining inter-population genetic cohesion; while restricted dispersion systems (in time and/or space) can alter the reproductive patterns and lead to the disruption of genetic connectivity even in large and well connected populations. Additionally, not only the quantity of gene flow itself prevents possible negative effects of genetic isolation (i.e. inbreeding depression, lack of diversity), but it is essential to also evaluate the quality of gene flow, which is determined by the effective number of pollen/seed sources. Therefore, for an effective characterization of dispersal, it is fundamental to take both components into account, in order to understand the dynamics of fragmentation and its consequences.

This Doctoral Thesis aimed to evaluate how habitat fragmentation (fragment size and connectivity) affects and determines both historical and contemporary gene flow and mating systems patterns, as well as the reproductive success of populations of the two common Mediterranean shrubs, *Myrtus communis* and *Pistacia lentiscus*. By comparing populations of these two species with contrasting mating and pollination systems, co-occurring in a diverse regional mosaic of Mediterranean forest patches in the Guadalquivir River Valley, this thesis highlights on the complexities surrounding fragmented landscapes and the influence of fragmentation on ecological interactions.

The importance of mating and pollination systems and the landscape context

Across all experimental studies of this Doctoral Thesis, mating and pollination system characteristics were fundamental in shaping the genetic diversity and variation in patterns of mating in both species. Accordingly, populations of the animal-pollinated and self-compatible species *Myrtus* are characterized by higher homozygosity values and genetic differentiation, stronger patterns of within-population spatial genetic structure than the populations of the wind-pollinated dioecious species *Pistacia*. Likewise, *Myrtus* also showed higher rates of correlated paternity, biparental inbreeding and global differentiation among pollen gene pools than *Pistacia,* which displayed an effective number of pollen donors similar to other wind-pollinated species (Dyer & Sork 2001; Pakkad *et al.* 2008). This is in agreement with the literature that discloses that wind-pollinated species have inherently less correlated paternity than those pollinated by animals, even when pollinator can transport pollen over long distances (Smouse & Sork 2004, Hughes et al. 2007).

The main goal of **Chapter 1** was to quantify the effects of the landscape configuration in determining the genetic diversity of shrub species that inhabit fragmented landscapes in order to infer patterns of historical gene flow of *Myrtus* and *Pistacia* populations under different landscape contexts. In general, *Myrtus* adult populations were apparently more susceptible to the negative effects of habitat fragmentation, by displaying lower values of effective population size and stronger evidence for recent genetic bottlenecks than *Pistacia*. However, even in *Pistacia*, a species that harbors a set of functional traits rendering it more resilient to the effects of fragmentation (Hamrick 2004), the fragmented-isolated populations displayed significant values of genetic differentiation than populations in other landscape contexts. Nevertheless, in both study species, it was found a rather population-specific reaction and weak responses to the landscape configuration per se (i.e. fragment size and/or isolation), which highlights the meaning of both historical and local factors, and emphasizes that both population history and species-specific characteristics are considered to shape genetic structure (Duminil *et al.* 2007).

Both **Chapters 2** and **Chapter 3** complemented each other in addressing contemporary pollen flow patterns with in a comparative approach that addresses whether different life history traits are reflected on species' reproductive success or common patterns can be detected across species regardless of their mutual differences. **Chapter 2** was mainly focussed in assessing the role of habitat fragmentation in conditioning plant's reproductive success either by disturbing directly plant's ability to reproduce and/or by changing plant's relationships with biotic and abiotic ecological components that are determinant to their reproductive success. While **Chapter 3** aimed to identify the ecological drivers that determine mating system patterns across different landscapes contexts. Rather than a direct effect of habitat fragmentation, it was detected that ecological components are more determinant to on species' reproductive success and mating system patterns and, interestingly, their influence vary along the fragmentation gradient. For instance, flowering synchrony had an important role in conditioning *Myrtus* fruit set in fragmented-isolated populations and also affecting *Myrtus* and *Pistacia* correlated paternity levels. Spatial arrangement of individuals is also a determinant factor affecting plant's reproductive success and patterns of pollen-mediated gene flow (Meagher & Vassiliadis 2003). Density of conspecific plants around maternal plants showed a direct relationship with fruit set in fragmented-connected populations of *Pistacia*, and also with correlated paternity levels in *Myrtus* populations. While reproductive success and mating systems patterns of *Myrtus* were mainly influenced by characteristics of the maternal plant local neighborhood, those of *Pistacia* were also influenced by habitat patch features involving population type and distance of the plant to the patch edge. For instance, in fragmented-isolated populations of *Pistacia* it was possible to detect a negative effect of habitat fragmentation on seed viability.

Moreover, by comparing both historical and contemporary patterns of gene flow, it is possible to gain some insights into the demographic history and futures of these populations. In an extensive population with reasonably homogeneous density, genetic estimates should be similar if populations and gene dispersal processes remain stable. However, recent disturbances, such as habitat degradation in quality and/or quantity should affect mostly contemporary estimates (Odduou-Muratorio & Klein 2008; Hamrick & Trapnell 2011). Chapter 3 revealed in both species a trend towards a decrease of genetic diversity in offspring when compared to adults in the analyses of genetic variation. This effect was particularly pronounced in *Myrtus*, which could indicate the occurrence of inbreeding depression in *Myrtus* populations, compromising the amount of genetic variation that will be available for the next generations in this species.

Finally, **Chapter 4** represents one of the few existing empirical examples that evaluate the role of the correlated paternity in shaping the early performance of maternal progenies (seedling emergence, seedling emergence time, seedling growth, biomass, lifetime and survival) sampled from contrasted landscape scenarios. The outcrossing-selfing paradigm has dominated research on plant mating systems (Barret & Harder 2017), however, correlated paternity received much less attention and limited analysis. The specific ecological mechanisms causing variation in correlated paternity are often poorly understood. In this study correlated paternity affected more to the progeny performance of the self-compatible *Myrtus* than that of the outcross *Pistacia*. High correlated paternity had a negative effect on the proportion and timing of *Myrtus* seedling emergence in natural field conditions and in the greenhouse sowing experiment, respectively. However, negatives effects of correlated paternity on *Pistacia* progeny emergence time and survival were also detected.

Overall, as expected due to its pollination syndrome, *Myrtus* populations presented more detrimental effects of habitat fragmentation, through all these experimental studies. Being an insect-pollinated species, makes *Myrtus* more vulnerable to habitat disturbance, because of its dependence on pollinator's abundance and diversity (Aizen & Feinsinger 2003; Winfree *et al.* 2009, 2011). However, a self-compatible with a generalized pollination system, species like *Myrtus* may show some resilience in a disturbed and changing environment (Johnson & Steiner 2000; Aguilar *et al.* 2006). Plus, recent evidence suggests that pollen limitation in wind-pollinated plants may be as common as it is in animal-pollinated species (Friedman & Barrett 2009; Barrett 2010). In fact, *Pistacia* populations also showed some negative signs of the habitat fragmentation effects.

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Conclusions

CONCLUSIONS

Listed below are the different conclusions obtained during the development of the present Doctoral Thesis:

(1) Habitat fragmentation consequences are not so straight forward and being common and long-lived species, regardless the pollination syndrome, is not a guarantee of the long-term survival of a population, emphasizing the importance of comparative studies (multi-species) across distinct landscapes contexts to evaluate habitat fragmentation effects on plant's reproductive success and mating system variation.

(2) In adults populations of *Myrtus* and *Pistacia* it was detected the influence of mating and pollination systems characteristics in shaping species' genetic patterns. The observed population-specific response in both study species could also be indicating a possible in influence of past events (e.g. historical connectivity, fluctuations in population size) and local factors (e.g. microhabitat availability for recruitment, habitat quality, plant density, native fauna). Although both species revealed weak responses to the landscape contexts, some detrimental effects of habitat fragmentation could be detected, such as low values of effective population size and evidence for recent genetic bottlenecks, more pronounced in *Myrtus* than in *Pistacia* populations.

(3) Rather than a direct effect of habitat fragmentation, we detected that ecological components are more determinant on *Myrtus* and *Pistacia* reproductive success and mating system patterns, and that their influence varies along the fragmentation gradient. These results highlight the complex scenario around habitat fragmentation where there are no single factors involved but the interplay between many factors responsible of the reproductive success and mating system patterns of plants.

(4) This Doctoral Thesis also demonstrates that the impacts of habitat loss and fragmentation, a part of being most of the times context and species dependent, they are often also more nuanced than simple and easy observed losses of genetic diversity and increase differentiation among populations. Some key consequences of forest fragmentation have now been identified as increased inbreeding depression in progeny sired in a fragmented landscape and decreased progeny fitness due to low numbers of effective pollen donors. It also confirms that variation in mating and pollination systems is an important factor in determining the type and magnitude of species response to habitat fragmentation.