1. SUMMARY OF THE PROPOSAL

MAIN RESEARCHER: Isabel Mateu Andrés

PROJECT TITLE: Comparative phylogeography of representative plants of Mediterranean flora. Data of ecological and forestry interest

SUMMARY:

The objective of the project proposed herein is to investigate the comparative phylogeography of species that share their circunmediterranean distribution by means of chloroplastic microsatellites (cpSSR). For this purpose, 8 species of ecologic and economic interest have been selected for their use in restoring ornamental and wood ecosystems, these being: *Arbutus unedo* L., *Celtis australis* L., *Myrtus communis* L., *Nerium oleander* L., *Pistacia lentiscus* L., *Quercus coccifera* L., *Rhamnus alaternus* L. and *Rosmarinus officinalis* L.

Forty populations of each species spread out over their area of distribution will be studied. The chosen markers are cpSSR since they are of monoparental heredity, suitable to learn the geographic distribution of their genetic variability, and they also present high levels of variability. With the purpose of not only verifying that the different sizes of each of the fragments corresponding with concordant *indels*, but also that the equal sizes of one fragment involve the same nucleotidic composition, samples of each different sized fragment will be sequenced. In order to know whether differences exist between populations as far as aspects of forest interest are concerned, such as the germination capacity of seeds and plant survival, tests will be conducted under homogeneous conditions with material from 20 populations among those studied for each species.

With the results obtained we will try to know if the phylogeographic patterns of the studied Mediterranean species fit with the previously described in European boreal and template species and to get data that uphold the identification of areas of special interest for the conservation of genetic diversity in the Mediterranean, supplies reliable molecular tools to define areas of seed sources and certification, and that upholds the regulations controlling the seed and plant trade.

2. Introduction

2.1. Project purpose.

The intention of this project is to carry out the comparative phylogeographic study of 8 plant species that are distributed throughout the whole Mediterranean basin: *Arbutus unedo* L., *Celtis australis* L., *Myrtus communis* L., *Nerium oleander* L., *Pistacia lentiscus* L., *Quercus coccifera* L., *Rhamnus alaternus* L. and *Rosmarinus officinalis* L. These are sexually-reproducing plants, and some also present vegetative multiplication; most are the shrub kind although one species is arboreal (*Celtis australis*). All the species are of important ecological and forest interest, and also of an economic interest mainly for their ornamental and wood use.

The markers selected are chloroplastic microsatellites given their uniparental transmission and at the same time, they present relatively high levels of variability.

Data on the phylography of species distributed around the Mediterranean Sea are scarce, such as those we wish to deal with herein; indeed, no phylogeographical studies exist on any of the chosen species, except the western *Quercus coccifera* populations.

Although the number of species may be considered as relatively low, these are practically all the shrubby species predominantly distributed throughout the Mediterranean Region. On the other hand, and since species of the same genus tend to present common models, we expect that the project results enable us to obtain phylographic patterns that are generalised to other species in the area. The resulting information will enable us to indentify migratory routes and possible Pleistocene areas of refuge, to indicate areas of particular interest for the conservation of the genetic diversity of Mediterranean plants, and to serve as a basis to define regions of source, certificate of quality and the commercial regulation of seeds.

2.2. Background and the present-day situation of scientific-technical knowledge

The Mediterranean Sea stretches in an easterly-westerly (E-W) direction from the Straits of Gibraltar to Palestine. It occupies a prime position between Africa and Eurasia, being delimited in its northern half by large peninsulas and islands, while its southern half is formed by rectilinear stretches.

Geologically speaking, it derives from the ancient Tethys Sea after tectonic movements which determined the present-day position of the two continents. The movement of Africa during the Miocene caused the closure of the Straits of Gilbraltar to the extreme west, the mountain ranges which surround its basin, as well as the loss of connexion with the Indian Ocean. During the transitional period between the Miocene and the Pliocene (6-5 my), the closure of the Straits of Gibraltar led to the Messiniense crisis in which the sea almost dried up completely except for a few marginal salt lakes. This had catastrophic consequences for the biota of the Mediterranean Sea. Subsequently, the later connexion with the Atlantic Ocean enabled their biological recolonization.

The Mediterranean basin actually presents a climate chiefly characterized by warm and dry summers with cold and wet winters, originated in the later Pliocene, having been previously of tropical character. On average, the climate is colder and more humid in the W (except in S-E Spain) in which the hydric deficit period lasts two months, and it is warmer and dryer in the E, where the hydric deficit period may prolong for 5-6 months. During the Pleistocene glaciations, most tropical phanerogamic plants disappeared, although some species survived mainly in Macaronesia, and were replaced by some Iranian-Turanian and Saharan-Arabian steppe species, boreal elements and autochthonous species (Blondel and Aronson, 1999). From a biogeographical point of view, the circummediterranean areas with this climate type are considered as the Mediterranean Region.

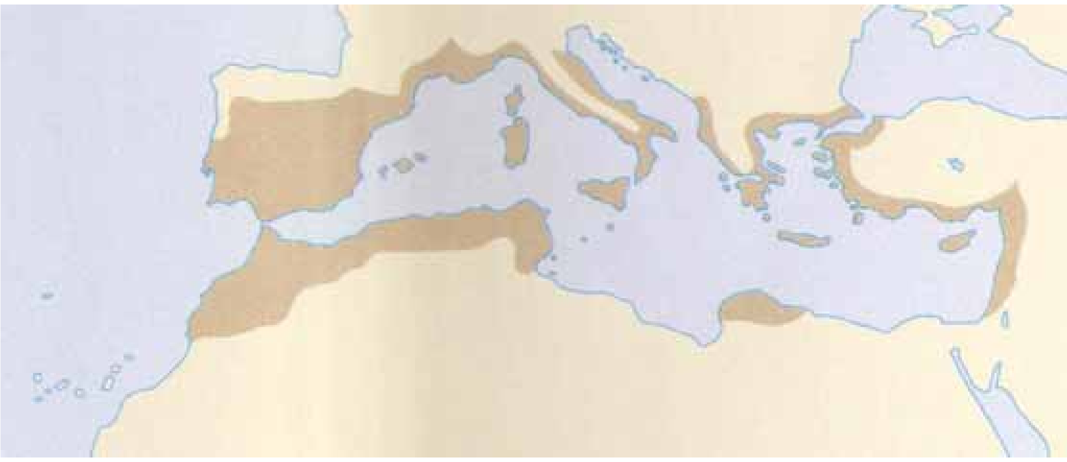

Figure 1: Areas of Mediterranean climate in the Mediterranean basin (taken from Folch et al., 1993)

Other important factors in present-day Mediterranean vegetation modelling have been the fire, either produced by natural or anthropogenic causes, and the historical deforestation of vast areas for agricultural use. Although the anthropogenic influence has been very important in the Mediterranean since ancient times, presently it is being made worse by intense farming and by vast urban development, especially in coastal areas.

The most characteristic natural vegetation of the Mediterranean area is the Mediterranean scrub (maquis and garriga); its name is designated to plant formations of sclerophyllous shrubs with a lower layer of annual herbaceous plants and associated perennials, and occasionally to species of the *Pinus* and *Quercus* genera. Another type of important vegetation is that which grows on soils that are either periodically or permanently humid in watercourses and brooks (edaphophyllous), and one of its most emblematic species is *Nerium oleander.*

Phylogeography is a part of biogeography which, in Avise's words (1998), the creator of the term, "it deals with the study relative to the principles and processes which govern the geographical distribution of lineages at an intraspecific level". Phylogeographic studies analyse the spacial distribution of alleles within and among populations to deduce their phylogenetic relations, although all those studies which deal with the spatial distribution of any genetic trait (morphological, behavioural, etc.) may be considered as phylogeographics in the broad sense (Avise, 1998). Most works on phylogeography have focussed on related species or groups of species (i.e. Tremblay and Schoen, 1999; Raspe *et al*., 2000; Hampe *et al*., 2003; Fineschi *et al*., 2003; Marcussen, 2003; Gardner *et al.*, 2004; Cieslak *et al.*, 2005).

As opposed to the abundant information available on phylogeography of European species (references in Aguinagalde *et al.*, 2005) which have extensively documented the role that refuges play in Mediterranean peninsulas during glaciations, works carried out on Mediterranean species are scarce.

The comparative phylogeography (Arbogast and Kenagy, 2001) is an extension of phylogeography which deals with the comparison between patterns exhibiting different *taxa* which share one distribution area. According to Bermingham and Moritz (1998), the comparative phylogeography may contribute to extending studies on ecology and evolution by providing an evolutionary and geographical context to species which share areas, by permitting the determination of historic factors and spacial influences which have determined the wealth of species, and by an understanding of the responses to changes in landscape as well as the identification of the evolutionary isolated areas by providing important information for conservation strategy design. Taberlet *et al.* (1998) published a study which compared the migration routes of various animal and plant species, in which common tendences between them are observed, as is the fact that this type of data have important implications in the conservation genetics since it is possible to establish areas in which efforts of this kind must be concentrated. According to these authors, the regions of Southern Europe are of a greater interest since most genetic variation is concentrated in them due to their refuge role during the ice ages.

In a very recent work, Aguinagalde et al.(2005) have demonstrated the influence of phylogeny in the geographic distribution patterns of genetic variability (in chloroplastic DNA), as well as the correlation between this and the species distribution type, whereas, on the contrary, no correlation with biological traits exists, such as the biologic type, form of reproduction, pollination and seed dispersal.

Within the European Economic Community (EEC), there is growing interest in restoring ecosystems, for which the use of autochthonous species and local materials is highly desirable since local genotypes are generally better adapted to their specific environmental conditions. With this purpose in mind, regions of forest species seeds within the EEC have been defined. These source regions are defined by their physiographic and ecologic data, and they are of a provisional nature since molecular data are expected which provide a more solid base for them to be definitely established.

While the flora distribution in the mild Europe follows certain latitudinal phylographic patterns, which have been extensively documented, the longitudinal orientation of the Mediterranean basin and its paleoclimatic history, marginally affected by glaciations presumably, suggest a longitudinal model which would correspond to colonisation phenomena across the migration routes. Nonetheless, the role that refuges play in the Mediterranean peninsulas and N Africa allow an alternative hypothesis to be considered; according to this later hypothesis, the genetic variability distribution would follow a mosiac model, a reflection of an ancestral fragmentation of populations.

If we base ourselves on what has been stated, the objective of this project is to carry out a comparative phylogeographic study of species which share their distribution around the Mediterranean Sea, with a view to:
1, To know the geographical distribution of genetic variability detecting possible phylogeographic patterns in such as way that it is possible to predict the organisation of the genetic diversity in other chloroplast species of maternal heredity.

2, To contrast possible phylogeographic patterns of the Mediterranean flora with previously observed by other authors for the boreal and template European flora.
3, Obtaining data which, along with those available in the bibliography (Fineschi *et al.*, 2005; Lumaret *et al.*, 2005; Jiménez *et al.*, 2004; González-Martínez *et al.*, 2004; Soranzo *et al.*, 2004), permit the indication of zones of essential interest to maintain the genetic diversity of Mediterranean plants.
4, Providing molecular data which are based on the definition of source regions of seeds of an ecological and forest interest, and which help regulate the seed and plant trade.

Choosing species:

Therefore, 8 species belonging to different families and with different biological characteristics have been selected which fulfil the following criteria:
- Of an autochthonous nature.
- Share a circummediterranean distribution area.
- Be of great ecological and economic interest: The chosen species are of an ample forest and ornamental interest, as they are used in gardening and to restore habitats, among other uses.

On the other hand, in order to collect the greatest taxonomic diversity and have more predictive value, and owing that the species of the same genus usually present common patterns (Aguinagalde *et al*., 2005), we have proceed to select only one species in the pluriespecific genus.

The selected species are: *Arbutus unedo* L., *Celtis australis* L., *Myrtus communis* L., *Nerium oleander* L., *Pistacia lentiscus* L., *Quercus coccifera* L., *Rhamnus alaternus* L. and *Rosmarinus officinalis* L.

They are sexually-reproducing plants, although several of them may multiply vegetatively (Table 1). Their distribution is mainly Mediterranean, although some species have marginally extended to other areas. *Celtis australis* is the only deciduous type tree, while the rest are evergreen shrubs, although they may reach a height of 4-6m. Other than *Nerium oleander* which inhabits phreatically humid soils, and *Celtis australis* which inhabits more xerophytic environments, grows in chasmophyte habitats with edaphic humidity, most are Mediterranean scrub species with a considerable ecologic expanse as far as soil types and altitudinal range are concerned, although they refuse continental environments.

Gymnosperms are not included because the chloroplasts within them are of a paternal hereditary, which would mean a different model (Burban and Petit, 2003). Species of the genera *Erica*, *Retama*, *Genista* and *Tamarix,* as well as *Ulex parviflorus*, *Phyllirea angustifolia* and *Viburnum tinus,* have been excluded since their distribution is limited to western basin area, as have species of the *Cistus* genera since they have already been studied (Vargas, 2005). Finally, *Phyllirea latifolia* and *P. media* have been excluded given their hybridization capacity with other species and their controversial taxonomic delimitation.

Other than the studies by Jiménez *et al.* (2004) and López de Heredia *et al.* (2005) on evergreen *Quercus* species (*Q. suber*, *Q. ilex* y *Q. coccifera*) of W Mediterranean, no previous phylogeographic studies have been conducted on any of these species. Werner *et al*. (2002) have studied the genetic diversity of *Pistacia lentiscus* in the southern Iberian Peninsula and North Africa by RAPDs, and a wide genetic difference was found among the populations of both zones. Portis *et al.* (2004) have published a work about the identification of varieties of the *Nerium oleander* culture by AFLPs. Angioni *et al.* (2004) have related the the results obtained with RAPDs with the chemical composition of essential rosemary oil in Sardinia, and its antimicrobian and antifungal activity. Hileman *et al*. (2001) have published a work on phylogenesis and biogeography of Arbutoideae which concludes the polyphyletic origin of the *Arbutus* genus. Traveset *et al.* (2001) have studied fruit dispersal-related aspects of *Myrtus communis*. Aronne and Wilcock (1994, 1995) demostrated how ants participated in the pollination and dispersal of *R. alaternus* fruits.

Table 1: The most significant biological characteristics of each species and its uses.

Species	Family	Type	Mult[1]	Sex	Pollin[2]	Fruit Disp[3]	Uses
Arbutus unedo	Ericaceae	Bush	R	H	Z	Z	Ornamental, wood, fruits
Celtis australis	Cannabaceae	Arbol	-	H	A	Z	Wood and ornamental
*Myrtus communis**	Myrtaceae	Bush	E	H	Z	Z	Ornamental, wood, tannery
Nerium oleander	Apocynaceae	Bush	E	H	Z	A	Ornamental
Pistacia lentiscus	Anacardiaceae	Bush	R	D	A	Z	Wood, resin
Quercus coccifera	Fagaceae	Bush	R	M	A	Z	Culture, cochineal, tennery
Rhamnus alaternus	Rhamnaceae	Bush	R	M	A, Z	Z	Ornamental, wood
Rosmarinus officinalis	Labiatae	Bush	E	H	Z	B	Ornamental, melliferous

* Protected species in the Balearics. H, Hermafrodita; D, Diocious; M, Monoecious. 1 Multiplication: E, cutting; R, sprout. 2, Pollination: Z, zoophilous; A, anemophilous. 3, Fruit dispersal: A, anemochorous; B, barochorous; Z, zoochore.

Choosing markers

In phylogeographic studies, chloroplastic or mitochondrial markers because they have a low mutation rate in relation to nuclear DNA and because of the uniparental heredity factor involved, which mean they are more geographically structured due to the restricted seed dispersal capacity in comparison with pollen. Mitochondrial DNA presents a very low mutation rate in plants, so we therefore centre on the cloroplastic DNA study that is maternally transmitted in angiosperms, except for very few exceptions.

The chosen markers are chloroplastic microsatellites (cpSSR), which present higher rates than other chloroplastic markers in plants (Provan et al., 1999), which is desirable when the genetic variability at an intraspecifc level has to be studied.

In the last ten years various universal primers have been described (Demesure *et al.*, 1995; Dumolin-Lapegue *et al.*, 1997; Grivet *et al.,* 2001; Taberlet *et al.*, 1991; Weising & Gardner, 1998), among which we have chosen those described by Weising & Gardner (1998). In 7 of them (ccpm1, ccpm2, ccpm3, ccpm4 ccpm7, ccpm9 and ccpm10) the cause of repetition are perfect microsatellites formed by mononucleotides (A or T), while the rest are compounds (ccpm5) or imperfect (ccpm6 and ccpm8), and are perfectly sited in chloroplastic DNA (Grivet *et al.*, 2001). The choice of these primers is motivated not only by their sound results with regards to the variability demonstrated in numerous plant groups, but also due to the fact that they have experience in the management and application of ash and other plant groups, the study of which is underway. In the possible case that the proposed primers do not present sufficient variability in any species, other primers will be used from the various types available in the above-mentioned literature.

The differences in the size of the fragments obtained by amplification with microsatellite primers is interpreted as different alleles. Nonetheless, these differences in the size of fragments may possibly be due to the number of repetitions of the microsatellite region, which either correspond to the different alleles in the strict sense, or to the differences of flanking region. On the other hand, equal sizes may present different compositions in nucleotides.

In order to verify that the differences in size of the PCR products are actually due to the number of repetitions of the motive, one sample of each different sized product will be sequenced with each primer and in all the species included in the study.

2.3. Most relevant bibliography

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Soranzo N, R Alia, J Provan, W Powell. 2000. Patterns of variation at a mitochondrial sequence-tagged-site locus provides new insights into the postglacial history of European *Pinus sylvestris* populations. *Molecular Ecology* 9: 1205-1211.
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Werner O, P. Sánchez-Gómez, M Carrión-Vilches, J Guerra. 2002. Evaluation of genetic diversity in *Pistacia lentiscus* L. (Anacardiaceae) from the southern Iberian Peninsula and NBorth Africa using RAPD assay. Implications for reafforestation policy. *Israel Journal of PlantSciences* 5:11-18.

2.4. National or international groups working on the same specific project subject, or on similar subjects.- Spanish groups:

R. Alía, alia@inia.es. INIA, CIFOR, PO 8111, Madrid 28080, Spain. For several years, he has been researching into the different aspects of differentiation and the genetic structure of the *Pinus* genus species, and also other aspects related to paternity, pollen flow and seeds, responses to sodium chloride (in *Populus* species).
P Goicoechea, pgoikoetxea@neiker.net. Neiker AB, Granja Modelo Arkaute, Apdo 46, Vitoria 01080, Spain. Although he has worked on various plant species, his most recent research is centred on phylogeography, genetic flow and the differentiation of the *Quercus* species.
P Vargas, vargas@ma-rjb.csic.es. CSIC, Real Jardín Botánico de Madrid, Plaza Murillo 2, E-28014 Madrid, Spain. Although his research has been concerned with plant phylogenesis, he has recently published a synthesis work on alpine plant diversification patterns in the European Mediterranean Region by ITS sequences.
J. Arroyo, Dpto. de Biología Vegetal y Ecología, Universidad de Sevilla, Aptdo. 1095, E-41080-Seville, Spain. He conducts research on the ecology of relict plant populations and phylogeography as a tool to study the evolutionary processes and their geographical patterns.
O. Werner & R. Ros, werner@um.cs , Fac Biol, Dept Biol Vegetal, Campus Espinardo, Universidad de Murcia, E-30100 Murcia, Spain; they have worked on the phylogeography of some Mediterranean moss species.

Foreign groups: Among the many groups which investigate on the phylogeography of plants, we indicate several European groups given their proximity and their better knowledge of flora from our territory:
RJ Petit: petit@pierroton.inra.fr. UMR Biodivers Genes & Ecosyst, 69 Route Arcachon, F-33612 Cestas, France. His extensive scientific production has covered several groups of animals and plants, mainly the species of the *Pinus* and *Quercus* genera, as well as a synthesis on the organisation of genetic diversity in nuclei and cytoplasmatic organelles, the correlation between biological traits and the distribution of species in the genetic structure through maternal heredity markers.
GG Vendramin: giovanni.vendramin@igv.cnr.it. CNR, Istituto di Genetica Vegetale, Via Madonna Piano, I-50019 Florence, Italy. He has produced ample studies about plant phylogeography and the distribution of the chloroplastic DNA-based genetic variation. He has worked on a wide diversity of species: *Fagus sylvatica*, *Zelkova abelicea*, *Fraxinus excelsior*, *Cupressus sempervirens*, and various *Quercus* and *Pinus* species.
P Taberlet: Univ Grenoble 1, CNRS, UMR 5553, Lab Ecol Alpine Genom Populat & Biodivers, F-38041 Grenoble 9, France. Although his works on phylogeography have mainly focussed on various animal groups (bears, donkey, salamanders, bovidae, etc.), his extensive production in this theme has led him to work in plants (*Rhododendron*, *Carex*, *Saxifraga*) and to carry out works on the synthesis concerning main postglacial migration routes in Europe.

RA Ennos: rennos@ed.ac.uk. Univ Edinburgh, Sch Biol Sci, Kings Bldg, Mayfield Rd, Edinburgh EH9 3JT, Midlothian, Scotland. Apart from works related to reproductive aspects and to plant phylogeography (*Calluna vulgaris, Ilex aquifolium*) he has published numerous works about the conservation of threatened species.

3. OBJECTIVES OF THE PROJECT

3.1. Initial hipótesis

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We will try to show if there are common phylogeographic patterns in the studied species and if those fit or differ with the previously described for the boreal and template European floras.

Plenty of bibliography exists on plant phylogeography which demonstrates the interest and viability of this type of works. The intention of the project proposed herein is to deal with the comparative phylogeographic study of 8 plant species distributed around the Mediterranean Sea: *Arbutus unedo*, *Celtis australis*, *Myrtus communis*, *Nerium oleander*, *Pistacia lentiscus*, *Quercus coccifera*, *Rhamnus alaternus* and *Rosmarinus officinalis*.

All of them are species of great ecological interest since they form an important part of the Mediterranean landscape, and they are also of an economic interest given their varied uses. The chosen species belong to a like number of families, and they have different vital characteristics.

For this work, chloroplastic microsatellites (cpSSR) will be used, as they have a maternally uniparental heredity, which is appropriate for the phylogeography purposes, and at the same time they show relatively high levels of variablity. We will try that the material comes from the same sampling stations.

The project results will be used to predict the organisation of the genetic diversity in other species with similar characteristics, to indicate areas of special interest for the genetic diversity maintenance of Mediterranean plants, and they will provide a valuable tool to certify the geographic source of seeds and will help to regulate the seed and plant trade.

3.2. Background and previous results to endorse the initial hypothesis.

◆

With the exception of the work which partially includes the area of distribution of *Quercus coccifera*, no phylogeographic data exist on the plants proposed herein, and works of this type on other Mediterranean species are scarce. However, the large number of species and groups studied with similar objectives and methodologies to those proposed herein endorse the project interest and viability.

Likewise, no foregoing comparative phylogeography studies exist on Mediterranean species, although some on flora from other territories exist of which the work by Taberlet *et al.* (1998) is stressed given its proximity, in which a study on comparative phylogeography is carried out and also on the postglacial recolonisation routes in Europe, where different animal and plant species are used. All this not only endorses the validity of the hypothesis presented herein, but also the possibility of obtaining results with the proposed methodology.

In addition, it has to be taken into account that the markers (cpSSR) to be used in this project are well-known and approved; they have been successfully used in a European project (FRAXIGEN) in which the leading investigator of this proejct has participated (IMA). On the other hand, the team members, which is entirely formed by people experienced in plant work (six of the members are lecturers in the Botany Department), along with the experience of some of the team members in this specific theme and in the use of other molecular markers (JP), are firm guarantees that this project will fulfil the objectives it proposes.

◆ 3.3. Specific objectives.

This project poses the following specific objectives:

1, **To know the geographic distribution of the genetic variability** of 8 Mediterranean species for it to be possible to predict the organisation of genetic diversity in other species with maternal transmitted chloroplasts.

2, **To compare the distribution patterns** of genetic varaibility in plants of an ecologic and economic interest, which are representative of Mediterranean flora to discover whether their phylogeographical patterns are generalized to other species that are not included in this work.

3, **To establish the areas where the greater genetic variability is concentrated of plants** round the Mediterranean, which may be taken as **areas of special interest** for genetic diversity maintenance of Mediterranean plants. For this purpose, sampled material from various species in these localities will be collected to conduct the molecular study.

4, **To make molecular tools available** to make the certification of the geographic source of seeds in species of an ecologic, forest and economic interest possible, as well as the regulation of the seed and plant trade.

5, **To obtain data of forestry interest**, in particular the rate of seeds germination and survival or plants between populations of the same species.

4. METHODOLOGY AND WORK PLAN

PERSONNEL INVOLVED IN THE PROJECT

Those tasks involving project development will be carried out by the following personnel:

Members of the investigation team

Dr. Isabel Mateu Andrés (IMA), Leading investigator. Full university teacher, appointed to the Dept. of Botany at the University of Valencia, and member of the Cavanilles Institute of Biodiversity and Evolutionary Biology. Along with JP, she will be in charge of preparing the protocols for DNA extraction and the amplification of various primers in all the species to be studied, as well as supervising cpSSR amplification reactions, and analysing the different generated fragments. She will also be in charge of analysing genetic data and of producing the informative book of results.

Dr. Miguel Guara Requena (MG), Full university teacher, appointed to the Dept. of Botany at the University of Valencia. Along with FB, MFP and AA, he will be in charge of collecting leaves and DNA extraction. He will participate in the collection of seeds in the more eastern regions along with FB, MFP and AA. He will be in charge of the informative results programme and the coordination with the EPOs.

Dr. Fernando Boisset López (FB), Full university teacher, appointed to the Dept. of Botany at the University of Valencia. He will be in charge of collecting leaves and DNA extraction along with MG, MFP and AA. He will collaborate with MG, MFP and AA in the collection of seeds in the more eastern regions.

Dr. Antoni Aguilella Palasí (AA), Full university teacher, appointed to the Dept. of Botany at the University of Valencia, and member of the Cavanilles Institute of Biodiversity and Evolutionary Biology. He will be in charge of collecting leaves and DNA extraction along with MG, FB and MFP. He will participate in the collection of seeds in the more eastern regions with MG, FB and MFP.

Dr. Maria Felisa Puche Pinazo (MFP), Full university teacher, appointed to the Dept. of Botany at the University of Valencia. She will be in charge of collecting leaves and DNA extraction along with MG, FB and AA. She will participate in the collection of seeds in the more eastern regions with MG, FB and AA.

Scientific collaborator:

Dr. Joan Pedrola Monfort (JP), An investigator who has been invited to the Dept. of Botany (Fac. of Biology) at the University of Valencia. He will be in charge of sequencing, and should it be the case, of cloning the fragments obtained with each of the primers in the different species. He will participate in optimising DNA extraction and in the amplification of microsatellite fragments.

Investigation team members belonging to the different applicant organisms.

Dr. Rafael Currás Cayón (RC), is a forestry expert. Director of CIEF (Regional Ministry of Environment,. Water, Urban Planning and Housing, G. V. - Valencian Autonomous Government) and part-time Associated Professor at the Dept. of Botany, University of Valencia. He will be in charge of collecting seeds from the most central and western area populations with EL and AM, and of conducting the germination and seedling survival tests.

Dr. Emilio Laguna Lumbreras, is a Biologist and Head of the Department of Protection of Natural Resources (Regional Ministry of Environment,. Water, Urban Planning and Housing, G. V. - Valencian Autonomous Government). He will be in charge of collecting seeds from the most central and western area populations with RC and AM, and of conducting the germination and seedling survival tests.

D. Antoni Marzo Pastor, is the Technical Director of the Forest Seed Bank Ministry of Territory and Housing, G. V. (Regional Ministry of Environment,. Water, Urban Planning and Housing, G. V. - Valencian Autonomous Government). He will be in charge of collecting seeds from the most central and western area populations with RC and EL, and of conducting the germination and seedling survival tests.

Requested personnel: This application includes expenses related to a temporary staff post (30 hours a week for 24 months), that of an average grade technician (CT) whose mission would consist in performing the experimental tasks required for the amplification of fragments with the different primers in each of the species to be studied (under the supervision of IMA). This person will also participate in the sequencing of the different fragments obtained (under the supervision of JP).

Due to high number of samples, amplification and sequencing of fragments are very laborious works. Sequencing of fragments needs a preparation process, and possibly complementary techniques, such as the cloning of small-sized fragments. Therefore, we consider that these tasks require a trained member of staff who has wide, demonstrated experience in molecular techniques. Thus, this post is considered essential to achieve the project objectives in the foreseen periods.

In addition, a grant from the Investigators Training Programme associated to the project is also requested . In the event of this grant being awarded, the selected grant holder will collaborate in the amplification of fragments . (cpSSR) and in analysing results (under the supervision of IMA).

<u>Previous tasks</u>

4.1. *Biological material collection*. Since the study to be undertaken is based on wild-type material, it will be necessary to collect material prior to undertaking any other part of this work.

We have to identify two aspects in this section:

1, collecting material for cpSSR amplification and sequencing.

2, collecting seeds to conduct germination and seedling survival tests.

For the first aspect, leaves of 8 individuals will be collected, separated by at least 50 m, in 40 natural populations of each of the species included in the project which cover the area of distribution. The material to be used will include healthy leaves with no signs of senescence, which will be dried in silicagel to avoid any DNA degradation. Once the leaves are in the laboratory, they will be stored in an ultra-freezer (-80ºC) until the DNA extraction process. Material will be collected from each population from the largest number of species possible with a view to achieving a greater integration of results.

For the second aspect, seeds from 20 populations of each species among the samplings will be collected for the first phase. The populations will be distributed throughout the various source areas and regions of each species. Seeds will be stored in the CIEF installations (Regional Ministry of Environment,. Water, Urban Planning and Housing, G. V. - Valencian Autonomous Government) by following the usual protocols used by staff in these installations and the indications by García-Fayos *et al*. (2001).

For the purpose of obtaining a greater integration capacity of results affecting the sampling populations, material of the different species within the same populations will be collected, as far as this possible, given the different ecology of *Nerium oleander* , and partly of *Celtis australis*.

Basically, material collection will be performed during the first project year. Nonetheless, the expanse of the area involved and the fact that seeds need to be collected, means having to organise collections in Autumn. In both cases, a logistic support is available which involves the participation of some INTERREG network team members.

Flowering and fructification seasons: ///// flowering season (yellow), ///// fructification season (red), ///// flowering+fructification (green)

Months:	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
*Arbutus unedo**	/////// (flowering+fructification)	///////							/////// (flowering+fructification)	///////	///////	///////
Celtis australis			/////// (flowering)	///////					/////// (fructification)	///////	///////	
Myrtus communis				/////// (flowering)	///////	///////	///////		/////// (fructification)	///////	///////	
Nerium oleander							/////// (flowering+fructification)	///////				
Pistacia lentiscus		/////// (flowering)	///////	///////					/////// (fructification)	///////		
Quercus coccifera			/////// (flowering)	///////					/////// (fructification)	///////	///////	
Rhamnus alaternus		/////// (flowering)	///////	///////					/////// (fructification)	///////	///////	
*Rosmarinus officinalis***	/////// (flowering+fructification)	///////	///////	///////	///////	///////	///////	///////	///////	///////	///////	///////

* In this species, the flowering season coincides with the ripening of fruits from the previous year.

** This species flowers and therefore fructifies throughout the year, especially after rainy seasons.

4.2. *Optimisation of the DNA isolation method.*

In order to amplify the microsatellite primers, quality, low degraded DNA preparations without contaminants are required to avoid inhibiting the amplification process.

The protocols that are normally used in other plant species will be followed, based on fractioned precipitations with cetyl-trimethyl-ammonium bromide (CTAB). If required, these will be modified for the phenolic compound absorption, by the addition of resins, in order to obtain the DNA purity level required.

4.3. Optimisation of the fragment amplification method.

Given the disparity among the species to be studied, it will be necessary to optimise protocols for the amplification by means of PCR reactions for each primer-species combination. These tests will be performed on 10 individuals of each species, and a wide range of annealing temperatures will be tested (between 38-58ºC), as will magnesium chloride concentrations (1-4mM), until fragments of the expected size are obtained.

<u>Objective 1</u>

4.1.1. Amplification and analysis of chloroplastic microsatellites (cpSSRs)

In order to perform this part of the work (Objectives 1 and 2), tests with 10 universal primers will be performed as described by Weising & Gardner (1998). Primers marked with different fluorochromes (HEX, NED, FAM Applied Biosystems) will be used to obtain fragments which may be analysed in a sequencer. If the fragment size range obtained with the different primers does not overlap, the multiplex technque will be used (simultaneous amplification with different primers).

The amplified fragments will be analysed in the Sequencing Service at the University of Valencia (SCIE), in a ABI Prism 3700 sequencer. The GENESCAN 3.1program will be used to determine the alleles. Different sized fragments will be taken as different alleles, assuming there is no homoplasy, since the individuals under study belong to populations of the same species.

For each species, initial tests will be performed with a sample of 50 individuals from different populations to select the primers which provide variability, and by ruling out those that do not. Should the proposed primers not present variability in a given species, or should the variability levels be low, other primers will be used from those available in the literature (Demesure et al., 1995; Dumolin-Lapegue et al., 1997; Grivet et al., 2001) until a minimum of 10 haplotypes are found per species.

4.1.2. Analysing genetic data

If the sequencing of fragments (Objective 3) confirm that the differences in a nucleotide are the result of a mutational event involving a single nucleotide, the statistically most parsimony haplotype network will be constructed, which will enable us to know the existing phylogenetic relations among them. The TCS program will be used for this purpose (Clement *et al*., 2000).

For each species, parameters related to genetic diversity of total and intrapopulation chloroplastic DNA will be obtained, based on unordered (h_S and h_T, respectively) and ordered (v_S and v_T, respectively) alleles. The unordered allelles model considers allelic frequencies, whereas the similarity among haplotypes is also taken into account in the ordered alleles model, that is, the proportion of shared fragments (Pons & Petit, 1996). In order to obtain the differentiation between populations, the rate of variation between populations will be calculated by following both models (G_{ST} and N_{ST}, respectively) with the PERMUTE program (pierroton.inra.fr/genetics/labo/Software) which, other than calculating genetic diversity and differentiation, also runs a test to establish whether any phylogeographic structure exists. In the absence of phylogeographic structure, then $G_{ST}=N_{ST}$.

Furthermore, the existence of phylographic structure will be checked by comparing the N_{ST} estimates with the N_{ST} values obtained after 10,000 permutations, using the SPAGEDI 1.1 program (Hardy & Vekemans, 2002). If $N_{ST}>N_{ST}$ is permuted, then phylographic structure exists. On average, this means that phylogenetically similar haplotypes are more frequently found in the same population than other randomly selected ones.

The geographic structure of genetic variation will be shown in a haplotypic frequency map in each of the populations studied by means of the MAPINFO 4.1 program (MapInfo Corp., NY).

In order to verify whether isolation by distance exists, the Mantel test will be performed by comparing the genetic distances matrix between the population pairs obtained by $G_{ST}/1-G_{ST}$, with the natural logarithm matrix of the geographic distances between them.

Through the SAMOVA algorithm (Dupanloup *et al*., 2002), the geographic groups of homogeneous populations with the maximum differentiation will be defined, as will the existence of genetic barriers between population groups.

4.1.3. Sequencing microsatellite fragments and their flanking areas.

The differences in the size of fragments obtained through the amplification of microsatellite primers are interpreted as different alleles. However, it is feasible that these differences in fragment size are due to the number of repetitions of the microsatellite region which, in the strict sense, would correspond to different alleles, or to the differences in the flanking region. On the other hand, equal sizes may present different compositions in nucleotides.

One sample from each different sized product will be sequenced (microsatellite region + flanking zones), with each primer from each species with a view to knowing whether the different sized fragments correspond, or not, to the different number of repetitions of the repetition motive. Although it is impossible to know *a priori* the number of samples to be studied, since we are unaware of the number of alleles we will find in each primer and species, we may make an estimate of between 2-4 alleles per primer, Therefore, the number of samples to be studied will vary between 20-30 per species, which means a total of 160-300 samples.

In order to carry out the sequencing of fragments, it is necessary to have purified material, and the nucelotides must be marked with a fluorochrome to make sequence reading possible. DNA fragment amplification will be performed by a two-step PCR procedure; pairs of primers and unmarked nucelotides will be used the first step, and the PCR products will be purified with conventional kits (QIAQUICK PCR PURIFICATION by Qiagen). In the second step, a second amplification will be performed by PCR using flurochrome-marked nucleotides (BigDye) from the fragments obtained and purified in the previous step.The ABI Prism 3700 sequencer at the Sequencing Service of University of Valencia (SCIE) will be used. The nucleotide sequences will be read and interpreted by the SeqEdit program. The automatic sequence alignment, along with its manual verification, will be performed with the BioEdit program's CLUSTAL X algorithm.

In the event of not being able to sequence any microsatellite fragment given its small size, this fragment will be cloned in a TA type vector, such as pGEM-T (Promega). It will subsequently be sequenced with the vector primers (T7 promotor and SP6).

<u>Objective 2</u>

Once we know the data on genetic variability levels and their structure both within and among the populations covering the whole area of study, and in the case of those species with a wider distribution, we know the data on the general species of each individual species, we will be in a position to make comparisons among them with a view to establishing phylogeographic patterns that are common to plants which, with their different biological characteristics, share the same autochthonous trait and circummediterranean distribution.

Development of this objective will not add work as it will be a conclusion of the analysis of the results obtained in objective 1.

<u>Objective 3</u>

For a best integration of the results of every species, and to detect areas with a high genetic diversity and singular haplotypes, samples of species for molecular analysis (objective1) and for germination assays and plants survival (objective 5) will be collected, as far as possible, in the same localities.

Development of this objective will not add work as it will be a conclusion of the analysis of the results obtained in objective 1.

<u>Objective 4</u>

The analysis of objective 1 results, will permit to establish if there are differences in haplotypes distribution in study area. If they will exist, it will be possible to know the seed geographic origin, and this aspect will be apply to establish the rules to control commercialisation of seeds and plants.

Development of this objective will not add work as it will be a conclusion of the analysis of the results obtained in objective 1.

<u>Objective 5</u>

4.5.1. Seed viability tests: Since the germination and dormancy requirements of seeds may vary among species populations (Gibson, 2002), the germination and seedling survival tests will be performed on seeds collected in 20 sampling populations for the molecular study of each species.

In the germination assays, the methodology proposed by the International Seed Testing Association (International rules for seed testing, 2006) will be used. Each assay will be carried out on 400 randomly taken seeds, distributed in four repetitions of 100 seeds in Petri dishes with dampened filter paper. The Petri dishes and paper will be autoclaved prior to the assay.

Assays will be performed in incubators or in germination chambers with the appropriate lighting and temperature conditions for each species. If required, seeds will be treated with acids or submitted to mechanical escarification of the testa, stratification or to embrion isolation, with a view to overcoming dormancy and to favouring rapid germination. Seeds will be considered as germinated when the root reaches the seed size. A count of germinated sedes will be performed on a daily basis until it is considered that the test is complete. An average of 4 repetitions will be considered as the result of germination. Assays are tolerable providing the deviation is produced between the extreme values and not the ISTA tolerance limits.

The germination tests will be repeated on a yearly basis to know the seeds variability throughout the study period.

In order to determine whether or not differences exist not only in the germination capacity of the seeds attributable to their different source, but also in the years within each population, both variance analyses will be performed.

4.5.2. Seedling/plant survival. As a natural continuation process to the study on seed germination capacity, seedling survival will be studied.

For this purpose, seedlings will be obtained through seed sowing in containers. Five seeds will be sown in each container and only one will be left after their germination. Seedlings will remain in the containers until after their first growth period. At this stage, 30 of them will be randomly selected, and transferred outdoors in plots at the CIEF installations inside their respective containers. Plants will be placed at equal distances of 30cm. No fertilisers will be used during the experiment, and plants will be watered homogeneously with an amount of water similar to the rainfall rate of their natural populations.

Seedling survival will be assessed by a direct twice-weekly count of surviving individuals.

The variance analysis applied to the results obtained for each parameter will enable to determine whether or not any differences exist that attribute to the different seed sources.

4.1 ORIENTATIVE SCHEDULE

<table>
<tr><th>Activities/Tasks</th><th>Executive Centre</th><th>Persone in charge and other people envolved</th><th>First year</th><th>Second year</th><th>Third year</th></tr>
<tr><td>Previous Works:
Collecting biological material</td><td>UVEG
GV</td><td>MG, FB, MFP, AA,
RC, EL, AM</td><td>| |x|x|x|x|x|x|x|x|x|x</td><td>| | | | | | | | |x|x|</td><td>| | | | | | | | | | |</td></tr>
<tr><td>Optimization of DNA isolation method.</td><td>UVEG</td><td>IMA, JP</td><td>x|x| | | | | | | | | |</td><td>| | | | | | | | | | |</td><td>| | | | | | | | | | |</td></tr>
<tr><td>Optimization of the amplification of fragments for each species-primer pair</td><td>UVEG</td><td>IMA, JP</td><td>| |x|x|x|x|x| | | | |</td><td>| | | | | | | | | | |</td><td>| | | | | | | | | | |</td></tr>
<tr><td>Objective 1.</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>DNA extraction</td><td>UVEG</td><td>MG, MFP, FB, AA</td><td>| | | | | | | |x|x|x|x</td><td>x|x| | | | | | | |x|x</td><td>| | | | | | | | | | |</td></tr>
<tr><td>Amplification and analysis of microsatellites fragments</td><td>UVEG</td><td>IMA, CT, becario</td><td>| | | | | | | | | | |x|x</td><td>x|x|x|x|x|x|x|x|x|x|x|x</td><td>x|x|x| | | | | | | | |</td></tr>
<tr><td>Secuenciación de fragmentos y análisis de secuencias.</td><td>UVEG</td><td>JP, CT</td><td>| | | | | | | | | | |</td><td>| | | | | | | | | | |</td><td>| | | | | | | | | | |</td></tr>
<tr><td>Objective 5.</td><td></td><td></td><td>| | | | | | | | | | |</td><td></td><td></td></tr>
<tr><td>Seed germination tests</td><td>GV</td><td>RC, EL, AM</td><td>| | | | | | | | | | |</td><td>| |x|x|x| | | | | | |</td><td>| |x|x|x| | | | | | |</td></tr>
<tr><td>Seedlings/plants survival</td><td>GV</td><td>RC, EL, AM</td><td>| | | | | | | | | | |</td><td>| | | | |x|x|x|x|x|x|x</td><td>x|x|x|x|x|x|x|x|x| | |</td></tr>
<tr><td>Reports and papers</td><td>UVEG, GV</td><td>IMA, JP, MG, FB MFP
AA, RC, EL, AM</td><td>| | | | | | | | |x|x|x</td><td>| | | | | | | | |x|x|x</td><td>| | | | | | | | |x|x|x</td></tr>
<tr><td>Web site production and maintenance.</td><td>UVEG</td><td>MG</td><td>| | |x|x| | | | | |x|</td><td>| | | | |x| | | | | |x</td><td>| | | | |x| | | | | |x</td></tr>
<tr><td>Informative book</td><td>UVEG, GV</td><td>IMA, JP, MG, FB MFP
AA, RC, EL, AM</td><td>| | | | | | | | | | |</td><td>| | | | | | | | | | |</td><td>| | | | | |x|x|x| | |</td></tr>
<tr><td>Objectives 2, 3 and 4 are not featured in this schedule as they no additional work (see Methodology section)</td><td></td><td></td><td>| | | | | | | | | | |</td><td>| | | | | | | | | | |</td><td>| | | | | | | | | | |</td></tr>
</table>

5. PROJECT BENEFITS, DIFFUSION AND EXPLOITATION OF RESULTS, SHOULD THIS BE THE CASE

Scientific and technical contributions

Many data on the previously described objectives are expected from the undertaking of this project. These data will be presented in those congresses and scientific meetings which cover similar themes. In addition, these data will be published in prestigious international journals in such areas of genetics, botany, forest cultures, etc. which may foreseeably cover the following specific aspects:

1 – Study of chloroplastics DNA genetic variability and its phylogeographic structure of each of the species studied.

2 – Comparative study of the different species to establish common patterns.

3- Germination capacity of seeds from different geographic origins, and seedling survival of different species.

It is expected that the results obtained from this project contribute to defining the source areas and zones of seeds of forest use, similarly to those established for arboreous species. This constitutes a task of essential importance which is still outstanding in Mediterranean scrub plants.

Likewise, it is expected that the project results will be directly applied to the public and private entities, which are interested for a variety of reasons, in the certification of origin of plants, either for their ornamental use or to restore ecosystems. Phylogeographic studies, the germination capacity of seeds, plant survival and the growth of important Mediterranean scrub plants will provide important data for the production and commercialisation of these plants, and for their appropriate use in restoration. Such interest has been shown by the support received by the different EPOs to the project.

Fitting of the project to the grant priorities

It is important to stress the project's adaptation to the priorities of this call for proposal, since Phylogeography is part of Biogeography; therefore, this project perfectly fits section 1.1 of the National Programme of Biodiversity, Earth Sciences and Global Change.

Plan for the dissemination of results

The project results will be diffused according to the scientific community through publications, and to the EPOs through meetings. Three one-day meetings with the EPOs are foreseen. The first will be held during the first 6-month project period when our approaches and methodology will be expounded, and comments, suggestions and requests will be attended to by people and groups with a great deal of practical experience in seed management and in the cultivation of the plant species included in this work. All this will provide us with essential information for the sound undertaking of this work. The second meeting will take place half-way through the second year, the purpose of which is to set out the results that are being obtained, along with the problems which we come across with a view to forming opinions to help us overcome them. The third meeting is foreseen at the start of the last six-month project period; the overall project results will be set out on this occasion.

In addition, an Internet web site will be set up, which will be updated every six months, where anyone interested could consult approaches, the project methodology and the results that are being obtained.

To culminate the project, a small explicative book will be produced in which the objectives, the methodology pursued and the results obtained from the project will be explained; special emphasis will be placed on those aspects that are more directly applied by the final users.

6. BACKGROUND OF THE APPLICANT TEAM OF THE PROPOSED THEME

As indicated in section 4, the investigation team is made up of :

Dr. Isabel Mateu Andrés (IMA), Leading investigator. Full university teacher, appointed to the Dept. of Botany at the University of Valencia, and member of the Cavanilles Institute of Biodiversity and Evolutionary Biology.

Dr. Miguel Guara Requena (MG), Full university teacher, appointed to the Dept. of Botany at the University of Valencia.

Dr. Fernando Boisset López (FB), Full university teacher, appointed to the Dept. of Botany at the University of Valencia.

Dr. Antoni Aguilella Palasí (AA), Full university teacher, appointed to the Dept. of Botany at the University of Valencia, and member of the Cavanilles Institute of Biodiversity and Evolutionary Biology.

Dra. Maria Felisa Puche Pinazo (MFP), Full university teacher, appointed to the Dept. of Botany at the University of Valencia.

Dr. Joan Pedrola Monfort (JP), Collaborating scientist of the University of Valencia, develops his research in IMA.'s laboratory.

Dr. Rafael Currás Cayón (RC), is the Director of CIEF (Regional Ministry of Environment,. Water, Urban Planning and Housing, G. V. - Valencian Autonomous Government)

Dr. Emilio Laguna Lumbreras (EL), is Head of the Department of Protection of Natural Resources (Regional Ministry of Environment,. Water, Urban Planning and Housing, G. V. - Valencian Autonomous Government)

D. Antoni Marzo Pastor, is Technical Director of the Forest Seed Bank (Regional Ministry of Environment,. Water, Urban Planning and Housing, G. V. - Valencian Autonomous Government)

These investigators form a multidisciplinary team including two people from Regional Ministry of Environment,. Water, Urban Planning and Housing, G. V. (Valencian Autonomous Government) (RC and EL), five people from the same Department (IMA, MG, AA, FB and MFP), as well as RC who is also a professor in this department, will significantly facilitate coordination. The participation of JP, who maintains a private professional activity which is totally compatible with the intensive collaboration with the investigation undertaken by IMA, strengthens the investigation team, particularly in relation to molecular work.. JP is also highly experienced in field work.

The investigation team is entirely made up of scientific personnel with the sufficient technical knowledge required to cover the various proposed project objectives, and whose experience endorses the attainment of results. The team includes members of staff experienced in the use of various molecular markers applied to the conservation objectives of both threatened plants and the characterisation of species (IMA); phylogenesis (JP); forest themes (MG); and the ample knowledge in the recognition of Iberian flora and vegetation (AA, FB, MFP, MG). We believe that the inclusion of specialists in non vascular flora does not reduce this group's efficiency since the tasks that they will be in charge of (material collection and DNA extraction) involve no difficulties. In fact, we believe the opposite occurs, that is, we consider this project an ideal occasion for a large group of investigators to be introduced to molecular work by extending the possibility of future investigations of a phylogeographic nature in other plant groups.

On the other hand, the team members have collaborated for a long time on different projects and investigation works (*see attached CVs*). Thus, even though they are an investigation team within this project, they have already been sharing professional activities for several years, which endorses team stability.

IMA has ample experience in the use of molecular markers applied to the systemised conservation of threatened species. She participates in project BFI2002-00664 and is in charge of applying microsatellites to the characterisations of rice varieties. She is the leading investigator of a coordinated European project in which nuclear microsatellites are applied to genetic diversity study and to the structure of European ash species (*Fraximus ornus, F. excelsior* and *F. angustifolia*), and chloroplastic microsatellites to establish the phylogeography of such species. As a result of this recently finished project (30th

June 2005), is the book entitled *Ash species in Europe* of which IMA is co-author and she has participated in the drafting of the aforementioned sections on *F. ornus* and *F. angustifolia*.

MG, has participated in more than 10 investigation projects (agreements and contracts) financed by public funds (Generalitat Valenciana (G.V.) and the Ministery of Education and Science: CICYT) which refer to the effects of forest fires and forest treatments, and the ecology and taxonomy of certain vascular species, as well as the taxonomy and chorologic treatment of several plants. He is highly experienced in the statistical and multivariate data treatment.

AA, is the director of the Botanical Gardens belonging to the University of Valencia. He is a specialist in Iberian flora and vegetation. He has participated in various projects financed by different public entities and organisms concerning the assessment of flora and phylogenetic resources. He currently participates in a European ENSCONET project, for the conservation of native European seeds.

FB, is a specialist in Mediterranean marine flora and he has participated in various projects financed with public funds (GV and CICYT), among which the Flora Phycologica Ibérica project stands out, the objective of which is to progressively update information on algal biodiversity along Iberian coastlines. Although his investigation activity covers the marine flora sphere, his teaching work in the Dept. of Botany provides him a sound knowledge of vascular flora, upon which he based his Degree Thesis.

MFP, is a specialist in Iberian bryophyte flora. She has participated in several investigation projects financed by various public organisms, among which the Flora Briofítica Ibérica stands out which has been underway for nine years.

JP has been director of the Marimurtra Botanical Gardens (Blanes, Gerona -Spain) and he has an extensive curriculum with regards to the application of various molecular markers (alloenzymes, RAPDs, microsatellites) in studies concerning evolutionary biology and plant phylogeensis. The techniques he manages include sequencing, and he is experienced in Bayesian methods with inference to plant phylogenesis and phylogeography.

RC is Director of the Centre for Forest Investigation and Experimentation of the Valencian Community. He is an associated professor at the Dept. of Botany at the Faculty of Biology. He has been Head of the Territorial Service Department at ICONA in the Valencian Community, and Head of the Forest Section of Valencia. He has ample experience in planning, drafting and project undertaking, and he is also experienced in works on hydrological-forest restoration, forest repopulation, forest management treatments, firefighting, restoration of degraded and burnt areas, and of Forest Care actions.

EL is Head of the Department of Protection of Natural Resources of the G,V., and he is in charge of the external relations in international projects and mutiregional or pluriregional networks for flora or habitat conservation programmes. It own the Silver Leaf Award Planta Europa on excellence in European Plant Conservation. It is expert member of the IUCN's Species Survival Commission.

AM is the Technical Director of the Forest Seed Bank (at CIEF) of the G.V. He is in charge of processing fruit, seed variability assays, and the cultivation of seedling under controlled conditions. He coordinates the European project P.I.C. IIIB Medoc, and he is in close contact with other Mediterranean centres dedicated to the conservation of genetic flora resources (the INTERREG network, the conservation centre network of Mediterranean flora genetic material).

6.1 PUBLIC AND PRIVATE FINANCING (I+D PROJECTS AND CONTRACTS) OF THE INVESTIGATOR TEAM MEMBERS

Project or contract title	Relation to the application presented herein (1)	Leading investigator	Subsidy awarded or requested EUROS	Financing entity and project reference	Period in force or appliance date (2)
Physiological and molecular markers of tolerance to abiotic factors generated by hydric deficit in cell lines and rice plants.	2	Dr. M.J. Cornejo	8,950,000pts	Ministry of Science and Technology PB 98-1428	01/ 2000-12/2002 C
FRAXIGEN. Ash for the future.	1	Dr. Janet Stewart/ Dr. I. Mateu (project coordinator)	135,960	European Economic Community EVK2-CT-2001-00108	11/2001-06/ 2005 C
Responses to saline and osmotic stress in rice (*Oryza sativa* L.): markers of crossed and specific tolerance.	3	Dr. M.J. Cornejo	116,150	Ministry of Science and Technology BF I 2002-00664	01/2003-12/2005 C
Biodiversity study of *Rosmarinus* populations in the Valencian Community.	1	Dr. P. Soriano	15,025	Ministry of Culture, Education and Science, GV GV00-060-3	1-1-2001 / 31-12-2002 C
Molecular characterisation of species and cultivars of the *Lavandula* L genus with an economic interest.	1	Dr. I. Mateu	12,000	University of Valencia	1-1-2006/ 31-12-2006 S
Controlling the pests of pine: study on the complex parasite in the municipality of Los Serranos (Valencia).	3	Dr. J. Selfa	10,818	Ministry of Culture, Education and Science GV99-129-1-3	1-1-2000/ 30-12-2001 C
Study of the complex parasite associated with “oruga de zurrón” *Euproctis chrysorrhea* (Lepidoptera, Lymantriidae) in autochthonous ferns.	3	Dr. J. Selfa	42,435	Ministry of Science and Technology BOS2002-03820	1-11-2000/ 30-10-2005 C
Determination of plant macrophytes in the study on the introduction of a vigilance water quality network by means of biotics indices in the sphere of the Hydric Confederation of el Júcar.	2	Dr. A. Aguilella	9,616	UTE Unima Control	2000-2001 C

Catalogue of macroscopic marine algae of the Valencian Community.	3	Dr. F. Boisset	12,500	Ministry of the Environment, GV 20020348	31-5-2002/ 31-12-2002 C
Flora Phycologica Ibérica. Bonnemaissoniales, Gracilariales, Palmariales and Rhodymeniales.	3	Dr. C. Rodríguez	13,216,35	Ministry of Science and Technology REN2001-1473-C03-02	28-12-2001/ 28-12-2004 C
"Flora Briofítica Ibérica" (2nd phase)	1	Rosa Mª Cros Matas	10,357	Ministry of Science and Technology BOS2000-0296-C03-03	2001-2003 C
"Flora Briofítica Ibérica"	1	Rosa Mª Cros Matas	40,000	Ministry of Science and Technology REN 2003-03131	2004-2006 C
"Flora Briófitica of the Macizo del Caroche and its surroundings".	1	Felisa Puche	16,279,66	Ministry of the Environment G.V. 99/03/070	Septiembre 1999 a Septiembre 2001 C
"Informatització i actualització de la Col.lecció de Briófits de l´Herbari VAL".	2	Felisa Puche	11,900	Minisitry of Territory and Housing (G.V.) 40/BD/05	Marzo 2005- Octubre 2005 C
"Flora briofítica Ibérica: Funariales (pro parte), Tatraphidales, Buxbaumiales, Diphysciales, Splachnales, Ditrichales".	1	Montserrat Brugués Doménech	75,000	Ministry of Education and Science	Enero 2006 S
Typification, cartography and assessment of Spanish pastures.	2	Dr. Alfonso Sanmiguel Ayanz	43,519,60 (Valencian Community)	INIA and all the autonomous communities except Catalonia OT00-037-C17	Enero 2001/ Diciembre 2003 C
Evolutionary Biology of *Androcymbium* from SW .Africa (alloenzymes and RFLPs).	2	Joan Pedrola	60,000	The Karl Faust Foundation	1-1-1998/ 31-12-2001 C
Phylogenesis and genetics of SE African population species of the *Androcymbium* genus (RAPDs, and cpDNA, mDNa and nDNA sequencing).	2	Joan Pedrola	80,000	The Karl Faust Foundation	1-1-2001/ 31-12-2003 C

Phylogenesis and Phylogeography of the *Lithodora* and *Merendera* genera in the Mediterranean (cpSTR and Sequencing).	1	Joan Pedrola	50,000	The Karl Faust Foundation	1-1-2002/ 31-12-2005 C
Phytoecological and Faunal Diagnosis of the Júcar river banks at the stretch between Carcaixent and the AP-7 motorway, and between la Gola de l'Estany and Cullera.	2	A. Aguilella	41,891,11	CEOP - Centre of Studies and Experimentation of Public Works (CEDEX)	2002 C
Activities on the germplasm of wild-type flora at the Botanical Gardens.	1	A. Aguilella	21,500	Ministry of the Environment – G.V.	2002 C
Collecting and processing seeds from the germplasm bank of threatened flora within the Valencian Community (LIFE Project on the conservation of priority habitats).	1	Antoni Aguilella	10,360,34	- Ministry of the Environment – G.V	2003 C
Production and scientific councilling of the exhibition of the LIFE Project on the conservation of priority habitats in the Valencian Community.	2	Antoni Aguilella	10,344	Ministry of the Environment – G.V	2003 C
Assessment of the ecological situation of the rivers in the hydrographic basin of el Júcar through the QBR index.	2	Antoni Aguilella	48,000	EPTISA, SERVICIOS DE INGENIERÍA, S. A.	2004 C
Biodiversity explanation and conservation: theme gardening and landscape restoration of the motorway areas between l'Hospitalet de Llobregat and Alicante: preliminary plan and LIFE funds application.	1	Antoni Aguilella	15,600	AUMAR Autopista del Mare Nostrum (Motorway services)	2004-05 C
CREATION D'UN RESEAU DE CENTRES DE CONSERVATION DU MATERIEL GENETIQUE DE LA FLORE DES REGIONS MEDITERRANEENNES DE L'ESPACE MEDOCC.(GENMEDOC). P.I.C. INTERREG III B – MÉDITERRANÉE OCCIDENTALE	1	Antoni Marzo	65,785	EEC	2004-06 C
ENSCONET - The European Native Seed Conservation Network VI PRGRAMME NETWORK, SUPORT FOR RESEARCH INFRAESTRUCTURES, INTEGRATING ACTIVITIES, CO-ORDINATION ACTION, EU, IIA-CA-506109/03 19 Participating entities (COORD. ROYAL BOTANIC GARDENS KEW, MSBP).	1	Roger Smith	364,607	EEC	2004-2009 C

(1)

0 = The same project
1 = It is very linked
2 = It is few linked
3 = It is not linked

(2) C=grant, S= application