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Influence of Phenological Barriers and Habitat Differentiation on the Population Genetic Structure of the Balearic Endemic *Rhamnus ludovici-salvatoris* Chodat and *R. alaternus* L.

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Running title: Population genetic structure of *Rhamnus ludovici-salvatoris*

Abstract. *Rhamnus ludovici-salvatoris*, endemic to the Gymnesian Islands, coexists with the related and widespread *R. alaternus* in Mallorca and Menorca. In both species, the population genetic structure using RAPD, and flowering during a three year period to check for possible phenological barriers, were analysed. *Rhamnus ludovici-salvatoris* showed lower genetic diversity and stronger population structure than *R. alaternus*, the Cabrera population being less diverse and the most differentiated. *Rhamnus ludovici-salvatoris* flowered one month later, although flowering of both species coincided sporadically. These congeners seem to have diverged through isolation by time and differentiation in habitat. The population genetic structure of *R. ludovici-salvatoris* could mainly be due to the existence of small populations on the one hand, and a gene flow caused by rare hybridization events on the other, which may also explain the presence of morphologically intermediate individuals in Menorca. The conservation of *R. ludovici-salvatoris* populations may include population reinforcements and other in-situ interventions.

Plant Systematics and evolution

Key words: AMOVA, Balearic Islands, endemic, isolation by time, population structure, RAPD markers, *Rhamnus alaternus* L., *Rhamnus ludovici-salvatoris* Chodat.

Endemism rates are especially high in islands due to their geographical isolation. Oceanic islands, which have never been connected to continents, exhibit the greatest proportions of endemic taxa of the world. These taxa are mostly ancient relict endemics or products of adaptative radiation (Cronk, 1997). In contrast, continental islands have lower endemism rates. Microendemism is usual and the distribution areas of some endemics are larger, including frequently continental areas, especially when isolation has occurred recently.

The Balearic Islands are the largest archipielago of continental islands in Spain. These islands display a complex paleogeography despite the fact that they have been isolated relatively recently. The eastern islands or Gymnesian (Mallorca, Menorca, and Cabrera) were connected to the western islands or Pithyusian (Ibiza and Formentera), and to the mainland several times during Miocene, and especially during the Messinian salinity crisis. In the early Pliocene (approx. 5.3 mya, Krijgsman et al., 1999), these three groups of emerged lands separated from each other as a result of the opening of the Gibraltar strait (Gautier et al., 1994). During the last 2.5 my, the Mediterranean area has been widely influenced by glaciations, allowing land bridges to form between the Gymnesian Islands, which have only been completely isolated from each other since the Würm glaciation, approx. 15.000 ya (Cardona and Contrandiopoulos, 1979).

Mainly as a consequence of its paleogeography, the Balearic archipielago is rich in endemic taxa, accounting to 6.7% of its flora. The origin of these endemic taxa is diverse. Most of them are schizoendemics from Spain and Pithyusan Islands, or Tirrenic endemics from Gymnesian Islands and Eastern Mediterranean islands, like Corsica and Sardinia.

However, other distributions exist, especially of those taxa which are exclusively present in the Gymnesian or Pithyusan Islands (Cardona, 1979).

The genetic evolutionary processes of some endemic species of the Balearic Islands and species of the islands and Western Mediterranean mainland have been studied in order to elucidate the structure and genetic diversity of populations, and the gene flow among them (Affre et al., 1997; Sales et al., 2001; Roselló et al., 2002; Juan et al., 2004). As a general conclusion, it has been shown that genetic diversity is positively correlated to population size. This is mainly due to genetic drift, founder effects, and inbreeding. A declining or fragmenting species may experience genetic changes including loss of differentiated populations, alteration of differentiation between populations, and loss of variation among members of the same population (Sherwin and Moritz, 2000). However, these genetic changes, especially in small and fragmented populations, are dependent on the amount of existing gene flow. This can occur if some pollinators or seed dispersers can overcome the distance among islands, or if the endemic can be crossed with other congener species occurring in sympatry. However, even if two different taxa are compatible and can hybridize, other mechanisms preventing gene flow and promoting genetic divergence may still exist, such as ecological isolation due to habitat preferences or differences in flowering times. Thus, the extinction risk of some populations depends on the interactions between genetic, demographic, and environmental factors (Gustafsson, 2003).

Random Amplified Polymorphic DNA (RAPD) has been used in some Balearic endemic species to perform population genetic studies. Although being dominant, RAPD markers have proven useful in these works (Sales et al., 2001; Roselló et al., 2002). In addition to their dominant nature, many researchers have argued some technical disadvantages in RAPDs, such as poor reproducibility, segregation distortion, or lack of homology between bands. However, improved laboratory techniques, band scoring

procedures, and numerous studies have minimized the effect of these problems, although other still occur, like competitive priming and artefactual bands. In relation to the efficiency of RAPD markers in studying the effects of life history traits on genetic diversity estimates in plants, it has been shown that RAPD can be a very sensitive method for detecting genetic structure according to the isolation-by-distance model, even if it is a less efficient method for estimating within-population diversity when compared to allozyme markers (Nybom and Bartish, 2000).

Rhamnus ludovici-salvatoris Chodat is an endemic of the Gymnesian Islands. This species is taxonomically related to *Rhamnus alaternus* L., whose late distribution excludes Cabrera. Both species are sclerophyllous shrubs, and can only be morphologically differentiated by a few macroscopical traits, leaf domatia, and leaf histology (Chodat, 1924; Martínez-Solis et al., 1993). Differences in flowering times also exist between them (Traveset et al., 2003; Gulías et al., 2004). Hybrid forms were first described in Mallorca as *Rhamnus x jacobi-salvadorii* O. Bolòs & Vigo. However, morphological, anatomical, and biochemical studies have shown that these supposed hybrids were a form of *R. alaternus* (Roselló and Sáez, 2000). In this context, the aim of the present study was (1) to analyse the population genetic structure of *R. ludovici-salvatoris* in comparison with *R. alaternus* and in relation to populations size; (2) to study isolation by time and phenological barriers between both species; (3) to elucidate the existence of gene flow and hybridization events between both species; and (4) to relate the existence of phenological barriers with the genetic population structure.

Materials and methods

Plant Material. *Rhamnus ludovici-salvatoris* grows preferentially in forest edges of *Quercus ilex* L. from sea level up to 1200 m a.s.l., but it can also occur in dry garrigues; while *R. alaternus* is more common in shrublands (Bolòs and Molinier, 1958). In recent decades, populations of *R. ludovici-*

salvatoris, unlike its congener, have been reduced in size and number (Gulías et al., 2002). As a consequence, the population of Menorca, which was even considered to have disappeared by some authors, was listed as endangered in 2005 (BOIB, Decreto 75/2005).

Seven populations of *R. ludovici-salvatoris* and *R. alaternus* were used in this study (Figure 1). The numbers of individuals sampled for each population are shown in Table 1. Three populations of *R. ludovici-salvatoris* were sampled. They were selected from all the locations where this species grows nowadays, as stated by Gulías et al (2002) and our personal observations: two populations in Tramuntana Mountains (Mallorca) and one in Cabrera. In both locations, population sizes are declining. Sampling of three populations of the widespread *R. alaternus* was undertaken in Mallorca and Menorca, where this species grows naturally. In addition, *R. alaternus* was also sampled along the east cost of the Iberian Peninsula. Cala Tirant of Menorca possesses a small population (no more than 18 individuals, from our personal observations) that displays a morphological gradation from *R. alaternus* to *R. ludovici-salvatoris*, including intermediate individuals. Individuals of this population were selected in order to represent the morphological variability. Furthermore, artificial hybridization was carried out between both species in March, 1991. Since *R. alaternus* flowers approximately one month before *R. ludovici-salvatoris*, parental plants were selected according to late or early cycles attributable to temperature and altitude. Mixed pollen of ten *R. alaternus* late-flowering plants (Calobra, Tramuntana Mountain Range, Mallorca, 680 m a.s.l.) were directly placed on gynoeciums of sixty female flowers of ten *R. ludovici-salvatoris* early flowering plants (Formentor, North of Mallorca, 50 m a.s.l.). Pollination was carried out in the early hours of the morning. Buds and flowers were bagged before and after pollination. Seeds of fifty-five resulting fruits of the total plants were collected. One of the hybrid individuals was included in the genetic study. An individual of *Rhamnus lycioides* L. was used as an out-group.

DNA extraction. Genomic DNA was isolated from young leaves by the modified CTAB (hexadecyltrimethylammonium bromide) method of Doyle and Doyle (1990). For each individual, 0.5 g of ground leaf tissue was suspended in 250 µl of extraction buffer [20 mM EDTA, 0.1 M Tris-HCl (pH 8.0), 1.4 M NaCl, 2% (w/v) CTAB, and 5 µl of beta-mercaptoethanol]. The suspension was mixed and incubated at 60ºC for 30 min. DNA was extracted by chloroform-isoamyl alcohol (24:1) and

precipitated with 0.67 vol isopropanol at -20ºC. The pellet formed after centrifugation (conducted at low speed for 5 min) was washed with 76% ethanol and 10 mM of ammonium acetate. The DNA was then suspended in TE buffer. The resulting DNA concentration was measured in a 1% (w/v) agarose gel stained with ethidium bromide using known size lambda DNA digested with Hind III.

DNA amplification using Random Amplified Polymorphic DNA (RAPD). Single arbitrary 10-base primers were initially tested to identify well-amplified and reproducible bands among individuals and populations. Primers resulting in faint or irreproducible bands were excluded from the analysis. Nineteen primers out of 26 were accepted for subsequent analysis (Table 2). Each 25-µl PCR reaction mixture consisted of 20 ng genomic DNA, 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.3 µM primer, 10x Taq buffer, and 1 unit of Taq polymerase (Boehringer Mannheim). Samples were subjected to the following thermal profile for amplification in an oven thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany): (1) 5 min of denaturing at 94ºC, (2) 40 cycles with three steps for each one: 1 min of denaturing at 94ºC, 1 min of annealing at 35ºC, and 2 min of elongation at 72ºC, and (3) a final elongation step of 5 min at 72ºC. Visualization of amplification fragments was accomplished on a 2% agarose gel in 1xTBE buffer stained with ethidium bromide, using GeneRuler™ 100bp DNA Ladder Plus (Fermentas, Ontario, Canada) as reference of DNA fragment sizes.

To test for reproducibility of amplification profiles, at least two replicates per sample were amplified. Furthermore, control samples containing all reaction products (except the DNA) were used to test whether self-amplification or DNA contamination occurred.

Molecular statistical analysis. In the RAPD analysis, data scored with presence (1) or absence (0) of amplification fragments were used to calculate genetic distances ($1 - S_{ij}$) among genotypes. This followed the Nei and Li (1979) similarity coefficient, $S_{ij} = 2a/(2a+b+c)$, where S_{ij} is the similarity between two individuals i and j; a is the number of shared bands; b is the number of bands exclusively amplified by i; and c is the number of bands exclusively amplified by j.

The distance matrix was subjected to cluster analysis by the Unweighted Pair-Group Method (UPGMA, Sneath and Sokal, 1973). Goodness of fit between the cluster and the data matrix was calculated by using the cophenetic coefficient. The statistical analyses were performed with NTSYS-

pc (version 2.0). The reliability and robustness of the dendrograms were tested by bootstrap analysis with 100 replications to assess branch support using PHYLIP 3.67 software.

The distance matrices among RAPD patterns were also used to calculate Nei's unbiased genetic distances between populations (Nei, 1978) using POPGENE 32. Additionally, gene diversity (Nei, 1973) was also estimated using POPGENE 32.

Analysis of molecular variance (AMOVA) was performed using ARLEQUIN 3.11 to study the genetic structure of *Rhamnus* populations. The dominant RAPD bands were analysed as phenotypes using the Euclidian distance matrix as an external file. This analysis allowed the total genetic diversity to be partitioned in variance components: within populations, among populations, and among groups (*R. ludovici-salvatoris* and *R. alaternus*).

Phenological analysis. The phenological analysis was performed in Mallorca since both species grow naturally on this island. For each species, natural populations situated within its normal distribution range were selected. The *R. ludovici-salvatoris* populations were located in Formentor, in northern Mallorca (at 50 m a.s.l., coordinates 39º56'04''N, 39º56'18''N, 3º08'30''E, and 3º08'39''E), and in Calobra, in the central zone of the Tramuntana Mountain Range (at 680 m a.s.l., coordinates 39º49'45''N, 39º49'53''N, 2º49'03''E, and 2º48'55''E). The *R. alaternus* populations were located in Calobra.

Phenological determinations were carried out weekly during flowering periods for three years, from March, 1991 to June, 1993. Ten plants were randomly selected per species and population. For each individual, the number of open male and female flowers on five branches selected at random was recorded through the flowering period.

In order to clarify the genetic vs. environmental components of the flowering phenology, and the reproductive isolation caused by time differentiation, seeds from five random plants of each population were collected from the original locations in 1991 (Calobra and Formentor for *R. ludovici-salvatoris* and Calobra for *R. alaternus*). The seeds were sowed in the experimental plots of the Balearic Islands University (UIB) (at 90 m a.s.l.). One individual was selected from the progeny of each parental plant. The five resulting progeny individuals per population were mixed and cultivated together for monitoring the flowering phenology. Three hybrid plants derived from artificial crosses

were also included in this analysis, as well as five *R. alaternus* individuals growing around UIB. Phenological determinations of hybrid and parental plants were performed weekly from 1st March, 1995 to 30th June, 1995, at full reproductive maturity.

A multifactor analysis of variance (ANOVA) was used to test for differences in means among species, sexes, years, and habitat altitudes. All factors were treated as fixed. Regarding the isolation by time, different parameters were considered, according to Lennartsson (1997). The mean isolation was calculated as the number of days between the mean date (the mean of individuals) for the last open flower of *R. alaternus*, and the mean date for the first open flower of *R. ludovici-salvatoris*. The absolute isolation was calculated as the number of days between the very last and very first flower of the two groups, respectively. Isolation parameters were measured between male flowers of *R. alaternus* and female flowers of *R. ludocivi-salvatoris*, and the reciprocal, as we do not know if both crossing directions can be achieved in nature. Only the populations from Calobra were used for the calculations in order to avoid the possible effect of the differences in habitat altitude. For the cultivated plants, three calculations were carried out: *R. alaternus* – hybrid offspring, *R. ludovici-salvatoris* – hybrid offspring, and *R. alaternus* – *R. ludovici-salvatoris*.

Results

RAPD analysis and population structure. Analysis with the 19 selected primers of the 51 *R. alaternus*, *R. ludovici-salvatoris* and the possible hybrid individuals identified a total of 125 reproducible RAPD fragments, ranging in size from 225 to 3050 bp (Table 2). Most of the bands (93.6%) were polymorphic among individuals. The number of fragments detected by an individual primer ranged from one (for OPF09) to 14 (OPG17 and OPG19), with an average of 6.6. The number of polymorphic fragments for each primer varied from one (OPF09) to 14 (OPG17), with an average of 6.2.

Species-specific bands were found for the totality of individuals. Fragments M20-1000, OPG13-950, and OPG19-775 were uniquely amplified by *R. ludovici-salvatoris* individuals, while fragment OPG13-1200 was uniquely amplified by *R. alaternus* individuals.

As RAPD are dominant markers, all these species-specific fragments were also amplified in the artificial hybrid between both species.

To analyse the percentage of variation between species, among populations within species, and within populations, only populations of the Balearic Islands were considered. AMOVA analysis revealed that 56.3% of the variation was between species ($P < 0.09$), 14.3% was among populations within species ($P < 0.001$), and 29.4% was within populations ($P < 0.001$) (Table 3).

The dendrogram obtained from the Cluster analysis is shown in Figure 2. The cophenetic coefficient was 0.86, indicating a good fit. The dendrogram grouped the different individuals in two major clusters, according to the species (bootstrap = 100). Cluster I included the *R. ludovici-salvatoris* individuals and cluster II the *R. alaternus* ones. The hybrid individual was included in Cluster I, but it was clearly separated from the *R. ludovici-salvatoris* populations (bootstrap = 89). The artificial hybrid clearly appeared as genetically intermediate between both species in a Principal Coordinate Analysis (PCoA) (data not shown). Within Cluster I, a subclustering of individuals according to their geographical origin could be observed. Individuals of Cabrera appeared to be separated from those of Mallorca (bootstrap = 41). However, in Mallorca, a grouping of individuals according to population could not be observed. Furthermore, one of the possible hybrid individuals growing in Menorca appeared intermingled with those of Mallorca, suggesting its adscription to the species *R. ludovici-salvatoris*.

In cluster II, no clear grouping of individuals according to their geographic origin, considering both island or peninsula and population location, could be observed, as confirmed by the low bootstrap values of the sub-clusters. Cluster II also included the remaining possible hybrids from Menorca, which also appeared intermingled with individuals from different

populations in the islands and the peninsula. This suggests again the adscription of these individuals to *R. alaternus* and the absence of frequent hybridization events.

The results of the cluster analysis and AMOVA corresponded with the unbiased genetic distances (Nei, 1978) obtained among populations. The average Nei's genetic distance between the populations of *R. ludovici-salvatoris* and *R. alaternus* was 0.31 ± 0.06. Within *R. ludovici-salvatoris*, the lowest genetic distance was found between the two populations of Mallorca (0.04), while the highest distances were found between the population of Cabrera and those of Mallorca (0.11 and 0.09). Within *R. alaternus*, the genetic distances among populations varied from 0.08 (between the population of the peninsula and that of Menorca in Cala en Porter) and 0.19 (between two populations of Menorca, in Cala Sant Climent and Cala en Porter). It is noteworthy that the average distance between the *R. ludovici-salvatoris* population of Cabrera and the whole of the *R. alaternus* populations (0.33 ± 0.08) was slightly higher than that between *R. ludovici-salvatoris* populations of Mallorca (Vall de Formentor and es Binis) and the whole of the *R. alaternus* populations (0.30 ± 0.06 and 0.31 ± 0.06 respectively). These unbiased genetic distances were positively correlated with the F_{ST} values obtained in the AMOVA analyses performed within each species, in order to evaluate the effect of population subdivision due to genetic drift (data not shown).

Genetic diversity of populations and species. The distribution of RAPD bands and gene diversity estimates by geographical locations and species is shown in Table 4. Considering only the populations of the Balearic Islands, the percentage of polymorphic fragments, as well as gene diversity, was greater in *R. alaternus* than in *R. ludovici-salvatoris* (82.0% and 69.4% respectively for percentage of polymorphic fragments; 0.165 and 0.096 respectively for gene diversity). At the population level within *R. ludovici-salvatoris*, the population from Cabrera showed a lower gene diversity than populations from Mallorca

(0.033 and a mean of 0.052, respectively). In contrast, within *R. alaternus*, populations of the islands (Mallorca and Menorca) showed similar gene diversities (0.082, 0.087, and 0.092).

Status of the Tirant population. The population from Tirant in Menorca (CT) is a particular case which included morphologically intermediate individuals between both species, and must be analysed separately. Cluster analysis showed the existence of individuals which were clearly intermingled with individuals of one of the two species, and thus the occurrence of hybrids was not detected. However, of the four species-specific bands found in the RAPD analysis, only two were amplified by Tirant individuals corresponding to the grouping obtained with the ordination analyses (OPG13-950 and OPG13-1200). In contrast, bands M20-1000 and OPG19-775 were randomly amplified by these individuals. Gene diversity of the whole population, consisting of one individual which appeared to be intermingled with *R. ludovici-salvatoris* and eight individuals intermingled with *R. alaternus*, was 0.173 ± 0.175, the highest of all populations. Considering only the individuals intermingled with *R. alaternus*, gene diversity was also high, 0.143 ± 0.169. Consequently, even if they were not detected in the cluster analysis, the occurrence of hybridization events at low frequency could not be discarded.

Phenology of *R. ludovici-salvatoris* and *R. alaternus* in the Balearic Islands. Flowering timing of *R. alaternus, R. ludovici-salvatoris,* and artificial offspring in relation to year, location, and sex is depicted in Figure 3. The statistical analysis of the data (ANOVA) revealed that year, locality (altitude), species, and sex had significant effects on flowering phenology timing (p-values < 0.01) (Table 5). The highest differences in the mean flowering date were recorded between species and localities. *Rhamnus alaternus* flowered 29.8 days earlier than R. *ludovici-salvatoris*. Similarly, at high altitudes (680 m a.s.l.), plants flowered 18.6 days later than low altitudes (50 m a.s.l.). The mean gap among years was 7.4 days and between sexes only 1.4 days.

In nature, *R. ludovici-salvatoris* and *R. alaternus* grow together at 680 m a.s.l., in Calobra. In this location, the mean isolation by time was 42.6 days between male *R. alaternus* and female *R. ludovici-salvatoris*, and 40.4 days reciprocally. When cultivated together at 90 m a.s.l., the mean isolation was 65.6 days between male *R. alaternus* and female *R. ludovici-salvatoris*, and 65.2 days reciprocally. In this case, three male hybrid individuals between both species were also evaluated, and the mean isolation between them and female *R. alaternus* and *R. ludovici-salvatoris* were 27.3 and 36.1, respectively.

In relation to the absolute isolation by time, in nature (Calobra, 680 m a.s.l.), the first female flowers of *R. ludovici-salvatoris* coincide with the last male flowers of *R. ludovici-salvatoris* on only one day in 1991 and 1993. For 1992, a gap of seven days was observed. Reciprocally, the first male flowers of *R. ludovici-salvatoris* coincide with last female flowers of *R. alaternus* on one day in 1992 and 1993, and an isolation of seven days was found in 1991. In cultivation, an absolute isolation of 14 days was observed in both directions. However, a coincidence of seven and 14 days were recorded between male flowers of the hybrid individuals and female flowers of *R. ludovici-salvatoris* and *R. alaternus* respectively.

Discussion

Relationship between *R alaternus* and *R. ludovici-salvatoris.* Our study showed that RAPD appear to be useful markers for discriminating individuals of *R. alaternus* and *R. ludovici-salvatoris*. Some species-specific bands were found. Similarly, using the same molecular markers, species-specific bands were also observed in other wild plant species which are morphologically similar, such as *Picea* spp. (Perron et al., 1995) and *Scutellaria* spp. (Hosokawa et al., 2000). These fragments are very useful for assigning some individuals to a given species, or for elucidating their hybrid nature (Collins et al., 2003; Ducarme and Wesselingh, 2005). In fact, all the species-specific bands were amplified in the artificial

hybrid between both species. These fragments also appeared to be very useful in the analysis of the Menorca population (Tirant), which includes some morphologically intermediate individuals between *R. alaternus* and *R. ludovici-salvatoris*, as will be discussed later.

Rhamnus alaternus and *R. ludovici-salvatoris* have slight morphological differences (Tutin et al., 2001). Furthermore, no genetic incompatibility exists between them, as manual pollination is sufficient to produce hybrid seeds. Consequently, both species may share a common ancestor, and isolation by time could have been the main cause of the sympatric speciation. Flowering plants are a group likely to manifest isolation by time because of their highly heritable flowering times (Hendry and Day, 2005). Other genera that include related species, which may also have evolved from isolation by time in sympatry, are: *Ferocactus* (McIntosh, 2002), *Banksia* (Lamont et al., 2003), and *Fraxinus* (Gerard et al., 2006). In addition, *R. alaternus* is more tolerant to drought and semi-arid habitats than *R. ludovici-salvatoris* (Gulías et al., 2002). Thus, in this case, differentiation by time is accompanied by a slight differentiation in habitat. Climate has been shown to be a selective factor involved in the evolution of timing of flowering (Harris, 1996; Lennartson, 1997). However, other selective factors have also been identified in plants, such as competition for pollinators or dispersal agents and reduced seed predation (Zimmerman, 1980a,b; Englishloeb and Karban, 1992). These factors cannot be ruled out and need deeper studies.

Genetic structure and gene diversity of *R alaternus* and *R. ludovici-salvatoris* populations. RAPD markers could also elucidate the population structure of the endemic and narrowly distributed *R. ludovici-salvatoris* in the Gymnesian Islands, and of the widespread *R. alaternus* in the Western Mediterranean. As expected in long-lived, outcrossing, and late successional taxa, AMOVA showed that most of the genetic variation is retained within populations (Nybom and Bartish, 2000). *Rhamnus alaternus* showed higher levels of genetic variation within and lower levels of genetic differentiation between populations than *R.*

ludovici-salvatoris, as shown by genetic distances among populations, F_{ST} values, and ordination methods. This is a common observation when widespread and narrowly distributed species are compared (Gustafsson and Sjögren-Gulve, 2002). The strong population structure according to the island of origin observed in *R. ludovici-salvatoris* when compared to *R. alaternus* also appears in other Balearic endemics, like *Hippocrepis balearica* Jacq. and *Medicago citrina* Font Quer (Greuter) (Roselló et al., 2002; Juan et al., 2004).

In relation to genetic diversity, and considering only the populations of the Balearic Islands, *R. alaternus* showed higher levels of gene diversity and polymorphic fragments than *R. ludovici-salvatoris*. As a consequence of small population size, endangered species are generally supposed to have lower levels of genetic variation than non-endangered species, because of higher effects of genetic drift and inbreeding. Thus, our results may reflect a higher decrease of the island population sizes in *R. ludovici-salvatoris* than in *R. alaternus*. This could be caused by deforestation and probably climatic change that occurred in Mediterranean regions during the Holocene (Traveset et al., 2003). *Rhamnus ludovici-salvatoris* showed a limited plant biomass production under an increasingly dry climate, displaying high ratios of respiration and photorespiration to photosynthesis, a low intrinsic water-use efficiency, a delayed stomatal closure at the onset of water deficit, and unfavourable photosynthetic traits (Gulías et al., 2002). Furthermore, drought probably cause a decrease in its potential seed production and seedling mortality (Traveset et al., 2003).

In the Balearic Islands, with an oceanic Mediterranean climate, *R. ludovici-salvatoris* flowers later and has narrower habitat amplitude than *R. alaternus*. In a similar study, where flowering-time variants of *Gymnadenia conopsea* were analysed, and the late-flowering variant has a smaller habitat distribution, some hypotheses were proposed (Gustaffson and Lönn, 2003). One possible explanation is that the low genetic diversity of *R. ludovici-salvatoris* gives a narrow ecological niche. However, the reciprocal could also be possible:

populations of *R. ludovici-salvatoris*, which grows in moister habitats that have been reduced over the last decades, may consequently reduce the size and then the genetic diversity by genetic drift. The phylogenetic relationship between *R. alaternus* and *R. ludovici-salvatoris* would certainly throw some light to the evolution of both species. However, although some works have been performed in order to elucidate the phylogenetic relationships among the members of *Rhamnaceae* and *Rhamnus* s.l., deeper studies are needed for analysing relationships within the *Alaternus* section of this genus (Richardson et al., 2000; Bolmgren and Oxelman, 2004).

Furthermore, within *R. ludovici-salvatoris*, populations from Cabrera showed lower genetic diversity than those of Mallorca. This could obviously be due to the smaller population size of Cabrera, when compared to the larger island of Mallorca. This is a general pattern that is also observed in other Balearic endemics (Affre et al., 1997; Sales et al., 2001; Juan et al., 2004). However, another possible explanation exists. *Rhamnus alaternus* is present in Mallorca and Menorca, but not in Cabrera. If hybridization can occur between this species and *R. ludovici-salvatoris*, gene flow would increase the gene diversity of both species, and especially of the more narrowly distributed *R. ludovici-salvatoris*. Over a long period, only one migrant per generation would counterbalance the effects of genetic drift (Wright, 1931). This seems to be a plausible hypothesis as genetic distances between *R. alaternus* populations and *R. ludovici-salvatoris* population from Cabrera were higher than those obtained between *R. alaternus* populations and *R. ludovici-salvatoris* populations from Mallorca.

Phenological barriers and gene flow between *R. alaternus* and *R. ludovici-salvatoris.* General patterns of the flowering traits of *R. alaternus* and *R. ludovici-salvatoris* obtained in the present study are in agreement with the results of Traveset et al. (2003) and Gulías et al (2004): the flowering peak occurs at least one month later in *R. ludovici-*

salvatoris; male and female individuals coincided in their flowering peak in both species; and flowering time varies from year to year depending mostly on climatic variability. However, the aim of their work was to study the critical stages from pollination to establishment in both species separately. To our knowledge, our study is the first that analyses possible phenological barriers in both species.

Although isolation by time may exist, results showed that, in nature, flowers of both species coincide sporadically in the same locality (only one day in some years). Pollination is carried out by bees and other insects that can visit flowers of both species, although wind pollination also occurs (Traveset et al., 2003; Gulías et al., 2004). Thus, rare hybridization events cannot be ruled out for these species. Furthermore, the hybrid progeny may act as a genetic bridge between both species, as the flowering time of each species and the hybrid individuals coincide at least during one week. These hybridization events could counter-balance the effects of genetic drift where both species occur in sympatry. Hybrid individuals could also displace the less competitive *R. ludovici-salvatoris* trough an occupation of the ecological niche and an increased fitness, threading its long term survival. As stated before, unlike Mallorca, individuals of both species co-exist in Menorca (Cala Tirant), and some morphologically intermediate individuals were observed. These individuals appeared in the cluster analysis as belonging to a given species, but they displayed random *R. alaternus* and *R. ludovici-salvatori*s specific bands. Consequently, they may be the progeny of hybridization events that were subsequently backcrossed to one of the parental species for some generations.

In conclusion, the present paper provides preliminary data suggesting that the related *R. alaternus* and *R. ludovici-salvatoris* seem to have diverged through isolation by time and differentiation in habitat preferences, although more studies are needed to confirm this hypothesis. In the endemic *R. ludovici-salvatoris*, both the consequences of small populations

and the existence of a gene flow may be the main causes for explaining the genetic structure of the populations in the Gymnesian Islands. This gene flow only exists in Mallorca and Menorca, but not in Cabrera, where *R. alaternus* is absent.

Conservation implications. Previous studies of the genetic structure and diversity of the populations of Balearic endemics have shown that molecular markers are a powerful tool for monitoring evolutionary processes that may threaten the viability of populations. Endemism in the Balearic Islands has a complex and diversified origin and consequently each taxon has to be studied in its own context (Affre et al., 1997; Sales et al., 2001; Roselló et al., 2002; Juan et al., 2004).

Tirant in Menorca is a very interesting population as individuals of *R. alaternus* and *R. ludovici-salvatoris* coexist in the same local area. Hybrid individuals were not clearly detected, but a thorough study of this population (or others that should exist) would help in understanding speciation caused by isolation by time. This would also help establish further conservation strategies for this population, which has been recently catalogued as endangered. It is important to screen for hybrids, conduct additional cross-pollinations, and germinate hybrid seed. However, this preliminary work suggests that the studied individuals from Menorca are genetically similar to the Mallorcan ones. If further studies confirm this fact, population reinforcements between both islands could be carefully evaluated to avoid possible future genetic impoverishment and local decline, as have been suggested for other species (Oostermeijer et al., 2002; Pierce et al., 2006).

Population from Cabrera was the most differentiated and was less diverse than those of Mallorca. However, population reinforcement from Mallorca or Menorca would not be a good solution for the Cabrera population because gene flow can avoid genetic drift, but can also be a constraining factor in this area of evolution. Natural selection tends to adapt a population to local environmental conditions (Huang et al., 2005). Thus, populations are

dynamic units genetically adapted to their environments and sensitive to any change in their environmental conditions. Therefore, if immigrants come to small populations with genes adapted to other conditions, this would counteract the ongoing selection process (Pease et al., 1989; Gustafsson, 2003). In our case, Cabrera is more arid than Menorca and Mallorca (Gil et al., 1995). Consequently, future conservation activities in Cabrera should be evaluated with care and include in-situ interventions.

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Table 1. Sampled populations of *Rhamnus alaternus* and *R. ludovici-salvatoris* with abbreviations, geographical location and number of individuals.

Table 2. Primers employed, and number of total and polymorphic fragments obtained for the 51 individuals studied using RAPD markers.

Table 3. AMOVA results comparing populations of *R. ludovici-salvatoris* and *R. alaternus*. Only populations of the Balearic Islands were considered and a population from Menorca including possible hybrids was excluded.

Table 4. Distribution of RAPD markers, and gene diversity estimates, for each population and species. For the calculations of *R. alaternus*, data of the peninsula was excluded and only populations from the islands were taken into account (aSSM, aSC, and aP).

Table 5. Multifactor analysis of variance carried out for the dependent variable phenology timing. Principal factors are year, species, locality (altitude) and sex.

Figure 1. Location of the populations of *Rhamnus ludovici-salvatoris* and *R. alaternus* included in the study (see Table 1 for population abbreviations). ▲ *Rhamnus ludovici-salvatoris*, □ *R. alaternus*, and ● morphologically intermediate forms.

Footnote: Population abbreviations: *Rhamnus ludovici-salvatoris*: lsVF: Vall de Formentor (Mallorca), lsEB: Es Binis (Mallorca), lsC: Cabrera; *Rhamnus alaternus*: aSSM: Son Serra de Marina (Mallorca), aSC: Sant Climent (Menorca), aEP: Cala en Porter (Menorca), aP: Peninsula; possible hybrids: CT: Cala Tirant (Menorca).

Figure 2. Dendrogram showing relationships among 51 individuals of *Rhamnus alaternu*s and *R. ludovici-salvatoris* using RAPD markers based on Nei and Li distance (1979) and UPGMA method. Bootstrap values over 40 are indicated and are based on 100 re-samplings of the data set. One individual of *R. lycioides* was used as outgroup.

Footnote: Population abbreviations: *Rhamnus ludovici-salvatoris*: lsVF: Vall de Formentor (Mallorca), lsEB: Es Binis (Mallorca), lsC: Cabrera; *Rhamnus alaternus*: aSSM: Son Serra de Marina (Mallorca), aSC: Sant Climent (Menorca), aEP: Cala en Porter (Menorca), aP: Peninsula; possible hybrids: CT: Cala Tirant (Menorca); artificial hybrid: lsxa; out-group: l: *Rhamnus lycioides*.

Figure 3. Relative frequency distribution of flowering observations for *R. ludovici-salvatoris*, *R. alaternus* and the hybrid offspring. In each diagram, observations from 3 consecutive years were depicted: 1991 (black lines), 1992 (grey lines), and 1993 (broken lines). Furthermore, for each year, male and female flowering were differentiated by round and triangular symbols, respectively. Data are divided into locations, categorizing each one to different altitudinal range and specific habitat. The last diagram shows the results in the field where *R. alaternus* (black lines), *R. ludovici-salvatoris* (grey lines) and the hybrid between them (broken line) were cultivated together. The locations and the altitudinal range are indicated in parentheses.

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