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Population genetic analyses of *Ferula* gr. *communis* and *Ruta* gr. *corsica* in the Tyrrhenian area

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Presentata da:	Caterina Angela Dettori
Coordinatore Dottorato:	Prof. Gianluigi Bacchetta
Tutors:	Prof. Gianluigi Bacchetta
	Prof.ssa Elena Conti
Co-tutor:	PhD. Marilena Meloni

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Caminante, son tus huellas
el camino, y nada más;
caminante, no hay camino,
se hace camino al andar.
Al andar se hace camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante, no hay camino,
sino estelas en la mar.

ANTONIO MACHADO, Proverbios y cantares - XXIX

If you want a happy ending,
that depends, of course,
on where you stop your story.

ORSON WELLES

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Abstract

The Western Mediterranean Islands represent a major hotspot of plant diversity in the Mediterranean area and are priority regions for conservation due to their high number of endemic plant species. However, information supporting human decision-making on the conservation of these species is still scarce, especially at the genetic level.

In this thesis the first assessment is reported of the genetic diversity and structure of the Corso-Sardinian endemic *Ferula arrigonii* (Apiaceae) and of *Ruta corsica* and *R. lamarmorae* (Rutaceae), endemic to Corsica and Sardinia, respectively. The main aim was to provide suggestions for the conservation of these rare species. In the case of the genus *Ferula*, the widespread *F. communis* was also analyzed in order to gain information on its genetic diversity and structure in the Tyrrhenian area and to compare it with the endemic *F. arrigonii*.

Nine populations (179 individuals) of *F. arrigonii* were investigated by means of AFLP (Amplified Fragment Length Polymorphism) markers. Results indicate that this species is characterized by high levels of genetic polymorphism (about 92% of the fragments were polymorphic), a low inter-population differentiation (G_{st} = 0.124) and a high intra-population variation (H_w = 0.317). PCoA, Bayesian analysis and neighbor-joining clustering were also employed to investigate the genetic structure of this species. Three genetically distinct groups were detected, although with considerable overlap between sampling sites.

The AFLP analysis of 12 populations of *F. communis* (168 individuals) from four different islands (i.e. Minorca, Corsica, Sardinia and Sicily) and the Tyrrhenian coast revealed that the populations form an homogeneous group irrespective of the geographic provenance, with the only exception of individuals belonging to *F. communis* subsp. *glauca*. Genetic diversity values were not significantly different with respect to those of the endemic *F. arrigonii*.

As regards the genus *Ruta*, a set of 11 microsatellite markers was used to assess the genetic diversity and the spatial structure of the genetic variation of 96 individuals of *R. corsica* (6 populations) and 63 individuals of *R. lamarmorae* (3 populations). The markers were highly polymorphic and detected 10 alleles per locus on average. Overall, results showed that both species have maintained relatively high levels of genetic diversity (H_e = 0.579 and 0.639, H_o =

0.558 and 0.591 for *R. corsica* and *R. lamarmorae*, respectively). Pairwise F_{st} values (0.035 – 0.351) indicated a low-moderate differentiation for most pairs of populations. AMOVA revealed that 80% of the genetic variation resides within populations, while only 4% is due to differences among the two species. The analyses of the spatial genetic structure suggested the clustering of the individuals into two groups, approximately corresponding to taxonomic affiliations.

Riassunto

I territori insulari del Mediterraneo Occidentale rappresentano un importante hotspot di biodiversità vegetale ed una regione prioritaria per la conservazione a causa dell'elevato numero di specie endemiche che li contraddistingue. Nonostante questo, le informazioni utili a supportare le azioni di conservazione di queste specie sono scarse, soprattutto a livello genetico.

In questa tesi viene riportata la prima valutazione sulla struttura e sulla diversità genetica della specie endemica Sardo-Corsa *Ferula arrigonii* (Apiaceae) e di *Ruta corsica* e *R. lamarmorae* (Rutaceae), endemiche rispettivamente della Corsica della Sardegna. L'obiettivo principale è quello di ottenere dati a livello molecolare da utilizzare come base per fornire dei suggerimenti per la conservazione di queste specie rare. Nel caso del genere *Ferula*, è stata analizzata anche la specie ad ampia distribuzione *F. communis* per ottenere informazioni sulla sua struttura e diversità genetica da comparare con quelle relative alla specie endemica *F. arrigonii.*

Nove popolazioni (179 individui) di *F. arrigonii* sono stati analizzate con marcatori AFLP (Amplified Fragment Length Polymorphism). I risultati indicano che questa specie è caratterizzata da un elevato polimorfismo genetico (circa il 92% di frammenti polimorfici), una differenziazione genetica inter-popolazionale sostanzialmente bassa (G_{st} = 0.124) ed una variabilità intra-popolazionale considerevole (H_w = 0.317). L'analisi della struttura genetica spaziale ha rivelato l'esistenza di tre distinti gruppi genetici, che hanno presentato comunque una notevole sovrapposizione tra i siti di campionamento.

L'analisi AFLP di 12 popolazioni di *F. communis* (168 individui) provenienti da quattro diverse isole (Minorca, Corsica, Sardegna e Sicilia) e dalla costa tirrenica ha rivelato che le popolazioni formano un gruppo genetico omogeneo che prescinde dalla localizzazione geografica delle popolazioni stesse, l'unica eccezione è rappresentata dagli individui di *F. communis* subsp. *glauca*. Inoltre, i valori di diversità genetica di *F. communis* non sono risultati essere significativamente diversi rispetto a quelli della specie endemica *F. arrigonii*.

Per quanto riguarda il genere *Ruta*, un set di 11 marcatori microsatelliti è stato utilizzato per investigare la diversità genetica e la struttura spaziale della variabilità genetica di 96 individui di *R. corsica* (6 popolazioni) e 63 individui di *R. lamarmorae* (3 popolazioni). I marcatori son

risultati essere molto polimorfici ed hanno generato una media di 10 alleli per locus. In generale, i risultati hanno mostrato che *R. corsica* e *R. lamarm*orae mantengono un livello relativamente alto di diversità genetica (rispettivamente H_e = 0.579 e 0.639, H_o = 0.558 e 0.591 in *R. corsica* ed in *R. lamarmorae*). I valori di F_{st} (0.035 – 0.351) hanno indicato nella maggioranza dei casi una differenziazione inter-popolazionale bassa-moderata. L'analisi AMOVA ha rivelato che l'80% della variabilità genetica risiede all'interno delle popolazioni, mentre soltanto il 4% è dovuto alle differenze inter-specifiche. L'analisi della struttura genetica spaziale ha suggerito il raggruppamento degli individui in due gruppi che corrispondono approssimativamente alle affiliazioni tassonomiche.

General introduction

Biodiversity

The term "biodiversity", which started to appear in the scientific literature in the 90s, is generally referred to the diversity of life on Earth, but there is no unambiguous definition of it. The word "biodiversity" or "biological diversity" was introduced by W.G. Rosen in 1988 (Frankel et al. 1995), it encompasses the totality and the variability of all living organisms of every source that can be found in the biosphere. Many authors started to deal with the term "biodiversity" in the second half of the 1980s, but without defining its precise meaning. It was Norse (1993) who gave the first formal definition, defining the concepts of "genetic diversity" and of "ecological diversity", after that Heywood (1995) differentiated a third level, the one of the "specific diversity", and considered the "cultural diversity" as the result of the anthropic interaction with the latter three. These three levels of diversity are recognized by Convention of Biological Diversity (CBD), signed by more than 150 nations in 1992 at the United Nations Conference on Environment and Development, held in Rio de Janeiro, which is up to now the most important initiative on biodiversity at a global scale: "Biological diversity means the variability among living organisms from all sources including, *inter alia*, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this included diversity within species, between species and of ecosystems" (CBD 1992).

Genetic diversity encompasses the components of the genetic coding that structures organisms (nucleotides, genes, chromosomes) and variation in the genetic make-up between individuals within a population and between populations. Specific diversity includes the taxonomic hierarchy and its components, from individuals upward to species, genera and beyond. Ecological diversity covers the scales of ecological differences from populations, through niches and habitats, on up to biomes. These groups are intimately linked, and in some cases share elements in common and 'population' is the unit common to all three components (Fig. 1).

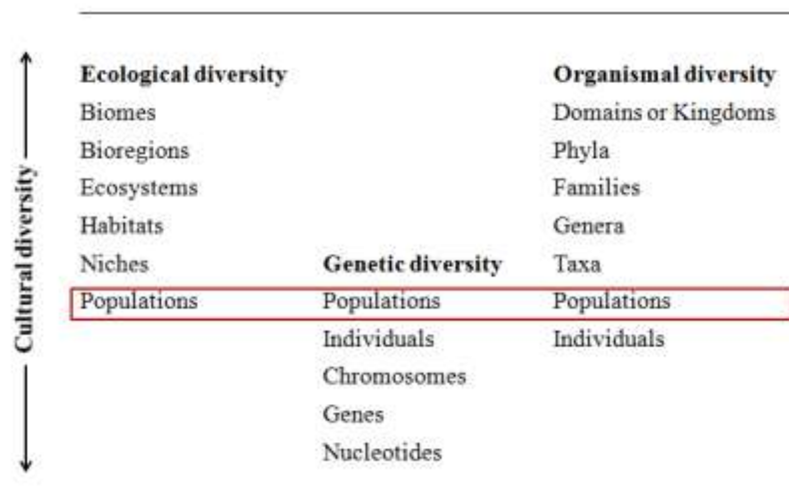

Fig. 1. Components of biodiversity (modified from Heywood 1995).

Genetic diversity

Genetic diversity refers to the total number of genetic characteristics in the genetic makeup of a species. The term was introduced by Norse & McManus in 1980. It was not until the 1970s that the need to conserve it was first acknowledged. Genetic diversity is fundamental for populations as it allows them, at an evolutionary scale, to cope with environmental changes and increases their chances of long-term persistence (Frankham et al. 2002).

Large populations which naturally present outcrossing are generally thought to have a high level of genetic diversity, while this is generally thought to be reduced in small populations, and especially in endangered species. Some of them may have experienced bottlenecks or long periods with a reduced population size, which might cause (in species with sexual reproduction) inbreeding depression phenomena and/or random genetic drift, thus leading to a decreased reproductive fitness and a decreased ability to evolve as a response to environmental changes (Frankham et al. 2002). However, this is not always the case: actually, nowadays the relationship between rarity, population size and genetic diversity is not considered to be direct, and some works (e.g. Bataillon & Kirkpatrick 2000; Hedrick 2002) indicate that the genetic

consequences of a small population size greatly depend on historical, evolutionary and reproductive characteristics.

Methods for detecting genetic diversity

Morphological characteristics were among the earliest genetic markers used for assessment of variation and are still of great importance. Usually, these characters are inexpensive and simple to score. The sharing of physical features is also often accepted as an indication of relatedness. There are several sets of physical character assessment for different crops at different developmental stages such as seed, juvenile, adult vegetative, flower and fruit. However, these sets of characters lack adequate coverage of the genome, are strongly influenced by environmental factors, and are apparently controlled by several genes (Wang & Tanksley 1989).

Molecular markers have been replacing or complementing traditional morphological and agronomic characterization, since they are virtually unlimited, cover the whole genome and are less time consuming. Each molecular marker has its advantages and drawbacks. Therefore, application of molecular marker techniques to diversity questions must take into account whether or not the data derived from a technique provide the right type of information for answering the question being addressed (Karp et al. 1997). This in turn depends on the taxonomic levels of the material being studied (different species, subspecies, varieties, populations and individuals). The closer the relationship of the materials to be studied, the more necessary it may be to consider highly discriminatory techniques. Besides, the choice of appropriate molecular markers also depends on the accessibility and cost effectiveness of the marker techniques.

The following section is a description of the main molecular techniques that have been, or can be, applied to the study of plant genetic diversity.

Protein-based molecular markers

For the generation of molecular markers based on protein polymorphisms, the most frequently used technique is the electrophoretic separation of proteins, followed by specific staining of a distinct protein subclass (Weising & al. 2005). Isozymes (Market & Moller 1959) are enzymes

that convert the same substrate, but are not necessarily products of the same gene. Isozymes may be active at different life stages or in different cell compartments. Allozymes are isozymes that are encoded by orthologous genes, but differ by one or more amino acids due to allelic differences (Prakash et al. 1969). Although some earlier studies focused on seed storage protein patterns, the majority of protein markers are derived from allozymes (Weising & al. 2005).

Allozyme analysis is relatively straightforward and easy to carry out (Murphy & al. 1996). A tissue extract is prepared and electrophoresed on a nondenaturing starch or polyacrylamide gel. The proteins of this extract are separated by their net charge and size. After electrophoresis, the position of a particular enzyme in the gel is detected by adding a colorless substrate that is converted into a dye under appropriate reaction conditions. Depending on the number of loci, their state of homo- or heterozygosity, and the enzyme configuration (i.e., the number of separable subunits), from one to several bands are visualized. The positions of these bands can be polymorphic and thus informative.

Protein-based markers have the advantages that they are cheap and need no sophisticated equipment; they are usually co-dominant making them appropriate for heterozygosity studies. However, the main drawbacks to their use are the limited number of enzyme systems available, the use of specific detection methods for each enzyme, and only genomic regions coding for expressed proteins can be analyzed resulting in low polymorphism.

DNA-based molecular markers

Plant DNA polymorphism assays are powerful tools for characterizing and investigating germplasm resources and genetic relatedness (Powell et al. 1996). These include sequencing of a known region of a genome; non-PCR-based DNA markers such as restriction fragment length polymorphisms (RFLP) and PCR based DNA markers.

With the development of the polymerase chain reaction, many PCR-based DNA molecular techniques have been, and still are being developed for plant genome analysis. These techniques include RAPD (random amplified polymorphic DNA) (Welsh & McClelland 1990; Williams et al. 1990), AFLP (amplified fragment length polymorphism) (Vos et al. 1995), ISSR (inter-simple

sequence repeat) (Zietkiewicz et al. 1994), SSR (simple sequence repeat) or microsatellite (Morgante & Olivieri 1993).

Random amplified polymorphic DNA (RAPD)

This technique employs single primers with 10 arbitrary nucleotide sequences and at least 50% GC content. PCR products are separated on agarose gels and detected by staining with ethidium bromide. To obtain an amplification product with only one primer, there must be two identical or at least highly similar target sequences in close vicinity to each other on different strands in an opposite orientation. RAPD polymorphisms can theoretically result from several types of events: (1) insertion of a large piece of DNA between the primer binding sites may exceed the capacity of PCR, resulting in fragment loss; (2) insertion or deletion of a small piece of DNA will lead to a change in size of the amplified fragment; (3) the deletion of one of the two primer annealing sites results in either the loss of a fragment or an increase in size; (4) a nucleotide substitution within one or both primer target sites may affect the annealing process, which can lead to a presence versus absence of polymorhism or to a change in fragment size (Weising et al. 2005).

The greatest advantage of the RAPD approach is its technical simplicity, paired with the independence of any prior DNA sequence information. One obvious disadvantage that RAPD share with other multilocus markers is their dominant nature, which limits their use in population genetics and mapping studies (Lynch & Milligan 1994). Besides, RAPD is sensitive to slight changes in reaction conditions, which interfere with the reproducibility of banding patterns between separate experiments, PCR instrumentation, and laboratories (Ellsworth et al. 1993; Muralidharan & Wakeland 1993; Penner et al. 1993). This high sensitivity is at least in part a consequence of the non-stringent PCR conditions, which are needed to allow for mismatch priming (Weising et al., 2005). An obvious measure to enhance reproducibility is to carry out replicate experiments, and exclude inconsistent bands from the analysis. Because reproducibility mainly depends on appropriate optimization of PCR components, it is advisable to determine optimal RAPD conditions empirically by performing a set of pilot experiments. Given that the outcome of RAPD experiments is influenced by many interacting variables, complete optimization can only be achieved if each component is tested independently and across a wide concentration range.

Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphisms are DNA fragments obtained from endonuclease restriction, followed by ligation of oligonucleotide adapters to the fragments and selective amplification by PCR. The PCR-primers consist of a core sequence (part of the adapter), a restriction enzyme specific sequence and 1–3 selective nucleotides. Typically two successive PCRs are performed on the restricted template, using specifically designed primers that allow only a subset of the restriction fragments to be amplified. In the standard procedure described by Vos et al. (1995) one of the selective primers is radioactively labeled, the amplification products are separated on highly resolving sequencing gels, and banding patterns are visualized by autoradiography. In the last years capillary electrophoresis of fluorescently labelled AFLPs have gradually replaced gel-based systems (Meudt & Clarke 2007). The other alternative method is using non-labeled primers and visualize the bands with silver staining technique. Polymorphisms between two or more genotypes may arise from sequence variation in one or both restriction sites flanking a particular fragment, insertion or deletions within an amplified fragment, and differences in the nucleotide sequences immediately adjacent to the restriction sites.

The advantages of the AFLP technology include no need of prior DNA sequence information, and the possibility of applying high stringency during PCR, which ensures high reproducibility of the method. Although it is a very powerful approach, it has a number of limitations such as dominance of markers, clustering of some markers in distinct genomic regions, limited levels of polymorphism in some cultivated species and the requirement of good quality DNA to ensure complete restriction (Weising et al. 2005).

Inter-simple sequence repeat (ISSR)

Inter simple sequence repeat (ISSR) is a PCR based method that involves amplification of DNA segments present at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites, usually 16–25 base pair long primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. The primers used can be either unanchored (Gupta et al. 1994; Wu et al. 1994) or anchored at the 3’ or 5’ end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz et al. 1994). The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide. ISSRs have

high reproducibility possibly due to the use of longer primers as compared to RAPD, which permits the subsequent use of a high annealing temperature (45–60° C) leading to higher stringency.

The sources of variation in ISSR markers could be: (1) mutations at the priming site (SSR), which could prevent amplification of a fragment as in RAPD markers and thus give a presence/absence polymorphism; (2) an insertion/deletion event within the SSR region or the amplified region would result in the absence of a product or length polymorphism depending on the amplifiability of the resulting fragment size.

The ISSR marker technique is simple, quick, and efficient. The primers are long resulting in high stringency and hence reproducibility. The amplified products are usually 200-2000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis. The technique is not without limitations. For instance, there is the possibility as in RAPD that fragments with the same mobility may originate from non-homologous regions, which can contribute to some distortion in the estimates of genetic similarities (Sanchez de la Hoz et al. 1996).

Microsatellites (simple sequence repeats)

Microsatellites or simple sequence repeats (SSRs) are tandemly repeated motifs of 1-6 bases found in all prokaryotic and eukaryotic genomes analysed to date. They are present in both coding and non-coding regions and are usually characterized by a high degree of length polymorphism. Slippage of DNA polymerase during DNA replication and failure to repair mismatches is considered as a mechanism for creation and hypervariability of microsatellites (Levinson & Gutman 1987). Microsatellites were widely employed in many fields soon after their first description (Litt & Luty 1989; Tautz 1989; Weber & May 1989). The large number of alleles and high level of variability among closely related organisms made PCR amplified microsatellites the marker system of choice for a wide variety of applications.

The popularity of microsatellite markers stems from a combination of several important advantages, namely their codominant inheritance, high abundance, enormous extent of allelic diversity, and the ease of assessing size variation by PCR with pairs of flanking primers (Weising et al. 2005). The major drawback of microsatellites is the necessity of sequence

information for primer design that they need to be isolated *de novo* from most species being examined for the first time. However, there is some cross species transferability of SSRs among closely related species that can help to defray the initial development costs (Peakall et al. 1998).

Study area

The Mediterranean basin, with its 11.8 endemic plants per 100 Km^2, hosts 10% of the world's higher plants, which can be found in an area representing only 1.6% of the Earth's surface (Médail & Quézel 1997, 1999; Médail & Diadema 2009). More than a half of its 25,000 known vascular plant species are endemic, of which 60% are restricted to small areas (Thompson 2005). For these reasons it has been recognised as a major centre of plant diversity and as one of the priority regions for conservation in Europe, as well as one of the 34 most important "biodiversity hotspots" of the planet (Médail & Quézel 1997, 1999; Myers et al. 2000; Mittermeier et al. 2005). To better assess plant conservation priorities, 10 Mediterranean hotspots were defined by Médail & Quézel (1997, 1999). Médail & Diadema (2009) recently updated the Mediterranean hotspots assessment including two new hotspots: the Dalmatian coast (Nikolic et al. 2008) and the Kabylies–Numidie–Kroumirie (Véla & Benhouhou 2007). In the same work they also identified 52 putative refugia for the Mediterranean region which could have allowed the long-term persistence of populations and the speciation of new *taxa* during the Pleistocene glaciations (Fig. 2). Sardinia, Corsica, Sicily and the Balearic Islands are the largest islands in the Mediterranean Sea and belong to the "Tyrrhenian islands" hotspot (Fig. 2), alternatively named "Western Mediterranean Islands" by Blondel & Médail (2009).

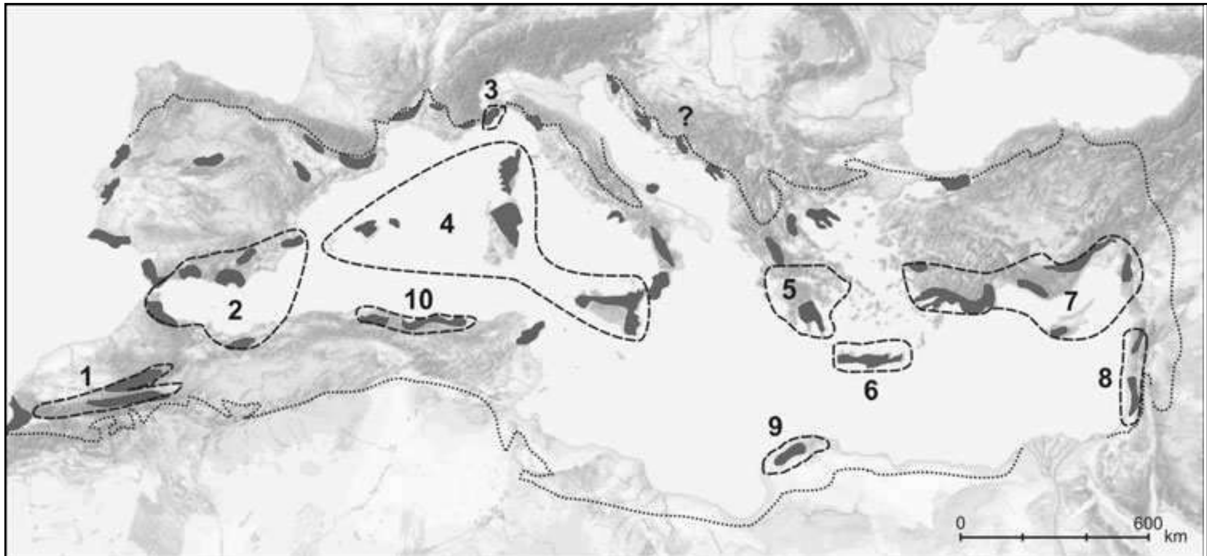

Fig. 2. Locations of the 52 putative refugia identified by Médail & Diadema (2009) (dark grey) and of the 10 biodiversity hotspots (large broken line): 1, High and Middle Atlas; 2, Baetic–Rifan complex; 3, Maritime and Ligurian Alps; 4, Tyrrhenian islands; 5, south and central Greece; 6, Crete; 7, south Anatolia and Cyprus; 8, Syria– Lebanon–Israel; 9, Mediterranean Cyrenaic; 10, Kabylies–Numidie–Kroumirie; ?, Dalmatian coast (from Médail & Diadema 2009).

All islands are characterized by a high floristic richness and high number of endemic species. The Sardinian flora consists of 2,498 *taxa* (Conti et al. 2005, 2007) comprising 347 endemics *sensu lato* (i.e. endemic of Sardinia and other insular territories of the western Mediterranean subregion, 13.9%), of which 171 are exclusive (endemic plants *sensu stricto*) to the island (updated from Bacchetta et al. 2012). Corsica is characterized by very similar figures, with a flora consisting of 2,325 *taxa*, of which 316 (13.59%) are endemic *sensu lato* and 146 are endemic *sensu stricto* (Jeanmonod & Gamisans 2007). About 2700 *taxa* are reported for Sicily, with ca. 426 (15.8%) being endemic (Di Martino & Raimondo 1979; Raimondo et al. 2001), while in the Balearic Islands approx. 7% of the flora is endemic (Alomar et al. 1997).

The high degree of local endemism in the Mediterranean basin (10%−20% of the local flora) concentrated in mountain chains and islands (Greuter 1991; Médail & Quézel 1997; Thompson 2005) is the result of its complex paleogeographic history. According to the geodynamic reconstructions of the Western Mediterranean during the last 30 million years southern France, the Balearic Islands, Corsica, Sardinia and Sicily, the Kabylies, northeastern Spain, the Rif/Baetic range and part of Calabria were connected together to form an Oligocenic land mass known as the Hercynian massif (Alvarez et al. 1974; Westphal et al. 1976; Rosenbaum et al. 2002; Speranza et al. 2002). This massif started to fragment and split away in the Late Oligocene, while during the Miocene the land messes rotated south-eastward leading to the

current position of the microplates. Only the Balearic Islands remained connected to the Iberian Peninsula, and were the last to break apart from mainland 1 mya. The formation of the islands promoted a disjunct distribution of populations either as a consequence of the fragmentation of ancient populations (Verlaque et al. 1991; Thompson et al. 2005), or due to dispersal and subsequent founder events among islands. The high level of endemism in the islands is also a result of its present isolation and high ecological diversity which have created a wide range of habitats, especially in their mountain massifs, where there are conditions of ecological insularity (Médail & Quézel 1997).

Despite the widely recognized conservation and evolutionary relevance and the long tradition of studies on Mediterranean endemics (e.g. Favarger & Contandriopoulos 1961; Contandriopoulos & Cardona 1984), studies on fine scale patterns of genetic variation of endemics inhabiting these islands were scarce until a few years ago. This trend has slowly started to change in recent years, and various endemics have been studied in Balearic Islands (e.g. Sales et al. 2001; Juan et al. 2004; Vilatersana et al. 2007; Molins et al. 2009; Orellana et al. 2009; Rosselló et al. 2009), Corsica-Sardinia (e.g. Bacchetta et al. 2008; Coppi et al. 2008; Mameli et al. 2008; Bacchetta et al. 2011; Garrido et al. 2012), Sicily (e.g. Sánchez et al. 2005; Bancheva et al. 2011; Raimondo et al. 2012).

Study species

Ferula communis L. and *F. arrigonii* Bocchieri

The genus *Ferula* L. (Apiaceae) is represented by 172 perennial herbaceous species occurring from central Asia westward throughout the Mediterranean region to northern Africa (Mabberley 2008). The genus is monophyletic and it belongs to the *Scandiceae* tribe and to the *Ferulinae* subtribe (Kurzyna-Młynik et al. 2008). The Mediterranean clade, including *F. communis* L. (the nomenclatural type of the genus), is distributed in the whole basin reaching as far as the Canary Islands to the west (*F. linkii* Webb) and Turkey and Israel to the east (*F. communis*; Kurzyna-Młynik & al. 2008; Fig. 3). The same authors also confirmed the origin of the Mediterranean group from Asian ancestors, this hypothesis being also supported by the general westward colonization of Asian steppe plants (Frantzke et al. 2004).

Two species are reported for the Tyrrhenian area: the Corso-Sardinian endemic *F. arrigonii* (Fig. 4) and the widespread *F. communis* L. (Fig. 5), which comprises the two widespread *F. c.* subsp. *communis* and *F. c.* subsp. *glauca* (L.) Rouy & Camus and the Minorcan endemic *F. c.* subsp. *cardonae* Sánchez Cuxart & M. Bernal. *F. c.* subsp. *communis* and *F. c.* subsp. *glauca* were originally described as autonomous species, and were later considered as being subspecies (Rouy & Camus 1901; Thellung 1926; Cannon 1968; Pignatti 1982; Sánchez-Cuxart & Bernal 1998; Bolòs de & Vigo 1984), varieties (Bertoloni 1837; Paoletti 1900; Fiori 1925) or even as being partially or totally synonymous (Caruel 1889). Other authors (Arcangeli 1882, 1894; Hayek 1927; Brilli Cattarini & Gubellini 1987; Anzalone et al. 1991; Conti et al. 2005; Jeanmonod & Gamisans 2007) have supported their specific status because of the several morphological, ecological, phenological, karyological, chorological and phytochemical differences between the two *taxa*. Both have morphological variants that have received a specific name in the past and that are now considered synonyms, e.g. *F. nodiflora* L., *F. brevifolia* Link, *F. neapolitana* Ten., *F. lobeliana* Vis. Sánchez-Cuxart & Bernal (1998) described a new subspecies from the island of Minorca (i.e. *F. c.* subsp. *cardonae*) based on morphological, phenological and karyological characteristics. In the same study the authors found great similarities between plants from Minorca and Sicily and the authors thus hypothesized they could belong to the same *taxon*. However, no specimens from Corsica and Sardinia were included in this work, and at present there are no available comparative studies comprising plants from the whole Tyrrhenian area.

F. arrigonii was described relatively recently as a distinct species with respect to *F. communis* L., from which it differs mainly by its smaller size and the dense and late flowering (Bocchieri 1988). Its distribution is highly fragmented and consists of a few relatively small and isolated populations located near the coasts and in small islands, either in rocky cliffs or at a few meters above the sea level and with no apparent substrate preference. Conti et al. (1997) suggest *F. arrigonii* should be included in the regional Red List as LR (lower risk).

A large amount of literature is available regarding the phytochemistry of both *F. arrigonii* (e.g. Delair et al. 1994; Filippini et al. 2000) and *F. c.* subsp. *communis.* In particular, two main chemotypes, one poisonous (with different degrees of toxicity, Sacchetti et al. 2003) and one non-poisonous, have been distinguished and studied within this latter *taxon* in Sardinia (Appendino 1997; Sacchetti et al. 2003; Arnoldi et al. 2004; Rubiolo et al. 2006). These two chemotypes are reported to be indistinguishable from a morphological and a karyological point

of view (Appendino 1997; Sacchetti et al. 2003; Rubiolo et al. 2006); their genetic differentiation has already been partially investigated by means of allozyme electrophoresis (Marchi et al. 2003). The germination ecophysiology of the *taxa* is also well known, as studies have been recently undertaken at the BG-SAR (Sardinian Germplasm Bank, Cagliari), revealing that their seeds germinate equally well in the light and in the darkness and reach the maximum germination percentages at 10-15 °C with no significant differences between the study *taxa* (Bacchetta et al., unpublished data).

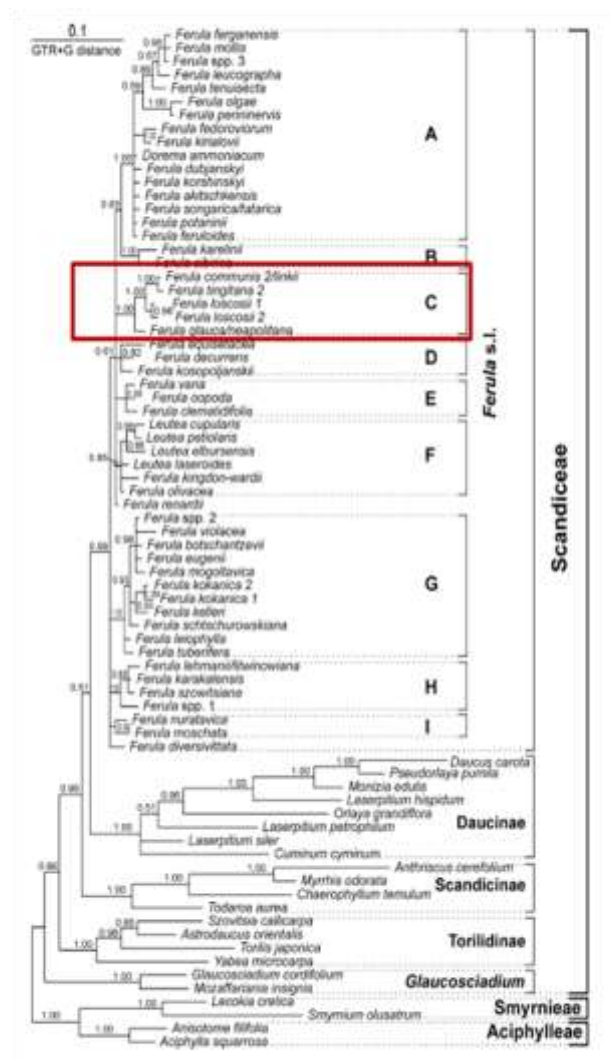

Fig. 3. Majority-rule consensus tree obtained from Bayesian analysis of ITS data. The Mediterranean clade is indicated in red (modified from Kurzyna-Młynik et al. 2008).

Fig. 4. *Ferula arrigonii* Bocchieri

Fig. 5. *Ferula communis* subsp. *communis* L.

Ruta corsica DC. and *Ruta lamarmorae* Bacch., Brullo & Giusso

Ruta L., the type genus of Rutaceae Juss., includes 10 *taxa* restricted to the Macaronesian islands, the Mediterranean basin and SW-Asia (Mabberley 2008). Four species are widespread (*R. chalepensis* L., *R. graveolens* L., *R. montana* (L.) L., *R. angustifolia* Pers.), two species are endemic to the Tyrrhenian islands (*R. corsica* in Corsica and *R. lamarmorae* in Sardinia; Bacchetta et al. 2006), one subspecies is endemic to Crete [*R. chalepensis* ssp. *fumariifolia* (Boiss. & Heldr.) Nyman] and three species are endemic to the Canary islands (*R. oreojasme* Webb & Berthel. in Gran Canaria, *R. microcarpa* Svent. in La Gomera, *R. pinnata* L.f. in La Palma and Tenerife; Bramwell & Bramwell 2001).

R. lamarmorae (Fig. 6), endemic to the Gennargentu massif in Sardinia (Bacchetta et al. 2006), is very localized and found only in three small stands, one of which has only recently been discovered. *R. corsica* (Fig. 7) is comparatively widespread and it can be found in all the main Corsican massifs. The two endemic *taxa* were considered the same species (*R. corsica*) until a few years ago, and only recently the Sardinian populations were ascribed to a new species (i.e. *R. lamarmorae*; Bacchetta et al. 2006), based on morphological and karyological differences, being *R. corsica* (2n=18, Contandriopoulos 1957) and *R. lamarmorae* tetraploid (2n=36, Honsell 1957). In a relatively recent phylogenetic analysis of Ruteae based on three cpDNA markers the accessions from Corsica and Sardinia formed two strongly supported clades, thus suggesting that the treatment of *R. lamarmorae* as a separate species should be warranted (Salvo et al. 2008). According to Contandriopoulos (1962), *R. corsica*, and consequently also *R. lamarmorae*, should be considered as relict species, taxonomically very isolated from the other known species belonging to the same genus. Both these species are morphologically well characterized and show some archaic features such as the pulvinate subspinescent habit, green-glaucescent leaves, and white to pale yellow petals, which are lacking in the other known species (Bacchetta et al. 2006).

In their work, Bacchetta et al. (2006) hypothesized that *R. lamarmorae* could have been arisen from the diploid populations of *R. corsica* and that the polyploidization event and the geographical isolation could have led to the morphological differentiation of the Sardinian populations. Ancestral area reconstruction and molecular dating (Salvo et al. 2010) estimated an age of 4-3 Myr for the split between the two species.

R. corsica is listed as LC (Least Concern) in the national red list (UICN France et al. 2012). *R. lamarmorae* is currently not included in any list of protected species, even though Bacchetta et al. (2006) suggested that *R. lamarmorae* should be listed in the IUCN regional red list as endangered (EN) according to the criteria B1ab(ii,iii,v)+2a(ii,iii,v); C2a(i), because of the strong pressure it suffers throughout its range due to overgrazing, fires, ski facilities and activities.

Fig. 6. *Ruta lamarmorae* Bacch., Brullo & Giusso

Fig. 7. *Ruta corsica* DC.

Aims of the thesis

The following main questions are addressed in this thesis:

- **What is the genetic diversity within and among the existing populations of the selected endemic species?** As more rare species are studied, our ignorance of them has been repeatedly highlighted, and perhaps the most crucial of our misconceptions is that, despite several arguments to the contrary (e.g. Drury 1974; Rabinowitz 1981), rare species are considered a relatively homogeneous assemblage of species with common characteristics that will become apparent given sufficient study. Plant species with small and isolated populations are more vulnerable to demographic, environmental and genetic stochastic events, and therefore face a higher risk of local extinction (Frankham & al. 2002; Ellstrand & Elam 1993). In addition, island endemics are thought to be more susceptible to extinction (Frankham 1997, 1998; Prohens et al. 2007). However, this general expectation has turned out not to be universally applicable. Moderate to high genetic diversity was found in other Mediterranean endemics, e.g. *Centaurea horrida* Badarò (Mameli et al. 2008), *Anchusa* sp. (Coppi et al. 2008), *Rhamnus persicifolia* Moris (Bacchetta et al. 2011), *Brassica rupestris* Raf. (Raimondo et al. 2012), and *Medicago citrina* (Font Quer) Greuter (Juan et al. 2004). Molecular markers and population genetic tools will be used in order to assess the level of genetic diversity of the selected endemic species.

- **What is the genetic diversity of rare and/or endemic species compared to their widespread congeners?** Upon reviewing the characteristics of endemic *taxa*, Kruckeberg & Rabinowitz (1985) concluded that future research should include more ‘‘comparative studies to contrast the biology of rare *taxa* with those of related common ones”. In the last two decades, many researchers have included widespread congeners when examining the genetic variation of rare species, and a considerable number of examples of highly polymorphic rare species has been reported (e.g., Ranker 1994; Lewis & Crawford 1995), and in some cases endemic species have shown to have equivalent or higher levels of diversity compared to their more widely distributed congeners (Gitzendanner & Soltis 2000; Ellis et al. 2006). Furthermore, in some genera both rare and widespread species turned out to have either very low or very high, but

similar, levels of polymorphism (e.g. Young & Brown 1996), thus suggesting that the classic view that rare species have less genetic variability than more widespread ones may be an overgeneralization. This hypothesis will be tested on the endemic *Ferula arrigonii* and on its widespread congener *F. communis*.

- **Is the genetic variation among populations spatially structured?** Island endemics usually exhibit high levels of spatial genetic structure; this pattern has been described in several cases, e.g. *Anchusa* sp. (Quilichini et al. 2004; Coppi et al. 2008) *Rhamnus persicifolia* (Bacchetta et al. 2011) and *Aquilegia* sp. (Garrido et al. 2012) in Sardinia and Corsica; *Medicago citrina* (Juan et al. 2004) and *Senecio rodriguezii* (Molins et al. 2009) in the Balearic Islands; *Nigella arvensis* (Bittkau & Comes 2005) in the Aegean region; *Primula sieboldii* in Japan (Kitamoto et al. 2005). The spatial structure of the genetic variation will be investigated in order to verify whether this applies to the endemic species *F. arrigonii*, *R. lamarmorae* and *R. corsica*.

- **Which possible conservation measures can be undertaken to protect the endemic species?** On the basis of the results, of the molecular evidence and/or of the possible taxonomic implications, suggestions will be provided regarding: i) *in situ* conservation actions (e.g. population reinforcements); ii) *ex situ* conservation protocols (e.g. collection and long term storage of high quality germplasm) in order to get to conserve the highest possible level of the genetic diversity of these endemic species in adequate structures.

References

Alomar G., Mus M., Rosselló J.A. 1997. Flora endemic de les Balears. Palma de Mallorca: Consell Insular de Mallorca.

Anzalone B., Lattanzi E., Leporatti M.L. 1991. Il gruppo di *Ferula communis* L. in Italia: ricerche sistematiche e corologiche. Archivio Botanico Italiano 67: 221-236.

Appendino G. 1997. The toxins of *Ferula communis* L. In: Verotta L. (ed.). Virtual ActiVity, Real Pharmacology, pp. 1-15. Research Signpost. Tivandrum, India.

Arcangeli G. 1882. Compendio della Flora Italiana, pp. 290-291.

Arcangeli G. 1894. Compendio della Flora Italiana (2nd ed.), pp. 608-609.

Arnoldi L., Ballero M., Fuzzati N., Maxia A., Mercallia E., Pagnia L. 2004. HPLC-DAD-MS identification of bioactive secondary metabolites from *Ferula communis* roots. Fitoterapia 75: 342-354.

Alvarez W., Cocozza T., Wezel F.C**.** 1974. Fragmentation of alpine orogenic belt by microplate dispersal. Nature 248: 309-314.

Bacchetta G., Brullo S., Giusso del Galdo G. 2006. *Ruta lamarmorae* (Rutaceae), a new species from Sardinia. Edinburgh Journal of Botany 63:153-160.

Bacchetta G., Coppi A., Pontecorvo C., Selvi F. 2008. Systematics, phylogenetic relationships and conservation of the taxa of *Anchusa* (Boraginaceae) endemic to Sardinia (Italy). Systematics and Biodiversity 6: 161-174.

Bacchetta G., Fenu G., Mattana E., Zecca G., Grassi F., Casazza G., Minuto L. 2011. Genetic variability of the narrow endemic *Rhamnus persicifolia* Moris (Rhamnaceae) and its implications for conservation. Biochemical Systematics and Ecology 39: 477-484.

Bacchetta G., Fenu G., Mattana E. 2012. A checklist of the exclusive vascular flora of Sardinia with priority rankings for conservation. Anales del Jardín Botánico de Madrid 69: 61-69.

Bancheva S., Geraci A., Raimondo F.M. 2011. Assessing the genetic diversity of *Centaurea parlatoris* group (sect. Dissectae, Compositae) in Sicily using isozymes. Plant Biosystems 145: 778-785.

Bataillon T., Kirkpatrick M. 2000. Inbreeding depression due to mildly deleterious mutations in finite populations: size does matter. Genetical Research 75: 75-81.

Bertoloni A. 1837. Flora Italica 3: 372-375.

Bittkau C., Comes H.P. 2005. Evolutionary processes in a continental island system: molecular phylogeography of the Aegean *Nigella arvensis* alliance (Ranunculaceae) inferred from chloroplast DNA. Molecular Ecology 14: 4065-4083.

Blondel J., Médail F. 2009. Biodiversity and conservation. In: Woodward J.C. (ed.). The physical geography of the Mediterranean. Oxford: Oxford University Press.

Bocchieri E. 1988. *Silene valsecchiae* e *Ferula arrigonii*, due specie nuove della Sardegna. Bollettino della Società Sarda di Scienze Naturali 26: 305-310.

Bolòs de O., Vigo J. 1984. Flora dels Paisos Catalans 2: 468-470.

Bramwell D., Bramwell Z.I. 2001. Wild flowers of the Canary Islands. Editorial Rueda, Madrid.

Brilli Cattarini A., Gubellini L. 1987. Segnalazioni floristiche italiane: 487. *Ferula communis* L. Informatore Botanico Italiano 19: 189.

Cannon J.F.M. 1968. Gen. Ferula. In: Tutin T.G., Burges N.A., Valentine D.H., Walters S.M., Webb D.A. (eds.). 1968. Flora Europaea 2: 358-359. Cambridge University Press, Cambridge.

Caruel T. 1889. Ferula. In: Parlatore F. Flora Italiana 8: 297-300.

CBD. 1992. Convention on Biological Diversity. 5 June 1992, Rio de Janeiro (Brazil).

Contandriopoulos J. 1957. Caryologie et localisation des espèces végétales endémiques de la Corse. Bulletin de la Societé Botanique de France 104: 53-55.

Contandriopoulos J. 1962. Réchèrches sur la flore endémique de la Corse et sur ses origines. Annales de la Faculté de Science de Marseille 32: 1-351.

Contandriopoulos J., Cardona M.A. 1984. Caractère original de la flore endémique des Baléares. Botanica Helvetica 94: 101-132.

Conti F., Manzi A., Pedrotti F. 1997. Liste rosse regionali delle piante d'Italia. WWF Italia, Società Botanica Italiana, TIPAR Poligrafica Editrice, Camerino.

Conti F., Abbate G., Alessandrini A., Blasi C. (eds.). 2005. An Annoted Checklist of the Italian Vascular Flora. Palombi Editori, Roma.

Conti F., Alessandrini A., Bacchetta G., Banfi E., Barberis G., Bartolucci F., Bernardo L., Bonacquisti S., Bouvet D., Bovio M., Brusa G., Del Guacchio E., Foggi B., Frattini S., Galasso G., Gallo L., Gangale C., Gottschlich G., Grünanger P., Gubellini L., Iiriti G., Lucarini D., Marchetti D., Moraldo B., Peruzzi L., Poldini L., Prosser F., Raffaelli M., Santangelo A., Scassellati E., Scortegagna S., Selvi F., Soldano A., Tinti D., Ubaldi D., Uzunov D., Vidali M. 2007. Integrazioni alla checklist della flora vascolare italiana. Natura Vicentina 10: 5-74.

Coppi A., Mengoni A., Selvi F. 2008. AFLP fingerprinting of *Anchusa* (Boraginaceae) in the Corso-Sardinian system: Genetic diversity, population differentiation and conservation priorities in an insular endemic group threatened with extinction. Biological Conservation 141: 2000-2011.

Delair P., Kann N., Greene A.E. 1994. Synthesis (in ent-form) of a Novel Jalcaguaianolide from *Ferula arrigonii* Bocchieri. Journal of the Chemical Society. Perkin Transactions 13: 1651-1652.

Di Martino A., Raimondo F. M. 1979. Biological and chorological survey of the Sicilian Flora. Webbia 34: 309-335.

Drury W.H. 1974. Rare species. Biological Conservation 6: 162-169.

Ellis J.R., Pashley C.H., Burke J.M., McCauley D.E. 2006. High genetic diversity in a rare and endangered sunflower as compared to a common congener. Molecular Ecology 15: 2345-2355.

Ellstrand N.S., Elam D.R. 1993. Population genetic consequences of small population size: implications for plant conservation. Annual Review of Ecology and Systematics 24: 217-242.

Ellsworth D.L., Rittenhouse K.D., Honeycutt R.L. 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. Biotechniques 14: 214-217.

Favarger C., Contandriopoulos J. 1961. Essai sur l’endémisme. Bulletin de la Société botanique de Suisse 71: 384-408.

Filippini M.H., Tomi F., Casanova J. 2000. Composition of the leaf oil of *Ferula arrigonii* Bocchieri. Flavour and Fragrance Journal 15: 195-198.

Fiori A. 1925. Nuova Flora analitica d’Italia 2: 63.

Frankel O.H., Brown A.H.D., Burdon J.J. 1995. The conservation of plant biodiversity. Cambridge University Press, Cambridge, UK.

Frankham R. 1997. Do island populations have less genetic variation than mainland populations? Heredity 78: 311-327.

Frankham R. 1998. Inbreeding and extinction: island populations. Biological Conservation 12: 665-675.

Frankham R., Ballou J.D., Briscoe D.A. 2002. Introduction to Conservation Genetics. Cambridge University Press, Cambridge, UK.

Franzke A., Hurka H., Janssen D., Neuffer B., Friesen N., Markov M., Mummenhoff K. 2004. Molecular signals for Late Tertiary/Early Quaternary range splits of an Eurasian steppe plant: *Clausia aprica* (Brassicaceae). Molecular Ecology 13: 2789-2795.

Garrido J.L., Mattana E., Fenu G., Bacchetta G. 2012. Spatial genetic structure of *Aquilegia taxa* endemic to the island of Sardinia. Annals of Botany 109: 953-964.

Gitzendanner M.A., Soltis P.S. 2000. Patterns of genetic variation in rare and widespread plant congeners. American Journal of Botany 87: 783-792.

Greuter W. 1991. Botanical diversity, endemism, rarity, and extinction in the Mediterranean Area: an analysis based on the published volumes of Med-Checklist. Botanika Chronica 10: 63-79.

Gupta M., Chyi Y.S., Romeroseverson J., Owen J.L. 1994. Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. Theoretical and Applied Genetics 89: 998-1006.

Hayek A. 1927. Prodromus Florae Peninsulae Balcanicae 1: 1028.

Hedrick P.W. 2002. Letals in finite populations. Evolution 56: 654-657.

Heywood V.H. (ed.). 1995. Global Biodiversity Assessment. Cambridge University Press, Cambridge, U.K.

Honsell E. 1957. Ricerche cario-embriologiche in Ruta corsica DC. Delpinoa 10: 141-153.

Jeanmonod D., Gamisans J. 2007. Flora Corsica. Edisud, Aix-en-Provence.

Juan A., Crespo M.B., Cowan R.S., Lexer C., Fay M.F. 2004. Patterns of variability and gene flow in *Medicago citri*na, an endangered endemic of islands in the western Mediterranean, as revealed by amplified fragment length polymorphism (AFLP). Molecular Ecology 13: 2679-2690.

Karp A., Kresovich S., Bhat K.V., Ayad W.G., Hodgkin T. 1997. Molecular tools in plant genetic conservation: a guide to the techniques. International Plant Genetic Resources Institute Technical Bulletin No. 2, pp. 5-47.

Kitamoto N., Honjo M., Ueno S., Takenaka A., Tsumura Y., Washitani I., Ohsawa R. 2005. Spatial genetic structure among and within populations of *Primula sieboldii* growing beside separate streams. Molecular Ecology 14: 149-157.

Kruckeberg A.R., Rabinowitz D. 1985. Biological aspects of endemism in higher plants. Annual Review of Ecology and Systematics 16: 447-479.

Kurzyna-Młynik R., Oskolski A.A., Downie S.R., Kopacz R., Wojewodzka A., Spalik K. 2008. Phylogenetic position of the genus *Ferula* (Apiaceae) and its placement in tribe Scandiceae as inferred from nrDNA ITS sequence variation. Plant Systematics and Evolution 274: 47-66.

Levinson G., Gutman G.A. 1987. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Molecular Biology and Evolution 4: 203-221.

Lewis P.O., Crawford D.J. 1995. Pleistocene refugium endemics exhibit greater allozymic diversity than widespread congeners in the genus *Polygonella* (Polygonaceae). American Journal of Botany 82: 141-149.

Litt M., Lutty J.A. 1989. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. American Journal of Human Genetics 44: 397-401.

Lynch M., Milligan B.G. 1994. Analysis of population genetic structure with RAPD markers. Molecular Ecology 3: 91-99.

Mabberley D.J. 2008. Mabberley's plant book: a portable dictionary of plants (3rd ed.). Cambridge University Press, Cambridge, UK.

Mameli G., Filigheddu R., Binelli G., Meloni M. 2008. The genetic structure of the remnant populations of *Centaurea horrida* in Sardinia and associated islands. Annals of Botany 101: 633-640.

Marchi A., Appendino G., Pirisi I., Ballero M., Loi M.C. 2003. Genetic differentiation of two distinct chemotypes of *Ferula communis* (Apiaceae) in Sardinia (Italy). Biochemical Systematics and Ecology 31: 1397-1408.

Markert C.L., Moller F. 1959. Multiple forms of enzymes: tissue, ontogenetics and species specific patterns. Proceedings of the National Academy of Sciences of the United States of America 45: 753-763.

Médail F., Diadema K. 2009. Glacial refugia influence plant diversity patterns in the Mediterranean Basin. Journal of Biogeography 36: 1333-1345.

Médail F., Quézel P. 1997. Hot-spots analysis for conservation of plant biodiversity in the Mediterranean Basin. Annals of the Missouri Botanical Garden 84: 112-127.

Médail F., Quézel P. 1999. Biodiversity Hotspot in the Mediterranean Basin: Setting Global Conservation Priorities. Conservation Biology 13: 1510-1513.

Meudt H.M, Clarke A.C. 2007. Almost Forgotten or Latest Practice? AFLP applications, analyses and advances. Trends in Plant Science 12: 106-117.

Mittermeier R.A., Robles Gil P., Hoffman M., Pilgrim J., Brooks T., Mittermeier C.G., Lamoreux J., da Fonseca G.A.B. 2005. Hotspots revisited: earth's biologically richest and most endangered terrestrial ecoregions. University of Chicago Press, Chicago, USA.

Molins A., Mayol M., Rosselló J.A. 2009. Phylogeographical structure in the coastal species *Senecio rodriguezii* (Asteraceae), a narrowly distributed endemic Mediterranean plant. Journal of Biogeography 36: 1372-1383.

Morgante M., Olivieri A.M. 1993. PCR-amplified microsatellites as markers in plant genetics. The Plant Journal 3: 175-182.

Muralidharan K., Wakeland E.K. 1993. Concentration of primer and template qualitatively affects products in random amplified polymorphic DNA PCR. Biotechniques 14: 362-364.

Myers N., Mittermeier R.A., Mittermeier C.G., Da Fonseca G.A.B., Kent J. 2000. Biodiversity hotspots for conservation priorities. Nature 403: 853-858.

Murphy R.W., Sites J.W., Buth D.G., Haufler C.H. 1996. Proteins: isozyme electrophoresis. In: Hillis D.M., Moritz C., Mable B.K. (eds.) Molecular Systematics, 2nd ed., pp. 51-120. Sinauer Associates, Sunderland, MA,

Nikolic T., Antonic O., Alegro A.L., Dobrovic I., Bogdanovic S., Liber Z., Resětnik I. 2008. Plant species diversity of Adriatic islands: An introductory survey. Plant Biosystems 142: 435-445.

Norse E.A. 1993. Global Marine Biological Diversity: a Strategy for Building Conservation into Decision Making. Island Press, Washington DC.

Norse E.A, McManus R.E. 1980. Ecology and living resources biological diversity. In: Environmental Quality 1980: The eleventh annual report of the Council on Environmental Quality: 31-80. Council on Environmental Quality, Washington DC.

Orellana M.R., Blanché C., Simon J., Bosch M. 2009. Genetic diversity within and among disjunct populations of the Mediterranean island endemic *Delphinium pictum* and *D. requienii* (Ranunculaceae). Folia Geobotanica 44: 47-63.

Paoletti G. 1900. *Ferula communis* L. In: Fiori A., Paoletti G. Flora Analitica d'Italia 2: 175-176.

Peakall R., Gilmore S., Keys W., Morgante M., Rafalski A. 1998. Cross-species amplification of soybean (*Glycine max*) simple sequence repeats (SSRs) within the genus and other legume genera – implication for the transferability of SSRs in plants. Molecular Biology and Evolution 15: 1275-1287.

Penner G.A., Bush A., Wise R., Kim W., Domier L., Kasha K., Laroche A., Scoles G., Molnar S.J., Fedak G. 1993. Reproducibility of Random amplified polymorphic DNA (RAPD) analysis among laboratories. PCR Methods and Applications 2: 341-345.

Pignatti S. 1982. Flora d'Italia 2: 229.

Powell W., Morgante M., Andre C., Hanafey M., Vogel J., Tingey S., Rafalski A. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Molecular Breeding 2: 225-238.

Prakash S., Lewontin R.C., Hubby J.L. 1969. A molecular approach to the study of genetic heterozygocity in natural populations. IV. Patterns of genetic variation in central, marginal and isolated populations of *Drosophila pseudobscura*. Genetics 61: 841-858.

Prohens J., Anderson G.J., Herraiz F.J., Bernardello G., Santos-Guerra A., Crawford D., Nuez F. 2007. Genetic diversity and conservation of two endangered eggplant relatives (*Solanum verpertilio* Aiton, and *Solanum lidii* Sunding) endemic to the Canary Islands. Genetic Resources and Crop Evolution 54: 451-464.

Quilichini A., Debussche M., Thompson J.D. 2004. Geographic differentiation of morphological traits and isozymes in the Mediterranean island endemic *Anchusa crispa*: implications for the conservation of a protected species. Biological Conservation 118: 651-660.

Rabinowitz D. 1981. Seven forms of rarity. In: Synge H. (ed.). The biological aspects of rare plant conservation, pp. 205–217. John Wiley & Sons, Chichester, UK.

Ranker T.A. 1994. Evolution of high genetic variability in the rare Hawaiian fern *Adenophorus periens* and implications for conservation management. Biological Conservation 70: 19-24.

Raimondo F.M., Schicchi R., Bazan G. 2001. (eds.) Azione Pilota Archi-med, progetto 2.1, Protezione delle specie endemiche minacciate, Rapporto finale. Tip. Luxograph, Palermo.

Raimondo F.M, Scialabba A., Zecca G., Grassi F., Casazza G., Minuto L. 2012. Genetic diversity in the endangered Sicilian endemic *Brassica rupestris*: Proposals for a conservation strategy. Plant Biosystems 146: 847-856.

Rosselló J.A., Cosín R., Bacchetta G., Brullo S., Mayol M. 2009. Nuclear and chloroplast DNA variation in *Cephalaria squamiflora* (Dipsacaceae), a disjunct Mediterranean species. Taxon 58: 1242-1253.

Rosenbaum G., Lister G.S., Duboz C. 2002. Reconstruction of the tectonic evolution of the western Mediterranean since the Oligocene. Journal of the Virtual Explorer 8: 107-130.

Rouy G., Camus E.G. 1901. Flore de France 7: 398.

Rubiolo P., Matteodo M., Riccio G., Ballero M., Christen P., Fleury-Souverain S., Veuthey J.L., Bicchi C. 2006. Analytical Discrimination of Poisonous and Nonpoisonous Chemotypes of Giant Fennel (*Ferula communis* L.) through Their Biologically Active and Volatile Fractions. Journal of Agricultural and Food Chemistry 54: 7556-7563.

Sacchetti G., Appendino G., Ballero M., Loy C., Poli F. 2003. Vittae fluorescence as a tool to differentiate between poisonous and non-poisonous populations of giant fennel (*Ferula communis*) of the island Sardinia (Italy). Biochemical Systematics and Ecology 31: 527-534.

Sales E., Nebauer S.G., Mus M., Segura J. 2001. Population genetic study in the Balearic endemic plant species *Digitalis minor* (Scrophulariaceae) using RAPD markers. American Journal of Botany 88: 1750-1759.

Sánchez J.L., Domina G., Caujapé-Castells J. 2005. Genetic differentiation of three species of *Matthiola* (Brassicaceae) in the Sicilian insular system. Plant Systematics and Evolution 253: 81-93.

Sánchez-Cuxart A., Bernal C.M. 1998. Estudi biosistemàtic de les poblacions de *Ferula communis* del NE de la península ibèrica i de les illes balears. Acta Botanica Barcinonensica 45: 217-239.

Sanchez de la Hoz M.P., Dávila J.A., Loarce Y., Ferrer E. 1996. Simple sequence repeat primers used in polymerase chain reaction amplifications to study genetic diversity in barley. Genome 39: 112-117.

Salvo G., Bacchetta G., Ghahremaninejad F., Conti E. 2008. Phylogenetic relationships of Ruteae (Rutaceae): New evidence from the chloroplast genome and comparisons with non-molecular data. Molecular Phylogenetics and Evolution 49: 736-748.

Salvo G., Ho S.Y.W., Rosembaum G., Ree R., Conti E. 2010. Tracing the Temporal and Spatial Origins of Island Endemics in the Mediterranean Region: A Case Study from the Citrus Family (*Ruta* L., Rutaceae). Systematic Biology 59: 705-722.

Speranza F., Villa I.M., Sagnotti L., Florindo F., Cosentino C., Cipollari P., Mattei M. 2002. Age of the Corsica-Sardinia rotation and Liguro-Provençal basin spreading: New paleomagnetic and Ar/Ar evidence. Tectonophysics 347: 231-251.

Tautz D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Research 17: 6463-6471.

Thellung A. 1926. *Ferula communis* L. In: Hegi G. Illustrierte Flora von Mitteleuropa 5: 1355-1356.

Thompson J.D. 2005. Plant Evolution in the Mediterranean. Oxford University Press Inc., New York.

UICN France, FCBN, MNHN. 2012. La Liste rouge des espèces menacées en France - Chapitre Flore vasculaire de France métropolitaine: premiers résultats pour 1 000 espèces, sous-espèces et variétés. Dossier électronique.

Véla E., Benhouhou S. 2007. Évaluation d’un nouveau point chaud de biodiversité végétale dans le bassin méditerranéen (Afrique du Nord). Comptes-Rendus Biologies 330: 589-605.

Verlaque R., Aboucaya A., Cardona A., Contandriopoulos J. 1991. Quelques exemples de speciation insulaire en Méditerranée occidentale. Botanika Chronika 10: 137-153.

Vilatersana R., Susanna A., Brochmann C. 2007. Genetic variation in *Femeniasia* (Compositae, Cardueae), an endemic and endangered monotypic genus from the Balearic Islands (Spain). Botanical Journal of the Linnean Society 153: 97-107.

Vos P., Hogers R., Bleeker M., Reijans M., Van De Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407-4414.

Wang Z.Y., Tanksley S.D. 1989. Restriction length polymorphism in *Oryza sativa* L. Genome 32: 1113-1118.

Weber J.L., May P.E. 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. American Journal of Human Genetics 44: 388-396.

Weising K., Nybom H., Wolff K., Kahl G. 2005. DNA Fingerprinting in Plants Principles, Methods, and Applications Second edition. Taylor & Francis Group, CRS Press, New York.

Welsh J., McClelland M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18: 7213-7218.

Westphal M., Orsini J., Vellutini P. 1976. Corsica-Sardinia microcontinent, its initial position — paleomagnetic data and geological fitting. Tectonophysics 30: 141-157.

Williams J.G.K., Kubelik A.R., Livak K.J., Rafalski J.A., Tingey S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic-markers. Nucleic Acids Research 18: 6531-6535.

Wu K., Jones R., Dannaeberger L., Scolnik P.A. 1994. Detection of microsatellite polymorphisms without cloning. Nucleic Acids Research 22: 3257-3258.

Young A.B, Brown A.H.D. 1996. Comparative population genetic structure of the rare woodland shrub *Daviesia suaveolens* and its common congener *D. mimosoides*. Conservation Biology 10: 1220-1228.

Zietkiewicz E., Rafalski A., Labuda D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain-reaction amplification. Genomics 20: 176-183.

Chapter 1

The genetic diversity and spatial genetic structure of the Corso-Sardinian endemic *Ferula arrigonii* Bocchieri (Apiaceae)

Caterina Angela Dettori[1], Simona Sergi[2], Elena Tamburini[2], Gianluigi Bacchetta[1]

[1] *Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente - Università degli Studi di Cagliari. Viale S. Ignazio da Laconi, 13 – I-09123 Cagliari, Italy.*

[2] *Sezione di Microbiologia e Virologia, Dipartimento di Scienze Biomediche - Università degli Studi di Cagliari. Sesta strada Ovest, Z.I. Macchiareddu – I-09010 Uta, Italy.*

Abstract

Corsica and Sardinia represent a major hotspot of plant diversity in the Mediterranean area and are priority regions for conservation due to their high number of endemic plant species. However, information supporting human decision-making on the conservation of these species is still scarce, especially at the genetic level. In this work the first assessment is reported the species-wide spatial genetic structure and diversity of *F. arrigonii* Bocchieri, a Corso-Sardinian endemic species located in a few coastal sites and islets. Nine populations were investigated covering the entire natural range of the species by means of AFLP (Amplified Fragment Length Polymorphism) markers. Results indicate that this species is characterized by high levels of genetic polymorphism (about 92% of the fragments were polymorphic), a low inter-population differentiation (G_{st} = 0.124) and a high intra-population variation (H_w = 0.317). PCoA, Bayesian analysis and neighbor-joining clustering were also employed to investigate the genetic structure of this species. Three genetically distinct groups were detected, although with considerable overlap between sampling sites. Finally, suggestions are provided for management strategies to facilitate the conservation of this rare species.

Key words: *Ferula*, AFLP, Corsica, Sardinia, genetic diversity.

Introduction

The genus *Ferula* L. (Apiaceae) is represented by 172 perennial herbaceous *taxa* distributed in the Mediterranean area and in Central Asia (Mabberley 2008) and is represented in Sardinia and Corsica by *Ferula arrigonii* Bocchieri and its widespread congener *F. communis* L. *F. arrigonii* is an herbaceous, rhizomatous perennial plant, 80-150 cm tall (Bocchieri 1988) displaying a diploid karyotype (2n=22; Bacchetta 2001). It was described relatively recently as a distinct species with respect to *F. communis* L., from which it differs mainly by its smaller size and the dense and late flowering (Bocchieri 1988). Its distribution is highly fragmented and consists of relatively small and isolated populations located near the coasts and in small islands, either in rocky cliffs or at a few meters above the sea level and with no apparent substrate preference (Fig. 1, Tab. 1). Seven populations are known for Sardinia and a new one (Isola dei Cavoli) is reported in this study. The only Corsican population (Bonifacio, southern Corsica) was originally reported by Bocchieri (1996) and later considered as a naturalized species of Sardinian provenance by Jeanmonod & Gamisans (2007). An introduction to Corsica from Sardinia was firstly hypothesized by Paradis & Piazza (2004), as an alternative hypothesis they suggested that the species could have diversified anciently, that it was once more widespread and that its distribution is currently reducing. However, the origin and phylogenetic position of *F. arrigonii* is still uncertain, since it was not included in previous phylogenetic (e.g. Kurzyna-Młynik & al. 2008) or biosystematic (e.g. Sánchez-Cuxart & Bernal Cid 1998) studies involving Western Mediterranean species of the same genus. The species has raised interest for its phytochemical properties; among others (Delair & al. 1994; Filippini & al. 2000) a study by Poli & al. (2005) revealed that daucane esters from *F. arrigonii* and from *F. communis* Sardinian plants might have therapeutic potential because of their antiproliferative effects on human colon cancer cells. *F. arrigonii* is currently not included in any list of endangered species, even though Conti & al. (1997) suggested it should be included in the regional Red List as LR (lower risk) already 15 years ago. A recent assessment of its status is lacking; however, the limited Extent Of Occurrence (EOO) of the species and the reduced size and Area Of Occupancy (AOO) of its populations are very likely to be critical factors for the persistence of *F. arrigonii*.

In this study, AFLP (Amplified Fragment Length Polymorphism) markers were used to provide baseline molecular data potentially useful to design conservation strategies for *F. arrigonii*. The following questions are addressed: i) what are the levels of the genetic diversity within and among populations of *F. arrigonii* populations? ii) how is genetic diversity spatially structured across the entire range of the species?

Materials and methods

Sampling sites and plant material

The study was conducted on nine populations covering the whole distribution of the species (Tab. 1): two from southeastern Sardinia (Isola di Serpentara and Isola dei Cavoli, henceforth SER and CAV), two from southwestern Sardinia [Buggerru (BUG) and Capo San Marco (SMA)], four from northern Sardinia [Capo Caccia (CAC), Isola di Budelli (BUD), Capo Testa (TES) and Isola di Tavolara (TAV)] and one from southern Corsica [Bonifacio (BON)]. Leaf material was collected during summer 2010 and 2011, dried with silica gel and stored at room temperature. Sampling of plants was done throughout the populations in order to cover the whole occupied area and to minimize sampling of related individuals; 20 individuals per population were included in the analysis.

Tab. 1. Sampling locations and their characteristics.

Population (code)	Coordinates	Mean elevation (m a.s.l.)	Lithology	Surface area	Population size	Legal protection of the site	N_i
Isola di Serpentara (SER)	39° 08' N - 9° 36' E	10	granites	1-5 ha	500-1000	PMA, SCI	20
Isola dei Cavoli (CAV)*	39° 05' N - 9° 31' E	15	granites	< 1 ha	50-100	PMA, SCI	20
Buggerru (BUG)	39° 24' N - 8° 24' E	55	limestones	< 1 ha	50-100	SCI	19
Capo San Marco (SMA)	39° 52' N - 8° 26' E	25	limestones	5-10 ha	500-1000	PMA, SCI	20
Capo Caccia (CAC)	40° 34' N - 8° 09' E	30	limestones	5-10 ha	500-1000	PMA, RP, SCI	20
Capo Testa (TES)	41° 14' N - 9° 08' E	10	granites	1-5 ha	500-1000	SCI	20
Isola di Budelli (BUD)	41° 17' N - 9° 20' E	20	granites	< 1 ha	250-500	NP, SCI	20
Isola di Tavolara (TAV)	40° 53' N - 9° 40' E	5	limestones	1-5 ha	250-500	PMA, SCI	20
Bonifacio (BON)	41° 23' N - 9° 09' E	25	limestones	1-5 ha	> 1000	NR, SCI	20

* newly reported population. PMA = Protected Marine Area; SCI = Site of Communitary Importance; RP = Regional Park; NP = National Park; NR = Nature Reserve. N_i = number of individuals analyzed.

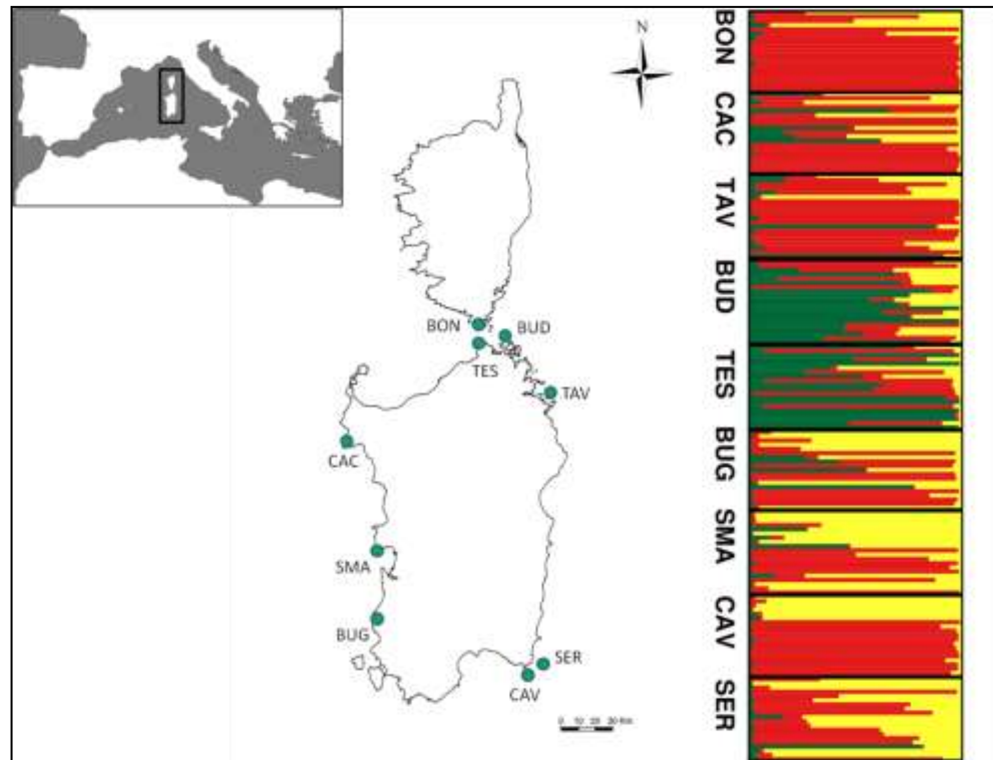

Fig. 1. Geographical location of study populations and spatial genetic structure analysis. Horizontal bars represent individuals and black lines delimit sites. The admixture diagram was redrawn from STRUCTURE results (see Materials and Methods for further datails).

AFLP analyses

Genomic DNA was extracted from 20 mg of silica gel-dried leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Italy) following the manufacturer's protocol. The AFLP technique was chosen because of its high reproducibility and no requirement of previous knowledge on DNA sequences. The original protocol by Vos et al. (1995) was followed with slight modifications. A total of 200 ng of genomic DNA was digested in a total volume of 20 µL for 2 h at 37°C with 5 U of *Eco*RI and 5 U of *Mse*I (New England Biolabs), followed by a 20 min enzyme heat inactivation at 65°C. Digestion products were then ligated for 2 h at 20°C by adding 5 pmol of *Eco*RI-adapter, 50 pmol of *Mse*I-adapter (Sigma-Genosys, Italy) and 1 U of T4 DNA ligase (Fermentas). 5 µL of 5-fold diluted ligated product were used as template in the preamplification step; the reaction mixture contained 10 pmol of *Eco*RI (+A)-primer and *Mse*I (+C)-primer (Sigma-Genosys, Italy), 1X PCR reaction buffer, $MgCl_2$ 1.5 mM, dNTPs 0.2 mM (Sigma-Aldrich, Italy) and 1.4 U of *Taq* polymerase (Fermentas) in a final volume of 20 µL. The

preamplification thermocycler profile was 20 cycles of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s.

A preliminary screening of 24 primer combinations was performed on 8 individuals. Two combinations giving the more informative and clearest readable fragment profiles were chosen for further analyses (*Eco*RI+ACC with *Mse*I+CAC and *Eco*RI+AAT with *Mse*I+CAG). The *EcoR*I primers were labeled with 6FAM and HEX, respectively). Each selective amplification reaction mixture contained 1 μL of undiluted preamplification product, 1X PCR reaction buffer, $MgCl_2$ 1.5 mM, 0.2 mM dNTPs (Sigma-Aldrich, Italy), 1 pmol of *Eco*RI-NNN primer, 5 pmol of *Mse*I-NNN primer (Sigma-Genosys, Italy) and 1.4 U of *Taq* polymerase (Fermentas). The selective amplification thermocycle profile was: 13 cycles of 94°C for 30 s, 65°C for 30 s (with a touchdown of -0.7°C per cycle), 72°C for 1 min, followed by 23 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. PCR products were run on an ABI310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with an internal size standard (GeneScan-500 ROX, PE Applied Biosystems).

To assess the reproducibility of the analysis the whole procedure (i.e. from DNA extraction to capillary electrophoresis) was repeated for 18 samples (about 10% of the total) and the error rate was calculated as the number of phenotypic differences related to the total number of phenotypic comparisons (Bonin et al. 2004).

<u>*Data analyses*</u>

Fragments between 100 and 500 bp were scored by means of GeneMarker v. 2.2.0 (Softgenetics LLC, PA USA) to produce a binary matrix. Input files for subsequent analysis were obtained either by using Transformer-4 (Caujapé-Castells et al. 2011) or edited manually.

Several parameters were computed to estimate the genetic diversity at the population level. Number and proportion of polymorphic loci ($Frag_{poly}$) were computed using AFLP-SURV v. 1.0 (Vekemans 2002). The same software was used to calculate H_j (Nei's gene diversity), H_t (the total gene diversity, i.e. expected heterozygosity or gene diversity in the overall sample), H_w (the average gene diversity within populations). Allele frequencies were generated using the default Bayesian method with non-uniform prior distribution (Zhivotovsky 1999). The number of private fragments was examined by means of FAMD v. 1.25 (Schlüter & Harris 2006). The

frequency and distribution of rare bands, i.e. those present in less than twenty individuals on the whole data set, was calculated following Stehlik et al. (2001). The frequency-down-weighted marker values (D_W; Schönswetter & Tribsch 2005) were also calculated considering 19 individuals from every population (being n = 19 the minimum number of individuals investigated per population; one individual that did not show amplification was excluded from the analysis).

To examine the significance of differences in genetic diversity and fragment rarity parameters between groups of populations an analysis of variance (ANOVA) and the Sheffé's *post hoc* test were performed using STATISTICA v. 7 software package (Statsoft). To compute the effective allele number (n_e), Shannon's information index (*I*; Lewontin 1972) at the population level and the Nei's (1973) fixation index G_{st} at the species' level we used POPGENE v. 1.32 (Yeh et al. 2000).

To investigate the global genetic structure, a principal co-ordinate analysis (PCoA) was computed from a matrix of Nei & Li distances (following Nei & Li 1979) among individuals using FAMD 1.25 (Schlüter & Harris, 2006). The same software was employed to further analyze the relationships among populations by constructing a chord distance matrix (single-locus chord distance; Cavalli-Sforza & Edwards, 1967) from allele frequency data (estimated in a Bayesian framework with a non-uniform prior derived from among-locus information; Zhivotovsky, 1999) which was visualized by neighbor-joining (NJ) clustering; support for branches was assessed by means of 10,000 bootstrap replicates. The genetic distance of Cavalli-Sforza & Edward's was chosen because it seems to be the most efficient distance to obtain a correct tree topology in closely related species and recently diverged populations (Takezaki & Nei 1996).

To quantify the amount of genetic differentiation attributable to geographic and population subdivision, both hierarchical and non-hierarchical analysis of molecular variance with 1023 permutations were performed (AMOVA; Excoffier et al. 2005) using Arlequin v.3.5 software. To test the existence of correlations between genetic (linear) and log-transformed geographic distances a Mantel test with 9999 permutations was carried out with GenAlex v. 6.5 (Peakall & Smouse 2006, 2012). To further investigate the genetic population structure of *F. arrigonii*, a Bayesian model-based approach was used to assign individual genotypes into genetically structured groups, as proposed in Pritchard et al. (2000) and implemented in the software

Structure v. 2.3 (Pritchard & al. 2000; Falush et al. 2007). Twenty independent runs for each *K* (from 1 to 11) were performed using 50,000 burn-in periods and 100,000 MCMC (Markov Chain Monte Carlo) repetitions, using no prior population information and assuming correlated allele frequencies and admixture. The most accurate value of *K* was evaluated following Evanno et al. (2005) and using the software STRUCTURE HARVESTER (Earl 2012). The program CLUMPP v. 1.1.2 (Jakobsson & Rosenberg 2007) was used to determine the optimum alignment of clusters across individual runs for each *K*; outcomes from CLUMPP were imported into Distruct v. 1.1 (Rosenberg 2004) for viewing the individuals' assignment probabilities.

Results

The error rate based on phenotypic comparisons among replicated individuals (Bonin et al. 2004) amounted to 3.9%; one individual that did not show amplification was not included in the analysis. The final dataset consisted of 179 individuals from nine populations surveyed for AFLP variation and of 262 fragments in the range of 100- 500 bp, of which 241 were polymorphic overall across populations. All 179 individuals investigated had a unique profile.

Genetic diversity

The degree of polymorphism was high for all populations, ranging from 88.5% (SER) to 95.4% (BON) (Tab. 2). The effective number of alleles (n_e) ranged from 1.37 (SER) to 1.50 (CAC). The AFLP variation within populations, estimated as H_j, ranged from 0.275 (SER), to 0.349 (CAC); the average gene diversity within populations (H_w) was 0.317 and the total gene diversity (H_t) was 0.336. The lowest value for the Shannon's information index (I) was also found in SER (0.330) and the highest in CAC (0.423). No population-specific fragment was detected in any of the nine populations. The frequency-down-weighted marker values (D_w) and the mean number of rare markers per individual were highest in BON (32.35 and 1.63, respectively) and lowest in SER (25.11 and 0.68, respectively). Slightly significant differences were found between the Southern populations and the group TAV-CAC-BON (identified by NJ, PCoA and STRUCTURE analysis) for the parameters H_j (ANOVA followed by the Sheffé's *post hoc* test; $p = 0.031$) and n_e ($p = 0.020$).

Tab. 2. Estimates of genetic diversity and fragment rarity in the investigated populations of *F. arrigonii*.

Population	$Frag_{poly}$ (%)	n_e	H_j	D_W	$Frag_{rare}$	I
SER	224 (85.5)	1.37 (±0.36)	0.275 (±0.0100)	25.11	0.68	0.330
CAV	246 (93.9)	1.42 (±0.38)	0.313 (±0.0092)	30.88	1.10	0.370
BUG	232 (88.5)	1.41 (±0.39)	0.303 (±0.0098)	26.73	0.79	0.358
SMA	244 (93.1)	1.40 (±0.36)	0.303 (± 0.0089)	26.44	1.10	0.359
CAC	249 (95.0)	1.50 (±0.37)	0.349 (±0.0086)	28.64	0.74	0.423
TES	243 (92.7)	1.46 (±0.37)	0.333 (±0.0088)	27.54	1.10	0.403
BUD	242 (92.4)	1.41 (±0.38)	0.307 (±0.0093)	25.97	1.0	0.365
TAV	247 (94.3)	1.47 (±0.37)	0.336 (±0.0089)	28.31	0.74	0.406
BON	250 (95.4)	1.45 (±0.39)	0.332 (±0.0087)	32.35	1.63	0.380

$Frag_{poly}$ = number and proportion of polymorphic fragments at the 5% level; n_e = effective number of alleles ± standard deviation; H_j = Nei's heterozygosity ± standard error ; D_w = frequency-down-weighted marker values; $Frag_{rare}$ = mean number of rare fragments per individual; I = Shannon's information index.

Population structure

Neighbour-joining analysis returned three clusters: one constituted by TAV, CAC and BON, one by TES and BUD and one constituted by the remaining populations (southern group), the latter being subdivided into two subclusters, one formed by CAV and SER populations and one by BUG and SMA (Fig. 2). Bootstrap support was higher for the TES + BUD cluster (100), followed by SER + CAV (84) and BUG + SMA (60). PCoA analysis using Nei & Li distances (Fig. 3) failed to group individuals belonging to the same population; the first axis only explained 11.66% of the variation, the second 6.06%, the third 4.64%.

The genetic differentiation among populations (G_{st}) was 0.124. Analysis of molecular variance (AMOVA; Table 3) confirmed these latter estimates and complemented the results, revealing that most of the genetic variation (about 89% in both the non-hierarchical and the hierarchical analysis) resides within populations, while a very small, but significant proportion of the variation was explained by between-group differences: southern *versus* northern populations only 1.68%, southern populations *versus* TES + BUD *versus* CAC + BON + TAV 4.42%, south-western *versus* south-eastern *versus* TES + BUD *versus* CAC + BON + TAV 5.23%. The Mantel test involving geographic and genetic distances returned a significant result (r_{xy} = 0.107, P < 0.001), providing evidence for an isolation-by-distance pattern. Structure analysis (Fig. 1) suggested an optimal value for K = 3 (Fig. 4); however, all populations except BON showed considerable admixture (Fig. 1).

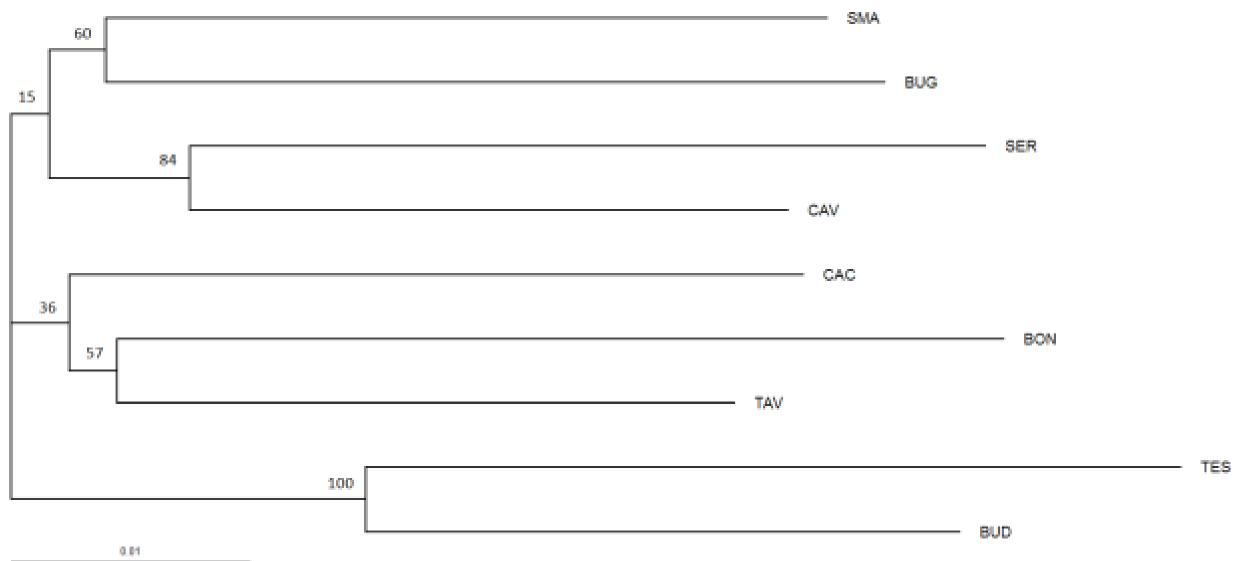

Fig. 2. Unrooted NJ tree based on Cavalli-Sforza's chord distance. Bootstrap values are indicated at each node based on 10,000 replicates.

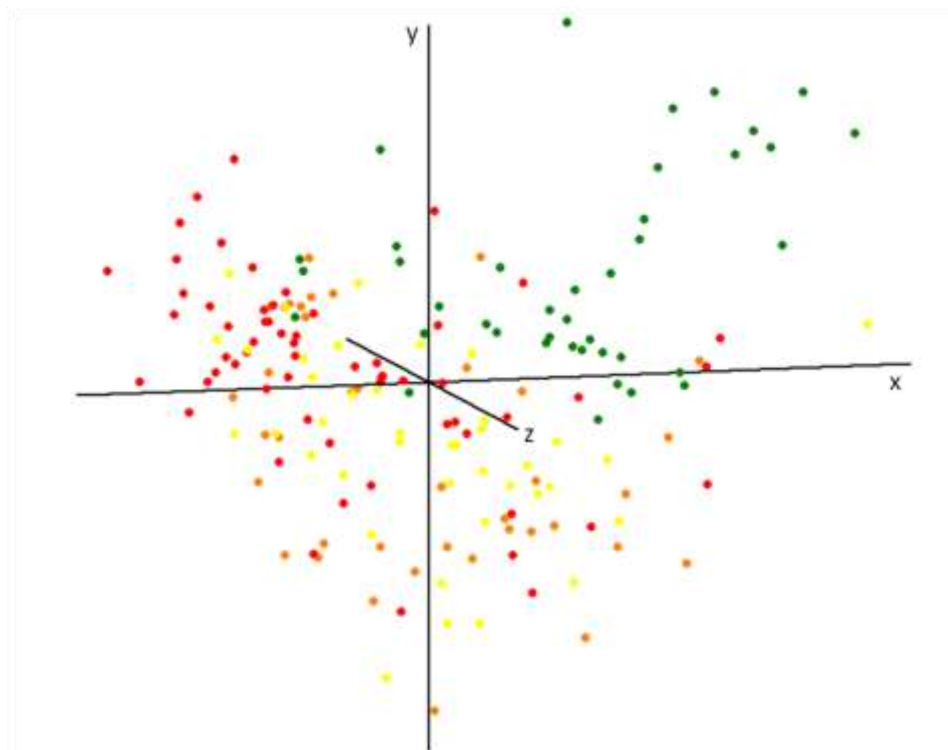

Fig. 3. PCoA based on Nei & Li distances. Yellow = SER + CAV; orange = BUG + SMA; red = CAC + BON + TAV; green = BUD + TES.

Tab. 3. Results of four analyses of molecular variance (AMOVA) of AFLP data (Squared Euclidean distance) from nine populations of *F. arrigonii*.

Grouping	N	Source of variation	d.f.	SS	Percentage of variance	*P*
No groups	9	Among populations	8	842	10.53	***
		Within populations	170	5359	89.47	
[CAC-BON-TAV-TES-BUD]	2	Among groups	1	151	1.68	***
[SER-CAV-BUG-SMA]		Among populations within groups	7	691	9.52	ns
		Within populations	170	5359	88.80	***
[CAC-BON-TAV] [TES-BUD]	3	Among groups	2	346	4.42	***
[SER-CAV-BUG-SMA]		Among populations within groups	6	495	7.20	**
		Within populations	170	5359	88.38	***
[CAC-BON-TAV] [TES-BUD]	4	Among groups	3	469	5.23	***
[SER-CAV] [BUG-SMA]		Among populations within groups	5	372	6.08	***
		Within populations	170	5359	88.69	***

d.f. = degrees of freedom; SS = mean sum of squares

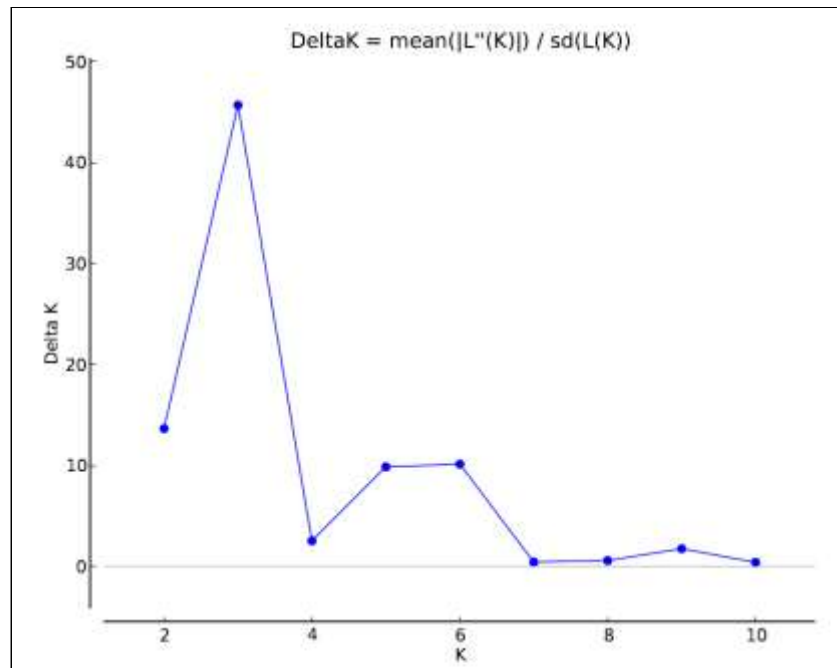

Fig. 4. DeltaK (ΔK) value calculated for assuming K=1-11 of nine populations of *F. arrigonii*.

Discussion

Genetic diversity

The analyses provided considerable information on the magnitude and pattern of genetic variation existing in nine natural populations covering the whole distribution of *F. arrigonii*. Overall, the results revealed high levels of genetic diversity and low levels of differentiation among populations. The high degree of genetic admixture observed in the analysis suggests that multiple connections exist between the currently fragmented populations.

First of all, results are clearly not in accordance with the general expectation that endemic species, and particularly island endemics exhibit low levels of genetic diversity (Frankham 1997, 1998). Species with large populations which naturally present outcrossing are generally thought to have a high level of genetic diversity, while this is generally thought to be reduced in small populations, and especially in endangered species. However, this is not always the case: actually, nowadays the relationship between rarity, population size and genetic diversity is not considered to be direct, and some works (e.g. Bataillon & Kirkpatrick 2000; Hedrick 2002) indicate that the genetic consequences of a small population size greatly depend on historical, evolutionary and reproductive characteristics. Moderate to high genetic diversity was found in other Mediterranean endemics, like *Centaurea horrida* Badarò (Mameli et al. 2008), *Rhamnus persicifolia* Moris (Bacchetta et al. 2011), *Brassica rupestris* Raf. (Raimondo et al. 2012), and *Femeniasia balearica* (J. J. Rodr.) Susanna (Vilatersana et al. 2007).

In 13 studies employing AFLP markers reviewed by Nybom (2004) the mean genetic variation within populations was 0.23, while it was much higher in *F. arrigonii* (0.317). The congener *F. loscosii* (endemic to the Iberian Peninsula) was studied by means of both allozymes (Pérez-Collazos & Catalán 2008) and AFLPs (Pérez-Collazos et al. 2009). In both cases the mean heterozygosity at the species level was lower (H_e = 0.125 and H_s = 0.171 with allozymes and AFLPs, respectively) than obtained in *F. arrigonii.* Some Sardinian populations of the widespread *F. communis* were also investigated by means of allozymes (Marchi et al. 2003), showing lower expected heterozygosity (ranging from 0.097 to 0.165).

The breeding system strongly influences genetic diversity (Charlesworth 2003; Loveless & Hamrick 1984). Nybom & Bartish (2000) concluded in their review that selfing *taxa* have a mean heterozygosity of around 0.09. In contrast, plant species with a mixed or outcrossing

breeding system show a heterozygosity of around 0.26, placing *F. arrigonii* clearly in this latter group. In a germination trial seeds of *F. arrigonii* (Bacchetta et al., unpublished data) exhibited high germination percentages (about 80%) with no dormancy and no particular pre-germination requirement and a high percentage of vital seeds, thus suggesting the potential presence of an efficient sexual reproduction. On the other hand, although specific pollinators have not been identified for *F. arrigonii*, insects have been observed to visit flowers of this species, mostly hymenopterans, coleopterans and dipterans (Bacchetta & Dettori, personal obs.). While pollen dispersal might represent an important connectivity factor between geographically closer populations (e.g. between CAV and SER, or TES and BUD), seed dispersal cannot be ruled out when considering major geographic distances. The latter can occur by either ectozoochory, thanks to the many bird species that either nest or use the sites as stopping areas during their migratory routes (especially *Phalacrocorax aristotelis dermarestii*, *Puffinus yelkouan*, *Laurus audouinii* and *L. cachinnans*); or by hydrochory, through sea current transport by floating.

Overall, northern populations were characterized by higher values for both the genetic diversity and the fragment rarity parameters, whereas values tended to get lower in Southern populations with the exception of CAV. Among the northern populations, the highest values for the genetic diversity parameters and the fragment rarity indexes were found in CAC and BON, respectively, suggesting that they may represent the current centers of *F. arrigonii* genetic diversity. This also indicates that it might be unlikely that the only population reported for Corsica (BON) results from the introduction of the plant from Sardinia and its subsequent naturalization, as firstly hypothesized by Paradis & Piazza (2004) and then reported by Jeanmonod & Gamisans (2007). Rather, the high values of genetic diversity support the alternative hypothesis of Paradis & Piazza (2004), which suggested that *F. arrigonii* was once more widespread than it is at present. Habitat deterioration or destruction, or the invasion of superior competitors could have led to its currently fragmented distribution. Further studies on the pollination biology and seed dispersal are needed in order to understand the factors that may explain the rarity and the currently fragmented distribution of this species.

<u>Population structure</u>

Island endemics usually exhibit high levels of spatial genetic structure; this pattern has been described in several cases, e.g. *Anchusa* sp. (Quilichini et al. 2004; Coppi et al. 2008) *Rhamnus*

persicifolia (Bacchetta & al. 2011) and *Aquilegia* sp. (Garrido et al. 2012) in Sardinia and Corsica; *Medicago citrina* (Juan et al. 2004) and *Senecio rodriguezii* (Molins et al. 2009) in the Balearic Islands; *Nigella arvensis* (Bittkau & Comes 2005) in the Aegean region; *Primula sieboldii* in Japan (Kitamoto et al. 2005).

Contrarily to the above-mentioned examples, *F. arrigonii* shows very little differentiation among populations, as shown by the G_{st} value, the AMOVA, the PCoA and the STRUCTURE analyses. This is somewhat surprising considering the particular location of the populations and the relatively large distance among some of them. The Mantel test indicates that there is a correlation between geographic and genetic distances, providing evidence for an isolation-by-distance pattern. This might probably be due to the relatively high distance between the northenmost and the southernmost populations.

Nevertheless, a weak pattern of genetic subdivision could be observed, and the neighbour-joining analysis indicated that the substrate might be an important factor in explaining it: all southern Sardinian populations clustered together but were furtherly subdivided into two subclusters formed by BUG + SMA (southwestern Sardinia, limestone substrate) and CAV + SER (southeastern Sardinia, granites). The Corsican and the northern Sardinian populations were divided into two clusters: TAV + BON + CAC (limestone substrate) and TES + BUD (granites).

In the Bayesian analysis carried out with STRUCTURE individuals were assigned to three different clusters (Fig. 1). Even if all populations showed a considerable level of admixture, the three genetic subgroups identified were in close agreement the overall topology of the neighbor-joining tree and the split roughly corresponded to individuals with positive versus negative values along the y axis of the PCoA plot. The close genetic association between BUD and TES is not surprising given their close proximity to one another. However, they are both geographically very close to BON (the Boniface Strait separates Corsica and Sardinia for only about 12 Km), and yet the analysis shows these populations to be genetically distinctive, with BON showing a stronger affinity for the more distant populations CAC and TAV. The biological significance of this is far from clear and the use of more robust genetic markers (e.g. microsatellites) and a deeper knowledge of the species biology is required to identify possible factors (other than substrate) that could explain the observed pattern.

Implications for conservation

The current study provides important base data for long-term conservation management of *F. arrigonii*. Despite its rarity, *F. arrigonii* has high genetic variability and it is far from being genetically depauperated. Currently, all the natural populations of *F. arrigonii* live in areas that are under regional or national protection from a legal point of view. Additionally, human activities are negligible in the most inaccessible sites (i.e. SER, CAV and BUD, which are uninhabited islets). However, the legal protection of the sites alone might not be so effective for preserving the populations, especially the smallest ones and those that live in coastal areas where touristic pressure is higher. The latter could face habitat reduction and increasing risks of genetic depauperation or extinction due to the combined effects of the fragmentation of populations, human impact and invasion of alien species, like many other coastal *taxa*. Consequently, active initiatives of *in situ* conservation, such as monitoring and limitation of invasive species, together with *ex situ* conservation of germplasm, should be undertaken. Future actions will thus be aimed at conserving the highest possible number of populations, with particular attention to the smallest ones to those that exhibit the highest genetic diversity.

Finally, it is inappropriate to consider conservation management initiatives from only neutral genetic data. Conservation strategies should involve several forms of data, including morphological traits, economic characters, chemical composition and, whenever possible, adaptive SNPs. Therefore, it is advisable that the representative sample of *F. arrigonii* is given synthetic considerations when an *in situ* or *ex situ* conservation strategy is developed.

References

Bacchetta G., Boscaiu M., Güemes J. 2001. Números cromosomáticos de plantas occidentales, 863-879. Anales del Jardín Botánico de Madrid 58: 341-342.

Bacchetta G., Fenu G., Mattana E., Zecca G., Grassi F., Casazza G., Minuto L. 2011. Genetic variability of the narrow endemic *Rhamnus persicifolia* Moris (Rhamnaceae) and its implications for conservation. Biochemical Systematics and Ecology 39: 477-484.

Bataillon T., Kirkpatrick M. 2000. Inbreeding depression due to mildly deleterious mutations in finite populations: size does matter. Genetical Research 75: 75-81.

Bittkau C., Comes H.P. 2005. Evolutionary processes in a continental island system: molecular phylogeography of the Aegean *Nigella arvensis* alliance (Ranunculaceae) inferred from chloroplast DNA. Molecular Ecology 14: 4065-4083.

Bocchieri E. 1988. *Silene valsecchiae* e *Ferula arrigonii*, due specie nuove della Sardegna. Bollettino della Società Sarda di Scienze Naturali 26: 305-310.

Bonin A., Bellemain E., Eidesen P.B., Pompanon F., Brochmann C., Taberlet P. 2004. How to track and assess genotyping errors in population genetics studies. Molecular Ecology 13: 3261-3273.

Caujapé-Castells J., Castellano J.J., Henríquez V., Ramos R., Sabbagh I., Quintana-Trujillo F.M., Rodríguez J.F. 2011. Transformer-4: a genotype matrix format translator. Jardín Botánico Canario "Viera y Clavijo"-Unidad Asociada CSIC, Instituto Tecnológico de Canarias, Jablesoft & Inventiaplus. Las Palmas de Gran Canaria, Spain. (http://www.demiurge-project.org/download_t4).

Cavalli-Sforza L.L., Edwards A.W.F. 1967 Phylogenetic analysis: models and estimation procedures. Evolution 32: 550-570.

Charlesworth D. 2003. Effects of inbreeding on the genetic diversity of populations. Philosophical Transactions of the Royal Society B 358: 1051-1070.

Conti F., Manzi A., Pedrotti F. 1997. Liste rosse regionali delle piante d'Italia. WWF Italia, Società Botanica Italiana, TIPAR Poligrafica Editrice, Camerino.

Coppi A., Mengoni A., Selvi F. 2008. AFLP fingerprinting of *Anchusa* (Boraginaceae) in the Corso-Sardinian system: Genetic diversity, population differentiation and conservation priorities in an insular endemic group threatened with extinction. Biological Conservation 141: 2000-2011.

Delair P., Kann N., Greene A.E. 1994. Synthesis (in ent-form) of a Novel Jalcaguaianolide from *Ferula arrigonii* Bocchieri. Journal of the Chemical Society. Perkin Transactions 13: 1651-1652.

Earl D.A., vonHoldt B.M. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4: 359-361.

Excoffier L., Laval G., Schneider S. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1: 47-50.

Evanno G., Regnaut S., Goudet J. 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. Molecular Ecology 14: 2611-2620.

Falush D., Stephens M., Pritchard J.K. 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Molecular Ecology Notes 7: 574-578.

Filippini M.H., Tomi F., Casanova J. 2000. Composition of the leaf oil of *Ferula arrigonii* Bocchieri. Flavour and Fragrance Journal 15: 195-198.

Frankham R. 1997. Do island populations have less genetic variation than mainland populations? Heredity 78: 311-327.

Frankham R. 1998. Inbreeding and extinction: island populations. Biological Conservation 12: 665-675.

Garrido J.L., Mattana E., Fenu G., Bacchetta G. 2012. Spatial genetic structure of *Aquilegia taxa* endemic to the island of Sardinia. Annals of Botany 109: 953-964.

Hedrick P.W. 2002. Letals in finite populations. Evolution 56: 654-657.

Jakobsson M, Rosenberg NA. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23: 1801-1806.

Jeanmonod D., Gamisans J. 2007. Flora Corsica. Edisud, Aix-en-Provence.

Juan A., Crespo M.B., Cowan R.S., Lexer C., Fay M.F. 2004. Patterns of variability and gene flow in *Medicago citrina*, an endangered endemic of islands in the western Mediterranean, as revealed by amplified fragment length polymorphism (AFLP). Molecular Ecology 13: 2679-2690.

Kitamoto N., Honjo M., Ueno S., Takenaka A., Tsumura Y., Washitani I., Ohsawa R. 2005. Spatial genetic structure among and within populations of *Primula sieboldii* growing beside separate streams. Molecular Ecology 14: 149-157.

Kurzyna-Młynik R., Oskolski A.A., Downie S.R., Kopacz R., Wojewodzka A., Spalik K. 2008. Phylogenetic position of the genus *Ferula* (Apiaceae) and its placement in tribe Scandiceae as inferred from nrDNA ITS sequence variation. Plant Systematics and Evolution 274: 47-66.

Lewontin R.C. 1972. The apportionment of human diversity. Evolutionary Biology 6: 381-398.

Loveless M.D., Hamrick J.L. 1984. Ecological determinants of genetic structure in plant populations. Annual Review of Ecology and Systematics 15: 65-95.

Mabberley D.J. 2008. Mabberley's plant book: a portable dictionary of plants (3rd ed.). Cambridge University Press, Cambridge, UK.

Mameli G., Filigheddu R., Binelli G., Meloni M. 2008. The genetic structure of the remnant populations of *Centaurea horrida* in Sardinia and associated islands. Annals of Botany 101: 633-640.

Marchi A., Appendino G., Pirisi I., Ballero M., Loi M.C. 2003. Genetic differentiation of two distinct chemotypes of *Ferula communis* (Apiaceae) in Sardinia (Italy). Biochemical Systematics and Ecology 31: 1397-1408.

Molins A., Mayol M., Rosselló J.A. 2009. Phylogeographical structure in the coastal species *Senecio rodriguezii* (Asteraceae), a narrowly distributed endemic Mediterranean plant. Journal of Biogeography 36: 1372-1383.

1973. Analysis of gene diversity in subdivided populations. Proceedings of the Natural Academy of Sciences of the United States of America 70: 3321-3323.

Nei M., Li W.H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the Natural Academy of Sciences of the United States of America 76: 5269-5273.

Nybom H. 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Molecular Ecology 13: 1143-1155.

Nybom H., Bartish I. 2000. Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. Perspectives in Plant Ecology, Evolution and Systematics 3: 93-114.

Paradis G., Piazza C. 2004. *Ferula arrigonii* en Corse: répartition, nombre d'individus and probabilità d'une introduction récente. Le monde des plantes 482: 15-17.

Peakall R., Smouse P.E. 2006. GENALEX 6: genetic analysis in Excel Population genetic software for teaching and research. Molecular Ecology Notes 6: 288-295.

Peakall R., Smouse P. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics 28: 2537-2539.

Pérez-Collazos E., Catalán P. 2008. Conservation genetics of the endangered Iberian steppe plant *Ferula loscosii* (Apiaceae). Plant Biology 10: 492-501.

Pérez-Collazos E., Sanchez-Gómez P., Jiménez J.F., Catalán P. 2009. The phylogeographical history of the Iberian steppe plant *Ferula loscosii* (Apiaceae): a test of the abundant-centre hypothesis. Molecular Ecology 18: 848-861.

Poli F., Appendino G., Sacchetti G., Ballero M., Maggiano N., Ranelletti F.O. 2005. Antiproliferative Effects of Daucane Esters from *Ferula communis* and *F. arrigonii* on Human Colon Cancer Cell Lines. Phytotherapy Research 19: 152-157.

Quilichini A., Debussche M., Thompson J.D. 2004. Geographic differentiation of morphological traits and isozymes in the Mediterranean island endemic *Anchusa crispa*: implications for the conservation of a protected species. Biological Conservation 118: 651-660.

Pritchard J.K., Stephens M., Donnelly O. 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945-959.

Raimondo F. M., Scialabba A., Zecca G., Grassi F., Casazza G., Minuto L. 2012. Genetic diversity in the endangered Sicilian endemic *Brassica rupestris*: Proposals for a conservation strategy. Plant Biosystems 146: 847-856.

Rosenberg N.A. 2004. DISTRUCT: a program for the graphical display of population structure. Molecular Ecology Notes 4: 137-138.

Sánchez-Cuxart A., Bernal Cid M. 1998. Estudi biosistemàtic de les poblacions de *Ferula communis* del NE de la península ibèrica i de les illes balears. Acta Botanica Barcinonensia 45: 217-239.

Schlüter P.M., Harris S.A. 2006. Analysis of multilocus fingerprinting data sets containing missing data. Molecular Ecology Notes 6: 569-572.

Schönswetter P., Tribsch A. 2005. Vicariance and dispersal in the alpine perennial *Bupleurum stellatum* L. (Apiaceae). Taxon 54: 725-732.

Stehlik I., Schneller J.J., Bachmann K. 2001. Resistance or emigration: response of the high alpine plant *Eritrichium nanum* (L.) Gaudin to the ice age within the Central Alps. Molecular Ecology 10: 357-370.

Takezaki N., Nei, M. 1996. Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. Genetics 144: 389-399.

Vekemans X. 2002. AFLP-SURV version 1.0. Distributed by the author. Laboratoire de Génétique et Ecologie Végétale, Université Libre de Bruxelles, Belgium.

Vilatersana R., Susanna A., Brochmann C. 2007. Genetic variation in *Femeniasia* (Compositae, Cardueae), an endemic and endangered monotypic genus from the Balearic Islands (Spain). Botanical Journal of the Linnean Society 153: 97-107.

Vos P., Hogers R., Bleeker M., Reijans M., Van De Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407-4414.

Yeh F.C., Yang R., Boyle T.J., Ye Z., Xiyan J.M. 2000. PopGene32. Microsoft Window-based freeware for population genetic analysis, version 1.32. Molecular Biology and Biotechnology Centre. University of Alberta, Edmonton, Alberta, Canada.

Zhivotovsky L.A. 1999. Estimating population structure in diploids with multilocus dominant DNA markers. Molecular Ecology 8: 907-913.

Chapter 2

The genetic diversity and structure of the *Ferula communis* L. complex (Apiaceae) in the Tyrrhenian area

Caterina Angela Dettori[1], Simona Sergi[2], Salvatore Brullo[3], Pere Fraga i Arguimbau[4], Elena Tamburini[2], Gianluigi Bacchetta[1].

[1] *Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente - Università degli Studi di Cagliari. Viale S. Ignazio da Laconi, 13 - I-09123 Cagliari, Italy.*

[2] *Sezione di Microbiologia e Virologia, Dipartimento di Scienze Biomediche - Università degli Studi di Cagliari. Sesta strada Ovest, Z.I. Macchiareddu - I-09010 Uta, Italy.*

[3] *Dipartimento di Scienze Biologiche, Geologiche e Ambientali - Università degli Studi di Catania. Via A. Longo 19 - I-95125 Catania, Italy.*

[4] *Departament d'Economia i Medi Ambient, Consell Insular de Menorca, Minorca, Balearic Islands, Spain.*

Abstract

The giant fennel group is a circum-Mediterranean complex characterized by a great morphological variability and comprising several species and subspecies with uncertain taxonomic boundaries. In this work AFLP variation has been used to: i) assess how the patterns of molecular differentiation among 15 populations from four different Tyrrhenian islands (i.e. Minorca, Corsica, Sardinia and Sicily) and the Italian Peninsula, belonging to different *taxa* (i.e. *F. communis* subsp. *communis*, *F. c.* subsp. *cardonae*, *F. c.* subsp. *glauca* and *F. arrigonii*), are related with geographic boundaries and current taxonomic affiliations; ii) compare the levels of genetic diversity of the Corso-Sardinian endemic *F. arrigonii* with that of the widespread congener *F. communis* s.l. Results indicate that *F. arrigonii* and *F. c.* subsp. *glauca* are well differentiated with respect to all other populations, which form an homogeneous group irrespective of the geographic provenance. Despite its endemicity, the values of genetic diversity for *F. arrigonii* were similar and not significantly different from those of *F. communis* s.l.

Keywords: *Ferulinae*, AFLP, Mediterranean basin, genetic differentiation, Tyrrhenian Islands.

Introduction

The genus *Ferula* L. (Apiaceae) is represented by 172 perennial herbaceous species occurring from central Asia westward throughout the Mediterranean region to northern Africa (Mabberley 2008). The genus is monophyletic and it belongs to the *Scandiceae* tribe and to the *Ferulinae* subtribe (Kurzyna-Młynik et al. 2008). The same authors also confirmed the origin of the Mediterranean group from Asian ancestors, this hypothesis being also supported by the general westward colonization of Asian steppe plants (Frantzke et al. 2004).

The focus of this work is on the *taxa* inhabiting the Tyrrhenian Islands and the Tyrrhenian coasts. Two species are reported for this area: the Corso-Sardinian endemic *F. arrigonii* Bocchieri and the widespread *F. communis* L., which comprises the two widespread *F. c.* subsp. *communis* L. and *F. c.* subsp. *glauca* (L.) Rouy & Camus, and the Minorcan endemic *F. c.* subsp. *cardonae* Sánchez Cuxart & M. Bernal. *F. arrigonii* was described relatively recently as a distinct species with respect to *F. communis* L., from which it differs mainly by its smaller size and the dense and late flowering (Bocchieri 1988). *F. c.* subsp. *communis* and *F. c.* subsp. *glauca* were originally described as autonomous species, and were later considered as being subspecies (Rouy & Camus 1901; Thellung 1926; Cannon 1968; Pignatti 1982; Sánchez-Cuxart & Bernal 1998; Bolòs de & Vigo 1984), varieties (Bertoloni 1837; Paoletti 1900; Fiori 1925) or even as being partially or totally synonymous (Caruel 1889). Other authors have supported their specific status because of the several morphological, ecological, phenological, karyological, chorological and phytochemical differences between the two *taxa* (Arcangeli 1882, 1894; Hayek 1927; Brilli Cattarini & Gubellini 1987; Anzalone et al. 1991; Conti et al. 2005; Jeanmonod & Gamisans 2007). Both have morphological variants that have received a specific name in the past and that are now considered synonyms, e.g. *F. nodiflora* L., *F. brevifolia* Link, *F. neapolitana* Ten., *F. lobeliana* Vis. Sánchez-Cuxart & Bernal (1998) described a new subspecies from the island of Minorca (i.e. *F. c.* subsp. *cardonae*) based on morphological, phenological and karyological characteristics. In the same study they found great similarities between plants from Minorca and Sicily and the authors thus hypothesized they could belong to the same *taxon*. However, no specimens from Corsica and Sardinia were included in this work, and at present there are no available comparative studies comprising plants from the whole Tyrrhenian area. None of these *taxa* has ever been studied from a molecular point of view with the only exception being represented by *F. c.* subsp. *communis* in Sardinia, which was investigated by means of allozymes (Marchi et al. 2003). Moreover, apart from this latter study, the only molecular data produced up to date regard the close Iberian endemic *F. loscosii* (Lange) Willk, which was investigated by

means of both allozymes (Pérez-Collazos & Catalan 2008) and AFLP markers (Perez-Collazos et al. 2009).

An important issue when investigating species complexes such as *Ferula* gr. *communis* is the comparison of the levels of genetic diversity between endemic and widespread species, especially when using a novel type of marker for which no previous term of comparison exists (Ellis et al. 2006). The general expectation is that endemic species, and particularly island endemics (Frankham 1997), exhibit lower levels of genetic diversity than widespread ones (Karron 1987; Hamrick & Godt 1989, 1996; Cole 2003). Upon reviewing the characteristics of endemic *taxa*, Kruckeberg & Rabinowitz (1985) concluded that future research should include more ‘‘comparative studies to contrast the biology of rare *taxa* with those of related common ones”. In the last two decades, many researchers have included widespread congeners when examining the genetic variation of rare species. In some cases endemic species have shown to have equivalent or higher levels of genetic diversity compared to their more widely distributed congeners (Karron 1988; Purdy & Bayer 1996; Gitzendanner & Soltis 2000; Dodd & Helenurm 2002; Ellis et al. 2006). Furthermore, in some genera both rare and widespread species turned out to have either very low or very high, but similar, levels of polymorphism (e.g., Young & Brown 1996; Whittall et al. 2010), thus suggesting that the classic view that rare species have less genetic variability than more widespread ones may be an overgeneralization (Mateu-Andrés 2004).

In this study, AFLP (Amplified Fragment Length Polymorphism) markers were used to address the following issues: i) which is the level of genetic diversity of *F. communis* and its subspecies (hereafter *F. communis* s.l., unless otherwise specified) in the Tyrrhenian area? ii) are the patterns of molecular variation spatially structured and how are they related with geographic boundaries and current taxonomic affiliations? iii) which are the relationships of *F. c.* subsp. *communis* with the Corso-Sardinian endemic *F. arrigonii*? iv) are the levels of genetic variability of *F. arrigonii* and *F. c.* subsp. *communis* comparable?

Materials and methods

Sampling sites and plant material

Leaf material was collected from 15 populations (Tab. 1, Fig. 1). Of these, three populations of *F. c.* subsp. *communis* were located in Sicily, three in Sardinia, two in Corsica and one from central Italy; one population of *F. c.* subsp. *cardonae* and one of were sampled in Minorca and one of *F. c.* subsp. *glauca* in central Italy. As regards *F. arrigonii*, one population was sampled in Corsica and two in Sardinia. Leaf material was collected insummer 2010 and 2011, dried with silica gel and stored at room temperature. Sampling of plants was done throughout the populations in order to cover the whole occupied area and to minimize sampling of related individuals; 12-20 individuals per population were analyzed.

Tab. 1. Sampling locations.

Population	*Taxon*	Code	Geographical location	Coordinates	Mean elevation (m a.s.l.)	N_i
Bonifacio	*F. arrigonii*	BON	Corsica	41° 23' N - 9° 09' E	25	20
Isola dei Cavoli	*F. arrigonii*	CAV	Sardinia	39° 05' N - 9° 31' E	15	20
Capo San Marco	*F. arrigonii*	SMA	Sardinia	39° 52' N - 8° 26' E	25	20
Restonica	*F. c.* subsp. *communis*	RES	Corsica	42° 15’ N - 09° 05’ E	830	14
Agheri	*F. c.* subsp. *communis*	AGH	Corsica	42° 07’ N - 9° 17’ E	520	15
Monte Crasta	*F. c.* subsp. *communis*	CRA	Sardinia	40° 21' N - 8° 40' E	540	14
Bindua	*F. c.* subsp. *communis*	BIN	Sardinia	39°17' N - 8°29' E	110	15
Monte Albo	*F. c.* subsp. *communis*	ALB	Sardinia	40° 27’ N – 9° 31’ E	1050	12
Monte Pizzuta	*F. c.* subsp. *communis*	PIZ	Sicily	37° 59' N - 13° 15' E	1065	14
Caltagirone	*F. c.* subsp. *communis*	CAL	Sicily	37° 15' N - 14° 30' E	480	14
Rometta	*F. c.* subsp. *communis*	ROM	Sicily	38° 09' N - 15° 24' E	450	12
Latina	*F. c.* subsp. *communis*	LAT	Peninsular Italy	41° 45' N - 12° 26' E	70	15
Carretera des Grau	*F. c.* subsp. *communis*	GRA	Minorca	39° 54' N - 4° 14' E	20	15
Cala en Blanes	*F. c.* subsp. *cardonae*	BLA	Minorca	40° 00' N - 3° 49' E	30	13
Monte Calvi	*F. c.* subsp. *glauca*	CLV	Peninsular Italy	43° 05' N - 10° 36' E	230	15

N_i = number of individuals analyzed.

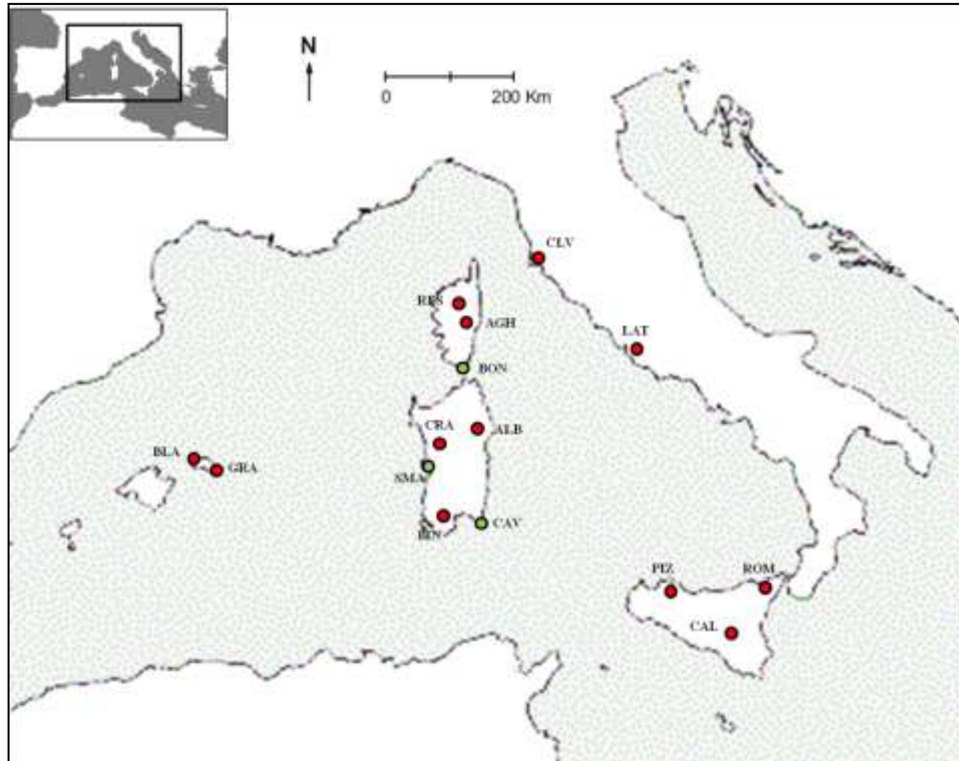

Fig. 1. Geographical location of the populations under study. Red dots = *F. communis* s.l.;

green dots = *F. arrigonii*

<u>AFLP analyses</u>

Genomic DNA was extracted from 20 mg of silica gel-dried leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Italy) following the manufacturer’s protocol. The AFLP (Amplified Fragment Length Polymorphism) technique was chosen to carry out the study because of its high reproducibility and no previous requirements of knowledge on DNA sequences. The original protocol by Vos et al. (1995) was followed with slight modifications.

A total of 200 ng of genomic DNA was digested in a total volume of 20 µL for 2 h at 37°C with 5 U of *Eco*RI and 5 U of *Mse*I (New England Biolabs), followed by a 20 min enzyme heat inactivation at 65°C. Digestion products were then ligated for 2 h at 20°C by adding 5 pmol of *Eco*RI adapter and 50 pmol of *Mse*I adapter (Sigma-Genosys, Italy) and 1 U of T4 DNA ligase (Fermentas). 5 µL of 5-fold diluted ligated product were used as template in the preamplification step; the reaction mixture contained 10 pmol of *Eco*RI (+A)- primer and *Mse*I (+C)- primer (Sigma-Genosys, Italy), 1X PCR reaction buffer, $MgCl_2$ 1.5 mM, dNTPs 0.2 mM (Sigma-Aldrich, Italy) and 1.4 U of *Taq* polymerase (Fermentas) in a final volume of 20 µL. The

preamplification thermocycler profile was 20 cycles of 94°C for 30 s, 56°C for 60 s and 72°C for 60 s.

A screening of 24 primer combinations was performed on 8 individuals. Two combinations giving the more informative and clearest readable fragment profiles (*Eco*RI+ACC with *Mse*I+CAC and *Eco*RI+AAT with *Mse*I+CAG). The *Eco*RI primers were labeled with 6FAM and HEX, respectively). Each selective amplification reaction mixture contained 1 μL of undiluted preamplification product, 1X PCR reaction buffer, $MgCl_2$ 1.5 mM, 0.2 mM dNTPs (Sigma-Aldrich, Italy), 1 pmol of *Eco*RI-NNN primer, 5 pmol of *Mse*I-NNN primer (Sigma-Genosys, Italy) and 1.4 U of *Taq* polymerase (Fermentas). The selective amplification thermocycle profile was: 13 cycles of 94°C for 30 s, 65°C for 30 s (with a touchdown of -0.7°C per cycle), 72°C for 1 min, followed by 23 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. PCR products were run on an ABI310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with an internal size standard (GeneScan-500 ROX, PE Applied Biosystems).

To assess the reproducibility of the analysis the whole procedure (from DNA extraction to capillary electrophoresis) was repeated for 16 samples. The error rate was calculated as the number of phenotypic differences related to the total number of phenotypic comparisons (Bonin et al. 2004).

Data analyses

In order to avoid excessive fragment size homoplasy (Vekemans et al. 2002) only fragments between 150 and 500 bp were scored by means of GeneMarker v. 2.2.0 (Softgenetics LLC, PA USA) to produce a binary matrix. Input files for subsequent analysis were obtained either by using Transformer-4 (Caujapé-Castells et al. 2011) or edited manually.

Several parameters were computed to estimate the genetic diversity at the population level. Number and proportion of polymorphic loci ($Frag_{poly}$) were computed using AFLP-SURV v. 1.0 (Vekemans 2002). The same software was used to calculate H_j (Nei's gene diversity), H_t (the total gene diversity, i.e. expected heterozygosity or gene diversity in the overall sample), H_w (the average gene diversity within populations). Allele frequencies were generated using the default Bayesian method with non-uniform prior distribution (Zhivotovsky 1999). The number of private fragments ($Frag_{priv}$) was examined by means of FAMD v. 1.25 (Schlüter & Harris 2006)

and considering 12 individuals from every population (being n = 12 the minimum number of individuals investigated per population) . The frequency and distribution of rare bands, i.e. those present in less than twenty individuals on the whole data set, was calculated following Stehlik & al. (2001). POPGENE v. 1.32 (Yeh et al. 2000) was used to compute the effective allele number (n_e) and Shannon's information index (*I*; Lewontin 1972) at the population level. Analysis of variance (ANOVA) and Sheffé's *post hoc* test were used to examine the significance of differences in genetic diversity and fragment rarity parameters between *taxa* based on linear model using STATISTICA v. 7 software package (Statsoft).

To explore the global genetic structure, a principal co-ordinate analysis (PCoA) was computed from a matrix of Nei & Li distances (following Nei & Li 1979) among individuals using FAMD 1.25 (Schlüter & Harris, 2006). The same software was employed to further analyze the relationships among populations by constructing a chord distance matrix (single-locus chord distance; Cavalli-Sforza & Edwards, 1967) from allele frequency data (estimated in a Bayesian framework with a non-uniform prior derived from among-locus information; Zhivotovsky 1999) which was visualized by neighbor-joining (NJ) clustering; support for branches was assessed by means of 1,000 bootstrap replicates.

To quantify the amount of genetic differentiation attributable to geographic and population subdivision, both hierarchical and non-hierarchical analysis of molecular variance with 1023 permutations were performed (AMOVA; Excoffier et al. 2005) using Arlequin v.3.5 software, the corresponding *F*-statistics were estimated: F_{st} (general fixation index), F_{ct} (*F*-statistic among regions) and F_{sc} (*F*-statistic among populations within regions). To further investigate the population structure, a Bayesian model-based approach was used, as proposed in Pritchard et al. (2000) and implemented in the software Structure v. 2.3 (Pritchard et al. 2000; Falush et al. 2007), to assign the genotypes into genetically structured groups. Twenty independent runs for each *K* (from one to 15) were performed using 50,000 burn-in periods and 100,000 MCMC (Markov Chain Monte Carlo) repetitions, using no prior population information and assuming independent allele frequencies and admixture. The same analysis was carried out using two partial datasets, one comprisingall populations of *F. communis* s.l. and one including only the populations of *F. c.* subsp. *communis* and *F. c.* subsp. *cardonae*. The most accurate value of *K* was evaluated following Evanno & al. (2005) and using the software STRUCTURE HARVESTER (Earl 2012). The program CLUMPP v. 1.1.2 (Jakobsson & Rosenberg 2007) was used to determine the optimum alignment of clusters across individual runs for each *K*; outcomes

from CLUMPP were imported into Distruct v. 1.1 (Rosenberg 2004) for viewing the individuals' assignment probabilities

Results

The information obtained by the analysis of the profiles is summarized in Tab. 2. The error rate based on phenotypic comparisons among replicated individuals amounted to 3.3%. The final dataset consisted of 228 individuals from 15 populations, generating 247 fragments in the range of 150-500 bp, of which 237 (96%) were polymorphic. The degree of polymorphism was relatively high for all populations, ranging from 71.3% (LAT) to 88.7% (BLA). The effective number of alleles (n_e) ranged from 1.29 (CLV) to 1.43 (BIN). Genetic variation within populations, estimated as H_j, ranged from 0.233 (CLV), to 0.313 (BLA); the average gene diversity within populations (H_w) was 0.276 and the total gene diversity (H_t) was 0.325. The lowest value for the Shannon's information index (I) was found in PIZ (0.261) and the highest in BIN (0.371). Only one allele exclusive to CLV was detected.

When grouping populations according to taxonomic affiliations a total of 12 and 10 exclusive fragments were reported for *F. arrigonii* and *F. communis* s.l., respectively, of which none was fixed. However, when grouping *F. communis* individuals into its subspecies, no private fragments was detected. There were no fragments exclusive to any particular island or group of islands. The mean number of rare markers per individual was higher in CLV (2.27) and lower in AGH (0.53). A significant difference was found between *F. c.* subsp. *glauca* and all other populations of *F. communis* for the $Frag_{rare}$ parameter ($P < 0.05$ by One-Way ANOVA followed by the Sheffé's *post hoc* test); all other comparisons were non-significant ($P > 0.05$).

Tab. 2. Estimates of genetic diversity and fragment rarity in the investigated populations of *Ferula*.

Population	*Taxon*	$Frag_{poly}$ (%)	n_e	H_j	$Frag_{rare}$	$Frag_{priv}$	I
BON	*F. arrigonii*	201 (81.4)	1.39 (±0.39)	0.280 (±0.0111)	1.85	0	0.332
CAV	*F. arrigonii*	197 (79.8)	1.37 (±0.38)	0.267 (±0.0111)	1.65	0	0.325
SMA	*F. arrigonii*	196 (79.4)	1.35 (±0.35)	0.259 (±0.0107)	1.48	0	0.315
RES	*F. c.* subsp. *communis*	194 (78.5)	1.37 (±0.38)	0.280 (±0.0107)	1.00	0	0.318
AGH	*F. c.* subsp. *communis*	194 (78.5)	1.36 (±0.37)	0.274 (±0.0106)	0.53	0	0.318
CRA	*F. c.* subsp. *communis*	212 (85.8)	1.40 (±0.38)	0.299 (±0.0108)	1.00	0	0.347
BIN	*F. c.* subsp. *communis*	197 (79.9)	1.43 (±0.38)	0.306 (±0.0110)	1.00	0	0.371
ALB	*F. c.* subsp. *communis*	204 (82.6)	1.39 (±0.38)	0.295 (±0.0110)	1.67	0	0.335
PIZ	*F. c.* subsp. *communis*	179 (72.5)	1.29 (±0.36)	0.235 (±0.0109)	1.07	0	0.261
CAL	*F. c.* subsp. *communis*	190 (76.9)	1.34 (±0.37)	0.270 (±0.0106)	0.64	0	0.302
ROM	*F. c.* subsp. *communis*	218 (88.3)	1.36 (±0.36)	0.297 (±0.0098)	1.25	0	0.319
LAT	*F. c.* subsp. *communis*	176 (71.3)	1.33 (±0.38)	0.252 (±0.0111)	1.20	0	0.287
GRA	*F. c.* subsp. *communis*	187 (75.7)	1.36 (±0.37)	0.272 (±0.0109)	0.87	0	0.322
BLA	*F. c.* subsp. *cardonae*	219 (88.7)	1.41 (±0.39)	0.313 (±0.0104)	1.38	0	0.353
CLV	*F. c.* subsp. *glauca*	181 (73.3)	1.29 (±0.34)	0.233 (±0.0107)	2.27	1	0.269

$Frag_{poly}$ = number and proportion of polymorphic fragments at the 5% level; n_e = effective number of alleles ± standard deviation; H_j = Nei's heterozygosity ± standard error ; $Frag_{rare}$ = mean number of rare fragments per individual; $Frag_{rpriv}$= number of private fragments per populations; I = Shannon's information index.

Neighbour-joining analysis returned two main clusters: a first one formed by the populations of *F. arrigonii* (CAV, BON, SMA) with 100% bootstrap support and a second one constituted by the populations of *F. c.* subsp. *communis* and *F. c.* subsp. *cardonae*., while *F. c.* subsp. *glauca* (CLV) was separated from all other populations with low support. The group formed by *F. c.* subsp. *communis* and *F. c.* subsp. *cardona*e showed a certain amount of substructuring, with ROM and CAL having 94% support and RES and AGH having 64%. (Fig. 2). The same three main groupings were evident in the PCoA: a first one formed by all individuals of *F. communis* s.l. except those belonging to *F. c.* subsp. *glauca* (CLV), which constituted a separate cluster, as did those of *F. arrigonii* (BON, CAV, SMA). The first axis explained 12.37% of the variation, the second 9.75%, the third 4.98% (Fig. 3).

The results of AMOVA analysis are presented in Table 3. Of the total genetic variation partitioned in the 15 *Ferula* populations, 25.74% was attributed to differences among populations and 74.26% to differences within populations. The percentage of variation among regions was maximised when grouping the populations of *F. arrigonii* (BON, CAV, SMA) vs. the population of *F. c.* subsp. *glauca* (CLV) vs. all other populations (Tab. 3); further subdivisions according to taxonomic affiliations and/or geographic location had a negligible effect on the partitioning of genetic variation.

The STRUCTURE analyses were coherent with previous results. A sharp signal was found at K = 2 (Fig. 4), the two clusters corresponded to individuals of *F. arrigonii* (BON, CAV snd SMA) and *F. communis* s.l. (all other populations), respectively (Fig. 5). RES, AGH, CRA, BIN and ALB populations (*F. c.* subsp. *communis* from Corsica and Sardinia) showed a slight degree of admixture with the cluster formed by BON, CAV and SMA (*F. arrigonii*). When individuals of *F. communis* s.l. were considered alone the best K was 2, the first cluster corresponded to individuals of *F. c.* subsp. *glauca* (CLV), while the second one comprised all other individuals. When excluding CLV population the best K was again 2, the first cluster comprised the individuals from Sicily (PIZ, CAL, ROM), Minorca (BLA, GRA) and central Italy (LAT), while the second one comprised the individuals from Corsica (RES, AGH) and Sardinia (BIN, CRA, ALB), with only CRA population showing a slight degree of admixture with the first cluster.

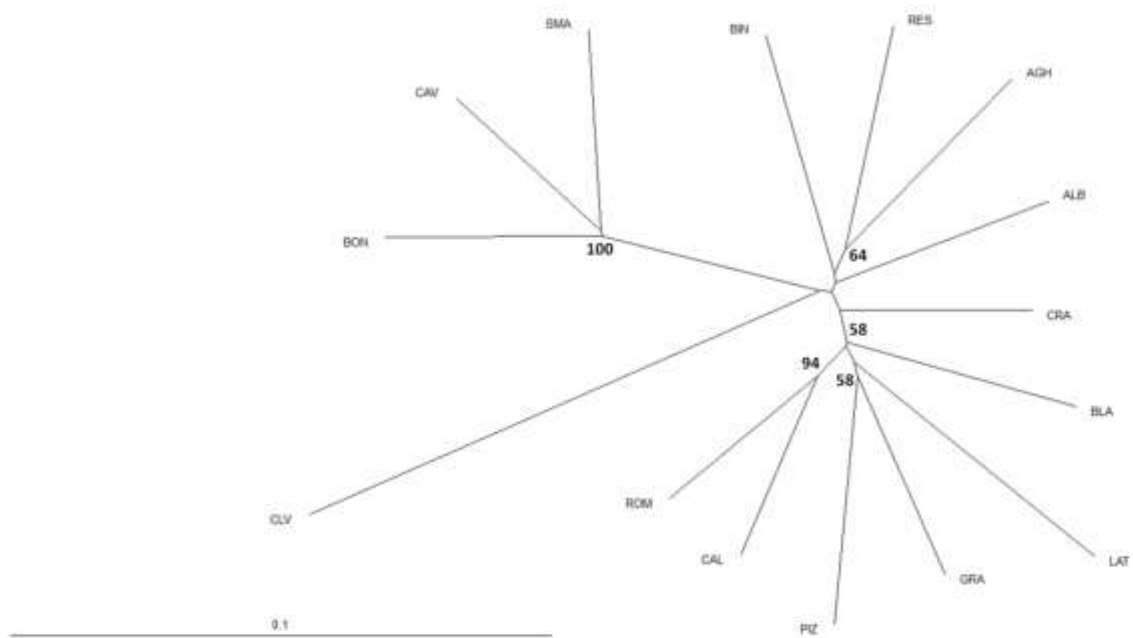

Fig. 2. Unrooted NJ tree based on Cavalli-Sforza's chord distance. Bootstrap values above 50% are indicated based on 1,000 replicates.

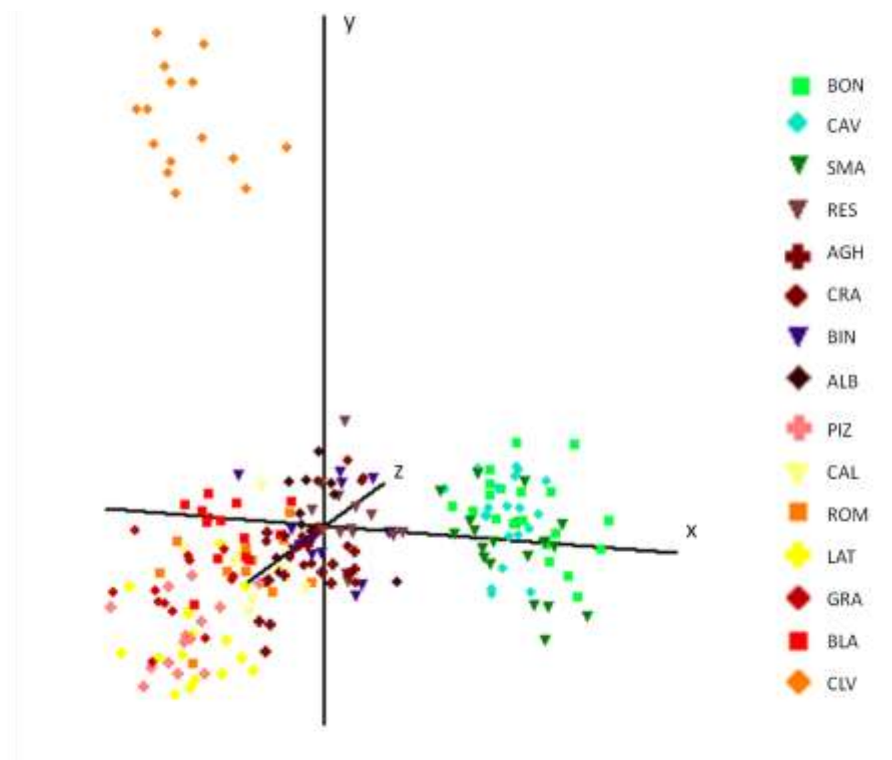

Fig. 3. PCoA based on Nei & Li distances.

Tab. 3. Results of three analyses of molecular variance (AMOVA).

Grouping	N	Source of variation	d.f.	SS	Percentage of variance	Fixation index
						F_{st} = 0.257 ***
No groups	15	Among populations	14	2363	25.74	
		Within populations	213	5745	74.26	
						F_{st} = 0.324 ***
[CLV] [CAV-BON-SMA]	3	Among groups	2	1044	18.56	F_{ct} = 0.185 ***
[CRA-ALB-BIN-RES-AGH		Among populations within groups	12	1318	13.92	F_{sc} = 0.170 ***
GRA-BLA-PIZ-CAL-ROM-LAT]		Within populations	213	5745	67.52	
						F_{st} = 0.271 ***
[CAV-BON-SMA] [CRA-ALB-BIN]	7	Among groups	6	1670	16.56	F_{ct} = 0.165***
[RES-AGH] [GRA-BLA] [LAT] [CLV]		Among populations within groups	8	693	10.55	F_{sc} = 0.126 ***
[PIZ-CAL-ROM]		Within populations	213	5745	72.89	

d.f. = degrees of freedom; SS = mean sum of squares; general fixation index (F_{st}), fixation index for the region (F_{ct}), and population within region (F_{sc}) level are shown.

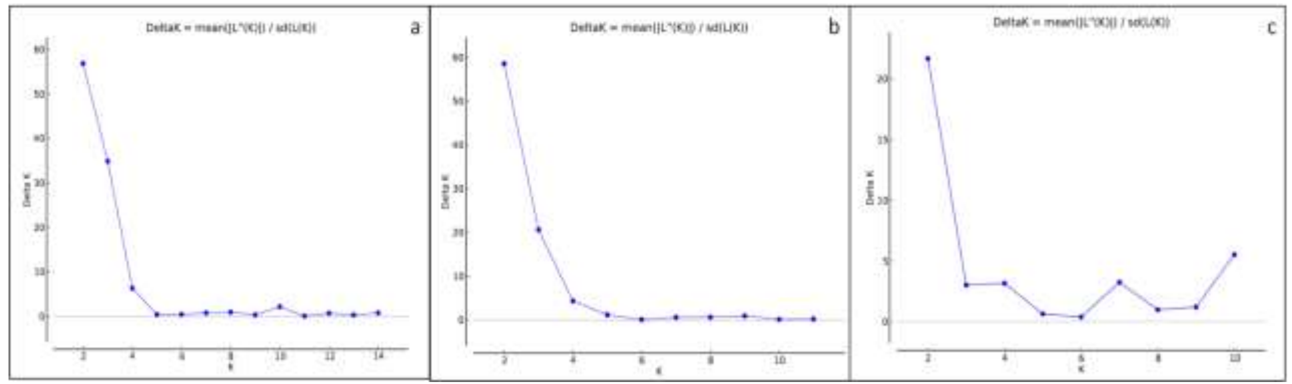

Fig. 4. DeltaK (ΔK) value calculated for assuming: a) K=1-15 of 3 populations of *F. arrigonii* and 12 of *F. communis* s.l. (b) K=1-12 of 12 populations of *F. communis* s.l.; c) K=1-11 of 10 populations of *F. c.* subsp. *communis* and 1 of *F. c.* subsp. *cardonae*.

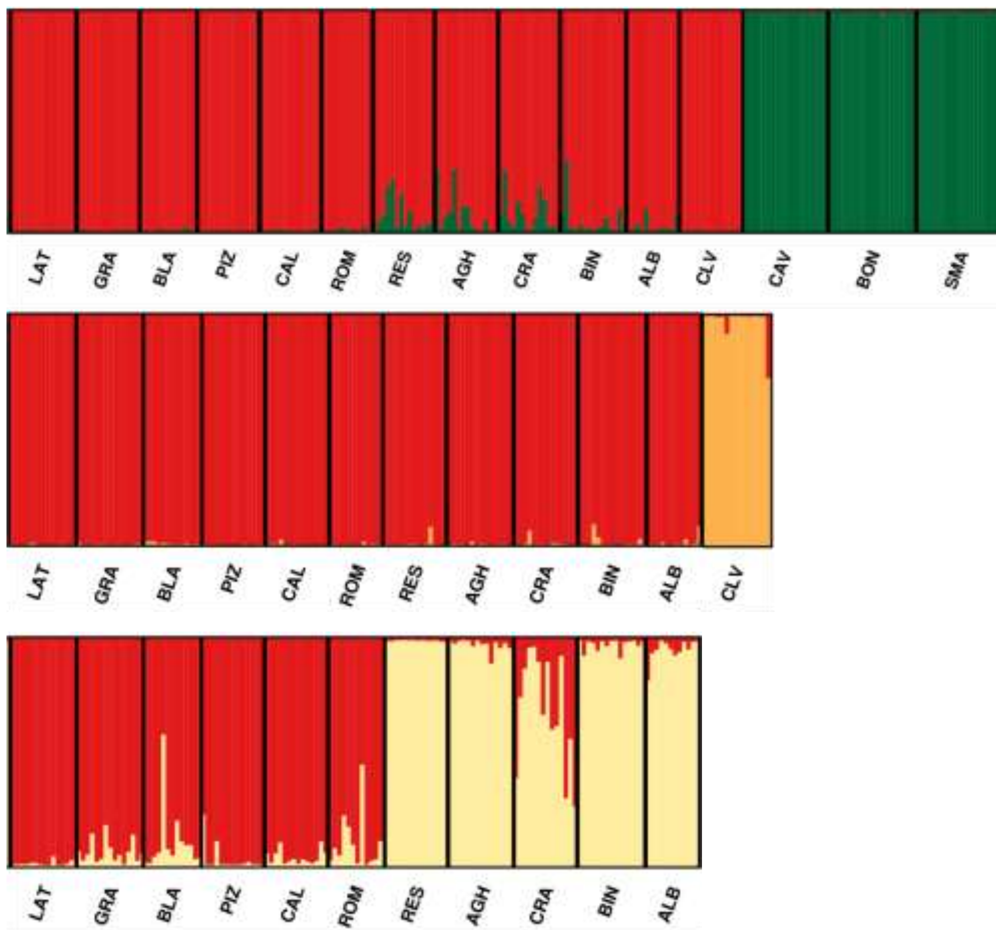

Fig. 5. Results of the STRUCTURE analysis. Each vertical bar represents an individual; black lines delimit sites. The diagram was redrawn from STRUCTURE (see Materials and Methods for further datails). a = results of the analysis considering individuals of *F. communis* s.l. and *F. arrigonii*; b = results of the analysis considering only individuals of *F. communis* s.l.; c = results considering only individuals of *F. c.* subsp. *communis* and *F. c.* subsp. *cardonae*.

Discussion

The present study using AFLP analysis provided information on the magnitude and spatial pattern of genetic variation existing in 15 natural populations of the *F. communis* group in the Tyrrhenian area.

Overall, the obtained results reveal high levels of genetic diversity and moderate levels of differentiation among populations. Some Sardinian populations of *F. c.* subsp. *ommunis* were also investigated by means of allozymes (Marchi et al. 2003), showing a similar differentiation among populations (mean F_{st} = 0.227) and a lower expected heterozygosity (ranging from 0.097 to 0.165). Both the levels of heterozygosity and the differentiation among populations were consistent with the trends of genotypic variation revealed through AFLP data in previous studies (Nybom et al. 2004). Genetic diversity values are similar and not significantly different between *F. arrigonii* and *F. communis* s.l., and are not in accordance with the theoretical expectations that endemic species (including island endemics; Frankham 1997) should exhibit lower levels of genetic diversity than widespread species (Karron 1987; Hamrick & Godt 1989, 1996; Cole 2003).

Many biological factors can influence both the species genetic diversity and its distribution among populations. Among these, the geographic distribution has been acknowledged to be one of the most important (Hamrick & Godt 1989). Mateu-Andrés (2004) argued that there are actually many studies reporting either low (e.g. Gemmill & al. 1998; Segarra-Moragues & Catalán 2002; Mateu-Andrés 2004) or high (e.g. Ranker 1994; Lewis & Crawford 1995; Young & Brown 1996) levels of genetic variation for rare plant species. Also, many other studies have reported either lower (e.g. Purdy & Bayer 1995; Maki et al. 2002; Franceschinelli et al. 2006; Moreira da Silva et al. 2007), similar or even higher (e.g. Karron et al. 1988; Purdy & Bayer 1996; Dodd & Helenurm 2002; Ellis et al. 2006) levels of genetic diversity for endemic and rare species with respect to their widespread congeners. Therefore a positive correlation between geographic range and genetic diversity must not be taken for granted. Recent bottlenecks and/or a high number of individuals, in spite of the limited distribution, could account for the high genetic diversity found in many narrowly distributed species (Maki 2003).

The neighbor-joining, the PCoA and the STRUCTURE analyses were highly coherent with one another. All of them indicated that *F. arrigonii* (BON, CAV, SMA populations) is well differentiated with respect to the widespread congener *F. communis* s.l. The latter was furtherly

subdivided into two genetic group, of which one was constituted by the individuals of CLV (*F. c.* subsp. *glauca*). This pattern was confirmed by the presence of a high number of private fragments at the specific level (12 for *F. arrigonii*, 9 for *F. communis* s.l.) and by the presence of a private fragment and of a significantly higher number of rare fragments per individual in *F. c.* subsp. *glauca*. Multiple evidence therefore indicates that this *taxon* is somewhat distinct within the *Ferula communis* s.l. populations analyzed in this study and suggest that the specific status for this *taxon* should be confirmed (Arcangeli 1882, 1894; Hayek 1927; Brilli Cattarini & Gubellini 1987; Anzalone et al. 1991; Conti et al. 2005; Jeanmonod & Gamisans 2007). However, since only one population has been investigated in this study, it might be worth analyzing more populations of this *taxon* across a wider distributional range.

All of the analyses, as well as the absence of fragments exclusive to any population, subspecies or island suggest that all investigated populations of *F. communis* s.l. except CLV are closely related. This is somewhat surprising considering the distances that separate the populations, their being located in different islands, and their taxonomic affiliations. In particular, the obtain data did not provide any evidence for the distinctiveness of BLA population, which is the *locus classicus* of *F. c.* subsp. *cardonae*, endemic to Minorca (Sánchex-Cuxart & Bernal 1998), but since only one population of this *taxon* was included in this study it might be appropriate to investigate a higher number of populations in order to confirm this result. Nevertheless, a certain amount of substructuring was detected by the STRUCTURE and the neighbor-joining analyses, showing that the Sardinian and Corsican populations are more closely related to one another than they are with all other investigated populations. From this point of view, this work confirms the findings of Sánchex-Cuxart & Bernal (1998). In their study, the authors found great similarities between plants from Minorca and Sicily based on morphological, phenological and karyological characteristics and they thus hypothesized they could belong to the same *taxon.* . A comparative morphological analysis on specimens from different islands comprising Corsica and Sardinia would be advisable to complement the current body of knowledge on this species complex in the Tyrrhenian area. Also, the use of more powerful molecular markers (e.g. microsatellites) could be crucial to unravel its genetic structure.

References

Anzalone B., Lattanzi E., Leporatti M.L. 1991. Il gruppo di *Ferula communis* L. in Italia: ricerche sistematiche e corologiche. Archivio Botanico Italiano 67: 221-236.

Arcangeli G. 1882. Compendio della Flora Italiana, pp. 290-291.

Arcangeli G. 1894. Compendio della Flora Italiana, 2nd ed., pp. 608-609.

Bertoloni A. 1837. Flora Italica 3: 372-375.

Bolòs de O., Vigo J. 1984. Flora dels Paisos Catalans 2: 468-470.

Brilli Cattarini A., Gubellini L. 1987. Segnalazioni floristiche italiane: 487. *Ferula communis* L. Informatore Botanico Italiano 19: 189.

Bocchieri E. 1988. *Silene valsecchiae* e *Ferula arrigonii*, due specie nuove della Sardegna. Bollettino della Società Sarda di Scienze Naturali 26: 305-310.

Cannon J.F.M. 1968. Gen. *Ferula*. In: Tutin T.G., Burges N.A., Valentine D.H., Walters S.M., Webb D.A. (eds.). 1968. Flora Europaea 2: 358-359. Cambridge University Press, Cambridge.

Caruel T. 1889. *Ferula*. In: Parlatore F. Flora Italiana 8: 297-300.

Caujapé-Castells J., Castellano J.J., Henríquez V., Ramos R., Sabbagh I., Quintana-Trujillo F.M., Rodríguez J.F. 2011. Transformer-4: a genotype matrix format translator. Jardín Botánico Canario "Viera y Clavijo"-Unidad Asociada CSIC, Instituto Tecnológico de Canarias, Jablesoft & Inventiaplus. Las Palmas de Gran Canaria, Spain. (http://www.demiurge-project.org/download_t4).

Cavalli-Sforza L.L., Edwards A.W.F. 1967. Phylogenetic analysis: models and estimation procedures. Evolution 32: 550-570.

Cole C.T. 2003. Genetic variation in rare and common plants. Annual Review of Ecology, Evolution and systematic 34: 213-237.

Conti F., Abbate G., Alessandrini A., Blasi C. 2005. An annotated checklist of the Italian vascular flora. Palombi Press, Rome.

Dodd S.C., Helernum K. 2002. Genetic diversity of *Delphinium variegatum* (Ranunculaceae): a comparison of two insulat endemic subspecies and their widespread mainland relative. American Journal of Botany 89: 613-622.

Earl D.A., vonHoldt B.M. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4: 359-361.

Ellis J.R., Pashley C.H., Burke J.M., McCauley D.E. 2006. High genetic diversity in a rare and endangered sunflower as compared to a common congener. Molecular Ecology 15: 2345-2355.

Excoffier L., Laval G., Schneider S. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1: 47-50.

Evanno G., Regnaut S., Goudet J. 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. Molecular Ecology 14: 2611-2620.

Falush D., Stephens M., Pritchard J.K. 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Molecular Ecology Notes 7: 574-578.

Fiori A. 1925. Nuova Flora analitica d'Italia 2: 63.

Franceschinelli E.V., Jacobi C.M., Gonçalves Drummond M., Silveira Resende M.F. 2006. The Genetic Diversity of Two Brazilian *Vellozia* (Velloziaceae) with Different Patterns of Spatial Distribution and Pollination Biology. Annals of Botany 97: 585-592.

Frankham R. 1997. Do island populations have less genetic variation than mainland populations? Heredity 78: 311-327.

Franzke A., Hurka H., Janssen D., Neuffer B., Friesen N., Markov M., Mummenhoff K. 2004. Molecular signals for Late Tertiary/Early Quaternary range splits of an Eurasian steppe plant: *Clausia aprica* (Brassicaceae). Molecular Ecology 13: 2789-2795.

Gitzendanner M.A, Soltis P.S. 2000. Patterns of genetic variation in rare and widespread plant congeners. American Journal of Botany 87: 783-792.

Hamrick J.L., Godt M.J.W. 1989. Allozyme diversity in plant species. In: Brown A.H.D, Clegg M.T, Kahler A.L, Weir B.S. (eds), pp. 43-63. Plant population genetics, breeding and genetic resources Sinauer Sunderland.

Hamrick J.L., Godt M.J.W. 1996. Conservation genetics of endemic plant species. In: Avise J.C., Hamrick J.L. (eds.) Conservation genetics. Chapman and Hall, New York.

Hayek A. 1927. Prodromus Florae Peninsulae Balcanicae 1: 1028.

Jakobsson M, Rosenberg NA. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23: 1801-1806.

Jeanmonod D., Gamisans J. 2007. Flora Corsica. Edisud, Aix-en-Provence.

Karron J.D. 1987. A comparison of levels of genetic polymorphism and self-compatibility in geographically restricted and widespread plant congeners. Evolutionary Ecology 1: 47-58.

Karron J.D., Linhart Y.B., Chaulk A.C., Robertson C.A. 1988. Genetic structure of populations of geographically restricted and widespread species of *Astragalus* (Fabaceae). American Journal of Botany 75(8): 1114-119.

Kruckeberg A.R., Rabinowitz D. 1985. Biological aspects of endemism in higher plants. Annual Review of Ecology and Systematics 16: 447-479.

Kurzyna-Młynik R., Oskolski A.A., Downie S.R., Kopacz R., Wojewodzka A., Spalik K. 2008. Phylogenetic position of the genus *Ferula* (Apiaceae) and its placement in tribe Scandiceae as inferred from nrDNA ITS sequence variation. Plant Systematics and Evolution 274: 47-66.

Lewis P.O., Crawford D.J. 1995. Pleistocene refugium endemics exhibit greater allozymic diversity than widespread congeners in the genus *Polygonella* (Polygonaceae). American Journal of Botany 82: 141-149.

Lewontin R.C. 1972. The apportionment of human diversity. Evolutionary Biology 6: 381-398.

Maki M., Horie S., Yokoyama J. 2002. Comparison of genetic diversity between narrowly endemic shrub *Menziesia goyozanensis* and its widespread congener *M. pentandra* (Ericaceae). Conservation genetics 3: 421-425.

Marchi A., Appendino G., Pirisi I., Ballero M., Loi M.C. 2003. Genetic differentiation of two distinct chemotypes of *Ferula communis* (Apiaceae) in Sardinia (Italy). Biochemical Systematics and Ecology 31: 1397-1408.

Mateu-Andrés I. 2004. Levels of allozyme variability in the threatened species *Antirrhinum subbaeticum* and *A. pertegasii* (Scrophulariaceae): implications for conservation of the species. Annals of Botany 94: 797-804.

Moreira da Silva R., Wilson Fernandes G., Lovato M.B. 2007. Genetic variation in two *Chamaecrista* species (Leguminosae), one endangered and narrowly distributed and another widespread in the Serra do Espinhaço, Brazil. Canadian Journal of Botany 85: 629-636.

Nei M., Li W.H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the Natural Academy of Sciences of the United States of America 76: 5269-5273.

Nybom H. 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Molecular Ecology 13: 1143-1155.

Paoletti G. 1900. *Ferula communis* L. In: Fiori A., Paoletti G. Flora Analitica d'Italia 2: 175-176.

Pérez-Collazos E., Catalán P. 2008. Conservation genetics of the endangered Iberian steppe plant *Ferula loscosii* (Apiaceae). Plant Biology 10: 492-501.

Pérez-Collazos E., Sanchez-Gómez P., Jiménez J.F., Catalán P. 2009. The phylogeographical history of the Iberian steppe plant *Ferula loscosii* (Apiaceae): a test of the abundant-centre hypothesis. Molecular Ecology 18: 848-861.

Pignatti S. 1982. Flora d'Italia 2: 229.

Pritchard J.K., Stephens M., Donnelly O. 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945-959.

Purdy B.G., Bayer R.J. 1995. Allozyme Variation in the Athabasca Sand Dune Endemic, *Salix silicicola*, and the Closely Related Widespread Species, *S. alaxensis*. Systematic Botany 20: 179-190.

Purdy B.G., Bayer R.J. 1996. Genetic variation in populations of the endemic *Achillea millefolium* ssp. *megacephala* from the Athabasca sand dunes and the widespread species ssp. *lanulosa* in western North America. Canadian Journal of Botany 74: 1138-1146.

Ranker T.A. 1994. Evolution of high genetic variability in the rare Hawaiian fern *Adenophorus periens* and implications for conservation management. Biological Conservation 70: 19-24.

Rosenberg NA. 2004. DISTRUCT: a program for the graphical display of population structure. Molecular Ecology Notes 4: 137-138.

Rouy G., Camus E.G. 1901. Flore de France 7: 398.

Sánchez-Cuxart A., Bernal C.M. 1998. Estudi biosistemàtic de les poblacions de *Ferula communis* del NE de la península ibèrica i de les illes balears. Acta Botanica Barcinonensica 45: 217-239.

Schlüter P.M., Harris S.A. 2006. Analysis of multilocus fingerprinting data sets containing missing data. Molecular Ecology Notes 6: 569-572.

Segarra-Moragues J.G., Catalán P. 2002. Low allozyme variability in the critically endangered *Borderea chowardii* and its congener *Borderea pyrenaica* (Discoreaceae), two paleoendemic relicts from the central Pyrenees. International Journal of Plant Sciences 163: 159-166.

Stehlik I., Schneller J.J., Bachmann K. 2001. Resistance or emigration: response of the high alpine plant *Eritrichium nanum* (L.) Gaudin to the ice age within the Central Alps. Molecular Ecology 10: 357-370.

Thellung A. 1926. *Ferula communis* L. In: Hegi G. Illustrierte Flora von Mitteleuropa 5: 1355-1356.

Vekemans X. 2002. AFLP-SURV version 1.0. Distributed by the author. Laboratoire de Génétique et Ecologie Végétale, Université Libre de Bruxelles, Belgium.

Vekemans X., Beauwens T., Lemaire M., Roldan-Ruiz I. 2002. Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. Molecular Ecology 11: 139-151.

Vos P., Hogers R., Bleeker M., Reijans M., Van De Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407-4414.

Whittall J.B., Syring J., Parks M., Buenrostro J., Dick C., Liston A., Cronn R. 2010. Finding a (pine) needle in a haystack: chloroplast genome sequence divergence in rare and widespread pines. Molecular Ecology 19: 100-114.

Yeh F.C., Yang R., Boyle T.J., Ye Z., Xiyan J.M. 2000. PopGene32. Microsoft Window-based freeware for population genetic analysis, version 1.32. Molecular Biology and Biotechnology Centre. University of Alberta, Edmonton, Alberta, Canada.

Young A.B., Brown A.H.D. 1996. Comparative population genetic structure of the rare woodland shrub *Daviesia suaveolens* and its common congener *D. mimosoides*. Conservation Biology 10: 1220-1228.

Zhivotovsky L.A. (1999) Estimating population structure in diploids with multilocus dominant DNA markers. Molecular Ecology 8: 907-913.

Chapter 3

Genetic diversity and differentiation of *Ruta corsica* DC. and *R. lamarmorae* Bacch., Brullo & Giusso (Rutaceae)

Marilena Meloni[1], Caterina Angela Dettori[2], Laetitia Hugot[3], Gianluigi Bacchetta[2], Elena Conti[1].

[1] *Institute of Systematic Botany - University of Zurich. Zollikerstrasse, 107– 8008 Zurich, Switzerland.*

[2] *Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente – Università degli Studi di Cagliari. Viale S. Ignazio da Laconi, 13 – 09123 Cagliari, Italy.*

[3] *Conservatoire Botanique National de Corse, Office de l'Environnement de la Corse. Avenue Jean Nicoli 14 – 20250 Corte, France.*

Abstract

Ruta corsica and *R. lamarmorae* are two species endemic to Corsica and Sardinia, respectively. To investigate the levels and the structure of genetic variation in natural populations of these species a set of 11 microsatellite markers was used to analyze 96 individuals of *R. corsica* (6 populations) and 63 individuals of *R. lamarmorae* (3 populations). The markers were highly polymorphic and detected 10 alleles per locus on average. Overall, results showed that both species have maintained relatively high levels of genetic diversity (H_e = 0.579 and 0.639, H_o = 0.558 and 0.591 for *R. corsica* and *R. lamarmorae*, respectively). Pairwise F_{st} values (0.035 – 0.351) indicated a low-moderate differentiation for most pairs of populations. AMOVA revealed that 80% of the genetic variation resides within populations, while only 4% is due to differences among the two species. The analyses of spatial genetic structure suggested the clustering of the individuals into two groups, approximately corresponding to taxonomic affiliations. Implications for the conservation of the two species are discussed based on the obtained results.

Key words: *Ruta*, Sardinia, Corsica, Biodiversity Hotspot, Mediterranean basin, SSRs, endemics, conservation genetics.

Introduction

The Mediterranean basin is one of 34 hotspots of biodiversity worldwide (Médail & Quézel 1997, 1999; Myers et al. 2000; Mittermeier et al. 2005). Its complex paleogeographic history has led to a high degree of local endemism (10%−20% of the local flora) concentrated in mountain chains and islands (Greuter 1991; Médail & Quézel 1997; Thompson 2005). Sardinia and Corsica are the second and third largest islands in the Mediterranean sea, respectively. Together with Sicily and the Balearic Islands they belong to the "Western Mediterranean islands" Biodiversity Hotspot, one of the most important ones within this region (Blondel & Médail 2009). Both islands are characterized by having a rich flora and a high endemism rate, with more than 6% of exclusive endemics (Jeanmonod & Gamisans 2007; Bacchetta et al. 2012); and together they host nine out of the 50 most threatened Mediterranean island plants (Montmollin de & Strahm 2005).

A characteristic element of the endemic component of the Mediterranean flora is that 60% are narrow endemic species, i.e., they have a distribution which is restricted to a single well-defined area within a small part of the Mediterranean region (Thompson 2005). Species with small and isolated populations are more vulnerable to demographic, environmental and genetic stochastic events, and therefore face a higher risk of local extinction (Frankham et al. 2002; Ellstrand & Elam 1993). In addition, island endemics are thought to be more susceptible to extinction (Frankham 1997, 1998; Prohens et al. 2007). In this framework, maintaining these species' genetic diversity is of prime importance as it could be related to population viability and the evolutionary potential to adapt to environmental change (Frankham et al. 2002; Reed et al. 2002; Reed & Frankham 2003). Therefore genetic data can be crucial when designing management strategies for both *in situ* and *ex situ* conservation.

The focus of this study is on *R. corsica* DC. and *R. lamarmorae* Bacch., Brullo & Giusso*,* endemic to Corsica and Sardinia, respectively (Bacchetta et al. 2006). *Ruta* L., the type genus of Rutaceae Juss., includes 10 *taxa* restricted to the Macaronesian islands, the Mediterranean basin and SW-Asia (Mabberley 2008). Four species are widespread (*R. chalepensis* L., *R. graveolens* L., *R. Montana* (L.) L., *R. angustifolia* Pers.), two species are endemic to the Tyrrhenian islands (*R. corsica* and *R. lamarmorae*), one subspecies is endemic to Crete [*R. chalepensis* subsp. *fumariifolia* (Boiss. & Heldr.) Nyman] and three species are endemic to the Canary islands (*R.*

oreojasme Webb & Berthel. in Gran Canaria, *R. microcarpa* Svent. in La Gomera, *R. pinnata* L.f. in La Palma and Tenerife; Bramwell & Bramwell 2001).

R. lamarmorae, endemic to the Gennargentu massif in Sardinia (Bacchetta et al. 2006), is very localized and found only in three small stands. *R. corsica* is comparatively widespread and it can be found in all the main Corsican massifs. The two endemic *taxa* were considered the same species (*R. corsica*) until a few years ago, and only recently the Sardinian populations were ascribed to a new species (i.e. *R. lamarmorae*; Bacchetta et al. 2006). *R. lamarmorae* is a perennial subspinescent half-shrub, 15-50 cm tall. Leaves are bipinnate and green-glaucescent, flowers are hermaphrodite. *R. corsica* is quite similar to *R. lamarmorae*, they share the same habit and leaves; however, they differ for several features: flowers, stamens and ovaries are smaller and sepals are narrower and shorter in *R. corsica* (Bacchetta et al. 2006) than in *R. lamarmorae*. Additionally, they differ in their chromosome number, as *R. lamarmorae* is tetraploid (2n=36; Honsell 1957) and *R. corsica* diploid (2n=18; Contandriopoulos 1957). They are both montane species, *R. lamarmorae* is orophilous and found at a range of 1500-1750 m a.s.l., while *R. corsica* occurs in a wider altitudinal range (1000-1900 m a.s.l.).

In their work, Bacchetta et al. (2006) hypothesized that *R. lamarmorae* could have been arisen from diploid populations of *R. corsica* and subsequent polyploidization events and geographical isolation could have led to the morphological differentiation of the Sardinian populations. In a relatively recent phylogenetic analysis based on three cpDNA markers, the accessions from Corsica and Sardinia formed two strongly supported clades, thus suggesting that the treatment of *R. lamarmorae* as a separate species might be warranted (Salvo et al. 2008). According to Contandriopoulos (1962), *R. corsica*, and consequently also *R. lamarmorae*, should be considered as relict species, taxonomically very isolated from the other known species belonging to the same genus. Both these species are morphologically well characterized and show some archaic features such as the pulvinate subspinescent habit, green-glaucescent leaves, and white to pale yellow petals, which are lacking in the other known species (Bacchetta et al. 2006).

R. corsica is listed as LC (Least Concern) in the national red list (UICN France et al. 2012). *R. lamarmorae* is currently not included in any list of protected species, even though Bacchetta et al. (2006) suggested that *R. lamarmorae* should be listed in the IUCN regional red list as endangered (EN) according, because of the strong pressure it suffers throughout its range due to overgrazing, fires, ski facilities and activities.

In the present study, newly developed polymorphic microsatellite markers are used to provide baseline molecular data potentially useful to design conservation strategies for *R. lamarmorae* and *R. corsica*. The following specific questions are addressed: i) what are the levels of genetic diversity within and among populations of *R. lamarmorae* and *R. corsica*? ii) how is genetic diversity spatially structured?

Materials and methods

Sampling sites and plant material

A total of 159 individuals were sampled from nine populations during summer 2010 (Fig. 1; Tab. 1). Specifically, material was collected from all the three known Sardinian populations, Bruncu Spina (henceforth BC), Su Sciusciu (SS) and Bacu Seardu (BS), the latter being newly reported in this study. Six populations of *R. corsica* were sampled across the whole distribution range of the species; out of these, four were from northern Corsica [Muvrella (MU), Monte Cinto (MC), Saltare (SA), Albertacce (AL)] and two from the central part of the island [Bastelica (BA) and Ghisoni (GH)]. Sampling was done throughout the populations in order to cover the whole occupied area and to minimize sampling of related individuals; 8-30 plants per populations were sampled, with the exception of BS site from which only 3 individuals could be sampled due to logistic constraints and small population size.

DNA isolation and microsatellite analyses

Genomic DNA was extracted from 20 mg of silica gel-dried leaf tissue using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol with slight modifications (i.e. 6 µL of RNAse A were used instead of 4 µL, and 200 µL of buffer AP2 were used instead of 130). Fifteen polymorphic SSR (microsatellites) markers were newly developed for *R. lamarmorae* and *R. corsica*. (unpublished data). Out of these, eleven were chosen to carry out the large-scale analysis based on their cross-amplifiability between the two species and their degree of polymorphism. SSR genotyping was performed using a modified version of the fluorescent labelling protocol of Schuelke (2000). PCR reactions consisted of a three-primer system: the forward primer with the 5′ end extended with a universal M13 tail, the reverse locus-specific

primer and a fluorescently labelled M13 primer. Amplification reactions were carried out in a total volume of 25 μL containing 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of reverse primer, 0.08 μM of M13-forward primer, 0.2 μM of M13 labelled primer (either with 6FAM, VIC, NED or PET fluorescent dyes; Applied Biosystems), 0.5 U *Taq* polymerase and 2 μL (5-15 ng/μL) of genomic DNA. The PCR conditions were as follows: an initial cycle at 94°C for 3 min, followed by 30 cycles at 94°C for 30 sec, 55°C for 45 sec and 72°C for 1 min, and by 8 cycles at 94°C for 30 sec, 53°C for 45 sec, 72°C for 1 min and a final extension step of 72° for 5 min. PCR products were loaded on an ABI Prism® 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA).

Data analyses

Alleles were scored using GeneMapper v. 4.0 (Applied Biosystems); *R. lamarmorae* exhibited a disomic pattern at all investigated loci and was subsequently analyzed as being effectively diploid.

Genotypic linkage disequilibrium was tested using Fisher exact test both for each pair of loci and within each population using GENEPOP v. 4.0.1 (Raymond & Rousset 1995; Rousset 2008) following the Markov chain method with 100 batches and 1000 iterations per batch. This software was also used to estimate deviations from Hardy–Weinberg equilibrium (HWE) both within each population and for each locus. The same package was used to calculate the following parameters: allele frequencies, the observed and expected heterozygosities (H_o and H_e, respectively) and the inbreeding coefficient (F_{is}; Weir & Cockerham 1984) at each locus for all populations. GenAlex v. 6.5 (Peakall & Smouse 2006, 2012) was used to compute P (percentage of polymorphic loci), the observed number of alleles per locus, Shannon's information index (I), the pairwise F_{st} measures between all populations and pairwise number of migrants per generation (N_m). For among-species comparison of the values of P, H_e, H_o, I and F_{is} an analysis of variance was performed using STATISTICA v.7.0 (Statsoft). Both a non-hierarchical and a hierarchical analysis of molecular variance (AMOVA) were performed to estimate the partitioning of genetic variability within and among populations and among species.

To explore the genetic and spatial grouping of individuals a principal co-ordinate analysis (PCoA) using GenAlex v. 6.5 (Peakall & Smouse 2006, 2012) and a Bayesian cluster analysis

using the software STRUCTURE (Pritchard et al. 2000) were carried out. Twenty independent runs for each K (from $K = 1$ to $K = 9$) were performed using 100,000 burn-in periods and 100,000 MCMC (Markov Chain Monte Carlo) iterations, using no prior population information and assuming correlated allele frequencies and admixture. The most accurate value of K was evaluated following Evanno et al. (2005) and using the software STRUCTURE HARVESTER (Earl 2012). The program CLUMPP v. 1.1.2 (Jakobsson & Rosenberg 2007) was used to determine the optimum alignment of clusters across individual runs for each K; outcomes from CLUMPP were imported into Distruct v. 1.1 (Rosenberg 2004) for viewing the individuals' assignment probabilities.

Results

A total of 159 individuals were genotyped at 11 loci detecting a total of 110 alleles. The number of alleles per locus across all populations ranged from 2 to 17, with a mean of 10 alleles/locus. The results of the genetic diversity parameters are summarized in Tab. 1. The percentage of polymorphic loci varied from 63.64% (SA) to 100% (BC, SS, MU and MC). Expected heterozygosity ranged from 0.409 (SA) to 0.697 (BS). The Shannon index (I) ranged from 0.540 (SA) to 1.309 (SS). No significant differences between *R. corsica* and *R. lamarmorae* were found for any of the diversity indexes ($P > 0.05$ in all cases).

At the population level, F_{is} values were slightly positive in four cases (BS, SS, MU and MC) and negative for all other sites, thus suggesting the presence of an excess of heterozygotes at different loci depending on the population considered, while significant departures from the HWE ($p < 0.05$) were detected for all populations except BS, MU and SA. Again, there were no significant differences between *R. lamarmorae* and *R. corsica* ($P > 0.05$ in all cases). Pairwise genetic differentiation among populations (F_{st}; Tab. 2) was highest between BS and SA (0.351) and lowest between MU and MC (0.009); the former was the only non-significant value ($p = 0.219$) while all other values were highly significant ($p < 0.01$). The highest number of migrants was found between MU and MC (26.15) and the lowest between BS and SA (0.46; Tab. 2).

The non-random association of the alleles at different loci, or linkage disequilibrium (LD), revealed that among all pairwise comparisons only 18 out of 495 were significantly associated

(three locus pairs in BC, seven in SS, one in MU, four in SA, one in GH, one in AL, three in BA).

Analysis of molecular variance (AMOVA; Tab. 3) confirmed and complemented the results, revealing that most of the genetic variation (about 80% in both the non-hierarchical and the hierarchical analysis) resides within populations, while a very small, but significant proportion of the variation was explained by differences between species.

Regarding the genetic clustering of individuals and their spatial genetic structure, the PCoA analysis (Fig. 3) returned three groups: two intermingled and not very well defined assemblages were formed respectively by individuals of *R. corsica* and *R. lamarmorae*, while a third group was constituted by individuals from SA populations (the first two axes explained 23.5 and 19.9% of the variation, respectively). Structure analysis (Fig. 1, 2) suggested an the optimal value for K=2, approximately corresponding to taxonomic affiliations; MC, MU and SA showed considerable admixture with the latter population displaying a higher genetic affinity with the cluster formed by *R. lamarmorae* individuals rather than with the one constituted mainly of *R. corsica* individuals.

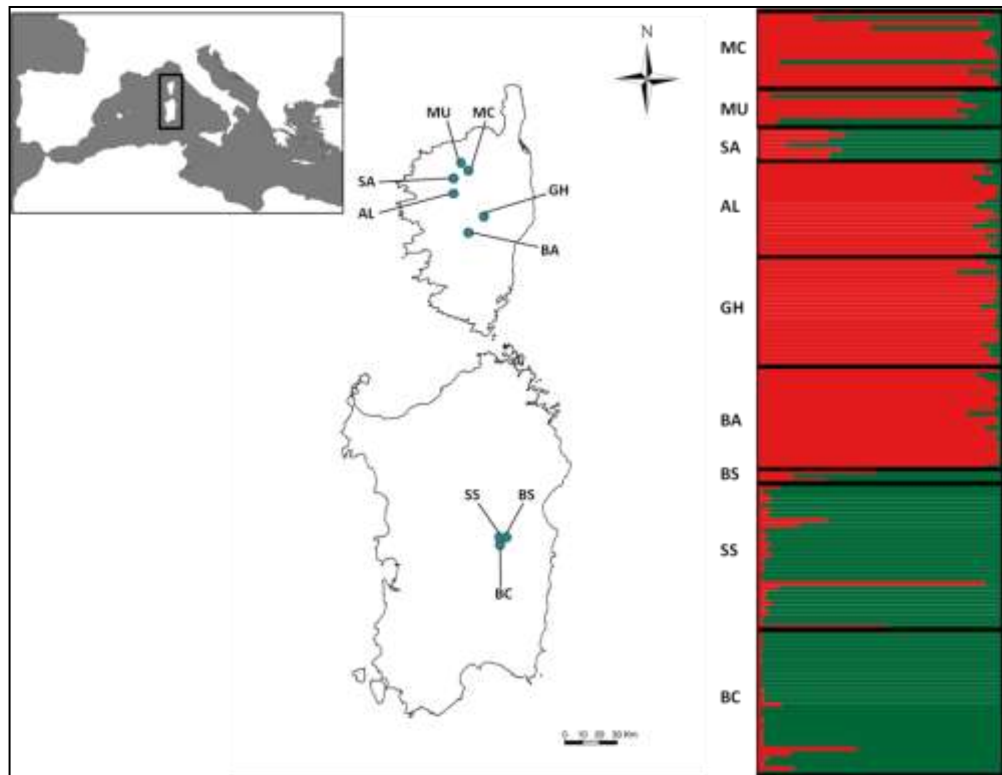

Fig. 1. Geographical location of study and genetic admixture individual profiles. Horizontal bars represent individuals and black lines delimit sites. The admixture diagram was redrawn from STRUCTURE results. (see Materials and Methods for further datails).

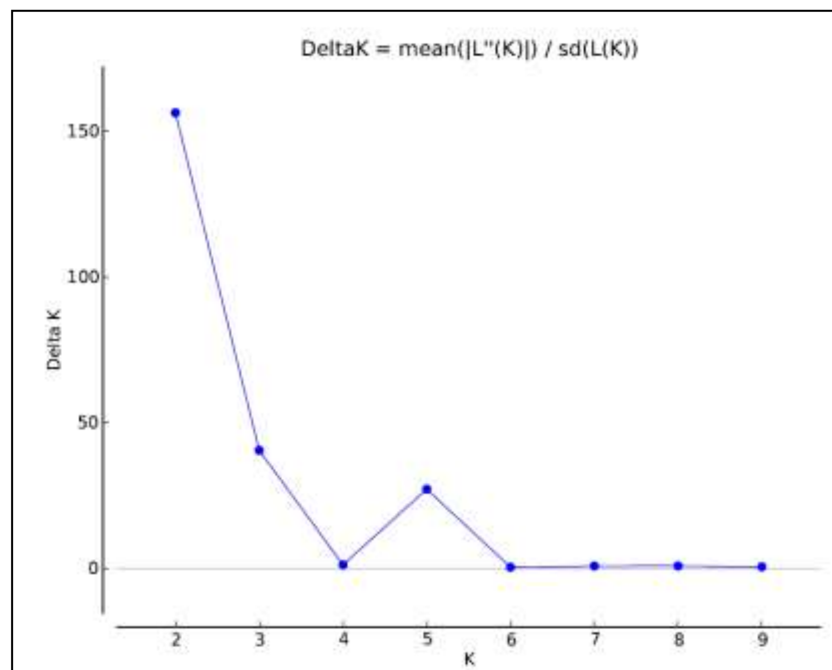

Fig. 2. DeltaK (ΔK) value calculated for assuming K=1-9 of three populations of *R. lamarmorae* and six of *R. corsica*.

Tab. 1. Sampling locations and genetic diversity parameters.

Population	Code	Coordinates	N_i	P (%)	H_e	H_o	I	F_{is}
Bruncu Spina	BC	40° 01' N - 9° 17' E	30	100.00	0.594	0.604	1.167 (±0	0.016
Su Sciusciu	SS	40° 00' N - 9° 19' E	30	100.00	0.625	0.626	1.309 (±0	0.001
Bacu Seardu	BS	40° 00' N - 9° 20' E	3	90.91	0.697	0.545	0.828 (±0	-0.278
Ruta lamarmorae			63	96.97	0.639	0.591	1.101	-0.087
Muvrella	MU	42° 24' N - 8° 54' E	8	100.00	0.659	0.670	1.249 (±0	0.017
Monte Cinto	MC	42° 23' N - 8° 55' E	16	100.00	0.617	0.618	1.210 (±0	0.001
Saltare	SA	42° 21' N - 8° 53' E	8	63.64	0.409	0.339	0.540 (±0	-0.206
Ghisoni	GH	42° 06' N - 09° 09' E	23	90.91	0.588	0.585	1.148 8±(	-0.005
Albertacce	AL	42° 17' N - 8° 52 E	20	90.91	0.659	0.612	1.248 (±0	-0.076
Bastelica	BA	42° 00' N- 09° 06' E	21	90.91	0.541	0.529	1.047 (± (	-0.023
Ruta corsica			96	83.39	0.579	0.558	1.074	-0.049
Average				91.92	0.599	0.570	1.082	-0.061

N_i = number of individuals analyzed; P = percentage of polymorphic loci; H_e = expected heterozygosity; H_o = observed heterozygosity; I = Shannon's information index; F_{is} = inbreeding coefficient.

Tab. 2. Pairwise F_{st} values (below the diagonal) and N_m (above the diagonal).

	BC	SS	BS	MU	MC	SA	GH	AL	BA
BC	_	2.847	1.040	3.086	2.331	0.961	1.752	1.769	1.384
SS	0.081	_	1.470	5.838	3.715	1.209	2.795	4.006	1.673
BS	0.194	0.145	_	1.890	1.238	0.461	1.642	1.329	0.981
MU	0.075	0.041	0.117	_	26.147	1.232	5.020	5.772	2.735
MC	0.097	0.063	0.168	0.009	_	1.217	6.892	5.796	3.460
SA	0.206	0.171	0.351	0.169	0.170	_	1.136	0.813	0.944
GH	0.125	0.082	0.132	0.047	0.035	0.180	_	2.848	2.758
AL	0.124	0.059	0.158	0.042	0.041	0.235	0.081	_	1.966
BA	0.153	0.130	0.203	0.084	0.067	0.209	0.083	0.113	_

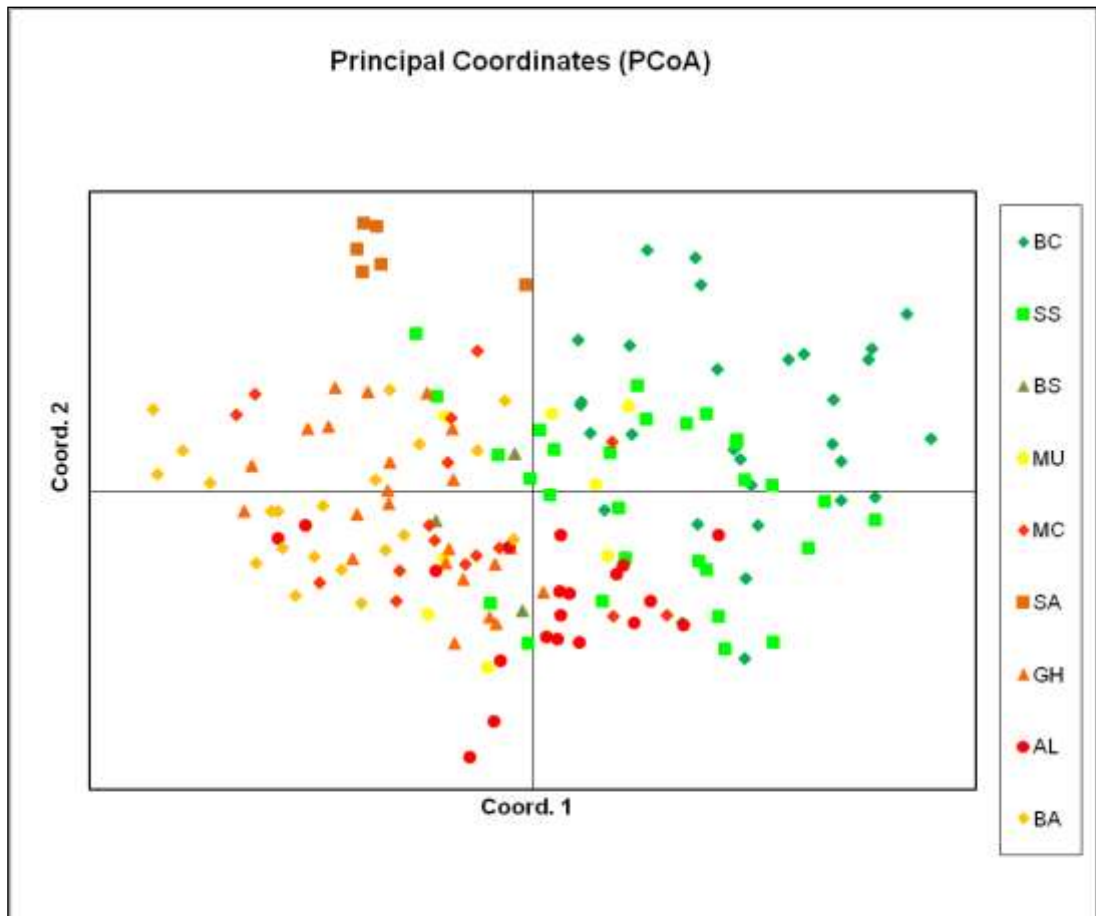

Fig. 3. Results of Principal Coordinate Analysis (PCoA) showing positions of all individuals along the first two axes (explaining 23.5 and 19.9 % of the total variation, respectively).

Tab. 3. Results of the analyses of molecular variance (AMOVA). Estimations are based on 999 permutations.

Grouping	n	Source of variation	df	SS	Percentage of variance	*P*
No groups	9	Among populations	8	264.728	19%	0.001
		Within populations	150	1006.228	81%	
[*R.lamarmorae*]	2	Among species	1	65.884	4%	0.001
[*R.corsica*]		Among Populations	7	198.845	16%	0.001
		Within Populations	150	1006.228	80%	0.001

Discussion

The set of 11 microsatellites used in this study provided highly informative data that were successfully used to characterize *R. corsica* and *R. lamarmorae* individuals collected from nine natural populations. Both species showed moderate-high level of genetic diversity. In particular, the levels of heterozygosity (H_e = 0.639 and 0.579 and H_o = 0.591 and 0.558 for *R. lamarmorae* and *R. corsica*, respectively) were consistent with the trends of genotypic variation revealed through microsatellite data in many long-lived, outcrossing flowering plants (Nybom et al. 2004). Surprisingly, *R. lamarmorae* showed slightly higher, but not significant values for most diversity parameters ($p > 0.05$ by One-Way ANOVA) than did its congener *R. corsica*. Given the small population sizes and the very restricted geographic distribution, *R. lamarmorae* was expected to be genetically impoverished (Hamrick & Godt 1989; Ouborg et al. 2006). Overall, results are not in accordance with the general expectation that endemic species, and particularly island endemics, have low genetic diversity (Frankham 1997). Much lower levels of heterozigosity than those of *R. corsica* and *R. lamarmorae* have actually been reported for other Mediterranean island endemics. As an example, a study on the Sardinian endemic *taxa* of the genus *Aquilegia* employing AFLPs yielded low genetic diversity values (Garrido et al. 2012). Results regarding both *R. corsica* and *R. lamarmorae* indicate that they rather belong to the same group of Mediterranean endemics for which high genetic diversity has been reported. High levels of genetic variability were detected in six natural populations of the Sicilian endemic *Brassica rupestris* Raf. by means of ISSR markers (Raimondo et al. 2012). In *Cedrus brevifolia* Henry, a narrow endemic island tree species of Cyprus, estimates of H_e by means of nuclear and chloroplast microsatellites yielded values in the range of 0.61 – 0.75 and 0.58 – 0.94, respectively (Eliades et al. 2011). In a study on the Sardinian endemic *Centaurea horrida* Badarò the average values of H_e ranged from 0.603 to 0.854 by means of microsatellites (Mameli et al. 2008). Moderate levels of genetic variability were also detected in the Sardinian endemics *Lamyropsis microcephala* (Moris) Dittrich & Greuter (Bacchetta et al. 2013). and *Rhamnus persicifolia* Moris (Bacchetta et al. 2011), both of them by means of ISSR markers. These examples not only regard species with different lifestyles, reproductive systems and ecological requirements, but are also based on different molecular markers, therefore are not directly comparable. Nevertheless, they provide further evidence of the fact that the general expectations on the genetic variability of endemic island species represent an overgeneralization.

Negative or slightly positive values of F_{is} indicate that populations are not suffering from inbreeding. The observed high within-population genetic diversity in these rare perennial plants might be explained by their presumably outcrossing behaviour, long-lived individuals and overlapping generations. Reproductive systems and the history of a species have often been regarded as the main factors affecting levels of genetic diversity and structure within and between plant populations (Loveless & Hamrick 1984; Hamrick & Godt 1989, 1996).

Genetic differentiation among populations was low-medium. The lowest F_{st} value (and the only one which was not significantly different from zero) was found between MU and MC, which is not surprising given the relatively low geographic distance between these two sites. The highest value was found between SA and BS; however, caution must be taken as this measure might be biased by the low sampling size of BS site. The AMOVA confirmed these results, revealing that a very small, but significant proportion (4%) of the genetic variation is explained by between-species differences, while most of the variation (about 80% in both the non-hierarchical and the hierarchical analysis) resides within populations, as it is expected for outcrossing species (Hamrick & Godt 1989).

Regarding the analyses of spatial genetic structuring of individuals, the PCoA identified three putative genetic clusters, while the STRUCTURE analyses indicated the occurrence of two distinct groups roughly – but not unequivocally - corresponding to the taxonomic affiliations of the individuals. Surprisingly, individuals from MU, MC and SA populations, the northernmost study sites, were partly assigned to the cluster formed mainly by individuals of *R. lamarmorae*. Individuals belonging to SA also clustered together and seemed to be distinctive with respect to both *R. corsica* and *R. lamarmorae* populations in PCoA analysis. Further genetic evidence, based on more markers and more individuals from this population, is therefore advisable.

Conclusions and implications for conservation

The levels of genetic diversity and the genetic differentiation observed among populations of *R. lamarmorae* indicate that management should aim to conserve all populations, with particular efforts on the two larger populations (BC and SS), since the loss of one of these would very likely result in substantial loss of genetic variability for the species. The pressure it suffers throughout its range due to overgrazing, fires, ski facilities and activities (Bacchetta et al. 2006) might realistically have an impact on the populations of this narrow endemic. Additionally,

mountain habitats are considered particularly sensitive to the influence of predicted climatic change and they are likely to show the deriving effects earlier and more clearly than some other ecosystems (Grabherr et al. 2000; Thuiller et al. 2005). Shrinking areas of currently rare climates may result in globally important centers of conservation interest becoming hotspots of extinction (Ohlemüller et al. 2008). The optimal germination protocol of the species (20° C with a photoperiod of 12/12 h and after 3 months of pre-chilling at 5°C; Bacchetta et al., unpublished data) actually indicates that a possible effect of warmer winters, as expected from IPCC projections (Alcamo et al. 2007), might lead to a failure of its seeds to receive sufficient chilling to satisfy the pre-germination vernalization requirement, and thus to a reduced reproductive capability of this species. This trend has already been observed for other Sardinian *taxa* living at medium-high altitudes, like *Lamyropsis microcephala* (Mattana et al. 2009), *Ribes multiflorum* subsp. *sandalioticum* (Mattana et al. 2012) and *Vitis vinifera* subsp. *sylvestris* (Orrù et al. 2012). While seeds from BC population have been already collected for long-term storage at the BG-SAR (the Sardinan Germplasm Bank, University of Cagliari), the collection of germplasm from SS and BS populations will be prioritary in the future. At the same time the designing of plant micro-reserves, a tool that has already been successfully applied (Laguna 1999, 2001; Laguna et al. 2004), would be advisable to protect *in situ* the populations of this rare species.

As regards *R. corsica*, being the species quite widespread across the main Corsican massifs and being its genetic diversity also quite high, it may be confirmed that the species is not in danger yet, and that the maintenance of an adequate number of populations might be effective in avoiding the impoverishment of its genetic resources.

Finally, it must be taken into account that molecular data alone do not accurately reflect the evolutionary potential of populations (Ouborg et al. 2006), even if they do represent a fundamental tool to address conservation issues (Tallmon et al. 2004; Segelbacher et al. 2010). It is therefore necessary to investigate other important factors of both species biology which may be crucial for their long-term survival.

References

Alcamo J., Moreno J.M., Nováky B., Bindi M., Corobov R., Devoy R.J.N., Giannakopoulos C., Martin E., Olesen J.E., Shvidenko A. 2007. Europe. Climate Change 2007: Impacts, Adaptation and Vulnerability. Contribution of Working Group II to the Fourth Assessment Report of the

Intergovernmental Panel on Climate Change. In: Parry M.L., Canziani O.F., Palutikof J.P., Van der Linden P.J., Hanson CE. (eds.), pp. 541-580. Cambridge University Press, Cambridge, UK.

Bacchetta G., Brullo S., Giusso del Galdo G. 2006. *Ruta lamarmorae* (Rutaceae), a new species from Sardinia. Edinburgh Journal of Botany 63:153-160.

Bacchetta G., Fenu G., Mattana E., Zecca G., Grassi F., Casazza G., Minuto L. 2011. Genetic variability of the narrow endemic *Rhamnus persicifolia* Moris (Rhamnaceae) and its implications for conservation. Biochemical Systematics and Ecology 39: 477-484.

Bacchetta G., Fenu G., Mattana E. 2012. A checklist of the exclusive vascular flora of Sardinia with priority rankings for conservation. Anales del Jardín Botánico de Madrid 69: 61-69.

Bacchetta G., Fenu G., Gentili R., Mattana E., Sgorbati S. 2013. Preliminary assessment of the genetic diversity in *Lamyropsis microcephala* (Asteraceae). Plant Biosystems, in press.

Blondel J., Médail F. 2009. Biodiversity and conservation. In: Woodward J.C. (ed.). The physical geography of the Mediterranean. Oxford: Oxford University Press.

Bramwell D., Bramwell Z.I. 2001. Wild flowers of the Canary Islands. Editorial Rueda, Madrid.

Contandriopoulos J. 1957. Caryologie et localisation des espèces végétales endémiques de la Corse. Bulletin de la Societé Botanique de France 104: 53-55.

Contandriopoulos J. 1962. Recherches sur la flore endémique de la Corse et sur ses origines. Annales de la Faculté de Science de Marseille 32: 1-351.

Earl D.A., vonHoldt B.M. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4: 359-361.

Eliades N.H., Gailing O., Leinemann L., Fady B., Finkeldey R. 2011. High genetic diversity and significant population structure in *Cedrus brevifolia* Henry, a narrow endemic Mediterranean tree from Cyprus. Plan Systematics and Evolution 294: 185-198.

Ellstrand N.S., Elam D.R. 1993. Population genetic consequences of small population size: implications for plant conservation. Annual Review of Ecology and Systematics 24: 217-242.

Frankham R. 1997. Do island populations have less genetic variation than mainland populations? Heredity 78:311–327.

Frankham R. 1998. Inbreeding and extinction: island populations. Biological Conservation 12: 665-675.

Frankham R., Ballou J.D., Briscoe D.A. 2002. Introduction to conservation genetics. University Press, Cambridge.

Garrido J.L., Mattana E., Fenu G., Bacchetta G. 2012. Spatial genetic structure of *Aquilegia taxa* endemic to the island of Sardinia. Annals of Botany 109: 953-964.

Grabherr G., Gottfried M., Pauli H. 2000. GLORIA: a global observation research initiative in alpine environments. Mountain Researche and Development 20: 190-192.

Greuter W. 1991. Botanical diversity, endemism, rarity, and extinction in the Mediterranean Area: an analysis based on the published volumes of Med-Checklist. Botanika Chronica 10: 63-79.

Hamrick J.L., Godt M.J.W. 1989. Allozyme diversity in plant species. In: Brown H.D., Clegg M.T., Kahler A.L., Weir B.S. (eds), pp 43-63. Plant population genetics. Breeding and Germplasm Resources, Sinauer, Sunderland, MA.

Hamrick J.L., Godt M.J.W. 1996. Conservation genetics of endemic plant species. In: Avise J.C., Hamrick J.L. (eds) Conservation genetics. Chapman and Hall, New York.

Honsell E. 1957. Ricerche cario-embriologiche in *Ruta corsica* DC. Delpinoa 10: 141-153.

Jakobsson M., Rosenberg NA. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23: 1801-1806.

Jeanmonod D., Gamisans J. 2007. Flora Corsica. Edisud, Aix-en-Provence.

Laguna E. 1999. The plant micro-reserves programme in the region of Valencia, Spain. Proceedings Planta Europa 1998. Second European Conference on the Conservation of Wild Plants: 181-185. The Swedish Threatened Species Unit and Plantlife, Uppsala and London.

Laguna E. 2001. The micro-reserves as a tool for conservation of threatened plants in Europe. Nature and Environment series n° 121. Council of Europe, Strasbourg.

Laguna E., Deltoro V.I., Pérez-Botella J., Pérez-Rovira P., Serra Ll., Olivares A., Fabregat C. 2004. The role of small reserves in plant conservation in a region of high diversity in eastern Spain. Biological Conservation 119: 421-426.

Loveless M.D., Hamrick J.L. 1984. Ecological determinants of genetic structure of plant populations. Annual Reviews of Ecology and Systematics 15: 65-95.

Mabberley D.J. 2008. Mabberley's plant book: a portable dictionary of plants (3rd ed.). Cambridge University Press, Cambridge, UK.

Mameli G., Filigheddu R., Binelli G., Meloni M. 2008. The genetic structure of the remnant populations of *Centaurea horrida* in Sardinia and associated islands. Annals of Botany 101: 633-640.

Mattana E., Daws M.I., Bacchetta G. 2009. Seed dormancy and germination ecology of *Lamyropsis microcephala*: a mountain endemic species of Sardinia (Italy). Seed Science & Technology 37: 491-497.

Mattana E., Pritchard H.W., Porceddu M., Stuppy W.H., Bacchetta G. 2012. Interchangeable effects of gibberellic acid and temperature on embryo growth, seed germination and epicotyl emergence in *Ribes multiflorum* ssp. *sandalioticum* (Grossulariaceae). Plant Biology 14: 77-87.

Médail F., Quézel P. 1997. Hot-spots analysis for conservation of plant biodiversity in the Mediterranean Basin. Annals of the Missouri Botanical Garden 84: 112-127.

Médail F., Quézel P. 1999. Biodiversity Hotspot in the Mediterranean Basin: Setting Global Conservation Priorities. Conservation Biology 13: 1510-1513.

Mittermeier R.A., Robles Gil P., Hoffman M., Pilgrim J., Brooks T., Mittermeier C.G., Lamoreux J., da Fonseca G.A.B. 2005. Hotspots revisited: earth's biologically richest and most endangered terrestrial ecoregions. University of Chicago Press, Chicago, USA.

Montmollin de B., Strahm W. (eds.) 2005. The Top 50 Mediterranean Island Plants: Wild plants at the brink of extinction, and what is needed to save them. IUCN, Gland and Cambridge.

Myers N., Mittermeier R.A., Mittermeier C.G., Da Fonseca G.A.B., Kent J. 2000. Biodiversity hotspots for conservation priorities. Nature 403: 853-858.

Nybom H. 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Molecular Ecology 13: 1143-1155.

Ohlemüller R., Anderson B.J., Araújo M.B., Butchart S.H.M, Kudrna O., Ridgely R.S., Thomas C.D. The coincidence of climatic and species rarity: high risk to small range species from climate change. Biology Letters 4: 568-572.

Orrù M., Mattana E., Pritchard W.H., Bacchetta G. 2012. Thermal thresholds as predictors of seed dormancy release and germination timing: altitude-related risks from climate warming for the wild grapevine *Vitis vinifera* subsp. *sylvestris*. Annals of Botany 110: 1651-1660.

Ouborg N.J., Vergeer P., Mix C. 2006. The rough edges of the conservation genetics paradigm in plants. Journal of Ecology 94: 1233-1248.

Peakall R., Smouse P.E. 2006. GENALEX 6: genetic analysis in Excel Population genetic software for teaching and research. Molecular Ecology Notes 6: 288-295.

Peakall R., Smouse P.E. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics 28: 2537-2539.

Pritchard J.K., Stephens M., Donnelly O. 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945-959.

Prohens J., Anderson G.J., Herraiz F.J., Bernardello G., Santos-Guerra A., Crawford D., Nuez F. 2007. Genetic diversity and conservation of two endangered eggplant relatives (*Solanum verpertilio* Aiton, and *Solanum lidii* Sunding) endemic to the Canary Islands. Genetic Resources and Crop Evolution 54: 451-464.

Raimondo F. M., Scialabba A., Zecca G., Grassi F., Casazza G., Minuto L. 2012. Genetic diversity in the endangered Sicilian endemic *Brassica rupestris*: Proposals for a conservation strategy. Plant Biosystems 146: 847-856.

Raymond M., Rousset F. 1995. Genepop (version. 1.2), a population genetics software for exact tests and ecumenicism. Journal of Heredity 86: 248-249.

Reed D.H., Briscoe D.A., Frankham R. 2002. Inbreeding and extinction: The effect of environmental stress and lineage. Conservation Genetics 3: 301-307.

Reed D.H., Frankham R. 2003 Correlation between fitness and genetic diversity. Conservation Biology 17: 230-237.

Rosenberg NA. 2004. DISTRUCT: a program for the graphical display of population structure. Molecular Ecology Notes 4: 137-138.

Rousset F. 2007. GENEPOP’007: a complete re-implementation of the GENEPOP software for Windows and Linux. Molecular Ecology Resources 8: 103-106.

Segelbacher G., Cushman S.A., Epperson B.K., Fortin M.J., Francois O., Hardy O.J., Holderegger R., Taberlet P., Waits L.P., Manel S. 2010. Applications of landscape genetics in conservation biology: concepts and challenges. Conservation Genetics 11: 375-385

Salvo G., Bacchetta G., Ghahremaninejad F., Conti E. 2008. Phylogenetic relationships of Ruteae (Rutaceae): New evidence from the chloroplast genome and comparisons with non-molecular data. Molecular Phylogenetics and Evolution 49: 736-748.

Schuelke M. 2000. An economic method for the fluorescent labeling of PCR fragments. Nature Biotechnology 18: 233-234.

Tallmon D., Bellemain E., Swenson J., Taberlet P. 2004. Genetic monitoring of Scandinavian brown bear effective population size and emigration. Journal of Wildllife Management 64: 960-965.

Thompson J.D. 2005. Plant Evolution in the Mediterranean. Oxford University Press Inc., New York.

Thuiller W., Lavorel S., Araujo M.B., Sykes M.T., Prentice C. 2005. Climate change threats to plant diversity in Europe. Proceedings of the National Academy of Sciences 23: 8245-8250.

UICN France, FCBN, MNHN. 2012. La Liste rouge des espèces menacées en France - Chapitre Flore vasculaire de France métropolitaine : premiers résultats pour 1 000 espèces, sous-espèces et variétés. Dossier électronique.

Weir B.S., Cockerham C.C. 1984. Estimating *F*-statistics for the analysis of population structure. Evolution 38: 1358-1370.

Conclusions

Our analyses provided considerable information on the magnitude and pattern of genetic variation of the study species.

First of all, none of the endemic *taxa* under study met the general expectation that endemic species, and especially island endemics (Frankham 1997), exhibit lower levels of genetic diversity than widespread species (Hamrick & Godt 1989, 1996; Gitzendanner & Soltis 2000; Ellis et al. 2006). The Corso-Sardinian endemic *F. arrigonii* and both *R. lamarmorae* and *R. corsica* (endemic to Sardinia and corsica, respectively) were far from being genetically depauperated despite their rarity and their current restricted distribution. Secondly, genetic diversity values of *F. arrigonii* were comparable to the widespread congener *F. communis*, contrarily to the theoretical expectation that endemic species should exhibit lower levels of genetic diversity than widespread species (Karron 1987; Hamrick & Godt 1989, 1996; Cole 2003). Finally, the spatial genetic structure was weak in the case of *F. arrigonii*, while in the case of *R. lamarmorae* and *R. corsica* the analyses suggested the clustering of the individuals into two groups, approximately corresponding to taxonomic affiliations..

On the basis of the obtained results, several considerations can be made on the possible measures that could be undertaken to maintain the species' genetic genetic diversity. As regards *F. arrigonii*, it must be taken into consideration that most of its populations currently live in areas that are under regional or national protection from a legal point of view. Additionally, human activities are negligible in the most inaccessible sites. However, the legal protection of the sites alone might not be so effective for preserving the populations, especially the smallest ones and those that live in coastal areas where touristic pressure is higher. The latter could face habitat reduction and increasing risks of genetic depauperation or extinction due to the combined effects of the fragmentation of populations, human impact and invasion of alien species, like many other coastal *taxa*. Consequently, active initiatives of *in situ* conservation, such as monitoring and limitation of invasive species, together with *ex situ* conservation of germplasm, should be undertaken. Future actions will thus be aimed at conserving the highest possible number of populations, with particular attention to the smallest ones to those that exhibit the highest genetic diversity and making sure that the three genetic groups identified in this study are adequately represented.

As regards *R. lamarmorae*, results indicate that management should aim to conserve all populations, with particular efforts on the two larger populations (BC and SS), since the loss of one of these would very likely result in substantial loss of genetic variability for the species. The pressure it suffers throughout its range due to overgrazing, fires, ski facilities and activities (Bacchetta et al. 2006) might realistically have an impact on the populations of this narrow endemic. Additionally, mountain habitats are considered particularly sensitive to the influence of predicted climatic change and they are likely to show the deriving effects earlier and more clearly than some other ecosystems (Grabherr et al. 2000; Thuiller et al. 2005). Shrinking areas of currently rare climates may result in globally important centers of conservation interest becoming hotspots of extinction (Ohlemüller et al. 2008). The optimal germination protocol of the species (20° C with a photoperiod of 12/12 h and after 3 months of pre-chilling at 5°C; Bacchetta et al., unpublished data) actually indicates that a possible effect of warmer winters, as expected from IPCC projections (Alcamo et al. 2007), might lead to a failure of its seeds to receive sufficient chilling to satisfy the pre-germination vernalization requirement, and thus to a reduced reproductive capability of this species. This trend has already been observed for other Sardinian *taxa* living at medium-high altitudes, like *Lamyropsis microcephala* (Mattana et al. 2009), *Ribes multiflorum* subsp. *sandalioticum* (Mattana et al. 2012) and *Vitis vinifera* subsp. *sylvestris* (Orrù et al. 2012). While seeds from BC population have been already collected for long-term storage at the BG-SAR (the Sardinan Germplasm Bank, University of Cagliari), the collection of germplasm from SS and BS populations will be prioritary in the future. At the same time the designing of plant micro-reserves, a tool that has already been successfully applied (Laguna 1999, 2001; Laguna et al. 2004), would be advisable to protect *in situ* the populations of this rare species.

As regards *R. corsica*, being the species quite widespread across the main Corsican massifs and being its genetic diversity also quite high, it may be confirmed that the species is not in danger yet, and that the maintenance of an adequate number of populations might be effective in avoiding the impoverishment of its genetic resources.

References

Alcamo J., Moreno J.M., Nováky B., Bindi M., Corobov R., Devoy R.J.N., Giannakopoulos C., Martin E., Olesen J.E., Shvidenko A. 2007. Europe. Climate Change 2007: Impacts,

Adaptation and Vulnerability. Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. In: Parry M.L., Canziani O.F., Palutikof J.P., Van der Linden P.J., Hanson CE. (eds.), pp. 541-580. Cambridge University Press, Cambridge, UK.

Bacchetta G., Brullo S., Giusso del Galdo G. 2006. *Ruta lamarmorae* (Rutaceae), a new species from Sardinia. Edinburgh Journal of Botany 63:153-160.

Cole C.T. 2003. Genetic variation in rare and common plants. Annual Review of Ecology, Evolution and systematic 34: 213-237.

Ellis J.R., Pashley C.H., Burke J.M., McCauley D.E. 2006. High genetic diversity in a rare and endangered sunflower as compared to a common congener. Molecular Ecology 15: 2345-2355.

Frankham R. 1997. Do island populations have less genetic variation than mainland populations? Heredity 78: 311-327.

Gitzendanner M.A, Soltis P.S. 2000. Patterns of genetic variation in rare and widespread plant congeners. American Journal of Botany 87: 783-792.

Grabherr G., Gottfried M., Pauli H. 2000. GLORIA: a global observation research initiative in alpine environments. Mountain Researche and Development 20: 190-192.

Hamrick J.L., Godt M.J.W. 1989. Allozyme diversity in plant species. In: Brown A.H.D., Clegg M.T., Kahler A.L., Weir B.S. (eds.) pp. 43-63. Plant population genetics, breeding and genetic resources. Sinauer Sunderland.

Hamrick J.L, Godt M.J.W. 1996. Effects of life history traits on genetic diversity in plant species. Philosophical Transactions of the Royal Society B:Biological Sciences 351: 1291-1298.

Karron J.D. 1987. A comparison of levels of genetic polymorphism and self-compatibility in geographically restricted and widespread plant congeners. Evolutionary Ecology 1: 47-58.

Laguna E. 1999. The plant micro-reserves programme in the region of Valencia, Spain. Proceedings Planta Europa 1998. Second European Conference on the Conservation of Wild Plants: 181-185. The Swedish Threatened Species Unit and Plantlife, Uppsala and London.

Laguna E. 2001. The micro-reserves as a tool for conservation of threatened plants in Europe. Nature and Environment series nº 121. Council of Europe, Strasbourg.

Laguna E., Deltoro V.I., Pérez-Botella J., Pérez-Rovira P., Serra Ll., Olivares A., Fabregat C. 2004. The role of small reserves in plant conservation in a region of high diversity in eastern Spain. Biological Conservation 119: 421-426.

Mattana E., Daws M.I., Bacchetta G. 2009. Seed dormancy and germination ecology of *Lamyropsis microcephala*: a mountain endemic species of Sardinia (Italy). Seed Science & Technology 37: 491-497.

Mattana E., Pritchard H.W., Porceddu M., Stuppy W.H., Bacchetta G. 2012. Interchangeable effects of gibberellic acid and temperature on embryo growth, seed germination and epicotyl emergence in *Ribes multiflorum* ssp. *sandalioticum* (Grossulariaceae). Plant Biology 14: 77-87.

Ohlemüller R., Anderson B.J., Araújo M.B., Butchart S.H.M, Kudrna O., Ridgely R.S., Thomas C.D. The coincidence of climatic and species rarity: high risk to smallrange species from climate change. Biology Letters 4: 568-572.

Orrù M., Mattana E., Pritchard W.H., Bacchetta G. 2012. Thermal thresholds as predictors of seed dormancy release and germination timing: altitude-related risks from climate warming for the wild grapevine *Vitis vinifera* subsp. *sylvestris*. Annals of Botany 110: 1651-1660.

Thuiller W., Lavorel S., Araujo M.B., Sykes M.T., Prentice C. 2005. Climate change threats to plant diversity in Europe. Proceedings of the National Academy of Sciences 23: 8245-8250.

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