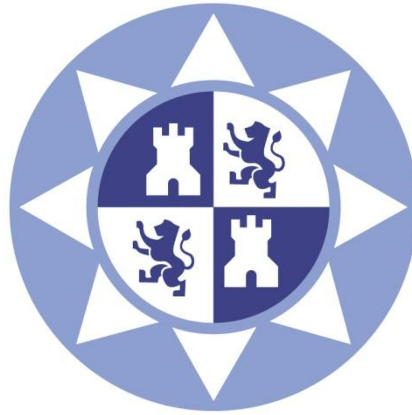


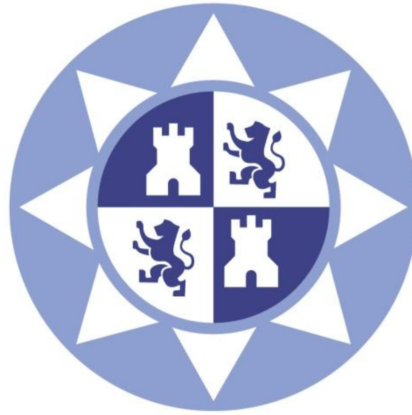
Universidad Politécnica de Cartagena

Departamento de Producción Vegetal

Estudio de la biología y ecología de *Astragalus nitidiflorus*

Francisco José Segura Carreras

2015



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Directores

María José Vicente Colomer

Juan José Martínez Sánchez

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ABSTRACT

Astragalus nitidiflorus (Leguminosae) is a rather short-lived, perennial herbaceous legume. Mature plants are generally < 30 cm high, with stems spreading from a caudex in a circular pattern up to 150 cm diameter. Its emergence period begins in autumn with seedlings developing until the summer. In summer, leaves and stems die and only a few buds remain at the base of the stem at ground level. After the autumn rains, the dormant buds sprout and begin a second stage of growth. Flowering begins in March up to the end of May, with fruits taking about two months to ripen. The life cycle continues in this way with successive periods of growth until the plant dies (maximum four years). This species is endemic to the province of Murcia (southern Spain), where it forms the only known metapopulation worldwide and it is classified as *Critically Endangered* according to the International Union for Conservation of Nature (IUCN) categories, and as *En Peligro de Extinción* in the Catálogo Nacional de Especies Amenazadas. Due to this fact and the little prior knowledge about the species, the aim of this thesis is to study biological and ecological traits of *A. nitidiflorus* in order to establish appropriate measures for its conservation. Thus, this work includes the study of: (1) location, habitat, demographic features and reproductive biology of the species; (2) the level of genetic diversity, including the genetic variation within and between natural populations of the species, using ISSR markers; (3) the ability of the species to form a persistent soil seed bank and the role that it can play in the maintenance of the population in an arid and unpredictable environment; (4) physical dormancy and germination characteristics of the seeds of *A.nitidiflorus* and the influence of some maternal and environmental conditions on them; and (5) suitability of the HRM technique using cpSSR markers to compare the genetic structure of different life stages of the species over the years the potential contribution of the soil seed bank for maintaining genetic diversity of the population.

According to the results obtained from the studies conducted we can affirm that the seedling stage is the most critical in the life cycle of *A. nitidiflorus*. Although the viability of the species is not limited by the flowering and fruiting process, the maintenance of the habitat in the early sucessional stages seems to be the critical point for long-term survival of the species. The adult plants show a long flowering season with a high degree of synchrony. The data also demonstrates that *A. nitidiflorus* is a facultative xenogamous species, but the presence of pollinators can enhance fruit set. Inter-simple sequence repeats (ISSR) markers indicate a low genetic diversity both at

the population and species level. The high genetic connectivity found among populations of *A. nitidiflorus* is an evidence of a recent habitat fragmentation. In addition, a bottleneck event in the past has been revealed, with a subsequent reduction of population size and a loss of genetic variation. Regarding soil seed bank, this species is able to form a short-term persistent soil seed bank that is strongly influenced by environmental factors and population fluctuations. Results show that most seeds are present on the soil surface, inside the fruit and close to the mother plant. In general seed longevity is low, but is higher for seeds protected by fruit than for single seeds after two years buried in the soil. The germination tests show a high variability over the years in the physical dormancy of *A. nitidiflorus* seeds because maternal environmental factors such as drought or mother plant age influence the proportion of seeds that enter dormancy. This in turn determines the proportion of seed that becomes part of the seed bank each year and also the age structure of the natural population. Finally, the use of the HRM technique with simple sequence repeats in chloroplast genomes (cpSSRs) proved to be suitable for studying the genetic diversity of the soil seed bank of *A.nitidiflorus*. Obtained results show that genetic diversity is higher in the soil seed bank than for above ground adults, although stored genotypes do not remain constant in the soil more than two years because of the low seed longevity. One of the highest genetic diversities is contained in the seedlings but nevertheless much of this genetic richness is not transmitted to adults due to the high mortality that occurs at early stages, hence this point also results to be determinant defining genetic characteristics of future populations.

In conclusion, this work goes into the biology of this threatened species and we hope that it will become a very useful tool for establishing appropriate measures for the species conservation when the Regional Administration carry out its recovery plan.

RESUMEN

Astragalus nitidiflorus es una leguminosa herbácea perenne de ciclo vital más bien corto. Por lo general, los individuos adultos no superan los 30 cm de altura, con tallos que se extienden desde el cáudice siguiendo un patrón circular que puede alcanzar hasta 150 cm de diámetro. El periodo de emergencia comienza en otoño y las plántulas se desarrollan hasta el verano. En verano las hojas y tallos mueren y sólo unas pocas yemas permanecen en la base del tallo a nivel del suelo. Tras las lluvias otoñales, las yemas latentes brotan y empieza un segundo periodo de crecimiento. La floración comienza en marzo y se alarga hasta finales de mayo, con frutos que necesitan aproximadamente dos meses para madurar. El ciclo vital continúa de este modo, con sucesivos periodos de crecimiento, hasta que la planta muere (máximo cuatro años). Esta especie es un endemismo de la Región de Murcia (sureste de España), donde forma la única metapoblación conocida a nivel mundial, estando catalogada como Critically Endangered por la International Union for Conservation of Nature (IUCN) y como en Peligro de Extinción en el Catálogo Nacional de Especies Amenazadas. Debido a este hecho y al escaso conocimiento previo sobre la especie, el objetivo principal de esta tesis es el estudio de las características biológicas y ecológicas de *A. nitidiflorus* a fin de establecer las medidas apropiadas para su conservación. Por lo tanto, este trabajo incluye el estudio de: (1) localización, hábitat, características demográficas y biología reproductiva de la especie; (2) el nivel de diversidad genética, incluyendo la variación genética dentro y entre las poblaciones naturales de la especie, usando marcadores ISSR; (3) la capacidad de la especie para formar un banco edáfico persistente y el papel que éste puede desempeñar en el mantenimiento de la población en un entorno medioambiental árido e impredecible; (4) latencia física y características germinativas de las semillas de *A.nitidiflorus* y la influencia de algunos factores maternos y medioambientales sobre las mismas; e (5) idoneidad del empleo de la técnica HRM con marcadores cpSSR a la hora de comparar la estructura genética de los distintos estadíos vitales de la especie a lo largo de los años y la contribución potencial del banco edáfico para el mantenimiento de la diversidad genética en la población.

Según los resultados obtenidos en los estudios realizados podemos afirmar que la etapa de plántula es la más crítica del ciclo vital de *A. nitidiflorus*. Aunque la viabilidad de la especie no se ve limitada ni por la floración ni por la fructificación, la conservación del hábitat en las primeras etapas sucesionales sí que parece ser un punto clave para la supervivencia de la especie a largo plazo. Las plantas adultas presentan un largo periodo de floración con un alto grado de sincronía. Los datos también

demuestran que *A. nitidiflorus* es una especie xenómaga facultativa, pero que la presencia de polinizadores puede favorecer el cuajado de los frutos. Los marcadores ISSR indican una baja diversidad genética tanto a nivel poblacional como de especie. La elevada conectividad genética detectada entre las poblaciones de *A. nitidiflorus* es una prueba de la reciente fragmentación del hábitat. Además, se ha detectado la existencia de un cuello de botella en el pasado, con la consecuente reducción del tamaño poblacional y la pérdida de variación genética. Referente al banco edáfico, esta especie es capaz de formar un banco edáfico persistente a corto plazo que está fuertemente influenciado por factores medioambientales y las fluctuaciones poblacionales. Los resultados muestran que la mayoría de las semillas se encuentran presentes en la superficie del suelo, dentro del fruto y distribuidas alrededor de la planta madre. En general la longevidad de las semillas es baja, pero mayor para las semillas protegidas por el fruto que para aquellas sueltas tras dos años enterradas en el suelo. Las pruebas de germinación muestran una gran variabilidad a lo largo de los años en la latencia física de las semillas de *A. nitidiflorus* debido a que factores maternos medioambientales como la sequía o la edad de la planta madre influyen en la proporción de semillas que adquieren dicha latencia. Esto a su vez determina la proporción de semillas que pasan a formar parte del banco edáfico cada año así como la edad de la población natural. Por último, el empleo de la técnica HRM con cpSSR resultó ser adecuada para estudiar la diversidad genética del banco edáfico de *A. nitidiflorus*. Los resultados obtenidos muestran que la diversidad genética es mayor en el banco edáfico que en los adultos superficiales, aunque los genotipos almacenados no se mantienen constantes en el suelo más de dos años debido a la baja longevidad de las semillas. Una de las mayores diversidades genéticas se encuentra contenida en las plántulas pero, sin embargo, gran parte de esta diversidad genética no se transmite a los adultos debido a la alta mortalidad que se da en las primeras etapas de desarrollo, de ahí que este punto también sea determinante definiendo las características genéticas de las futuras poblaciones.

En conclusión, este trabajo se adentra en la biología de esta especie amenazada y esperamos que se convierta en una útil herramienta a la hora de establecer las medidas apropiadas destinadas a la conversación de la especie cuando la Administración Regional establezca el Plan de Recuperación.

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GENERAL INTRODUCTION

1. Systematics and description of the species

The word legume is derived from the Latin verb *legere* which means to gather. Legumes are flowering plants in the Leguminosae family. This family is also known as Fabaceae, and both terms can be used interchangeably to indicate the some 690 genera and 18,000 species therein (Morris, 2003). The Leguminosae family is classified into three sub-families: Papilionoideae, Caesalpinioideae, and Mimosoideae. Each sub-family is identified by its flowers.

Like many flowers, those found on legume plants are hermaphroditic, containing both the stamen and pistil. This makes the plants self-fertile, meaning that an individual plant is able to reproduce by itself which can have the effect of limiting genetic diversity. However, hybridization occurs frequently in nature due to this characteristic, as any plant can pollinate another due to the hermaphroditic properties therein (Weaver, 2003). The flower typically has five petals and an ovary with one carpel, cavity, and style (Morris, 2003). The petals of the legume plant are shaped into a cup. One large petal, the 'banner' or 'standard' folds over the rest for protection. In front of this petal are two narrower petals called 'wings,' between which two other petals unite. Due to their shape these petals are referred to as the keel. Within that fold are the stamens and pistil (Earle, 1971). After pollination the flower will die and reveal the growing ovary which becomes the pod.

The distinctive fruit is the most ready resource by which to identify members of the Leguminosae family. This fruit principally grows into a pod that contains the seeds of the plant. The legume pod is a one-celled seed container formed by two sealed parts called valves. Legume pods always split along the seam which connects the two valves. This characteristic is called dehiscent, from the Latin word meaning to gape or burst open (Earle, 1971). However, not all pods are shaped the same. The thickness, length, curve, and fleshy nature of the pods can vary between species. In addition, some pods are winged or indehiscent (meaning the pods do not split open at maturity) (Morris, 2003).

Legumes convert atmospheric nitrogen into nitrogenous compounds useful to plants. Root nodules containing the bacteria *Rhizobium* fix free nitrogen for the plants. And in return, the legumes then supply the bacteria with valuable carbon produced by photosynthesis. Thus this symbiotic relationship between plant and bacteria facilitates

the unique process of nitrogen fixation in legumes (Morris, 2003).

The flowering plant genus *Astragalus*, the common name "milkvetch", containing upwards of 2500, mostly perennial species, are distributed primarily around the northern hemisphere and South America. *Astragalus* species are found mostly in cool temperate semi-arid and arid continental regions of the world. Many species are narrow endemics, often found in marginal habitats, while relatively few are widespread. *Astragalus* is especially diverse in southwest Asia, the Sino-Himalayan region, western North America, where we have about 400-450 species, and along the Andes in South America. *Astragalus* is also diverse in Mediterranean climatic regions, along the Pacific coasts of North and South America, and in southern Europe and northern Africa. The Platyglottis section is formed by 10 species, all of the Middle East, Libya, Egypt and western Asia - except *A.verrucosus* Moris, Sardinia; *A.peregrinus* Vahl, which goes up to Algeria and *A.gines-lopezii*, *A.devesae* and *A.nitidiflorus* Jimenez Mun. & Pau located in Spain.

Astragalus nitidiflorus is a rather short-lived, perennial herbaceous legume. Mature plants are generally <30 cm high, with stems spreading from a caudex in a circular pattern up to 150 cm diameter. The leaves are imparipinnate and measure up to 14 cm and leaflets are villous. The flowers appear in racemes (up to 300 racemes in the biggest plants) of up to 30 yellow flowers, with a corolla of up to 2.1 cm. Fruits are curved and acuminate legume of 18 mm × 7 mm, approximately.

The life cycle of *A. nitidiflorus* begins with seeds, which germinate in autumn and winter. Seedlings develop until the summer. In summer, leaves and stems die and only a few buds remain at the base of the stem at ground level. After the autumn rains, the dormant buds of the plants that have survived the summer sprout and begin a second stage of growth. Flowering begins in March up to the end of May, with fruits taking about two months to ripen. The life cycle continues in this way with successive periods of growth until the plant dies. Some plants survive for four years.

A. nitidiflorus is an endemism of southern Spain with an only known population (worldwide) located in the Campo de Cartagena (Murcia). It was collected in 1909 by Francisco de Paula Jimenez Munuera and described by him and Carlos Pau. Until 2004 only scarce fruiting herbarium material (Royal Botanical Garden of Madrid, MA 66838) was known. This species went unnoticed until Vazquez et al. ascribed to this species samples collected in Badajoz and Avila, but later was assigned to another species: *A.*

gines-lopezii. In May 2004 it was re-discovered in the locality of Tallante by a biologist who took some samples to identify them, confirming later that collected samples belonged to *Astragalus nitidiflorus* (Dirección General del Medio Natural, 2004).

Picture 1. Detailed view of imparipinnate leaves (left), postrate habit (above center), indehiscent fruits (below center) and inflorescences of *Astragalus nitidiflorus*.

2. Background and justification

The population of *Astragalus nitidiflorus* is practically entirely located in the Lugar de Interés Comunitario ES6200040 "Cabezos del Pericón" situated in the locality of Los Puertos de Santa Barbara in the municipal term of Cartagena. This area has been integrated with other LICs and a protected natural area (La Muela-Cabo Tiñoso) in the development of a Plan of Arrangement of the Natural Resources currently in the process of realization. In the newly discovered population have been counted more than 2,000 individuals spread over around 13 population nuclei.

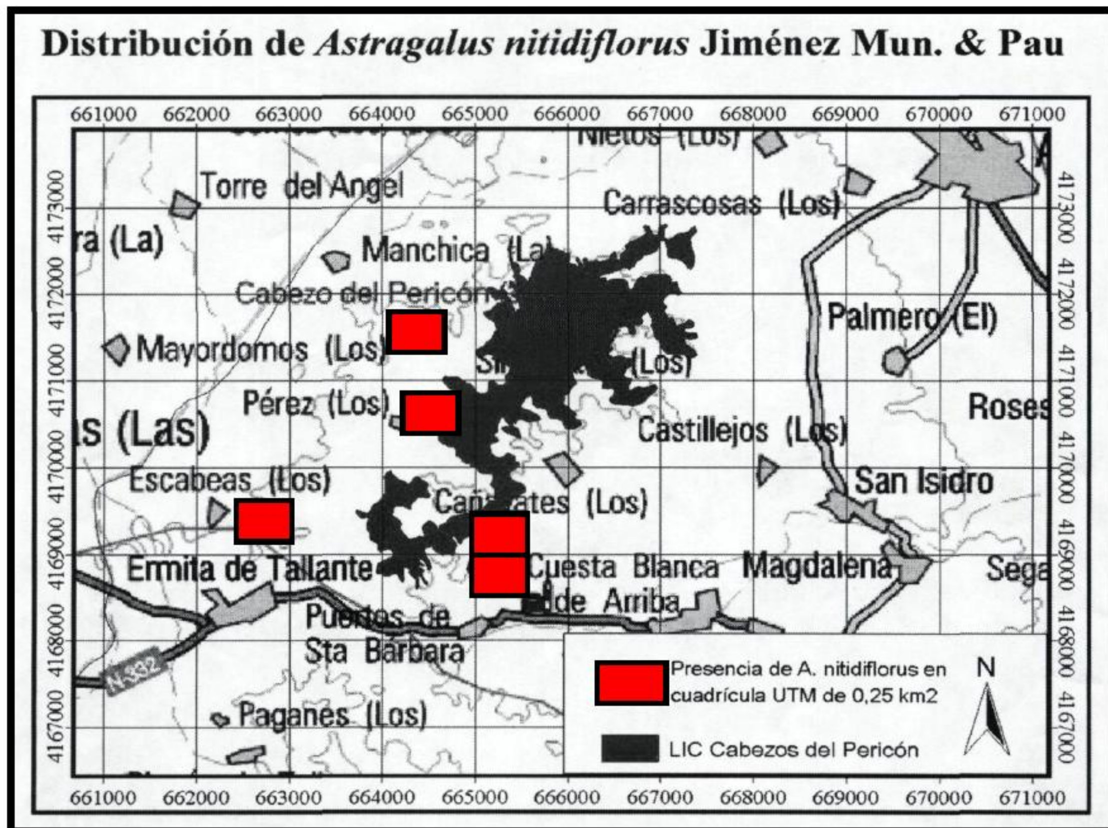

Figure 1. Distribution of population nuclei around the Lugar de Interés Comunitario Cabezos del Pericón.

Regarding protective legal actions, *A. nitidiflorus* is classified as “endangered” in the Catálogo Nacional de Especies Amenazadas by order of the Ministerio de Medio Ambiente 2231/2005, of June 27th 2005. A taxon or population must be considered "endangered" (E), when its survival in the short term is unlikely. To be included in this category must have been or be in serious demographic decline, known or inferred, in the recent past or that this regression is predictable in the near future, a scenario that easily meets the species. It is also included in the Catálogo Regional de Flora Silvestre Protegida de la Región de Murcia (Decreto 50/2003, of June 10th 2003) with the category of "special interest". In Extremadura was included in the Catálogo Regional de Especies Amenazadas (Decreto 37/2001, of March 13th 2001) classified as "endangered" on the grounds that individuals found there belonged to *Astragalus nitidiflorus*, although in reality were *Astragalus gines-lopezii*. In the Atlas y Libro Rojo de la Flora Vascular Amenazada de España (2004) and the Lista Roja de Flora Vascular Española (2010) it is listed as critically endangered (CR), as assessed by IUCN categories.

Threatened plant species are considered a priority in the field of biodiversity conservation because of its restricted geographical distribution, reduced ecological amplitude and vulnerability to processes of genetic drift, inbreeding depression of close relatives and random environmental events. According to Falk (1992), there is an urgent need to improve the knowledge of their biology, especially in the areas of population and reproductive biology, genetics and ecology, as an inevitable step to prevent their extinction.

Despite the degree of threat to this species, there are few references to its distribution and the plant communities in which it appears and no reports on its biology and ecology, prior to the commencement of this doctoral thesis. However, in order to establish appropriate measures for the conservation of this species, we thought it necessary to determine its reproductive biology and ecology.

3. Features of the distribution area in Cartagena

The Region of Murcia lies within the scope of the Baetic Ranges, which are divided in three areas: Pre-baetic, Sub-baetic and Baetic, with materials that belong to each one. The area where *A. nitidiflorus* is distributed is characterized by the outcrop of alkaline basaltic rocks belonging to the Plio-Quaternary volcanic region of the northwest of Cartagena, the most recent eruptions area of the Spanish southeast (approximately 2.6 million years ago). Volcanism was strombolian with eruptions where pyroclastic materials and low power lava flows were thrown. The area is one of the few places where thorn of jujubes (pre-desertic arborescent scrubs as *Ziziphus lotus* exclusive of the arid southern region of the Iberian Peninsula and considered to be of Interés Comunitario Prioritarios by Directive 92/43/EEC) are present. The flatter areas and foothills are cultivated mainly for rainfed crops such as almond and carob trees, some of them already abandoned. All this forms the traditional agricultural and rural environment of the Campo de Cartagena, constituted by rainfed fields (Red Natura, 2000).

Picture 2. Location of tertiary and quaternary volcanic outcrops of Southeast Spain (left) and natural environment where the species grows (right).

Regarding the climatic conditions, this Region presents the characteristics of the semi-arid subtropical Mediterranean climate. Precipitation is low throughout the regional territory (approximately 300–350 mm per year), but frequently concentrated in periods such as spring (April) and autumn (October), the summer being predominantly dry. However, within the regionwe can distinguish three rainfall areas; sub- humid (defined by rainfall of around 500 mm), semi-arid (between 500 and 300 mm), and arid (300 mm or less). Cartagena lies within the arid area. The Region of Murcia, as with the whole of southeastern Spain, is characterized by rainfall irregularity among years. Between 1692 to 1985, averages of 88 mm in 1945 and 765 mm in 1884 were recorded, even 1000 mm in mountainous areas (Mas et al., 1986). 1961, 1978, 1983, and 1984 were particularly dry years, especially 1978 in Cartagena, with less than 100 mm. Regarding temperature, the region has an annual average temperature of 18ºC, with hot summers (absolute maxima of 40ºC) and mild winters (11ºC average temperature in January and December). More specifically, the local area of Cartagena is characterized by the even milder temperatures. The mean annual air temperature is 17.6ºC, and the mean maximal and minimal temperatures are 23.4 and 6ºC, respectively, lying within the Thermo-Mediterranean bioclimatic level (Rivas–Martínez, 1987).

The vegetation landscape found by primitive man in Cartagena was probably extensive areas of *Pistacia lentiscus* L., *Chamaerops humilis* L., *Ziziphus lotus* (L.) Lam., *Periploca angustifolia* Labill., *Pinus halepensis* Mill., and *Tetraclinis articulata* (Vahl) Masters. In the watercourses there was *Nerium oleander* L., in salt marsh areas *Tamarix boveana* Bunge. and *Tamarix canariensis* Willd., and so on. The destruction of that old vegetation has given rise to the current vegetation and many of the processes that originated from the massive destruction (erosion, decline in rainfall, desertification…) are largely irreversible (Mas et al, 1986). Despite all of this, the Region of Murcia is squarely in the territorial area of the Mediterranean basin, a hot spot of world plant-diversity. In the Mediterranean basin there are nearly 25,000 species of vascular plants, value which represents at least 10% of the world plant diversity. In the Region of Murcia, plants with flowers (Angiosperms) encompass virtually all of the vascular flora, with more than 1,900 species. More than 50% of the flora is in only 8 families, the more diversified being the Asteraceae, Poaceae, and Leguminosae (11.5%, 9.2%, and 8.8% of the total, respectively). The species that set this region apart from others are those with a smaller geographic range, which is called, although not rigorously, the endemic component. Within this endemic component, the majority corresponds to elements of Iberian distribution (19%), such as *Ferula loscosii,* and Ibero-African elements (12.7%) such as *Tetraclinis articulata* (Vahl.) Masters and *Anthemis chrysantha* J. Gay, while some species have their sole population in Murcia, such as *Astragalus nitidiflorus* Jiménez Mun. et Pau, *Cistus heterophyllus* Desf. subsp. *carthaginensis* (Pau) Crespo & Mateo, or *Limonium carthaginense* (Rouy) C. E. Hubb. & Sandwith**,** which are endemic to Cartagena (Martínez–Sánchez et al, 2008).

Astragalus nitidiflorus inhabits grasslands and thermophilic grasslands rich in perennial grasses and legumes, dominated by *Hyparrhenia synaica*, adjacent to species such as *Lotus edulis, Ononix natrix,* and *Bituminaria bituminosa* (*Aristido coerulescentis-Hyparrhenietum hirtae*; Clase *Lygeo sparti-Stipetea tenacissimae*). Among the clear patches of these lush grasslands one can find arvense therophyte communities, cereal and optimum spring crop weeds such as *Ruderali–Secalietea cerealis* (*Galium verucosum, Sherardia arvensis, Geranium molle, Vicia sativa, Trifolium stellatum, Trifolium campestre, Bromus rubens, Bromus tectorum, Nigella damascena, Erodium neuradifolium, Vulpia ciliata, Cerastium glomeratum, Leontodon taraxacoides, Rostraria cristata* y *Bellardia trixago*), and other species, therophytes

also, very ephemeral and xerophytic belonging to *Tuberaietea guttate* Class (*Linum strictum, Trifolium scabrum, Euphorbia exigua, Valantia hispida, Asrerolinum linum-stellatum, Neatostema apulum, Helianthemum salicifolium* and *Hedysarum spinosissimum*).

Regarding the soil characterization of the habitat of the species, in 2008 soil samples in all populations of the species were taken and analyzed in the laboratory of Soil Science of the UPCT. Based on its physical and chemical characteristics soils can be classified as Regosols. They are basic soils with pH values around 8, free of salts, with low content of carbonates and sandy texture having a high content of organic matter and C/N ratio indicative of humification processes.

4. Endangered species conservation

Indigenous plants have been declining at an alarming rate. They face an ever-increasing range of threats, from the fragmentation of their habitats to pressures resulting from agriculture, forestry, and urban sprawl. Climate change and the spread of invasive alien species are additional threats. Conservation management oriented to the conservation of species is a consequence of the finding that the rate of species extinction is reaching levels not seen throughout history and that man is mainly responsible for this situation. However, the starting point (i.e., what are the current species extinction rates?) is an aspect of enormous controversy. Probably the most important effort in this sense comes from the IUCN Red List Programme. Thus, scientists (including botanists, taxonomists, and ecologists) are working hard to catalogue and describe the biology of plants throughout the world before they disappear, often using a method called Rapid Ecological Assessment. This activity is especially important in highly diverse regions of the globe with high numbers of rare species. However, uncertainty remains high, especially if we take into account that the material evaluated so far is a small fraction of the existing biodiversity and is far from representing the necessary basic knowledge (Escudero et al., 2002). Global efforts are underway to protect the remaining areas on Earth that harbor high diversity of both common and rare plants (called biodiversity hotspots). The goal is to provide habitat in perpetuity for as many species of plants as possible. Another approach is to educate people about the economic, utilitarian, and intrinsic values of biodiversity. Finally, national and international legislation is

necessary to prevent the trafficking of rare species such as orchids. For example, the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES) prohibits or limits the trade of almost 28,000 species of rare plants worldwide (Farnsworth and Sarkar, 2012).

In Spain, starting with the 4/89 Nature Conservation Law, public regional administrations have been obliged to establish recovery plans for threatened species, since the 1990s. Since then, some scientists have tackled research lines about inventories of endangered species (for example, Sánchez Gómez, Hernández, López, Vera Pérez and Carrión); about genetic diversity (Caujapé–Castells and Sosa); and about the biology and ecology of threatened species, in which many scientists are working in Spain, perhaps following Iriondo and Escudero.

In this context, since 2005, we have been studying the ecology and biology of some endangered species endemic to the Southeast of Spain, such as *Astragalus nitidiflorus*. Thanks to the Consejería de Agricultura y Agua of the Comunidad Autónoma de la Región de Murcia and others organisms, such as the Fundación Séneca of the Región de Murcia, some important projects have been carried out and the data obtained from them will be crucial for the future recovery of plants of the studied species.

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GENERAL OBJETIVES

The high degree of threat to *Astragalus nitidiflorus* amply justifies its study in depth. However, until the beginning of this work there were only few references to its distribution and the plant communities in which it appears, and no reports on its biology and ecology. For this reason, the goal of this study is to understand the biological traits of the species, in order to establish appropriate measures for its conservation. The specific aims of this study are:

1. To study the life story, demographic features and reproductive biology of *A. nitidiflorus* trying to identify possible weaknesses that might contribute to its rarity and hamper its conservation.

2. To assess the level of genetic diversity within and among natural populations of the species by using ISSR markers and to provide elementary information for future conservation strategies.

3. To evaluate the ability of the species to form a persistent soil seed bank and understand the role that it can play in the maintenance of the population in an arid and unpredictable environment.

4. To analyze the physical dormancy and germination characteristics of the species' seeds and verify whether they are affected by some maternal or seasonal environmental conditions.

5. To compare the genetic structure of different life stages of the species over the years and examine the potential contribution of the soil seed bank for maintaining genetic diversity of the population.

CHAPTER 1

Life history and demographic features of *Astragalus nitidiflorus*, a critically endangered species

Published in Flora

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Abstract

Astragalus nitidiflorus is an endangered short-lived legume of SE Spain, which have been re-found after 100 years. To identify possible weak points that might contribute to its rarity and hamper its conservation, this paper presents data concerning location, habitat, demographic features and reproductive biology of the species. Censuses of three cohorts of seedlings show that the seedling stage is the most critical in the life cycle. The adult plants show a long flowering season with a high degree of synchrony. Despite the low reproductive success of the species, the annual seed production is very high due to the high floral production. The data show that *A. nitidiflorus* is a facultative xenogam species, but the presence of pollinators can enhance fruit set. The viability of the species is not limited by the flowering and fruiting process, however the maintenance of the habitat in the early successional stages seems to be the critical point for long-term survival of the species.

1. Introduction

The genus *Astragalus* is represented by ca. 3000 taxa in the world and is distributed in semi-arid steppe regions. In the Iberian Peninsula there are 41 described species of *Astragalus*, nine of which are endemics (Podlech, 1999). Among these endemic species, *Astragalus nitidiflorus* Jiménez Mun. et Pau (Fabaceae) is a endemism of the province of Murcia (SE Spain). It is a perennial herbaceous species that was collected in 1909 and described by Pau (1910). Since then it has not been recorded until a few individual plants were observed in 2003 near Cartagena (Murcia province). This species was first classified as extinct (Galicia and Sánchez, 2003) and is now listed as Critically Endangered (Sánchez et al., 2004) in accordance with IUCN criteria (2001). It is currently protected by a national law (BOE, 2005).

The reappearance of this species has awakened the interest of environmental managers because the reasons for its situation are unknown. Following Schemske et al. (1994), narrow endemics are susceptible to extinction for a variety of reasons, such as habitat destruction, biotic interactions and genetic collapse. Hence, those reasons need to be considered when designing conservation strategies because they may affect reproductive success (Godt and Hamrick, 1995). Several authors have described how many factors may affect reproductive success by influencing the processes of flowering, fruit and seed production: plant size (Bishop and Schemske, 1998; Torres et al., 2002; Albert et al., 2008), phenological traits (Sobrevilla, 1988; English-Loeb and Karban 1992; Gómez, 1993; Giménez-Benavides et al., 2007), the breeding system of species (Debandi et al., 2002; Torres et al., 2002; Albert et al., 2008; Hill et al., 2008) and the genetic structure (Hamrick and Godt ,1990).

For most plant populations with overlapping generations, mortality, survival and reproduction tend to vary with age or the size of individual plants (Harper, 1980; Hutchings, 1987; Hegazy, 1990). Quantitative data on population survival, mortality and reproduction throughout life may provide essential information on which conservation management can be based (Hegazy, 1992). However, no information exists about the effect of these factors on this species because it has been overlooked by investigators for almost a hundred years. In order to design and implement an effective conservation programme for this species, understanding demographics features and insight into the life history of *A. nitidiflorus* is needed. In the present paper, the location,

habitat, demography and reproductive biology of *A. nitidiflorus* is described and preliminary data are presented about biotic interactions with other species. More specifically, this study will aim to (1) describe the life history of the species, (2) describe the flowering and fruiting phenology, determining the reproductive success and the factors that influence it, (3) describe the patterns of seedling recruitment, and (4) discuss the implications for the management and conservation of the species.

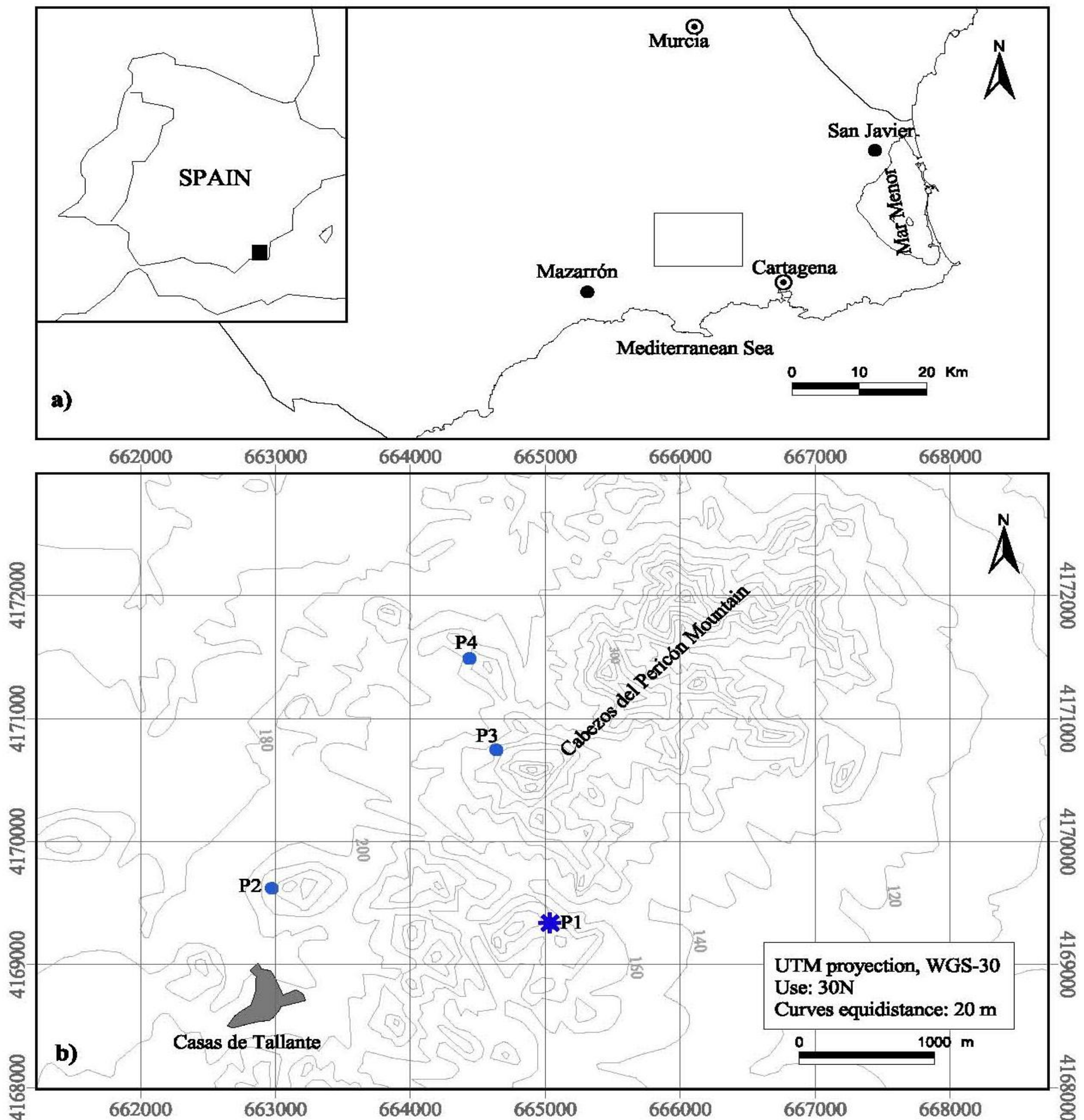

Figure 1. A) Geographical distribution range of *Astragalus nitidiflorus* in south-eastern Spain; B) Location of the population of *Astragalus nitidiflorus* subdivided into four subpopulations (P1, P2, P3, and P4).

2. Materials and methods

2.1. Species and study site

Astragalus nitidiflorus is a short-lived herbaceous legume. Mature plants are generally < 30 cm high, with stems spreading from a caudex in a circular pattern up to 150 cm diameter. The leaves are imparipinnate and measure up to 14 cm and leaflets are villous. The flowers appear in racemes (until 300 racemes in the biggest plants) of up to 30 yellow flowers, with a corolla of up to 2.1 cm. Fruits are curved and acuminate legume of 18 x 7 mm, approximately.

Currently there is only one population of *A. nitidiflorus,* located near Cartagena, in the Cabezos del Pericón Mountain Range (Murcia province) (Fig. 1). The site contains four subpopulations. The first subpopulation, found in 2003, is annotated in Fig. 1 as P1, and was chosen for the present study, which began in 2005. Subpopulation P2 was found in 2007 and its individuals have been monitored for demographic studies. Subpopulations P3 and P4 were found in June 2008 in previously unexplored habitats and they were not monitored for the study.

The species grows in shallow soil from metamorphic and volcanic rocks in the interface between mountain and cultivated areas, from which it colonises old fields. Soils are Leptic Regosols (Eutric) (WRB, 2006) with pH values around 8 and a sandy loam texture. Altitude in the specific distribution area is from 200 to 260 meters above sea level. *Astragalus nitidiflorus* occurs in pastures with *Lotus edulis* L., *Astragalus sesameus* L., *Scorpiurus sulcatus* L., *Brachypodium dystachion* (L.) Beauv., *Bellardia trixago* (L.) All., *Hyparrhenia synaica* (Delile) Llauradó *ex* G. López, *Ononis natrix* L. among which are some chamaephytes such as *Lavandula multifida* L. and *Teucrium capitatum* L. and nanophanerophytes such as *Thymelaea hirsuta* (L.) Endl. The study area has a Mediterranean type climate with semiarid conditions. The mean annual rainfall is 246 mm and annual ETP of 1319 mm. The annual drought period lasts normally 5 months. The mean annual air temperature is 17.6º C, the warmest month being August (monthly mean temperature 26.1ºC, mean maximal temperature 28.9ºC, and mean minimal temperature 23.4ºC), and the coldest month is January (mean monthly temperature 10.4ºC, mean maximal temperature 15.26ºC, and mean minimal

temperature 6ºC). Data of precipitation and temperature during the study period are shown in Fig. 2.

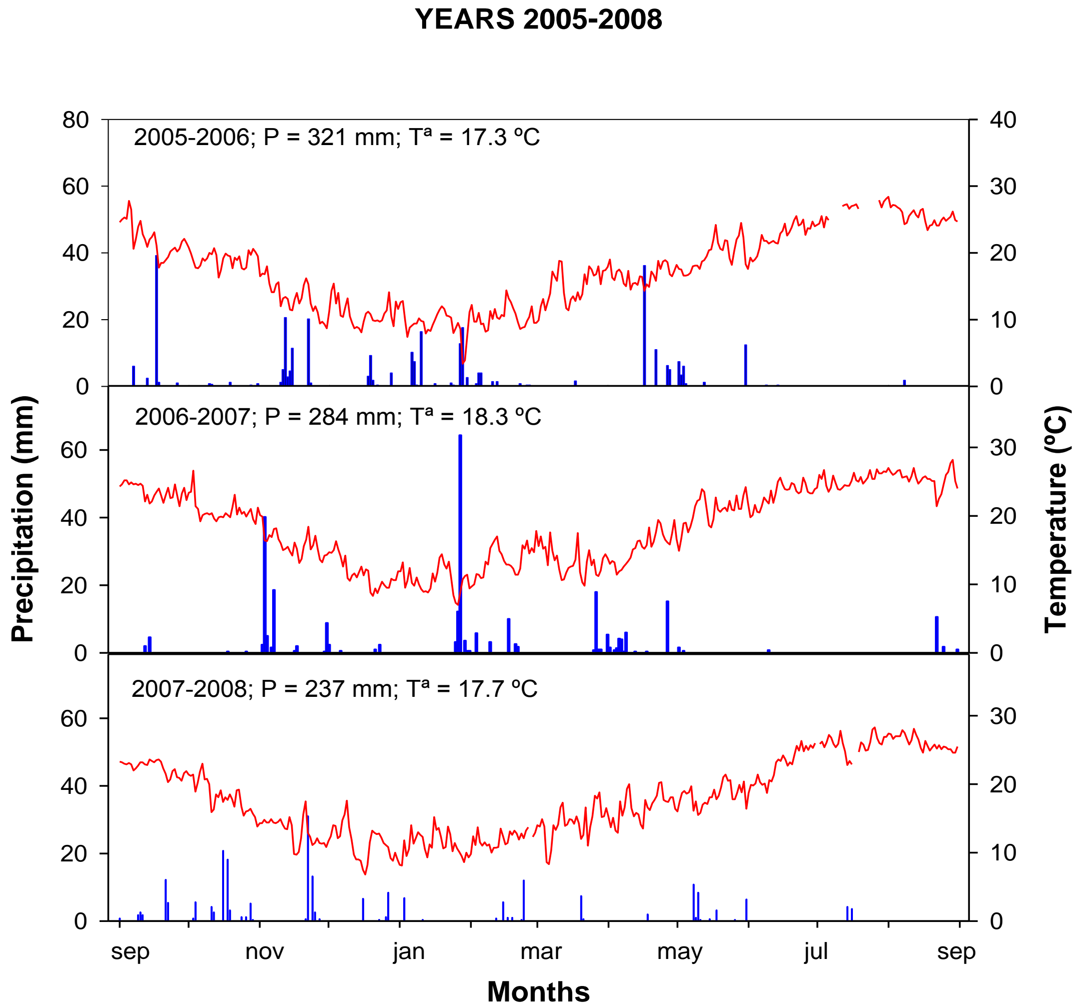

Figure 2. Daily precipitation amount (bars) and mean daily temperatures (lines) for the periods from September 2005 to August 2006; September 2006 to August 2007 and September 2007 to August 2008 in the studied area. P = total precipitation in each period; T = mean temperature for each period.

2.2. Demographic studies

In order to know the life history and population dynamics of *A. nitidiflorus*, all individuals growing in subpopulation P1 were monitored (censused and labeled) from October 2005 to September 2009. In 2007 all plants of P2 were also censused. In order to register all individuals, several visits to the study area in the different seasons of the year were made. Seedlings and the autumn sprout of adult plants that appeared in autumn-winter were recorded and monitored until the end of the study. For the

demographic study, plants monitored throughout the study were grouped into cohorts so that all the individuals that germinated in the same autumn-winter period belonged to the same cohort (e.g. all the individuals germinated in autumn 2006-winter 2007 belong to 2006 cohort). From the beginning of the study (September 2005) a total of 390 individuals were labeled and monitored, of which 20 were already adults when the study began. These 20 adult individuals were assigned to a specific cohort based on their phenological stage, so that one was assigned to 2003 cohort and the other nineteen to 2004 cohort (Table 1).

In order to analyse the population demographic data, and following Pielou (1977) and Hegazy (1992), a life table with the following parameters is shown:

Percentage of the original cohort surviving to the start of each stage (l_x), calculated as follows:

$$l_x = (N_x / N_1)100$$

where x is the age in years, N_1 is the original number of seedlings in each cohort and Nx the surviving individuals in each age.
Percentage of the original cohort dying during each stage (d_x), calculated as:

$$d_x = (l_x - l_{x-1})100$$

Then, the stage-specific mortality percentage (q_x) was calculated as:

$$q_x = (d_x / l_x)100$$

The expectation of future life (e_x) in age units was estimated as:

$$e_x = \sum_{j=x}^{\infty} l_j / l_x$$

2.3. Growth and flowering attributes

Plant diameters were measured at the end of the growing season for each tagged plant. This is considered an appropriate measure of size in plants that are prostrate and whose planar projection is roughly circular (Bishop and Schemske, 1998). To study the flowering phenology, in 2006, maximum flowering moment, flowering intensity, flowering duration and flowering synchrony were obtained in all nine reproductive adult plants in bloom in subpopulation P1. They were monitored every 3-4 days and all

inflorescences were labeled when they appeared. Also, the number of flowers and fruits were monitored at each census, and, at the same time, the number of open flowers in each inflorescence was recorded to know the flowering intensity. In 2007, flowering duration and flowering synchrony were also studied in the eleven flowering plants.

For each plant, maximum flowering moment was calculated as the number of days from the first open flower in the population to the day of maximum flower count on each plant (Bishop and Schemske, 1998). Flowering duration was calculated as the number of days the plant remained in bloom. Intensity was estimated as the maximum number of simultaneously open flowers on one plant. Synchrony is described as the number of days when the flowering of one individual overlaps with the flowering of every other plant in the population (Gómez, 1993). This variable was calculated as follows:

$$S_i = \frac{1}{n-1}\left(\frac{1}{e_i}\right)\sum_{\substack{j=1 \\ j \neq i}}^{n} e_j$$

where: n is the number of plants in the population, e_j is the number of days in which i and j individuals flower simultaneously, and e_i is the number of days individual i is in flower. This index ranges from zero when there is no synchrony to one when flowering overlap is complete.

2.4. Reproductive success

Following Charlesworth (1989), the reproductive success of a plant species is defined as the product of the number of fruits/number of flowers (fruit set) and number of seeds/number of ovules (seed set) ratios. These ratios range from 0 to 1. In 2006 and 2007 fruit set was calculated in the labeled inflorescences, in which the number of flowers and fruits were recorded. To know the seed set, fruits were opened to estimate the number of seeds, which were then related with the mean number of ovules per ovary previously obtained to calculate *P/O* ratio (number of pollen grains/number of ovules).

Table 1. Life table calculated for the studied population of *Astragalus nitidiflorus*: x, age in years; N_x , number of individual per stage; l_x, proportion of original cohort surviving to the start of each stage; d_x, proportion of the original cohort dying during each stage; q_x, stage-specific mortality rate; e_x, expectation of future life. The questions marks in Cohorts 2003 and 2004 indicate unknown number of seedlings (P1) in N_x column, and then incalculable values l_x and d_x columns.

Stage of cohort	Growth period	x (years)	N_x	l_x (%)	d_x (%)	q_x (%)	e_x (years)
Cohort 2003							
P1 (Seedlings)	oct-03/sep-04	1	?				
P2	oct-04/sep-05	2	?				
P3	oct-05/sep-06	3	1	?	?	0	
P4	oct-06/sep-07	4	1	?	?	100	
P5	oct-07/sep-08	5	0	?			
Cohort 2004							
P1 (Seedlings)	oct-04/sep-05	1	?				
P2	oct-05/sep-06	2	19	?	?	31.58	
P3	oct-06/sep-07	3	13	?	?	53.85	
P4	oct-07/sep-08	4	6	?	?	100	
P5	oct-08/sep-09	5	0	?			
Cohort 2005							
P1 (Seedlings)	oct-05/sep-06	1	64	100	71.87	71.87	1.58
P2	oct-06/sep-07	2	18	28.12	3.12	11.11	2.06
P3	oct-07/sep-08	3	16	25.00	20.31	81.25	1.19
P4	oct-08/sep-09	4	3	4.69	4.69	100	1
P5	oct-09/	5	0				
Cohort 2006							
P1 (Seedlings)	oct-06/sep-07	1	292	100	79.11	79.11	1.35
P2	oct-07/sep-08	2	61	20.89	13.36	63.93	1.67
P3	oct-08/sep-09	3	22	7.53	0.98	12.96	1.87
P4	oct-09	4	4	6.56			
Cohort 2007							
P1 (Seedlings)	oct-07/sep-08	1	14	100	100	100	1
P2	oct-08/sep-09	2	0	0			
P3	oct-09	3	0				
Cohort 2008							
P1 (Seedlings)	oct-08/sep-09	1	8	100	100	100	1
P2	oct-09/	2	0	0			
Total cohorts (2005-08)							
P1 (Seedlings)	1th growth period	1	378	100	79.10	79.10	1.33
P2	2th growth period	2	79	20.90	10.85	51.90	1.60
P3	3th growth period	3	38	10.05	8.20	81.58	1.18
P4	4th growth period	4	7	1.85	1.85	100	1
P5	5th growth period	5	0	0			

2.5. Breeding system

To determine the breeding system of *A. nitidiflorus*, we calculated the Outcrossing Index (OCI) and the *P/O* ratio, following Cruden (1977). According to this author, the OCI is the sum of assigned values for three characteristics of the flower and floral behavior: 1) diameter of the flower (corolla up to one mm wide = 0; 1-2 mm wide = 1; 2-6 mm wide = 2; more than 6 mm wide = 3), 2) temporal separation of anther dehiscence and stigma receptivity (homogamy and protogyny = 0, protandry = 1) and 3) spatial relationship of stigma and anthers (if the stigmas and anthers were at the same level and contact between anther and stigma seemed possible = 0, if the stigmas and anthers were spatially separated and contact seemed unlikely = 1). Cruden took account also of the type of habitats: from highly disturbed (value 1) to late successional stages in which pollinator activity was unreliable (value 4).

To calculate pollen production and abundance, eight plants were randomly chosen, from which three inflorescences each with closed flowers were collected. Inflorescences were stored in FAA (formaldehyde acetic alcohol) and taken to the laboratory, where the three closed flowers were excised from each inflorescence for pollen measurements (Burne et al., 2003). The anthers were manually extracted and the pollen grains from each anther were washed into 2 ml soap solution (Tween 80) and then stirred to ensure adequate mixing of pollen in the solution. From this solution 0.01 mL was taken to calculate the amount of pollen per anther. Thereafter the *P/O* ratio was determined by dividing the average number of pollen grains by the average number of ovules per flower. The ovule number per ovary was counted under a stereo microscope in 30 randomly collected flowers.

Pollen viability was estimated using the method initially proposed by Heslop-Harrison and Heslop-Harrison (1970), based on the fluorochromatic reaction that viable pollen grains exhibit when they are incubated with fluorescein diacetate (FDA test). For this determination, 2 mg fluorescein diacetate and 1.71 g sucrose were disolved in 10mL distilled water and the pollen was homogeneously immersed in this solution. All pollen grains, which fluoresced brightly in a fluorescene microscope were scored as viable.

Since some field observations indicated that the species might be self-compatible, three cultivated plants were individually bagged during their flowering

period. At the end of this period, plants were uncovered and the average number of mature fruit in 126 randomly chosen inflorescences was calculated. Data were compared with those obtained from 119 inflorescences of uncovered plants. The average number of seeds per fruit was also recorded in these fruits.

2.6. Statistical analysis

All statistical analyses were performed using the statistical package SPSS 15.0 for Windows (SPSS. Inc., 2006). ANOVA and GLM were used to test for differences in the growing, flowering and reproductive parameters. We used linear regression to test for an association of plant size with age, and plant size with flower production, fruit production, seed production and reproductive success. Before analysis, all the proportions were arcsine transformed to ensure homogeneity of variance. To describe the symmetry of the flowering curves the Weibull function was used, whereby the values lower and higher than 3.6 indicate asymmetry to the left or right, respectively.

3. Results

3.1. Life stage

The plant occurs in the first stages of the plant succession in old fields and bordering intensively tilled almond crops. Tillage practice prevents the expansion of the species. When agricultural activity ceases, *A. nitidiflorus* forms part of the herbaceous colonizer communities in the old fields. However, no *A. nitidiflorus* plants have been found when succession progresses to produce a dense matorral.

The life cycle of *A. nitidiflorus* begins with seeds, which germinate in autumn and winter. Seedlings (henceforth P1) develop until the summer. In summer, leaves and stems die and only a few buds remain at the base of the stem at ground level. After the autumn rains, the dormant buds of the P1 plants that have survived the summer sprout and begin a second stage of growth (P2). The life cycle continues in this way with successive periods of growth (P3, P4) until the plant dies. Some plants survive for four years (P4) (Fig. 3).

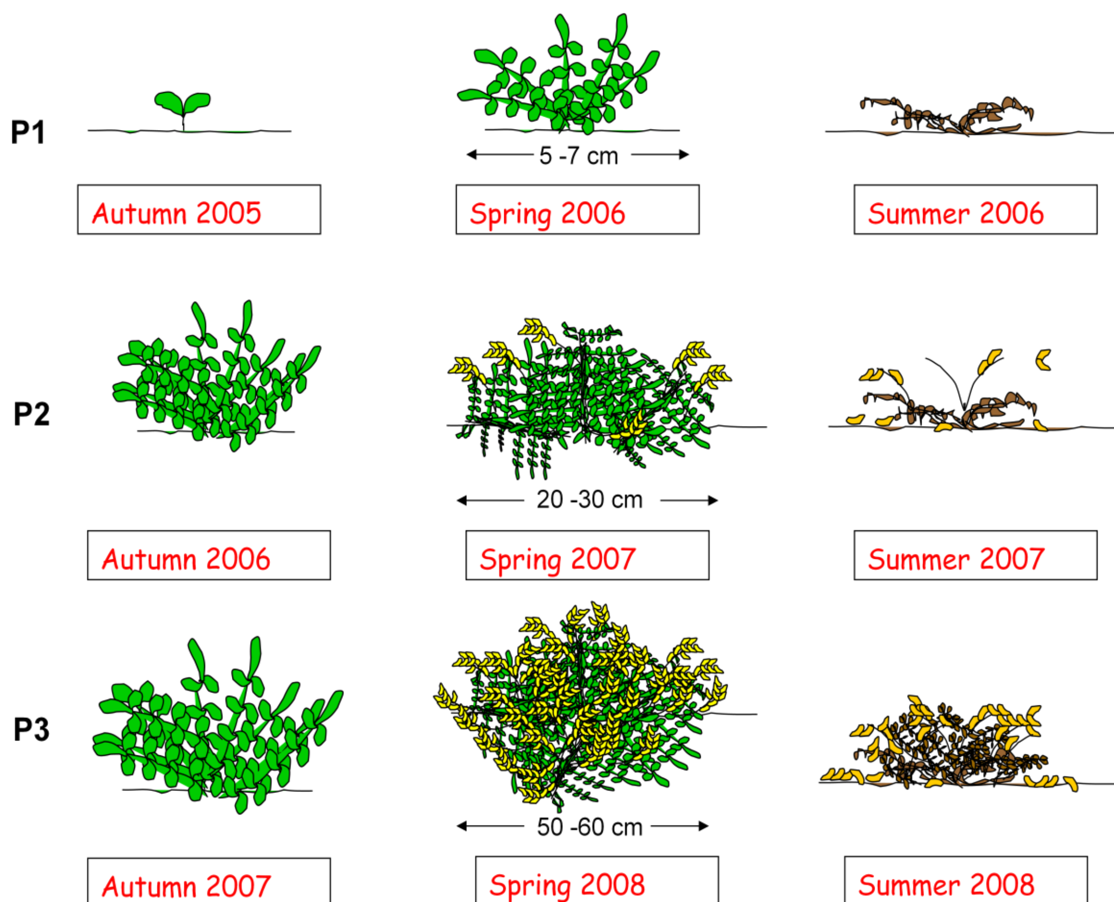

Figure 3. Life cycle of *Astragalus nitidiflorus*.

Until plants reach the stage P2, they are not able to reproduce. In the older plants flowering begins in March and continues until the end of May. The flowers are grouped into racemes that appear from the axillae of the apical leaves on the stem; new inflorescences appearing on the stem as it grows. During six to seven days (exceptionally up to 9 days), in each inflorescence two to four flowers appear per day. Three days elapse between the time the flower corolla appears and the time it withers, so that the inflorescence has open flowers for a total of 9-10 days. The number of flowers per inflorescence is about 20, sometimes reaching 30. The flowers are visited by bees such as *Osmia tricornis* Latreille and *Apis mellifera* L. The fruits take about two months to ripen. Dispersion unit is the fruit, an indehiscent legume that contains up to 19 seeds. The prostrate plant growth habit and weight of the fruits on the infructescences bring these into contact with the soil and the mature fruits drop in the same place as the inflorescences develop (autochory). In this way, the fruit shadow occupies the whole area covered by the mother plant, under which fruits of different

years can be found. Secondary agents (not studied here) must be responsible for fruits being transported far from the mother plant.

As regards biotic interactions in the species, apart from the presence of the above mentioned pollinators, pre-dispersive predation of the seeds by *Bruchophagus astragalii* Fedoseeva, a parasitic hymenoptera whose larvae develop inside the seeds, has been observed. At the beginning of vegetative growth (autumn), the leaves are consumed by larvae of the butterfly *Colias crocea* Furcroy, although little damage results. Generally, cattle avoid the plant, as do small wild rodents such as rabbit.

3.2. Demographic studies

From October 2005 to the end of the study period the seedling emergence varied according to the year, 64 seedlings per population appearing in 2005 cohort, 292 in 2006 cohort, 14 in 2007 and 8 in 2008 cohort (N_1 in Table 1). At the end of the first growing period, only 20.9% of the 378 seedlings developed from October 2005 to September 2009 survived (see *Total cohorts 2005-2008* in Table 1). At the end of the second growing period, only 10.1 % of those seedlings had survived, and at the end of the third period 1.9 % of the original 378 seedlings were still alive. The highest mortality rate (q_x) was recorded during the seedling stage and at the end of the third growing stage (P3). In all cohorts (2005-2008), the P2 plants show lower mortality rates than any other stage and greater life expectancy than P1 and P3 (Table 1). In the analysis of the data for individual cohorts, the seedling stage was the most critical, with mortality reaching 100% in some years (Table 1). Life expectancy also differs according to the cohort to which an individual belongs.

3.3. Growth and flowering attributes

Plant size increased with plant age, and there was a positive relationship between both parameters (r^2 = 0.536, P = 0.0001). Seedlings (P1) showed a mean size of 9.1 ± 4.4 cm (range 3-17.5 cm, median = 9.5) and P2 plants showed a mean size of 51.9 ± 27.6 cm (range 14-106.5 cm, median = 49.5). P3 individuals were significantly larger than P2 (F = 35.88; P = 0.000) reaching a size of 80.4 ± 33.2 cm (range 20-153.5 cm, median = 83). P4 plants did not differ in size from P3 (98.14 ± 30.4 cm, with a range

from 51 to 134 cm, median = 109.5). All the adult plants studied (from P2 stage onwards, N=28) flowered in 2006 and 2007.

The population showed marked interannual differences for the flowering period (Fig. 4). In 2006 the flowering season started on 21 March and lasted until 19 May (60 days). However, in 2007 the flowering period was much longer (84 days) because it started 13 days earlier (8 March) than in 2006 and finished 11 days later (30 May). In 2006, 100% of plants flowered simultaneously during 7 days (8 to 14 April), while in 2007 the simultaneous flowering was observed for a longer period (two weeks, approximately from 19 April to 3 May) (Fig. 4). The pattern of the percentage of flowering plants through time was symmetrical in 2006 (c = 5.50 in the Weibull distribution function), but asymmetrical to the left in 2007 (c = 2.44).

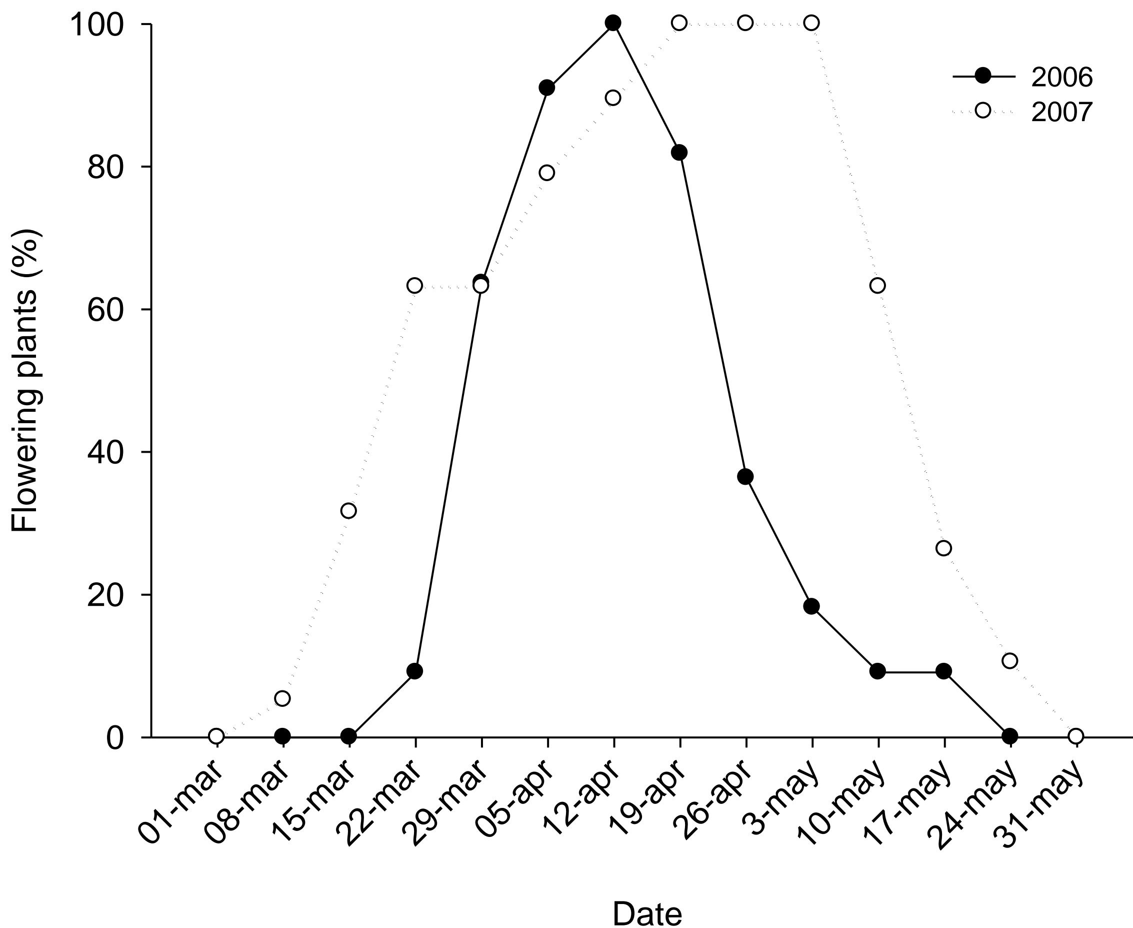

Figure 4. Flowering phenology in the studied population of *Astragalus nitidiflorus* in 2006 (closed circles) and 2007 (open circles).

Mean duration of the flowering period in P2 plants varied significantly (F = 9.99; P = 0.006, N = 19) from 27.9 ± 9.6 days in 2006 to 47.2 ± 15.1 days in 2007 (Table 2). The only P3 plant in the population in 2006 flowered for 36 days, and in 2007 the P3 plants flowered for a mean of 59.7 ± 5.9 days (N=7). There were no significant differences between P2 (N=11) and P3 (N=7) in 2007 (F = 4.30; P = 0.054). Flowering duration of the only plant P4 was 64 days (Table 2).

Maximum flowering moment occurred between 3 and 13 April (13 and 24 days after flowering started (Table 2), a few days after the middle of the mean flowering period.

The maximum number of open flowers per plant (flowering intensity) showed a mean of 101 ± 70.4 flowers in P2, with a very variable range of 27-219 flowers (Table 2). The only P3 plant in 2006 showed a flowering intensity of 479 flowers. There was a strong positive relationship between flower production and flowering intensity (r^2 = 0.964, P = 0.0001, N = 9). In most of the plants the patterns of the flower production were significantly asymmetrical to the left ($c < 3.6$ in the Weibull distribution function), showing a sudden increase in flower production at the beginning of the flowering period, reaching a peak and then a gradual descent until the end of the flowering period (Fig. 5).

As regards the flowering synchrony values there were not significant differences between years (F = 0.01; P = 0.913) and adult types (F = 1.41; P = 0.252), with values of between 0.77 and 0.82 (Table 2).

Picture 1. Detailed view of *Astragalus nitidiflorus* inflorescences.

Table 2. Values of phenological variables in different *Astragalus nitidiflorus* adult plants (P2, P3 and P4) in 2006 and 2007.

Flowering moment (days)		Flowering intensity (flowers)		Flowering duration (days)					Flowering synchrony				
2006		2006		2006		2007			2006		2007		
P2	P3	P2	P3	P2	P3	P2	P3	P4	P2	P3	P2	P3	P4
17	14	122	479	25	36	54	67	64	0.90	0.68	0.78	0.73	0.77
14	-	27	-	18	-	29	51	-	0.93	-	0.97	0.84	-
14	-	172	-	29	-	61	64	-	0.84	-	0.78	0.76	-
13	-	27	-	22	-	54	64	-	0.92	-	0.81	0.77	-
24	-	81	-	25	-	51	57	-	0.76	-	0.82	0.80	-
17	-	41	-	29	-	62	61	-	0.84	-	0.68	0.77	-
14	-	120	-	25	-	22	54	-	0.93	-	0.99	0.72	-
21	-	219	-	50	-	26	-	-	0.47	-	0.90	-	-
-	-	-	-	-	-	52	-	-	-	-	0.78	-	-
-	-	-	-	-	-	65	-	-	-	-	0.70	-	-
-	-	-	-	-	-	43	-	-	-	-	0.78	-	-
16.7±3.9	-	101±70.4	-	27.9±9.6	-	47.2±15.1	59.7±5.9	-	0.82±0.15	-	0.82±0.1	0.77±0.04	-

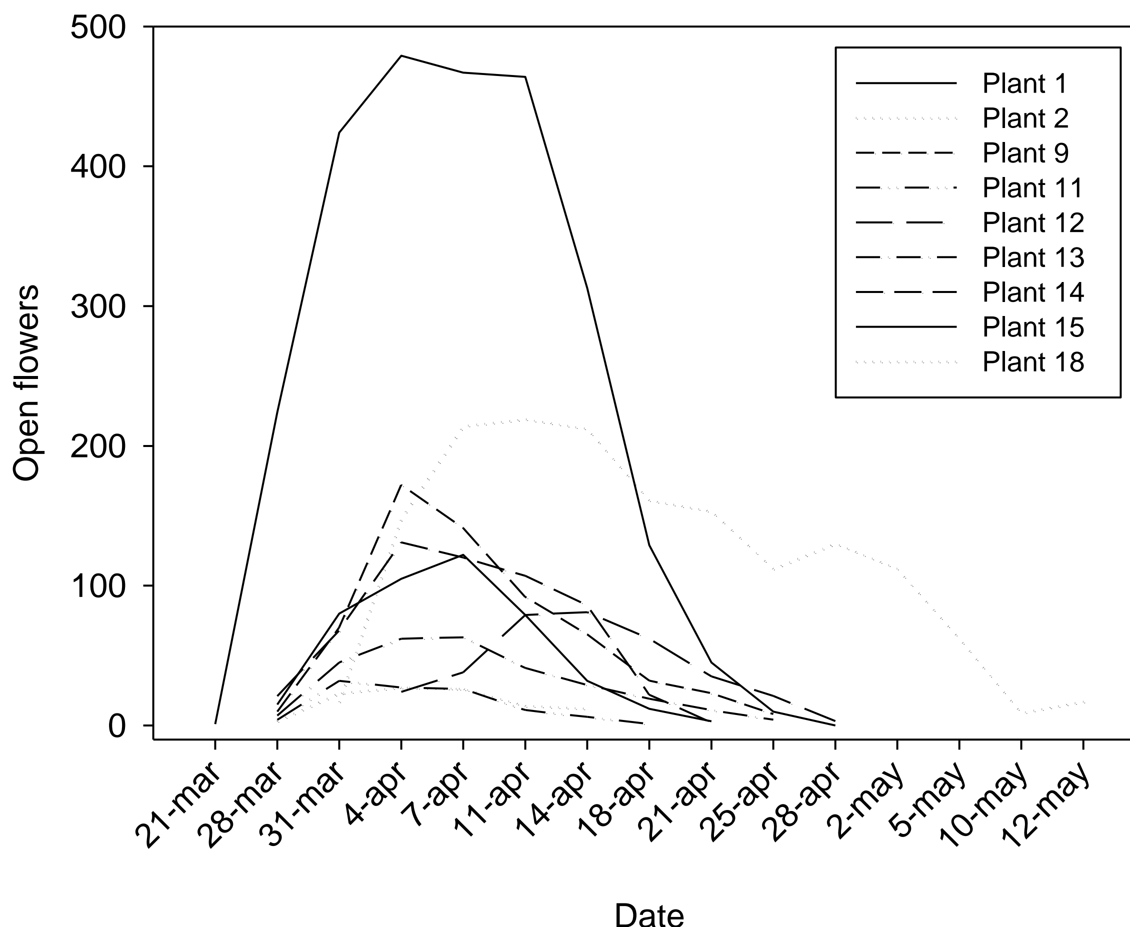

Figure 5. Flowering curves of *Astragalus nitidiflorus* individuals in studied population in 2006.

3.4. Reproductive success

Mean number of inflorescences in P2 plants varied from 33.9 to 32.5, with very high range of variation within the same year (from 2 to 118 per plant, median = 13), but no differences between years (Table 3). In P3 plants from 2007, the mean value reached 179.5 ± 108 inflorescences (range 36-339, median = 163.5). The only P3 plant alive in 2006 produced 219 inflorescences, and 166 in the following year when the plant had become P4 (Table 3), both values within the range shown by P3 in 2007.

The number of flowers per plant varied greatly within each adult type studied. In P2 plants, the number of flowers per plant ranged between 690 ± 539.8 and 673.3 ± 843 (in 2006 and 2007 respectively, with no significant differences), while in P3 plants the

mean value was significantly higher (F = 24.97; P = 0.000) reaching 3715.6 ± 2236.3 flowers per plant. The P4 plant produced 3436 flowers, which was within the range of variation shown by P3 plants (Table 3).

The mean number of ripe fruits per inflorescence (3.5-4.5) did not depend on the age of the plants (Table 3). The number of fruits produced by P2 plants hardly reached the mean value of 150 in either of the years studied (133.5 ± 114.3 and 148.5 ± 201.2 in 2006 and 2007 respectively). P3 plants produced a significantly higher number of fruit than P2 (800.2 ± 617) (F = 16.06; P = 0.001), with a very high range of variation (132 to 2017 fruits per plant) (Table 3).

The proportion of flowers setting fruits (fruit set) was always very low (mean values varying between 0.173 ± 0.06 and 0.214 ± 0.09), with no significant differences between years and type of individual. The number of seeds per fruit did not depend on the plant age, either, ranging from 10.1 ± 2.9 to 13.3 ± 2.8 seeds (Table 3). The number of ovules per ovary did not vary greatly (15-18 ovules; mean value 16.6 ± 0.93). The average proportion of ovules setting seed in each ripe fruit (seed set) was similar among adult plant types and years, varying between 0.788 and 0.610. Reproductive success was very low and was also very similar among adult plant types (mean values from 0.109 to 0.177) (Table 3). The seed production capacity of plants varied. In P2 plants, values ranged from 577 to 7741 seeds, while in P3 the range was 1386 to 27855. The estimated production for the only P4 plant studied in 2007 was 6238 seeds (Table 3).

The absolute reproductive variables as number of flower per plant, number of fruits and number of seeds were related to the size of the individuals (Number of flowers = $164 - 13x + 0.37x^2$, $r^2 = 0.83$, $P < 0.0001$, N = 34; Number of fruits = $154.75 - 8.20x + 0.12x^2$, $r^2 = 0.88$, $P < 0.0001$, N = 34; Number of seeds = $6{,}563.67 - 186.51x + 1.74x^2$, $r^2 = 0.62$, $P = 0.0003$, N =20) (Fig. 6). However, fruit set, seed set and reproductive success were not related to plant size ($r^2 = 0.09$, $P = 0.06$, N = 35; $r^2 = 0.02$, $P = 0.053$, N = 20 and $r^2 = 0.006$, $P = 0.73$, N = 20, respectively).

Table 3. Flower, fruit and seed production of different *Astragalus nitidiflorus* adult types (P2, P3 and P4) in 2006 and 2007 (mean ± S.D and range of values between parentheses).

Plant Stage and year	Number of Inflorescences	Total flowers	Number fruits/ inflorescence	Total fruits	Fruit set	Number seeds/fruit	Seed set	Reproductive success	Total seeds per plant
P2 - 2006 (N=8)	33.9 ± 27.0 (7-87)	690 ± 539.8 (150-1790)	3.7 ± 1.4 (0-14)	133.5 ± 114.3 (6-367)	0.173 ± 0.06 (0.04-0.239)	13.3± 2.8 (6-18)	0.788*	0.177*	1519.6*
P2 - 2007 (N=17)	32.5 ± 40.7 (2-118)	673.3 ± 843 (41-2443)	4.5 ± 2 (0-15)	148.5 ± 201.2 (5-656)	0.214 ± 0.09 (0.06-0.416)	10.6 ± 1.3** (1-16)	0.636 ± 0.08** (0.518-0.729)	0.174 ± 0.08** (0.079-0.303)	3021 ± 2558** (577-7741)
P3 - 2006 (N=1)	219	4939	4.0 ± 4.1 (16-0)	832	0.168	12.6 ± 1.7 (16-8)	0.757	0.109	10483.2
P3 - 2007 (N=8)	179.5 ± 108 (36-339)	3715.6 ± 2236.3 (745-7017)	4.2 ± 0.9 (0-22)	800.5 ± 617 (132-2017)	0.204 ± 0.04 (0.156-0.287)	10.1 ± 2.9 (1-19)	0.610 ± 0.17 (0.252-0.832)	0.127± 0.06 (0.054-0.239)	8031.2 ± 8259.8 (1386-27855)
P4 - 2007 (N=1)	166	3436	3.5 ± 3.2 (0-12)	583	0.170	10.7 ± 2.9 (5-15)	0.645	0.110	6238

* N=1; ** N=7.

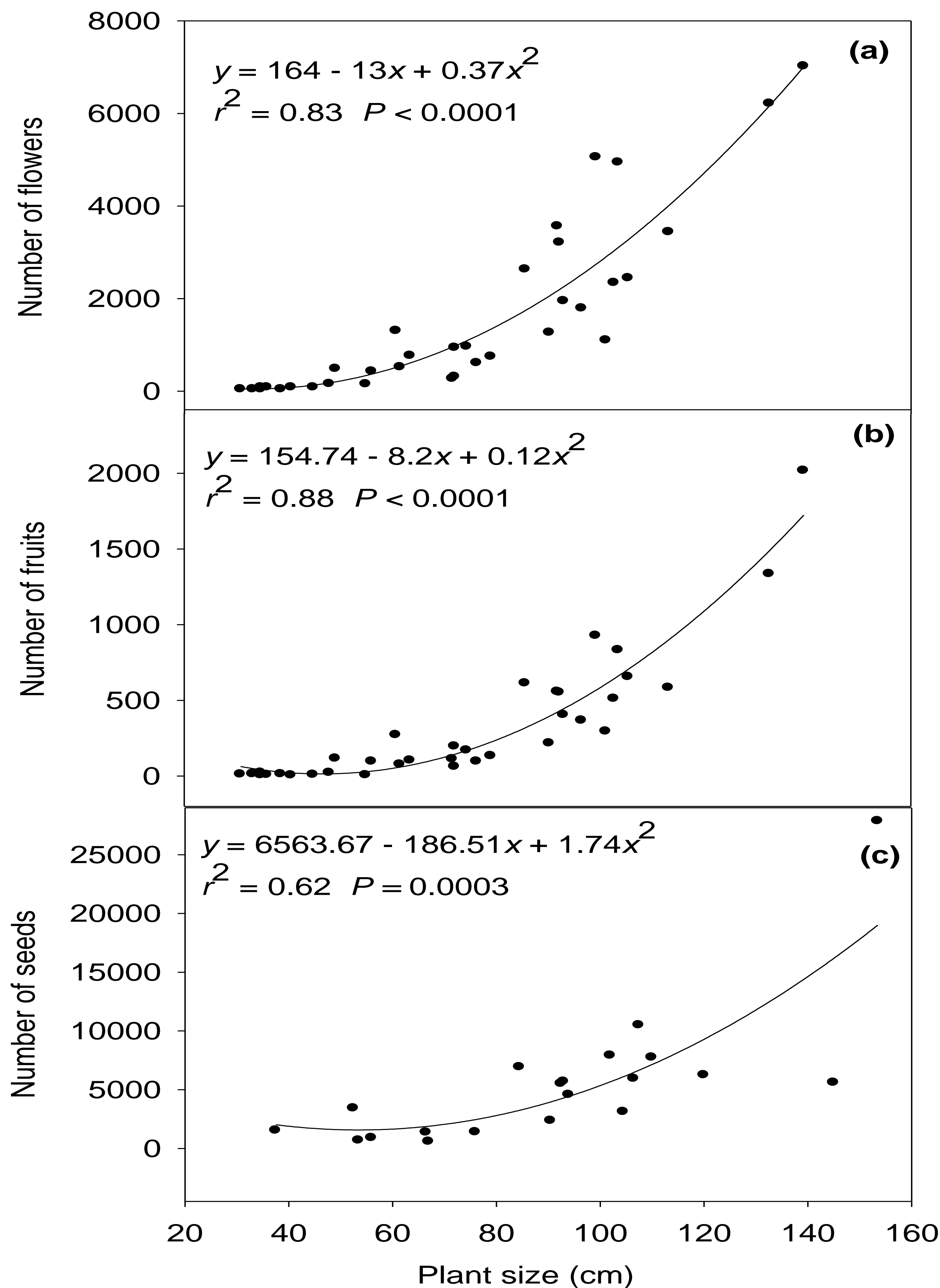

Figure 6. Flowers, fruits and seed production *Astragalus nitidiflorus* plants as a function of size of individuals.

3.5. Breeding system

Taking into account that the corolla diameter of the species is slightly greater than 2 mm (value = 2 following Cruden (1977)), that there is no contact between anthers and stigma (value = 0) and that the species is typical of early plant successional stages (value = 2), the OCI value was equal 4.

The mean number of pollen grains per stamen was 1893± 174. Taking into account that each flower has ten stamens, the number of pollen grains estimated per flower would be 18937. The mean number of ovules per ovary was 16.6 ± 0.93, and the *P/O* ratio was 1,140.8. Pollen viability was 98.8 ± 1.33 %.

Uncovered plants produced a statistically higher number of fruits per inflorescence than covered plants (7.1 ± 4.34 compared with 4.6 ± 2.8; F = 29.32, P = 0.000). However, no significant differences were found in the seed set between flowers from covered plants (12 ± 3.1) or from uncovered plants (10.8 ± 3.7; F = 3.53, P = 0.063).

4. Discussion

4.1. Life stage

Field observations indicate that *Astragalus nitidiflorus* must be considered a short-lived perennial herb (four years old and maximally three flowering periods) that lives in border strips of crops or in old-fields undisturbed by tilling for several years although often with sheep grazing. The species is unable to colonize arable lands that are frequently disturbed (at least twice every year), nor can it colonize areas that have developed late successional vegetation, with higher amounts of nanophanerophytes.

The species is adapted to severe summer drought conditions by forming vegetative buds at the base of the plant at ground level (hemicryptophyte). The vegetative period of the species begins after the first autumn rains (September-October) with the appearance of new seedlings from seeds germinating from the soil seed bank or the resprouting of individuals from previous years that have spent the summer with their dormant vegetative buds at ground level.

In all the cohorts, mortality at the seedling stage was very high (71-100% of seedlings died at the end of the first growth period with the arrival of the summer drought). Therefore this is the most vulnerable stage of the species' life cycle. Although a proper test was not carried out, field observations would suggest that only plants of the first year's cohort which have a sufficiently developed deep root system or which grow in the shade provided by bushes have any possibility of surviving beyond the beginning of summer, especially when spring rainfall has been scarce. Adult P2 plants show a much lower mortality rate, although this varies according to the year (11-64% according to the cohort). Another critical time is the end of the third growth stage (P3), when plants have exhausted their reserves following two intense flowering periods: approximately 82% of P3 plants did not survive the summer season. It is therefore very difficult to find plants that have undergone four growth periods (P4) and which will flower for a third time (only 7 out of the 378 seedlings recorded since 2005 reached P4 stage). No P4 survives to begin another growth period.

Low plant recruitment due to high seedling mortality, probably as a result of the adverse arid conditions prevailing in the region, and the high mortality of adults leads to an age population structure in which adult plants are scarce. This low life expectancy of adult plants, together with the low or null recruitment of new individuals to the population in dry years, could lead to the extinction of individual patches or small subpopulations. Similar observations can be read in classic literature on rare *Astragalus* (Moseley and Popovich, 1995; Kaye,1999). High germination rates are necessary to increase recruitment and to ensure the survival of a population. However, despite the production of a high number of viable seeds (ca. 100%), very few seedlings emerge, presumably due to the physical dormancy of the hard coated seeds, which, also, are protected inside an indehiscent fruit. The absence of seedlings recruitment can threaten the maintenance of the population, though the reproductive effort of the adults is high, as mentioned by Hegazy (1990, 1992) for other, similarly rare taxa.

The long time that *A. nitidiflorus* seedlings remains in a vulnerable juvenile stage, where mortality tends to be relatively high, is an adaptative disadvantage in face of therophytic species in Mediterranean areas with long dry summer (Hegazy, 1992). In the case of *A. nitidiflorus*, we have already mentioned the high number of plants that die in the first year of life before they can flower. This is a disadvantage when they are competing with autumn therophytic species that germinate at the same time as *A.*

nitidiflorus and compete for the same scarce resource that water represents in spring. However, this could turn into an advantage for the few plants that survive the summer, since these plants sprout strongly after the first autumn rains and could compete advantageously against to the newly generated therophytes, taking up sufficient reserves for a prolonged and intense flowering period. That is, to prepare for such a long flowering period, the plants sacrifice flowering the first year, since this would be of little benefit (indeed, poorly developed P2 plants produce hardly any seeds) and involve a high expenditure of their scant reserves, harming their capacity to resprout in the following growing season.

4.2. Flowering period

The population showed a long flowering period lasting two to three months. The flowering period began in late winter and finished in late spring, with an optimum in April. This may be considered as an adaptive advantage in Mediterranean ecosystems, where water stress would restrict the reproductive success of later-flowering plants (Johnson, 1992; Copete et al., 2008). In fact, the data we have concerning fructification in 2008 (not shown) indicate a reproductive failure due to an earlier drought period that occurred in 2008. The rainfall may be extremely scarce both during the flowering period, and in the period between the beginning of sprouting (September) and the time when flowering begins.

An extended blooming period can increase the individual's chance of having a large number of mates both as pollen donor and recipient (Torres et al., 2002) and it reduces the risk of reproductive failure (Bawa, 1983). Furthermore, the individual flowering pattern produced right-skewed flower production curves, which implies the rapid appearance of flowers at the beginning of the flowering season and a gradual, slowly falling away towards the end of the flowering period. This phenological pattern has also been documented in other species (Thomson, 1980; Torres et al., 2002) and it has been suggested that it may be an adaptive response to attract pollinators that usually visit other species (Thompson, 1980). A high flowering intensity (up to 479 flowers open at the same time) positively correlated with flower production is very attractive for a pollinator, and can be added to the strategy that the above mentioned flowering pattern represents (Iriondo et al., 1998).

The high degree of flowering synchrony in the species also may be related with attracting pollinators or simply due to the fact that plants live in very homogeneous habitats as regards ecological conditions (Thompson, 1980). Strong flowering synchrony implies that each plant can exchange genes with most plants of the population, increasing the genetic diversity of the same.

4.3. Reproductive success

The reproductive success of the species is very low and does not depend on plant size. Individual analysis of the components of reproductive success showed that the proportions of flowers setting fruits (fruit set) were always very low regardless of the year and age of the plants (no more than 17% of the flowers produced ripe fruit). However, the average proportion of ovules setting seeds (seed set) was relatively high and was also independent of the year studied and age of the plants (ca. 60 to 80%). Despite the low reproductive success of the species as a result of a low fruit set, seed production capacity may be very high.

This high level of seed production, despite the poor reproductive success of the plants, can be explained by the high number of flowers produced. Large floral displays represent larger energy investments in reproduction (Udovic, 1981), but they attract more pollinators, allow versatility of the plants in face of variable pollinator and improve male fitness of hermaphroditic flowers (Stephenson, 1981; Sutherland, 1986).
The low dispersal capacity of the seeds, which are contained inside indehiscent fruits, implies a high concentration of seeds in the immediate surroundings of the plant. Taking into account the low emergence rate of seedlings near the mother plant (personal observation) and the fact that the seeds have a hard water-proof cover that hinders germination, the seeds must be concentrated in the soil seed bank, as preliminary data suggest (data not shown). The fact that not all the seeds dispersed in one year germinate the following year may prevent high seedling mortality in a year with a favourable autumn but unfavourable (dry) spring, which would lead to the extinction of the population, particularly if we remember that the plants do not produce seeds until the second year of life.

4.4. Breeding system

According to Cruden (1977), a *P/O* ranging from 800 to 5800 would normally point to facultative xenogamy, but OCI values of 4 would indicate obligate xenogamy. The OCI calculated for *A. nitidiflorus* is 4 and the P/O ratio of 1140 suggests that the species is xenogamous. In obligate xenogamous plants, pollination occurs when the pollen from the stamens of a plant reach the stigma of another plant. However, facultative xenogamous plants are regularly self-compatible and although some species require in any case a pollinator, most are able to self-pollination when the flowers close (Cruden, 1977). The mean value of flowering synchrony in *A. nitidiflorus* (ca. 80%) would also suggest xenogamy, although the high fruit set in inflorescences of plants that flower alone during some time (e.g. plant 18 in Fig. 5) would indicate the facultative character of this crossing system. Also, the exclusion of pollinators from *A. nitidiflorus* inflorescences reduced fruit set per inflorescence but not seed set per fruit, suggesting that plants are facultative xenogamous (typically outcrossed, but genetically self-compatible). The same suggestion has been made for other common and rare species of *Astragalus* (Green and Bohart, 1975; Karron, 1989; Kaye, 1999). This phenomenon is facilitated by the simultaneous ripening of anthers and stigmas in the genus *Astragalus* (Barneby, 1964). Among the evolutionary advantages of self-compatibility, Bawa (1973) mentions that it increases the probability of successful pollination and represents a lower cost of reproduction because the fruit set ratio is much higher in self-compatible than in self-incompatible species. Therefore, selective pressure for high levels of seed production may also be responsible for the evolution of self-compatibility. This self-compatibility may provoke an inbreeding depression in seedlings (although not observed in *A. nitidiflorus* seedlings growing in the greenhouse). However, when population sizes are small the genetic load may be largely eliminated (Lande and Schemske, 1985; Schemske and Lande, 1985) and self-compatibility may increase. Then, self-compatibility, which is a vital life-history strategy for many rare and locally endemic species that might otherwise be particularly vulnerable to inconsistent availability of pollinators (Karron, 1987; Stebbins, 1957), may be the result of natural selection during repeated population bottlenecks (Kaye, 1999), and the phenomenon of self-compatibility in *A. nitidiflorus* suggests that the taxon might be able to survive future bottlenecks.

4.5. Implications for conservation and management

In this paper we provide evidence for a facultative xenogamous breeding system in *A. nitidiflorus*, which suggests that the plant does not exclusively depend on pollinators, although their activity would favour fruit production. The results also show that the investigated population of *A. nitidiflorus* is presently not limited by the flowering and fruiting process. Plants are able to produce a sufficiently high number of viable seeds, which because of their low germination rate and hard coats, will form a seed bank which helps dampen the effect of missing recruitment in “bad” years. Detailed studies on the role of the soil seed bank are necessary to fully understand the life cycle of the species. Nevertheless, due to the small size of the subpopulations of the species, demographic stochasticity and extremes of natural climatic conditions (e.g. severe drought periods) could easily lead to a decrease in the number of individuals or even the disappearance of small population patches. In addition, the restricted range of the species makes it vulnerable to catastrophic events.

The intense tillage in the dispersal area of the species prevents the establishment of new patches that would ensure its continued presence. In addition, advanced stages of plant succession (dense formations of nanophanerophytic species) do not favour its presence. Given that *A. nitidiflorus* must be considered typical of early-successional stages, maintenance of this early successional habitat seems to be the critical point to preserving the species. So, suitable agricultural management practices would needed to avoid both excessive disturbance of the soil and the prolonged advance of successional stages of the vegetation. Further studies are needed to look into other factors, both biotic (grazing, symbiosis, competition) and abiotic (land management), which may affect the conservation of the species.

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CHAPTER 2

Genetic diversity of *Astragalus nitidiflorus*, a critically endangered endemic of SE Spain, and implications for its conservation

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Abstract

Inter-simple sequence repeats (ISSR) markers were used to assess the genetic diversity and population structure in five populations of *Astragalus nitidiflorus*, a critically endangered species endemic to southeast Spain. Eight primers amplified 78 bands with 40 (51.3%) being polymorphic. Statistical results indicated a low genetic diversity at the population and species level, with percentages of polymorphic bands (PPB) ranging from 28.2 to 37.2% (an average of 31.8%), and means of gene diversity (H_E) of 0.129 and 0.171 respectively. The Shannon's index (SI) ranged from 0.160 to 0.214 at the population level and was 0.260 at the species level. A low level of genetic differentiation among populations was detected, based on the Shannon's information index (0.297), the coefficient of genetic differentiation between populations (G_{ST} = 0.2418) and AMOVA analysis (Φ_{ST} = 0.255). The estimated gene flow (*N*m) was 0.789. The high genetic connectivity found among populations of *A. nitidiflorus* is an evidence of a recent habitat fragmentation. In addition, a bottleneck event in the past has been revealed, with a subsequent reduction of population size and a loss of genetic variation. Based on these results, the conservation strategy of *A. nitidiflorus* was proposed.

1. Introduction

Astragalus nitidiflorus Jiménez Mun. et Pau (Leguminosae) is a perennial herb endemic to the province of Murcia (SE Spain). This species was classified as Critically Endangered (Sánchez et al., 2004) in accordance with IUCN criteria (2001). It is distributed in five spatially separated populations with around two thousand specimens. The weakest points of the life cycle of the species are the low germination rate and the scarce recruitment of adult individuals in some years (Martínez-Sánchez et al., in press). Therefore, demographic stochasticity and extremes of climatic conditions could lead to a decrease in the number of individuals or the disappearance of small populations.

Preserving the genetic diversity of endangered species is one of the primary goals in conservation strategies, because long term survival and evolution of species depends on the maintenance of sufficient genetic variability within and among populations to accommodate new selection pressures brought about by environmental changes (Barrett and Kohn, 1991). Genetic collapse, that is characteristic of species with a history of fragmented populations and small population sizes, is believed to have a dramatic impact on the ability of a species to survive environmental changes (Wise et al., 2002), if associated with certain fitness traits (Paschke et al., 2002). Thus, assessment of levels and patterns of genetic variation within threatened plant species is essential for the development of effective conservation strategies (Holsinger and Gottlieb, 1991). However, for *A. nitidiflorus*, such information remains unknown.

Though the inter-simple sequence repeat (ISSR) technique (Zietkiewicz et al., 1994) has some problems as a dominant marker, it has proved an effective tool in addressing problems of systematic and hybridization, as well as use in population genetics (Reddy et al., 2002). ISSR markers have been succesfully used to determine the genetic diversity of others narrow endemic species of *Astragalus* (Alexander et al., 2004).

In the present study, using ISSR markers, we aimed to assess the level of genetic diversity including the genetic variation within and between natural populations of *A. nitidiflorus* and provide elementary information for future conservation strategies.

2. Material and methods

2.1. Plant materials

Astragalus nitidiflorus is a short-lived legume, which germinates in autumn and winter; the seedling develops until the summer if there are no water limitations. In summer, the leaves and stems die and only a few buds remain at the base of the stem at ground level. After the autumn rains, the dormant plants that have survived the summer sprout and begin a second stage of growth, showing full flowering capacity. The life cycle continues in this way with successive periods of growth until the plant dies. Some plants survive for four years (Martínez-Sánchez et al., in press).

A. nitidiflorus is located near Cartagena, in the Cabezos del Pericón Mountain Range (Murcia province) (Fig. 1). The site contains a classical metapopulation with five known populations. The first, found in 2003, is annotated in Fig. 1 as P1. Since early 2005, demographic studies of *A. nitidiflorus* carried out by Martínez-Sánchez et al. (in press) have identified a total of 69 individual adult plants. Populations P2 and P3 were found in 2007; since their discovery, only 10 and 20 adult individuals have been identified, respectively. In 2008 the two largest populations were found, P4 y P5, with a population size of around 1000 individuals in each. The maximum and minimum distances between populations are approximately 2600 m (between P1 and P5) and 700 m (between P1 and P2), respectively (Fig. 1). The species grows in shallow soil from metamorphic and volcanic rocks in the interface between mountain and cultivated areas, from which it colonizes abandoned agricultural soils.

In this study a total of 75 individuals of *A. nitidiflorus* from five populations were sampled. Plant material was collected in November 2008, following the sprouting of *A. nitidiflorus* individuals that had survived the summer. Due to the small size of P1, P2 and P3 populations, all adult individuals were selected, while those in P4 and P5 were selected at random, a total of 10 and 15 adult individuals respectively. Voucher specimens are on deposit in the Universidad Politécnica de Cartagena Herbarium (Herbarium code and voucher numbers: UPCT-1937, UPCT-1938, UPCT-1939). The details of the sampling populations are shown in Table 1. Young leaf material from selected plants was collected from each population and stored at -20 ºC with silica gel in plastic bags until DNA extractions were carried out.

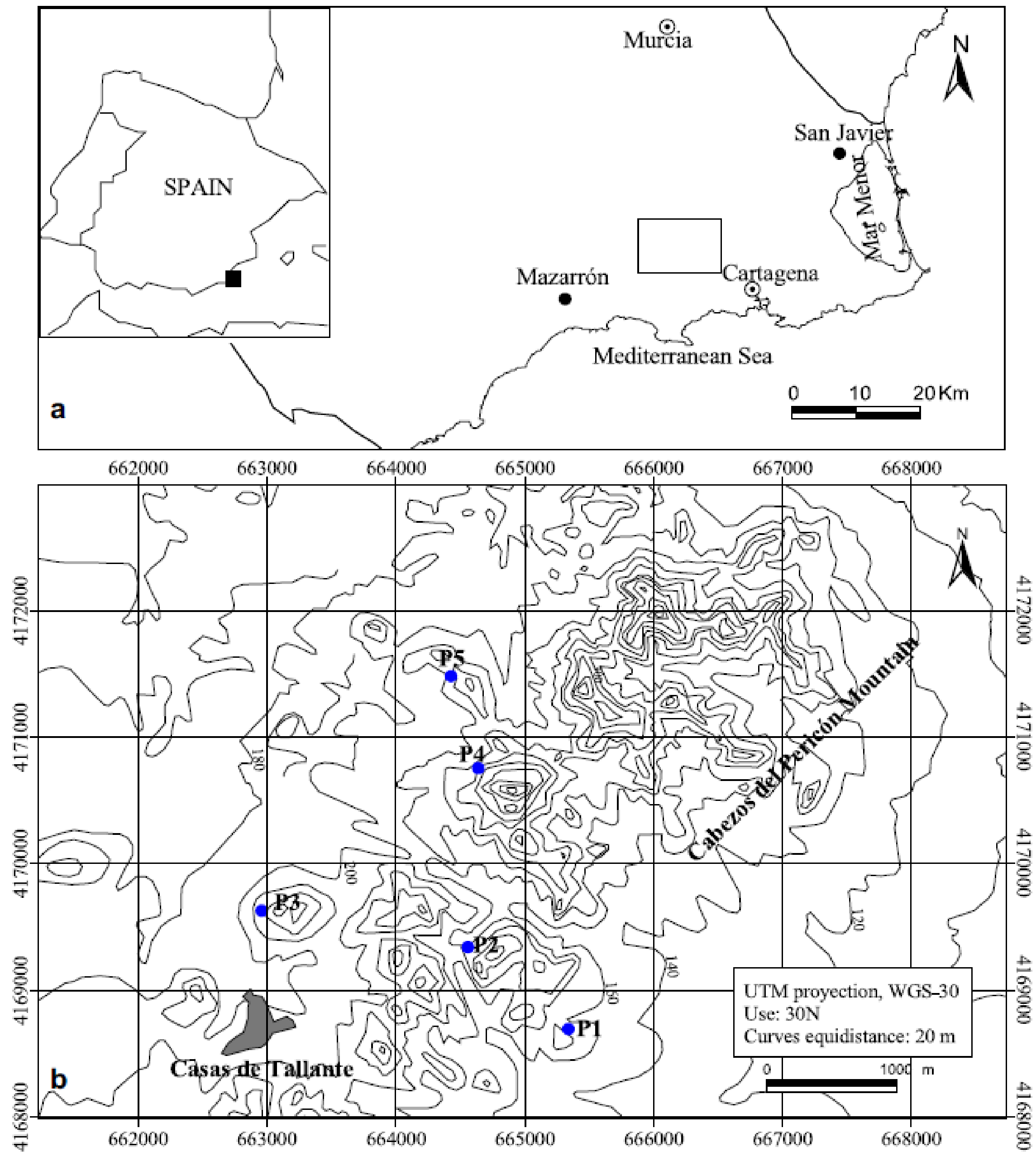

Figure. 1. a) Geographical distribution range of *Astragalus nitidiflorus* in south-eastern Spain; b) Location of the 5 sampled populations of *Astragalus nitidiflorus* (P1, P2, P3, P4 and P5).

Table 1. Sampling details of *Astragalus nitidiflorus* populations used in the present study.

Population	Latitude and longitude	Population size	Sample size
P1	37º39´13.2´´N, 1º07´34.6´´E	28	28
P2	37º39´23.5´´N, 1º08´00.1´´E	8	8
P3	37º39´34.1´´N, 1º09´08.5´´E	14	14
P4	37º40´06.8´´N, 1º08´00.6´´E	~1000	10
P5	37º40´33.2´´N, 1º08´12.1´´E	~1000	15

2.2. Extraction of total DNA and ISSR amplification

Total genomic DNA was extracted individually from young leaves using a ‘‘Danapure. Plantas y hongos’’ extraction kit (GeneDan, Barcelona, Spain). DNA concentration was assessed spectrophotometrically and by 1% agarose gel electrophoresis. The DNA samples were then diluted to 20 ng/µL prior to polymerase chain reaction (PCR) amplification. A total of 30 ISSR primers (UBC primers set #9, University of British Columbia, Vancouver, Canada) were tested for DNA amplification. Eight primers were chosen for ISSR analyses of genetic diversity, based on band reproducibly (Table 2). PCR reactions were carried out using a single primer at a time, in 25 µL reaction mixture containing 40 ng of template DNA, 1 x reaction buffer, 200 µM of each of the four dNTPs, 1 U of Taq DNA polymerase (ECOGEN, S.R.L.), 1.5 mM $MgCl_2$ and 0.5 µM of primer. Amplification was performed using a thermal cycler (Bio-Rad, California, USA) programmed for an initial denaturation step of 5 min at 94 ºC followed by 35 cycles of 45 s at 94 ºC, 1 min at the specific annealing temperature and 1 min at 72 ºC, ending with a final extension step of 7 min at 72 ºC. Each reaction was repeated at least twice, and only reproducible bands were scored. ISSR markers were resolved on 1.5% agarose gels with ethidium bromide detection. Gels were documented using a gel documentation and image analyzer (Vilber Lourmat, Germany).

Table 2. ISSR primers used in this study and analysis of ISSR-generated banding patterns.

Primers	Sequence (5´— 3´)	Annealing temperature (ºC)	No. of bands scored	No. of polymorphic bands
UBC 825	$(AC)_8T$	49.2	9	0
UBC 826	$(AC)_8C$	53.3	10	1
UBC 827	$(AC)_8G$	54.9	16	11
UBC 840	$(GA)_8(CT)T$	48.2	12	10
UBC 841	$(GA)_8(CT)C$	48.5	9	6
UBC 844	$(CT)_8(AG)C$	49.3	10	5
UBC 850	$(GT)_8(CT)C$	55.2	6	3
UBC 855	$(AC)_8(CT)T$	57.1	6	4
Average			9.75	5
Total			78	40

2.3. Genetic diversity analysis

ISSR bands were used to assign loci for each primer and scored as presence (1) and absence (0). Only data from intensely stained, unambiguous, clear bands were used for statistical analysis. The band presence/absence data matrix was analyzed by POPGENE version 1.32 (Yeh et al., 1997), making the assumption that the subpopulations are in Hardy–Weinberg equilibrium at these ISSR marker loci. Genetic diversity within and among populations was measured by the percentage of polymorphic bands (PPBs), the observed number of alleles (Ao), effective number of alleles (Ae), Nei's (1973) gene diversity (H_E, also called expected heterozygosity) and Shannon's information index (SI) (Lewontin, 1972). Within-population genetic diversity (H_{pop}), total genetic diversity (H_{sp}), within-population gene diversity (H_S), total gene diversity (H_T), genetic differentiation coefficient among populations (G_{ST}), Nei's (1972) genetic identity and genetic distance (D) between populations were also calculated. The amount of gene flow among these populations was estimated as $Nm = (1/G_{ST} - 1)/4$ (Slatkin and Barton, 1989). The additional measurement of partitioning genetic variation was obtained with the hierarchical analysis of molecular variance (AMOVA) analysis, using GENALEX 6 (Peakall and Smouse, 2006) with 9999 permutations.

To visualize the genetic relationship among populations, a dendrogram was constructed based on Nei's genetic distance (D) by an unweighted pair-group method of cluster analysis using arithmetic averages (UPGMA) using the software NTSYS pc2.02 (Rohlf, 1998). To test the correlation between Nei's genetic distance (D) between populations and geographic distances (in km) among populations, a Mantel test was performed using GENALEX 6.

The program BOTTLENECK (Piry et al., 1999) was used to test whether populations have recently passed through a bottleneck. Both the stepwise mutation model (SMM) and the infinite allele model (IAM) were run to calculate the heterozygosity (H_{eq}) expected at mutation–drift equilibrium, because ISSR markers evolve under a true model intermediate between them (Di Rienzo et al., 1994; Godwin et al., 1997; Hassel et al., 2005). The sing test was conducted to determine the significance of heterozygosity excess (Cornuet and Luikart, 1996).

3. Results

3.1. Genetic diversity within populations

Of the 30 ISSR primers screened, 8 produced clear and reproducible fragments and were chosen for further analysis. A total of 78 bands ranging from 300 to 1500 bp were scored, corresponding to an average of 9.75 bands per primer (Table 2). The percentage of polymorphic bands (PPB) for a single population ranged from 28.2 to 37.2% (31.8 ± 3.55%), while at the species level this value was the 51.3% (Table 3). The average effective number of alleles per locus (*Ae*) at the population level was 1.233, and 1.301 at the species level (Table 3). The average gene diversity (H_E) was estimated to be 0.129 within populations, and 0.171 at the species level. The Shannon's index (SI) ranged from 0.160 to 0.214 (0.183 ± 0.019) at the population level and was 0.260 at the species level (Table 3). The population P2 had low samples and showed the lowest genetic variation (PPB: 28.2%, *Ae*: 1.192, H_E: 0.109, SI: 0.160), while the highest level of genetic variability occurred in population P1 (PPB: 37.2%, *Ae*: 1.251, H_E: 0.145, SI: 0.214) as shown in Table 3.

Table 3. Genetic variability within populations of *Astragalus nitidiflorus* detected by ISSR analysis.

Population	PPB (%)	A_o	A_e	H_E	SI
P1	37.18	1.3718 ± 0.4864	1.2512 ± 0.3567	0.1457 ± 0.1976	0.2146 ± 0.2867
P2	28.21	1.2821 ± 0.4529	1.1926 ± 0.3397	0.1094 ± 0.1863	0.1604 ± 0.2679
P3	33.33	1.3333 ± 0.4745	1.2334 ± 0.3661	0.1314 ± 0.1986	0.1918 ± 0.2846
P4	29.49	1.2949 ± 0.4589	1.2438 ± 0.4016	0.1297 ± 0.2089	0.1848 ± 0.2944
P5	30.77	1.3077 ± 0.4645	1.2440 ± 0.3892	0.1326 ± 0.2069	0.1899 ± 0.2937
Average	31.80 ± 3.55	1.3179 ± 0.0355	1.2330 ± 0.0234	0.1298 ± 0.0130	0.1833 ± 0.0193
Species	51.28	1.5128 ± 0.5031	1.3010 ± 0.3783	0.1712 ± 0.1999	0.2608 ± 0.2872

PPB, percentage of polymorphic bands; *Ao*, number of alleles per locus; *Ae*, effective number of alleles per locus; H_E, Nei's genetic diversity (assuming Hardy–Weinberg equilibrium); SI, Shannon's information index.

3.2. Between populations diversity

The coefficient of genetic differentiation between populations (G_{ST}) was 0.2418 (Table 4), indicating that about 24% of the total ISSR variation was found among

populations. The ratio of genetic diversity among populations as estimated by Shannon's information index was 0.297 (Table 4). The AMOVA analysis showed that 75% of the total variation occurred between populations and only 25% occurred among populations (P = 0.001) (Table 5). The nearly identical Φ_{ST} from the AMOVA analysis (Table 5) and the G_{ST} from the PopGene analysis demonstrate the robustness of ISSR markers used in this study. The estimated number of migrants per generation (Nm) was 0.789 (Table 4), which suggested that the gene flow in *A. nitidiflorus* was low. Estimates of Nei's genetic distance (D) ranged from 0.026 between populations P1 and P3 to 0.075 between populations P2 and P5 (Table 6). The UPGMA dendrogram (Fig. 2) revealed that the 5 populations were separated into two main groups with a 100% bootstrap support: P1, P2 and P3 populations were in one group, P4 and P5 formed the other group. The Mantel test indicated that there was no significant correlation between genetic variation and geographic distance (r = -0.179, P = 0.273). Bottleneck analysis showed that only the P2 population does not deviated significantly from mutation–drift equilibrium, according to the sing test under both IAM and SMM assumptions (Table 7). The rest of the populations had gone through a bottleneck effect in the past, with the heterozygosity deficiency and excess ratio ranging from 1/23 (P = 0.000) to 4/22 (P = 0.000) under IAM, and from 2/22 (P = 0.000) to 5/21 (P = 0.010) under SSM in the populations P5 and P3 respectively (Table 7).

Table 4. Genetic differentiation within and among populations of *Astragalus nitidiflorus*.

Shannon´s information index		Nei´s gene diversity	
H_{pop} (S.D)	0.1833 (0.0193)	H_S	0.1298 (0.0130)
H_{sp} (S.D)	0.2608 (0.2872)	H_T	0.1712 (0.1999)
H_{pop}/H_{sp}	0.7028	H_S/H_T	0.7582
$(H_{sp} - H_{pop})/H_{sp}$	0.2972	G_{ST}	0.2418
-		Nm	0.789

H_{pop}, within-population genetic diversity; H_{sp}, total genetic diversity; H_{pop}/H_{sp}, ratio of genetic diversity within population; $(H_{sp} - H_{pop})/H_{sp}$, ratio of genetic diversity among population. H_S, within-population gene diversity; H_T, total gene diversity; H_S/H_T, ratio of gene diversity within population; G_{ST}, genetic differentiation coefficient; Nm, gene flow estimated from G_{ST}; S.D., the standard deviation.

Table 5. Analysis of molecular variance (AMOVA) within and among *Astragalus nitidiflorus* populations.

Source of variation	d.f.	SSD	MSD	Variance component	Percentage	Φ_{ST}	*P* value
Among populations	4	89.318	22.330	1.305	25%	0.255	<0.001
Within populations	70	266.975	3.814	3.814	75%		<0.001

d.f., degree of freedom; SSD, sums of squares; MSD, mean square deviations; variance component estimates; percentage of total variance (% total) contributed by each component and significance of variance (P value). Significance test after 9999 permutations.

Table 6. Nei's (1978) unbiased estimates of genetic identity and genetic distance among populations of *Astragalus nitidiflorus*.

Population	P1	P2	P3	P4	P5
P1	-	0.955	0.974	0.949	0.958
P2	0.046	-	0.955	0.937	0.928
P3	0.026	0.046	-	0.941	0.962
P4	0.052	0.065	0.061	-	0.948
P5	0.043	0.075	0.038	0.053	-

Genetic similarity is listed above the diagonal and genetic distance is listed below the diagonal.

Table 7. Results of bottleneck test in the five populations sampled of *Astragalus nitidiflorus*.

Population	Bottleneck test				
		IAM		SMM	
	PL	H_d/H_e	*P*	H_d/H_e	*P*
P1	29	2/27	0.000	3/26	0.000
P2	22	6/16	0.060	6/16	0.281
P3	26	4/22	0.000	5/21	0.010
P4	23	3/20	0.003	3/20	0.000
P5	24	1/23	0.000	2/22	0.000

PL, number of polymorphic loci used in the test; H_d/H_e, number of loci with heterozygosity deficiency and heterozygosity excess under the infinite allele model (IAM) and the stepwise mutation model (SMM); P, probability.

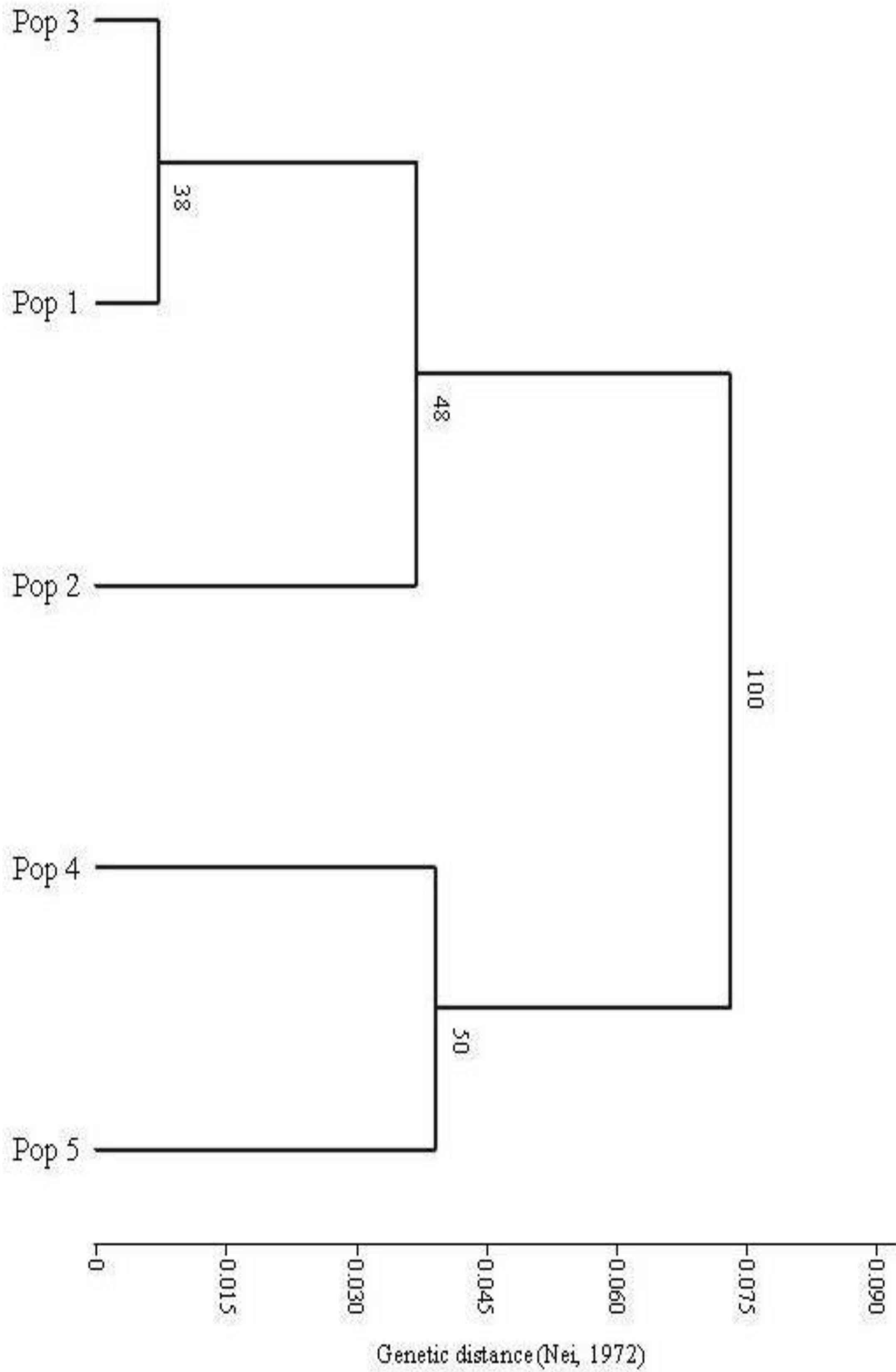

Figure. 2. Dendrogram of five populations of *Astragalus nitidiflorus* using UPGMA cluster analysis of ISSR data. The numbers marked on the branches are bootstrap values (%) out of 1000 bootstrapping.

4. Discussion

4.1. Genetic diversity within populations

The genetic diversity indices based on ISSR markers showed that genetic variation of *A. nitidiflorus* was low both at the species and population level. These results are consistent with some traits of its life history, such as geographical distribution and breeding system. Endemic species have significantly lower within-population diversity than all other species (Gitzendanner and Soltis, 2000). The Nei diversity value at the population level in *A. nitidiflorus* (H_E = 0.1298) was lower than the average plant species with restricted distribution (H_E = 0.191) using RAPD markers (Nybom and Bartish, 2000). On the other hand, the breeding system of a species is a major factor in explaining levels of genetic variability at both the species and population level (Nybom and Bartish, 2000), with selfing taxa being the least diverse and outcrossing taxa the most diverse. Martínez-Sánchez et al. (in press) estimated that *A. nitidiflorus* was a facultative xenogamous plant. These plants are regularly self-compatible and although some species require a pollinator, most self-pollinate when the flowers close (Cruden, 1977). The genetic diversity found in *A. nitidiflorus* at the population level is lower than the average outcrossing-animal species (H_E = 0.260) but higher than self-pollinating plants (H_E = 0.091) using the same data source (Nybom and Bartish, 2000). This is consistent with the mixed breeding system found in this species.

When compared with other narrow endemic *Astragalus* species, the Nei diversity value of *A. nitidiflorus* was similar to *A. albens* (H_E = 0.139) although using allozyme markers (Neel, 2008), and *A. oniciformis* (H_E = 0.150) using ISSR markers (Alexander et al., 2004). Like *A. nitidiflorus*, both species are short-lived perennial plants with a prostrate growth habit, existing in patchily distributed populations in disturbed habitats. Extensive reviews of allozyme-based studies have demonstrated that life form is highly associated with the within-population diversity (Hamrick and Godt, 1989). None the less, *A. nitidiflorus* possessed a lower PPB and Shannon's index compared to both *Astragalus* species, suggesting a relatively lower level of genetic variation within-population in *A. nitidiflorus*.

4.2. Genetic structure of populations

All the estimates of genetic differentiation between populations obtained in this study were very similar and revealed a low level of genetic differentiation among populations of *A. nitidiflorus*. On the other hand, the low G_{ST} value obtained indicates considerable genetic connectivity among populations, which could be explained by medium-high levels of genetic flow or a recent species fragmentation (Caujapé-Castells, 2006). In this case, the low level of gene flow (Nm = 0.789) found in *A. nitidiflorus* is evidence that the high genetic connectivity of the populations is the result of a recent fragmentation, probably due to the habitat fragmentation by agricultural development during the past century. In population genetics, a value of gene flow (Nm) < 1.0 is generally regarded as the threshold quantity beyond which significant population differentiation occurs (Slatkin, 1987). In addition, genetic drift can affect the genetic structure and increase differentiation among populations of a species with small and isolated populations (Ellstrand and Elam, 1993). At present, the small, patchy and isolated populations of *A. nitidiflorus* are probably subject to such genetic drift. The effective gene flow for this species (Nm = 0.789) was slightly lower than one successful migrant per generation among populations. This value is not enough to overcome the diversifying effects of random drift, and therefore the populations of *A. nitidiflorus* could lose the genetic connectivity they have at present, and evolve independently if perpetuated over time. The Mantel test and the UPGMA dendrogram indicate that genetic differentiation among populations of *A. nitidiflorus* does not seem to be correlated with geographic distance among populations, which provides further evidence of genetic drift (Shah et al., 2008).

According to our field observations, we propose that gene flow among populations of *A. nitidiflorus* occurs mainly through pollinators (the flowers are visited by bees such as *Osmia tricornis* Latreille and *Apis mellifera* L.), because the fruit, an indehiscent dry legume, is dispersed by gravity (autochory), and the existence of different hydrologic micro-basins among populations prevents fruit dispersal among them by hydrochory. On the other hand, inbreeding is likely due to the fruit being gravity-dispersed and the seeds falling around mother plants (Keppel et al., 2002). This type of seed dispersal might be in part responsible for the low genetic diversity at the population level found in this species (Hamrick and Godt, 1989).

Finally, our results show that in general, the populations of *A. nitidiflorus* have gone through a bottleneck effect in the past, and a subsequent reduction in population size. This bottleneck has resulted in a loss of genetic variation, as revealed by the low genetic diversity at the population level (H_E = 0.1298) found in this species. Because genetic erosion in fragmented habitats should be more pronounced after several generations, it is expected to find stronger negative effects on the adult generation of short-lived species compared to long-lived species (Young et al., 1996). In addition, the population sizes of *A. nitidiflorus* range from 8 to 1000 individuals, which make this species extremely vulnerable to fluctuations in climate and habitat disturbance (Travis et al., 1996). This data indicates that *A. nitidiflorus* is in a very vulnerable situation.

4.3. Conservation implications

Based on the results reported here, in situ conservation strategies should be adopted to protect and restore all existing populations of *A. nitidiflorus*. Anthropogenic habitat destruction should be prevented, in order to help seedling establishment and recruitment of new individuals, increasing the population size through natural regeneration. A further management measure should aim at increasing the number of plants in small populations by reintroduction from nursery. Ex situ strategies are also needed for the species conservation. In this case, due to the significantly low genetic diversity at the species level, we suggest that the samples for ex situ conservation are collected from all populations of this species, and the seed sample size of each populations should be as large as possible to ensure the diversity of populations. In order to increase the genetic diversity and the adaptation capacity, the transfer of germplasm between populations must be taken into consideration, and new individuals should be established into populations of *A. nitidiflorus* with seeds from multiple sources. Moreover, this measure would limit the loss of genetic connectivity due to low gene flow among populations of this species. New studies on land management are needed to complete the knowledge about the conservation of *A. nitidiflorus*.

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CHAPTER 3

Could recently locally extinct population patches of *Astragalus nitidiflorus* regenerate from the soil seed bank?

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Abstract

Persistence, distribution, and dynamics of *Astragalus nitidiflorus* soil seed bank, a critically endangered species of southern Spain, were studied during four consecutive years to determine their importance to regenerate locally extinct patches of the only known *A. nitidiflorus* population worldwide. The spatial distribution of seeds in the soil was highly influenced by the presence/absence of adult plants and by the indehiscent trait of the fruit. Results showed that most seeds were present on the soil surface, inside the fruit and close to the mother plant. Seed longevity was low, but higher for seeds protected by fruit than for single ones after two years buried in the soil. This species is able to form a short-term persistent soil seed bank that is strongly influenced by environmental factors and population fluctuations. Based on these results, natural regeneration of local patches where plants of *A. nitidiflorus* are no longer present is unlikely from the soil seed bank and population recovery should be attempted by sowing seeds or planting new specimens.

1. Introduction

Astragalus nitidiflorus Jiménez Mun. et Pau (Leguminosae) is a perennial herb endemic to the province of Murcia (southern Spain), where it forms the only known metapopulation worldwide. This species is classified as Critically Endangered in accordance with IUCN (2001) criteria. Although their life history and demographic features has been studied (Martínez-Sánchez et al., 2011), there is no information about its ability to form soil seed banks. The formation of a permanent soil seed bank is favorable in semi-arid ecosystems or in habitats where environmental conditions change dramatically and with unpredictable time patterns (Bonis et al.,1995), ensuring the maintenance of a population in an area even in years with no seed production (Aguado et al., 2012). Seed dormancy and longevity should be extremely important in the maintenance of soil seed banks, but their formation is probably also highly influenced by seed input in the soil or seed production. Hard-seededness is a widely occurring feature in the Leguminosae family that imposes physical seed dormancy allowing the longterm burial of seeds and consequently the formation of persistent soil seed banks (Baskin and Baskin, 1998). Taking into account that plant populations of most semi-arid regions are good candidates for the maintenance of seed banks (Caballero et al., 2003), and the physical dormancy evidence observed in seeds of *A. nitidiflorus*, we hypothesized that this species could form a persistent soil seed bank. Moreover, seed banks in arid ecosystems are characterized by high spatial and temporal variability (Kemp, 1989), presumably in response to the dynamics of processes regulating seed additions and depletions (Cabin and Marshall, 2000), and most arid plants have short-range dispersal (Chambers and MacMahon, 1994). However, there is poor knowledge of such critical aspects of the seed ecology of this species. Therefore, the aims of this study were to analyze: (i) if *A. nitidiflorus* has the ability to form a persistent soil seed bank; (ii) the quantitative importance of the soil seed bank over time; (iii) the seed pool distribution in the soil. This analysis may provide enough information to provide a protocol aimed at preserving small *A. nitidiflorus* population patches that have disappeared and to recover them.

2. Plant material, soil seed bank sampling and burial experiments

A. nitidiflorus is a short-lived legume that colonizes old fields on volcanic soils. The dispersion unit is the fruit, a woody indehiscent legume (ca. 2.5e3.0 cm), being barochory the main dispersal mechanism. The species constitutes a classical metapopulation with five small patchily distributed populations located near Cartagena (Murcia province), having a Mediterranean-type climate with semi-arid conditions. The mean annual rainfall is 246 mm and annual potential evapotranspiration 1319 mm.

Soil samples were collected from forty 1-m^2 permanent plots in June, from 2009 to 2012, in the largest known population (37º40´006.800"N, 1º080´00.600"E). Plots were 5-m apart from each other along four transects established perpendicularly to the maximum slope (Fig. 1).

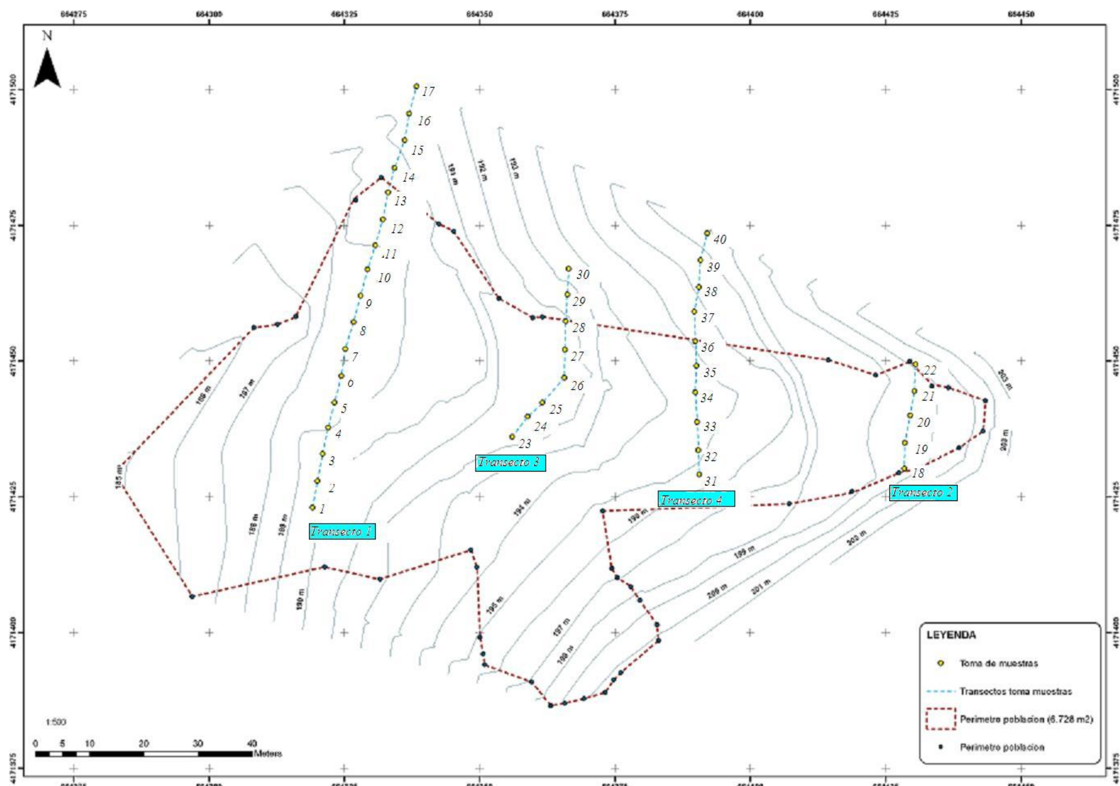

Figure 1. Distribution of plots and transects where samples were taken across the area of appearance of *A. nitidiflorus* largest known population.

In each plot two cores (20 cm x 20 cm) were sampled at two different depths: 0-5 and 5-10 cm, being both cores belonging to the same depth mixed in a single sample. Simultaneously, we measured the distance between the center of the plot and the nearest adult plant of *A. nitidiflorus*. Apparently healthy seeds content in soil samples were separated by physical separation method (Ferrandis et al., 1999a). To test seed viability,

seeds extracted from soil samples were scarified with sandpaper and incubated in a growth chamber (Sanyo MLR-351H, Osaka, Japan) at 15 ºC and 12-h photoperiod. To test if the presence of fruits and seeds in the soil was related to the proximity of adult plants, three plot categories were defined based on the presence/absence of adult plants: (i) presence of the adult near the plot (PA), (ii) absence of the adult near the plot since last year (ALY) and (iii) absence of the adult near the plot for at least two years (ATY). Considering barochory as the dispersal mechanism, a plant was present when it was in a radius ≤2 m to the center of the plot.

Soil seed bank data did not fit a normal distribution, so comparisons of seed density, vertical distribution of fruits and seeds in the soil profile and its relation with presence or absence of adult plants were performed using Wilcoxon, Mann-Whitney U, Friedman or Kruskal-Wallis non-parametric tests at $P < 0.05$. To evaluate the statistical independence between samples the Spearman's rank correlation coefficient was calculated. A chi-squared test was performed to analyze the percentage of plots with seeds or fruits over the years.

To determine the viability over time of seeds from the soil seed bank, two burial experiments were designed using fruits and seeds extracted from fruits (henceforth ‘single seeds’). Two depths were tested: 2 and 7 cm. Sixty four lots containing 10 fruits each were introduced in small aluminum trays with drainage, which were filled with nearby field soil until reaching the desired depth. Another 64 lots of 25 apparently healthy single seeds were put into square nylon bags (5 cm x 5 cm) of 0.1-mm mesh filled with soil, and bags were buried into trays following the same procedures as with experimentally buried fruits. The experiments were placed in a non-heated mesh irrigated with natural rainfall during two consecutive years. At the end of each season, four replicates of both buried fruits and single seeds were exhumed to count physically undamaged seeds. All apparently healthy seeds were tested for germination at 15 ºC and 12-h photoperiod for 30 days, and those germinated were considered as the non-dormant viable seed fraction. To know the initial non-dormant viable seed fraction before burial four replicates of 25 apparently healthy seeds were tested for germination at the same conditions. Then, ungerminated seeds were dried for 1 d and then slightly scarified with sandpaper, and incubated for an additional 30 days period to determine the initial dormant viable seed fraction. Viability was evaluated by the final cumulative germination percentage. The same test was repeated at the end of the burial experiment to determine the viability of seeds after two years buried. Also, before the burial

experiment, 80 fruits were opened and their seeds analyzed to determine the initial percentage of apparently healthy seeds (some seeds are parasitized by *Bruchophagus astragalii*).

Picture 1. Burial experiments carried out with fruits (left) and extracted seeds (right).

A MANOVA was used to evaluate the changes in germinability and viability of seeds in the burial experiment. Prior to analysis, data were root square arcsine transformed and the normality and homoscedasticity of the variables were checked. Differences were tested by Tukey's multiple comparison tests at $P < 0.05$. Regression curve analyses were performed. All data was analyzed using the statistical package SPSS 20.0 (IBM Corp., Armonk, NY) for Windows, and the graph-analysis performed by the software Sigmaplot Version 10.0 (Systat Software Inc., Point Richmond, California).

3. Soil seed bank

Most seeds and fruits were distributed in the soil surface layer (Table 1), and, except for 2011, most seeds were found inside fruits. This is explained by the type of fruit of *A. nitidiflorus*, an indehiscent and hard legume that prevents rapid release of seeds, which remain some years inside the fruit. Moreover, the considerable size of these fruits (ca. 1.9 cm x 0.8 cm) hampers their burial, and nearly 100% them were on the soil surface.

Table 1. Vertical distribution of seeds and fruits extracted from the soil throughout the study period, and proportion of seeds found inside fruits and single seeds. Different lowercase letters denote significant differences between categories in each year (P: non–parametric test significance). M–W: Mann–Whitney U test. W: Wilcoxon signed–rank test. n: number of positive cases of total analyzed. rho: Spearman's rank correlation coefficient. Significance was considered at 0.05(*), 0.01(**) and 0.001(***) P–levels.

Year	Distribution of total seeds layer 0-5 (layer 5-10)			Distribution of single seeds layer 0-5 (layer 5-10)			Distribution of fruits layer 0-5 (layer 5-10)			Seeds found in fruits (single seeds)		
	%		*Stat.*	%		*Stat.*	%		*Stat.*	%		*Stat.*
2009	93.55b n=14	(6.45 a) n=4	$P_{M\text{-}W} = 0.004$ rho = 0.215	84.21b n=10	(15.79a) n=4	$P_{M\text{-}W} = 0.016$ rho = 0.203	100b n=4	(0a) n=0	$P_{M\text{-}W} = 0.041$ rho = 0	59.14b n=4	(40.86a) n=14	$P_{M\text{-}W} = 0.022$ rho = -0.237
2010	95.83b n=8	(4.17a) n=3	$P_W = 0.013$ rho = 0.407**	90.48b n=7	(9.52a) n=3	$P_W = 0.021$ rho = 0.436**	96.72a n=3	(3.28a) n=1	$P_W = 0.109$ rho = 0.592**	62.5a n=4	(37.5a) n=10	$P_W = 0.532$ rho = 0.429**
2011	70.69a n=5	(29.31a) n=2	$P_{M\text{-}W} = 0.231$ rho = 0.286	62.64a n=4	(37.36a) n=2	$P_W = 0.498$ rho = 0.325*	100b n=4	(0a) n=0	$P_{M\text{-}W} = 0.042$ rho = 0	21.55a n=3	(78.45a) n=6	$P_W = 0.128$ rho = 0.431**
2012	100b n=4	(0a) n=0	$P_{M\text{-}W} = 0.042$ rho = 0	100b n=4	(0a) n=0	$P_{M\text{-}W} = 0.041$ rho = 0	100a n=3	(0a) n=0	$P_{M\text{-}W} = 0.079$ rho = 0	95.39a n=3	(4.61a) n=4	$P_W = 0.144$ rho = 0.862**

The highest density of seeds was in PA plots and the lowest density in ATY plots (Fig. 2A). Most plots with seeds or fruits were PA and 83.6% seeds and 89.2% fruits were extracted in PA plots (Fig. 2B). So, the spatial distribution of fruits and seeds on the ground was very heterogeneous and highly influenced by the presence or absence of adult plants, showing the typical contagious distribution pattern observed in other semi-arid sites (Pugnaire and Lázaro, 2000). These data were expected because fruit dispersal happens by gravity from prostrate stems. It has been demonstrated that restricted spatial dispersal could be selected under certain conditions to ensure in situ plant survival and establishment (Lavorel et al., 1994), to facilitate plant coexistence (Green, 1989) and to reinforce spatial aggregation of species inhabiting arid environments (Puigdefabregas and Pugnaire, 1999).

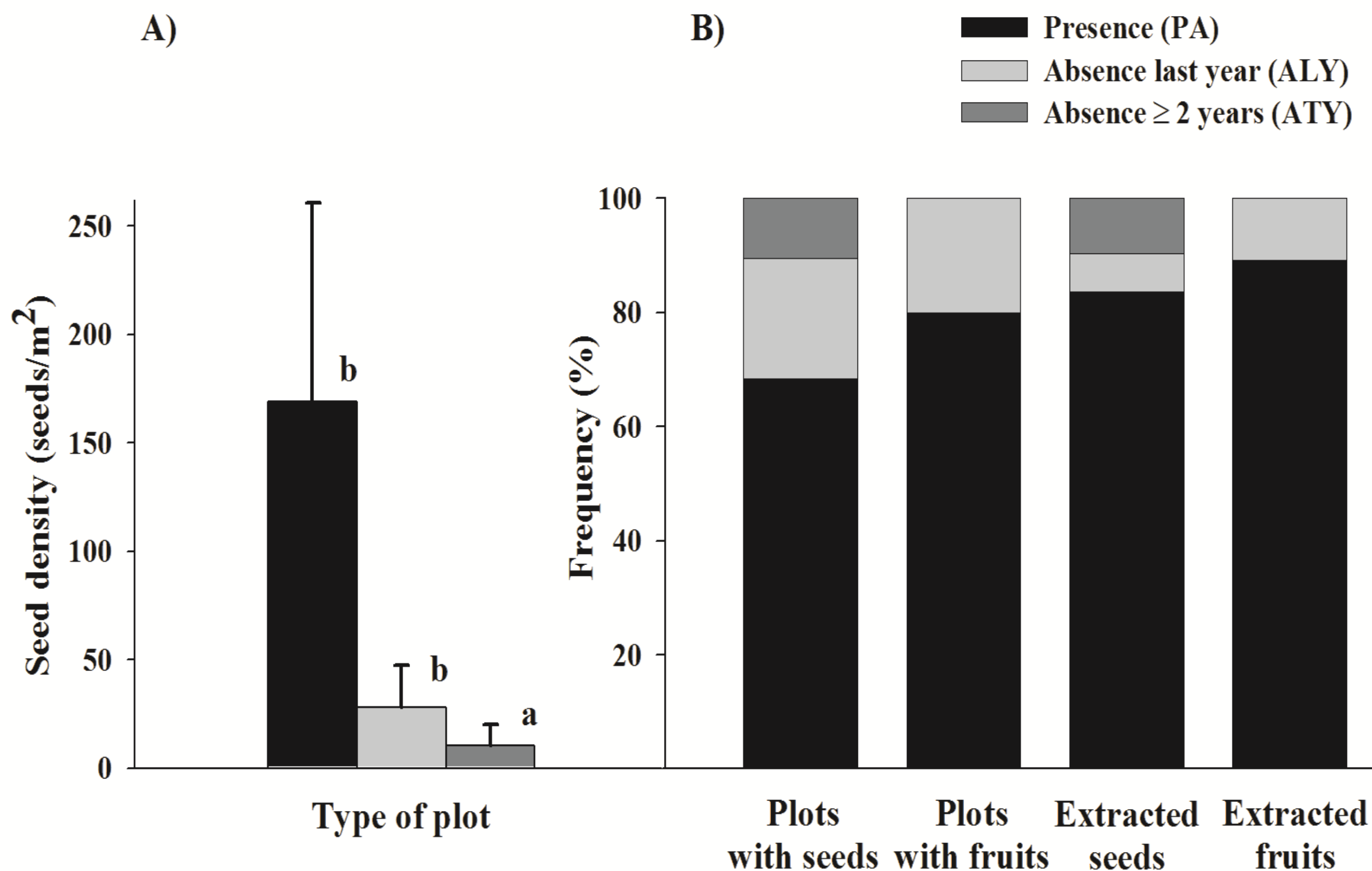

Figure 2. Seed density (A), frequency of plots with seeds or fruits and proportion (%) of total seeds or fruits extracted in each plot type (B). Bars show standard errors. Different lowercase letters denote significant differences of seed density between types of plots.

Table 2. Changes recorded in mean seed density (viable seeds/m^2 ± standard error) and percentage of plots with seeds and fruits of *A. nitidiflorus* in the soil seed bank throughout the study period (2009–2012). Different capital letters denote significant differences between years and different lowercase letters between layers. (P: non–parametric test significance). M–W: Mann–Whitney U test. W: Wilcoxon signed–rank test. F: Friedman test. n: number of positive cases of total analyzed. rho: Spearman's rank correlation coefficient. χ^2: chi–squared value. Significance was considered at 0.05(*), 0.01(**) and 0.001(***) P–levels.

Year	Percentage of plots with seeds	Percentage of plots with fruits	Mean seed density in plots ± S.E. (maximum density found in a plot)		
			Layer 0–5	Layer 5–10	*Stat.*
2009	37.5B n=15	10A n=4	208.81 ± 73.75Bb n=14 (2240)	12.73 ± 7.46Aa n=3 (203.5)	$P_{M\text{-}W}$ = 0.004 rho = 0.015
2010	22.5AB n=9	7.5A n=3	15 ± 8.48Ab n=7 (312.5)	1.25 ± 0.75Aa n=2 (25)	P_W = 0.024 rho = 0.436**
2011	15AB n=6	10A n=4	11.56 ± 8.29Aa n=4 (325)	8.44 ± 8.12Aa n=2 (325)	P_W = 0.500 rho = 0.325*
2012	10A n=4	7.5A n=3	55 ± 43.7Aa n=3 (1725)	0 ± 0Aa n=0 (0)	$P_{M\text{-}W}$ = 0.079 rho = 0
Stat.	χ^2 = 9.722*	χ^2 = 0.313	P_F = 0.003	P_F = 0.256	

The soil seed bank density drastically decreased during 2009-2012 and the percentage of plots with seeds also showed a continuous decline (Table 2). In the best year a mean of 221.54 viable seeds/m^2 was estimated (Table 2), similar to that obtained for the shrub legume *Echinospartum algibicum*, another endemic threatened species (Aparicio and Guisande,1997). Although in most years studied the amount of seeds found in soil was very low, and scarcer than other species that tend to form persistent seed banks such as shrubs of the genus *Cistus* (500-9000 seeds/m^2) or some sprouting shrubs of the genus *Erica* (8500 seeds/m^2), abundant in disturbed forest or scrubland areas (Ferrandis et al., 1999b). This decrease may be a direct consequence of the exceptionally rainy month in September 2009 (222.4 mm) that resulted in a massive emergence of seedlings from the soil seed bank (although most of them died before reaching the adult stage). Moreover, the dynamics of the population in the sample site showed a great reduction in the number of adult plants, declining from almost 2000 plants in 2008 to 73 and 82 individuals in 2010 and 2012 (personal observation). Taking into account that the soil seed bank was so sensitive to environmental factors and changes in population size, and that the seed density values in soil were not maintained

constant over time, we should consider that this species has a short-term persistent seed bank (sensu Thompson and Grime, 1979).

4. Seed longevity in the soil

The percentage of apparently healthy seeds was significantly affected by the time of burial ($P < 0.001$), but not by depth. The initial percentage of apparently healthy seeds in fruits was only 76.6 ± 3% (Fig. 3A) due to seeds parasitized by *B. astragalii*. The percentage of apparently healthy seeds extracted after each season showed a progressive decrease without significant differences between exhumed single seeds and those contained in fruits (Fig. 3A). In both cases, the major decrease was during the first season of burial remaining around 35-30% apparently healthy seeds after two years of being buried (Fig. 3A). The percentage of non-dormant viable seeds was significantly affected by time of burial ($P < 0.001$) and type of seeds ($P < 0.001$), and their interaction ($P < 0.001$). Depth of burial had no effect. Before being buried, the percentage of non-dormant viable seeds was 29 ± 5.3% (Fig. 3B). After burial, the percentage of non-dormant viable single seeds decreased according to an inverse curve ($F_{1,7} = 632.54$, $P < 0.001$, $R^2 = 98.9\%$) (Fig. 2B). However, in seeds extracted from buried fruits the decline was more gradual, following a linear function ($F_{1,7} = 26.3$, $P = 0.001$, $R^2 = 79\%$) (Fig. 2B).

Picture 2. Unburied fruit containing apparently healthy seeds inside.

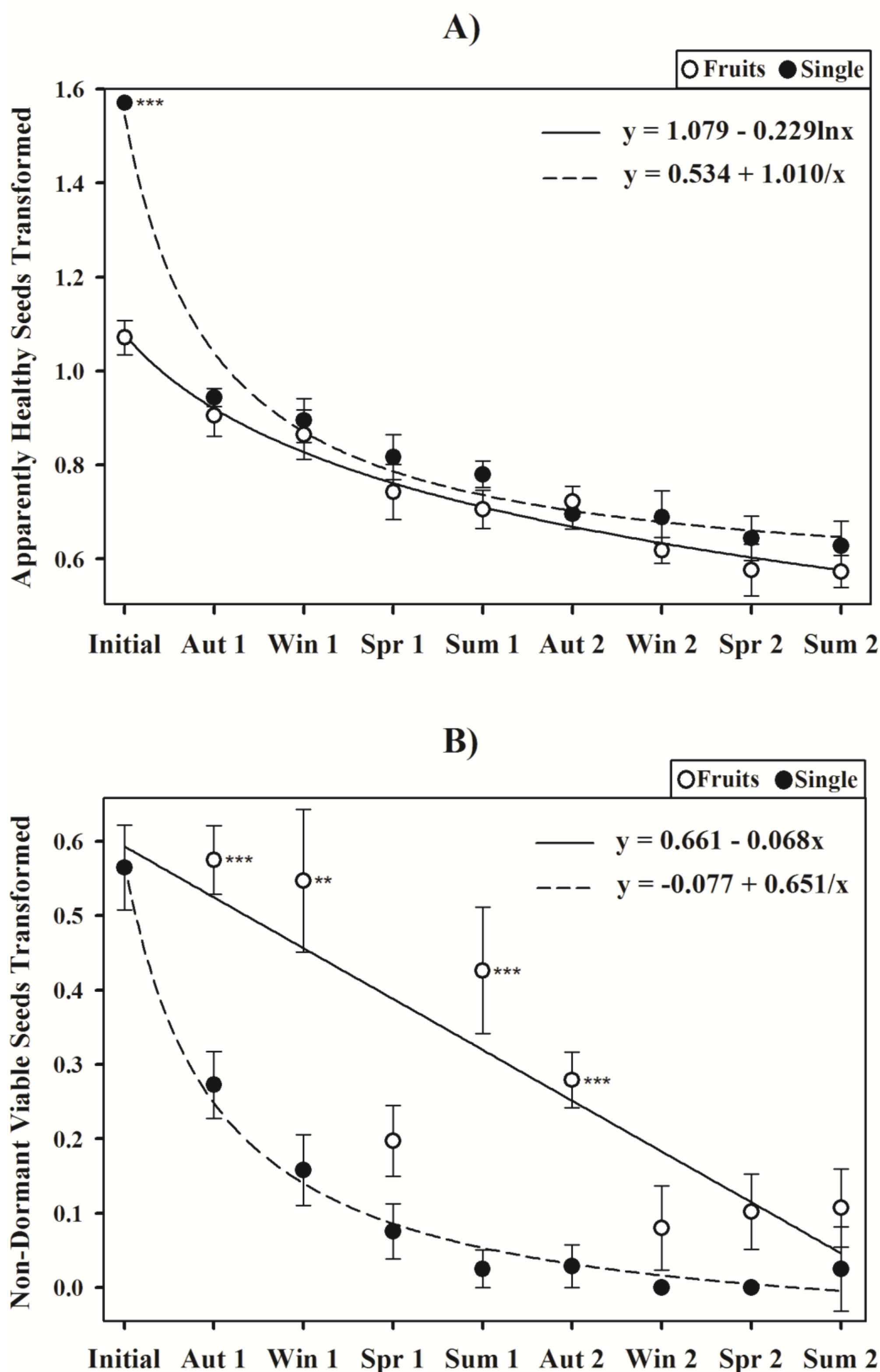

Figure 3. Lost of apparently healthy seeds (A) and non-dormant viable seeds (B) during the seed burial experiment. Mean values are root arcsine transformed. Bars represent the standard error. Significant differences between seeds contained in fruits or single seeds were considered at 0.05(*), 0.01(**) and 0.001(***) P-levels. Equations of significant regression models are also shown.

Single seeds showed a significantly higher initial viability than those contained in fruits (80.7 ± 5.9% versus 61.8 ± 2.5%, P = 0.005) (Fig. 4), mainly because all single seeds used in this experiment were apparently healthy (parasitized seeds were discarded). However, after two years of burial the most significant decline in the viability was for single seeds (Fig. 4): only 5.2 ± 0.6% remained viable compared to 29.3 ± 3.1% seeds contained in fruits (P < 0.001).

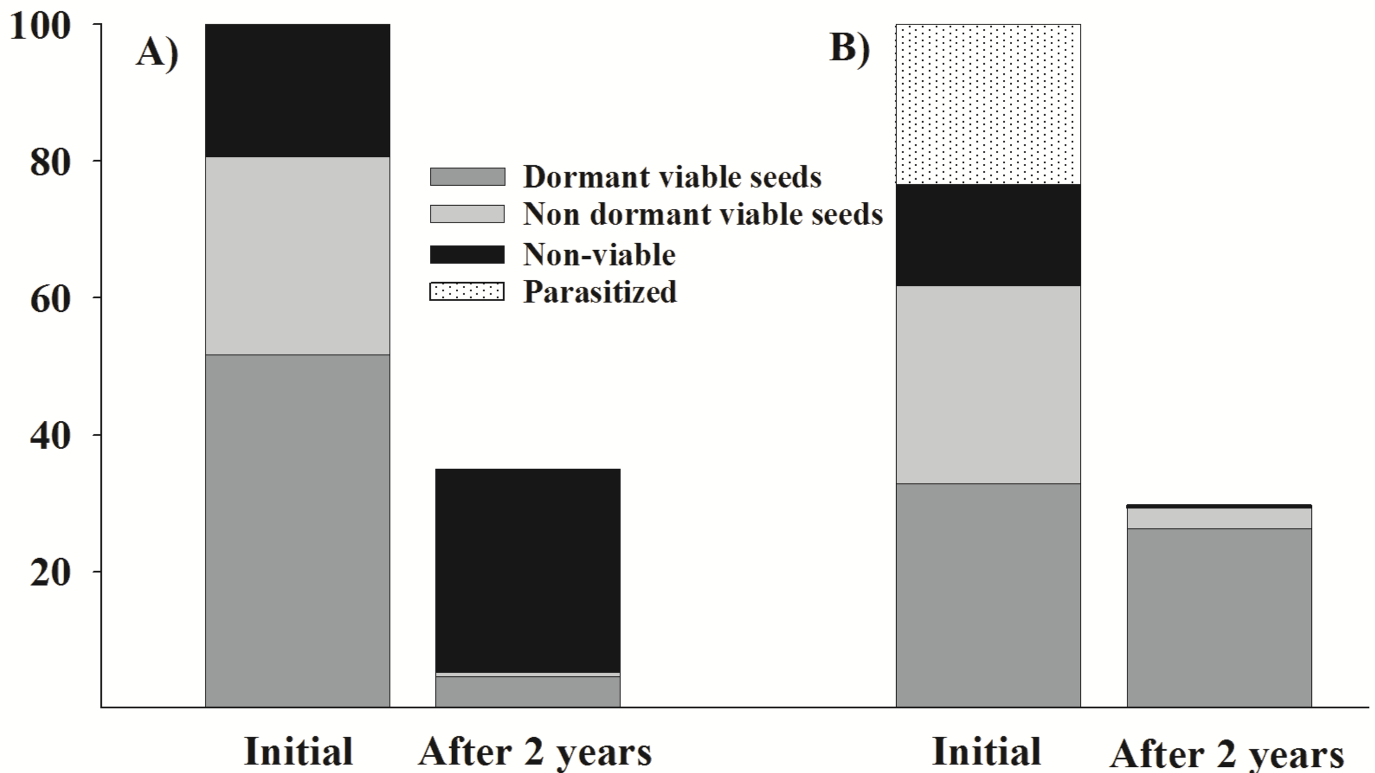

Figure 4. Evolution of the viability and percentage of parasitized, non–viable, dormant and non-dormant viable seeds for single seeds (A) and those contained in fruits (B) after two years of burial.

The woody indehiscent fruits appeared to slow down the loss rate of seed viability, probably due to enhanced protection against fungal and non-fungal diseases (Beckman and Muller-Landau, 2011), against predators (Ellner and Shmida, 1981), prevention of seed scarification by mechanical friction, and also protection of seeds from exposition to climatic factors, e.g. heat, which is one of the principal drivers of seed senescence (Walters, 1998). Initially, around 30% seeds were non-dormant viable, but 100% germinated once they were scarified in the laboratory. This fact, and the seed coat formed by a palisade-cell epidermis (personal observation under scanning electron microscope) like other Leguminosae with hard coats (Patanè and Gresta, 2006), indicate

that seeds of *A. nitidiflorus* have physical dormancy. However, if we consider the high rates of loss of viability experienced in just two years of burial, the hardness of these seeds was not as strong as in other legumes or Cistaceae seeds that have long lifespan once buried in soil (Ferrandis et al., 1999a).

All the evidence suggests that *A. nitidiflorus* has the ability to form a short-term persistent soil seed bank, since it was verified that some seeds remained viable in the soil for at least two years. Viability of seeds from the soil seed bank is strongly influenced by environmental factors and population fluctuations though. Based on this conclusion, natural regeneration from the soil seed bank is not expected for patches where plants of *A. nitidiflorus* have been absent for >5 years as, probably, there will be no new input of seeds. Recovery of such locally extinct patches should be attempted by sowing seeds or planting new specimens. Knowledge of the morphological and dispersal fruit traits can be as necessary for explaining the demographic patterns of the species as classical soil seed bank studies.

Acknowledgements

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CHAPTER 4

Effects of maternal environmental factors on physical dormancy of *Astragalus nitidiflorus* seeds (Fabaceae), a critically endangered species of SE Spain

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Abstract

The viability and seed dormancy of the critically endangered species *Astragalus nitidiflorus* were tested during eight consecutive years (2006–2013) in order to determine if the high physical seed dormancy described in a previous study is affected by annual or seasonal environmental conditions. Also, the effects of maternal factors – such as fruit position in the inflorescence or seed position in the fruit, the age of them other plants, and water stress – on seed viability and dormancy were tested. In order to determine the variation in the degree of germinability between years, ripe seeds were harvested each July from 2006 to 2013 and their viability and dormancy were tested. Moreover, in 2013 new seeds were collected to check the effects of the maternal factors mentioned above. A trial with potted plant in greenhouse was performed to corroborate the observed field data about the effect of water stress suffered by the mother plant on seed dormancy. The results show a high variability over the years in the physical dormancy of *A.nitidiflorus* seeds because maternal environmental factors such as drought or mother plant age influence the proportion of seeds that enter dormancy. This in turn determines the proportion of seed that becomes part of the seed bank each year and also the age structure of the natural population. The conservation programs for this critically endangered species should consider these results to implement measures to prevent the extinction of the species.

1. Introduction

In most plant species, seeds vary in their degree of germination between and within populations and between and within individuals. Some of these variations may be genetic, but many of them are known to be phenotypic (Gutterman, 2000). The so-called maternal environmental effects on germination are considered to be the phenotypic effects caused by the environmental conditions that produce seeds that reach different germination percentages and/or have different germination requirements (Shem-Tov and Gutterman, 2003; Donohue, 2009). According to Gutterman (2000) these conditions consist of a combination of the microenvironment experienced by the seed due to its position on the mother plant and the abiotic environment of the plant (temperature, light, water availability, etc.). As a result, seeds from a single species can vary greatly in dormancy status, depending on when and where they were collected (Hoyle et al., 2008). The maturation of seeds with different germinability and dormancy on one mother plant has a very important ecological advantage, especially in arid ecosystems (Gutterman, 2000). Seed dormancy allows the long-term burial of seeds and consequently the formation of a persistent soil seed bank (Baskin and Baskin, 1998), enhancing seedling survival by delaying germination to avoid competition from established plants or unfavourable weather conditions (Fenner and Thompson, 2005).

Astragalus nitidiflorus Jiménez Mun. et Pau (Leguminosae) is a perennial herb endemic to the province of Murcia (Southern Spain) that was declared extinct in 2003 after nearly 100 years without being observed, but was rediscovered in 2004. Currently it is listed as Critically Endangered, in accordance with IUCN (2001) criteria. After the rediscovery of the species, the first seeds collected (a scarce number in 2005) were analysed and most of them (ca. 90%) showed physical dormancy imposed by a hard coat (Martínez-Sánchez et al., 2011). Hardseededness is a widely occurring feature in Leguminosae (Herranz et al., 1998; Ooi, 2012), that imposes physical seed dormancy by preventing embryo hydration and radicle expansion (Rolston, 1978), and particularly in the genus *Astragalus* (Eisvand et al., 2006; Bacchetta et al., 2011; Martínez-Fernández et al., 2014). In fact, there are evidences in the literature on seed dormancy in 33 *Astragalus* perennial taxa and all of them were reported to have water-impermeable seeds and thus physical dormancy (PY) (Long et al., 2012). Ecologically, physical dormancy has long been proposed to enables plants to survive in unpredictable environments and to prevent seeds from predation, mainly through the formation of

persistent seed banks in soil (Baskin and Baskin, 1998). So we considered the physical seed dormancy in *A.nitidiflorus* as a proven fact. However, in 2005 studies on the biology of this species began (Martínez-Sánchez et al., 2011; Vicente et al., 2011; Segura et al., 2014), the latest one showing that the seeds have a hard coat but the species is unable to form a long-term persistent soil seed bank. So, new evidence about the physical dormancy of seeds and the maternal factors that may influence this dormancy is needed to understand the population dynamics, and would contribute to sustainable management and preservation strategies for this species. Also, the seedling stage is the most critical part of the life cycle of *A. nitidiflorus*, since between 70 and 100% of the seedlings which emerge in autumn do not survive the following summer (Martínez-Sánchez et al., 2011). If there were such an effective mechanism, allowing seeds to 'predict' their future success by utilising information about the environment of their mother plant, as proposed by Tielbörger and Petru (2010), it would be very interesting to know whether it exists in this critically endangered species.

The aim of this paper was to analyse the physical dormancy and germination of *A. nitidiflorus* seeds from different harvests (2006–2013) and to study the effects on seed dormancy of some maternal factors such as (i) fruit position in the inflorescence and seed position in the fruit, (ii) the age of the mother plant and (iii) water stress in the mother plant.

2. Materials and methods

2.1. Plant material and study site

A. nitidiflorus is a perennial herbaceous legume that is short-lived (the plants do not live more than four years) and germinates in autumn. In mature plants (two years old) flowering begins in March and continues until the end of May, being maximal in April. The flowers appear in racemes of up to 30 flowers. The fruits are indehiscent legumes (18 mm × 7 mm) that take about two months to ripen and dispersion take place from early July. The unit of dispersion is the fruit, that may contain up to 19 seeds (Martínez-Sánchez et al., 2011). Currently there is only one metapopulation, composed of four populations very close together (less than 2 km between them). The study area has a Mediterranean type climate with semi-arid conditions. The mean annual rainfall is around 300 mm and the annual potential evapotranspiration 1319 mm. The annual

drought period normally lasts five months. The mean annual air temperature is 17.6 ºC, the warmest month being August (mean monthly temperature 26.1 ºC) and the coldest month January (mean monthly temperature 10.4 ºC). The precipitation and temperature data during the study period are shown in Fig. 1.

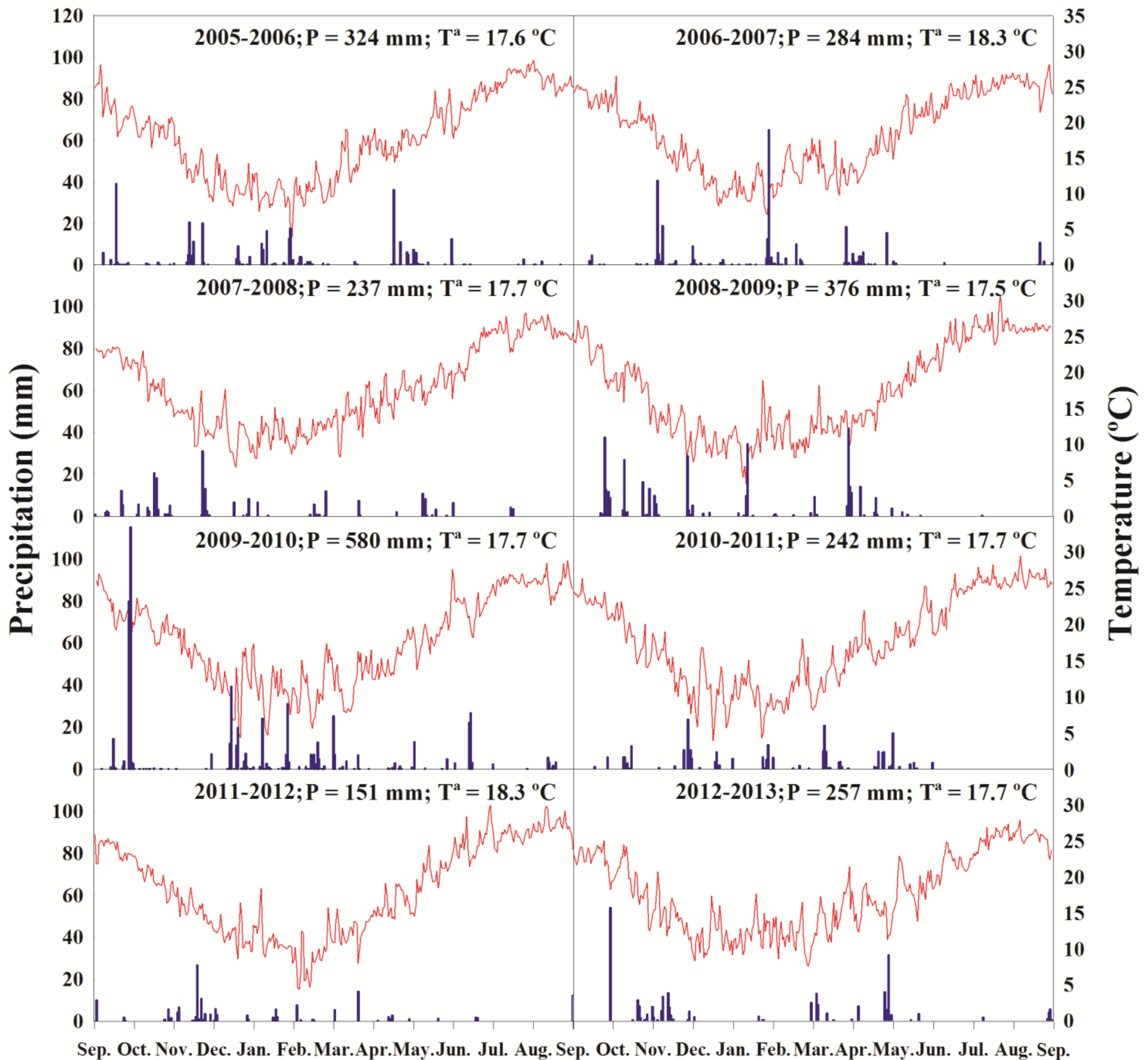

Figure 1. Daily precipitation (bars) and mean daily temperature (lines) for the periods from September of one year to August of the following year (for September 2005 to August 2013) in the studied area. P = total precipitation; T = mean temperature.

2.2. Germination experiments

To examine the variation in the degree of germinability between years, ripe fruits were harvested each July from 2006 to 2013—from 20 fruiting individuals in the largest known population (37º40´N, 1º08´W). The seeds were removed from the fruits by hand and conserved in the Seed Bank of the Technical University of Cartagena at 4 ºC. In July 2013 four replicates (25 seeds each) per year were placed on moistened filter paper in Petri dishes and incubated in a growth chamber with digital temperature and light control system (±0.1 ºC, cool white fluorescent light, 20,000 lx) (Sanyo MLR-351H, Osaka, Japan) at 15 ºC and 12-h photoperiod. The number of germinated seeds (2 mm radicle emergence) was registered every two days for one month and recorded as non-dormant viable seeds (N-DVS). From the same test, ungerminated seeds were scarified with sandpaper, to break physical dormancy, and then placed back on moist filter paper for 15 days. Then, the scarified seeds that germinated were recorded as dormant viable seeds (DVS) and those that did not germinate as non-viable seeds (N-VS).

Picture 1. Impermeable seed coat structure of *A. nitidiflorus* captured by using electron microscope.

2.3. Maternal environmental effect experiments

To evaluate the effects on germinability of the position of the fruit, positions in the inflorescence and seed positions inside the fruit, 20 two-year-old plants were randomly selected from the field population and their ripened fruits collected in July 2013. From each plant, fruits were collected from five different inflorescences in two morphological positions: the basal and apical positions of the inflorescence. From each fruit, healthy seeds were collected separately from the middle and from the apical end of the fruit (two seeds per fruit and position). At the same time, fruits from 10 two-year-old plants and from the same number of three-year-old plants were collected to test the effect of the age of the mother plant on germinability. The germination response was tested as described above for the seed germination experiments, using four replications of 25 seeds each.

Considering that our study on the germination characteristics was always conducted with seeds collected in the same location and that environmental factors such as temperature, light, altitude and mineral nutrition remained practically constant over the years, we focused on the only environmental factor that varied significantly between years, the rainfall (Fig. 1). So, to check the effect of rainfall on seed dormancy a correlation was made between seed germination and the annual amount of rainfall, but no correlation was obtained ($F_{1,6} = 1.153$, $P = 0.324$, $R^2 = 0.161$). However, according to Donohue and Schmitt, 1998, germination is highly responsive to environmental stress experienced before dispersal, during seed maturation on the maternal plant, and at least in some species the last 5–15 days of seed maturation is the critical time (Gutterman, 2000). Taking into account that the maximum flowering moment occurs in April and that the fruits take about two months to ripen, the period of maximum influence of the water availability on *A.nitidiflorus* germinability is most likely to occur during May and June. So, another correlation analysis was made between seed germination and the amount of rainfall in May–June.

Also, one experiment was carried out in a greenhouse to test the effect of maternal drought on seed dormancy. For this, *A. nitidiflorus* plants were grown in a greenhouse at the Tomás Ferro Agrifood Experimental Station in Cartagena (37º41´N, 0º57´W). Plants grown from seed collected in July 2011 in the field population were planted in 48 2.5-L PVC pots filled with a mixture of black peat and soil from the habitat (6:1 vol.) and arranged on metal crop tables in six rows of eight pots each. All

pots were well irrigated until April 2012, when two irrigation treatments were implemented: “well irrigated plants” (WIP) and “plants stressed by drought” (PSD). For each irrigation treatment, three replicates (eight pots each) were considered. The irrigation was controlled by a system identical to that described by Valdés et al. (2012) at the same experimental station. This system was composed of a CR 1000 data logger, balances (Analytical Sartorius, Model 5201) and an Agrónic 4000 (Sistemes Electrònics PROGRÉS, S.A. Bellpuig, Spain). Each pot had an emitter (2 L h^{-1}). The CR1000 recorded the weight of three pots (one pot per balance) per treatment every 30 min. The substrate water available was estimated at 35% by volume (De Boodt and Verdonck, 1972). So, when the balances detected a loss of weight of 500 mg (a 20% reduction of the water available) in the WIP treatment an irrigation event was triggered while for the PSD treatment this occurred when the pot weight fell by 850 mg (35% of the water available). At the fruiting stage (late July) fruits from both treatments were collected separately (five per pot) and seeds were removed by hand in order to test the germination behaviour. Then, four replicates (of 25 seeds each) per irrigation treatment were plated and their germination responses were tested as in the germination experiments described above.

2.4. Statistical analysis

The germination data did not fit a normal distribution, so comparisons of non-dormant viable seeds (N-DVS), dormant viable seeds (DVS) and non-viable seeds (N-VS) between years were performed using the Kruskal–Wallis non-parametric test at $P < 0.05$. To analyse the effects of fruit and seed position, plant age and water availability on these germination variables a Mann–Whitney U non-parametric test at $P < 0.05$ was performed. A logarithmic transformation was performed with percentages of N-DVS, DVS, N-VS in order to calculate the correlation between the proportion of these type of seeds and rainfall amount. After data transformation regressions were analyzed using the Curve Estimation Method (SPSS, 2011).

All data were analysed using the statistical package SPSS 20.0 (IBM Corp., Armonk, NY) for Windows, and the graph-analysis was performed by the software Sigmaplot Version 10.0 (Systat SoftwareInc., Point Richmond, CA).

3. Results

The seed germination of *A. nitidiflorus* varied greatly depending on the year in which the seeds were collected (P < 0.001). In 2007, 2012 and 2013 the proportions of seeds with physical dormancy (DVS) were 97%, 73% and 87%, respectively, but in 2010 and 2011 only 13% and 16% (Fig. 2).

The results also show that fruit position on the inflorescence had no effect on the proportion of dormant seed (P = 0.095) and the same happened regarding the seed position in the fruit (P = 0.663) (Table 1). However, the age of the mother plant had a significant effect on the proportion of non-dormant viable seeds (P = 0.018) and plants at the first fruiting produced seeds with germination percentages (19 ± 6.8%) higher than those of the oldest plants (1 ± 2%; Table 1). But the most influential effect on seed germination characteristics was the availability of water at the fruiting stage. Seeds from well irrigated plants showed a total absence of dormancy (all viable seeds – 87% – were non-dormant) while those from plants stressed by drought showed lower viability (only 68% were viable) and the proportion of non-dormant seed did not reach 20% (Table 1).

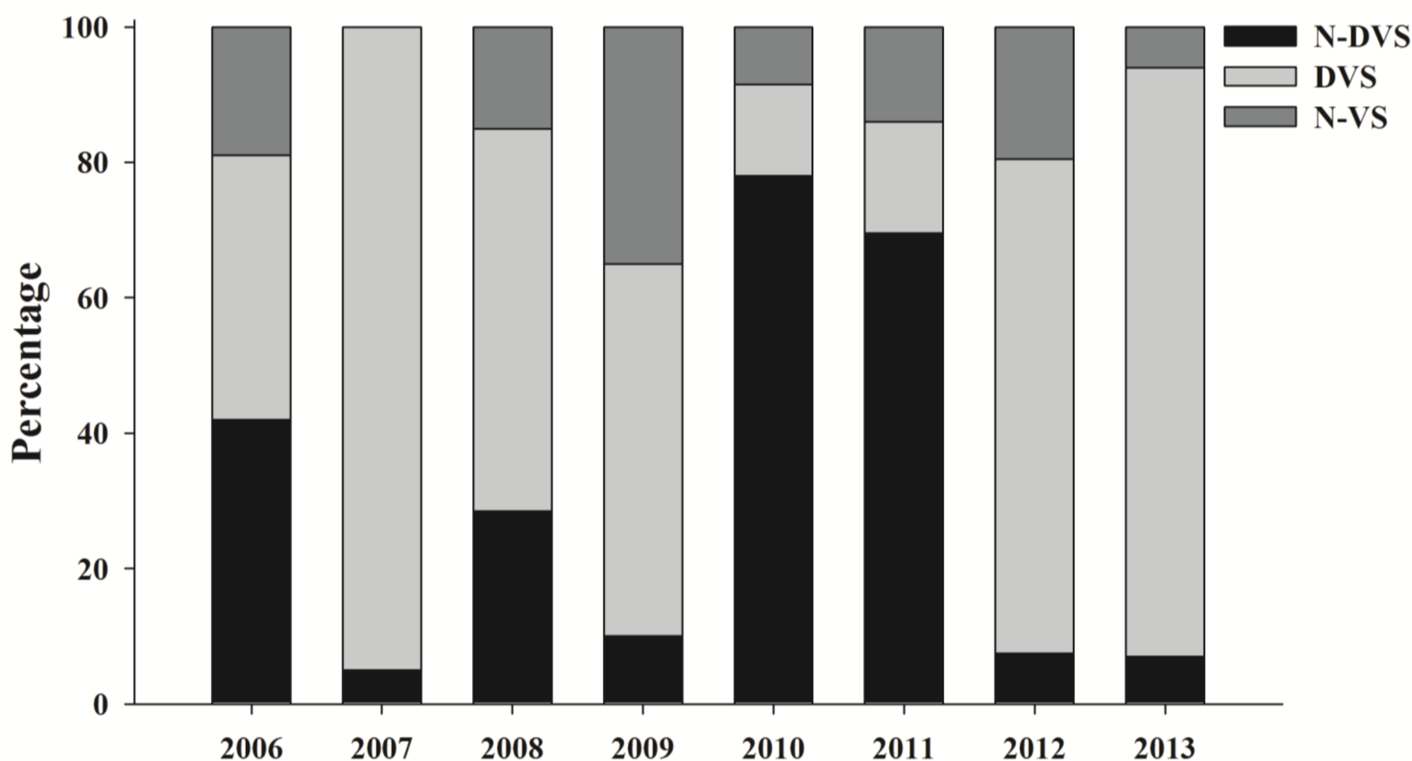

Figure 2. Proportion of dormant seeds collected in the field population in each year from 2006 to 2013. N-DVS = non-dormant viable seeds; DVS = dormant viable seeds; N-VS = non-viable seeds.

In the natural population, the percentage of germinated seeds was also significantly affected by the amount of rainfall during the May–June period ($P < 0.001$), and proportion of N-DVS increasing with the amount of rainfall according to a power curve ($F_{1,6} = 110.005$, $P \leq 0.001$, $R^2 = 0.948$; Fig. 3A). When the rainfall was less than 10 mm in the period from May to June ("dry springs" in 2007, 2009, 2012 and 2013) the proportion of N-DVS was 7.4 ± 1%; when rainfall exceeded 25 mm ("wet springs" in 2006, 2008, 2010 and 2011) this value increased to 54.5 ± 11.6%. However, the proportion of N-DVS was not affected by the total annual rainfall ($F_{1,6} = 1.153$, $P = 0.324$, $R^2 = 0.161$; Fig. 3B). Proportions of N-VS and DVS were not significantly affected by the May–June rainfall ($P = 0.926$; $R^2 = 0.161$ and $P = 0.409$; $R^2 = 0.479$ respectively).

Table 1. Effect of fruit position in the inflorescence, seed position in the fruit, the age of the mother plant and maternal drought on seed dormancy and viability. Stat. (M-W).

Studied factor	Non-dormant viable seeds (%)	Dormant viable seeds (%)	Non-viable seeds (%)
Fruit position			
Basal	13.1 ± 16.6	51.9 ± 38.3	35.1 ± 41.9
Apical	10.9 ± 26.5	73.2 ± 35.8	15.9 ± 29
	P = 0.296	P = 0.095	P = 0.291
Seed position			
Middle	3 ± 2	62 ± 22.7	35 ± 22.5
Outermost	2 ± 2.3	68 ± 19.9	30 ± 19.2
	P = 0.495	P = 0.663	P = 0.770
Plant age			
Two years	19 ± 6.8B	59 ± 9.4	22 ± 14
Three years	1 ± 2A	74 ± 18	25 ± 18.3
	P = 0.018	P = 0.180	P = 1.000
Maternal drought			
Well-irrigated	87 ± 5B	0A	13 ± 5A
Stressed	19 ± 8.9A	39 ± 2B	42 ± 8.3B
	P = 0.020	P = 0.011	P = 0.020

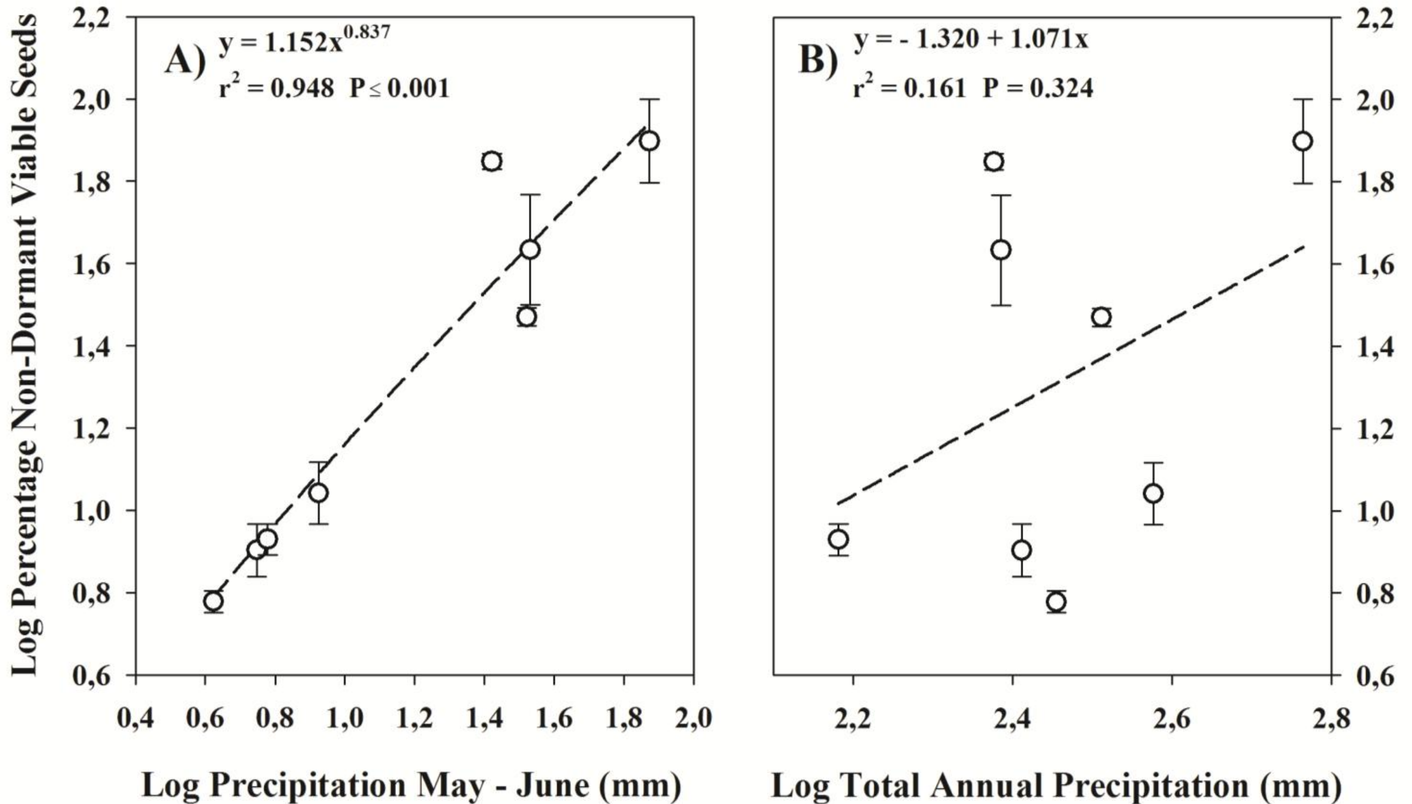

Figure. 3. Relationship between proportion of Non-dormant viable seeds and the precipitation recorded in the May–June (A) or annual (B) period in the eight years sampled.

4. Discussion

Results obtained in this work concerning seed germination are in agreement with results obtained for other species of the genus *Astragalus* in which most of seeds are dormant due to the impermeability of the seed coat to water (Eisvand et al., 2006; Kim et al., 2008; Bacchetta et al., 2011; Long et al., 2012). Also, Martínez-Fernández et al., (2014) showed that seeds from different plants of *Astragalus gines-lopezii* (a species closely related with *A. nitidiflorus*) have not the same germination behaviour even though seeds were collected in the same population and year. But this study has not explained the causes of this intrapopulation variation in seed germination because they did not studied the age effects or water stress effects. However, our results show that the age of the mother plant has an effect on seed germination: older plants produce viable seeds with lower germination percentages than those from younger plants. This is known to occur for a large number of annual species and many examples are cited in Gutterman (2000), supporting the idea that senescence influences seed dormancy. But

the senescence effect is not a good explication in the case of *A. nitidiflorus* because it is a perennial herbaceous species and seeds from plants of different ages were collected at the same time. Taking into account that *A. nitidiflorus* plants at the first fruiting stage (two years old) produced an average of 1500–3000 seeds per plant while plants at the second fruiting stage (three years old) produced 8000–10,500 seeds (Martínez-Sánchez et al., 2011), we can explain our result by taking into account the likely negative relationship between maternal fecundity and offspring germination fraction because the risk of sibling competition increases with increasing seed family size (the sibling competition hypothesis in Eberhart and Tielbörger, 2012). But the same authors have shown that in semi-arid land there is no relationship between maternal fecundity and germination. Regardless of the factors that cause the differing germination behaviour of seeds from plants of different age, our results suggest that the age structure of the population should be an important factor to be considered when estimating the annual replenishment of soil seed banks. At the same time, in perennial species, maternal effects have the potential to influence the age structure of populations, which in turn would influence the projected population growth rates, probability of population extinction and genetic variation (Tonsor et al.,1993).

The seed coat is considered one of the main ways in which information is transmitted to the following generation, as it develops entirely from maternal tissues (Luzuriaga et al., 2006). Changes in the structure and thickness of the seed coat have been described by Lacey et al. (1997), among many other authors. But there are discrepancies among the reports on the impact of drought on seed dormancy (Baskin and Baskin, 1998). The type of response to drought conditions during seed development seems dependent on the kind of dormancy involved. Seeds of *A. nitidiflorus* are known to have physical dormancy due to their suberized and water impermeable seed coat; this is commonly known as hardseededness and is typical of many species from families such as the Leguminosae, Malvaceae, Chenopodiaceae and Liliaceae (Copeland and Mcdonald, 2001). When dormancy is imposed mechanically by a thick seed coat, as in our case, drought usually increases its thickness, thereby contributing to reduced germinability (Fenner, 1991; Baskin and Baskin, 1998). In fact, our results are supported by some studies in which parental drought increased dormancy in other legumes such as *Glycine max* (Dornbos and Mullen, 1991), *Arachis hypogea* (Pallaset al., 1977) and *Acacia saligna* (Tozer and Ooi, 2014). However, other studies found that parental drought decreased dormancy in *Sorghum halepense* (Arnold et al., 1992),

Raphanus rapahnistrum (Eslami et al., 2010) and *Sinapis arvensis* (Wright et al., 1999; Luzuriaga et al., 2006) among many other species. Tielbörger and Petru (2010) assumed that competition is the ultimate reason for the existence of such maternal environmental effects and hypothesised that there is a negative relationship between favourable seasons and seed germination; then, the offspring may reduce the negative effects of crowding in the following year (Tielbörger andValleriani, 2005; Valleriani and Tielbörger, 2006). However, this hypothesis is valid in climates with high precipitation but not in semi-arid ecosystems where plant interactions can be neutral or positive (Holzapfel et al., 2006). The total annual rainfall, the amount of rain in each rainfall event and the time between rainfall events are highly variable in semi-arid areas (Shem-Tov and Gutterman, 2003). This is the case for our study, where the annual rainfall from 2005 to 2013 averaged 306 mm (Fig. 1) and massive offspring germination was observed in some autumns. The ecological implication of these differences is that seeds produced in a dry spring are likely to have a slower rate of dormancy release than those produced in a wet spring. Thus, seeds from a dry year maybe more likely to become part of the persistent soil seed bank than those produced in a wet year, which are more water permeable and will be able to germinate when the environmental conditions are favourable. This mechanism is responsible for long-term seed dormancy but also provides sufficient germinable seeds for colonisation when conditions become favourable (Eslami et al., 2010). Our findings show that drought from May to June could prevent the emergence of most of the offspring with the first autumn rains. The results shown in this paper are consistent with the population dynamics of *A. nitidiflorus*, characterised by highly variable population censuses between years and specific episodes of massive emergence of seedlings as well as great oscillations of the soil seed bank, depending on the demographic fluctuations in the short-term population shown by Segura et al. (2014). The maximum life span of an *A. nitidiflorus* plant is four years and in that time the plant is fruitful three times, so the probability that different seed yields are affected by plant age or different drought periods is very high. Then, the offspring will have a very high plasticity, to closely match the changing conditions of this unpredictable environment.

In conclusion, the production of dormant seed in *A. nitidiflorus* could be very variable over the years because maternal environmental factors, such as drought or mother plant age, influence the proportion of seeds that enter dormancy and become part of the seed bank. So, the age structure of the population is an important factor to be

considered when estimating the annual replenishment of soil seed banks. Besides, the amount of rainfall in late spring needs to be considered in order to plan strategies to manage the endangered populations of *A. nitidiflorus* and of other species with similar characteristics.

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CHAPTER 5

Potential contribution of the soil seed bank for maintaining genetic diversity in a rare perennial species (*Astragalus nitidiflorus*) measured by HRM

Abstract

Genetic diversity of the soil seed bank, seedlings and above ground adults of *Astragalus nitidiflorus*, an endangered rare species of southern Spain, were studied during three years in the most largest known population to check the potential of seeds stored in the soil to maintain genetic diversity. For this, we used the new HRM technique employing chloroplast markers (cpSSRs). Results showed that genetic diversity was higher in the soil seed bank than for above ground adults, although stored genotypes not remained constant in the soil more than two years because of the short-term persistence character of *A.nitidiflorus* soil seed bank. Despite the continuous reduction of population size, soil seed bank genetic diversity increased over time probably due to pollen exchange between more physically and genetically distant individuals. One of the highest genetic diversities was contained in the seedling stage although much of this genetic richness is not transmitted to adults due to high mortality that occurs at early stages, hence this point results to be determinant defining genetic characteristics of future populations.

1. Introduction

When plants face stressful environments, many can survive temporally by producing seeds that are stored in the soil where they can await favorable conditions for germination (Cabin, 1996; Thompson et al., 1997). Longevity of seeds in the soil is the major characteristics of persistent seed banks, and the greater the longevity of seeds in the soil, the greater the evolutionary and ecological potential of seed banks (Venable and Brown, 1988). The persistent soil seed bank with different genotypes from multiple generations may establish a very diverse gene bank, potentially exceeding the genetic diversity of the above ground population (Templeton and Levin, 1979; Brown and Venable, 1986; Mandak et al., 2006). Thus, the seed bank is thought to be a way of conserving and restoring the genetic diversity of above ground plant populations (Templeton and Levin, 1979; Honnay et al., 2008). The genetic variation stored in persistent soil seed banks can function as a genetic buffer, counteracting genetic drift and genetic differentiation in plant populations (Honnay et al., 2008). When the above ground plants are destroyed, the seed bank could provide enough "sleeping genes" and the subsequent regeneration from the seed bank of a population may be an important determinant of the success of a species at a given locality (Mandak et al., 2006). This is often the case for small fragmented habitats and rare plant populations. Fragmented plant populations are expected to be much more susceptible to extinction than large populations as they may suffer from genetic erosion and environmental and demographic stochasticity (Ellstrand, 1992; Young et al., 1996; Honnay et al., 2005).

Microsatellites, also called simple sequence repeats (SSRs), are abundant polymorphic elements of eukaryotic nuclear genomes and consist of tandemly reiterated, short DNA sequence motifs (Wang et al., 1994). Simple sequence repeats in chloroplast genomes (cpSSRs) provide a powerful tool to study the genetic variation and evolution of plants. Owing to their relatively high mutation rates, haploid nature and high copy number of the chloroplast genome, the cpSSRs have been used for the study of intraspecific genetic diversity in plant species such as *Glycine* (Xu et al., 2002), *Hordeum* (Provan et al., 1999), *Oryza* (Ishii and McCouch 2000), *Pinus* (Cuenca et al., 2003), *Solanum* (Sukhotu et al., 2006) and *Vitis* (Arroyo-García et al., 2002). High resolution melting (HRM) analysis allows genotyping by the discrimination of DNA sequence variant such as single point mutations and small insertion and deletions (INDELs) based on the shape of melting transitions (Tm) of the PCR products (Wittwer

et al., 2003; Zhou et al., 2005; Wittwer, 2009). The methods generally involve the gradual denaturation (melting) of the PCR amplicons and real-time detection of the subtle changes in fluorescent signal over temperature by double-strand DNA-binding fluorescent dyes present in the amplification reaction (Erali and Wittwer, 2010). Genotyping by HRM analysis is beneficial to investigators primarily due to its cost-effectiveness. This technique is also faster and simpler than alternative approaches requiring post-PCR processing enzyme restriction and electrophoresis. The use of HRM in genotyping and gene scanning have been reported in several crops including rice (Li et al., 2011), tomato (Bae et al., 2010), pepper (Jeong et al., 2010), melon (An et al., 2010) and almond (Wu et al., 2008).

In this study we examine the potential contribution of the soil seed bank for maintaining genetic diversity in a rare perennial species. *Astragalus nitidiflorus* exhibits many characteristics that make it susceptible to loss of genetic diversity. The populations are narrowly distributed and the number of individuals in these populations is generally small. The populations are subject to frequent fluctuations in population size, sometimes producing almost no adults in a season but then reestablishing in a following season (Martinez-Sánchez et al., 2011). Second, we check the suitability of the new HRM technique using cpSSRs to compare the genetic structure of the soil seed bank relative to the seedlings and above ground adults in the populations. If the seed bank has the potential to maintain genetic diversity we would expect to see as much or more genetic diversity in the seed bank as in the standing crop of adults. The results presented here will contribute to our empirical understanding of population processes and will have management implications for *A. nitidiflorus* and other rare species.

2. Materials and methods

2.1. Plant materials

To obtain samples from different phases of the life history cycle for genetic analysis, we sampled the soil seed bank, seedlings and above ground adult plants in the largest known population (37°40´06.8"N, 1º08´00.6"E). Soil samples were collected from forty 1–m^2 permanent plots in June, from 2010 to 2012, to see how the soil seed bank pattern of genetic diversity changes over time. Plots were 5–m apart from each other along four transects established perpendicularly to the maximum slope. In each

plot two cores (20 cm × 20 cm) were sampled at two different depths: 0–5 and 5–10 cm, being both cores belonging to the same depth mixed in a single sample (Segura et al. 2014). Seeds extracted from soil samples were scarified with sandpaper and incubated in a growth chamber (Sanyo MLR–351H, Osaka, Japan) at 15°C and 12–h photoperiod. Upon germination, seedlings were planted into individuals pots filled with peat mixed with soil from the natural habitat and placed in a greenhouse. Once the plants reached the two leaf stage, we collected tissue samples from the most recently fully expanded leaf for cpSSRs analysis. On autumn of 2011, twenty eight non flowering mature plants were randomly selected and a young expanded leaf of each plant was sampled. Finally, on autumn of 2012, twenty five seedlings were sampled in the same way. In total, 139 individuals of different life history cycle and different year were analyzed.

2.2. cpSSR analysis

Total genomic DNA was extracted individually from young leaves using a ‘‘Danapure. Plantas y hongos’’ extraction kit (GeneDan, Barcelona, Spain).We tested 32 primer pairs to validate the potential of cpSSRs to study the genetic diversity in *Astragalus nitidiflorus*. Nine were universal primer pairs designed by Weising and Gardner, 1999 that were tested on *Pisum sativum*. Three of them (ccmp1, ccmp2, ccmp3) were also used to study the genetic diversity in the domesticated common bean from central Italy (Sicard et al., 2005). Eighteen were universal primer pairs designed by Chung and Staub, 2003 that were tested on *Pisum sativum* and *Phaseolus vulgaris*. We also included two primer pairs of Xu et al., 2002 and three primer pairs of Angio et al., 2008 that were specifically developed for analyses in soybean and *Phasoleus spp.* respectively.

PCR reactions were carried out in a 25 µL reaction mixture containing 40 ng of template DNA, 1 x reaction buffer, 200 µM of each of the four dNTPs, 1 U of Taq DNA polymerase (ECOGEN, S.R.L.), 1.5 mM MgCl2 and 0.5 µM of each primer. Amplification was performed using a thermal cycler (Bio-Rad, California, USA) with an initial 5 min at 94 ºC, followed by 35 cycles of: 1 min at 94 ºC, 1 min at annealing temperature, 1 min at 72 ºC, and a final extension step for 7 min at 72 ºC. Amplification products were resolved on 1.5% agarose gels with ethidium bromide detection. Gels were documented using a gel documentation and image analyzer (Vilber Lourmat, Germany).

2.3. HRM analysis

PCR amplification and HRM analysis were performed using a QIAGENS´s real time PCR cycler, the Rotor-Gene® Q, using 15 µl reactions in a 96-well plate. PCR consisted of 1 × HRM PCR Master Mix, 25 ng of genomic DNA, and each forward and reverse primer at 10 µM. PCR amplifications were conducted by touchdown cycling conditions as described above and were immediately followed by high resolution melting step: ramp from 65 ºC to 95 ºC, rising by 0.1 ºC each step to cover the full range of expected melting points. When each tube aligns with the detection optics, the sample is illuminated and the fluorescent signal is rapidly collected from a single, short optical pathway. Melting curve analysis was performed by using the gene scanning software module of the Rotor-Gene® Q system. After data acquisition, fluorescent levels were normalized to correct for nonspecific signal, and Tm was converted to the negative derivate. Melt curves were transformed for easier read out to either temperature shifted melting curves or difference plots. In all analyses, each DNA sample was replicated in the same PCR run to validate the reproducibility of melting curves. Finally, Rotor-Gene® Q software was used for the genotyping of individuals by comparing their melting curves.

2.4. Data analysis

Haploid data matrix was analyzed by POPGENE version 1.32 (Yeh et al., 1997), making the assumption that the subpopulations are in Hardy–Weinberg equilibrium at these cpSSRs marker loci. Genetic diversity within/among life story stages and years was measured by the percentage of polymorphic loci (PPL), the mean number of alleles per locus (*Ao*), the effective number of alleles per locus (*Ae*), the number of private alleles per locus (*Ap*), Nei's (1973) gene diversity (*H)* and Shannon's information index (SI) (Lewontin, 1972).

Genetic divergence among classes was measured by Nei´s unbiased genetic identities. The genetic composition was further investigated through measurement of partitioning genetic variation (PHI_{PT}) obtained with the hierarchical analysis of molecular variance (AMOVA) analysis, using GENALEX 6 (Peakall and Smouse, 2006) with 9999 permutations. Estimate of gene flow (*N*m, number of migrants per generation = $0.25[(1-PHI_{PT})-1]$) was also calculated in GENALEX.

Finally, the genetic relationship between life story stages and years was estimated using un-weighted pair-group method with arithmetic average (UPGMA) clustering analysis. Dendrograms were drawn using UPGMA based on Nei´s (Nei, 1978) unbiased genetic distance which was calculated in the TFPGA software program (Miller, 1997).

3. Results

Of the 32 cpSSRs primer pairs tested in *Astragalus nitidiflorus*, three primer pairs (9.4%) (ccmp3, ccmp7, ccSSR19) gave no amplification products, three (9.4%) (ccmp9, ccSSR4, gmcp4,) did not give a clear banding pattern (fancy bands), and 13 (40.6%) (ccmp1, ccmp2, ccSSR2, ccSSR7, ccSSR14, ccSSR15, ccSSR16, ccSSR17, ccSSR20, ccSSR22, cp1, cp3, gmcp2) gave multiple bands. Therefore, these 19 primer pairs were not considered in the subsequent analyses. On the other hand, 13 (40.6%) primer pairs produced a single and clear amplification product, from which we selected five (ccmp5, ccSSR8, ccSSR9, ccSSR11, cp2) with similar band size to perform the genetic analysis by HRM. These five selected cpSSRs primers pairs generated a total of 104 genotypes (Table 1), with an average of 20.8 genotypes per primer pair. All genotypes were polymorphic.

Table 1. cpSSR markers used in this study and analysis of HRM-generated loci.

Locus	Sequence	Tm (ºC)	NL	PPL(%)
ccmp-5	F 5´-TGTTCCAATATCTTCTTGTCATTT-3´	55	16	100
	R 5´-AGGTTCCATCGGAACAATTAT-3´	55.4		
cp-2	F 5´-TCTGTTTTGACCATATCGCACT-3´	48	23	100
	R 5´-GTCCATAAATAGATTCCCGAAAAA-3´			
ccSSR-8	F 5´-TTGATCTTTACGGTGCTTCCTCTA-3´	54	29	100
	R 5´-TCATTACGTGCGACTATCTCC-3´	52		
ccSSR-9	F 5´-GAGGATACACGACAGARGGARTTG-3´	56-59	16	100
	R 5´-CCTATTACAGAGATGGTGYGATTT-3´	52-54		
ccSSR-11	F 5´-TTGGCTACTCTAACCTTCCC-3´	52	20	100
	R 5´-ACCATAGAAACGAWGGAACCCACT-3´	56		

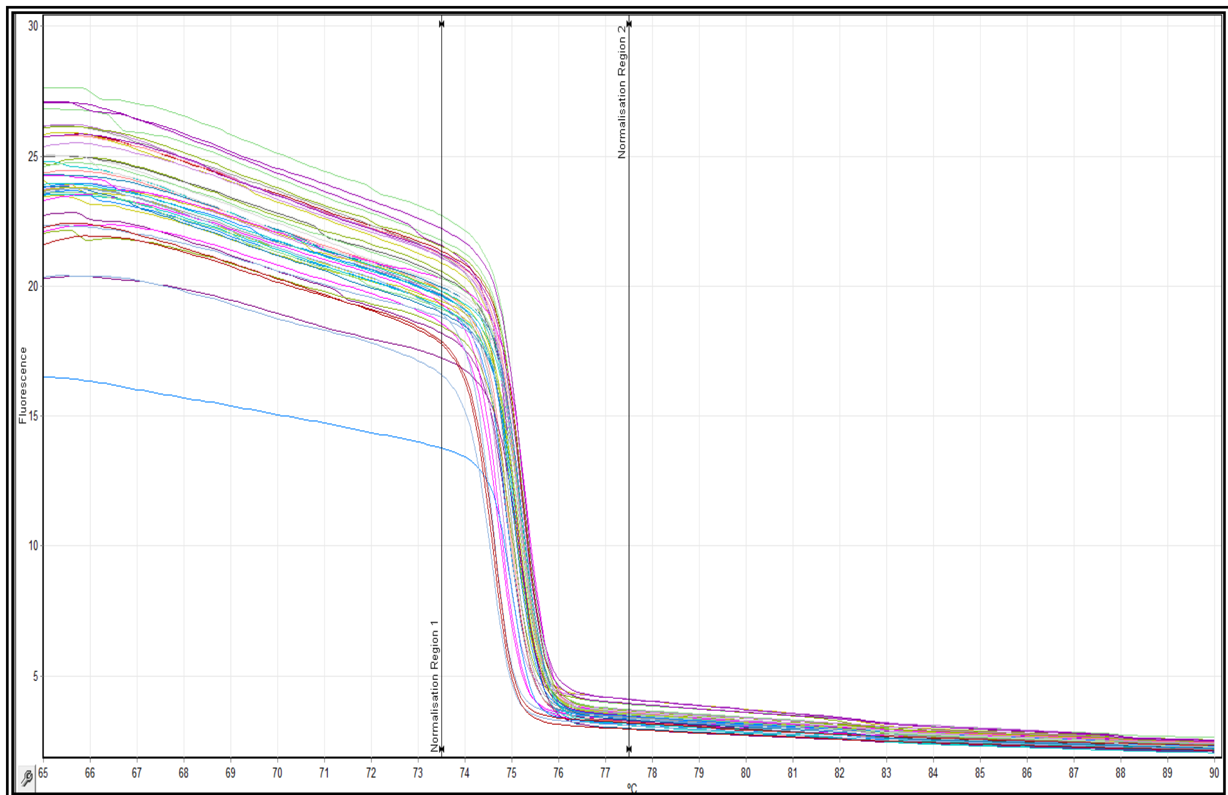

Figure 1. HRM dissociation curves generated by the ccSSR-8 marker.

The parameters of genetic diversity in all life story stages and years analyzed of *A. nitidiflorus* are listed in Table 2. As mentioned above, the percentage of polymorphic loci was 100% in all cases. The mean number of alleles (*Ao*) per life story stage/year ranged from 5.2 (SB10) to 7.6 (SB12). The effective number of alleles (*Ae*) was always lower than the observed number of alleles. The greatest number of private alleles was found in the seedlings (5.4) and the lowest in the seed bank collected in 2011 (0.6). The value of Nei´s *H* per population varied from 0.800 (SB12) to 0.642 (AG11) and the mean Shannon´s information index accordingly from 1.780 (SB12) to 1.299 (AG11). Therefore, summarizing, the above ground adults showed the lowest genetic variation while the highest level of genetic variability occurred in the soil seed bank collected in 2012 as shown in Table 2.

Table 2. Genetic variability within life stages-years of *Astragalus nitidiflorus* detected by HRM analysis.

Code	Nº samples	Description	PPL(%)	Ao	Ae	Ap	I	h
SB10	30	Seed bank collected in 2010	100	5.2	3.5	1.2	1.383	0.690
SB11	28	Seed bank collected in 2011	100	6.2	3.8	0.6	1.463	0.711
AG11	28	Above ground adults sampled in 2011	100	5.4	3.5	1.6	1.299	0.642
SB12	28	Seed bank collected in 2012	100	7.6	5.2	4	1.780	0.800
SL12	25	Seedlings sampled in 2012	100	7.4	4.9	5.4	1.725	0.781

PPL, percentage of polymorphic loci; Ao, mean number of alleles per locus; Ae, the effective number of alleles per locus; Ap, number of private alleles per locus; I, Shannon's information index; h, Nei's genetic diversity (assuming Hardy–Weinberg equilibrium)

The genetic differentiation index (PHI_{PT}), according to the AMOVA analysis results, ranged from 0.074 within seed bank collected in 2010 and 2011 to 0.252 within above ground adults sampled in 2011 and seedlings sampled in 2012 (Table 3). Estimated number of migrants per generation (*N*m) was greater than 1 in all cases (Table 3), which suggested that the gene flow between life story stages and years was high in general although lower between seedlings sampled in 2012 and the rest of the classes.

Table 3. Measurement of partitioning genetic variation and estimation of gene flow among life stages-years of *Astragalus nitidiflorus*.

		Nm				
PHI_{PT}		**SB10**	**SB11**	**AG11**	**SB12**	**SL12**
	SB10	♦♦♦	3.190	2.910	1.938	1.612
	SB11	0.074	♦♦♦	2.423	2.044	1.805
	AG11	0.147	0.171	♦♦♦	1.901	1.483
	SB12	0.205	0.197	0.208	♦♦♦	2.411
	SL12	0.237	0.217	0.252	0.172	♦♦♦

Estimation of gene flow is listed above the diagonal and partitioning genetic variation is listed below the diagonal.

The UPGMA dendrogram (Fig. 2) revealed that the 5 classes were separated into two main groups with a 100% bootstrap support: SB10, SB11, AG11 SB12 were in one group and only SL12 was separated from the rest. SB10, SB11 and AG11 tended to cluster together while SB12 and particularly SL12 showed a farther relationship.

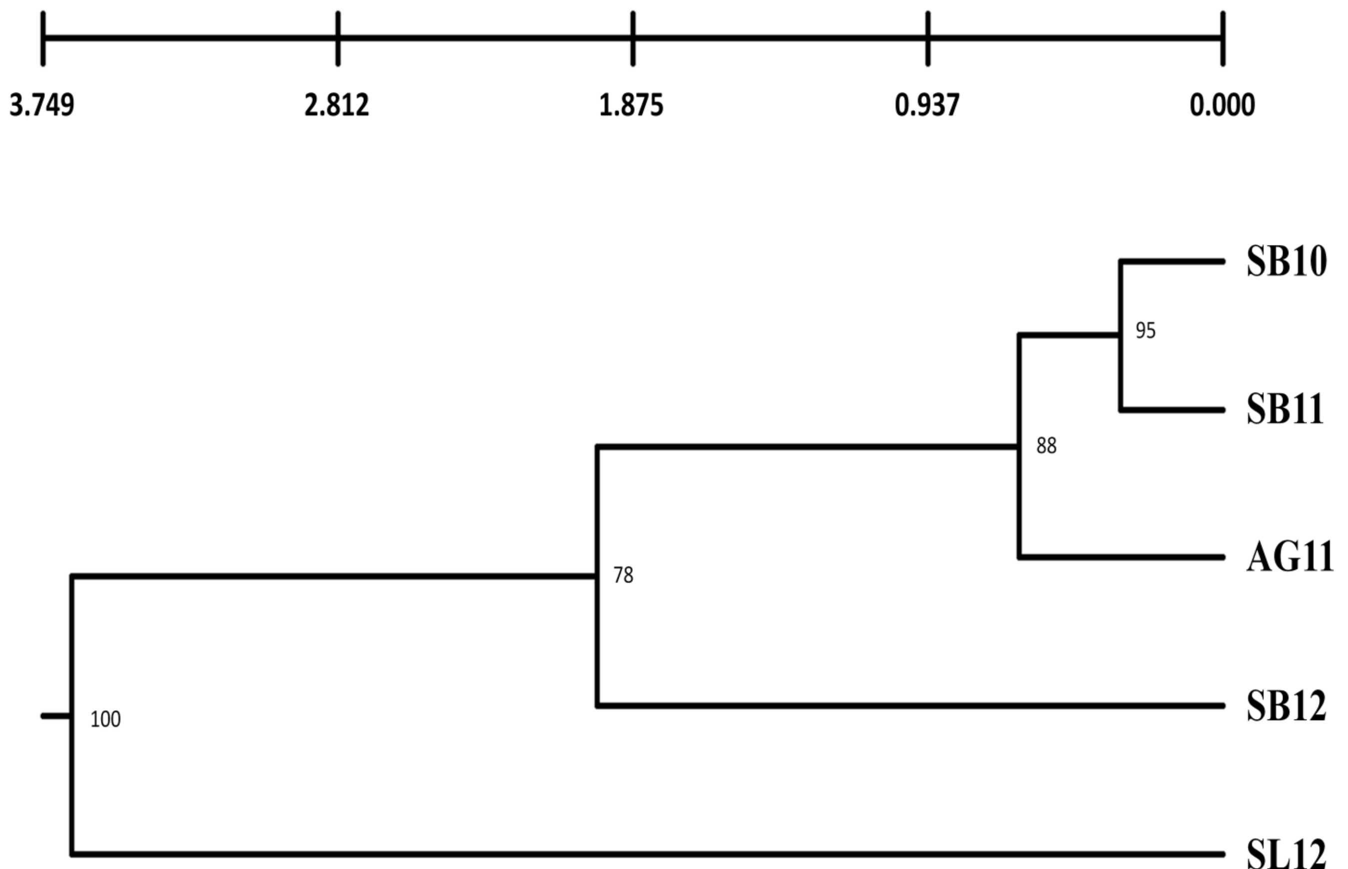

Figure 2. UPGMA dendogram based on Nei´s genetic distance among life stages-years of *Astragalus nitidiflorus*.

4. Discussion

High resolution melting curve analysis (HRM) has been used as an efficient, accurate and cost-effective tool to detect single nucleotide polymorphisms (SNPs) or insertions or deletions (INDELs). Despite its efficiency, accuracy and applicability to discriminate microsatellite polymorphism have not been extensively assessed. The traditional protocols used for simple sequence repeats genotyping include PCR amplification of the DNA fragment and the separation of the fragments on electrophoresis-based platform. However, post-PCR handling processes are laborious

and costly. In our study, HRM was demonstrated a good alternative to the electrophoresis-based method. The results showed that the five selected cpSSR markers produced distinct polymorphic melting curves among the different life stages of *A.nitidiflorus* investigated through HRM analysis. Moreover, the method presented in this study, i.e., HRM analysis of cpSSR markers adapted from publications or developed *de novo*, can be widely used in all the plant and animals species in the areas such as biodiversity analysis, genetic mapping and breeding programs.

It has been suggested (Templeton and Levin, 1979) that seed banks may act as reservoirs of genetic variation. Results obtained for *A. nitidiflorus* are consistent with such a hypothesis since Nei´s *H* value was lower in the above ground adults than in the soil seed bank and seedlings. Nevertheless, results from the literature are conflicting with regards to a general pattern of genetic differentiation between extant and seed bank populations. Genetic markers have revealed lower (Tonsor et al., 1993; Cabin et al., 1998) higher (McCue and Holtsford, 1998; Morris et al., 2002) and equal (Mahy et al., 1999) levels of genetic variation in the seed bank compared with adult populations, suggesting that the genetic consequences of a seed bank may vary between species and populations depending on a variety of potential genetic and demographic factors including genetic drift, micro-environmental selection, gene dispersal, plant density, mortality rates and possibly many other details of regeneration process. The capacity of seed banks to retain higher levels of genetic diversity may be dependent on seed dormancy characteristics. Species with strong germination barriers would be expected to have seed banks that contain a wider range of seed age classes and to contain genetic variants from a larger number of vegetative generations than species with seeds that germinate within a few years (Templeton and Levin, 1979). Previous studies (Segura et al., 2014) indicated that *A. nitidiflorus* has the ability to form a short-term persistent soil seed bank, since it was verified that some seeds are able to remain viable in the soil for at least two years. Results in current study supports this assumption, since soil seed banks collected in 2010 and 2011 were genetically closely (PHI_{PT} = 0.074) but quite far from the one collected in 2012 (PHI_{PT} = 0.205 and 0.197 respectively). In other words, the soil seed bank of *A. nitidiflorus* experienced a significant renovation after only two years. This means that seeds present in the soil in 2010 and 2011 were different from those of 2012 probably due to mortality or germination thereof.

We also noticed that there are significant differences in allele frequencies among the different life stages and years studied. There are more alleles present in the soil seed

bank and seedlings of 2012 than in previous soil seed banks and above ground adults, and results also suggests that the absent alleles are mainly rare alleles. A possible explanation for this situation could be related with the breeding system of *A. nitidiflorus* and its facultative xenogamus character (typically outcrossed, but genetically self-compatible) (Martínez-Sánchez et al., 2011). We hypothesize that this results are likely to reflect changing patterns of mating within the study population over time, whereby pollen exchange took place between more genetically similar individuals earlier in the population´s history. It has also been demonstrated that pollinators are more likely to move between adjacent or neighbouring plants within populations (Collins and Rebelo, 1987). During the last years, the number of individuals in the study population have been slowly lost from family patches after the onset of reproductive maturity, increasing the physical distance between plants and as well as genetic distance separating potential mates, thus probably generating the pattern observed here. On the other hand, the relative size of the seed bank population compared to the vegetative population may also determine its capacity to act as a genetic reservoir. Depending on dormancy characteristics, large seed bank populations may be more likely to sequester rare genetic variants that are not present in the respective vegetative population. However, as pointed out by Cabin et al., 1998, the large size and aggregated spatial distribution of seed bank populations render them inherently difficult to sample. Hence, it is likely that even relatively large sample sizes will miss much of the variation present, and this may explain why some studies have no detected higher levels of seed bank diversity. It is notable that the studies that did find seed banks that were genetically diverse compared to the extant vegetative populations (i.e., the present study) were on endemic species with relative small population sizes.

The ability of a seedling to survive the most hazardous life stage of a plant may create fine spatial genetic variability in relation to local ecological conditions. In *A. nitidiflorus* the low plant recruitment due to high seedling mortality as a result of the adverse arid conditions prevailing in the region is a common occurrence (Martínez-Sánchez et al., 2011). This factor could act as strong selective force, causing that mature plants that survive the establishment process may differ genetically from those that appear at the seedling stage. Vitalis et al., 2004 pointed out that "whenever such differences are found, they are likely to be the consequence of selective pressure that follows germination rather than the buffering effect of the seed bank." High mortality rates during the first life cycle stage prevents that much of the genetic diversity stored in

the soil seed bank and seedlings reaches later stages. Seed ageing has also been shown in some species to be associated with delayed germination and reduced competitive ability, and to ultimately result in a reduction in the relative fitness of older cohorts in the seed bank (Rice and Dyer, 2001). Older seeds are therefore likely to have a significantly smaller presence in established populations, potentially rendering genetic variation in older seeds largely inconsequential.

To conclude, the use of the HRM technique with simple sequence repeats in chloroplast genomes (cpSSRs) proved to be suitable for studying the genetic diversity of the soil seed bank of *A.nitidiflorus* compared to seedlings and above ground adults, providing a high percentage of polymorphisms and consistent genetic differentiation at individual level. Results confirmed that the soil seed bank contains more genetic diversity than above ground adults, although due to the relatively low seed longevity in the soil stored gene pool was not constant over time (changing after only two years). Despite the continuous reduction in the number of individuals that has experienced the population, the levels of genetic diversity of the soil seed bank increased over time (reaching the highest value in 2012) probably due to the exchange of pollen between more physically and genetically distant adults. One of the highest genetic diversities was found in the seedling stage, but unfortunately this genetic richness is not transmitted to adults owing to the high mortality experienced by seedlings, which generates a strong loss of genetic diversity, surviving only the best adapted genotypes. Therefore, researchers and conservationists should focus in this critical phase since it determines the number of individuals that will conform the future population as well as its genetic characteristics.

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GENERAL CONCLUSIONS

Astragalus nitidiflorus is a *Critically Endangered* species but this thesis shows it has many survival strategies that let it to maintain in the unpredictable habitat in which it occurs.

The first chapter shows that *Astragalus nitidiflorus* must be considered a short-lived perennial herb (four years old and maximally three flowering periods) that lives in border strips of crops or in old-fields being unable to colonize areas that have developed late sucessional vegetation. Given that *A. nitidiflorus* is typical of early-successional stages, maintenance of this early successional habitat seems to be the critical point to preserving the species. Low plant recruitment due to high seedling mortality, probably as a result of the adverse arid conditions prevailing in the region, could lead to the extinction of individual patches or small subpopulations. However, flowering and fruiting process are not a limitation in this species. We provide evidence for a facultative xenogamous breeding system in *A. nitidiflorus*, which suggests that the plant does not exclusively depend on pollinators, although their activity would favour fruit production. Plants are able to produce a sufficiently high number of viable seeds, which because of their low germination rate and hard coats, will form a seed bank. This will help dampen the effect of missing recruitment in “bad” years.

The second chapter shows that genetic variation of *A. nitidiflorus*, measured by ISSR markers, is low both at the species and population level which is a typical feature in species with restricted distribution. All the estimates of genetic differentiation revealed a low level of genetic differentiation among populations. The low level of gene flow found in *A. nitidiflorus* is evidence that the high genetic connectivity of the populations is the result of a recent fragmentation. The current gene flow is not enough to overcome the diversifying effects of random drift, and therefore the populations of *A. nitidiflorus* could lose the genetic connectivity they have at present, and evolve independently if perpetuated over time. Finally, our results demonstrate that the populations of *A. nitidiflorus* have gone through a bottleneck effect in the past that has resulted in a loss of genetic variation, as revealed by the low genetic diversity at the population level.

In the third chapter we confirm that *A. nitidiflorus* has the ability to form a short-term persistent soil seed bank, since it was verified that some seeds remain viable in the soil for at least two years, although strongly affected by environmental factors and population fluctuations. Most seeds and fruits are distributed in the soil surface layer inside fruits. This is explained by the type of fruit of *A. nitidiflorus*, an indehiscent and

hard legume that prevents rapid release of seeds. The spatial distribution on the ground is very heterogeneous and highly influenced by the presence or absence of adult plants due to barochory dispersal mechanism, showing the typical contagious distribution pattern observed in other semi-arid sites. Based on these results, natural regeneration of local patches where plants of *A. nitidiflorus* are no longer present is unlikely from the soil seed bank.

In the fourth chapter we detected that the physical dormancy of *A. nitidiflorus* seeds is highly variable among years because maternal environmental factors (such as age of the mother plant or drought) influence the proportion of seeds that enter dormancy. Older plants produce viable seeds with lower germination percentages than those from younger plants hence age structure of the population should be an important factor to consider when estimating the annual replenishment of the soil seed bank. The type of response to drought conditions during seed development seems dependent on the kind of dormancy involved. When dormancy is imposed mechanically by a thick seed coat, as for *A.nitidiflorus*, drought increases its thickness, thereby contributing to higher proportion of dormant seeds in the offspring. This mechanism is responsible for long-term seed dormancy but also provides sufficient germinable seeds for colonisation. when conditions become favourable.

In the fifth chapter results prove that use of the HRM technique with cpSSRs is a good alternative to the electrophoresis-based method for studying the genetic diversity of the species. Results showed that the soil seed bank of *A.nitidiflorus* contains more genetic diversity than adults, although due to low longevity of the seeds in the soil the stored gene pool is not constant in the long term (being renewed every two years). Seedlings also contain higher genetic diversity than adults but unfortunately this genetic richness is not transmitted to older life stages owing to the high mortality experienced by seedlings, perpetuating only the best adapted genotypes.

We hope that the conclusions of our studies can serve to aid the recovery plan for *A. nitidiflorus,* which will be the basic tool to protect the species.