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Phylogeographic and conservational studies of an Iberian endemism, *Carduncellus dianius* (Compositae)

Studio filogeografico e conservazionistico di un endemismo iberico, *Carduncellus dianius* (*Compositae*)

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I. INTRODUCTION

I.1. The Mediterranean Basin: a plant diversity cradle

Endemic species are of great interest to the student of plant life for they help to elucidate both the past history of the flora and its continuing development. Endemics may also be distinctive and of considerable beauty, and enhanced by their uniqueness they are often sought after by naturalists, who in consequence have a special responsibility to ensure that they continue to survive.

O. Polunin (1980, p. 23-26)

Nowadays the number of species threatened with extinction far outstrips available conservation resources, thus it is required a delineation of priorities. A promising approach is to identify 'hotspots', or areas featuring exceptional concentrations of endemic species and experiencing exceptional loss of habitat. As many as 44% of all species of vascular plants and 35% of all species in four vertebrate groups are confined to 25 hotspots comprising only 1.4% of the land surface of the Earth, including the Mediterranean Basin (Myers et al. 2000; **Fig. I.1**).

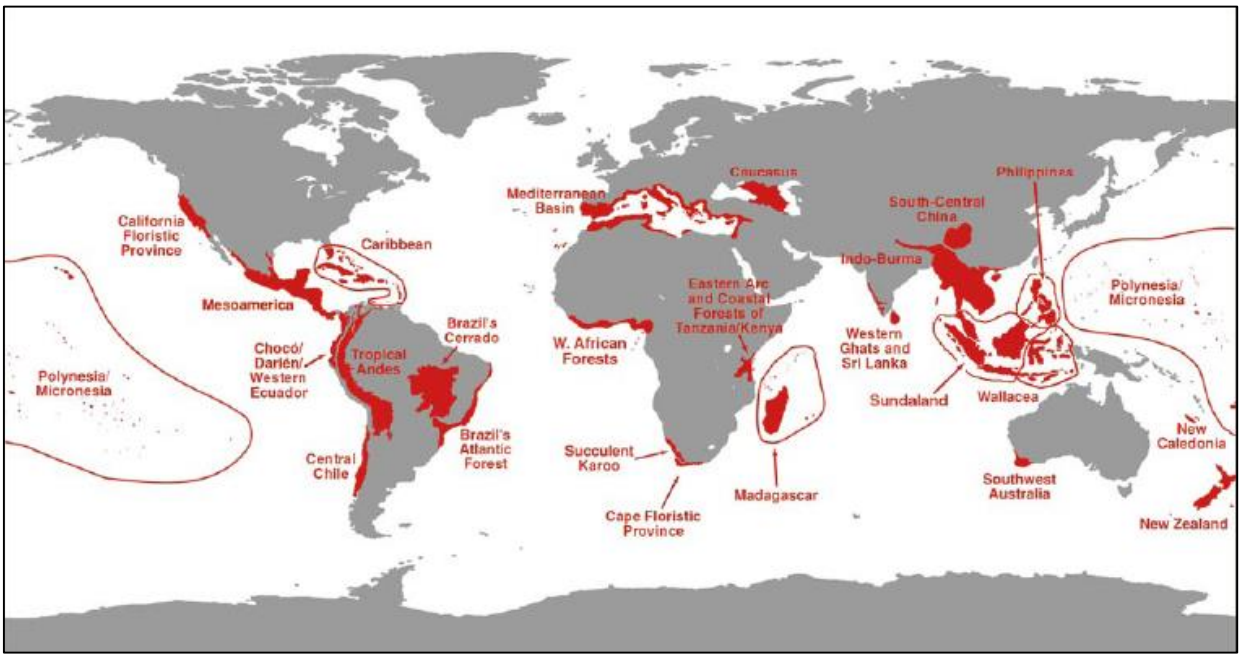

Figure I.1. The 25 biodiversity hotspots of the world (extract from Myers et al. 2000)

The Mediterranean Basin (**Fig. I.2**), along with parts of south-western Australia, the south-western Cape of South Africa, western California, and central Chile is one of the five Mediterranean-climate regions of the world. These five regions only occupy ~5% of the land surface but harbor 20% of known vascular plant species (Cowling & Arianoutsou 1996). They also contain a large number of endemic species and show strong patterns of local or regional differentiation.

Although many endemic species are unlikely to be those whose removal and loss has the greatest impact on ecosystem function, they do provide an objective means of assessing the conservation value of particular habitats and thus the establishment of habitat directives. In his book "Plant evolution in the Mediterranean" (2005), John D. Thompson argues that "conservation plans directed at endemic species are an essential step in the conservation of whole ecological systems" (p. 241). Moreover, by definition (see I.4. Narrow endemism), endemic plants do not occur elsewhere, hence they are priorities of conservation. Lastly, narrow endemism is universally considered a key feature of high species richness in the Mediterranean Basin, thus scientists, as the citation suggests, should have a critical responsibility at least to draw attention to key issues for future conservation plans.

Particularly, the flora of the Mediterranean Basin contains ~24,000 plant species in a surface area of about 2.3 million km^2 (Greuter 1991), that is 10% of known plant species in a small part of the world. In addition, ~60% of the native species in the Mediterranean flora are endemic to the region (Quézel 1985; Greuter 1991), making it one of the world's 'hot spots' of plant species diversity (Myers et al. 2000).

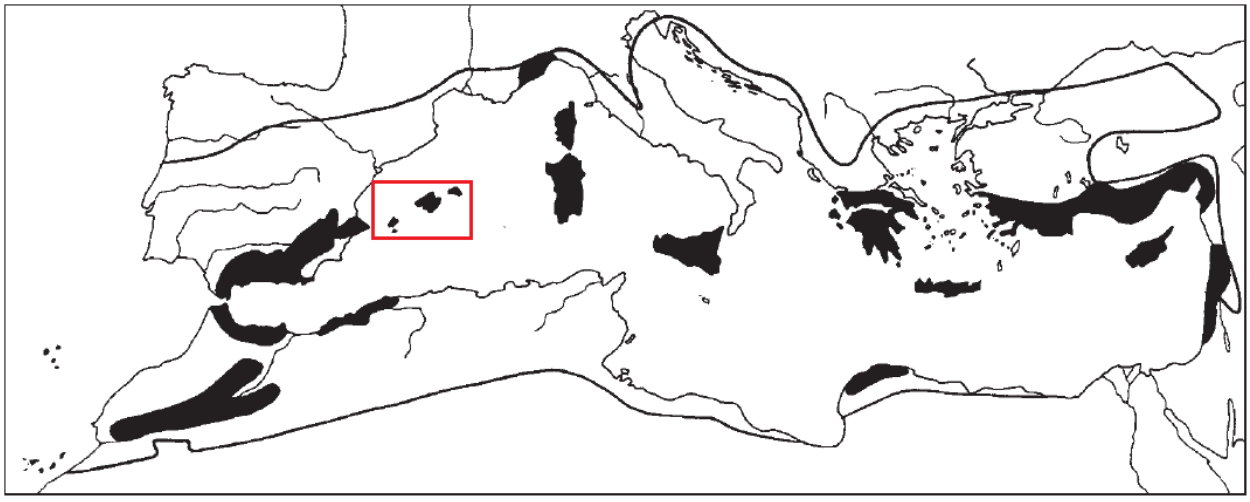

Figure I.2. The black line delimits the Mediterranean region. The areas of the Mediterranean Basin (shaded areas), including the Balearic Archipelago (red square), recognized as 'hot spots of biodiversity' by Médail & Quézel (1997), where rates of endemism exceed 10% of the local flora (modified from Thompson 2005)

Being itself as a whole a plant diversity cradle, the Mediterranean Basin includes regions with a higher level of biodiversity. Médail & Quézel (1997) proposed the delimitation of ten 'hot spots' of biodiversity within the Mediterranean region (**Fig. I.2**), which are areas where species diversity and endemism are high, exceeding 10% of the local flora. It is on mountains and several of the islands previously connected by mountain chains – notably the Balearic Archipelago – that rates of endemism (Médail & Quézel 1997) and species diversity (Lobo et al. 2001) are at their highest.

I.2. The Balearic Islands: a hot spot of a hot spot

The Balearic Archipelago (*Illes Balears* in Catalan, *Islas Baleares* in Spanish) is located in the western Mediterranean Sea, lying 80 to 300 km east of the Spanish mainland. The archipelago consists of 5 main islands and about 100 small islets, which are conventionally regrouped in two ensemble of islands. The eastern and larger group forms the Gymnesian Islands (*Illes Gimnèsies* officially in Catalan), and includes the principal islands Majorca (*Mallorca*) and Minorca (*Menorca*) and the small island of Cabrera. The western group is known as the Pitiusic Islands (*Illes Pitiüses)* and includes the islands of Ibiza (*Eivissa*) and Formentera (Rodriguez 2013; **Fig. I.3**).

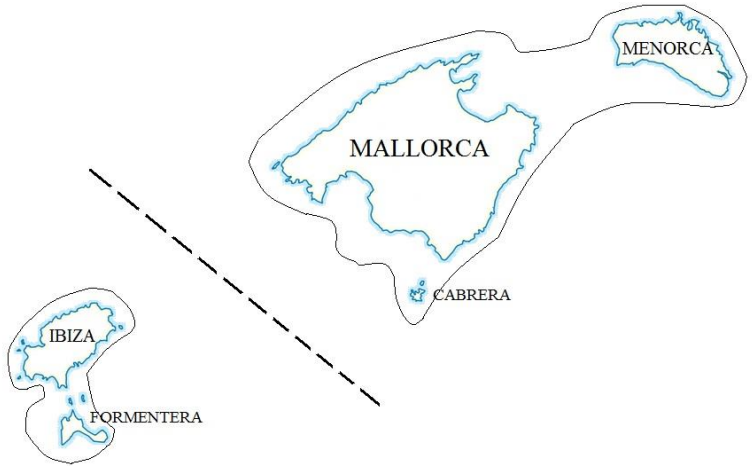

Figure I.3. Representation of the Balearic Archipelago and approximate profile of the Balearic coastal area (exterior line) during the maximum sea regression (~150 m) at the end of Middle Pleistocene (redrawn from Cuerda 1975)

The Balearic Islands are home of an extraordinary and unique biodiversity. Such richness is largely due to the fact that they are the most isolated archipelago in the Mediterranean. This archipelago constitutes a territory where climatic, geological, biogeographical and historical conditions

converge to produce environmental heterogeneity, large biological diversity, and a remarkable species and ecosystem richness (Benayas-Rey & Scheiner 2002). The diverse geology, substrates, and climate of the Iberian Peninsula and Balearic Islands are paralleled by their botanical richness: 1,258 species or subspecies are endemic to this region (Gómez-Campo & Malato-Beliz 1985). Half of these belong to only 27 genera, while the other half are distributed across no less than 286 genera, among which is found the *Carduncellus-Carthamus* complex (Aedo et al. 2013).
Given its high level of endemism and ecosystem variety, even when compared to the rich plant diversity of the Mediterranean Basin, the Balearic Archipelago can be described 'as a hot spot of a hot spot', one of the major poles of Mediterranean plant diversity (Thompson 2005).

I.3. The "mould and sculptors" of Mediterranean and Balearic plant diversity

In his book "Plant evolution in the Mediterranean" (2005), John D. Thompson identifies the "mould and sculptors of Mediterranean plant diversity" (p. 10-18) in three different elements: the geological history, the oscillations of the climate, and the impact of human activities.

I.3.1. Geological history

One of the instruments in shaping patterns of Mediterranean plant distribution has been its complex geological history (**Table I.1**). The Mediterranean is the largest inland sea in the world. From Gibraltar in the west, the Mediterranean Sea stretches eastwards for over 3,500 km (**Fig. I.2**). Trapped in a collision zone between the African and Eurasian plates, the Mediterranean Sea has only one narrow natural outlet, via the Straits of Gibraltar, which provides an exchange with the oceans outside.
The Mediterranean has been fashioned by the meeting of Eurasia and Africa. With the opening of the Atlantic Ocean in the Early and Middle Jurassic (~165 Ma), Eurasia and Africa began convergence motion, which was to shape the early orogeny of the Alps and the initial formation of the Mediterranean Sea as deep basins bordered by shallow sills (Biju-Duval et al. 1976; Rosenbaum et al. 2002) (**Fig. I.4.a-b**).
Since at least the Paleogene, the dispersal of plates – among which the Iberian microplate – have played a major role in the tectonic evolution of the Mediterranean Basin (Biju-Duval et al. 1976;

Rosenbaum et al. 2004). The Iberian microplate is located at the western extremity of the contact zone between the African and European plates. It was initially attached to Europe, but following the movement of the African plate, it was pushed northeastwards (~70 Ma), causing the uplift of various mountain ranges, notably the Pyrenees (**Fig. I.4.c**).

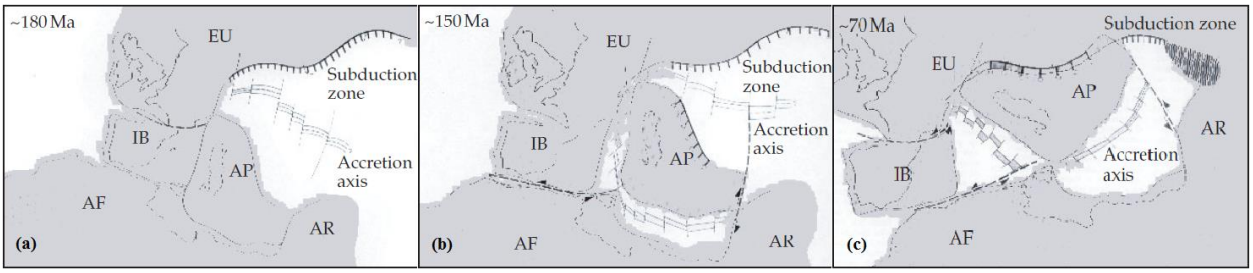

Figure I.4.a-b-c. Historical movements of plates during the development of the Mediterranean. AF: African plate, AP: Apulian microplate, EU: European plate, AR: Arabian plate, IB: Iberian microplate. Arrows represent plate movements (redrawn from Thompson 2005)

During the **Paleogene** (~65-25 Ma), the area now occupied by the Western Mediterranean was a continent, the Tirrenida, or almost a continent, if the possibility remains that the land-mass was a group of islands. During the first part of Paleogene (~63 Ma), an entry of the sea was establish from Betic Corridor to central Europe (Golpe-Posse 1981).

It is during the **Eocene** (~56-34 Ma) that the Primitive Balearic Area (PBA) emerged from the sea in conjunction with the Sardo-Corsican microplate. The origin of the native Balearic biota dates back from the late **Oligocene** (~36 Ma) when the dismembration of mainland Tirrenida (**Fig. I.5.a**) allowed the colonization of marginal areas – such as the PBA – and thus, permitted the independent evolution of populations. There is general agreement that this region (PBA) was populated by organisms adapted to a tropical climate (Colom Casasnovas 1978). At ~30 Ma the emerged land-mass that would eventually become the Balearic Archipelago detached from Sardinia and adjacent lands, starting evolution in isolation of the species living there (Altaba & Ponsell 2001) (**Fig. I.5.b**).

Burdigalian Sea transgression (~20.5-16 Ma) submerged all the Primitive Balearic Area. This great transgression caused a mass-extinction, triggering a dramatic reduction in number of species of the ancient Balearic tropical flora and fauna (Colom Casasnovas 1978). The following regression and the formation of the Arc of Gibraltar (**Serravalian**, ~14 Ma) allowed the emergence of the Ibiza-Majorca massif (Balearic Promontory) and part of Menorca, even if in an irregular form. It is certain that this promontory was linked to the Iberian Peninsula through the Alicante region during the latest phase of the **Tortonian** (~11.5-7 Ma; Colom Casasnovas 1978);

this connection was relatively brief, although it allowed the arrival of some species from the mainland to the Gymnesian group (Altaba 1997). Till this period, the Pitiusic Islands were never directly connected with continents, thus they show an ecology quite similar to oceanic islands (Paul & Altaba 1992; Alcover et al. 1994).

In the **Late Miocene** (~8 Ma), marine passages in southern Spain and northern Morocco linked the Mediterranean Sea to the Atlantic Ocean. Starting from ~5.96 Ma, this marine gateway from the Mediterranean to the Atlantic, precursor of the Straits of Gibraltar, was closed as a result of uplifting along the African and Iberian plate margins in association with mantle processes (Duggen et al. 2003). Marked regional aridity followed, as a results of the high levels of evaporation from the closed Mediterranean Sea, which led to a basin-wide lowering of sea level; the Mediterranean Sea became a disjointed mosaic of large lakes. This event is referred to as '**Messinian Salinity Crisis**' (~5.5-4.5 Ma) and was, to quote Duggen et al. (2003: 602) "one of the most dramatic events on Earth during the Cenozoic era". During this period, the Mediterranean became almost a desert (Hsu 1973) with land-bridge connections between current islands and mainland. It is during the Messinian that Greuter (1994) suggests a major increase in Balearic flora after the Burdigalian crisis, assuming that the islands colonization was allowed by the connection between the archipelago and the continent due to extremely low sea level (**Fig. I.5.d**).

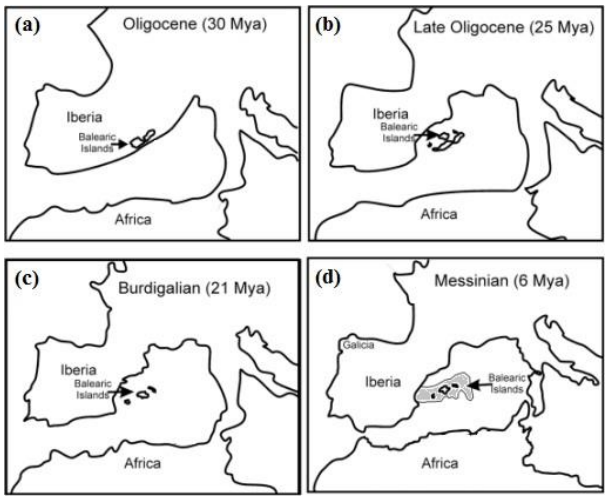

Figure I.5.a-b-c-d. Paleomaps of the developing western Mediterranean through the Early Oligocene **(a)**, Late Oligocene **(b)**, Burdigalian **(c)** and Messinian **(d)**. In **(d)**, the grey area indicates the position of the land bridge connecting the Balearic Islands to the Iberian Peninsula (redrawn from Pérez-Losada et al. 2011)

The end of the Messinian and the beginning of the **Pliocene** occurred suddenly at 5.33 Ma (Krijgsman et al. 1999) with the opening of the Gibraltar Straits and the establishment of a

permanent connection between the Atlantic and the Mediterranean. The consequent increase of the sea level led to the isolation of the Gymnesian and Pitiusic Islands (Gautier et al. 1994); this separation was fully achieved during the **Upper Pliocene** (~2.6-1.8 Ma).

Throughout the **Quaternary**, even during marine regression periods, the Balearic Islands continued to be isolated and without connections neither with the mainland nor with other archipelagos. Since then, sea levels were subjected to a trend of oscillations (+90/-180 meters from the current level), declining during the different Quaternary glaciations and uplifting afterward, shaping Balearic coastline into the present configuration (Cuerda 1975; Vesica et al. 2000). When sea level was at its lowest – ~150 m less than the present, during the last glacial period of Riss and Würm glaciations (Kaiser 1969) – many land-bridge connections were allowed to form between Majorca and Menorca, and among the smallest islets and the main island groups of the Balearic Archipelago. At the time, its shape consisted in two great islands: the Pitiusic (Ibiza and Formentera) and the Gymneasian group (Majorca, Menorca and Cabrera) (**Fig. I.3**). This configuration may explain the notable uniformity of biodiversity of the former three islands. Furthermore, the distance that separated Ibiza from the Iberian Peninsula was reduced to approximately half of the current (~50 km). The complete separation of Majorca and Menorca occurred in recent times, during the latest phases of **Würm glaciation** (~110,000-12,000 Ma) (Contandriopoulos & Cardona 1984). In conclusion, climatic and sea level fluctuations throughout the Quaternary furnished many opportunities for the evolution of a remarkable biota, as proved by the many highly restricted endemism (Altaba & Ponsell 2001).

Cenozoic	**Quaternary**	Holocene		
		Pleistocene	Würm glaciations (110-12 Ka)	Majorca-Menorca separation
	Neogene	Pliocene	Upper Pliocene (2.6-1.8 Ma)	Gymnesian-Pitiusic separation
		Miocene	Messinian (5.5-4.5 Ma)	Gymnesian-Pitiusic sub-archipelagos and Pitiusic-Iberian approach
			Tortonian (11.5-7 Ma)	Alicante-Balearic connection
			Burdigalian (20.5-16 Ma)	Submersion of PBA
	Paleogene	Oligocene (~36 Ma)		Origin of native Balearic biota
		Eocene (~56-34 Ma)		Emersion of PBA
		Paleocene		

Table I.1. Geologic time scale in reference to relevant phases in Balearic geological history. PBA stands for "Primitive Balearic Area"

I.3.2. Climate oscillation and the onset of Mediterranean climate

During the **Early Miocene** and beyond (< ~23 Ma), Mediterranean vegetation was subjected to subtropical conditions, characterized by hot, humid 'summers' and generally mild 'winters', with a fluctuation in temperature so moderate, it almost implies the absence of seasonality. Mild seasonal climatic contrasts began to develop only after the **Middle Miocene** (~15 Ma) (Thompson 2005). According to Thompson (2005), the development of contemporary Mediterranean-type vegetation occurred in the **Late Pliocene** (~2-3 Ma) as the highly seasonal Mediterranean climate with summer-drought regime set in. The essential characteristic of this seasonality is that the warmest season is associated with a drought that limits plant growth, while the cool or cold moist season presents unpredictable and often intense rainfall events, from autumn through spring. In an insular context, close to sea, the moist season is mild compared to inland, where freezing temperature occur in winter; thus archipelagos as the Balearic favored the survival of various plant species acting as glacial refugia, isolated by the harsh climate of **Pleistocene glaciations** (~2.5-0.12 Ma). During periods of Quaternary glaciations, many other parts of Mediterranean region acted as a refuge. Climatic oscillations caused plant species ranges to contract and then to expand again as the climate warmed. These oscillations opened the way for hybridization and evolution in new environments and have been fundamental for patterns of diversification in many groups of plants (Thompson 2005).

The Iberian Peninsula was one of the most important Pleistocene glacial refugia in Europe (Hewitt 1999, 2001). In spite of its geographically isolated position on the westernmost point of Europe, several characteristics favored survival in the Iberian Peninsula throughout the Pleistocene. First, the Iberian Peninsula possesses high physiographic complexity, with several large mountain ranges primarily oriented East-West. This mountain range orientation offers a number of microclimatic areas, and permits survival of populations by altitudinal shifts, allowing the plants to choose the most suitable microclimate up or down mountains as the general climate worsens or ameliorates (Hewitt 1996). Second, due to its geographical position, the Iberian Peninsula is under the influence of both the North Atlantic and the Mediterranean, and enjoys a wide range of climates, including desert, Mediterranean, Alpine, and Atlantic. Interestingly, these very same characteristics, together with its large area (580,000 km^2), make it unlikely that Iberia offered a single homogeneous and continuous refugial area throughout the Pleistocene. Instead, the differential distribution and fragmented nature of suitable habitats favor the occurrence of multiple glacial refugia isolated from one another by the harsh climate of the high central Iberian plateau (Weiss & Ferrand 2007).

The present-day flora of the Iberian Peninsula and Balearic Islands is located in two biogeographical-climatic regions: Eurosiberian and Mediterranean. The first includes the territories located to the North and the Northwest of the Peninsula, with wet, cool climate and without marked summer drought, whereas the rest of the peninsular territory enters within the domain of the Mediterranean region, with warm, dry summers and relatively cool, wet winters (Alcaraz Ariza et al. 1987). Phytogeographical hotspots of the Iberian Peninsula and Balearic Islands all lie in the Mediterranean region (Médail & Diadema 2009).

I.3.3. Human impact

The Mediterranean is also the home of many human civilizations. Human activities – burning, pastoralism, mining, coppicing, slash, ploughing by agrarian and metallurgic societies – have been modifying natural habitats and the spatial distribution of species for thousands of years (Carrión et al. 2010). Thus, anthropogenic disturbances cause evolutionary pressures and adaptive variation in natural populations, or, in the most extreme circumstances, mass-extinction. Moreover, under migration, trade and economy, humans have been altering gene flow and seed dispersal through 6-8 millennia. Man as a sailor has provided the travelling means for plant diasporas, and, as shepherd and peasant, he opened the ground for new species to prosper (Greuter 1995).
Finally, a most relevant human activity exerting deep transformations is the tourist sector. Unquestionably, the Balearic are a leader in vacational tourism, which had promoted a conspicuous economic progress (Mayol & Machado 1992; Conselleria de Turisme del Govern de les Illes Balears 2000). There are, however, negative aspects to all this development. For example, the massive destruction of the coastline has yielded the term “Balearization”. This environmental problem is indeed taken seriously, since current wealth is based mostly on tourism: 84% of the Balearic GNP (Gross National Product) is related to it (Verd 2000). In conclusion, by modifying the action of selection and gene flow, human activities have become a key element of the process of population differentiation, but also a source of extinction and endangerment.

I.4. Narrow endemism: the cornerstone of Mediterranean plant diversity

The Mediterranean region is an ideal place to study plant endemism. What is perhaps the major characteristic of the Mediterranean flora is the fact that its great diversity (24-25 thousand plant

species) is to a large part due to the high incidence of species turnover (β-diversity) and regional endemism, somewhere close to 60% of all native taxa in the whole Mediterranean area (Greuter 1991). These local endemics are defined narrow endemic species, that is, "species whose distribution is restricted to a single well-defined area within a small part of the Mediterranean region" (Thompson 2005, p. 39). In contrast, only 28% of non-endemic species (e.g. species that also occur outside the Mediterranean region) occur in just a single region within the Mediterranean. So more than one-third of the native flora (~37%) have restricted distribution patterns. Thus, narrow endemism represent an important trend to the Mediterranean flora and it is an integral component of endemism in the Basin (Thompson 2005).

Types of endemism	Brief description
Paleo-endemics	▪ isolated taxa, which are clearly ancient and usually show little variability; they are probably relict taxa that have persisted through long periods of time ▪ they do not necessarily occupy the region in which they originated
Patro-endemics	▪ diploid endemic taxa, which represent the progenitors of now more widespread polyploid entities ▪ strong reproductive isolation between the two ploidy levels can occur due to the formation of sterile triploids
Apo-endemics	▪ endemic polyploids whose distribution is a small portion of the range or a disjointed isolate of a more widespread ancestral diploid (reverse case of patro-endemics); in some cases the diploid ancestor is unknown ▪ polyploid evolution and endemism may be recurrent, causing multiple origins of endemic polyploids from a single widespread diploid
Schizo-endemics	▪ endemic taxa differentiated due to the fragmentation of the range of a widespread ancestral taxon; thus, the pattern of distribution is disjointed ▪ closely related species with the same chromosome number

Table I.2. Different types of endemism (Thompson 2005)

Endemic species are not a homogeneous group, other than the fact that they can all be classified as endemics because their distribution is limited to a particular area. The different types of endemic taxa are resumed in **Table I.2**.

The above classification oversimplifies in some ways the complexity of endemism. It should not be forgotten that endemism is a feature of distribution that says little about local population

characteristics in terms of abundance across the regional landscape and numbers of individuals in local populations. An endemic species is not necessarily rare in terms of local abundance, indeed some endemic species have large populations and/or many populations in the region in which they occur (Thompson 2005). Another problem is that in many schizo-endemic groups of species, although ploidy level may be constant, different endemic species may vary in chromosome number, structure, or types of rearrangements (Favarger & Siljak-Yakovlev 1986). This variation in karyotype may be quite common in Mediterranean endemic species. Hence, the category of schizo-endemics may encompass a rather diverse and heterogeneous group of endemics.
Finally, in a given region the diversity of endemic species may not only be high, but also may contain a range of endemic species with very different evolutionary patterns, hence local differences in endemic species (even within a single genus) reflect different episodes of Mediterranean history (Thompson 2005).

I.4.1. Evolution of endemism

Endemism concept is strictly connected with speciation process, which may be described as the differentiation of discrete entities (taxa), starting from genetic changes of ancestral populations (Alomar Canyelles et al. 1997). These changes have multiple causes, which can be considered as barriers that restrict or suppress gene flow, and agents that re-shape the genetic pools of ancestral populations. In Mediterranean contest, these factors are listed below (Thompson 2005).

- Geographical barriers to dispersal, favored by the complex geological history of the Mediterranean (geographical isolation, allopatric speciation)
- Modification of distributions in association with climate change, notably the Mediterranean glacial refugia (geographical and ecological isolation)
- Genetic factors (hybridization, polyploidy, mating system evolution) that cause karyotype differentiation (reproductive isolation, often a sub-cause of sympatric speciation)
- Interactions with and/or absence of common pollinators and dispersal agents, and species traits which restrict dispersal (ecological isolation)
- Long-distance dispersal (especially for colonization of remote localities, such as oceanic islands, or for promotion of gene flow between close insular areas, such as Mediterranean islands; see I.4.3. Endemism and insularity)
- Localized dispersal following range restriction in almost contiguous areas
- Migration over historical land-connections or migration of an ancestral stock into two different areas (with possible extinction in different parts of the range)

In addition to evolution via large-scale isolation, local speciation may be common in plants due to their sedentary nature and the high frequency of polyploidy and potential for hybridization (Levin 1993). Local speciation is the end-result of the micro-evolutionary processes that promote population differentiation. Random genetic drift and the fixation of new gene combinations and the selection of novel variants if they have an adaptive advantage (e.g. reproductive assurance in the absence of pollinators) in novel ecological conditions, or if they provide reproductive isolation from ancestral species, are at the heart of this process. Genetic factors (e.g. hybridization, polyploidy, and mating system evolution) can greatly affect the capacity for adaptation to new ecological conditions, hence, the process of divergence of endemic plants may be connected both to ecological and genetic differentiations (Rosenbaum et al. 2002; Thompson 2005).

These processes may be particularly important in populations at the geographic periphery of species ranges due to their small size, potential isolation, reduced gene flow and faster population turnover (extinction and colonization) than in the central part of the range (Thompson et al. 2005). Thus, in the Mediterranean, especially on mountains and islands, where widespread species show marked differentiation in geographically isolated and marginal populations, endemism is a predictable evolutionary outcome (Fréville et al. 1998).

Dispersal is the movement of individuals from their source location – birth or breeding site – to another location where they might establish and reproduce (Clobert et al. 2001; Bullock et al. 2002). In plants, as in other sessile organisms, dispersal is mostly passive: seeds or other diasporas (dispersal units) are transported away from the parent plant by various vectors, which are mechanisms that take it from one place to another. Nathan (2006) suggests six major long-distance dispersal (LDD) mechanisms or favorable conditions in plants: open terrestrial landscapes (e.g. areas with relative lack of obstacles to movements of seeds and their vectors); large animals; migratory animals; extreme meteorological events (e.g. tropical cyclones); ocean currents; and intentional or accidental human transportation.

Even if LDD is typically rare and it is subjected to many stochastic factors (e.g. fecundity and seed-to-adult survival probability), effective LDD events do occur far beyond the expected distances and rates. This evidence comes from ecological studies of multiple colonization of remote islands such as Hawaii (Carlquist 1981) and intercontinental disjunctions across the Atlantic Ocean (Givnish & Renner 2004). Moreover, LDD events play a major role in determining large-scale dynamics of plant populations and communities such as population spread, the flow of individuals between populations, the colonization of unoccupied habitats and the assembly of local communities from the metacommunity (Hubbell 2001; Levin et al. 2003). Furthermore, it is LDD,

rather than local dispersal, that determines large-scale phenomena of greater conservational concern, such as the spread of invasive plants, range shifts following climate change and the persistence of species in fragmented landscapes (Trakhtenbrot et al. 2005).

LDD is extremely important for the colonization of islands. Studies of the colonization of islands provide evidence of the most impressive seed dispersal distances known. Ridley (1930) shows that plants with a broad range of dispersal modes, including those with no obvious means of LDD, reached Krakatau (Indonesia) in 50 years after the volcanic eruption of 1883. To reach the Krakatau Archipelago, the seeds of these species probably traveled 12-40 km from Java, Sumatra, and several smaller islands (Thornton et al. 1996). Another example is the rapid colonization of Surtsey Island, an oceanic island of volcanic origin, 32 km offshore Iceland. After two years from its emergence (1965), the first vascular plant appeared on the island (Fridriksson 1992); in 2008, the vegetation already amounted to 69 species of vascular plants (Blask 2008).

I.4.2. Endemism and insularity

Even though islands represent only 5% of the earth's surface, they are considered one of the most important ecosystems of the world; indeed, they host a significant proportion of global biodiversity, from 1/6 to 1/4 of total vascular plant species known (Kier et al. 2009). Furthermore, many insular species have narrow distributions and high endemicity level (Whittaker & Fernández-Palacios 2007). Vascular plant endemic richness has been inferred to be 9.5 times higher on islands than on mainland areas (Kier et al. 2009), and 20 of the 34 biodiversity hotspots defined by Myers et al. (2000) are islands or have a remarkable insular component. The main reason for this high endemism can be searched in the isolation barrier in terms of distance from the mainland, sea depth and time of separation. Among these centers of diversity, there are major Mediterranean islands and island groups: Corse, Sardinia, Kriti, Cyprus and the Balearic Islands (Domina et al. 2012).

With regard to the origin and evolution of the Mediterranean flora, the hypothesis of insular colonization assumes the main influence of demographic and genetic bottleneck due to founder events. Conversely to this hypothesis, a number of Mediterranean insular species and complexes (e.g. *Limonium*; Domina et al. 2012) shows pattern of geographical vicariance rather than sympatric niche differentiation and following speciation. These studies suggest that plant endemics from Mediterranean islands have conspicuous genetic diversity levels, which are also highly structured (López de Heredia et al. 2005), indicating that gene flow is scarce, even within species with a high dispersal capacity.

Regarding to these two hypotheses (**Table I.3**), the different geological origins of islands may have important biological consequences. Continental islands derive from the tectonic fragmentation of continental plates (see I.3.1. Geological history). Therefore, many islands of the Mediterranean Sea split off from the mainland already carrying their own, fully adapted and diversified flora; they present a subset of the flora that already existed on the mainland when the separation took place, whereas the component derived from long-distance dispersal is not known. Hence, the distance of islands from each other and from continental areas is poorly correlated with species richness for Mediterranean islands, even if the mainland is the source of new immigrants to the island (e.g. Médail & Vidal 1998). By contrast, oceanic islands have always been islands. They are the product of volcanic activity; originally devoid of life, they become populated by long-distance dispersal through oceanic barriers (Whittaker & Fernández-Palacios 2007).
In the Mediterranean, islands are for the most part fragments of mainland. Thus, genetic drift may have acted as one of the main evolutionary forces conditioning the genetic variation patterns and structuring of species endemic to Mediterranean islands. The following evolutionary process in such insular systems may also implies restricted gene flow (Holzapfel et al. 2002)

Hypotheses	**Insular colonization**	**Native mainland flora**
Type of islands	Oceanic islands	Continental islands
Origin of islands	Volcanic activity	Tectonic split off from the mainland
Genetic diversity	Low	High
Genus : species ratio	High	Low
Evolutionary forces	Sympatric speciation (bottleneck by founder event with long-distance dispersal)	Mainly genetic drift with scarce gene flow

Table I.3. Main differential characteristics between the two hypotheses of the origin of Mediterranean insular flora

Traditionally, continental and larger islands have been considered relatively poor in species, rich in endemic taxa, and hosting a flora of a relictual nature and at least ancient as the islands themselves (Greuter 1995); for the Balearic Islands about 5-6 million years, dating back to the post-Messinian transgression (see I.3.1. Geological history).
As explained above, the age of the insular flora is due to the geological origin of its continental island. Since Mediterranean islands were refugia during the climatic upheavals of the Pleistocene (see I.3.2. Climate oscillation and the onset of Mediterranean climate), their flora presents mostly

a relictual nature (Greuter 1995). Hence, paleo-endemic species may be more prevalent in the endemic flora of islands compared to continental areas (Verlaque et al. 1997).

According to the equilibrium theory of island biogeography (MacArthur & Wilson 1967), the number of species on an island is in a state of dynamic equilibrium: diversity eventually stabilizes but turnover remains high as species continuously colonize and go extinct (**Fig. I.6**). As stated above, the mainland is still considered the source of new immigrants to continental islands, however, this contribute is poor. Hence, the assumption that Mediterranean islands flora is relatively poor in species appears well founded. In spite of enrichment due to immigration by long-distance dispersal (Quézel & Médail 2003), it is expected that the original species pool of insular flora will become considerably impoverished before immigration can balance extinction (Runemark 1969).

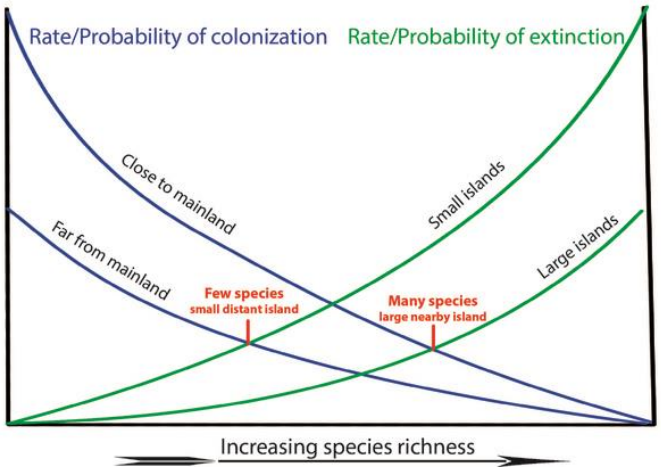

Figure I.6. Expected number of species on an island if correlated with rates of immigration and extinction according to the modified classical island equilibrium model proposed by MacArthur & Wilson (1967) (extract from http://www.islandbiogeography.org/)

Although island floras may actually have low levels of biodiversity in terms of species number, the proportion of endemic species that occur on islands is unrivalled by continental areas, illustrating the importance of isolation for the presence of endemic taxa.

MacArthur & Wilson (1967) pointed out that the insularity prevents common species – which can be seen as non-endemic species – from being present on a large number of islands since dispersal among islands is less probable than dispersal among contiguous patches of mainland. High dispersal rates will thus distribute common species more widely on continental areas, where endemic species richness will be reduced due to a higher rate of competition and thus extinction of rare species across the landscape (Hubbell 2001).

I.5. The Balearic flora

The Balearic Islands are home to 1,521 species of vascular plants, representing a percentage somewhat higher than 20% of all Spanish species, which contains 53.2% of the whole European plant diversity (Tutin et al. 1980; Aedo et al. 2013); of this 1,500 species (8.5%) are endemic to the archipelago (**Table I.4**; Domina et al. 2012). The analysis of phytogeographical composition of the Balearic flora shows that 61.9% of the species are Mediterranean plants, while the Eurosiberian component does not exceed the 5.4%. Finally, the pluriregional group is represented by 32.6% of the species, which form a diverse group in terms of their geographic origin (Bolòs 1996; Larrucea & Coll 2006; Aedo et al. 2013).

Area km^2	**N° of species**	**Species / area**	**N° of endemics**	**Endemics / area**	**% of endemics**
4996	1,500	0.30	121	0.02	**8.5**

Table I.4. Number of species and endemic in relation with their area in the Balearic Archipelago (data from Domina et al. 2012)

The paleographic history of the Western Mediterranean has ensured that the Balearic Islands were connected to other areas throughout theirs geological history, allowing ample and mutual colonization. The complex biogeographic pattern of theirs endemism can be essentially classified in three elements: Tyrrhenian, Iberian and Balearic (Alomar Canyelles et al. 1997; Pla et al. 1992). The **Tyrrhenian element** includes all plants that are distributed in Western Mediterranean islands (Gymnesias, Pitiusics, Îles d'Hyères, Corsica, Sardinia, Tuscany islands up to the islets of Sicilian coastline). All these territories were originally conjunct in the Tirrenida microplate (see I.3.1. Geological history), which had later separated (Oligocene, ~36 Ma), allowing the colonization of marginal areas, such as the Primitive Balearic Area (PBA). When the region detached from Sardinia and adjacent lands (~30 Ma), the evolution of the species living there started and these originally single-unit populations differentiated in vicariant taxa as a consequence of the isolation of the PBA (Alomar Canyelles et al. 1997). The existence of these vicariants follows a pattern of Balearic geographic divergence; the rest of the Tyrrhenian area harbors other vicariant elements, although with some exceptions (e.g. *Delphinium pictum* Willd. has two subspecies, subsp. *requieni* (DC.) C. Blanché & Molero that is located in Îles d'Hyères, and subsp. *pictum* in Balearic Islands, Corsica and Sardinia). The parallel of Balearic and other Tyrrhenian regions subspecies, as in

Cephalaria squamiflora (Sieber) Greuter subsp. *balearica* (Coss. ex Willk) O. Bolòs & Vigo - subsp. *mediterranea* (Viv.) Pignatti, proves the divergence and vicariant differentiation of biodiversity in these territories. An example of widespread Tyrrhenian element is *Arenaria balearica* L. presents on Gymnesias, Corsica, Sardinia and Tuscany islands. In general, the Tyrrhenian element express the antiquity of the flora, as it comprises taxonomic relics and species very much isolated from theirs congeners (e.g. *Naufraga balearica* Constance & Cannon).

The **Iberian element** contains species that are localized both in the Iberian Peninsula (mainly its eastern half) and in the Balearic Archipelago. *Silene cambessedesii* Boiss. & Reut. and *Carduncellus dianius* Webb are endemic to the Pitiusic Islands and the eastern Iberian Peninsula.

The **Balearic element** represents the native part of the Balearic flora. Few endemic taxa of this element show a widespread distribution throughout the archipelago, since the majority can be restricted to a single island (e.g. *Daphne rodriguezii* Texidor on Minorca, *Globularia cambessedesii* Willk. on Majorca, *Viola stolonifera* J. J. Rodr. on Minorca, and *Allium grosii* Font Quer on Ibiza). Sometimes, the distribution is limited to one of the two sub-archipelagos (e.g. *Thymelaea velutina* Meisn. and *Rhamnus ludovici-salvatoris* Chodat on the Gymnesias).

The difference between the Eastern and Western Balearic Islands regarding the composition, diversity and relationships of their endemic floras have been highlighted by various authors (Thompson 2005).

The Gymnesian islands have a significantly more diverse endemic flora: 120 endemic taxa (105 of which are exclusive), whereas in the Pitiusic islands there are 35 endemic taxa, 20 of which are restricted to this sub-archipelago. Although there are some exceptions, these distribution patterns illustrate the different biogeographical affinities of taxa on the Gymnesian and Pitiusic islands: endemic taxa shared between these two sub-archipelagos are scarce (15 taxa, 14.5%; **Fig. I.7**).

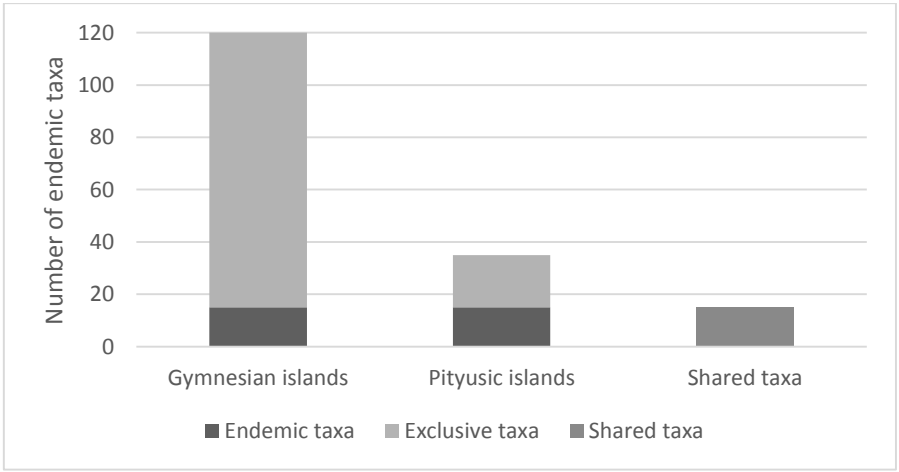

Figure I.7. Percentage of endemic taxa in the Balearic Islands on each specific sub-archipelago (Gymnesian and Pitiusic) and shared taxa (draw from data by Sáez et al. 2011)

A most relevant biogeographical fact shown in **Fig. I.8** is the affinity of the Gymnesian flora with the eastern territories (the Tyrrhenian element), conversely to the Pitiusic islands. Whereas endemic taxa on the Gymnesian islands tend to be endemic to this subset of islands or have distributions that encompass the Tyrrhenian islands and surrounding continents, endemic plants on the Pitiusic islands are either endemic to these islands or have distributions that extend to the eastern Iberian Peninsula (Iberian element). There is a marked break across the Balearic Islands through the middle of the archipelago – a sort of Wallace-line type separation between the Gymnesian and Pitiusic islands – which strictly resembles the geological condition of the archipelago during the maximum sea regression at the end of Middle Pleistocene (see I.2. The Balearic Islands: a hot spot of a hot spot, **Fig. I.3**).

During this period, the shortening in distances helped to foster the passage of species among island units and between mainland and the archipelago. This phenomenon may explain the notable uniformity of the Gymnesian islands biodiversity and its marked difference with the plant species of the Pitiusic islands, which remained isolated from the continent (see I.3.1. Geological history); thus, they show an ecology quite similar to oceanic islands (Paul & Altaba 1992; Alcover et al. 1994).

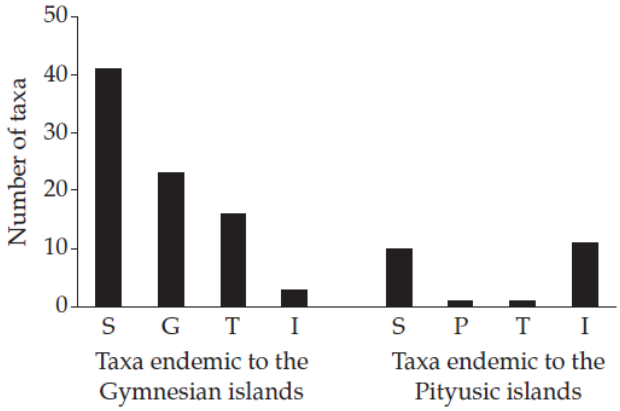

Figure I.8. The distribution patterns of endemic taxa in the Balearic Islands. Not included in this graph are taxa whose endemic distribution encompasses the Gymnesian and Pitiusic Islands (18 taxa). **S**: single island endemics, **G**: taxa endemic to two or more Gymnesian Islands, **P**: taxa endemic to two Pitiusic Islands, **T**: taxa whose distribution extends to the Tyrrhenian Islands and/or surrounding continents, **I**: taxa whose distribution extends to the eastern Iberian Peninsula (extract from Thompson 2005)

Paleo-endemics are isolated taxa, which are clearly ancient, probably relict taxa that have persisted through long periods of time (see I.4. Narrow endemism). Examples of these endemics

on the Balearic Islands are *Naufraga balearica*, *Daphne rodriguezii* and *Pimpinella bicknellii* Briq. The latter two species belong to the Balearic element, whereas the former is Tyrrhenian. The stronger historical affinities of the Gymnesian islands to the ancient microplate means that paleo-endemic taxa occur primarily on the islands of Majorca and Minorca and only rarely on the Pitiusic islands (Cardona & Contandriopoulos 1977).

As paleo-endemics, **patro-endemics** (e.g. *Paeonia cambessedesii* Willk., a Balearic element) are well represented in Balearic flora and are an index of its antiquity.

Schizo-endemics or **endemo-vicariants** characterize the most represented group of Balearic endemic flora. The formation of schizo-endemics is indeed accelerated by insularity, since it constitutes an isolation barrier that suppress gene flow and thus, contributes to speciation of endemism (see I.4.1. Evolution of endemism). Schizo-endemics are primarily Tyrrhenian elements (see above) of ancient origin: *Erodium reichardii* DC. (Balearic), *E. corsicum* Léman (Sardo-Corsican) and *Pastinaca lucida* L. (Balearic), *P. latifolia* DC. (Corsican) are examples of these intratyrrhenian species. Whereas differentiation produced by geographic isolation is dated in ancient geological times as happened for Tyrrhenian elements, micro-differentiation of vicariant taxa harbored on the same island is a more recent phenomenon. An example of this endemo-vicariant Balearic element is *Teucrium subspinosum* Pourr. ex Wild. on the Gymnesias with the var. *spinescens* Porta solely on Minorca (Cardona & Contandriopoulos 1977).

Lastly, **apo-endemics** (reverse case of patro-endemics) are poorly represented in Balearic flora, but examples still can be found: *Genista cinerea* (Vill.) DC. subsp. *leptoclada* (Willk) O. Bolòs & Vigo is a tetraploid Balearic plant, whilst subsp. *cinerascens* (Lange) Rivas Mart. shows diploidity in central-western Iberian Peninsula (Cardona & Contandriopoulos 1977).

In summary, even if the essential trait of Balearic Islands flora is its Paleogenic origin with *in situ* development, its endemism is not solely relictual as the importance of paleo- and patro-endemics as much as schizo-endemics of Paleogenic origin might points out. Despite the undeniable antiquity of this flora, apo-endemics and vicariants of recent differentiation suggests an on-going evolutionary activity that allows process of micro-speciation (Cardona & Contandriopoulos 1977).

I.6. *Carduncellus dianius*: an Iberian endemism

Carduncellus dianius (Cardueae, Compositae) is an endemism of the *comarca* of Marina Alta in the province of Alicante, northern Ibiza and the *Illa de s'Espartar* (Espartar Islet), a small

uninhabited island of the North West seaboard of Ibiza (**Fig. I.9**). *Carduncellus dianius* is a diploid (2*n* = 24; Vilatersana et al. 2000*a*) perennial plant that can reach one meter in height, with basal lanceshaped leaves, large and separate, very close to the base . Its capitulum presents large tubular flowers of whitish color; this inflorescence surrounded by involucral bracts – main feature of the family Compositae – is formed between April and July. The ovoid achenes have a width of about 7-7.5 mm, with little angular edges and a small apical tuft of pale yellow, spotted with black at times (Vilatersana 2008). *Carduncellus dianius* presents a high *ex situ* germination capacity (R. Vilatersana, com. pers.).

The pollination mechanism of *C. dianius* is not known in detail, but it is presumed that, as it is the case for the rest of the Compositae, the plant is pollinated by means of Hymenoptera and Diptera (Jeffrey 2009). In fact, studies conducted on the genera *Femeniasia* Susanna (Conesa et al. 2003) and *Carthamus* L. (Pandey et al. 2008) emphasize the role of Hymenoptera as pollinators (**Fig. I.10**).

Figure I.9. Specimens of *C. dianius* at Alicante region, Marina Alta. (Photos by J. López-Pujol)

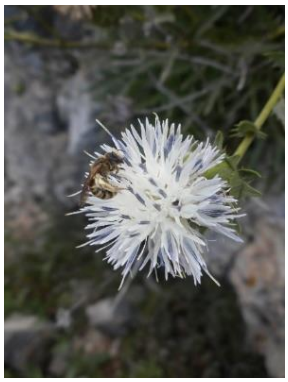

Figure I.10. Hymenoptera pollinating an individual of *C. dianius*. (Photo by J. López-Pujol)

Carduncellus dianius lives on limestone rock formation at a height between 0 and 500 meters above sea level (**Fig. I.11**), mainly on the coastal slope and accompanied by *Scabiosa saxatilis* Cav., *Teucrium buxifolium* Schreb. subsp. *hifacense* (Pau) Fern. Casas, *Asperula paui* Font Quer subsp. *dianensis* (Font Quer) De la Torre, Alcaraz & M. B. Crespo, *Melica minuta* L. and *Chiliadenus glutinosus* (L.) Fourr., among other species. Secondly, it can be found in inland groves with *Teucrium flavum* L. subsp *glaucum* (Jordan & Fourr.) Ronninger, *Pistacia terebinthus* L., *P. lentiscus* L., *Rhamnus alaternus* L. and *Coronilla juncea* L. The number of active populations is scarce, finding most of these in the area of the Montgó Massif (Marina Alta).

Figure I.11. Different populations of *C. dianius* at Marina Alta. To the left, individuals on a limestone walls at the quarry of Gata de Gorgos. At the upper right, mountain walls of Bèrnia where *C. dianius* is located. At the bottom right, *C. dianius* individual on the southern side of Montgó. (Photos by J. López-Pujol)

I.6.1. Phylogeny and Taxonomy

The genus *Carduncellus* Adans. belongs to the **tribe Cardueae** (Cass. in J. Phys. Chim. Hist. Nat. Arts 8:155. 1819 – Type: *Carduus* L., Sp. Pl. 2: 820. 1753), **subtribe Centaureinae** (Susanna & Garcia-Jacas 2009). On the basis of different surveys, some informal groups have been recognized in the subtribe Centaureinae, among whom the ***Carduncellus-Carthamus* complex**. The genera included in this complex share a native distribution ranging from the Iberian Peninsula and Morocco to West Asia, but the core of *Carduncellus* centers in the western Mediterranean (Iberian Peninsula and North Africa, eastward to Greece and Egypt), while *Carthamus* centers in the eastern Mediterranean Basin, except for a small section endemic to southern Spain and Morocco (**Fig. I.12**).

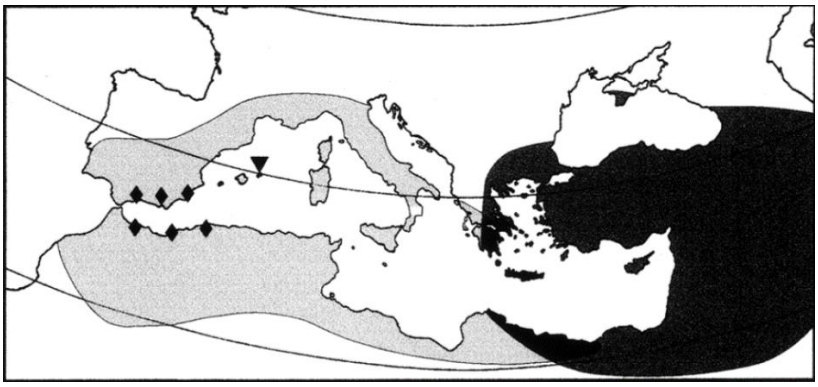

Figure I.12. Geographic distribution of the genera of the *Carduncellus-Carthamus* complex. Light grey: *Carduncellus.* Dark grey: *Carthamus* (excluded the cosmopolitan section *Atractylis).* Diamonds: *Phonus (=Carthamus* sect. *Thamnacanthus).* Inverted triangle: *Femeniasia* (extract from Vilatersana et al. 2000*b*)

Within this Mediterranean group, taxonomic classification has proven problematic. Attempts to clarify the generic delimitation and phylogeny of *Carduncellus-Carthamus* complex have included surveys on molecular studies based on ITS sequences (Vilatersana et al. 2000*b*), karyology (Vilatersana et al. 2000*a*) and morphoanatomy of the achene (Vilatersana et al. 2007). Vilatersana et al. (2000*b*) results indicate that the complex should be classified into four genera: *Carduncellus, Carthamus, Femeniasia* and *Phonus* Hill; thus, the delineation of *Carduncellus* coincided with the conclusions of Hanelt (1963, 1976): split of *Carduncellus* and *Carthamus* genera, with the later including five sections. The survey suggests that speciation in *Carduncellus* is very recent relative to other Centaureinae. Vilatersana et al. (2007) assessed a comparison of morphonatomic

characters with the phylogeny ITS previously cited, finding in the features of the apical plate a diagnostic factor that differentiates *Carthamus* genus from *Carduncellus s. l.*

Barres et al. (2013) dated the differentiation of the *Carduncellus*-*Carthamus* complex – considering the four genera of *Carduncellus, Carthamus, Femeniasia* and *Phonus* (Vilatersana et al. 2000*b*) – in the Late Miocene, approximately 6.82 Ma (with 4.79-9.28 Ma as 95% interval confidence), whereas the differentiation of *Femeniasia*-*Carduncellus* genera was dated approximately at 5.71 Ma (3.92-7.95 Ma). These two events occurred during the Messinian Salinity Crisis (5.96-5.33 Ma), when land connections between Iberia and North Africa would have allowed migration across North Africa and the eastern Mediterranean (see I.3.1. Geological history).

Massó (2011) used chloroplast DNA markers (*rpl*32-*trn*L, *trn*K^{UUU}-*rps*16 and *ycf*3-*trn*S intergenic spacers) to assess genetic diversity and relationships of 48 accessions representing 16 populations of *C. dianius*, most of them in the area of Montgó (Marina Alta). Firstly, a high genetic diversity was detected in this study, contrary to what expected from a species that grows in a restricted area such as *C. dianius*. Secondly, *C. dianius* presented different patterns of genetic diversity according to its regional distribution (Marina Alta and Ibiza). Thirdly, the study identified in the Montgó populations the ancestral group from which others have diversified, expanding to Marina Alta and Ibiza. It is plausible to assume that this spread has reverted its course, returning to the Iberian Peninsula. Lastly, this survey recommends the conservation of all populations of *C. dianius* sampled to preserve the maximum genetic diversity of this species, and, particularly, it suggests the protection of Montgó population due to its genetic singularity as ancestral group.

I.6.2. Protection

The IUCN (International Union for Conservation of Nature) Species Survival Commission has identified plant conservation and red listing as a regional priority for action; its ongoing work in the Mediterranean includes the project "Mediterranean Plant Red List" to assess the status of steno-endemic plants. Several taxa in the Balearic Islands are on the brink of extinction. Their threats have been assessed according the criteria of the IUCN (2001) (**Fig. I.13**); the number of threatened taxa is 180 (11.6% of all native taxa; **Fig. I.14**). Among these endangered taxa, 63 (35%) are endemic to the Balearic Archipelago, including *C. dianius*.

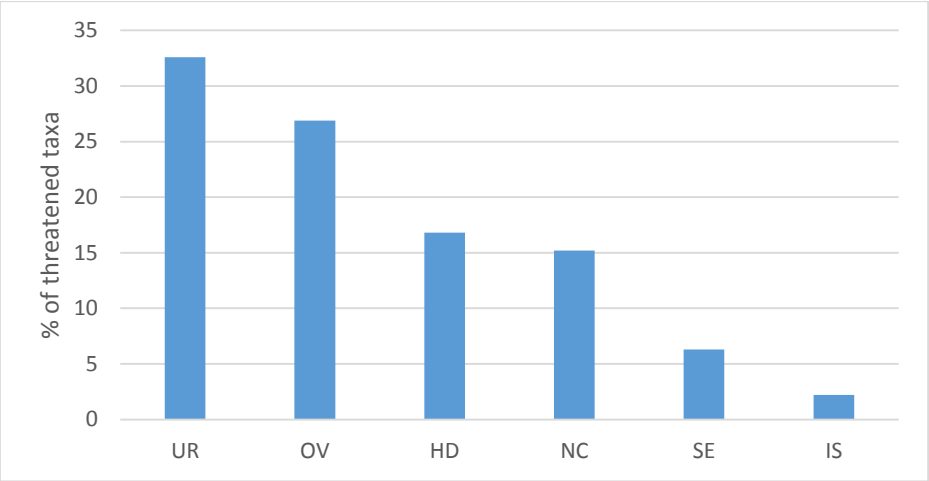

Figure I.13. The percentage of species threatened by urbanization and infrastructure (UR), overgrazing (OV), human disturbance (HD), natural causes (NC), stochastic events (SE) or introduced species (IS) in the Balearic Islands (redrawn from Sáez et al. 2011)

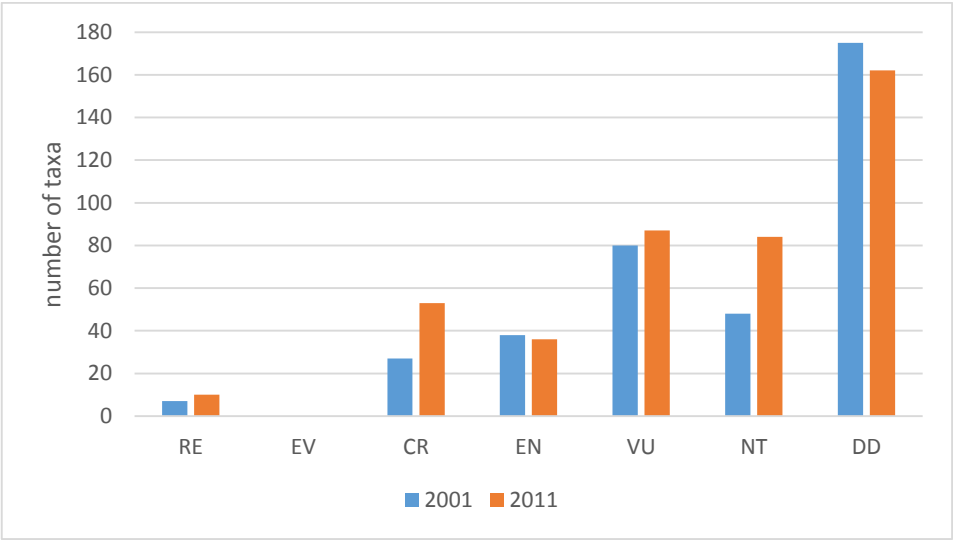

Figure I.14. Comparison of number of taxa assigned to the IUCN category in 2001 and 2011: Regional Extinct (RE), Extinct in the Wild (EW), Critically Endangered (CR), Endangered (EN), Vulnerable (VU), Near Threatened (NT), Data Deficient (DD) (redrawn from Sáez et al. 2011)

Balearic flora is strictly protected under European, national and local laws. The main standards of protection are:

- *"Real Decreto 139/2011",* 4th February, *para el desarrollo del Listado de Especies Silvestres en Régimen de Protección Especial y del Catálogo Nacional de Especies Amenazadas* (BOE, 2011), to develop some of the contents of the law, *Ley 42/2007*, 13th December, *del Patrimonio Natural y de la Biodiversidad*

- *"Decreto 75/2005"*, 8th July, *por el cual se crea el Catálogo Balear de Especies Amenazadas y de Especial Protección, las Áreas Biológicas Criticas y el Consejo Asesor de Fauna y Flora de las Islas Baleares* (BOIB, 16/07/2005).

The legislation established conservation tools such as recovery plans or managements that identify threats, objectives and conservation actions to promote the stability and increase of endangered species.

Specifically, *C. dianius* is a species listed as Vulnerable (VU) in the IUCN *Lista Roja 2008 de la Flora Vascular Española* (Moreno 2008). In legal terms, the populations of the Balearic Islands are included in the *Listado de Especies Silvestres en Régimen de Protección Especial* ("List of Wild Species in the Special Protection Scheme"; Royal Decree 139/2011; see above), while the population of the province of Alicante has lost the protection guaranteed by the old *Catálogo Nacional de Especies Amenazadas* ("National Catalogue of Endangered Species"; BOE 2011).

I.7. Phylogeography and conservation

Phylogeography is the study of the historical processes that may be responsible for the contemporary geographic distributions of individuals. This is accomplished by considering the geographic distribution of individuals in light of the patterns associated with a gene genealogy. This term was introduced to describe geographically structured genetic events within and among species, such as population expansion, population bottleneck, vicariance or migration (Avise 2000). Phylogeography can help in the prioritization of areas of high value for conservation. Genetic diversity is an important issue in terms of the conservation of many wild plant species endangered due to human activity and habitat destruction. The loss of genetic diversity within endangered populations can decrease adaptability to environmental changes and eliminate unique characteristics crucial for survival. Therefore, assessment and maintenance of genetic diversity are important in endangered species. Fast and cost-effective assessment of genetic diversity between individuals or population can be determined using phenotypic variation and/or molecular markers. Phylogeographic analyses based on molecular markers are now widely used in conservation studies to identify unique evolutionary lineages. These analyses can clarify the evolutionary context of organismal diversification especially when combined with various geological and climatic events. Examination of the spatial patterns of intraspecific gene flow can also lead to the discovery of cryptic but genetically distinct populations (Heled & Drummond 2010). In addition,

molecular phylogeographies can be used to obtain a temporal context for major population subdivision and facilitate inferences of the historical forces that have produced contemporary patterns of population structure (De Guia & Saitoh 2007). Determining the distinctiveness and age of populations especially in highly threatened habitats is essential to both managers and policy makers attempting to identify the population units most in need of conservation. Genetically identifying unique, persistent lineages of organisms can also address the impact that the loss of particular populations would have on overall biodiversity (Bradic et al. 2012).

I.8. Molecular markers: general introduction

In the recent years, molecular markers and especially DNA-based markers, have been extensively used in many areas such as gene mapping and tagging (Karp & Edwards 1997), characterization of sex (Flachowsky et al. 2001), analysis of genetic diversity (Martínez-Palacios et al. 1999; Erschadi et al. 2000) or genetic relatedness (Mace et al. 1999). In population genetic, protein-based markers (allozymes) were the first markers developed and widely used (Hamrick & Godt 1990). DNA-based methodologies are now the method of choice to differentiate closely related organisms (Ouborg et al. 1999). Moreover, the use of DNA-based markers allows efficient comparisons because genetic differences are detectable at all stages of development of the organism unlike allozymes, which may show age dependent changes. Molecular markers are useful for the assessment of genetic diversity and relatedness between or within different species, populations, or individuals (Weising et al. 2005). According to Elrod & Stansfield (1983), the term "marker" is usually used for "locus marker". Each gene has a particular place along the chromosome called locus. Due to mutations, genes can be modified in several forms mutually exclusives called alleles. Genetic biodiversity is detected by every nucleotide, gene or chromosome variation, considering the entire genome of the studied organism. Each one of these variations is detected as a different allele or polymorphism. Thus, molecular markers are all loci markers related to DNA.

I.8.1. Molecular markers: the ideal marker

Any chromosomal locus with an allelic variation has the potential to be used as a molecular marker, but some specific characteristics are required for a practical application, which is the trademark of a good molecular marker.

An ideal molecular marker should be/have:

1. Mendelian inheritance: transmit from one generation to another
2. Polymorphic: present several alleles at the locus investigated (multiallelic)
3. Codominant: allow the discrimination between homozygotes and heterozygotes
4. Neutral: all alleles have the same fitness
5. Not epistatic: one can determine the genotype of a phenotype irrespective of the genotype of the other loci
6. Independent of environment: no phenotypic plasticity
7. Frequent occurrence in the genome
8. Even distribution throughout the genome
9. Highly reproducible

No marker with all the listed requirements does exist, hence a series of techniques and genetic markers have been developed to estimate genetic diversity, but no single technique is universally ideal; each available technique exhibits both strengths and weaknesses (**Table I.5**). Therefore, the choice of technique is often a compromise that depends on the research question pursued and the genetic resolution needed, as well as on financial constraints and the technical expertise available. The most frequently used markers in population genetics are allozymes (biochemical), RAPD (Random Amplified Polymorphic DNA; Williams et al. 1990), RFLP (Restriction Fragment Length Polymorphism; Botstein et al. 1980), AFLP (Amplified Fragment Length Polymorphism; Zabeau & Vos 1993), minisatellite fingerprints, microsatellite and SSR (Single Sequence Repeats; Tautz & Renz 1984) and SNPs (Single Nucleotide Polymorphism; Nachman 2001).

I.8.2. AFLP markers

To study *C. dianius* genetic diversity, we choose to use Amplified Length Polymorphism (AFLP) molecular marker. This technique is a recent DNA fingerprinting technique developed by Zabeau & Vos (1993), Vos et al. (1995) and Vos & Kuiper (1997). It is based on PCR amplification of selected restriction fragments of a total digested genomic DNA (Mullis & Faloona 1987).
The invention of polymerase chain reaction, PCR (Saiki et al. 1988) allowed the targeted amplification of specific sections of genomes from small amounts of DNA. PCR is essentially an *in vitro* version of natural DNA replication except that only a small section of the DNA is replicated. The reaction is subjected to repeated temperature phases; usually 25-40 cycles are performed allowing amplification of targeted sequences from small starting amounts of DNA. The

PCR starts first with a high temperature phase (denaturation) that produces single-stranded DNA. Then, once temperature has reached the TM (annealing temperature; **Fig. I.15**), primers will bind to the template DNA. The *Taq* polymerase recognizes each double-stranded DNA as a start of synthesis and will continue the polymerization reaction in the direction 5'-3' as soon as the temperature has reached 72°C (optimal elongation temperature). Therefore, in order to design specific primers, the sequence of the flanking regions of the target DNA must be known. This supposes detailed knowledge about the genome or further elaborated investigations to get it. This step usually requires high laboratory and is most time-consuming.

The originality of the AFLP method was to design and synthesize arbitrary primers (adaptors) first, and then to ligate them to target DNA fragments generated by restriction enzymes.

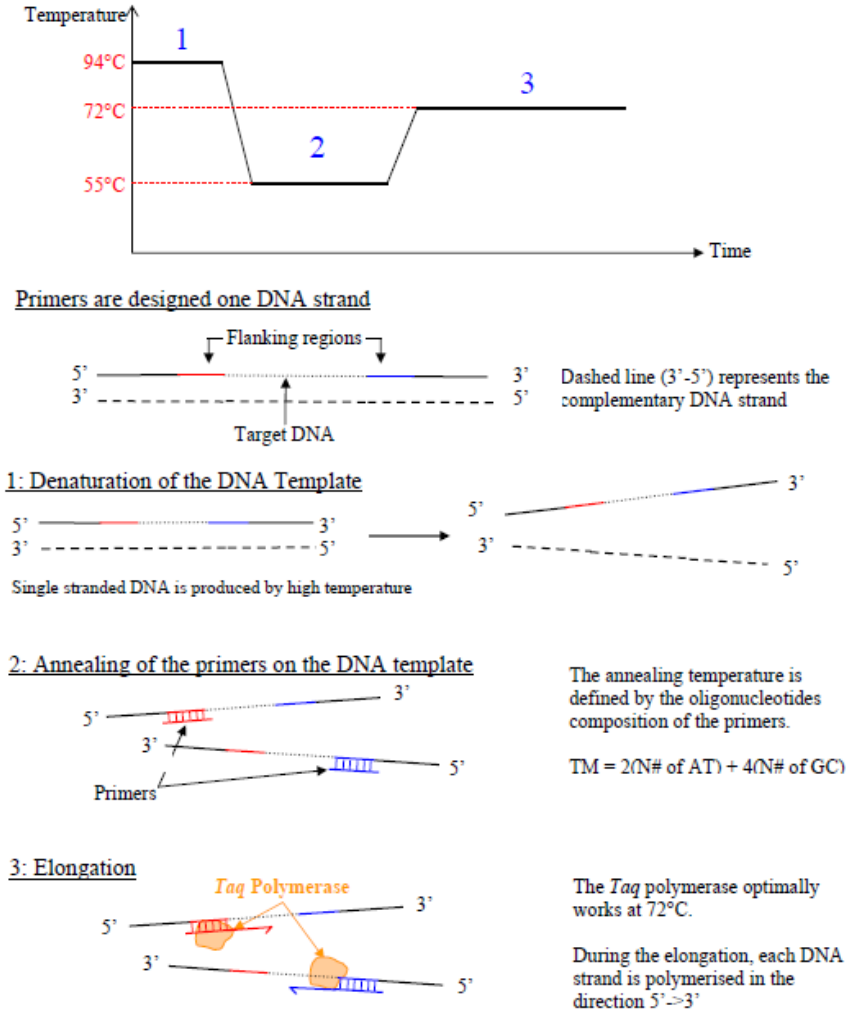

Figure I.15. Illustration of the Polymerase Chain Reaction

Restriction and ligation phases are followed by selective PCR amplification of restriction fragments using a limited set of primers (Vos et al. 1995) in order to generate a unique fingerprint

of a particular genome (Mueller & Wolfenbarger, 1999). During the selective amplification, the fragments are labelled by the incorporation of radioisotopes or fluorescent dyes. The labelled amplified fragments are usually resolved by electrophoresis through acrylamide gels and scored either manually or automatically. A pair of primers will amplify a large number of AFLP loci.

I.8.3. AFLP markers: advantages and disadvantages

AFLP markers fill all the previous characteristics of the "ideal molecular marker" (**Table I.5**) except for the codominance. AFLP loci are dominant markers and are scored as either present or absent. As the present allele is dominant to the absent allele, it is not possible to distinguish heterozygotes from homozygotes. These features mean that the per locus information content of AFLP markers is lower than that of co-dominant markers like microsatellites. Co-dominant markers enable for an easy estimation of allele frequencies and, therefore, they are suitable to estimate gene flow between populations. On the other hand, dominant markers can estimate genotypes but not allele frequencies. Thus, dominant markers are preferably used as fingerprints (Mueller & Wolfenbarger 1999). Moreover, the large number of fragments generated by AFLP gives an estimate of variation across the entire genome, which thus gives a good general picture of the level of genetic variation of the studied organism.

	AFLPs	**RAPDs**	**SSRs**	**RFLPs**	**Allozymes**
Type of inheritance	Dominant	Dominant	Codominant	Codominant	Codominant
Replicability	High	Variable	High	High	High
Prior knowledge[1]	No	No	Yes	No	No
Quantity of information	High	High	High	Low	Low
Resolution[2]	High	Moderate	High	High	Moderate
Ease of use and development[3]	Moderate[a]	Easy	Difficult	Difficult	Easy
Development time	Short	Short	Long	Long	Short
DNA quantity	Low	Low	Low	High	Moderate

Table I.5. Comparison of five popular genetic markers by seven criteria. [1]requirement of prior information about an organism's genome; [2]provide adequate resolution of genetic differences; [3]require little molecular expertise. [a]Analysis of AFLP markers is easy with the help of an automated genotyper, or when using low-resolution agarose gel electrophoresis, but manual polyacrylamide electrophoresis requires a certain amount of experience.

With reference to the characteristic of a good molecular marker, AFLP offers several advantages:

1. **Non-priori information**. AFLP markers can be amplified in any species without any prior DNA sequence knowledge. As the same protocol is used regardless of species – from bacteria (Siemer et al. 2004) and fungi (Rosendahl & Taylor 1997), to diverse groups of animals and plants (Ajmone-Marsan et al. 2001) – there is no requirement for optimization.
2. **Quantity of tissue or DNA**. AFLP markers are generated by PCR, therefore they can be amplified from less than 50 ng of DNA. Only small amounts of tissue are required and even very small organisms can be genotyped. Rosendahl & Taylor (1997) successfully amplified AFLP markers from single fungal spores.
3. **Resolution**. Because of the nearly unlimited number of markers that can be generated with AFLP-PCR, using a series of different primer combinations, at least some AFLP markers will be located in variable regions (Vos et al. 1995) and thus reveal even minor genetic differences within any given group of organisms.
4. **Error levels**. AFLP amplifications are performed under conditions of high selectivity (at high stringency), thus eliminating the artifactual variation that is seen routinely in RAPD-PCR. Repeated AFLP amplifications show near perfect reliability (Vos et al. 1995), and overall errors (including mispriming and scoring error) generally amount to less than 2% (Tohme et al. 1996).
5. **Time efficiency.** AFLP markers can be generated at great speed, as illustrated by the high ratio of polymorphisms generated per PCR experiment (multiplex ratio) and by the high percentage of polymorphism in all markers generated (% polymorphism; Mueller & Wolfenbarger 1999).
6. **Mendelian inheritance**. AFLP markers segregate in a mendelian fashion (Vos et al. 1995) and can be used for population genetic and QTL analyses.
7. **Cost and training efficiency**. Single-nucleotide differences between AFLP fragments can be resolved with either manual polyacrylamide gel electrophoresis or with the help of automated genotypers. These high-resolution methods do require training and some laboratory setup costs, but it is also possible to analyze AFLP markers with technically simpler agarose gel electrophoresis as done in this study.

So far, AFLP has proved to be a powerful marker technique to distinguish plant genotypes (Milbourne et al. 1997). We judged this marker to be the most suitable to conduct the present survey.

II. AIMS

The aim of this study was to investigate the extent and distribution of genetic variability in *Carduncellus dianius* within and among sixteen sampled populations, which include the whole of the known areal of the species partitioned in two main regions (Marina Alta and Ibiza). This survey was carried out using AFLP with the following objectives:

(i) to infer historical processes of origin, expansion and diversification of *C. dianus*;

(ii) to investigate the current genetic structure of the populations;

(iii) to assess the extent and distribution of diversity among and within populations;

(iv) to clarify the genetic pattern of *C. dianius* at total and regional level;

(v) to determine some biological traits of *C. dianius* based on genetic predictors;

(vi) to provide data for the formation of conservation strategies.

Moreover, in combination with previous morphological and biomolecular studies, these new AFLP data will be used as supplement for a survey with complete sampling and analyses of *C. dianius* populations.

III. MATERIAL AND METHODS

III.1. Sampling

Sampled material was collected in the known *C. dianius* areal according to d'Arellano & Torres (1981) from Ibiza to Espartar, including Marina Alta (province of Alicante) as indicated by Soler et al. (1999). Sampled populations are 16: 9 of Marina Alta, 6 of Ibiza and 1 of Espartar Islet, for a total of 146 individuals (**Fig. III.1**; **Table III.1**).

Sampled plant material was mainly collected from tender leaves of young individuals without evident phythosanitary problems. Collected material was preserved in silica gel at room temperature.

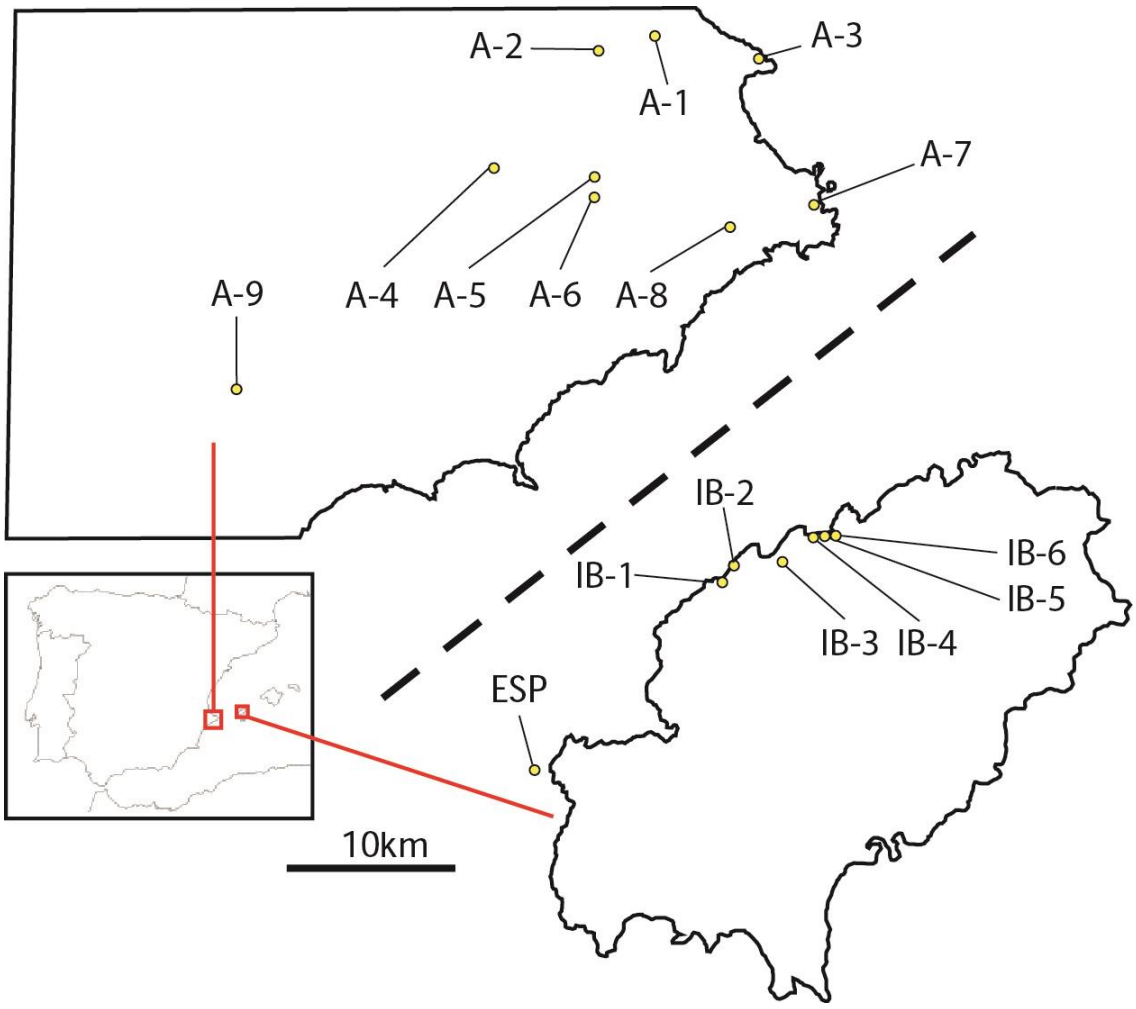

Figure III.1. Localization of the sampled populations (code names) of *Carduncellus dianius* (redrawn from Massó 2011)

	Code	Region	Locality	Latitude	Longitude	N	Collectors
1	A1	Marina Alta	Montgó - Pare Pere	38º49'01.5" N	0°06'21.4" E	10	G. & V.
2	A2	Marina Alta	Montgó - Jesús Pobre	38º48'45.9" N	0º05'19.5" E	10	L.-P., M., S. & V.
3	A3	Marina Alta	Cap de Sant Antoni	38º48'16.3" N	0°11'31.4" E	10	G. & V.
4	A4	Marina Alta	Llosa de Camatxo	38º46'07.5" N	0º00'02.3" W	10	L.-P., M., S. & V.
5	A5	Marina Alta	Gata de Gorgos	38º45'52.4" N	0º04'03.9" E	9	L.-P., M., S. & V.
6	A6	Marina Alta	Pedrera de Gata de Gorgos	38º44'53.6" N	0º06'28.6" E	10	L.-P., M., S. & V.
7	A7	Marina Alta	La Falzia - Xàbia. Caramull dels cingles	38º44'52.7" N	0º13'24.8" E	10	L.-P., M., S. & V.
8	A8	Marina Alta	Barranc d'Orxella - Xàbia. Penya-segats	38º44'10,7" N	0º10'21,9" E	3	L.-P., M., S. & V.
9	A9	Marina Alta	Serra de Bèrnia	38º39'55,5" N	0º04'51.7" W	9	L.-P., M., S. & V.
10	IB1	Ibiza	Penyals de Cala Sardina	39º03'11,2" N	1º20'21,1" E	10	G. & V.
11	IB2	Ibiza	Punta ses Torretes	39°03'44.2" N	1°20'46.4" E	5	G. & V.
12	IB3	Ibiza	Racó Sa Punta	39º03'42.0" N	1º22'16.8" E	10	G. & V.
13	IB4	Ibiza	S'Àguila	39º04'38,0" N	1º24'36,0" E	10	G. & V.
14	IB5	Ibiza	Penyal s'Àguila	39º04'37,5" N	1º24'42,1" E	10	G. & V.
15	IB6	Ibiza	Cala Ximena	39º04'34.6" N	1º24'55.7" E	10	G. & V.
16	ESP	Espartar	Illa s'Espartar	38°57'30.7" N	1°11'40.2" E	10	B. & S.
Tot.						**146**	

Table III.1. Codes of *Carduncellus dianius* sampled populations, related regions, localities, geographic coordinates, number of sampled individuals (N) for each population and total, and collector acronyms: "G. & V." Garnatje & Vilatersana "L.-P., M., S. & V." López-Pujol, Massó, Soler & Vilatersana "B. & S." Blanché & Sáez

III.2. DNA extraction

Murray & Thompson (1980) originally developed the cetyl trimethylammonium bromide (CTAB) method, which is still widely used for DNA extraction from plants. CTAB is a detergent that forms insoluble complexes with nucleic acid, leaving carbohydrate, protein and many other contaminants in solution. The insoluble precipitate is collected by centrifugation and resuspended in a salt solution, which causes the complex to break down, releasing the purified DNA (Giles & Brown 2008). The CTAB method have been used to obtain good quality total DNA for PCR based downstream applications (such as AFLP), and it is easily modified based on the plant tissue type and species. The following protocol (Doyle & Doyle 1990, modified following Cullings 1992) is an example of a modification from the original CTAB method in which additional wash buffer and cleaning cycles are used to grant good quality DNA isolation from recalcitrant material.

- Weigh approximately 150-200 mg of silica gel dried and clean plant tissue, giving priority to the leaves; put the material in a 1.5 ml microtube
- Grind the material using a mechanic homogenizer (Retsch Ball Mill MM301) with a frequency of 30 hertzes for the time necessary to obtain a powder (usually 5 min, depending on material)
- Add to the powder 1 ml of sorbitol/mercaptoethanol solution; this wash buffer is obtained by adding just before use 0.2% of 2-mercaptoethanol, that has an antioxidant action, to sorbitol buffer stored at 4°C (100 mM Tris-HCl pH 8.0, 0.35 M sorbitol, 5 mM EDTA) (Tel-Zur et al. 1999)
- Shake the solution vigorously by hand to suspend the plant material and leave to stand for 20 min in the ice
- Spin tubes at maximum speed (13,300 rpm) for 4 min to promote decanting of the supernatant
- Add 1 ml of sorbitol/mercaptoethanol solution (1:2), vortex vigorously and spin tubes at maximum speed for 3 min (repeat this step another time)
- Add 700 µl of CTAB, shake and put on heating bath for 2 hours (65°C)
- Add 500 µl of chloroform/isoamylalcohol (24:1) and gently agitate the tube until the solution is homogeneous; allow to stand for 5 min
- Spin tubes at maximum speed for 10 min to allow the phase separation (two phases neatly divided by a more or less thick interphase should be observed at the end of centrifugation)
- Remove 500 µl of (or more, accordingly to quantity available) of supernatant (superior clear phase) and transfer in a new sterile tube, taking care not to draw the interphase, or the organic phase below

- Add 350 µl of isopropanol to supernatant in a 3:2 proportion (for example, 500 µl supernatant : 350 µl isopropanol); shake and put in fridge (-72°C) for about 10 min; the isopropanol is a drying agent which causes nucleic acid precipitation combined with the action of cations in the solution, that neutralize negative charges on DNA; this process allows to remove the compounds which could hinder the next steps of DNA purification
- Spin tubes at maximum speed for 3 min to decant DNA on the tube bottom; remove supernatant with Pasteur's pipettes
- Add 1 ml of ethanol (70%) to the pellet of DNA; shake and spin at maximum speed for 3 min
- Remove ethanol to avoid interference in the RNase action: for the purpose, Pasteur's pipettes are initially used, then ethanol is completely vapored by drying in vacuum-centrifuge for 1 hour
- Resuspend in 50 µl of TE-buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0)
- Add 2.5 µl of RNase (1 mg/ml), vortex and allow to stand at 37°C (block-temperature) for at least 30 min in order to break down any trace of RNA in the solution
- Before quantification or other processes, DNA samples must incubate at 55°C for nearly 1 hour or, alternatively, put in fridge for about all night before adding RNase
- The samples were stored at -20°C

III.3. DNA quantification and quality evaluation

The DNA quality and quantity were assessed by NanoDrop technology (NanoDrop® ND-1000 UV-Vis Spectrophotometer, Thermo Scientific). The NanoDrop ND-1000 is a full-spectrum (220-750 nm) spectrophotometer that can measure concentrations in microvolumes of sample (1-2 µl) with good accuracy and reproducibility, and without dilution (75x higher concentrations than can be measured in a standard cuvette spectrophotometer). The Nanodrop uses an innovative sample retention technology that employs surface tension alone to hold the sample in place when it is placed between two close surfaces. To operate the Nanodrop you pipette a drop of sample onto the end of a fiber optic cable (receiving surface) after calibration with water and TE; a second cable (source surface) is lowered close to the first and causes the liquid to form a column between the two surfaces. A pulsed xenon flash lamp provides the light source and a spectrometer using a linear CCD array analyzes the light (reflection and transmission properties) after it passes through the sample in order to assess its quantity and other information. This technology eliminates the need for cumbersome cuvettes and other sample containment devices and allows for clean up in

seconds; moreover, it saves a considerable amount of time in preparation processes. The analysis of the sample is carried out quickly and simply.

This technology not only allows to assess DNA concentration (as well as other nucleic acids), but also its quality: a measurement of the sample at 260-280-230 nm wavelength, its concentration, and associated ratios may be performed automatically in order to evaluate the purity of the sample. The OD_{260}/OD_{280} ratio (R) is considered a sound DNA purity (quality) index. Indeed, whereas absorbance measure at 260 nm is an indication of nucleic acid presence, 280 nm measure points to impurities contamination (mainly proteins). A R value in the range from 1 to 2 means a good quality of the DNA sample. In this study, R value was considered acceptable if not less than 1, because of the marker used.

III.4. AFLP (Amplified Fragment Length Polymorphism)

III.4.1. Template preparation: Restriction and Ligation

For the initial reaction of the AFLP protocol, it is required a 250 ng DNA concentration of a final solution of 5 µl; DNA volume to add is determined by the DNA quantity previously detected with NanoDrop technology as follows:

DNA (ng/µl) in final solution = 250 ng / DNA (ng/µl) in each sample

The solution has been made up to 5 µl volume with distilled water.

AFLP fragments were generated using standard approaches (Vos et al. 1995). For each individual sample, genomic DNA was digested with *Eco*RI and *Mse*I restriction enzymes (**Fig. III.2**; **Table III.2**) in a reaction mixture (**Table III.3**) which was incubated 3 hours at 37°C.

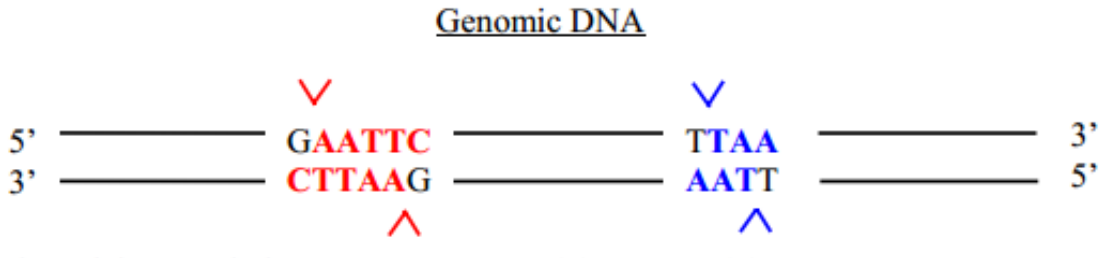

Figure III.2. Action of the restriction endonucleases *Eco*Ri (>) and *Mse*I (>). Restriction results in three different types of fragments base on the terminal site: *Eco*RI-*Eco*RI, *Eco*RI-*Mse*I, *Mse*I-*Mse*I

Enzymes	Cut-off frequency	Recognition sequence
MseI	Low. Cut once every 4096 bp (*rare cutter*)	5'-G//AATTC-3'
EcoRI	High. Cut once every 300 bp (*frequent cutter*)	5'-T//TAA-3'

Table III.2. Restriction enzymes with associated cut-off frequencies and recognition sequences ("//" indicates the cut site). The frequent cutter (*Eco*RI) serves to generate small fragments, which amplify well and which have the optimal size range for separation on a sequence gel, whereas the rare cutter (*Mse*I) limits the number of fragments to be amplified

Reagent	Reaction conditions	Volume
dH_2O	-	3.63 µl
EcoRI buffer	-	1 µl
BSA	1 mg/ml	0.1 µl
*Eco*RI enzymes	40 U/µl	0.07 µl
*Mse*I enzymes	50 U/µl	0.2 µl
DNA	250 ng	5 µl
Total		10 µl

Table III.3. Restriction reaction of AFLP protocol

Next, double-stranded adaptors (**Table III.4**) were ligated in a mixture (**Table III.5**) to the resulting restriction fragments for 16 hours at 16°C. Adaptors consist of a core sequence (striped boxes) and an enzyme-specific sequence for either the *Eco*RI (red) or the *Mse*I (blue) site. The enzyme-specific sequence allows the ligation of the adaptors to the matching restriction fragments without restoring the original restriction sites (**Fig. III.3**). In this way, ligated adaptors create a target template for the AFLP primers in the subsequent amplifications.

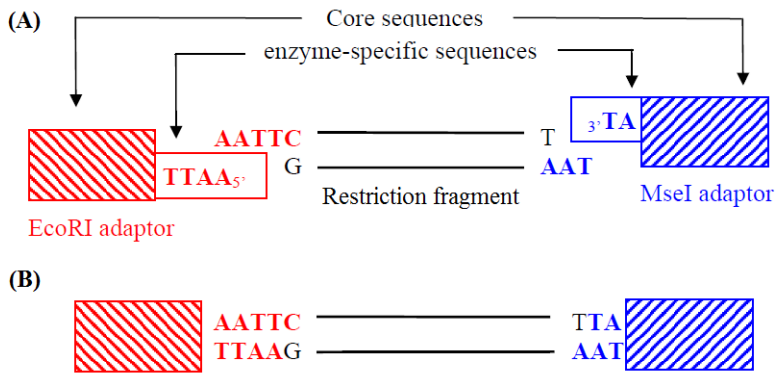

Figure III.3. Ligation reaction **(A)** and formation of RL target template **(B)**

Adaptors	Sequence (5'-3')		Volume	Final concentration
*Eco*RI-1	CTCGTAGACTGCGTACC	100 µM	10 µl	10 µM
*Eco*RI-2	CATCTGACGCATGG	100 µM	10 µl	
dH_2O	-	-	80 µl	
*Mse*I-1	GACGATGAGTCCTGAG	100 µM	50 µl	50 µM
*Mse*I-2	TACTCAGGACTC	100 µM	50 µl	

Table III.4. Preparation and oligonucleotide sequences of double-stranded adaptors. *Eco*RI-1 and -2 were annealed to generate a double-stranded adaptor. Likewise for *Mse*I-1 and -2. Preparation was optimized in Thermal Cycler PTC-200 Peltier as follows: 95°C for 5 min – go down 0.1°C each second until 2°C – 21°C for 10 min – 4°C forever storage

Reagent	Reaction conditions	Volume
dH_2O	-	1.30 µl
X10 T4 buffer	-	1 µl
BSA	1 mg/ml	0.50 µl
*Eco*RI adaptor	10 µM	1 µl
*Mse*I adaptor	50 µM	1 µl
T4 ligase	5 U/µl	0.20 µl
Restriction DNA	-	7.50 µl
Total	-	12.50 µl

Table III.5. Ligation reaction of AFLP protocol

In order to verify the success of the digestion of fragments, samples exposed to restriction-ligation treatment (RL products) were run on a 1.2% agarose gel stained with SYBR® Safe DNA Gel Stain (Life Technologies), a less hazardous alternative to ethidium bromide which, when intercalated into DNA, fluoresce under ultraviolet light. 2 µl of a gel loading buffer (10X solution of 0.2% bromophenol blue and xylene cyanol dyes, and 30% glycerol in a Tris-EDTA buffer) was mixed with 5 µl of RL product: the glycerol renders samples denser than the running buffer so that they sink directly into the wells of the gel, whereas "dyes" aid monitoring the migration of DNA during an electrophoretic run. A size marker (HyperLadder^{TM} 100bp, Bioline) was loaded in another lane of the gel; its bands of known size (10 bands from 100bp to 1013bp) can be compared to those of the unknown samples in order to determine their size. The distance a band travels is approximately inversely proportional to the logarithm of the size of the molecule.

An ideal profile of DNA restriction fragments in gel electrophoresis is shown in **Fig. III.4**. Once assesed the succes of the reaction, RL samples were diluited 2:20 with distilled water.

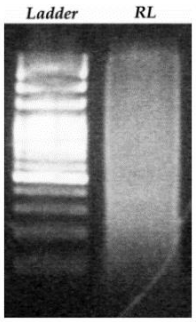

Figure III.4. Agarose gel electrophoresis of a RL product (*RL*) and size marker (*Ladder*). The restriction produces a large quantity of small size DNA fragments

III.4.2. Template amplifications (pre-selective and selective PCRs)

The fragments were then filtered based on terminal sequences and amplified using two successive PCR reactions (pre-selective and selective PCRs).

Preamplification allows a first selection of RL products by only amplifying the DNA restriction fragments that have ligated an adaptor to both extremities (RL target templates). For this purpose, primer design matches the core, the enzyme-specific and the restriction-site remnant sequence. Additionally to the adaptor sequences, the primers used for the pre-selective amplification have a supplementary 3'-terminal base, called the selective nucleotide (represented by N, in yellow; **Fig. III.5**). This extra base enables another first selection by amplifying ¼ of the RL target templates. As at the end of ligation reaction, it is required an electrophoretic run on agarose gel to confirm the good outcome of pre-selective amplification (**Fig. III.6**) and a 2:20 dilution with distilled water.

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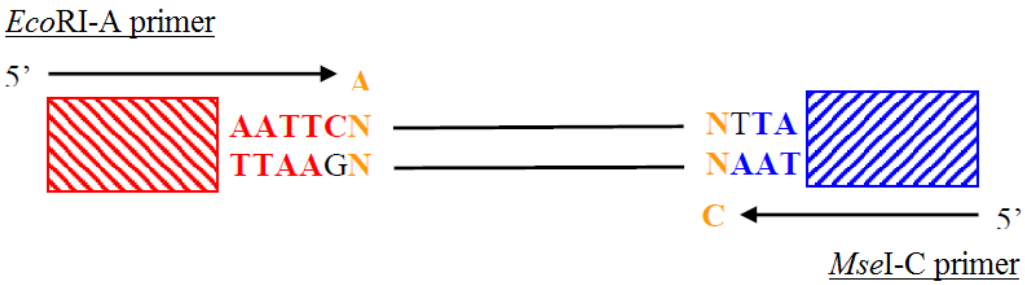

Figure III.5. Preamplification reaction

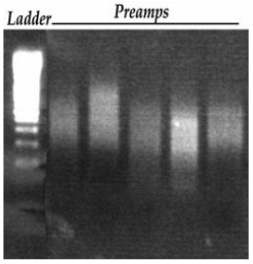

Figure III.6. Agarose gel electrophoresis of some preamplification products (*Preamps*) with size marker (*Ladder*). The preamplification leads to a homogeneous DNA smear from 100 to 800 bp

The aim of the selective amplification is to restrict the level of polymorphism and to label the DNA. For this step, we added three more nucleotides (e.g. ATA for *Eco*RI primer) at the 3' end of the pre-selective primer sequence; thus, we obtain a selective primer (**Fig. III.7**). The two additional nucleotides (e.g. TA) make the amplification more selective and will decrease the number of restriction fragments amplified. Moreover, *Eco*RI primer (E-primer*) is labelled with a fluorescent dye, so that the resulting selective products could be detected on an automated DNA analyzer (see III.4.3. DNA sequencer). To verify the success of the amplification, selective products were run on a 1.2% agarose gel (**Fig. III.8**). Thermal cycling and chemistry parameters differed for pre-selective and selective PCR amplifications (**Tables III.6**, **III.7**, **III.8** and **III.9**).

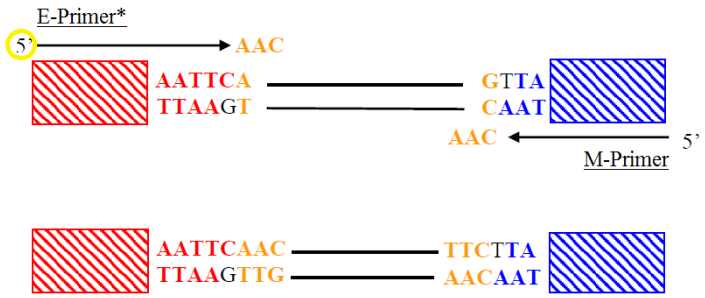

Figure III.7. Selective amplification reaction. "Yellow circle" at 5' end of E-primer* indicates the fluorescent dye: it could be blue (FAM™), green (VIC®) or yellow (NED™)

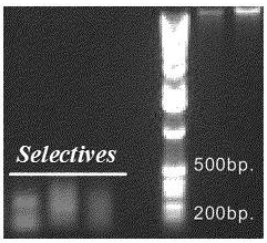

Figure III.8. Electrophoresis of selective amplification products (*Selectives*) with size marker (to the right). Successful selective reaction resulted in a series of distinct bands from 100-500 bp

III.4.3. DNA sequencer

Selective products were analyzed with a DNA sequencer (ABI 3730, Applied Biosystems®) in which a thin capillary containing a polymer serves as "running gel" for the electrophoresis. Samples are loaded in a track, and run one after the other through the capillary. All fragments are separated with regard to length, smaller fragments running first. Once passing the laser, the dye attached to the *Eco*Ri primer (E-primer*) is excited and emits a fluorescent signal that is then collected by a computer. In order to assign sizes to DNA fragments by comparison, a DNA size standard (GeneScan^{TM} 500 LIZ^{TM} dye Size Standard, Applied Biosystems®) is added to each sample. It is designed for sizing DNA fragments in the 35-500 nucleotides range and provides 16 evenly distributed single-stranded fragments labeled with the LIZ® fluorophore.

Reagent	**Reaction conditions**	**Volume**
dH_2O	-	6.82 µl
Taq buffer	-	1.25 µl
$MgCl_2$	-	0.75 µl
dNTP	10 mM	0.63 µl
*Eco*RI-A primer	10 µM	0.25 µl
*Mse*I-C primer	10 µM	0.25 µl
Taq polymerase	5 U	0.05 µl
RL product	-	2.50 µl
Total	-	12.50 µl

Table III.6. Pre-selective PCR amplification reaction of the AFLP protocol

Step	**Temperature (°C)**	**Time**	**Number of cycles**
1	72	2 min	1
2	94	30 s	25
	56	30 s	
	72	2 min	
3	60	10 min	1
4	4	∞	1

Table III.7. AFLP thermal cycler parameters for the pre-selective amplification reaction

Reagent	Reaction conditions	Volume
dH_2O	-	6.45 µl
Taq buffer	-	1.25 µl
$MgCl_2$	-	0.75 µl
dNTP	10 mM	1 µl
BSA	1 mg/ml	1 µl
E-Primer*	10 µM	0.10 µl
M-Primer	10 µM	0.25 µl
Taq Gold polymerase	5 U/µl	0.20 µl
Presel product	-	2.50 µl
Total	-	12.50 µl

Table III.8. Selective PCR amplification reaction of the AFLP protocol * fluorescent dye

Step	Temperature (°C)	Time	Number of cycles
1	95	10 min	1
2	94	20 s	13
	65-56*	30 s	
	72	1 min	
3	94	30 s	23
	56	30 s	
	72	1 min	
4	72	10 min	1
5	12	∞	

Table III.9. AFLP thermal cycler parameters for the selective amplification reaction. *Step 2 The annealing temperature consists of touch-down cycles with a decrease of 0.7°C each cycle

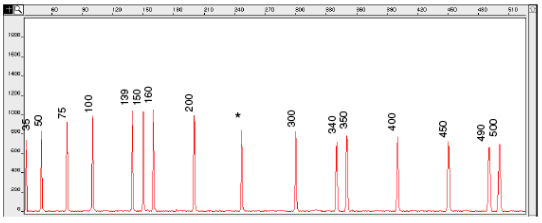

Figure III.9. Electropherogram of the GeneScan-500 size standard run under denaturing conditions on the ABI PRISM 310 Genetic Analyser

This dye results in a single peak when run under denaturing conditions (**Fig III.9**). The results of fluorescence are visualized on the computer as peaks, called electropherograms. Each peak corresponds to a band on a normal electrophoretic gel.

III.4.4. Choosing selective primer combinations

Choice of number and sequence of selective primers is an important step of the AFLP process because they will later determine the level of polymorphism accessible in the studied species.
To test for good primer combination, we choose randomly among eight *Mse*I primers and eleven *Eco*RI-labelled primers. Thus, 88 selective primer combinations were available.
An initial screening was performed on nine pairs on a random sample of eight individuals across all sampled populations. Even if combination were chosen randomly, we considered that yellow dye (NED) is usually the worse among the three available fluorescent dyes, whereas blue one (FAM) is more efficient. Thus, we tested mainly FAM-labelled *Eco*RI primers.
Three selective primer combinations were finally chosen based on their electropherogram profile with (i) good number of peaks (above 50), (ii) little background noise, (iii) well separated and (iv) wide-rage (70-500 bp) distributed peaks:

Primer pair code	**E*-M-primer Combination**	**E*- fluorescent dye (channel name)**	**Best attribute of the Electropherogram profile**
P1	ACA-CAC	Green (VIC)	High number of peaks (>70)
P2	AGC-CTG	Blue (FAM)	Well separated peaks
P6	ACT-CTG	Blue (FAM)	Well separated peaks and minimal background noise

Table III.10. The three primer combinations used in this survey with related proprieties

Moreover, a good primer discriminates individuals coming from different populations, but it is still polymorphic enough at the population level to precisely identify individuals; thus, chosen primer pairs must show a good level of inter- and intra-population polymorphism.

Once the validity of selective primers were assessed in the studied species, AFLP protocol was performed on all sampled individuals using P1, P2 and P6 for each one. Selective products were then elaborate on DNA sequencer in order to obtain data to analyze.

III.5. Data analysis

III.5.1. Matrix generation

GeneMarker 1.9 fragment analysis software (SoftGenetics LLC) analyzes AFLP data obtained with DNA sequencer. The data processed by the program are shown in electropherograms (**Fig. III.10**).

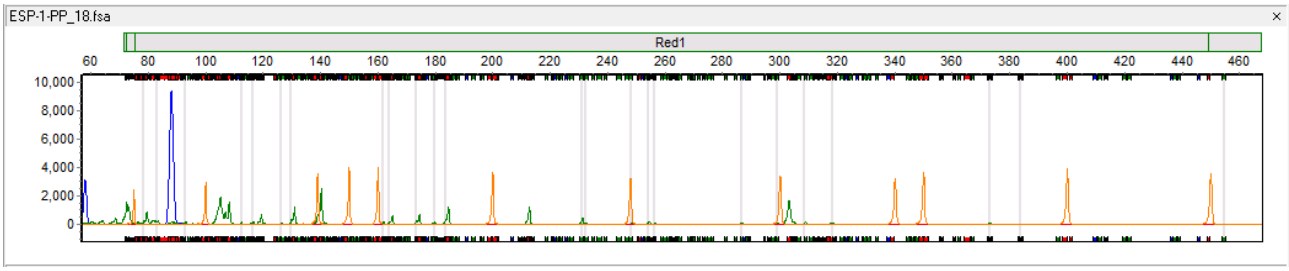

Figure III.10. Electropherogram as shown in GeneMarker 1.9

These graphics represent fluorescent signal intensities corresponding to dyes (orange, blue, green, yellow) in an interval measured in base pair (bp). The data were filtered by color according to the chosen selective primer combinations (**Table III.10**) of samples. The signal intensities, recorded in Relative Fluorescent Units (RFLUs), are displayed in the vertical axis. Each peak corresponds to a DNA restriction fragment of a precise length: the location of peaks is indicated by the bp position (Bin on x axis). The program was run with standard size parameter of GS500-1 and default criteria; the chosen length setting was from 50 to 500 bp.

The program was initially run without a panel in order to check the quality of the amplifications. I assess this either by discarding individuals without or with very few peaks, or by keeping samples with both ideal profiles and with noisy background signals; I separated these two sample types classifying them as ‘good’ and ‘dirty’ respectively. The ‘dirty samples’, alongside repetitions, are very important because they later are used to discard alleles and/or individuals that could be causing biases in the analyses.

Next, I used a pair of ‘good samples’ from each population to create a Panel. The purpose of a Panel is to outline the position of expected alleles. The ‘Loci’ parameters give a range where a group of alleles is expected to appear and ‘Bins’ indicate the specific bp position of the expected allele. In GeneMarker, Loci are indicated by a horizontal gray bar, while Bins by the dye-colored brackets at the bottom of the electropherogram (**Fig. III.11**).

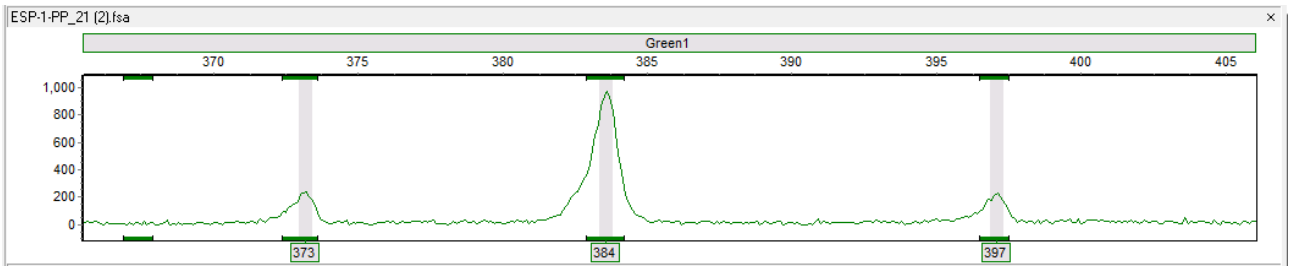

Figure III.11. Electropherogram of a scored sample amplified with P1

Post-processing of electropherograms (scoring) focuses on checking each fluorescence peak of each sample of each primer to assess if it is a valid allele, using the Panel as guideline. This checking process, even with the help of the Panel, is time-consuming and comprises substantial subjectivity. Thus, it is important to be rigorous and to follow a standard criterion.

The main issues in scoring AFLP electropherograms are varying fluorescence intensities and size of peaks among samples, and artefactual peaks. This variation in AFLP electropherograms may lead to erroneous assignment of peaks to bins (Paris et al. 2010), as each of these parameters influences the number of characters available for phylogenetic analysis and whether or not these characters represent homologous fragments; homoplasy is the occurrence of two different, but equally sized fragments in different individuals (Gort et al. 2009).
Introducing more homologous characters should lead to higher resolution, but in practice by including more characters we also risk introducing errors. First, lowering the minimum fragment length will increase the number of characters, but these characters may be of lower quality, as previous studies (Vekemans et al. 2002) indicate that smaller fragments are more likely to be homoplasious than larger fragments. Second, reducing the bin width will increase the number of characters, but as bin width is reduced, single characters may split into pairs of characters, which at the very least can reduce branch support in the resulting tree but could also potentially introduce error into the data set. Conversely, as we increase bin width, separate characters will be amalgamated. If these characters are not really identical, this could create character conflict in the resulting data matrix, which may reduce resolution. Third, lowering the peak height threshold will increase the number of characters. If peak height threshold is set too low, we will, by scoring background noise, call peaks present when they are really absent, and if it is set too high we will call peaks as absent when they are really present.

The effect of these issues can, however, be reduced by choosing a set of appropriate scoring parameters. The essential criterion I used is that a peak is considered valid if it has at least 25% of

the intensity of the peak immediately before and immediately after. I have generally not considered valid peaks with an intensity of less than 50, but this may vary with the case, for example if the profile has a distinctive low signal as it happens with dirty samples. Finally, I vertically checked each bin in every sample to assess if the criteria were effectively carried out.

If a peak is detected in a sample (line), the bin table report style (**Fig. III.12**) will report the presence (1) of the peak at that position (bin). The resulting AFLP profiles are recorded in a binary presence-absence (1/0) character matrix within each bin (column) and for each sample. The Bin Table was subsequently reported as an Excel file.

		Green1	Green1	Green1	Green1	Green1	Green1	Green1	Green1	Green1	Green1	Green1	Green1	Green1	Green1	G
		107	108	113	114	116	117	118	120	125	126	127	129	131	133	1
1	ESP-1-PP_18.fsa	0	0	1	0	0	1	0	0	0	0	1	1	0	0	
2	ESP-1-PP_21 (1).	1	1	1	0	1	1	0	0	0	0	0	0	1	1	
3	ESP-1-PP_21 (2).	1	1	1	0	1	1	0	0	0	0	1	0	1	1	
4	ESP-8-PP_21 (1).	1	1	1	1	1	1	0	0	0	0	0	1	1	1	
5	ESP-8-PP_21 (2).	1	1	0	1	1	1	0	0	0	0	0	1	1	1	
6	V2305-1-PP_21 (2	1	1	0	0	1	1	1	1	0	0	1	1	1	1	
7	V2305-1-PP_21.fs	1	1	0	0	1	1	1	1	0	0	0	1	1	1	
8	V2313-6-PP_21 (2	1	1	1	0	1	1	0	1	0	0	0	0	1	0	

Figure III.12. Part of bin table report as shown in GeneMarker 1.9

The last step of matrixes generation is to estimate the genotyping error specific for each primer. Genotyping errors occur when the genotype determined after molecular analysis does not correspond to the real genotype of the individual under consideration. Calculating the error rates per locus (bin) makes sense for AFLP studies because a single PCR generates many dominant alleles (Pompanon et al. 2005). This procedure was carried out by scoring pairs of replicate samples, which have to be at least 10% of the total number of individuals available for each primer (N). The replicates were checked bin by bin to locate any differences in allele presence/absence within the pair. Next, the absolute number of these mismatches was divided by N to calculate the percentages of errors for each pair. The genotyping error rate for a specific primer is the arithmetical mean of these.

Estimation of genotyping error is also a final scoring quality check as the mismatches among replicates could be artefactual errors of scoring (Bonin et al. 2007). Although these artefacts cannot be scientifically calculated, errors occurring with frequency in a certain bin may point out to the susceptibility of this bin to error scoring. Thus, the bin must be re-checked in all samples to assess if its scoring problem is widespread; if this is the case, the bin will be eliminated from the final matrix. After this error cleaning, the genotyping error rate can be re-calculated. At this point, the

rate in percentage indicates the minimum acceptable number of alleles detected for each bin if we consider all individuals. For example, a 2% error rate indicates that if we detect a bin that has two or less alleles transversally to all the samples, it shall be effaced from the matrix. Long last, we have obtained fairly cleaned matrixes for each primer.

Lastly, the binary matrixes generated for each primer were manipulated to include only the samples common in all primers. Next, the matrixes were joined horizontally in an Excel file in order to obtain the dataset for successive molecular analyses.

III.5.2. Molecular analyses

The analyses of the molecular data were carried out with two different data sets: one comprising all primer combinations for phylogenetic studies (P1-P2-P6), and the other including two out of three primer combinations for conservational aims (P1-P2) (**Table III.11**). This choice was dictated by the necessity of conservational studies to include in the analysis only populations with at least three individuals ($N \geq 3$). The "two primers choice" allows to include an higher number of samples and of population of significant size, among which Montgó ones; these populations were considered genetically important in a previous study (Massó 2011). The unfinished set of molecular data will be subsequently integrated. In the final publication, all samples of the total number of primers will be used to complete the survey.

The software PAUP* 4.0 (Phylogenetic Analysis Using Parsimony; Swofford 2002) was used to infer phylogenetic relationships between individuals. The resulting evolutionary tree, an unrooted phylogram, was constructed with neighbor-joining (NJ) analysis; the branch support was assessed by bootstrap analysis (1000 replicates). The input data file format (.nexus) was edited with AFLPdat package (Ehrich 2006) conducted in the R program (R Development Core Team 2012), which converts data from a standard tab separated table (usually .txt) to the input matrix of many programs, among which the following used in this work: ARLEQUIN, AFLP-SURV, AFLP-DIV, BAPS.

In order to examine the relative position of populations, the genetic relationship among them was represented graphically by principal coordinate analysis (PCoA); this analysis was carried out on the basis of Nei's genetic distance (Nei & Feldman 1972) using GenAlEx 6.5 software (Peakall & Smouse 2012).

	P1-P2-P6		P1-P2	
	Population code	*Individuals (N)*	*Population code*	*Individuals (N)*
1	A1	4	A1	5
2	A2	6	A2	8
3	A3	2	A3	5
4	A4	7	A4	7
5	A5	5	A5	5
6	A6	9	A6	9
7	A7	7	A7	8
8	A8	2	A8	3
9	A9	3	A9	5
10	IB1	4	IB1	7
11	IB2	1	IB2	2
12	IB3	5	IB3	7
13	IB4	3	IB4	8
14	IB5	7	IB5	7
15	IB6	6	IB6	6
16	ESP	8	ESP	9
Tot.		79		101

Table III.11. The two different data sets used in this survey for phylogenetic (P1-P2-P6) and conservational studies (P1-P2). "*Individuals (N)*" indicates the population size

Significance in the isolation by distance relationship between populations can be tested statistically using a Mantel test (Mantel 1967). This test assesses whether the pairwise genetic distance matrix (F_{ST}) is correlated with the pairwise geographic distance matrix; it was performed on the total data and for each region separately (Marina Alta and Ibiza), using 1000 permutations and the default parameter of the Isolation by Distance Web Service (IBDWS 3.23; Jensen et al. 2005).

Pairwise F_{ST} values between populations were estimated through AFLP-SURV 1.0 software, while Nei values were the output data of GenAlEx 6.5 software. The Geographic Distance Matrix Generator (Ersts 2011) was used to compute all pairwise distances from a simple list of geographic coordinates, where latitudes and longitudes are expressed in degrees with decimals.

To analyze molecular variance, AMOVA, was used to examine within and among different populations based on the complete sample set and different groups separately using ARLEQUIN 3.5.1.2 (Excoffier & Lischer 2010). The AMOVA analyses were performed at different hierarchical levels. The significance levels of the variance components were obtained by nonparametric permutation using 10,000 replicates.

BAPS 6.0 (Bayesian Analysis of Population Structure) is a program for Bayesian inference (Corander et al. 2008) of the genetic structure in a population. The program output represents the most probable clustering solution where the clustering sampling units (individuals) are shown as colored vertical bars with the color determining the cluster membership (groups). The program asks for the number of clusters (K) and replicate runs, which increases the probability of finding the posterior optimal clustering with the K value specified. For each analysis, 10 independent runs (repetitions) per K value of K = 4 and K = 10 were performed. The most likely K was chosen according to the highest log marginal likelihood [log(ml)] values. The relationships among the identified clusters were represented using Phylips 3.695 package (Felsetein 2005).

For each population and regions, allele frequencies were estimated using the Bayesian approach (Zhivotovsky 1999) based on nonuniformation prior distribution of nulle-allele frequencies and Hardy-Weinberg genotypic proportions as implemented in the program AFLP-SURV 1.0 (Vekemans 2002). The program then calculates the proportion of polymorphic markers (PLP), estimates of gene diversity (Hj), and between-population unbiased estimates of Nei's (1972) using the approach of Lynch & Milligan (1994). Moreover, we calculated the proportion of private markers (e.g. bands unique to a population or region but not detected in all individuals) and fixed private markers (e.g. exclusive bands displayed by all individuals of a particular population or region) using Microsoft Excel.

As difference in sampling intensity between populations could bias the comparation of genetic diversity, we computed the band richness (Br) standardized to the smallest population size (S) by means of a rarefaction methods with the AFLP-DIV 1.0 program (Coart et al. 2005). Initially, we computed four combination of parameters (all-primers / two-primers data set; S = 5 / S = 3) for the purpose of assessing the better pair of parameters with regards to our conservational studies. In order to include the maximum number of population, we chose the two-primers data set, and S = 5 since the Br value didn't differ much when calculated with a S of 3.

IV. RESULTS

IV.1. Phylogeographic analyses

Within dataset 1 (**Table III.11**) a total of 338 AFLP bands – whereof 110 of P1 primer, 101 of P2 primer, and 127 of P6 primer – were scored in the 79 individuals of 16 populations, and within regional units. Considering the regional bipartition, the values of polymorphic bands detected were 242 (71.6%) and 273 (80.8%) for Ibiza and Marina Alta respectively. At intraregional level, the number of polymorphic loci varied from 248 (73.4%) in Ibiza (less Espartar) populations to 287 (84.9%) in Marina Alta populations except for Montgó ones (**Table IV.1**). The number of polymorphic bands per population varied from 185 (54.7%) in A3 population to 240 (71.0%) in A4 population. The mean number of polymorphic bands per population was 212.5 (± 18.7) (**Table IV.2**). At population level, the highest number of fixed private markers was found in A6 population (3), followed by ESP population (2); only one fixed private marker was detected in A3, A4, IB1 and IB4 populations. Private markers were found only in four populations (ESP, A5, IB4, IB6); in each of these populations only one private marker was detected (**Table IV.2**). At regional level (Ibiza *vs.* Marina Alta), we found no fixed private markers, whereas private markers were detected both in Ibiza (16) and Marina Alta (34) regions (**Table IV.1**).

Region	n	loci_P	PLP(%)	n° fixed private markers	n° private markers
Ibiza	34	242	71.6	-	16
M.A.	45	273	80.8	-	34
Ibiza (-ESP)	26	248	73.4	-	7
ESP	8	221	65.4	2	1
M.A. (-Mont)	33	287	84.9	-	10
Montgó	12	271	80.2	-	2

Table IV.1. Values of genetic diversity for 79 *C. dianius* individuals within regional bipartition (Ibiza *vs.* Marina Alta) and within intraregional units of Montgó, Marina Alta populations except for Montgó ones "M.A. (-Mont)", Ibiza populations "IB (-ESP)", and Espartar Islet "ESP"; **loci_P**: number of polymorphic loci; **PLP**: percentage of polymorphic loci at the 5% level (e.g. loci with allelic frequencies lying within the range 0.05 to 0.95)

Population	n	loci_P	PLP(%)	n° fixed private markers	n° private markers
ESP	8	221	65.4	2	1
A1	4	215	63.6	-	-
A2	6	224	66.3	-	-
A3	2	185	54.7	1	-
A4	7	240	71.0	1	-
A5	5	217	64.2	-	1
A6	9	238	70.4	3	-
A7	7	227	67.2	-	-
A8	2	186	55.0	-	-
A9	3	200	59.2	-	-
IB1	4	216	63.9	1	-
IB2	1	-	-	-	-
IB3	5	187	55.3	-	-
IB4	3	188	55.6	1	1
IB5	7	235	69.5	-	-
IB6	6	209	61.8	-	1
Mean (SD)	-	212.5 (18.70)	62.9 (5.54)	-	-

Table IV.2. Values of genetic diversity at population level for 79 *C. dianius* individuals within 16 populations; **loci_P**: number of polymorphic loci; **PLP**: percentage of polymorphic loci at the 5% level (e.g. loci with allelic frequencies lying within the range 0.05 to 0.95)

The Neighbor Joining (NJ) unrooted tree of the "P1-P2-P6" dataset (**Fig. IV.1**) suggests a regional clustering in two groups: Marina Alta (indicates in red) and Ibiza/Espartar Islet (in light blue). Only A8 population seems to deviate from the well-defined regional division with a high bootstrapping (BS) value (100). All individuals cluster into their own group of population; some of these clusters show high BS values (e.g. A3, A6 and A9 populations have respectively 98, 96 and 98 BS values) with the exception of A4 and A5 populations, which mix in a single cluster without branch support value. Ibiza populations have lower (e.g. IB3 with BS = 56) or not significant branch support indices, with the exception of Espartar Islet individuals (ESP) and IB5 individuals, which form well clustered groups with BS values of 89 and 65 respectively.

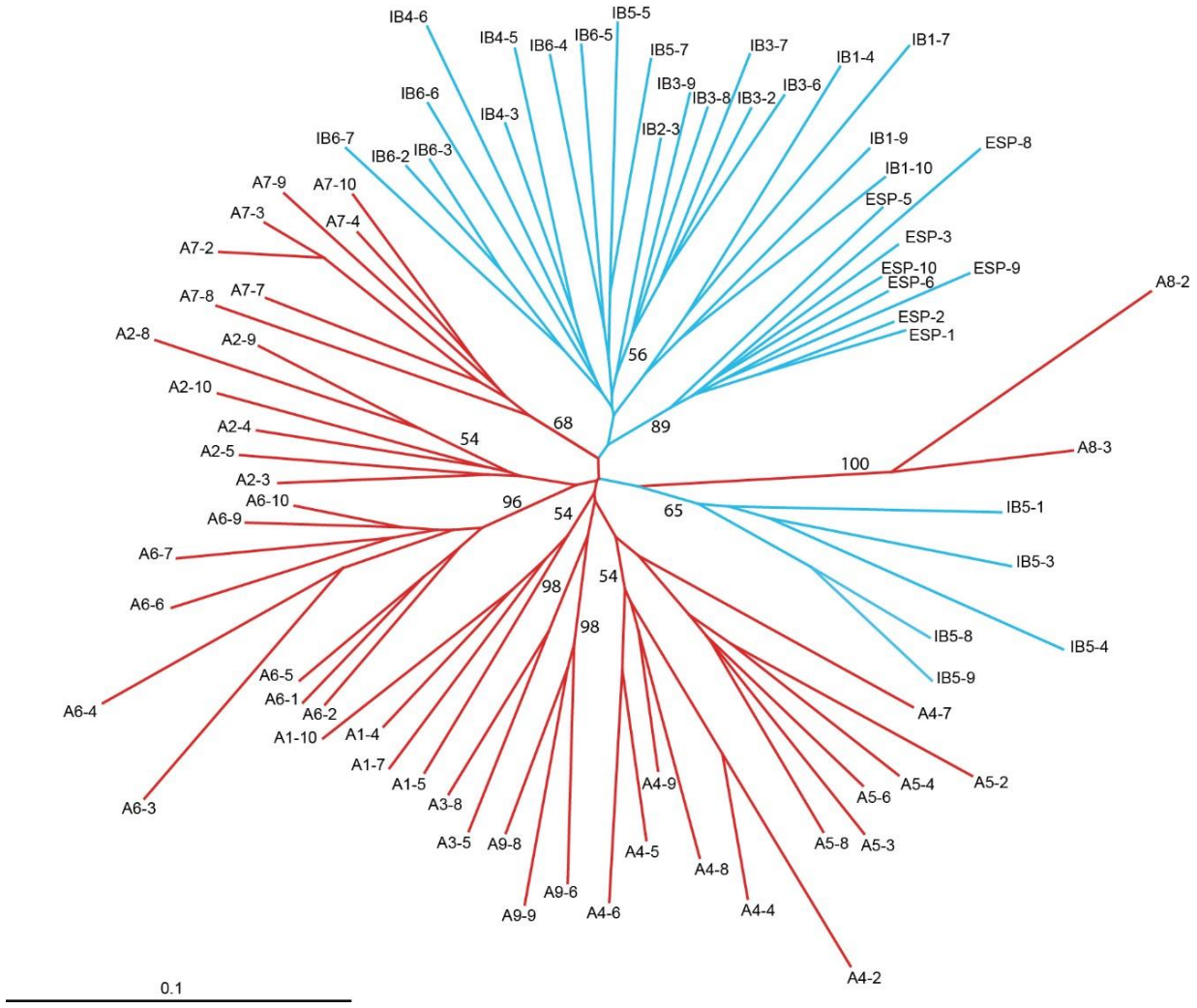

Figure IV.1. Neighbor Joining unrooted tree of 1000 replicates bootstrap analysis (branch support values) based on 338 markers. The two regional groups, as identified in **Table III.1**, are indicated by color: red = Marina Alta; light blue = Ibiza (including Espartar Islet)

The Principal Coordinates Analysis (PCoA) using the relative position of all populations of *C. dianius* according to Nei's genetic distance with two dimension projections is drawn in **Fig. IV.2.a**. This figure represents all populations of *C. dianius* mainly scattered along axis 1, showing a significant separation in two groups according to regional partition: Ibiza with concentrated dots (except for isolated IB2 and IB5 populations), and Marina Alta that presents a more dispersed pattern of populations. Along axis 2, A8 population is clearly separated from the remaining groups. This pattern is mirrored in the NJ tree (**Fig. IV.1**).

The **Fig. IV.2.b** focus on the structural pattern of Ibiza populations of *C. dianius*. These populations do not form any obvious intra-grouping. They are uniformly dispersed along axis 1 and 2, with the exception of IB2 along axis 1 (eigenvalue = 0.141) and IB5 along axis 2 (eigenvalue

= 0.113). Both of these populations show a significant distance from the others as previously observed in **Fig. IV.2.a**, and individuals of IB5 population clearly form a cluster in the NJ tree (BS value = 65). The general indistinct uniformity of Ibiza scatterplot is mirrored by the undifferentiated Ibiza cluster (except for the aforementioned IB5 and ESP) of NJ tree with low or not significant branch support values.

The **Fig. IV.2.c** shows a pattern of Marina Alta populations of *C. dianius*. These populations are dispersed with less uniformity than Ibiza populations (**Fig. IV.2.b**), thus, we may identify more populations that significantly distance themselves from the others. The most evident isolated dot along axis 1 (eigenvalue = 0.211) represents A8 population, which ubiquitously shows a pattern of segregation from the other groups. Axis 2 shows significant separation of A5 (eigenvalue = 0.131), A3 (-0.094) and A9 (-0.063) populations. Individuals of all these populations clustered in the NJ tree with high bootstrapping values (e.g. both A3 and A9 with 98). Axis 1 produces less separation, as Marina Alta populations are roughly lined up vertically, except for A8 and A5 (eigenvalue of axis 1 = -0.069) populations.

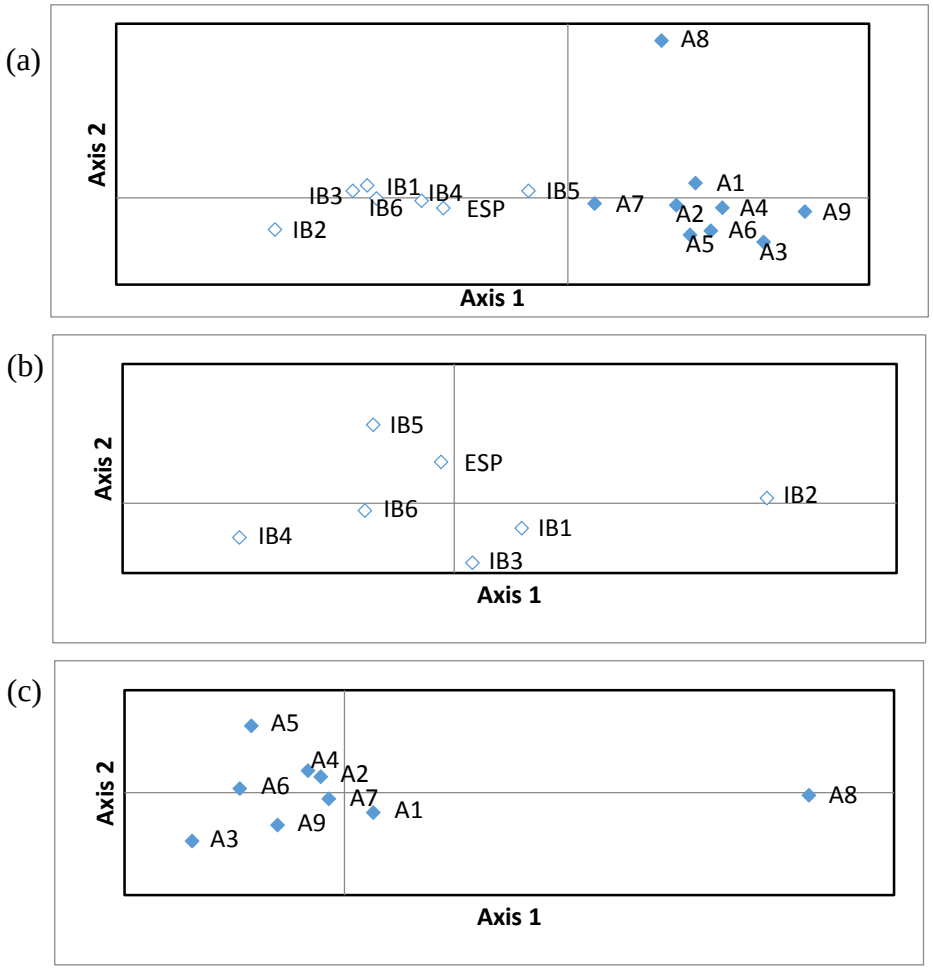

Figure IV.2.a-b-c. Principal Coordinates Analysis (PCoA) scatterplots in two dimension projections of all populations (**a**), Ibiza (**b**) and Marina Alta (**c**) datasets of *Carduncellus dianius*

Table IV.3 show three axes of the PCoA analyses of all populations (according to "P1-P2-P6 dataset"), Ibiza and Marina Alta that cumulatively account for 41.85%, 62.05% and 55.53% of the variation respectively. The eigenvectors were plotted in two-dimensional scatter plots based on all populations (**Fig. IV.4.a**), Ibiza (**Fig. IV.4.b**) and Marina Alta (**Fig. IV.4.c**) datasets.

	All populations			**Ibiza**			**Marina Alta**		
Axis	%	Cum	Eigen	%	Cum	Eigen	%	Cum	Eigen
1	17.12	17.12	0.048	24.49	24.49	0.033	26.33	26.33	0.055
2	14.54	31.66	0.041	20.02	44.52	0.027	16.53	42.86	0.035
3	10.19	41.85	0.029	17.54	62.05	0.024	12.67	55.53	0.026

Table IV.3. Eigenvalues and percentage of variation expressed by each axis for all populations, Ibiza and Marina Alta datasets. "Cum" cumulative; "Eigen" eigenvalue

In the AMOVA analyses based on "P1-P2-P6" dataset (**Table III.11**), excluding IB2 population with only one individual, was represented in **Table IV.4** and **Table IV.5**, according to different hierarchical levels.

	d.f.	**Sum of squares**	**Variance components**	**% variation**	**F_{ST}**
Two hierarchical levels					
All populations					0.314
Among populations	15	1320.941	12.49	31.41**	
Within populations	63	1718.198	27.27	68.59**	
Ibiza					0.233
Among populations	6	410.142	8.56	23.24**	
Within populations	27	762.917	28.26	76.76**	
Marina Alta					0.324
Among populations	8	707.163	12.71	32.39**	
Within populations	36	9555.282	26.54	67.61**	

Table IV.4. Analysis of molecular variance (AMOVA) at two hierarchical levels based on 338 polymorphic markers of *Carduncellus dianius*. "d.f." degrees of freedom; "**" $P < 0.0001$ (after 1023 permutations)

	d.f.	Sum of squares	Variance components	% variation	F_{ST}
Several groups of populations					
M.A.* vs *Ibiza					0.337
Among groups	1	203.636	2.87	6.97**	
Among populations within groups	14	1117.305	10.97	26.68**	
Within populations	63	1718.198	27.27	66.35**	
Montgó* vs *M.A. (-Mont)* vs *Ibiza					0.325
Among groups	2	275.058	1.97	4.88**	
Among populations within groups	13	1045.883	11.17	27.64**	
Within populations	63	1718.198	27.27	67.48**	
Montgó* vs *M.A. (-Mont)					0.311
Among groups	1	71.422	-1.37	-3.56 ns	
Among populations within groups	7	635.741	13.33	34.63**	
Within populations	36	955.282	26.54	68.93**	

Table IV.5. Analysis of molecular variance (AMOVA) with populations nested analyses within regions (Ibiza, Marina Alta, Montgó), based on 338 polymorphic markers of *C. dianius*. "d.f." degrees of freedom; "**" $P < 0.0001$ (after 1023 permutations); "ns" P value not significant

In the case of a simple hierarchical analysis (two levels), the genetic structure consists of one group with high within population variability (68.59%). In all of the three groups considered (all populations, Ibiza, Marina Alta), the highest percentage of genetic variability was found also within populations, especially in the Ibiza group (76.76%) (**Table IV.4**).

Next, a hierarchical AMOVA with populations nested analyses within regions (Ibiza, Marina Alta, Montgó) was performed to examine the distribution of variation (i) among groups, which presents the lowest values (4.88 and 6.97%); (ii) among populations within groups (from 26.68 to 34.63%), and (iii) within populations, where the highest percentages were found with values ranging from

66.35 to 68.93%. All performed analyses are statically significant with the exception of the percentage of genetic variability among Montgó and Marina Alta groups (**Table IV.5**).

The fixation index (F_{ST}) is a measure of population differentiation due to genetic structure. Values can range from 0 to 1, where 0 means complete sharing of genetic material (population are interbreeding freely) and 1 implies no sharing (populations are fixed).

The pairwise F_{ST} values used to generate the genetic distance for Mantel test were computed with AFLP-SURV with 1000 permutations and using the "P1-P2-P6" dataset minus IB2 population. To do analysis of isolation by distance, the half matrixes of F_{ST} values matrixes (**Tables IV.6.a-b-c**) were completed with semi matrixes of geographical distance.
The highest significant distances (values closer to 1) were found between A3 and A8 considering all sampled populations of *C. dianius* (F_{ST} = 0.432; **Table IV.6.a**) and Marina Alta populations (**Table IV.6.c**), between IB3 and ESP considering Ibiza populations (F_{ST} = 0.173; **Table IV.6.b**). The lowest F_{ST} values were found between IB4 and IB6 (F_{ST} = 0.056), for all sampled populations and Marina Alta populations; and between A2 and A6 (F_{ST} = 0.121) among Ibiza populations.

(a)	*ESP*	*A7*	*A8*	*A5*	*A6*	*A4*	*A2*	*A9*	*IB6*	*IB5*	*IB4*	*IB3*	*IB1*	*A3*	*A1*
ESP	0														
A7	0.180	0													
A8	0.357	0.337	0												
A5	0.218	0.200	0.368	0											
A6	0.210	0.197	0.369	0.206	0										
A4	0.189	0.180	0.297	0.123	0.164	0									
A2	0.158	0.150	0.294	0.179	0.121	0.133	0								
A9	0.278	0.222	0.364	0.265	0.249	0.179	0.196	0							
IB6	0.133	0.174	0.330	0.214	0.217	0.176	0.176	0.278	0						
IB5	0.143	0.185	0.289	0.184	0.165	0.142	0.104	0.223	0.092	0					
IB4	0.143	0.164	0.31	0.212	0.203	0.172	0.171	0.225	0.056	0.117	0				
IB3	0.173	0.169	0.351	0.257	0.241	0.218	0.195	0.307	0.091	0.168	0.106	0			
IB1	0.124	0.150	0.296	0.239	0.222	0.156	0.143	0.235	0.066	0.111	0.101	0.102	0		
A3	0.324	0.258	0.432	0.301	0.249	0.232	0.243	0.240	0.301	0.282	0.270	0.335	0.276	0	
A1	0.200	0.167	0.274	0.208	0.197	0.146	0.127	0.182	0.185	0.170	0.157	0.211	0.154	0.213	0

Table IV.6.a. The output from AFLP-SURV displays F_{ST} at each scale among all sampled populations of *Carduncellus dianius*. The value in a given cell indicates the genetic distance among units of the corresponding column compared to the corresponding row. The most relevant values, highest and lowest, are boxed

(b)	*ESP*	*IB6*	*IB5*	*IB4*	*IB3*	*IB1*
ESP	0					
IB6	0.133	0				
IB5	0.143	0.092	0			
IB4	0.143	0.056	0.117	0		
IB3	0.173	0.091	0.168	0.106	0	
IB1	0.124	0.066	0.111	0.101	0.102	0

Table IV.6.b. The output from AFLP-SURV displays F_{ST} at each scale among Ibiza populations of *Carduncellus dianius*. The value in a given cell indicates the genetic distance among units of the corresponding column compared to the corresponding row. The most relevant values, highest and lowest, are boxed

(c)	*A7*	*A8*	*A5*	*A6*	*A4*	*A2*	*A9*	*A3*	*A1*
A7	0								
A8	0.337	0							
A5	0.210	0.368	0						
A6	0.197	0.369	0.206	0					
A4	0.180	0.297	0.123	0.164	0				
A2	0.150	0.294	0.179	0.121	0.133	0			
A9	0.222	0.364	0.265	0.249	0.179	0.196	0		
A3	0.258	0.432	0.301	0.249	0.232	0.243	0.240	0	
A1	0.167	0.274	0.208	0.197	0.146	0.127	0.182	0.213	0

Table IV.6.c. The output from AFLP-SURV displays F_{ST} at each scale among Marina Alta populations of *Carduncellus dianius*. The value in a given cell indicates the genetic distance among units of the corresponding column compared to the corresponding row. The most relevant values, highest and lowest, are boxed

Levels of genetic differentiation among-populations were moderate, with global F_{ST} among all sampled populations, Ibiza's and Marina Alta's being 0.214, 0.115, and 0.237 respectively (**Table IV.7**). All of these F_{ST} estimates show a significant P-value (*high*), lower than 0.05 (P = 0.000); thus, it can be concluded that the actual populations are more genetically differentiated than random assemblages of the individuals.

We tested whether geographical distance was correlated to pairwise genetic differentiation (F_{ST}) by simple Mantel test; thus, we conducted an isolation-by-distance (IBD) analysis for all of the three units of analysis (**Table IV.7**). Using Marina Alta as the unit of analysis, there was no significant pattern of IBD detected (P = 0.127; **Fig. IV.3.c**). However, IBD was significant both

among Ibiza populations (P = 0.013; **Fig.** I**V.3.b**) and all sampled populations (P = 0.017; **Fig.** I**V.3.a**) with *r* values (Pearson correlation measure) of 0.698 and 0.215 respectively.

Unit of analysis	**F_{ST} (± SD)**
All populations	0.214 (± 0.123)
Ibiza	0.115 (± 0.128)
Marina Alta	0.237 (± 0.155)

Table IV.7. Global estimates of genetic differentiation (F_{ST}) among populations of complete "P1-P2-P6" dataset minus IB2 ("*all populations*"), Ibiza and Marina Alta populations sub-datasets. Standard deviation of F_{ST} (± SD) was assessed based on 1000 permutations

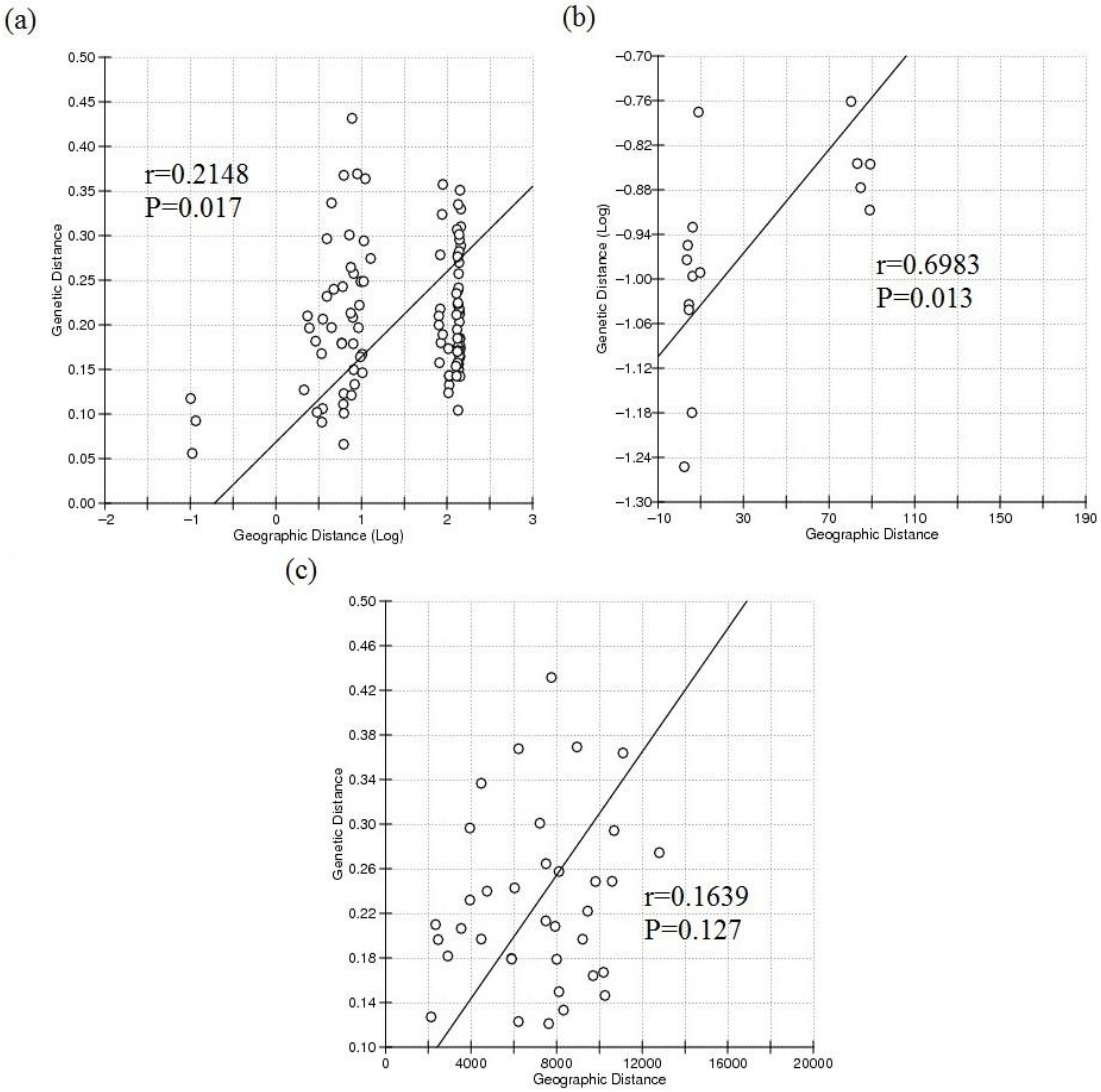

Figure IV.3.a-b-c. Correlation of genetic differentiation (F_{ST}) and geographic distance among *Carduncellus dianius* all sampled populations (**a**), Marina Alta (**b**), and Ibiza (**c**)

BAPS 6.0 program found the most probable solution of population genetic structure in 4 groups [(log of marginal likelihood) log(ml) = -10058.5013; **Fig. IV.4**]. There is a good reliability as other independent runs (e.g. K = 10) also resulted in the same 4 classes. The probability that the samples would form 4 clusters was calculated to be around 0.999, whereas a value of 0.0002 was assigned to 3 clusters solution. Thus, the 4 groups result is highly probable. The genetic divergence of the estimated 4 clusters is represented in **Fig. IV.5**.

Cluster 1 (yellow) includes exclusively A6 individuals. Indeed, on the NJ unrooted tree (**Fig. IV.1**) this population was well clustered with a high bootstrapping value of 98. The reported changes in log(ml) if individuals of A6 are moved to cluster 3 – that includes all the Marina Alta populations – range from 35 to 60 in absolute values. Allocation in another cluster is quite improbable as only small absolute values of the changes (< 2.3, see Kass & Raftery 1995) indicates a significant alternative cluster, whereas changes to cluster 2 and 4 especially show higher absolute values, hence less probability. Similar high values of the changes are reported in the other clusters. Thus, the suggested 4 groups repartition is quite well-defined.

Cluster 2 (blue) comprises all the Ibiza populations, including Espartar Islet.

Cluster 3 (red) includes only Marina Alta populations with the exceptions of A6 (cluster 1) and A8 (cluster 4) populations.

Cluster 4 (green) corresponds to A8 population. Similarly, in NJ unrooted tree A8 individuals form a well clustered group with a high bootstrapping value of 100.

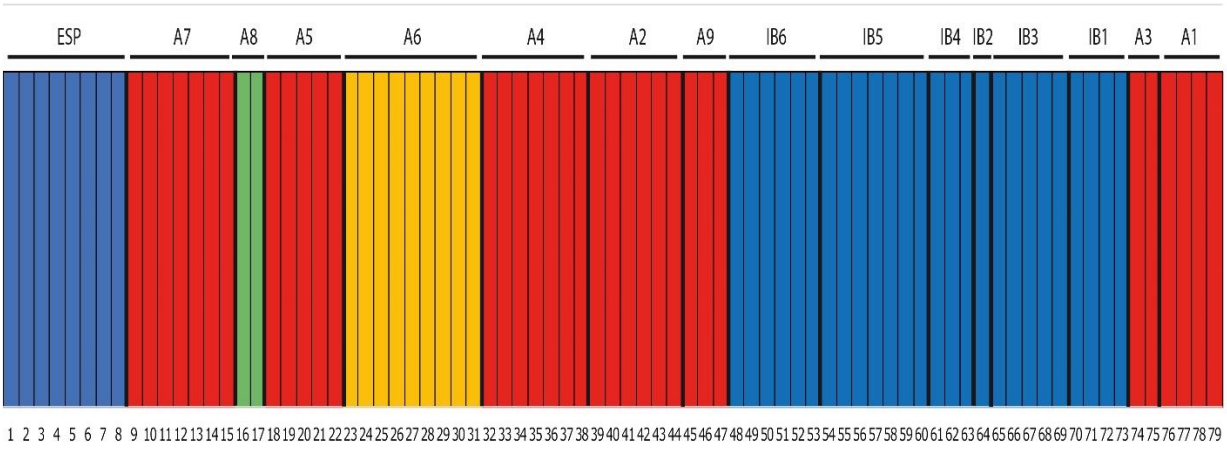

Figure IV.4. Each cluster is assigned a unique color: yellow = cluster 1; blue = cluster 2; red = cluster 3; and green = cluster 4. Every vertical colored bar represents an individual

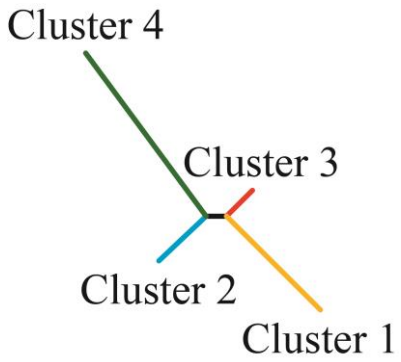

Figure IV.5. Representation of the divergence of the 4 groups calculated by BAPS. Each cluster, as identified in **Fig. IV.4**, is indicated by branch color

IV.2. Conservation analyses

We estimated the levels of within-population genetic variation of *C. dianius* using the "P1-P2" dataset (**Table III.11**); thus, we considered 111 individuals parted in 16 populations, and 211 total number of loci. We used the percentage of polymorphic loci (PLP) at 5% level and expected heterozygosity over all loci (H_j) and band richness (Br) as measures of within-population genetic variation for each of the 16 populations.

The percentage of polymorphic loci ranged from 50.2% (IB2) to 73.5% (A7) in the individual populations. The average PLP of all 16 populations was 64.13% ± 1.46% (mean ± SD). The level of expected heterozygosity within the populations ranged from 0.187 (A9) to 0.248 (IB2). The mean H_j was 0.220 ± 0.005 (**Table IV.8**).

Genetic diversity was also calculated using regions as units of analysis. Firstly, Marina Alta (all A coded populations) and Ibiza (IB and ESP) regional division was considered as indicated in **Table III.11** (under "P1-P2" caption). Secondly, the inter-regional genetic diversity analysis was performed by separating Montgó cluster (A1, A2, A3) from the other Marina Alta populations. This choice was dictated by the fact that Montgó populations were considered genetically important in a previous study (Massó 2011), hence we wish to submit them to further analysis.

Considering the **(ii)** analysis unit (two regions: Ibiza and Marina Alta), we found 73.5% and 77.7% percentages of PLP for Ibiza and Marina Alta respectively, and H_j values of 0.219 and 0.232 (**Table IV.8**). Considering the **(iii)** analysis unit (three regions: Ibiza, Marina Alta and Montgó), the highest levels of genetic diversity were reported within Marina Alta (PLP = 79.1%; H_j = 0.243 ± 0.010; Br[18] = 1.839; **Table IV.8**).

(i) Population	n	loci_P	PLP(%)	H_j	SD (H_j)	Br[5]
ESP	9	140	66.4	0.203	0.012	1.231
A1	5	140	66.4	0.229	0.012	1.167
A2	8	134	63.5	0.208	0.012	1.225
A3	5	130	61.6	0.199	0.011	1.162
A4	7	150	71.1	0.243	0.012	1.209
A5	5	125	59.2	0.205	0.012	1.142
A6	9	145	68.7	0.213	0.012	1.247
A7	8	155	73.5	0.239	0.011	1.234
A8	3	122	57.8	0.235	0.013	-
A9	5	126	59.7	0.188	0.012	1.163
IB1	7	146	69.2	0.233	0.012	1.208
IB2	2	106	50.2	0.248	0.013	-
IB3	7	128	60.7	0.196	0.011	1.182
IB4	8	139	65.9	0.228	0.012	1.220
IB5	7	145	68.7	0.232	0.012	1.207
IB6	6	134	63.5	0.227	0.012	1.164
Mean (± SD)	-	135.313 (± 11.930)	64.131 (± 5.700)	0.220	0.018	1.197 (± 0.032)
(ii) Region	**n**	**loci_P**	**PLP(%)**	**H_j**	**SD (H_j)**	**Br[46]**
Ibiza	46	155	73.5	0.219	0.011	1.921
M.A.	55	164	77.7	0.232	0.011	1.943
(iii) Region	**n**	**loci_P**	**PLP(%)**	**H_j**	**SD (H_j)**	**Br[18]**
Ibiza	46	155	73.5	0.219	0.011	1.830
M.A. (-Mont)	37	167	79.1	0.243	0.010	1.839
Montgó	18	158	74.9	0.223	0.011	1.682

Table IV.8. Values of genetic diversity for 211 *C. dianius* individuals according to three different units of analysis: **(i)** within the 16 populations; **(ii)** Marina Alta and Ibiza regions; **(iii)** Montgó, Marina Alta (-Montgó), and Ibiza populations; **loci_P**: number of polymorphic loci; **PLP**: percentage of polymorphic loci at the 5% level (e.g. loci with allelic frequencies lying within the range 0.05 to 0.95); **H_j**: expected heterozygosity under Hardy-Weinberg genotyping proportions; **SD (H_j)**: standard error of H_j; **Br**: band richness

Band richness (Br) can be interpreted as an allelic richness analogue (Coart et al. 2005; Honnay et al. 2006). Br[S] indicates the number of bands expected at each locus in a sample size of S individuals. Normally S value is equal to the population with the smallest sample size (Petit et al. 1998; Coart et al. 2005), but we decided to assign a value of S = 5 for the 16 populations analysis unit, thus excluding A8 and IB2 populations, in order to increase the resolution of the analysis. Considering the **(ii)** analysis unit, we found 1.921 and 1.943 Br values for Ibiza and Marina Alta respectively. In the **(iii)** analysis unit, the values of Br reported were 1.682 for Montgó, 1.839 for Marina Alta and 1.830 for Ibiza. At level of populations, Br values ranged from 1.162 (A3) to 1.247 (A6) (**Table IV.8**).

V. DISCUSSION

V.1. Phylogeography

Current populations of *C. dianius* are geographically located in the eastern part of the Iberian Peninsula, in Ibiza island and in near sited Espartar Islet. This population structuring has been modulated by a balance between historical processes linked to the dynamic Quaternary paleogeography of the western Balearic Islands and to past and contemporary recurrent gene flow involving long-distance dispersal (range expansion, colonization).

Barres et al. (2013) dated the differentiation of the *Carduncellus-Carthamus* complex – considering the four genera of *Carduncellus, Carthamus, Femeniasia* and *Phonus* (Vilatersana et al. 2000*b*) – in the Late Miocene, approximately 6.80 Ma, whereas the differentiation of *Femeniasia-Carduncellus* was dated approximately at 5.70 Ma. These two events occurred during the Messinian Salinity Crisis (5.96-5.33 Ma), when the Balearic Archipelago formed a single mass which had connections to the mainland (see I.3.1. Geological history). Results from Massó (2011) point both to a recent geographical expansion and diversification of *C. dianius*, which have occurred most likely during the Quaternary when oscillation of marine level due to glacial cycles (sea regression) favored the passage of species across the Iberian Peninsula toward the Balearic Archipelago. Patterns of differentiation and expansion across the Mediterranean Basin during the Quaternary have already been documented in other species (e.g. *Senecio*; Comes & Abbott 1999).

A disjoint distribution pattern can be traced both to dispersal and vicariance events. Vicariance is described as the process of disruption of a formerly continuous distribution area (Schönswetter & Tribsch 2005). This would be the case of *C. dianius* if its expansion and diversification have taken place during the Messinian, since the Balearic Archipelago formed a single mass with connections to the mainland and the following increase of sea level would have led to the separation of Alicante and Ibiza subsets as vicariants (see I.4.2. Endemism and insularity). Dispersal, on the other hand, occurs when a species spreads from one area to another through a barrier (see I.4.1. Evolution of endemism). Indeed, long-distance dispersal (LDD) is the most probable mechanism explaining the expansion and diversification of *C. dianius* through the Quaternary as even during the most prominent marine regressions, the Balearic Archipelago continued to be isolated and lacked connections with the mainland (see I.3.1. Geological history); thus, only LDD would have allowed

the colonization of Ibiza island. This hypothesis is confirmed also in genetic terms, as vicariance results in significant genetic divergence of disjointed species, whereas dispersal may lead to genetic depauperation due to founder effects but to little or no genetic divergence (Schönswetter & Tribsch 2005). Indeed, Ibiza populations show less diversity but are not neatly differentiated from Marina Alta group (see below). The identification of LDD as a major factor in the generation of disjoint distribution areas has already been documented in many Mediterranean species. For example, the presence of *Naufraga balearica* in Majorca may be the result of LDD through Quaternary glacial cycles (Fernández-Mazuecos et al. 2014).

Higher levels of genetic diversity and diversification of Marina Alta group related to the rest of populations (see below) may indicate that Marina Alta is the center of origin of the current *C. dianius* populations. This hypothesis is supported also by a higher number of private markers of the region (34) compared to Ibiza (16) (**Table IV.1**). Assuming that private markers accumulate through time, they can be used to estimate the antiquity of populations (Schönswetter & Tribsch 2005); thus, it appears that Marina Alta is the most ancient group of all sampled. According to Massó (2011), both location of the putative basal haplotype, levels of genetic diversity and subsequent genetic singularity suggest specifically Montgó as the main diversification area, as higher levels of genetic diversity are generally associated to diversification centers (Hewitt 2001). This hypothesis is not supported by the data of the present survey. The discrepancy may be due to the different type of DNA used for our phylogenetic analyses as Massó (2011) relied on chloroplast (cp) genome whereas my analyses are based on nuclear DNA (nDNA). These DNAs differ in their modes of inheritance, the information they provide, and the assumptions their analyses carry. Most critically, nuclear and chloroplast genomes are inherited differently and thus trace potentially different evolutionary histories. The nuclear genome is always inherited in a biparental, Mendelian fashion, whereas cpDNA is often inherited maternally (Palmer 1985). Overall, in terms of its size, organization and sequence, cpDNA is the most conservatively evolving genome known (Palmer 1985). The conservative evolution of the cpDNA provides it with advantages in phylogenetic studies regarding ancestral history compared to the much more dynamic and diverse nuclear genome of plants; thus, cpDNA is more reliable in terms of predicting the exact origin of one species. Moreover, Massó (2011) has had access to an outgroup (*Femeniasia balearica* J. J. Rodr.) which I lack. On the other hand, nDNA is quite useful for studies of genetic variation at the interspecific level and below regarding the current genetic arrangement of the species, a rather unsuitable target for slower mutational cpDNA (Palmer 1985). Despite difference between cpDNA

and nDNA that can potentially contribute to phylogenetic disagreement, discrepancies may be due to other genetic processes (e.g. bottleneck; see V.2. Diversity and conservation).
Accepting that the center of diversity of *Carduncellus* genus is located at Montgó area, the species probably has become widespread through dispersal to other areas in Marina Alta and then expanded toward Ibiza region as reported by Massó (2011).

Ibiza populations do not appear strongly divergent and their moderate bootstrap support in the NJ tree (**Fig. IV.1**) can be explained by their low genetic diversity (see V.2. Diversity and conservation). Thus, for the Ibiza populations recent, late or postglacial dispersal is a possible scenario (Schonswetter & Tribsch 2005). It seems likely that populations from Ibiza island originated via dispersal from source populations in Marina Alta through Espartar Islet since ESP population has much more private fragments (1), some of which are fixed (2) (**Table IV.1**), than the rest of Ibiza populations; thus, it appears that Espartar Islet is the most ancient populations of Ibiza group (Schonswetter & Tribsch 2005).

Massó (2011) suggests a return of the species to the mainland from Ibiza island, following the opposite direction of its first expansion. A second introduction in Marina Alta through LDD is also supported by my results. Both in the NJ tree (**Fig. IV.1**) and **Fig. IV.5**, the common origin of A8 population (a ubiquitously differentiated population from Marina Alta) and Ibiza group (most prominently IB5 populations) is observed; even in the PCoA analysis (**Fig. IV.2.a**), IB5 population appears closer to Marina Alta cluster and rather distinct from the other populations of Ibiza group. These results seem to point to recent seed dispersal between genetically different groups (Ibiza *vs.* Marina Alta) rather than to progenitor-derivative relationships among Ibiza area in the case of IB5 population (Stehlik et al. 2001). Moreover, AMOVA analysis lack a net regional differentiation being among-variation of Marina Alta and Ibiza just 7% of the total variation of the species (**Table IV.5**); this is in pronounced contrast with genetic cluster analyses by NJ tree (**Fig. IV.1**), PCoA (**Fig. IV.2.a**) and BAPS (**Fig. IV.4**), which results in groups being congruent with geographical regions. This loss of interregional differentiation can be explained by recent dispersion of A8 population to Marina Alta from Ibiza, which would have undermined a sharp regional bipartition. Thus, it is not possible to reject the second introduction hypothesis.

All phylogeographic analyses mark more diversified structure within Marina Alta region compared to near genetic uniformity in Ibiza group. Ibiza populations do not show any obvious intra-grouping with lower or not significant branch support in the NJ tree (**Fig. IV.1**), and

representation in the PCoA scatterplot as a concentrated cloud of dots uniformly distributed (**Fig. IV.2.b**), whereas Marina Alta presents a more dispersed pattern of populations (**Fig. IV.2.c**) and the highest bootstrapping (BS) values (e.g. A3 with BS = 98). Likewise, in reference to global estimates of genetic differentiation, Marina Alta shows the highest value (F_{ST} = 0.237 ± 0.155), whereas Ibiza the lowest (F_{ST} = 0.115 ± 0.128; **Table IV.7**). Thus, samples from different regional groups (Ibiza *vs.* Marina Alta) show different genetic patterns.

Island and mainland populations of plants often differ in their reproductive biology and genetics, depending on population size, distribution range, as well as historical and ecological factors e.g. climate, plant-pollinator interactions, substrate preference and interspecific competition (Fernández-Mazuecos et al. 2014). These factors are known to determine the distribution of other Balearic endemics. For example, low diversity of narrow endemic species of Apiaceae from Majorca *Coristospermum huteri* (Porta) L. Sáez & Rosselló has been explained by a founder effect resulting from hypothetical recent dispersal from the continent (López-Pujol et al. 2013). Indeed, island populations contain less diversity and are usually more differentiated than comparable mainland samples that had acted as source populations. However, exception to this pattern occur, as in the Mediterranean endemic *Cyclamen balearicum* Willk. Affre et al. (1997) found population differentiation to be significantly higher among mainland populations in southern France than among those occurring on the Baleric Islands. This is also the case with the current survey on *C. dianius*.

Our results for Ibiza indicate relatively low genetic diversification (NJ tree, PCoA, BAPS analyses), contrary to Massó (2011) which detected unique haplotypes per population, which is an evidence of high differentiation. This discrepancy may be explained by processes of Ibiza colonization resulting in a genetic bottleneck, which would have produced a fixation of haplotypes, and by a subsequent increase in gene flow in recent times. Only our nuclear marker would have detected tracks of gene flow, especially by pollen, since cpDNA used by Massó (2011) is maternally inherited and pollen is the male gametophyte involved in genes plant dispersal (Petit 2004). Overall, the mode of inheritance has a major effect on the partitioning of genetic diversity, with studies based on maternally inherited markers having considerably higher population differentiation than those based on biparentally inherited markers (Petit et al. 2005; see V.2. Diversity and conservation).

Being isolated on coastal cliffs and the smallest in size of the total sampled, Ibiza populations are likely to show a high level of inbreeding. Baker's Rule (1955) proposes that self-compatible rather

than self-incompatible plants will be favored in establishment following LDD to islands with subsequent reduction in floral size and adaptation to promote pollination (Barrett et al. 1996). Significant reduction of dispersal ability may develop relatively quickly in small, isolated populations such as *C. dianius* individuals on Ibiza, and has been documented for species living on continental islands (Cody & Overton 1996). Furthermore, selection against dispersal in insular species might be enhanced in strictly coastal species such as *C. dianius*, since diasporas with high dispersal ability may be blown off the islands or dispersed to unsuitable habitats surrounding the existing populations (Olivieri et al. 1995).

A high level of self-pollination may be also due in part to the lack of specialized pollinators on Ibiza region. Islands usually support fewer animal species than occur on comparable mainland areas (MacArthur & Wilson 1967), including pollinator faunas (Howarth & Mull 1992).

It must also be considered the significance of isolation-by-distance (IBD) in Ibiza region, as this population genetic pattern results from spatially limited gene flow. In the case of Ibiza group, the Pearson correlation (*r*) value is rather high: 0.698 (**Fig. IV.3**). Thus, there is a strong positive relationship between geographical and genetic distances; the genetic correlation between populations declines as a function of geographic distance between them (Wright 1943). Most probably, higher significance and value of IBD pattern in Ibiza region is mainly due to Espartar Islet. Notably, the highest F_{ST} values among Ibiza populations are to be found in pairs that include ESP population (**Table IV.6.b**). Moreover, Espartar Islet is the most geographical isolated location in Ibiza area. ESP population extreme values, both in pairwise F_{ST} and in geographical coordinates, are likely to produce a strong correlation between genetic and geographical distances, hence, a high IBD value. Finally, IBD pattern in near distributed populations produces a result resembling the stepping-stone model (Kimura & Weiss 1964). According to this pattern, organisms with relatively low effective rates of dispersal – as previously hypothesized for *C. dianus* – will exchange their genes between neighboring demes. This pattern of migration is common for species distributed in linear separated habitats such as coastal cliffs as observed in Ibiza populations of *C. dianius*.

With regard to the mainland group, the NJ tree (**Fig. IV.1**), PCoA (**Fig. IV.2.c**), BAPS (**Fig. IV.4**) and gene diversity (see V.2. Diversity and conservation) indices show that the Marina Alta populations have the highest genetic variability of all sampled populations. This pattern may be due to easier connectivity among populations on a continental area as it presents continuity of habitat (rocky limestone massifs are common in Alicante region), which allows for populations establishment across a large range; thus, there is greater potential for gene flow (Massó 2011).

Moreover, active dispersal is enhanced by increased presence of pollinator insects (e.g. Hymenoptera; **Fig. I.10**) in a continental area that can favor genetic exchange within and among populations. Most samples from different Marina Alta populations are grouped without any clear geographical pattern as IBD is not significant among Marina Alta populations (P = 0.164; **Fig. IV.3**). The absence of an IBD pattern may be caused by high levels of gene flow and outcrossing among and especially within populations of Marina Alta (see V.2. Diversity and conservation). Despite easier connectivity granted by Alicante continuity of habitat and a subsequent favored level of gene flow, high F_{ST} value (0.237 ± 0.155; **Table IV.7**) indicates low connectivity among Marina Alta populations. Indeed, this result reflects more differentiated populations structure in Marina Alta region compared with Ibiza group as aforementioned. On the contrary, Massó (2011) reported a nearly haplotype uniformity in Marina Alta populations. This discrepancy may be due again to different assumptions based on nuclear and chloroplast DNA.

V.2. Diversity and Conservation

Population genetics parameters are comparable across studies and are closely associated with various life history traits (e.g. Hamrick & Godt 1996). Life form, geographic distribution range, breeding system and seed dispersal have proved to be closely associated with the amount of total genetic variation and its partitioning among and within population (Nybom & Bartish 2000). Genetic diversity is usually thought of as the amount of genetic variability among population of a species that is best described by AMOVA-derived estimates F_{ST} (Nybom 2004). Several different methods to estimate genetic within-population variation have been applied in molecular markers studies. One of the most commonly employed is the expected heterozygosity (Nybom & Bartish 2000).

Estimates of among-population diversity show significant variation for life form (annual, short-lived perennial, long-lived perennial) with the lowest values assigned to long-live perennials (Nybom 2004). On the other hand, life form had no significant impact on within-population diversity in Nybom (2004) study. Still, long-lived perennials present the highest mean value, as reported also by Hamrick & Godt (1996). These predictions are consistent with the results of our AMOVA analysis, which shows that genetic diversity is partitioned more within than among populations (**Table IV.4**). Indeed, *C. dianius* is a perennial plant (see I. 6. *Carduncellus dianius*: an Iberian endemism).

Among-population diversity show a positive association with maximum geographical distance between populations (Nybom 2004); thus, endemic species should present the lowest among-population values. On the other hand, geographic range show no association at all with within-population diversity in Nybom (2004). Being *C. dianius* an endemic species, this life trait predictor is congruent with our AMOVA findings, especially in regard to Ibiza group which presents lower percentage of among-population diversity (23.24%) than Marina Alta region (31.41%; **Table IV.4**) and thus the lowest F_{ST} value (0.115 ± 0.128; **Table IV.7**). Indeed, F_{ST} values appear to be very sensitive to variation in the maximum distance between sampled populations (Nybom & Bartish 2000). Several studies have shown that differentiation generally increases with distance (e.g. Dumolin-Lapègue et al. 1997), so detecting lower F_{ST} values in closely located populations, such as in Ibiza region compared to Marina Alta area, makes sense.

Breeding system is a major factor in explaining levels of within-population genetic variability with selfing plants being the least diverse and outcrossing the most diverse (Hamrick & Godt 1996). As previously hypothesized, Ibiza group is likely to show higher levels of inbreeding (see V.1. Phylogeography) than Marina Alta region, whose populations are easily connected by various ecological characteristics associated with a continental area. Indeed, Marina Alta region contains the highest amount of genetic diversity for all indices (PLP, H_j and Br), whereas the least amount of diversity is attributed to Ibiza region (**Table IV.8**). Thus, Marina Alta populations would show more pronounced outbreeding genetic pattern (e.g. high level of gene flow). According to a number of surveys (e.g. Lanner-Herrera et al. 1996), outbreeders often show much more gradual changes among populations, hence lower F_{ST} values than self-pollinating individuals. If we assume that Marina Alta has more pronounced outcrossing breeding system than Ibiza, our findings point to the opposite F_{ST} pattern (Marina Alta > Ibiza; **Table IV.7**). However, as aforementioned, this genetic parameter is also closely correlated with maximum geographic distance between the analyzed populations, especially in outcrossing individuals, namely Marina Alta group (Nybom & Bartish 2000). The discrepancy is thus explained by correlation between higher divergence among Marina Alta group (highest F_{ST} value) with larger geographical distance between Marina Alta populations, despite outcrossing system.

Both the genetic diversity within populations and the genetic differentiation among populations are closely related to seed dispersal mechanism. Seeds that are either ingested or dispersed by wind or water show the highest mean within-population diversity, whereas higher among-population values were noted for gravity and attached seed dispersal modes (Nybom 2004). However, these

genetic predictions focus only on mechanisms of seed dispersal. Seed plants present more complex and asymmetrical dispersal behaviors, since the dispersal function – hence gene flow – is mediated by two very distinct vehicles that operate in sequence, the male gametophyte (pollen) and the young sporophyte (seed). The contrasted patterns of inheritance of organelle (maternal cpDNA in Massó 2011) and nuclear genes (biparental nDNA in the present study) can be used to unravel the complexity of gene flow in plants, as they are predicted to result in very different distribution of genetic diversity within and among populations (Petit et al. 2005). Comparison between Massó's (2011) and my results on Ibiza group diversification suggests presence of gene flow by pollen, although seed dispersal may also act at short-distance. Pollen flow between populations is estimated to have, on average, greater potential of dispersion in area than seed flow (Petit 2004), especially of a short-range kind as that estimates by life trait predictors for Ibiza region, namely seed dispersal by gravity. Indeed, barocory is the reported dispersal mechanism of *C. dianius* (e.g. mature diasporas emerge from the mother and fall to the ground under their own weight), which probably contributes to the lack of gene flow in the Ibiza region according to Massó (2011). Thus, we may conclude that gene flow by seeds is highly restricted in Ibiza populations. It is likely that longevity (see before), high germinability and moderate pollen flow compensate seed dispersal inefficiency (with major loss of seeds due to gravity mode) and habitat fragmentation in Ibiza territory (Massó 2011).

With respect to Marina Alta, gene flow vectors must cover greater distances, due to larger distribution of populations in this area, in order to affect among-population diversity. It is likely that pollen would play a major role in a continental area such as Marina Alta, even if Massó (2011) detected quasi null diversification among this populations (with the exception of Montgó group) using cpDNA, which cannot perceive male gametophyte pollen. Almost haplotype uniformity in the rest of Marina Alta populations may be due to rapid expansion from Montgó (Massó 2011). Genetic life traits predictors indicate seed dispersal by wind as possible agent of Marina Alta gene flow (Nybom 2004). However, predictions based on a wide range of supporting data suggest that wind-dispersed taxa should exhibit low values of population differentiation, at least for nuclear markers (Nybom & Bartish 2000); Marina Alta group displays the opposite case. Moreover, dimensions (7-7.5 x 4.5-5.5 mm) and weight of *C. dianius* seeds (Vilatersana 2008) make wind dispersal unlikely especially at long-range. Similarly, the pappus, that enables the seed to be carried by wind in some species, seems unfit to accomplish this task in long distance in the case of *C. dianius*. Morphology of the species renders it unsuitable to seed dispersal by wind. Furthermore, it is not possible to exclude zoochory (e.g. seed dispersal by animals), maybe with birds as vectors, which would results in LDD.

Lastly, the use of these estimators further emphasizes the high level of geographical structure found in most plant species when using maternally inherited markers, in stark contrast with biparentally inherited nuclear markers (Petit et al. 2005).

While all components of genetic diversity are affected by changes in population structure, bottlenecks are predicted to have a larger immediate effect on allelic diversity than on estimating heterozygosity. Large losses of heterozygosity are most likely if the recovery of a population after a bottleneck is slow or delayed for several generations (Rao & Hodgkin 2002). Thus, in our study, the most sensitive tool for detecting recent genetic bottlenecks is provided by band richness (Br) value that can be interpreted as an allelic richness analogue, since we cannot discern heterozygotes with a dominant marker (Leberg 2002).

When an outbreeding population passes through a bottleneck originated by a founder effect (i.e. new populations are established with small number of progeny plants), first there is decrease in Br value, then genetic variation declines in proportion to the severity of the bottleneck (Leberg 2002). This is due to a primary loss of low frequency (hence rare) alleles as a consequence of decrease of population size, namely loss of individuals. Therefore, it may happen to detect different values of genetic diversity per population, accordingly to the occurrence (low Br values) or not (higher Br values) of a genetic bottleneck. Low Br values are found in A1 and A3 populations of Montgó area and in some other populations of Marina Alta (A5 and A9). These areas are subject to frequent forest fires, a typical occurrence of Mediterranean climate that dramatically alters the number of individuals per population through time. Disturbance by fire thus enhances the beginning of ecological successional dynamic having removed a pre-existing ecological community. Reduced number of individuals both decreases competition and increase available resources and area for settlement. General increase of accessible ecological niches would enhance rapid establishment of new individuals, first grass and other herbaceous plants. In the case of *C. dianius*, an efficient and quick recolonization after disturbance is particularly favored by high rates of germinability. Lately, fast growing trees would develop to their fullest, restoring above cover and the major mass forest lost by fire. With the development of aboveground competition, further vegetation establishment would stop and the number of individuals would decrease (J. Soler, com. pers.). Fire is thus considered an enhancer of genetic bottleneck for certain Marina Alta populations, which are subject to size reduction with loss of genetic diversity detected by low Br values. An example of this successional cycle is noted in A9 population (Serra de Bèrnia). Soler et al. (1999) has described this population with an amount of near 500 individuals; however, it was arduous to

collect an average of a dozen individuals during the sampling for the current study (**Table III.1**). This pronounced oscillation in A9 population size combined with its low Br value (**Table IV.8**) is likely to be a valid indicator of a genetic bottleneck.

In theory, considering their larger distribution, Marina Alta populations should be more subject to genetic drift, namely a shift in gene pool content caused, for example, by a genetic bottleneck. However, genetic drift effect in Marina Alta region (e.g. higher diversification; see IV.1. Phylogeography) is compensated both by greater size of populations and higher level of gene flow among them compared to Ibiza's. This combined effect tends to increase within-population variability and to suppress genetic diversity among populations, as it is the case of our AMOVA findings (**Table IV.4**). Higher diversification is also the main cause of greater genetic diversity detected in Marina Alta group compared to Ibiza.

A moderate bottleneck after founder effect may have also occurred in Ibiza region through the colonization from Marina Alta during the Quaternary as previously hypothesized by other indices (e.g. private markers; see V.1. Phylogeography); indeed, we detect a slight lower genetic diversity in Ibiza region than Marina Alta as indicates by Br value (**Table IV.8**). Moreover, the size of populations plays an important role in relation to genetic diversity; the smaller the size of a population and the longer it remains small, the more genetic variation it will lose (Luikart et al. 1998). Indeed, Ibiza populations are the smallest of the total sampled; moreover, they are also fragmented as the area of suitable habitat is severely limited (only on coastal cliffs).

As observed in Massó (2011), Ibiza region displays a pronounced different genetic pattern than Marina Alta. Ibiza within-population diversities at different hierarchical levels tend to be higher than Marina Alta and mean value of all sampled individuals (**Table IV.4**), despite the limited size of Ibiza populations, with the exception of Espartar Islet. As aforementioned, ESP population is probably the main source of differentiation in Ibiza region as it is associated with the highest pairwise F_{ST} in this area and clusters separately in various phylogeographical analyses (NJ tree, PCoA and BAPS; see V.1. Phylogeography). However, it must be observed that Ibiza populations are located at a distance less than 10 km in a straight line (**Fig. III.1**) and, as previously stated, geographical distribution severally affects F_{ST} index by decreasing its value (hence amortizing diversification) in closely located populations.

The use of genetic variability to elucidate patterns of colonization or geographical distribution has been reported for other endangered plants (e.g. White et al. 1999) and contributes to the knowledge of conservation of genetic resources. The present study provides important base data for conservation management of *C. dianius*.

Generally, sampled populations as a whole are not depauperated, thus it is recommended to preserve the maximum number of individuals from both regions (Ibiza and Marina Alta) since each area displays its own genetic singularity. However, we would give priority to the tutelage of Marina Alta which presents the largest, most diverse and ancient populations. In Ibiza area, protection should be prioritized in Espartar Islet, that is the most genetically unique in the region, shows higher levels of genetic diversity and has the largest population. Furthermore, we should consider Massó (2011) cpDNA findings, which recommend tutelage of Ibiza populations as they display unique haplotypes.

VI. CONCLUSIONS

Contrary to what is expected by plants of restricted area, *C. dianius* is a genetically structured species showing different traits according to the geographical area (Marina Alta or Ibiza). This scenario appears to be modulated by Quaternary paleogeography (e.g. sea regression fostering *C. dianius* expansion) and past and contemporary recurrent gene flow involving long-distance dispersal (range expansion and colonization).

Higher levels of genetic diversity and diversification of Marina Alta group compared to the rest of sampled populations may indicate this region as the center of origin of *C. dianius* as observed by Massó (2011) specifically in the case of Montgó area.

Larger distribution of Marina Alta populations and recurrent fires in the area favored by Mediterranean climate are expected to increase the probability of genetic drift. However, both population size of Marina Alta, gene flow and outcrossing behavior favored by a continental area setting (e.g. habitat continuity, abundance of pollinators) act as buffer to a potential marked diversification, suppressing among-population diversity as observed in our AMOVA findings.

The expansion toward Ibiza through the Quaternary probably resulted in a moderate bottleneck after founder effect; this process combined with closer distribution of its populations (which buffer F_{ST} value) is likely the cause of lower genetic diversity and diversification of Ibiza populations compared to Marina Alta.

Comparison with cpDNA analyses (Massó 2011) allowed us to unravel the complexity of recent Ibiza gene flow that seems partitioned in moderate pollen flow and short-distance seed dispersal, most probably barocory. Selection may have acted against long-range dispersal by lack of pollinators in an insular environment, favoring an inbreeding pattern especially in coastal, isolated and small Ibiza populations.

It seems likely that the expansion in the Ibiza area first went through Espartar Islet being ESP population the most ancient of the Ibiza group as predicted by its genetic singularity (e.g. private markers, phylogeographical clusterings) and higher genetic diversity. Moreover, being the most isolated location in Ibiza area, Espartar Islet is likely the main source of Ibiza diversification (higher pairwise F_{ST}) and subsequent significance of its IBD pattern. Furthermore, it is not possible to exclude the second introduction hypothesis (to Marina Alta from Ibiza) both suggested by cpDNA (Massó 2011) and supported by my data.

The description of the extent and distribution of the different aspects of genetic diversity in *C. dianius*, and of the way in which it is structured, allows us to discern its priority of conservation. Thus, we suggest to preserve the maximum number of populations per region, as they both possess genetic singularity, but giving priority to Marina Alta higher structured and richer genetic populations, and to Espartar Islet in Ibiza region.

VII. BIBLIOGRAPHY

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