UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO

DIPARTIMENTO DI TECNOLOGIE, INGEGNERIA E SCIENZE DELL'AMBIENTE E DELLE FORESTE

DOTTORATO DI RICERCA IN SCIENZE E TECNOLOGIE PER LA GESTIONE FORESTALE E AMBIENTALE

XXII CICLO

BIOLOGICAL STUDIES AIMED AT THE RE-INTRODUCTION OF ENDANGERED ENDEMIC FOREST SPECIES OF THE AZORES ARCHIPELAGO

SETTORE SCIENTIFICO DISCIPLINARE AGR05

COORDINATORE

PROF. Gianluca PIOVESAN

TUTOR

PROF. Bartolomeo SCHIRONE

DOTTORANDA

DOTT.ssa Raquel Gomes Caetano Ferreira

To my Parents and brother

To Marco Paulo

RIASSUNTO

L'arcipelago delle Azzorre ospita un particolare tipo di flora, la flora della Macaronesia, che raggruppa una vasta serie di elementi relitti risalenti al Terziario attualmente sotto minaccia di estinzione. L'arcipelago rappresenta pertanto un eccellente campo di studi mirati allo dinamiche evolutive di tali specie ed allo sviluppo di strategie di conservazione. Questo è proprio il caso delle due specie scelte in questo lavoro, una endemica dell'arcipelago attualmente definita "Critically Endangered", *Picconia azorica* (Tutin) Knobl. (Oleaceae) e l'altra, *Taxus baccata* L. (Taxaceae), recentemente classificata come "probabilmente estinta". Nel presente studio, per l'urgenza di promuovere i programmi di conservazione della *Picconia azorica,* sono stati trattati: (a) la biotassonomia dettagliata della specie (inerente le ipotesi di origine e le relazioni filogenetiche con l'unica altra specie congenerica di *P.azorica*: *P. Excelsa*, endemica delle isole Canarie e dalll'arcipelago di Madeira); (b) la valutazione della diversità genetica intra-specifica di *P.azorica*. I metodi usati per questo scopo sono stati l'analisi delle sequenza del DNA plastidiale e l'analisi con i marcatori molecolari (PCR-RFLP e SSR). I dati filogenetici suggeriscono la monofilia del genere *Picconia* e sostengono la divergenza del genere *Picconia* dalle altre Oleaceae nel tardo Miocene. Tre marcatori cpSSR polimorfici hanno permesso l'identificazione dei cinque aplotipi diversi della *P. azorica.* La specificità e la relittualità delle discendenze di *P. azorica* vengono presentati e discussi. I profili di diversità intra-specifica della *P. azorica* dimostrano una debole struttura genetica. La sopravivenza della specie è a rischio e viene suggerita una gestione dei popolamenti concentrata sulla conservazione *ex situ* ed *in situ*, basata sui dati genetici ottenuti. Inoltre, sono stati effettuati la caratterizzazione del legno della specie e diversi studi biologici volti ad aumentare le scarse conoscenze disponibili su questa specie.

Nonostante la specie venga ritenuta attualmente estinta, la presenza di *Taxus baccata* nelle Azzorre è stata documentata e ne ha permesso la prima caratterizzazione per mezzo di analisi morfometriche e genetiche. Gli individui ritrovati sono molto pochi ma hanno dimostrato delle peculiarità estremamente relevanti per la biodiversità della specie e per le sue implicazioni

filogeografiche. Tutti gli individui analizzati sono stati trovati nell'isola di Pico e mostrano caratteri micromorfologici fogliari diversi, e probabilmente "primitivi", rispetto a quanto riportato in letteratura. Questa assunzione è stata confermata dallo studio genetico effettuato, per mezzo dell'analisi della sequenza di un frammento genico plastidiale, che ha dimostrato che gli individui dalle Azzorre rappresentano una linea evolutiva diversa entro il genere *Taxus*, suggerendo la derivazione più diretta dai progenitori rispetto alle altre provenienze analizzate (appartenenti alle regioni Mediterranee e nord-Europee). Gli individui trovati nell'isola di Pico potrebbero essere gli ultimi discedenti sopravissuti di progenitori estremamente antichi, preservati nell'arcipelago di Azzorre insieme con la flora Macaronesiana. Anche in questo caso, viene suggerita un'attività di conservazione urgente, focalizzata sulla protezione *in situ* ed *ex situ* (in particolare, mediante propagazione clonale analizzata durante lo studio presente) per la salvaguardia di questa rara e preziosa discendenza.

Parole chiave: Flora Macaronesica, Arcipelago delle Azzorre, Specie endemiche, Specie minacciate, Diversità genetica, Conservazione

ABSTRACT

A particular flora, the Macaronesian flora, enclosing Tertiary relict elements, characterizes Azores archipelago. Thus, the archipelago provides an excellent field test for research activities aimed at developing conservation strategies for endangered species. This is the case for two forest species, an endemic *Picconia azorica* (Tutin) Knobl. and yew (*Taxus baccata* L.), formerly widely distributed in the Archipelago, that was recently classified has "probably extinct". In this study, the urgency to promote *Picconia azorica* conservation programs addressed (a) insights into the biotaxonomy of the species (including an evaluation of its origin and relationships with the only congeneric species of *P. azorica*: *P. excelsa* from the Canary and Madeira islands), and (b) the evaluation of the species genetic diversity. Plastid DNA sequence analysis and molecular markers (RFLP and SSR) were used for this purpose. Phylogenetic data suggest monophyly of *Picconia* and support a late Miocene divergence of the two species. Three polymorphic cpSSR loci allowed the identification of five different haplotypes in *P. azorica*. Uniqueness and relictuality of lineages are presented and discussed. *P. azorica* intra-specific diversity patterns revealed low genetic diversity and a weak genetic structure. The species survival is at risk, and it is suggested management practices focusing on *ex situ* and *in situ* conservation units based on genetic data. Additionally, a wood characterization of the species was conducted as well as biological studies. *Taxus baccata* occurrence in Azores was documented and provides its first characterisation, by means of morphometric and genetic analyses. Although the population entity is critically low, it shows some extremely relevant peculiarities for the species' biodiversity and its phylogeographical implications. All the surveyed yew individuals were found in Pico Island and present leaves smaller than all other *Taxus baccata* described in literature; in addition, Azores provenance has a higher stomata density and displays higher number of stomata rows. These features are all "primitive", according to suggested morphological evolutionary trends. In line with this assumption, the phylogeographical investigation accomplished through the sequence analysis of the chloroplast *trnS-trnQ* intergenic spacer demonstrates that the

Azorean provenance represents a different evolutionary line within *Taxus*, suggesting more direct derivation from yew ancestors within those examined from Mediterranean and European regions. The individuals found in Pico Island may be the last survivors of an extremely ancient lineage, preserved in Azorean archipelago along with Macaronesian flora. Extremely urgent conservation actions focusing on site protection and *ex situ* (namely, clonal propagation which was tested) reproduction strategies are suggested to save this lineage from a short-time extinction.

Key-words: Macaronesian Flora, Azores archipelago, Endemic species, Endangered species, Genetic diversity, Conservation

ACKNOWLEDGMENTS

I am indebted to my supervisor, Prof. Bartolomeo Schirone, for his encouragement and for the time and effort he devoted to my project, as well as for the opportunity of integrating the team of the European project Pre-Forest, in which I have learned so much. I am also grateful for Dr. Marco Simeone co-supervision, interest and support on my project and for discussing this research. I would like to thank Prof. Rosanna Bellarosa for her support during my project. I am also thankful to Prof. Maria Helena Almeida (Technical University of Lisbon, ISA) for the encouragement and friendly advices.

I am thankful to Dr. Avra Schirone, Dr. Angela Lo Monaco and Dr. Rodolfo Picchio for all the support and for teaching me the concepts of wood research. To Dr. Anna Rita Taddei for assisting with the SEM analysis to both *P.azorica* and *T.baccata*. Special thanks my Parents for giving *P. azorica* wood samples.

Special thanks to Dr. Roberta Piredda for the bioinformatics support. I also thank Dr. Silvia Fineschi and Dr. Francesca Bagnoli for the collaboration and the support given and to Dr. Giovanni G. Vendramin for the critical discussion of the results of *P.azorica* genetic studies.

I am also thankful to Prof. Carla Ceoloni for permitting the use of her photo-camera for the PCR-RFLP gels and to Dr. Lijliana Kuzmanovic for assisting me.

I thank Prof. Rosario Muleo for his helpful discussions concerning DNA extraction in Oleaceae species. To Dr. Beti Piotto from ISPRA (Italy), Dr. Gavril Spyroglou from the National Agricultural Research Foundation (N.AG.RE.F. – Greece) and to Fernando Clerici my sincere thanks for their advices and ideas concerning seed pre-treatments.

I would like to thank my colleges Dr. Antonello Sallis, Dr. Rita Bosi, Dr. Laura Armenise, Dr. Federico Vessela, Giulia Sandoletti, Mariolina Morucci, Cinzia De Cicco, Armando and Luigi for their support whenever it was necessary.

I thank the University of the Azores, Department of Agricultural Sciences, especially to Prof. Alfredo Borba who allowed me to use the University laboratory facilities. I would like to thank Prof. David Horta Lopes, Prof. Carlos Vouzela and Prof. Jorge Pinheiro for the use of their equipments and laboratories. I would like

to thank Eng. Reinaldo Pimentel for his help on elaborating some maps and to Mr. Mário Rui Silva for all his help regarding bureaucracies.

A special thanks to MSc Benilde Pereira for her precious help, committement and support during the field visits to Pico Island. My sincere gratitude to Prof. Miguel Sequeira (University of Madeira) for his precious clarification regarding the biological features of *Picconia azorica*.

Thanks are also due to Prof. José Maria Fernandèz-Palacios and Dr. Manuel Arteaga (University of Laguna, Tenerife Island, Canary Islands), to Dr. Francisco Fernandes, (Botanical Garden of Madeira Archipelago) for collecting *Picconia excelsa* samples; to Eng. José Maria Freitas (Flores Forest Services), to Eng. Carla Moutinho (Nordeste Forest Services), to Eng. Francisco Sardinha (S.ta Maria Forest Services) for collecting *Picconia azorica* samples and to Eng. Isabel Ramos and Eng. João Belchiorinho (Portugal); to Dr. Andrès Espejo (Spain), to Dr. Richard Michalet (France) and to Dr. Giuseppe Puddu (Italy) for collecting Oleaceae samples from Sardegna.

To Eng. Eduardo Franco and Dr. Eduardo Guimarães of S.Jorge Island, to Eng. João Melo of Faial Island and to Mr. Fernando Pereira of Terceira Island for accompanying me during the field work, to all my sincere thanks.

I thank my friend Joana Cunha for her precious help formating the text.

I thank also Eng. Jorge Belerique, Eng. Elsa Silva and Eng. Paulo Barcelos for sharing with me their knowledge about the Azorean endemic forest species.

I would like to thank all my friends that accompanied me during my stay in Italy, Ljiliana Kuzmanovic, Arnada Cakrani, Alba and Anisa Oxa and Farida for all their support and friendship. I thank my friends Ana Bettencout and Marta Bastos for their support and friendship for all these years.

I am very grateful to my Family and Marco Paulo for all their support, advices and encouragement.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

1. INTRODUCTION

1.1 Biodiversity

Biologists estimate that everyday species become prematurely extinct; this is so because the extinction rate and the pace, at which natural environments are being destroyed, are increasing on a daily basis. This biodiversity loss makes the conservation of the world's remaining species an extremely important issue. The term "biodiversity" many times is defined in an ambiguous meaning. In Article 2 of the Convention on Biological Diversity, the global legally-bounding instrument for ensuring biological diversity, in 1993, defined biodiversity as follows: "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 includes diversity within species, between species and of ecosystems (…)" (Turok and Geburek, 2000). European policy-makers have recognized the need to preserve biodiversity, seeing that loss of biodiversity makes ecosystems more vulnerable (Fontaine et al., 2007). This is expressed in the strategic direction of the EU's environmental policy (Sixth Environmental Action Programme) with the objective *"To protect and restore the functioning of natural systems and halt the loss of biodiversity in the European Union and globally"*. Moreover, the EU's Development Strategy identifies biodiversity loss as being one of the main threats to sustainable development, and similarly includes the following objective: *"Protect and restore habitats and natural systems and halt the loss of biodiversity by 2010."* Biodiversity, or biological diversity, can be evaluated at smaller scales, such as the genetic diversity of a species, or at larger scales, such as the variety of community types present in a region (Begon et al., 1996). It can be expressed at three major levels – the diversity of ecosystems, the diversity of species and the diversity of genes.

But what leads to the loss of biodiversity, to the loss of species, which are the major causes? The principal cause is habitat destruction, degradation and fragmentation, this means the reduction

of continuous habitat into several smaller spatially isolated remnants. Other causes are over-exploitation, the proliferation of invasive alien species and pollution. Unfortunately, every time we lose a species, a set of genes particular to that species is irreversibly lost. This shortfall has serious implications, for example, the loss of rare forms of a gene (alleles) with no present advantage, could be valuable to overcome, for example, a pest in the future. A reduced genetic variation is expected to diminish the species ability to adapt to changing environments, limiting the species populations potential for adaptation to global and local changes, therefore the evolutionary potential of the taxon (Huenneke, 1991). In sum, the species long-term survival will be most likely compromised, as genetic variability confers a species with the capacity for adaptation to pests and diseases, competitors or environmental and climate changes (Begon, 1996; Rajora and Mosseler, 2001). Moreover, seeing that genetic variability is the basis for tree improvement programs, these will also be limited.

The aim of biological conservation is to avoid individual species from becoming extinct either regionally or globally, which requires a sound basis in scientific understanding of the entities being protected. This is crucial in conservation efforts for rare and endangered species, many of which are endemic (one in three of all known endangered plants is an insular endemic, World Conservation Monitoring Center, 1992). The risk of extinction is higher according to the species status. A species is considered vulnerable if there is 10% probability of extinction within 100 years, endangered if the probability is 20% within 20 years or ten generations and critical if the probability of extinction is at least 50% within five years or two generations (Begon, 1996). With the shortfall of each single individual within a species population, the variety of genes found within the remaining population also decreases. Such decrease leads to the decrease in heterozygosity that is translated in reduced individual fitness and population viability and limited allelic diversity, which in the end, restricts the species ability to respond to evolutionary forces (Gemmill et al., 1998; Storfer, 1999). This loss is exacerbated on endemic species due to their reduced genetic diversity expressed in a lower proportion of polymorphic loci, many times with fewer alleles per polymorphic locus and less even allele frequencies within populations (Hamrick et al., 1991).

Accurately characterize genetically the available resources (populations, individuals, etc) is prime in the development of a conservation programme, as it will allow to prioritize populations in terms of conservation (Marrero-Gomèz, 2003). The population genetic parameters relevant in gene conservation, and therefore, relevant in biodiversity, are those that describe population genetic structure and diversity. Genetic diversity evaluation is determined by allelic richness (e.g., number of alleles per locus), allelic evenness (e.g., heterozygosity) and genotypic richness (e.g., genotype additivity, genotype multiplicity) (Rajora and Mosseler, 2001). The genetic structure of a species is how alleles and genotypes are distributed among and within populations, which is its genetic diversity, structured over space and time (Eriksson et al., 2006; Mohapatra et al., 2009), being the result of both history and present evolutionary processes (mutation, natural selection, migration and genetic drift). Within the geographical distribution of a particular species, it is likely that its genetic structure be determined by the variable ecological conditions encountered.

The quantity of genetic diversity and the existence of a spatial correlation are estimated by several parameters, namely h_T, the amount of genetic diversity found in a given species or population when compared with the total genetic diversity of the all the populations of such species or all the individuals of a population; G_{ST}, measures the proportion of genetic diversity that is distributed among populations; N_{ST}, measures the relative differentiation of each population that accounts for the total diversity (Pons and Petit, 1995; Eriksoon et al., 2006). G_{ST} and N_{ST} values vary between 0 and 1, with higher values indicating a high genetic diversity, with all intra and inter-population components in equilibrium.

Genegeography is the method that allows to comprehend the genetic variability of a given species within its geographical distribution, providing us with vital information to delineate for such species and its population's conservation and management programmes (Simeone, 2007). This author points out the importance for forest plant species the relation between genetic variability, space and the capacity to adaptation, as these species are characterized by very long life cycles, therefore with a high probability of being affected by environmental changes.

1.2 Forest genetic resources

Forests are economically, ecologically, environmentally, and aesthetically important. Forest genetic resources, meaning the genetic diversity in thousands of forest tree species, constitute an intergenerational resource of vast social, economic and environmental importance (Amaral et al., 2004). Forest tree species are typically long-lived, long generation time, highly heterozygous organisms, which maintain high levels of intra-specific variation due to high rates of outcrossing and the dispersal of pollen and seeds over wide areas. Such intra-specific genetic variation is needed to ensure the future adaptability of the species to new diseases or climate change, amongst others, but also to allow artificial selection and breeding programmes. Therefore, assuring the continued existence, evolution and availability of such resources for present and future generations is a worldwide objective. Such objective has been expressed in varied international conferences namely Rio de Janeiro (1992), Kyoto (1997), Johannesburg (2002), as well as in four Ministerial European Conferences (Strasbourg, 1990; Helsinki, 1993; Lisbon, 1998; Vienna, 2003). All of which stipulated the urgency of adopting sustainable forest management measures, expressed by the implementation of forest policies and initiatives. These species are absolutely essential if one bears in mind that 10% of the World's 240.000 plant species are under threat of extinction (Begon, 1996), and in Europe alone, about 800 plant species are at risk of global extinction (Planta Europa, 2009).

The major threats to Europe's forest are the loss, the erosion of their genetic resources, reason for which it was created the European Forest Genetics Programme – EUFORGEN. This programme is a collaborative mechanism among European countries to promote conservation and sustainable use of forest genetic resources. The Programme was established in October 1994 to implement the Strasbourg Resolution S2 (Conservation of forest genetic resources) of the first Ministerial Conference on the Protection of Forests in Europe (MCPFE), held in France (Strasbourg, 1990).

Experts defend a new policy, a sustainable one, to preserve the forest genetic resources, which ideally should be developed according to the following: 1) list and map the geographical distribution of

the species; 2) evaluate the genetic variation present at intra-specific level; 3) identify the various genetic variations of each forest population; 4) define the geographical structure of the existent genetic variability; 5) sustainable forest management with the maintenance of the existent genetic variability by evaluating the present situation and the impact of each action within the management; 6) conservation of rare species and populations through protection *in situ* and conservation *ex situ* (Simeone, 2007).

Moreover, particular attention should be given to forestry management practice itself, being recognized that sustainable forestry practices are a critical component of forest genetic resources and biodiversity conservation. Forest management has several critical issues to take into account, as it creates diverse and complex ecological effects in forest ecosystems that may affect regeneration, abundance and mating systems of species in different ways. Such forest ecosystems are composed of species, which in turn are composed of populations, both of which take part in a dynamic evolutionary process driven by mutation, natural selection, migration and random genetic drift and the mating system, governing the genetic variability of a species. For example, activities that reduce population size will normally increase the rate of inbreeding; some forms of harvesting can favour or disfavour certain alleles and so change allelic frequencies; the use of clonal seedlings in forest plantations can reduce the genetic diversity. On the other hand, the promotion of natural regeneration and the use of seedling material in plantations, can contribute with the "building blocks" the species needs for adaptation. In sum, one must guarantee the potential for adaptation, starting from the present adaptedness and benefit from natural selection, securing the species regeneration (Eriksson et al., 2006).

Altogether, it is absolutely vital to increase our knowledge on the genetic structure of forest tree species as well as their evolutionary dynamics.

1.3 Phylogeography as a study method

Phylogeography is the study of the historical processes that may have influenced the geographic distributions of gene lineages, particularly within and among closely related species (Avise, 2007).

This discipline allows inferring past episodes of population expansion, bottlenecks, vicariance and colonization through the reconstruction of genealogical history (Whittaker and Fernández-Palacios, 2007). For example, in an island, it could allow to determine the sequence of interisland dispersal events or help in the prioritization of areas of high value for conservation.

The phylogeographic method has demonstrated its usefulness in evolutionary research across a vast range of taxa, being dominated by studies employing single-locus uni-parentally inherited markers, as are mtDNA and cpDNA genes (Weiss and Ferrand, 2007). However, plastid genomes are the most frequently used for phylogeographical studies in plants (Schaal et al., 1991; Newton et al., 1999; Petit and Vendramin, 2007).

Thus, molecular markers play an important role, allowing evaluating the genetic diversity of a species, as they are capable of revealing and quantifying the variation between two or more individuals. Such is achieved by studying the DNA (nuclear or organelle) with molecular techniques, all of which have benefited from the Polymerase Chain Reaction (PCR), a technique that allows to isolate and study the DNA of any organism, even in diminutive quantities. These molecular techniques can be grouped: those that reveal polymorphism in terms of length of the specific DNA fragment (is the case of RAPD, RFLP, AFLP and SSR) and those that register the polymorphism present in the nucleotide sequence of particular genes. From within these methods, the most commonly used are Restriction Fragment Length Polymorphism (RFLP) and Simple Sequence Repeats (SSR), both of which obtained through the PCR technique. The importance of the maternal contribution is in the fact that it cannot recombine, making it an excellent marker capable of detecting hybridization and potential introgression (Schaal et al., 1991).

1.4 Macaronesia

1.4.1 The Macaronesian Region

The Macaronesia concept was used for the first time by the botanist Philip Barker Webb (Ceballos, 1953; Sunding, 1979) nearly 150 years ago. The term defines an area of natural delimitation as

regards its plant and animal life. Such area includes five archipelagos: Azores, Madeira and the Selvagens Islands (Portugal), the Canary Islands (Spain) and Cape Verde (Cape Verde). Sunding (1979) defends that from a biogeographical point of view a limited area of the African mainland – South Morocco and Spanish West Africa – should also be included as a "Macaronesian Enclave" on the mainland (Fig.1.1).

Figure 1.1 - The biogeographical region of Macaronesia (Sunding, 1979).

A total of 30 islands comprise the five Macaronesian archipelagos all of which differ in latitude, altitude, area, geological age and distance from the nearest continent. All archipelagos are situated in the North Atlantic Ocean, extending from 15° to 39° N latitude, with distances from the European or African continents varying from 95 to 1450 km. The Canary Islands have a total land surface of 7447 km^2 (with seven islands), much larger than the 2328 km^2 of Azores (with nine islands) and Madeira (801 km^2, including Madeira Island, Porto Santo Island, Desertas and Selvagens) (Silva et al., 2008). The Canary Islands are only 95 km from the African Continent (North Africa), Madeira is about 660 km from North Africa, 980 km from Lisbon, 400 km from Gran Canaria Island (Canary Islands) and 880 km from Azores archipelago (Sta. Maria Island is the nearest one). Azores archipelago is about 1450 km away from the European Continent (Portugal mainland).

The Azores archipelago is geologically the youngest Macaronesian archipelago (Borges et al., 2005). Madeira oldest sub-aerial geological formations are known to be around 5.6 million years (Capelo et al., 2007), while in the Canary Islands it varies between the individual islands from 0.8 to 21 million years (Carracedo et al., 2002) (See Fig. 1.2).

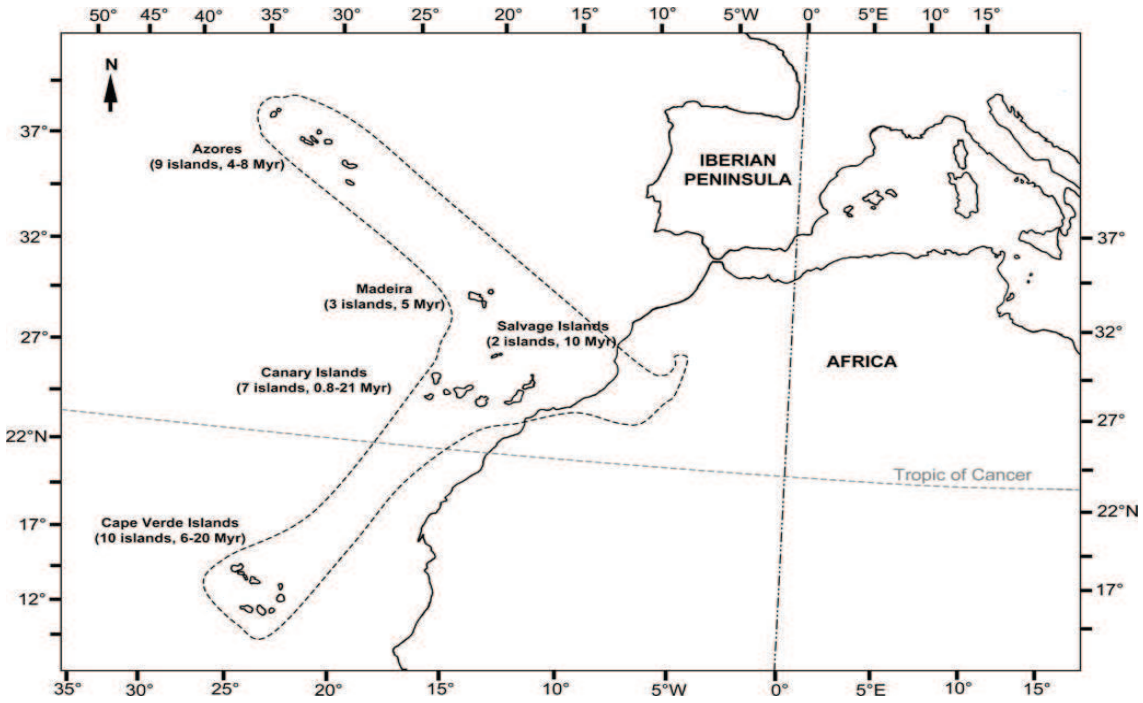


Figure 1.2 - The Macaronesian Region, with the five Atlantic volcanic archipelagos (the Azores, the Madeira, the Salvage Islands, the Canary Islands, and the Cape Verde Islands) indicating the age of current above-sea landmass for each archipelago. (Adapted from Kim et al., 2008).

Climate varies within the Macaronesian archipelagos. The dominant factor shared by all three archipelagos (Azores, Canary Islands and Madeira) is the influence of the Azores anti-cyclone regime. It influences each of them in a distinct way with weather conditions that are prevalent within the islands being conditioned by the anti-cyclone. Due to its significance in the precipitation regime and humidity is prime the influence of trade winds, with predominant direction N and NE, affecting similarly all the archipelagos considered, principally Canary Islands and Madeira and with principal components NW and SW in Azores (Santos, 1990). One of the most peculiar aspects of each archipelago is its precipitation regime. These winds are responsible for a "sea of clouds" that has more impact within the N and NE facing areas, having also some impact in the climate conditions of the meridian areas; acquiring higher relevance in Madeira Island, allowing the development of laurel arboreal communities in such areas. In the Canary Islands, these conditions are formed in areas facing NE or where humidity and shade conditions are appropriate (Santos,

1990). In the Azores, climatic conditions permit the development of laurel forest in whatever its aspect and altitude, if below 1350 meters.

Precipitation values of Azores coastal areas are higher than the precipitation values registered in the rainiest areas of Canary Islands. The laurel forest in the Canary Islands can be found in areas with 400 mm, in the lower areas (facing the N and NE), up to 3000 mm or more in the very-humid Azores areas. High relative humidity values are one of the constants more significant for laurel forest. It varies between 70%, in the driest areas of Canary Islands, around 400-500 meters a.s.l., whereas in Azores can be as high as 80-90%, nearly the saturation level. Temperatures within the three archipelagos tend to be more uniform. Temperature at sea level in the Canary Islands fluctuates between 17º and 25ºC and rainfall ranges between 100 mm near the coast up to 300 or 700 mm per year at higher altitudes (Silva et al., 2008). Madeira Island annual temperature is about 20ºC at sea level and rainfall ranges from 500 mm up to 2000 mm or higher (at the highest altitudes). In the Azores, mean annual temperature at sea level is 17^0C, with rainfall reaching values as high as 3000 mm, at the highest altitudes. All factors combined create, within each island, conditions that will support part of the structural and floristic characteristics of the vegetation.

Taken as a whole, Madeira climate is classified as a moist temperate climate with moderate winters, including however two main types: mediterranean and oceanic (Cardoso et al., 2007). Canary Islands present great climatic contrasts. In the west and northwest sides of the islands one can find an oceanic humid and fresh climate, whereas the East sides of the islands that face towards Africa are characterized by an arid climate. Finally, the Azores climate is classified as an oceanic one.

1.4.2 The Macaronesian Flora

The Macaronesian region is characterized by the Macaronesian flora, very rich in species and endemisms representative of vegetation types, such as the laurel-type forest, formerly distributed in vast regions of Europe, Northern Africa and North America, but now disappeared elsewhere.

The Macaronesian biogeographical area can be subdivided according to the flora origin. Hence, the northern archipelagos, Azores, Madeira and Canary Islands, compose one sub-region and

the other sub-region is Cape Verde Archipelago (Sunding, 1979). According to this author, flora arrival to the northern archipelagos occurred most probably during the Cretaceous, Tertiary and Quaternary.

It occurred so, as during the Tertiary the climate gradually became cooler, pressing the climatic zones in Europe, and thus their vegetation zones, southwards towards the Tethys, later the Mediterranean Ocean (Bramwell and Richardson, 1973; Axelrod, 1975; Bramwell, 1976). In the late Cretaceous, broadleaved sub-sclerophyllous vegetation, characterised by evergreen trees and shrubs, representing numerous Lauraceae, was widespread across lands marginal to the Tethys Sea, and captioned it Tertiary-Tethyan vegetation (Takhtajan, 1969; Axelrod, 1975). Propagules are believed to have arrived either by long-distance dispersal (Guppy, 1917; Axelrod, 1975) or by a "stepping-stone" system that enabled, for example, connectivity between the Canary Islands, Madeira and the Iberian Peninsula; connectivity between the Dacia and Concepción banks linking Madeira with Africa (Whittaker and Fernández-Palacios, 2007). Melville (1979) argues the relevant part birds play linking the Miocene laurel forests of Europe and the Macaronesian islands, when sea passages were not very wide as a result of to the stepping-stone system.

The reason why so great a part of the Tethys countries flora has survived in the Macaronesian islands is due to two main reasons, one the buffering effect of the Atlantic Ocean and the Gulf Stream, with constantly mild temperature, high rainfall and high relative humidity; the second is the fact that these islands offered a wide range of ecological niches (Sunding, 1979; Vargas, 2007). The prevalent climate has conferred Macaronesia with suitable characteristics for sheltering subtropical Tertiary plant groups, in contrast to mainland dryness and cold during the late Tertiary (Miocene and Pliocene) and the Quaternary glaciations (Hewitt, 2000). Consequently the region functioned as a refugium for this ancient flora that went extinct on the continent after late Tertiary and Quaternary climatic deterioration (Ceballos, 1953; Tutin, 1953; Takhtajan, 1969; Bramwell and Richardson**,** 1973; Sunding, 1979; Hewitt, 2000). However, the surviving Macaronesian flora does appears to be only a fraction of the Tethyan-Tertiary flora formerly found in the Mediterranean basin (Bramwell, 1976; Vargas, 2007).

Indeed, a close relationship between the Macaronesian and the Mediterranean floras is indicated by the species with a Mediterranean affinity among endemics as well as by the largest group of non-endemic species being Mediterranean ones (Sunding, 1979). Moreover, this author argues the four Lauraceae genera (*Apollonias*, *Ocotea*, *Persea* and *Laurus*) were represented in the Tertiary flora of the Mediterranean by taxa identical or related to those of the present-day Macaronesian flora. Other links are *Ilex*, *Myrica*, *Juniperus*, *Rhamnus*.

The Miocene and Pliocene fossil floras of Southern Europe have been investigated by Saporta (1865), Depape (1922), Takhtajan (1969), Axelrod (1975). Depape (1922) and Saporta (1865) have detected in the Tertiary deposits of species such as *Woodwardia radicans*, *Myrica* sp., *Laurus azorica*, *Persea indica*, *Ocotea foetens*, *Phillyrea latifolia* and *Picconia excelsa* (reported from the Rhone valley as Notelea excelsa by Depape, 1922).

Picconia Genus is at present-day endemic to the Macaronesian flora. As other taxa, it reached the Macaronesia by long-distance transport. This endemic Genus indicates earlier migrations that were followed by evolution in isolation or survival under the insular maritime climate and extermination on the continent (Axelrod, 1975). As a result of being a remnant of the once richer forest flora, *Picconia excelsa*, as *Persea indica*, *Laurus azorica* and *Ilex canariensis* are considered palaeoendemic species (Whittaker and Fernández-Palacios, 2007).

For its absolute importance, the Macaronesian flora is acknowledged and protected under the Habitat Directive (EC, 1992). Moreover, the three European archipelagos (Azores, Canary Islands and Madeira) constitute one of the ten acknowledged hot-spots in the Mediterranean basin (Médail and Quézel, 1997), probably the most important biodiversity centre of the Mediterranean bioclimatic region, and one of the 25 Biodiversity hot-spots known to exist (Martín *et al.,* 2008). Presently, the Macaronesian Region has 211 sites acknowledged in Natura 2000 network, hosting no less than 19% of the habitat types in Annex I of the Habitats Directive and 28% of the plants Annex II.

The Macaronesian laurel forest cannot be considered a homogenous formation due to its climatic and floristic variety (Santos, 1990; Pena and Cabral, 1997). It is possible to differentiate three main types: dry laurel forest, humid laurel forest and very-humid laurel forest.

1 – Dry laurel forest, this type is predominant in the Canary Islands. It is characterised by a mean annual precipitation ranging from 350 to 500 mm; mean annual temperatures slightly higher than in other types, high floristic diversity but small number of *Pteridophyta* species. The main arboreous species are *Apollonias barbujana*, *Visnea mocarena* and *Maytenus canariensis*, sometimes with a significant presence of *Picconia excelsa*, *Ardisia bahamensis* and *Myrsine canariensis*.

2 – Humid laurel forest, this type is predominant in Madeira Archipelago, being also found in the Canary Islands and at Azores lowest altitudes. It is characterised by a mean annual precipitation ranging from 500 mm up to 1000-1200 mm. Represents the woods with higher richness in terms of fauna and flora, occupying the areas with higher precipitation in the Canary Islands and Madeira Archipelago. Tree species found in this type are *Clethra arborea*, *Persea indica*, *Ocotea foetens*, *Erica azorica* and *Laurus azorica*. In Azores *P. azorica* is found in this laurel forest type.

3 – Very-humid laurel forest, this type is predominant in Azores Archipelago, in areas were mean annual precipitation is higher than 2000 mm and mean annual humidity is as high as 80%. These conditions are also found in the north coast of Madeira Island. Such special conditions allow the development of tree species as *Juniperus brevifolia*, *Frangula azorica* and *Picconia azorica*, amongst others.

In the Azores, Madeira and Canary Islands more than 5300 endemic species (fauna and flora) are identified. While Canary Islands contain the richest flora and surely the uppermost percentage of endemisms (Sunding, 1979; Martín et al., 2008). Canary Islands have 570 plant endemic species, representing 40% of its flora (Francisco-Ortega et al., 2000), Madeira Archipelago, including the Selvagens Islands, has nearly 13% of flora endemisms (154 species) (Martín et al., 2008), whereas Azores possesses only 68 endemic plant species representing 7,2% of its flora (Schafer, 2002). Such low endemism percentage is mainly because of its uniform wet and coldest climatic conditions leading to a great homogeny between islands, thus accounting for a smaller number of

microhabitats (Humphries, 1979; Hobohm, 2000). Therefore, Azorean plant communities could be more fragile and species more vulnerable under anthropic impacts.

Nowadays a considerable number of species of Azores flora are under the category "endangered" as their populations are subjected to several sources of pressure, e.g. agriculture expansion to higher lands, invasive species proliferation, habitat fragmentation, amongst others (SRAM, 2005).

1.5 The Azores Archipelago

Azores discovery exact date is not known. However, it is agreed that from 1420 onwards the islands started to be visited regularly by Portuguese ships (Drummond, 1859). By 1440 the Azores settlement was progressing quite fast. The archipelago was named after a large species of hawk, by the time thought to be *Buteo vulgaris*, "Azores" or Hawk Islands (Watson, 1870), now identified as *Buteo buteo* ssp. *rothschildi* (Martins et al., 2002).

Azores first descriptions mention that forests had a high density and were composed by very tall and wide diameter trees, making very difficult the passage into the island's interior (Frutuoso, 1583). The first measure accomplished by Portuguese settlers was to clear areas to allow animals, namely, goats, cows, pigs, etc, to feed and reproduce. This was the first negative impact in the Azores flora.

Presently, Azores archipelago has, roughly 242.000 inhabitants with an average density of 104 inhabitants/km^2. Agriculture, dairy and beef production, has a profound impact in the archipelago economy and landscape with about 65% of the soil area within the category "agriculture and pastures". Forest have, over the centuries lost their dominance in terms of area.

1.5.1 Geographic localization

The Archipelago is located on the North Atlantic Ocean roughly between the latitudes of 36º 55'N and 39º 43'N and the longitudes 24º 46'W and 31º 16'W (Fig. 1.3 and Fig. 1.4). The islands are spread along a NW-SE trending strip 600 km long, in a complex geodynamic setting (Cruz et al.,

2006). Composed of nine islands and small islets, all oceanic, Azores islands are mid-ocean ridge ones in association with a triple junction where the North American, Eurasian and African plates meet. These islands were formed recently, not contemporaneous, chiefly through volcanic activity due to tectonic movements (Martins, 1993), arising from the ocean floor and have never been connected to any continent by a land bridge (Borges, 1997). The archipelago lies over two tectonic plates: the westernmost islands of Flores and Corvo lie over the American plate and are separated from the eastern islands by the Mid-Atlantic Ridge (MAR); the other seven main islands are located in the "Azores microplate" (França et al., 2003). Geologically, the Archipelago comprise a 20-36 million year old volcanic plateau; the youngest is Pico island, with the oldest sub-aerial lava flows being dated from about 0.3 millions years (Van Riel et al., 2005) whereas the oldest island is Santa Maria with rocks being dated to the Miocene, eight million years (Martins, 1993; Van Riel et al., 2005). The triple junction dynamic is responsible for tectonic earthquakes and many volcanic eruptions. The Azores are characterized by high volcanic activity, with twenty-six volcanic eruptions been registered after the settlement (França et al., 2003).

Figure 1.3 - World map evidencing the position of Azores Archipelago (unknown source).

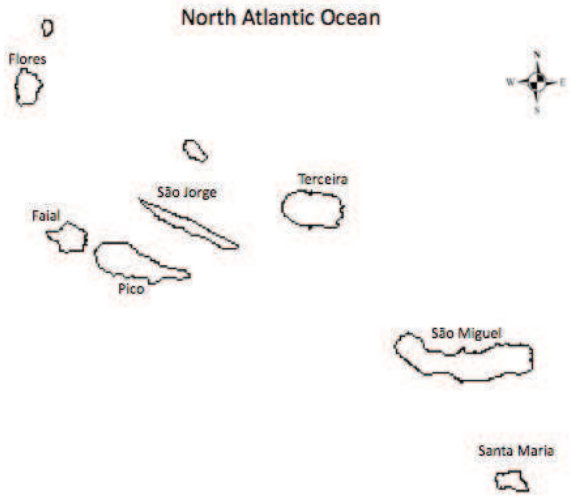


Figure 1.4 - The Azores Archipelago map with the nine islands.

The archipelago is divided in three groups: Eastern (São Miguel and Santa Maria islands), Central (Terceira, Graciosa, São Jorge, Pico and Faial islands) and Western (Flores and Corvo islands). The lowest island is Graciosa with a maximum altitude of 402 meters above sea level (a.s.l.) and Azores highest altitude is 2351 meters a.s.l. in Pico Island (Table 1.1 and Table 1.2). Curiously, Pico Mountain is Portugal highest peak. The Archipelago is far out from the European Continent 1400 km, whereas the North African and North America Continent are at a distance of 1450 Km and 3900 km, respectively. Corvo island lies approximately at the same distance from the Iberian Peninsula (Europe) and from Newfoundland (North America – Canada). The maximum inter-island distance is roughly 600 km, between Sta. Maria and Corvo islands and the minimum is six km, between Faial and Pico islands. The total sea surface of the archipelago is 181.500 km^2 (Fernandes, 1985). Azores comprises a total area of 2328 square kilometres, representing 2,5% of Portugal total area and 0,06% of EU 25 total area.

Table 1.1 - Physical features of Azores islands. (Long. = longitude; Lat. = latitude; d.n.m. = distance from the nearest mainland). Adapted from Borges (1997).

Island	Long. (W)	Lat. (N)	d.n.m. (Km)	Area (Km^2)	Maximum Altitude (m)	Geological age (Ma. B.P.)
Sta. Maria	25.1	36.9	1588	97	587	8.120
S. Miguel	25.5	37.7	1584	757	1103	4.010
Terceira	27.2	38.7	1764	402	1023	2.000
Graciosa	27.8	39.1	1844	62	402	2.500
São Jorge	27.9	38.7	1832	246	1053	0.550
Pico	28.2	38.5	1860	433	2351	0.300
Faial	28.5	38.6	1908	172	1043	0.730
Flores	30.9	39.4	2152	142	915	2.900
Corvo	30.8	39.7	2148	17	718	?
Total	--	--	--	2328	--	--

Table 1.2 - Altitude zones for each individual island (% of total area).

Island / Altitude zones (m)	Santa Maria	São Miguel	Terceira	Graciosa	São Jorge	Faial	Pico	Flores	Total
0 – 300	85.4	50.5	55.9	94.9	29.9	53.6	41.0	32.8	49.1
300 - 600	14.6	36.5	37.6	5.1	48.5	34.2	25.1	48.0	34.5
600 - 800	-	11.0	4.6	-	17.9	7.8	17.5	18.0	11.4
> 800	-	2.0	1.9	-	3.7	4.4	16.4	1.2	5.0

1.5.2 Climate

The climate in this part of the World is shaped by the Azores anti-cyclone, always present in the eastern part of the North Atlantic Ocean, determining the islands weather conditions. The Archipelago is situated in a transition and confrontation zone of air masses with tropical and temperate or polar origin (Azevedo, 1996). The influence of the warm Gulf Stream is also important,

allowing temperatures at sea level to be quite similar in the south-eastern and in the north-western islands, as well as the humidity (Agostinho, 1966). Overall, the climate is mild and agreeable.

The archipelago oceanic climate main characteristics are small temperature annual range (annual average temperature around 9-11ºC at high altitudes and around 15-17ºC at lower ones), high rainfall spread throughout the year (300-400 mm during winter months and 100-150 mm during summer months, at medium altitude), high relative humidity, rarely below 75% (IM, 2008), and persistent wind. The mean temperature values for each month changes regularly during the year, with a maximum in August and a minimum in February. Temperature decreases 0,9ºC for each 100 meters in altitude up to 400 meters a.s.l., and decreases 0,6ºC for each 100 meters increment in altitude (Azevedo, 1996). Nearly 75% of the annual precipitation occurs between October and March (DROTRH/INAG, 2001). The precipitation seems to be directly dependent on the air circulation caused by the trade winds (SW, NW), making the rainiest islands the western ones (Flores and Corvo) and the driest one Sta. Maria (Agostinho, 1941). Average rainfall therefore increases from east (Sta Maria Island – 981 mm) to west (Flores and Corvo Islands – 1430 mm), additionally increases 25% per each 100 meters increment in altitude (Martins et al., 2002), up to 3000 mm. The percentage of insolation in the Azores is very low, with a minimum in February (30%) and a maximum in August (45%). As a result, nebulosity remains stable throughout the year. Sjogren (1990) points out the importance of such fact, which allows for each island to define at high altitudes a "cloud-zone forest", leading to a fog interception phenomenon. Such horizontal rain has significant ecological meaning and is a consequence of the Fohen effect (Dias, 1996). In these cloud-zones, the rainfall (vertical and horizontal) is high, leaving the air saturated with moisture and the soil waterlogged. Due to these conditions anoxia occurs in the soil.

Most of the available data concerned with the Azorean climate are from a restricted low altitude band, the climate of high altitudes being still unrecorded. However, climatic models, namely CIELO (Clima Insular à Escala LOcal) a local climate model for island environments (Azevedo, 1996; Azevedo et al., 1999), permits estimates for such high altitudes.

1.5.3 Soils

Despite the fact that soils in Azores are not fully studied, the vast majority of Azorean soils are under the category of Andosols, a consequence of the islands volcanic origin (Pinheiro, 2007). Such soils are, in general, modern soils, developing under oceanic climate conditions (Madruga, 1986), from airborne volcanic deposits, in particular basic ashes (Schafer, 2002). These soils are characterised for high permeability, high organic matter content (reaching about 23%) and potassium-rich due to the predominance of basaltic rocks (Tejedor et al., 1985; Pinheiro, 2007) and have a high ion exchange capacity, making them very fertile (Schafer, 2002); are also rich in unstructured weathering products and volcanic glass and therefore finely coarsed, with low bulk density, but still quite adhesive. The high infiltration capacity and good percentage of organic matter allow them to have a low risk of erosion; nevertheless, on slopes and in coastal areas such risk is relatively high. In Azores these soils are intensively cultivated and mostly allocated to agriculture. Therefore, natural vegetation is mainly found on Incipient or Organic Soils, which are found predominantly in marginal sites.

1.5.4 Vegetation

Azores islands were covered with forest when discovered. Forests were composed by tall and wide trees, which formed dark and very dense sites (Frutuoso, 1853). Soon after Azores settlement, the archipelago forest cover and its native flora started to diminish as a result of man action, mainly to generate land for agricultural purposes, as well as fuel, to construct farm equipment and ships. In particular, primeval forests were negatively affected by sugar cane cultivation (need of space and fuel) and orange cultivation (need of space and of material to construct wood boxes for exportation) (Sacuntala, 1995) and by shipbuilding and repairing (Boid, 1832). Not surprisingly, numerous historical descriptions mention wood as one of the economic capital of the Azores islands. Indeed, the trees constituted a very important resource, for the wealth of Azores first inhabitants and surely the main source of energy (Dias et al, 2007). Unfortunately, today it is not possible to say whether

the local limitation to native flora distribution have always existed in some areas, as the five centuries of pressure disrupted their habitat and original distribution within each island.

Azores natural vegetation area comprises about 13% of its total area, whereas forest area, consisting of production forest and plantations represents nearly 22% of the archipelago total area (Tab. 1.3) (SRAM, 2007). In sum, roughly 35% of the Archipelago's area is allocated to natural vegetation and forest cover.

Table 1.3 - Natural vegetation and forest area for each Azorean island, expressed in percentage of total area (Adapted from SRAM, 2007).

Island	Natural Veg. Area (%)	Forest Area (%)
Sta. Maria	7.75	21.13
S. Miguel	8.69	21.54
Terceira	14.15	14.35
Graciosa	0.38	12.37
São Jorge	14.71	26.37
Pico	13.69	32.47
Faial	9.35	17.90
Flores	32.94	22.06
Corvo	51.33	1.61
Total	12.78	22.23

The lack of knowledge of forest management and production of Azorean endemic forest species and the fact that some exotic species present high growth rates in the archipelago climate are the chief reasons for reforestation to take place mostly with exotic species. Such species are *Cryptomeria japonica* (L.f.) D. Don, *Acacia melanoxylon* and *Eucalyptus globulus* (DRRF, 2007). The main forest species is *Cryptomeria japonica* (L.f.) D. Don occupying nearly 12.400 hectares, representing about 58% of Azores production forest. This species is planted in monoculture forming dark stands where only very few other species can survive, and is susceptible to strong winds. Followed by *Acacia melanoxylon* with 4.300 hectares (in some cases can become an invasive),

then *Eucalyptus globulus* with 3.500 hectares and finally *Pinus pinaster* with 900 hectares (DRRF, 2007). The production management of such forest favours wood in terms of quantity (in function of total volume), but not in terms of quality (Barcelos, 1996). Schafer (2002) stated, about forestry in the Azores "The quick profit with rapidly growing exotic trees of low quality timber instead of a farsighted, careful use of precious, native species comes with an immeasurable liability which threatens the practice itself".

In the Macaronesian archipelagos significant problems due to exotic species, both fauna and flora, have been identified in recent studies. This is more serious in the case of invasive species. Azores is no exception, which can be exacerbated given that 60% of its vascular flora is exotic (Silva and Smith, 2004, 2006). Several plant species are presently considered as invasive species in Azores and are a serious threat to the archipelago natural ecosystems and endemic plant species but also to the conservation of bird species, as the Passeriformes *Pyrrhula murina* Godman (Critically Endangered - IUCN 2009). A considerable number of the Azorean endemic tree species are under the risk of extinction, namely *Picconia azorica* (Tutin) Knobl., *Erica azorica* Hochst., *Prunus azorica* (Hort. Ex Mouil.) Rivas Mart., Losa Fer. Prieto, E. Dias, J. C. Costa, C. Aguiar and *Frangula azorica* V. Grubow. The negative effects of the invasive alien species aggravate this status. Moreover, "the continuous expansion of some invasive plants like *Hedychium gardneranum* Sheppard ex Ker Gawl., *Pittosporum undulatum* Vent. and *Hydrangea macrophylla* (Thunb.) Ser., is threatening several fragments of native vegetation, leading to the prediction that several communities of lichens, vascular plants, molluscs, and arthropodes native and endemic to the Azores might be endangered" (Silva et al., 2008). According to these authors, *Hedychium gardneranum* Sheppard ex Ker Gawl. can already be found in small gaps in the middle of large fragments of otherwise pristine native forest. The area occupied by a very aggressive invasive species is absolutely striking, *Pittosporum undulatum* Vent. occupies approximately 24.000 hectares in Azores. Just to have an idea, it occupies nearly 11.000 hectares more than *Criptomeria japonica*. This aggressive species, which was introduced for sheltering S.Miguel's orange orchards in the XVIII century, has profound impacts in terms of biodiversity as well as landscape, amongst others. Moreover, Azores

is the Macaronesian archipelago with the highest ratio exotic/indigenous plant species per square kilometer, being the number of exotic species one or two times higher than the indigenous ones per square kilometer.

Recently the Environment Agency of Azores developed a Program for the Control or Eradication of Invasive Plants in Sensitive Areas (PRECEFIAS). It was delineated to recover priority habitats and species, consisting of actions for controlling or eradication of the invasive alien species mentioned above.

Curious is the fact that the species *Hydrangea macrophylla* (Thunb.) Ser. known as the Azores symbol is indeed an invasive alien species, which threatens the archipelago indigenous vegetation. Eradication in some areas is needed, and most likely a new symbol for Azores.

1.5.5 Endemic vegetation

Nearly 95% of Azores territory is within the range of 800 meters (see Tab. 1.2), the potential area for laurel forest (Santos, 1990). Simultaneously, each different species has its own potential area, where the species finds ecological conditions for its existence and reproduction. The different types of association of species according to their ecological preferences allows to define, in Azores as for the Macaronesian region, three main types of natural forests. These are dry laurel forest, humid laurel forest and very-humid laurel forest (Dias et al., 2007):

A – The dry laurel forest, is present at low altitudes and is dominated by *Myrica faya* and *Picconia azorica*, sometimes *Laurus azorica* is also present. This type of forest is almost extinct nowadays as a consequence of the strong competition this habitat suffered from agriculture and urban pressure. Additionally, wood exploitation of this community main species, as *Myrica faya* and specially *Picconia azorica*. Moreover, the recent negative impacts cause by the aggressive invasive alien species, *Pittosporum undulatum* has lead to the fragmentation of this habitat. As a result of its reduced species number, its structure is very simple, with a high canopy formed by the characteristic species, *Myrica faya* and *Picconia azorica*. This community is adapted to mean annual minimal temperatures of 14ºC and mean annual precipitation of around 350 – 500 mm;

B – Humid laurel forest is present within the range of 400 and 650 meters altitude. As defined by Santos (1990) is characterised by a great structural and floristic biodiversity. This type of forest is characterized by evergreen tree species, with large leaves, glabrous or sub-glabrous and coriaceous. Climatic conditions are mild, with moderate winters, without frosts and snow, and with summers without water stress. The *Laureaceae* family characterizes this type of forest, which in Azores is represented by a single species, *Laurus azorica*. It is dominated by *Laurus azorica* with *Picconia azorica* and *Myrica faya* at lower altitudes, and *Frangula azorica* at higher ones. Species such as *Prunus azorica*, *Erica azorica* and *Ilex perado* ssp *azorica* are also present. These formations are very rare now; again the pressure for agricultural land and the invasive alien species are amongst the causes for its rarity;

C – Very-humid laurel forest can be found above the clouds. Trees are small due to the lack of nutrients and action of strong winds. It develops in areas subjected to intensive fogs and wet winds, with an impermeable soil, reason for which water logging is frequent and in some areas permanent. It is dominated by *Ilex perado* ssp. *azorica*, *Laurus azorica*, *Frangula azorica* and *Vaccinium cylindraceum.* It can also be dominated by *Juniperus brevifolia* and *Sphagnum* spp. Presently it is the most frequent type of laurel forest, mostly because it persists in marginal sites for agriculture.

Historical data points out the evidence that some endemic forest formations have become extinct. Guppy (1917) identifies that species such as *Prunus azorica*, *Picconia azorica* and *Taxus baccata* are being over-exploited and becoming rare. In fact, such formations are currently extinct; it is the case for *Prunus azorica*, *Picconia azorica* and *Laurus azorica* formations at lower altitudes (Dias, 1996; SRAM, 2005) and the case of *Taxus baccata* (Dias, 1996).

It is urgent to protect and preserve the remaining endemic flora and areas were it was identified to avoid permanent losses. In consequence, presently nearly 20% of Azores territory is legally protected as Sites of Community Interest (SCIs), Special Areas of Conservation (SAC) and Special Protection Areas (SPAs) altogether integrating Natura 2000 network. A total of 26 habitat types are protected under the Annex I of the Habitats Directive and 26 plants under the Annex II. Other areas

can be found in the islands, in particular Natural Reserves, Natural Monuments, and Protected Landscapes and others (Fig. 1.5). Recently in 2009, for each island was created a Natural Park, the Island Park, which will have a specific management plan according to its particularities and priorities.

Figure 1.5 - Image from the Island Park of Terceira Island, where can be seen several endemic species, namely *Juniperus brevifolia*, *Erica azorica* and others.

The Azores protected areas include the most important terrestrial habitats, from the sea coast up to the mountain areas, including the Laurel forest which currently only persists relatively intact in high altitude areas in Terceira, Pico and Flores Islands. A known fact for most Azores islands is that below 300 meters, areas are predominantly agriculture ones, so rarely natural habitats can be found (Borges et al., 2005).

Azores flora is characterized by a small number of native and endemic species, being clearly poorer than Madeira and Canary Islands floras. The Azorean vegetation reveals affinities with the European SW, noticeable by the abundance of Ericaceae (*Calluna, Erica, Daboecia*), the diversity of bracken species (*Pteris, Osmunda, Polystichum*, etc.) and by the presence of species

typically euro-atlantic and western mediterranean such as *Taxus baccata*, *Daphne laureola*, *Myrtus communis*, *Viburnum tinus*, etc. (Ceballos, 1953).

With a total of 947 *taxa* of vascular plants (Pteridophyta and Spermatophyta) has only 7.2% of endemism, which represents 68 endemic *taxa*, (68 species and 12 subspecies) includes 71 pteridophytes (9.9% of endemism), five gymnosperms (20% of endemism), 643 dicotyledons (6.8% of endemism) and 228 monocotyledons (7% of endemism) (Borges et al., 2005). Azores has only one endemic genus of vascular plants – Azorina, with the species *Azorina vidalii* (Wats.) Feer. (Fig.1.6). As pointed out by the authors, some endemic Macaronesian *taxa* can also be found in Azores flora, namely five pteridophytes, three dicotyledones and four monocotyledons.

Figure 1.6 - An *Azorina vidalii* (Wats.) Feer. specimen (Unknown source).

Endemic species can be found on several or all nine islands of the archipelago, growing in different kinds of habitats from sea level up to the top of Pico Mountain, with 2351 meters high. As can be seen in the Figure 1.7, the number of taxa per island varies between 335 for Corvo and 706 for S.Miguel, but Flores, Faial, Pico, Terceira and Sta. Maria Islands have more than 500 species (Borges et al., 2005). Corvo island retains the higher percentage of endemism, in Flores, Pico and S.Jorge this percentage is equal or superior to 10%, it is inferior to 10% in Faial, Terceira, S.Miguel and Sta. Maria islands, whereas Graciosa possesses the lowest percentage (Borges et al., 2005).

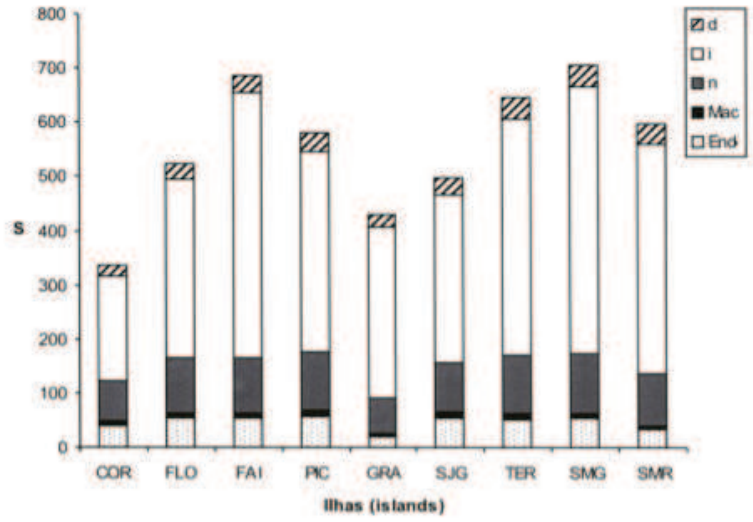


Figure 1.7 – Number of vascular plant species (S) endemic to Azores flora (End), Macaronesian endemic (Mac), Azores native (n), introduced (i) and of doubtful status (d) in each of the Archipelago nine islands (Source: Borges et al., 2005).

The absence of some endemic tree species from a particular island is most likely to be a result of its over-exploitation regarding the soil use for agriculture, which has lead to local extinctions. This is specifically true for Graciosa Island.

1.5.6 Endangered vegetation

From within the endemic vascular plants from Azores, 50% a are priority in terms of future conservation actions, representing a total of 37 species (Cardoso et al., 2008). However, such actions are partially limited due to population's isolation, some species occur in small areas and their density is low. Their main threats are habitat degradation, soil use conversion, agriculture as well as the competition with invasive alien species. For each *taxa* there is a necessity to draw a specific recovery plan and special care must be taken as most being endemic and endangered, there

is not much margin for mistakes. The recovery plan should take into consideration the species genetic diversity, assess the threats it's subjected to and combine the available resources to provide the highest possible degree of protection.

From within the endangered tree species it is important to note that a considerable number is under such status due to its over-exploitation, as their wood was a valuable resource and source of income. This is the case for the following species: *Juniperus brevifolia* (Seub.) Antoine, *Picconia azorica* (Tutin) Knobl., *Prunus azorica* (Hort. Ex Mouil.) Rivas Mart., Losa Fer. Prieto, E. Dias, J. C. Costa, C. Aguiar, *Frangula azorica* V. Grubow and *Taxus baccata* L. Apart from *Juniperus brevifolia* and *Taxus baccata* L., the other two species are protected under the Directive Habitats (Annexes II and IV) (EC, 1992) and Bern Convention (Annex I) (Council of Europe, 1993).

a)

b)

c)

d)

Figure 1.8 – a) *Frangula azorica* tree with nearly ripen red fruits; b) *Prunus azorica* tree; c) *Juniperus brevifolia* and d) *Ilex perado* spp. *azorica* with ripen red fruits.

The two species studied in this research are *Picconia azorica* (Tutin) Knobl. and *Taxus baccata* L.

Picconia azorica (Tutin) Knobl.

1.6 The species

The species synonym name is *Notelaea azorica* (Tutin, 1953) and its vernacular name is "pau-branco", in Portuguese, meaning literally "white-wood". Its wood colour is indeed white to pinkish, though it becomes darker with time. *Picconia azorica* is an evergreen tree with slightly smooth and pale bark.

1.6.1 Morfology

This perennial tree can reach up to 15 meters height and the maximum diameter registered is 53 cm (undeterminated age; Dias, 2001) but for a specimen with a diameter of 22 cm, an age superior to 64 years was given (Museu Carlos Machado, Ponta Delgada, Azores). Leaves are opposite, entire, lanceolate, simple, glabrous measuring up to 6x3 cm. Flowers are white in short axillary clusters; corolla white, 4-lobed. It begins fruiting from the age of 10 to 15 years. The fruit is an ovoid dark blue - purple drupe up to 1-1,5 cm, fleshy when young, later dry and brown (Fig. 1.9); it is collected during the months of July/August, when it presents a dark colour (Fagundo and Isidoro, 2006). The species is a xerophytic organism apt to colonize dry environments and is resistant to sea spray (Dias, 2001).


a)


b)


c)

d)


e)

Figure 1.9 – *Picconia azorica* (a) flowers; (b) non-ripen fruits; (c) ripen fruits; (d) seedlings and (e) adult tree.

1.6.2 Uses

P. azorica played an important social value in the past, being used to build wooden wagons, agriculture equipment and tools (Frutuoso, 1583), house beams as well as fine furniture (Fig. 1.10) (Dias et al, 2007), specially during the period 1580 – 1640 (Martins, 1984). On historic references the wood hardiness and the difficulty to work it are constant facts (Dias et al., 2007). Therefore, this species wood, in the past, corresponded to a supplementary income to households. Additionally, its foliage was also used to feed animals in silvopastoral systems. Nowadays, the scarcity of individuals of considerable dimensions as well as the populations low numbers and its legal status (EC, 1992; Council of Europe, 1995) forbids current generations to profit from its value.

Figure 1.10 – A fine piece of furniture built from house beams of destroyed ceiling structures after the 1980 earthquake in Terceira Island (private collection).

1.6.3 Taxonomy

Genus *Picconia* (dedicated to the memory of J. B. Picconi, Italian horticulturist of XIX century) is endemic to the Macaronesian Flora with *Picconia excelsa* endemic to Madeira and Canary archipelagos and *Picconia azorica* endemic of the Azorean Flora. *Piccconia* Genus belongs to the *Oleaceae* Family (with about 600 species in 25 Genera). *Picconia* closest relatives can be found in Eastern Australia, *Notelaea* Genus, spaced out approximately 19.000 km from the Macaronesian Region.

The two taxa, *P.azorica* and *P.excelsa*, are morphologically very similar, and their taxonomic ranking has been long debated (Seubert 1844; Knoblauch 1895), until Tutin (1933) proposed a specific rank for *P. azorica.* This author identified the following different of *Picconia azorica* in relation to *P.excelsa*: similar plant, that differs by having a form closer to a shrub, with smaller leaves (measured), with a proeminent nervure in the abaxial side; smaller flowers and ripen fruit with 1.3 cm long and generally more pronounced sclerophyll habitus.

1.6.4 Geographical distribution

P. azorica when first mentioned in the Azores, one century after the islands settlement was present in all nine islands, in dense and high pure stands (Frutuoso, 1583). Presently *P.azorica* is extinct in Graciosa, nearly extinction in S.Miguel, and despite the fact that GEVA map lists some populations on Corvo Island, the local Forest Service stated that in the present time it is not possible to find individuals of this species. Therefore it is present only in seven islands. Clearly, the 500 years of pressure towards these species populations unables one to say which were the habitat limits and if the present have continually existed. Guppy (1917) reported that the species was found at altitudes between 600 and 750 meters, though its rareness was already documented. According to Dias et al. (2007) the species distribution potential area, simulated through GIS models, excludes areas above an altitude of 800 meters. The actual distribution area is far less extensive and it is associated to

well drained and developed soils, not water logged (Dias et al., 2007), at the altitude range of 300 to 600 meters (Palhinha, 1966). *P. azorica*, once a dominant component of forests between 300 and 600 meters a.s.l. on all of the Azores, is now scattered in small patches of coastal forests and marginal sites, consisting of more than one thousand reproductive individuals dispersed all across the archipelago (Cardoso et al., 2008). In contrast, the occurrence of *P. excelsa* in the Canary and Madeira Islands is much better preserved; though fragmented, its distribution appears to be generally widespread, and populations are relatively frequent in areas of cloud forest above 1000 m on all the Canary Islands (except Lanzarote), and on a slightly wider altitudinal range in the laurel forest of Madeira, which still occupies up to 22% of the island area (Capelo et al., 2007). Wood over-exploitation, habitat loss, and massive introduction of allochtonous species represent the historical causes of the decline of Azorean native forests, and the current most dangerous threats to *P. azorica* (Martin et al., 2008). Moreover, it is subjected to the strong competition of invasive species, namely *Pittosporum undulatum* and *Hedychium gardneranum*.

1.6.5 Ecology

The majority of *P. azorica* populations develops in full sunlight and, as said before it occurs in areas up to 600 meters high, in areas with up to 20º slopes. Most generally occurs on hillsides, in areas having as predominant geological materials basalts and developed soils. Four types of habitat have been identified for this species (SRAM, 2005).

1 – A typical hillside habitat consists on the lowest altitude areas close to the coast, that are characterised by developed soils. Geological materials are basalts and clays. It is the driest area where the species can be found. Nearly all S.ta Maria island populations can be found in this habitat. Small patches and a reduced number of individuals can be found in this low altitude habitat that is quite modified due to external pression, namely the expansion of exotic species. The species that dominate woodlands is the invasive alien

species, *Pittosporum undulatum*, and in some cases plantations of *Cryptomeria japonica*, *Acacia melanoxylon* and *Eucalyptus globulus*.

2 – This habitat is also a hillside one, however at a superior altitude than the last one. As before it still suffers pression due to invasive species, namely *P. undulatum* and *Pteridium aquilinum* (bracken). Basalts are the predominant geological materials. This habitat is slighty "more natural" than the previous one, being dominated by *Myrica faya* and *Erica azorica* woodlands.

3 – A hillside habitat at higher altitudes, with saxicolas as dominant geological materials, though still quite variable. Exotic species again dominate; nonetheless woodlands of *Laurus azorica*, *Erica azorica*, *Myrine africana* and *Frangula azorica* can be present.

4 – The mountain habitat, found in lava areas, in streams and calderas. Basaltic geological materials are dominant, but developed soils are also significant. Sometimes humidity conditions are so extreme that plants from the Genus *Sphagnum* spp. can be found. Its is far away from man action, therefore better preserved and composed by natural woodlands dominated by *Laurus azorica*, *Juniperus brevifolia* and *Ilex perado* ssp. *azorica*. Woodlands dominated by *P. azorica* are frequent.

1.6.6 Current status in the Archipelago

Nowadays, *P. azorica* status is classified as Endangered (IUCN, 2009) protected under the Directive Habitats (Annexes II and IV) (EC, 1992) and Bern Convention (Annex I) (Council of Europe, 1993). Approximately 70% of the species populations are well preserved, whereas the remaining ones are not as well preserved (SRAM, 2005). *P. excelsa* is not included on these species lists, though it is classified as Vulnerable on the 2008 IUCN Red List of Threatened Species (IUCN, 2009). *Picconia azorica* fragile status lead it to be included on the 100 taxa (endemic and non-endemic) list of Azores priority species (fauna and flora) for which management and conservation actions are crucial for their survival and populations increment (Martin et al, 2008). This group of authors classified the species with an ecologic value of "structuring species".

Additionally, the species plays an important ecological role as part of the diet of two protected Azorean endemic bird species (Dias et al., 2007; SPEA, 2008), both species are protected under the Birds Directive (Annex I) (EC 1979) and the Bern Convention (Council of Europe 1998). These species, a pigeon sub-species, *Columba palumbus azorica*, and a Passeriformes, *Pyrrhula murina* (Critically Endangered - IUCN 2009, the Passeriforme species most endangered in Europe and one of the world's most endangered), feed on *P.azorica* fruits and have an important contribution to its dissemination within and among islands.

Conservation plans to prevent erosion of *P. azorica* genetic resources are thus necessary, primarily because of its endangered status, for its biological relevance and for its major ecological importance. Cardoso et al. (2008) believe that the species would have the capacity to double its population figures in less than ten years, provided that specific long-term management plans are implemented.

***Taxus baccata* L.**

1.7 The species

Taxus baccata L., known as the European or common yew, currently is considered rare or endangered in Austria (Dhar et al., 2008), in Denmark (Svenning and Magard, 1999), Spain (García and Obeso, 2003) and elsewhere within its geographic distribution (Thomas and Polwart, 2003). This species is currently the focus of considerable investigation to better understand its complex bio-ecology and to assure the conservation of the species (Svenning and Magård, 1999; Dhar et al., 2008; Iszkulo et al., 2009; Piovesan et al., 2009).

Yew is an ancient tree that can live for centuries. Such fact gave the species the reputation that it can live to a greater age than most other tree species (Savill, 1991), some trees have been calculated

to be more than a thousand years old. Hence yew became a symbol of everlasting life. Yews are important symbolic trees that pre-date the Christian era. Pre-Christian burial sites often had yews to protect against evil spirits. Early Christian churches were built on pagan sites often including a yew tree. In many parts in Europe, namely Spain, United Kingdom and France it has been planted in churchyards for many centuries. All parts of the plant, except a bright red cup (called the aril) that resembles a berry, are poisonous (Savil, 1991). Yew toxicity is due to alkaloids, which are very poisonous. The chief consequence of the taxine action of this species in humans and other mammals is heart failure and death (Thomas and Polwart, 2003). It is very shade tolerant and is capable to withstand salty winds. Rarely planted in commercial plantations as a consequence of its extremely slow grow rate. It achieves maturity at about 70 years of age.

1.7.1 Morphology

Yew is an evergreen conifer, small in height (12-15 meters) when growing in woodland (see Fig. 1.11). However trees growing individually of this non-resinous gymnosperm species can reach up to 20 – 28 meters tall. Trees have conical, rounded or pyramidal canopy and spread out to a great width. It has the capacity to regenerate from old branches, trunks and sometimes stools. The species tends to have a shallow root system. The dense mass of leaves does not allow other vegetation to grow underneath it. The bark is reddish-brown or grey with peeling red strips. As the tree ages, the heartwood rots and it becomes hollow. Leaves are linear, needle-like, acute, spirally attached but on lateral shoots twisted more or less into two ranks; dark green 10-30(45) mm long and 2-3mm wide (Thomas and Polwart, 2003). Yew is normally a dioecious species, with green reproductive structures, borne in leaf axils near the end of the previous summer's growth. It flowers in spring. Male structures in small cones, which consist of small yellow spheres, producing masses of pollen in February; each consisting of 6-15 anters, clustered near the branches ends. Female flowers are very small and green, being wind-pollinated; solitary or in pairs in leaf axils on underside of shoots, with one fertile scale surrounded by several sterile scales (Fig. 1.12). It flowers from February to

April and seeds ripen sequentially from late July to October (García and Obeso, 2003). Yew does not actually bear its seed in a cone. The fruit is a hard seed, ovoid, enclosed in a sweet-tasting aril. Seeds should be collected from the female trees in November. Seeds are also dispersed by birds, which are attracted to the fleshy red arils. Seedlings are tolerant to deep shade.

Figure 1.11 - The dark glossy green tree is a *T.baccata* individual growing under the shade of a *Fagus sylvatica* L. woodland in Greece Mountains.

Figure 1.12 – *Taxus baccata* female and male structures, 1) globose male cone; 2) male cone shed its pollen in early spring; 4 and 5) immature seed cone with one single seed partly surrounded by a modified scale that develops into a soft bright red berry-like structure aril; 6 and 7) mature seed cone and its transversal section; Dark-green yew shoot with lanceolate leaves with (A) male and (B) female structures (Source: Thomé, 1885).

1.7.2 Uses

The oldest archaeological artefact found made of yew wood was discovered in Clacton, Essex, with an approximate age of 250.000 years! It was a spear (Mabey, 1996). This species wood was also used to build the Egypt's Pharaohs sarcophagus (Catarino, 2007).

Yew wood is the densest of all conifers reaching density values of 670 kg/m^3 (Hoadley, 1990) and is extremely durable (Lo Monaco and Bernabei, 2003) as well as flexible. Produces strong decay-resistant wood. Its distinctive reddish – orange to russet heartwood colour, extremely fine texture and irregular growth rings providing decorative value made it an attractive wood for fine furniture making in the Azores (Fig. 1.13). In the Azores, as well as in Italy, it was also used to build ceiling structures, which were said to be very strong, resistant and durable. In the past, because of its wood strength and flexibility, in the United Kingdom was used for making longbows (the weapon of choice before guns) and knife handles (Manzi, 2003).

Figure 1.13 – Table made of yew grown in the Azores islands (private collection).

Additionally, it's a species worldwide studied due to other economical reasons, namely medicine applications (Goodman and Walsh, 2001). *T. baccata* has been known as a poisonous plant for over 2000 years (Itokawa, 2003). The Genera name (Taxus) itself, is also the origin of the word "toxic". Thus, the first taxoids were isolated owing to the interest in the toxic constituents of the species. Taxoids are contained only in Taxus species, most of them are difficult to synthesize industrially and taxol and related compounds are medically useful (Jennewein and Croteau, 2001;

Kikuchi and Yatagai, 2003). The isolation of paclitaxel (synonym taxol) from the bark of Taxus brevifolia, proved to exist also in Taxus baccata fresh needles (Kikuchi and Yatagai, 2003), and the discovery of its useful physiologically active substances, made it a species of scientific and economic interest. Placitaxel (Taxol®), is the yew-derived anti-cancer agent used clinically against a variety of cancer diseases, namely breast and ovarian cancer (Parmar et al., 1999; Cragg and Newman, 2005; Khosroushahi et al., 2006).

1.7.3 Taxonomy

Taxales is a small Order composed by a single Family (Taxaceae), five Genera and approximately 20 species. One of the Genera is *Taxus* with approximately 10 species widely distributed in the Northern Hemisphere, in which *Taxus baccata* L. is included.

Two *Taxus baccata* varieties are recognised for Portugal territory and islands, both in the Baccata group (Spjut, 2006). One variety is *T. baccata* var. *erecta* Loudon known to exist in Portugal mainland and Spain. The second variety is *T. recurvata* var. *linearis* (Carrière) Spjut ineditus, which the author states to exist in Madeira Island, as well as in England, Germany, Hungary, Bulgaria, Austria and then in SW Asia in Syria and Turkey. This variety is categorized under the Baccata Group, in the Baccata Alliance under the *T. recurvata* complex.

The Azores yew should fit under the denomination *T. baccata* var. *erecta* Loudon according to Spjut (2006) classification of *Taxus* (Taxaceae) species and varieties. However, in a recent work by Spjut (2007) an anatomical evaluation was carried out on two samples of *T. baccata* from Pico. According, to this last work, Taxus Azorean provenance is classified has *T. baccata* var. *variegata* under the Elegantissima Complex of Taxus Baccata Alliance. As main characteristics is pointed out an abaxial leaf margin of four cells across without papillae, followed by 10 rows of papillose cells and with 10 to 12 rows of stomata.

1.7.4 Geographical distribution

Taxus baccata L. has a widespread distribution (see Fig. 1.14). According to Thomas and Polwart (2003) it is present in "Norway and Sweden, eastwards to Estonia, Poland, to the Caspian Sea and Turkey, and southwards to Greece", northern Spain and into Algeria. Additionally, large populations can be found in Ukraine, Poland, Hungary, Slovakia, Romania and the Caucasus Mountains. These authors stated that the species "is absent from the most continental climatic regions of Europe and also northern, south-eastern divisions of Russia, Crete, Faeroes and Iceland". The species is also found in Portugal mainland and along times have been described as part of the Azorean flora (Drouet, 1866; Watson, 1870; Trelease, 1897; Guppy, 1917; Dias, 1996). Nevertheless, due to its over-exploitation it became so rare that it was thought to be extinct (Thomas and Polwart, 2003; Cardoso et al., 2008). In Portugal mainland it can be found at Gerês National Park, about 10.000 individuals, and in "Serra da Estrela" an approximate number of 50-55 (Dr. Alexandre Silva, pers. comm.)

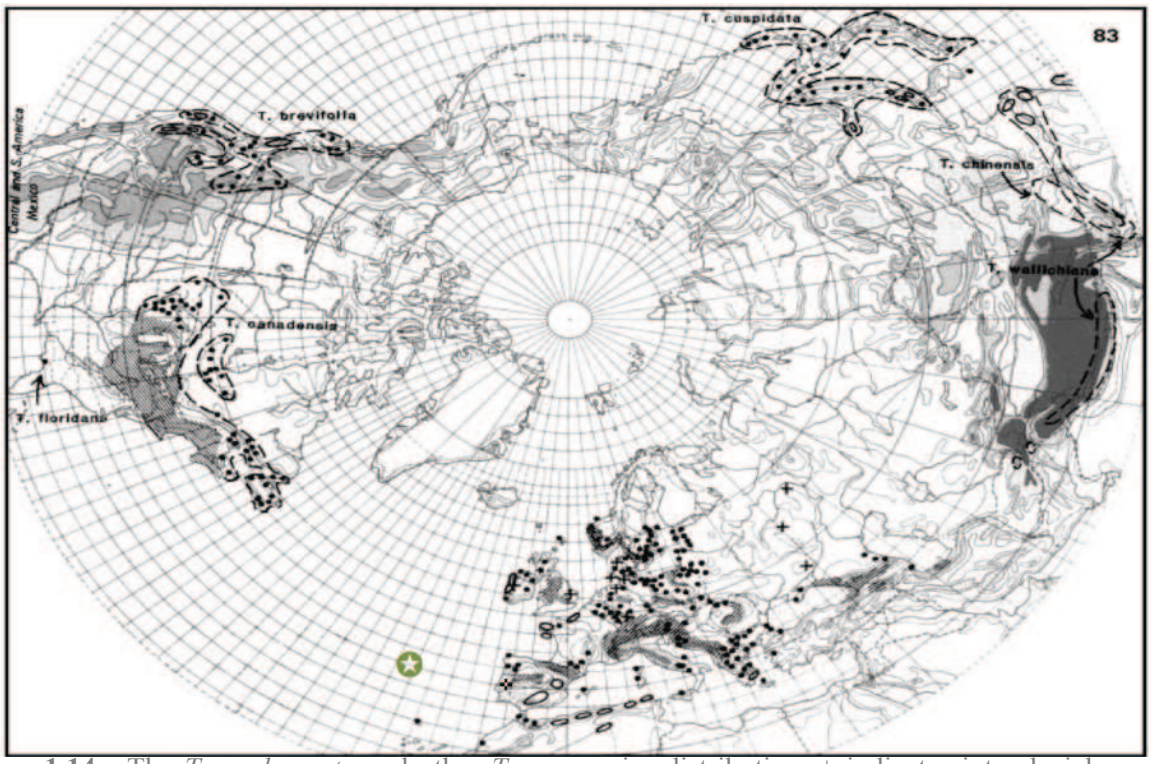


Figure 1.14 - The *Taxus baccata* and other *Taxus* species distribution. + indicates interglacial records; ● indicates isolated occurrences; ☒ indicates Azores archipelago position; ☒ indicates Serra da Estrela position; hatched areas indicate regions of common or fairly common occurrence. Broken lines outline the distribution of *Taxus* species other than *T. baccata*. (Adapted from Thomas and Polwart, 2003).

1.7.5 Habitat

The species grows best in high humidity of mild oceanic climates (Thomas and Polwart, 2003). The ecological barriers that limit the species distribution are low temperatures, severe continental climate, long droughts and drought and high temperatures. According to the same authors, in mountainous areas of Europe, the species tends to grow on shaded north-western or north-eastern slopes. Slope does not limit the species distribution, occurring on moderate to very steep slopes.
Yew grows on most soils as long as not waterlogged (Savill, 1991). In Europe best growth (in terms of growth rates and largest dimensions) are observed on deep, moist sandy loams and well-drained clays, and worst on dry, rocky and sandy soils, where it occurs as dispersed and underdeveloped individuals (Thomas and Polwart, 2003).

1.8 State of the art – the island paradigm

Charles Darwin foundations of his evolutionary theory were established from the insight of oceanic isolated islands namely Galápagos. Others like Wallace, Mayr, Wilson and Diamond were also inspired by oceanic islands systems. Isolated oceanic islands are not easily reached by propagules, being generally species-poor and disharmonic, yet can be rich in species found nowhere else, i.e., endemic to those islands (Whittaker and Fernández-Palacios, 2007). There are mainly two types of islands, true islands and habitat islands. The first ones differ greatly from the second ones in their dynamics. Consequently true islands ecosystems have been long recognized as ideal natural laboratories for the study of evolution namely for: (i) being discrete geographic entities; (ii) island isolation leads to reduced gene flow; (iii) often small size that allows to better quantify species numbers; (iv) can contain a diversity of habitats and; (v) frequently geologically dynamic with historical and contemporary volcanic and erosional activity (Emerson, 2002).
As true islands, oceanic islands provide exceptional models to examine patterns of organism dispersal, isolation and diversification (Emerson, 2002). Patterns of genetic variation in island

wild organisms may be inspected at different levels of isolation: mainland-archipelago (Inoue and Kawahara, 1990; Chiang et al., 2006), among islands (Gómez et al., 2003), and among populations within an island (Maki, 2001; Nielsen, 2004). In general, we assume that island populations and taxa are highly differentiated from continental closest relatives (Böhle et al., 1996; Helfgott et al., 2000; Bromham and Woolfit, 2004) and display lower levels of genetic diversity (Frankham, 1997; Maki, 1999; Nielsen, 2004), being more prone to be genetically impoverished, as consequence of possible founder effects, isolation from the source population, and stochastic processes triggered by limited population size (Maki, 2001; Garcia-Verdugo et al., 2009). Nevertheless, genetic mechanisms may be also affected by a series of eco-geographical factors driving the patterns of colonization and local extinction–recolonization cycles (Juan et al., 2000, Moore et al., 2002; Burns and Neufeld, 2009; Borges and Hortal, 2009), not excluding recent anthropic disturbance (Vaxevanidou et al., 2006).

Given that true islands have clearly defined limits and properties, and so are discrete objects for investigation, these characteristics make them remarkable to explore their endemic species of both fauna and flora providing insights into the species ecology and guidelines for their conservation.

Many studies have been focused on the genetic diversity of endangered plant species (Falk, 1990; Fontaine et al., 2007; Mohapatra et al., 2009), since not considering the genetic factors may lead to inadequate recovery strategies (Newton et al., 1999; Frankham, 2005; Avise, 2007). This has a greater impact if it's an endemic species (Gemmill et al., 1998; Wolf, A., 2001; Pereira et al., 2002; Wesche et al., 2006), which tends to have a limited geographical distribution. Moreover, about one in six known vascular plant species grows on oceanic islands, and one in three of all known endangered plants is an insular endemic (World Conservation Monitoring Center, 1992).

So far neither the Azorean endemic, *Picconia azorica*, nor the Azorean provenance of *Taxus baccata*, have been objects of biological studies (at least accessible for consultation).

1.9 The research main objectives

As pointed out before, from within the endemic vascular plants from Azores, 50% are priority in terms of future conservation actions, representing a total of 37 species. This is the case for *Picconia azorica* (Tutin) Knobl. and *Taxus baccata* L., that will be studied in this research project. These species in the past were over-exploited due to their economical importance and face now a series of threats that compromise their future existence.

The need to adopt re-introduction strategies, for conservation or for forest production objectives, lead to the evaluation of the genetic variability of *Picconia azorica* (Tutin) Knobl. and *Taxus baccata* L., as well as their phylogeny. Propagation trials (seminal for *P.azorica* and clonal for *T.baccata*), were conducted and *Picconia azorica* wood characterization was done.

In this study, was undertaken a molecular study at the population level on *P. azorica*, with the aim to describe macroevolutionary and phylogeographical patterns. The objectives were (1) to assess the biological significance of *P. azorica* by inspecting the evolutionary history of the genus and the systematic relationships between *P. azorica* and *P. excelsa*, and (2) to explore the species' genetic diversity in order to formulate appropriate management and conservation strategies.

The Azorean *T. baccata* provenance, can be seen as a marginal population, as a result of having survived under particular ecological conditions, far from its core geographical distribution. This population due to the evolutionary processes might contain unusual adaptations and constitute a valuable genetic resource, being therefore an important population to be investigated.

The research will be based on historical information, field surveys and laboratory work. It aims to document the occurrence and current status of *Taxus baccata* in the Azores, in order to (a) evaluate some "basic" characteristics that might imply special bio-ecological features; (b) trace back some useful inferences on the evolutionary history of the genus *Taxus* and the colonization processes of the Macaronesian islands; and, finally, (c) suggest a conservation plan for its reliable preservation.

2. MATERIALS AND METHODS

Picconia azorica (Tutin) Knobl.

2.1 Plant material

Picconia azorica leaf samples were collected according to the species distribution map (Fig. 2.1) elaborated by GEVA (Gabinete de Ecologia Vegetal e Aplicada, University of Azores). Due to the loss of the species on Graciosa and Corvo, only seven islands were sampled: S.ta Maria, S. Miguel, Terceira, S. Jorge, Pico, Faial and Flores.

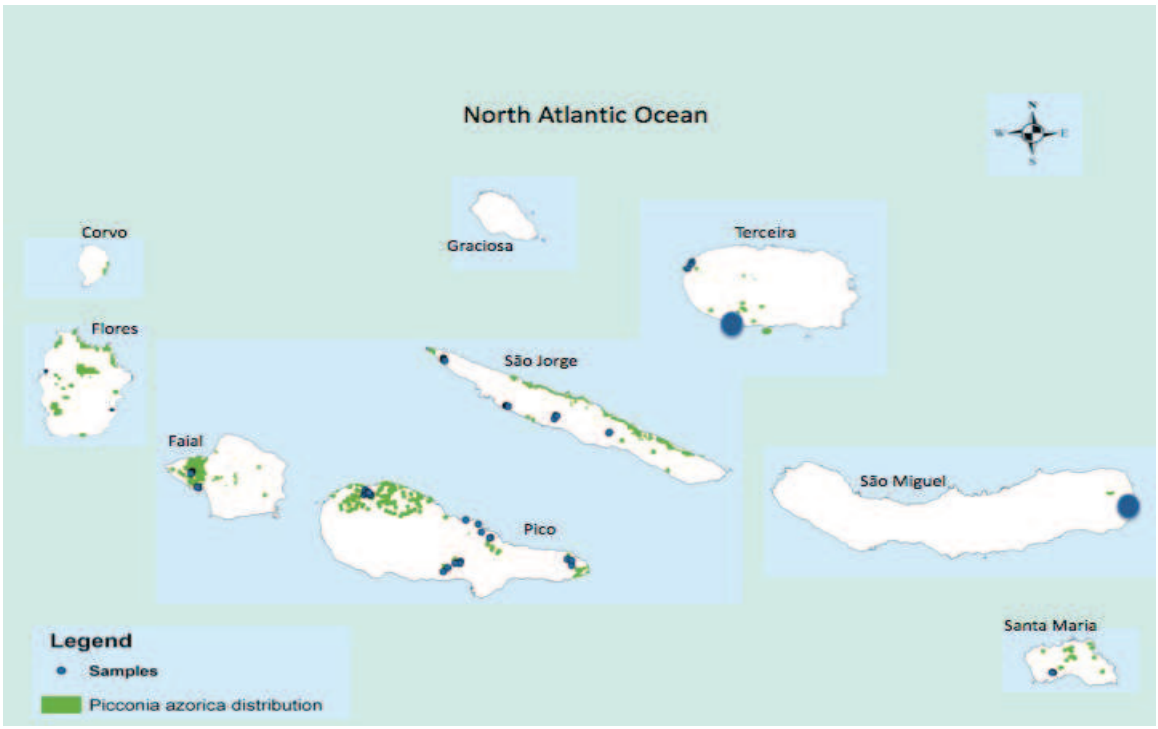


Figure 2.1 - Present *Picconia azorica* distribution map (green) and sampled populations (blue). Note: Although indicated in Corvo Island, presently it is not possible to find *P.azorica* individuals in this island (Adapted from GEVA).

Picconia excelsa was sampled in Madeira Island (Madeira archipelago) and Tenerife Island (Canary Islands). Other *Oleaceae* species were also collected in Portugal, Spain, France and Italy and used in the phylogenetic analysis and in the inter-/intraspecific genetic diversity study. A minimum of three individuals was randomly sampled from each population (Pons and Petit, 1995),

with the exception of Varadouro, the largest site, where eight individuals were sampled in two subpopulations. It is worth emphasizing that, because of its patchy distribution, *P. azorica* trees are extremely sparse and it was not easy to find many individuals in some populations; in the few cases where needed, 100 m minimal distance separated each sample, to decrease the odds of analyzing closely related individuals. Whenever possible, the GPS (Global Position System) coordinates were registered (see Annex I).

Plant material, a total of 10-15 leaves per individual, was labelled and placed in plastic bags, removing the excess of air, to avoid its degradation. After which it was sent by air-mail to DAF Laboratory, Università degli Studi della Tuscia and conserved at -20ºC. Leafs were trimmed to remove their veins and damaged tissues and lyophilized in a FreeZone 2,5 liter - Labconco. The lyophilized material was reduced to a powder by using a mortar and pestle and sterile quartz sand (silicon dioxide, Sigma S9887) (see Fig. 2.2). Lyophilized tissue can be stored for several years with a minor loss of DNA quality (Sharma et al., 2002). For this reason all lyophilized plant material became part of the Forest DNAbank (La Banca del DNA forestale), belonging to Università degli Studi della Tuscia, Viterbo. As a result, each sample has a voucher with institutional accession numbers.

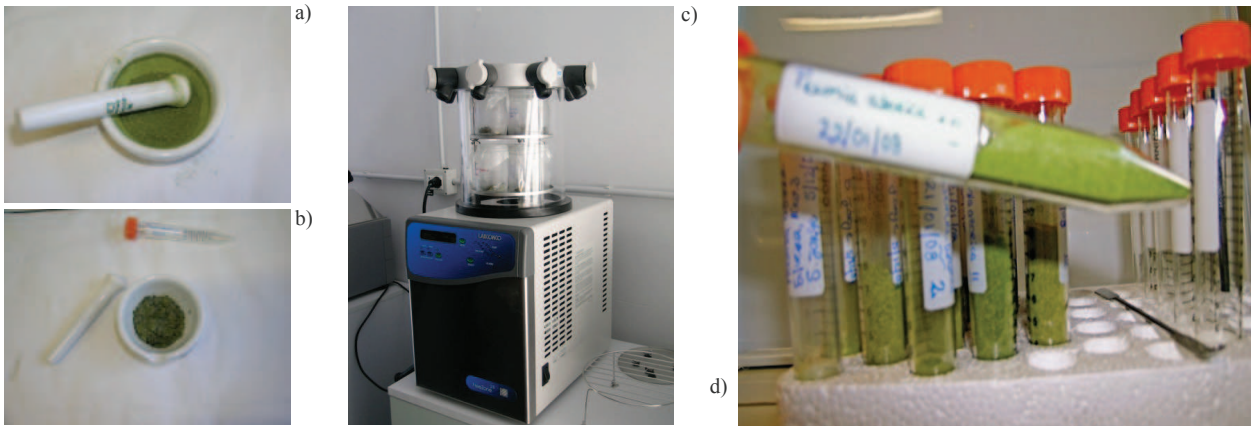


Figure 2.2 - a) *P. azorica* leaf material during the lyophilisation process; b and c) lyophilized material prior (b) and after (c) being grinded; d) labelled samples prior do DNA extraction.

2.2 DNA extraction

Extracting DNA from *Oleaceae* species encounters as main constraints the presence of high concentrations of polyphenols, oils, waxes as well as tannins, polysaccharides, that are present on mature leaves (Varma, 2007). Moreover, in this case, the difficulty increases due to the fact that the plant material is lyophilized and the DNA to be extracted is chloroplast DNA - cpDNA (Prof. Rosario Muleo, persn. com.).

The initial DNA extraction protocol followed was the one from a commercial kit, the DNeasy Plant Tissue Mini Protocol, Qiagen. This kit is characterized by the absence of toxic chemicals, as chloroform and phenol, allows both high DNA quality and quantity and is accomplished in about two hours. However, with the *Oleaceae* species the amount of DNA obtained was very reduced, not allowing subsequent PCR (Polymerase Chain Reaction) analysis.

Therefore, several small modifications were made in the Mini Protocol Qiagen namely, the incubation period was increased from 10 to 30 minutes at 65ºC; the sample was grinded again with a mortar and pestle with 600 µl Buffer AP1 adding also PVP (polyvinylpyrrolidone). A last variation was on the sample weight, it was increased and 100 mg of grinded plant material was used. Nevertheless, with none of these alterations was possible to extract DNA as desired and needed to proceed with the cpDNA analysis.

Due to the facts exposed it was not possible to extract DNA from *P.azorica* and the other *Oleaceae* samples with the Qiagen kit. As a result, the Doyle and Doyle (1990) DNA extraction protocol was tailored with modifications being made to overcome the restrictions already mentioned. Accordingly, the adjustments should: 1) eliminate the secondary metabolites (oils, waxes, polyphenols and others); 2) allow the re-hydration of the lyophilized plant tissue and, 3) the complete disruption of the tissue to obtain the cpDNA. Having that in mind the protocol modifications included:

(i) - plant material weight was about 150mg;

(ii) - re-hydration was made by grinding gently, with a mortar and pestle, the sample with CTAB, PVP and β-mercaptoethanol;

(iii) - immersion at 65ºC was done for one hour agitating the samples every 10-15 minutes in a vortex.

To help prevent the formation of polyphenolic compounds, plant material was frozen, at -20ºC, prior to DNA extraction and PVP was added when samples were mechanically grinded with a mortar and pestle (Porebski et al., 1997). PVP, by forming complex hydrogen bonds with polyphenolic compounds allows their elimination by centrifugation (Maliyakal, 1992). The buffer CTAB contains sodium chloride (NaCl), that allows removing polysaccharides, when used in a concentration higher than 0.5M (Varma et al., 2007), which is the case. β-mercaptoethanol was used in order to inactivate the DNase, and so the DNA oxidation as well as to remove proteins. Chloroform also permits proteins elimination.

The major drawback these modifications imply is the use of toxic reagents such as chloroform, isoamyl alcohol and β-mercaptoethanol. Hence, all laboratory safety rules were followed to avoid any hazards.

The protocol adopted for the DNA extractions required was as follows:

-Homogenization of the lyophilized plant material with mortar and pestle in the presence of sterile quartz sand;

- Weight 150 mg ground plant material in an Eppendorf tube;

- Re-hydration and new homogenization with 1.5ml CTAB, 5 µl β-mercaptoethanol and PVP with a mortar and pestle;

- One hour waterbath at 65ºC, shaking every 10-15 minutes in a vortex;

- Centrifuge for five minutes at maximum velocity (15.000 rpm);

- Recover the liquid phase to a new Eppendorf;

- Add 400 µl of chloroform:isoamyl alcohol (24:1) and mix thoroughly;

- Centrifuge for five minutes at 9000 rpm;

- Transfer top aqueous solution to a new Eppendorf tube and avoid disturbing the interface. Add 400 µl of chloroform:isoamyl alcohol, and mix thoroughly;

- Recover once again the top aqueous solution to a new Eppendorf tube and repeat the above

step. On the final upper DNA containing phase recover avoid pipetting chloroform:isomayl alcohol as it will interfere negatively on DNA precipitation;

- Add 50 µl potassium-acetate 3M, pH 4.8 and 500 µl cold isopropanol (-20ºC), mix gently, these reagents will allow the DNA pellet precipitation;
- Centrifuge for 10 minutes at maximum velocity (15.000 rpm);
- Verify the DNA pellet presence and pour off supernatant;
- Add 250 µl of 70% cold ethanol (-20ºC) to wash the DNA pellet, mix gently and centrifuge for five minutes at maximum velocity (15.000 rpm);
- Eliminate the liquid phase (ethanol) and without touching the DNA pellet, clean with tissue the particles of ethanol that remain in the Eppendorf;
- Air-dry the DNA pellet for 15-20 minutes at room temperature to allow ethanol evaporation;
- Add 150 µl TE buffer and 4µl RNAse (10mg/ml);
- Incubate the sample at 4ºC overnight, after which DNA concentration should be controlled.

Reagents and chemicals required:

- Extraction buffer: 100mM Tris-HCl, 20mM EDTA, 1.5M NaCl, pH 8.0, 2% CTAB
- CTAB (cation hexadecyltrimethyl ammonium bromide)
- Polyvinylpyrrolidone (PVP) Sigma P-9003-39-8 (40.000)
- Chloroform:isoamyl alcohol 24:1 (v/v)
- Sterile quartz sand (silicon dioxide, Sigma S9887)
- β-mercanptoethanol
- Potassium-acetate 3M
- Isopropanol
- Ethanol 70% pure

To verify the presence and DNA concentration, 5µl aliquots of extracted volumes were loaded on a 1% agarose gel and subjected to an electrophoresis (Gene Power Supply GPS 200/400, Pharmacia) at about 70 V for half hour. Genomic DNAs were visualised by UV fluorescence after staining with ethidium bromide. DNA samples were then quantified using a DNA Molecular Weight Marker (Qiagen Gel Pilot 50bp). Subsequent to DNA concentration quantification and evaluation of purity degree and integrity, DNA samples were preserved at -20ºC to be used later on cpDNA analysis.

2.3 Molecular analysis - cpDNA analysis

Genetic variation of *Picconia ssp.* will be evaluated through the study of chloroplast DNA - cpDNA. *Picconia* intra-species genetic diversity at the plastid genome level was evaluated by use of PCR-RFLP (Polimerase Chain Reaction – Restriction Fragment Length Polymorphism) and SSRs (Simple Sequences Repeats) variation in 17 populations, for a total of 90 individuals. Additional two populations of *P. excelsa* (eight individuals) and eight populations (35 individuals) of *Olea europaea* var. *sylvestris*, *Phillyrea latifolia* and *P. angustifolia* from France, Portugal, Spain and Italy were analysed for comparison.

2.3.1 The PCR- RFLP technique

The PCR- RFLP technique was applied to study the cpDNA variability. This technique consists on three main steps:

1. DNA fragment amplification by a primer pair;
2. Digestion of the fragment with a restriction enzyme;
3. Separation of the digestion fragments on an acrylamide gel.

The PCR is a powerful technique that allows the amplification of specific DNA sequences *in vitro* using appropriate primers. The reaction mixture is set up containing a sample of DNA that includes the region to be amplified, the primers in large molar excess, deoxynucleoside triphosphates (dNTPs) and a heat-stable DNA polymerase. In the present case it was used the *Taq* polymerase,

which is purified from the thermophilic bacterium *Thermus aquaticus*. Additionally, specific DNA segments with a small number of nucleotides are added. These segments, the *primers*, guide the replication process. This is so because they contain sequences complementary to the target region of the DNA, *primers* along with the *Taq* polymerase are key components to enable selective and repeated amplification. The DNA polymerase will then catalyze the synthesis of a new DNA strand complementary to a template DNA from 5'→ 3' direction by primer extension reaction (Wu et al., 2004). Consequently the DNA region flanked by the two primers will be produced. Then, each new strand produced is used as a template for replication, setting in motion a chain reaction in which the target DNA sequence is exponentially amplified. The reaction is therefore repeated in cycles through alternating periods of thermal denaturation, annealing, and extension.

The denaturation implies the separation of the DNA double helix, annealing consists in primers targeting their homologue regions and amplification is the production *in vitro* of new copies of the target DNA sequence allowing in this way the production of millions of copies. The final product is a large sample of the copies of the gene of interest.

In the laboratory, the first step was to perform a screening of the primer/taxon combination on a subset of 13 individuals belonging to the genus *Picconia* (one individual per island), *Phillyrea* and *Olea* to test the efficiency of every primer/enzyme combination to use on the study. Accordingly to the results, the six pairs of primers specific for the chloroplast genome used in the screening were employed to amplify their corresponding cpDNA regions.

Consequently, PCR-RFLP analysis was performed with the following primer/enzyme combinations: CD/TaqI, SR/HinfI, TF/HinfI, DT/TaqI, AS/HinfI, FV/TaqI (Taberlet et al., 1991; Demesure et al., 1995; Dumolin-Lapegue et al., 1997). Amplification and restriction conditions followed the protocols of the above referenced works.

DNA fragment amplification was performed by the PCR technique and took place on a MJ Mini Thermal Cycle (Bio-Rad). DNA aliquots reacted with a lyophilized commercial bead, which contains all the elements necessary for the amplification reaction. For the present work every PCR reaction mix contained 50 ng of DNA from each sample, 2.5 μl (2 μM) of each primer (forward

and reverse), a puRe Taq Ready-To-Go PCR bead (Amersham) that contains the Taq polymerase enzyme, reaction buffer, nucleotides and 1.5mM $MgCl_2$ (for the primer TF an additional 1.0 mM $MgCl_2$ was added) and sterile water enough to realize a total volume of 25 µl.

As said before, six molecular markers were used, meaning that six different regions of the chloroplast DNA were investigated through copies of six different primers: AS, CD, DT, FV, SR and TF. The CpDNA regions investigated are responsible for codifying transfer RNA (tRNAs) of several amino acids or enzymes of the chloroplast photosystem. In Table 2.1 can be seen some information regarding the *primers* used, as well as the dimensions of the fragments obtained with the PCR reaction/technique.

Table 2.1 - Homologous region for each primer used in the PCR reactions. The synthesis of a new strand is made in the 5'→ 3' direction.

Primers designation	Homologous region 5'	Homologous region 3'	Distance (bp)
CD	trnC [tRNA – Cys(GCA)]	trnD [tRNA – Asp(GUC)]	3290
DT	trnD [tRNA – Asp(GUC)]	trnT [tRNA – Thr(GGU)]	1730
TF	trnT [tRNA –Thr(GUC)]	trnF [tRNA – Phe(UGU)]	1840
AS	psaA [PSI (P700apoproteinaA1)]	trnS [tRNA – Ser(GGA)]	3510
SR	trnS [tRNA – Ser(GCU)]	trnR [tRNA – Arg(UCU)]	2050
FV	trnF [tRNA – Phe(UUG)]	trnV [tRNA – Val(UGU)]	3492

PCR amplifications began with an initial denaturation step of four minutes at 94ºC, after which the PCR consisted of 30-35 cycles with two minutes at 94ºC, an annealing phase and an amplification

phase at an optimal temperature according to the primer pair (see Table 2.2), followed by a final elongation step of 10 minutes at 72ºC.

Table 2.2 - PCR conditions for the annealing and amplification phases for each primer used.

Primers designation	Annealing temperature (ºC)	Annealing duration	Amplification temp. (ºC)	Amplification duration	No. of Cycles
CD	58	2 min.	72	3min. 30seg.	30
DT	54,5	45 seg.	72	2 min.	30
TF and SR	56	2 min.	72	2 min.	30
AS and FV	61	2 min.	72	2 min.	35

To evaluate PCR products concentration, an electrophoresis took place within the same conditions as to verify DNA concentration. Thus, PCR products (2µl) were loaded on a 1% agarose gel. Once completed the electrophoresis, the gel was ethidium bromide stained, allowing to ascertain the fragments concentration for later enzyme digestion.

Each different restriction enzyme recognizes a specific and characteristic nucleotide sequence, and any alteration on just one nucleotide on that sequence will originate a variation on the number of cutting sites (White and Matise, 2001). As a result, "polymorphism is observed between individuals in the positions of cutting sites and the lengths of DNA between them, resulting in restriction fragments of different sizes" (Jones et al., 1997). The different fragment lengths will be observed on the acrylamide gel, allowing one to evaluate the genetic variation. This is the essence of the RFLP method.

The following step was PCR fragments digestion with two restriction enzymes, *TaqI* and *HinfI,* in a total volume of 15 µl. Therefore, for each PCR sample an aliquot was obtained for a reaction with the restriction enzyme recommended (Jimenez et al., 2004) according to the following protocol:

PCR product	5ml
Restriction enzyme	0.25 U
Reaction buffer	1,5 ml
Water	volume needed to the final volume of 15ml.

The combination primer/restriction enzyme is indicated in Table 2.3, with the indication of the reaction temperature and duration.

Table 2.3 - The primer/restriction enzyme combination and reaction conditions.

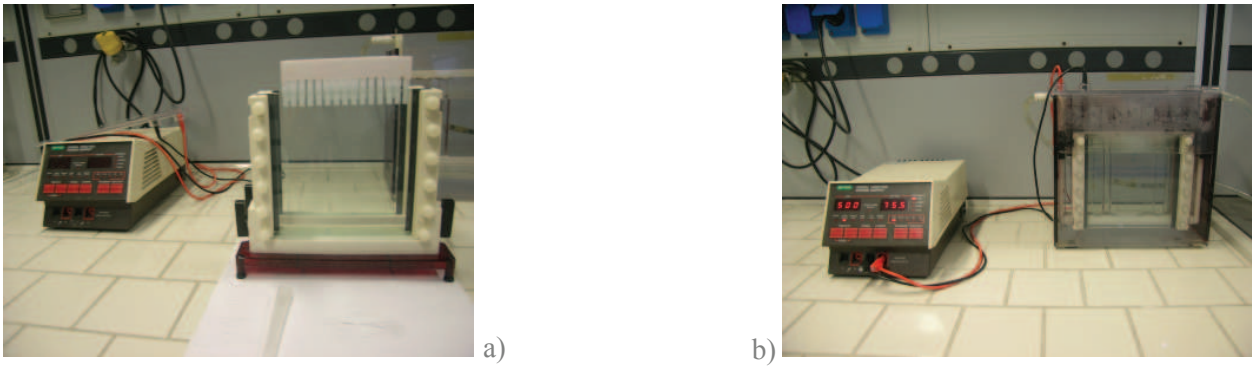
Primer Set	Restriction Enzyme	Temperature (ºC)	Duration (hours)
CD / DT / FV	TaqI	65	6
TF / AS / SR	HinfI	37	12

The digested cpDNA fragments (PCR-RFLP) were separated under electrophoresis on an 8% acrylamide gel. Two gels were prepared, simultaneously, on the afternoon prior to the electrophoresis and left to polymerase for the entire night. Every two acrylamide gels were prepared with the following amount of reagents:

Distilled water	54 ml
Acrylamide (sol. Rapid Gel 40%) (USB)	18 ml
TBE 10x	9 ml
TEMED	72 µl
APS (Ammonium Persulphate) 10%	432 µl

This electrophoresis took place on a vertical apparatus (HOEFER SE 600), allowing two gels to run at the same time, each being loaded with 13 samples and two positions loaded with DNA Molecular Weight Marker (Qiagen GelPilot 50 bp) (see Fig. 2.3). Each sample was loaded with 7.5 µl of digested DNA and 2.5 µl of a biological colorant (bromophenol blue). Migration occurred

due to an electric current (constant voltage 300V, current 50-70mA per gel) passing through the gels for about three to four hours, according to the PCR-RFLP fragments.



a) b)

Figure 2.3 - (a) Vertical apparatus (HOEFER SE 600), with two gels prior to electrophoresis; (b) Electrophoresis progress with 13 samples running in each acrylamide gel.

Once finalised the electrophoresis, gels were immersed and agitated on distilled water for 10-15 minutes to remove any buffer particles. After which, gels were stained in 150 ml distilled water containing 6 µl ethidium bromide and agitated again for 10-15 minutes. Restriction patterns were visualised by UV transillumination and recorded with a Kodak Edas 290 photocamera. Subsequently, polymorphisms were scored based on bands presence/absence or different sizes.

2.3.2 DNA sequencing

DNA sequencing similarly to RFLPs allows studying DNA variation. This method determines the order of the nucleotide bases (adenine, guanine, cytosine and thymine) of the DNA fragment. It will enable to evaluate similarities and differences, at the level of individual's bases/nucleotides, with the objective of inferring structural, functional and evolutionary relationships among the sequences under study (Schuler, 2001). To complement this study two loci where investigated, the non-coding plastid *trnH-psbA* intergenic spacer region and the *rbcL-a* gene that encodes the ribulose – 1,5 – bisphosphate carboxylase/oxygenase large subunit. According to Kress and Erickson (2007) combining a subunit of the coding gene *rbcL* with the non-coding *trnH-psbA* spacer provides good

differentiation between species, making this two-locus barcode an advantageous selection to land plants DNA barcoding. Moreover, these fragments were suggested to be the more efficient within the few available and within *Oleaceae* (Kress and Erickson, 2007; Shaw et al., 2007).

The aim of DNA sequencing was to find more evidences for phylogenetic relationships of *Picconia* genus with other *Oleaceae* species. Allowing this way to compare phylogenetic relationships analysed at two complementary levels of cpDNA variation: RFLPs and DNA sequencing.

A non-transcribed (*trnH-psbA*) and a transcribed region (*trnL-a*) were chosen as the two kinds of regions have different mutation rates, with the non-transcribed region having higher mutations rates. Non-coding regions of cpDNA tend to evolve more rapidly than do coding sequences, becoming good candidates to investigate phylogenetic relationships among closely related taxa (Gielly and Taberlet, 1994; Gielly et al., 1996). This will permit acquiring a comprehensive view of the differences within the genotypes under study.

As done with PCR-RFLP, a DNA aliquot was used on a PCR reaction. For both *rcbL-a* and *trnH-psbA* regions the PCR amplification was identical, although each had its own site-specific primer. After an initial denaturation step of three minutes at 94ºC, the PCR consisted of 35 cycles with 30 seconds at 94ºC, an annealing phase of 30 seconds at 53ºC and an amplification phase of one minute at 72ºC, followed by a final elongation step of 10 minutes at 72ºC.

Afterwards, as for the other PCR products and DNA samples, an electrophoresis was made to verify the success of the amplification. Subsequently, PCR products were purified prior to sequencing with the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) following the manufacture's protocol. And again, the purified PCR products were controlled on an electrophoresis in order to ascertain its quantity for successive sequencing. From each purified sample, an aliquot was used, according to its concentration; the DNA template was dissolved in Tris-HCl, pH 8.0, 10mM that was added up to 20 µl total volume. Samples and primers were sent to Eurofins MGW Operon (www.mwg-biotech.com), company that processed the sequencing reactions.

Sequences consisted on one sample of *Picconia azorica* from each Azorean island where it's present

and one sample of *Picconia excelsa* from Madeira and from Canaries Archipelagos. Additionally samples of other Oleaceae were sequenced (see Annex II).

2.3.3 Microsatellites

All populations sampled were analyzed at the "Consiglio Nazionale delle Ricerche" (C.N.R.), Protection Plant Institute laboratory (Istituto per la Protezione delle Piante – I.P.V.) at Florence, within a scientific collaboration with Dr. G. G. Vendramin and Dr. Silvia Fineschi.

Microsatellites (simple sequences repeats, SSRs or tandem repeats) are powerful markers in population genetics. The reason for such is their codominant inheritance in combination with a large number of alleles, enabling in forest tree populations the study of contemporary gene flow as mediated by pollen and seeds (Schueler et al., 2003). These markers consist of small sequences of mono - di and trinucleotides. Microsatellites from organelle DNA are quite stable and are vastly used in inter- and intra-specific studies.

The objective of these microsatellite analyses was to evaluate the variability of eight loci of chloroplastic DNA, which allowed the DNA amplification with eight specific primers. The chloroplast microsatellite markers (cpSSRs) used: ccmp1, ccmp2, ccmp5, ccmp6, ccmp7, ccmp10, cmcs8 and cmcs13 (Weising and Gardner, 1999; Sebastiani et al., 2004). Polymerase chain reactions (PCRs) were performed in 12.5 µl containing 10 ng of template DNA, 1x PCR reaction buffer (Promega), 200 µM of each dNTPs, 0.5 U *Taq* polymerase (Go*Taq*, Promega), 1.5 mM MgCl2, and 0.4 µM of each primer. The 5' ends of the forward primers were labelled with FAM, HEX or TMR, in order to allow multiplexing of PCR products. All cpSSR markers were amplified on an Eppendorf thermal cycler (Mastercycler), following a touchdown PCR protocol: 3 min at 94°C, 10 touchdown cycles of 94°C 30s, 60°C 30s (-1°C/cycle), 72°C 30s; 27 cycles of 94°C 30s, 50°C 30s, 72°C 30s and a final extension at 72°C 10 min. The fluorescently labelled PCR products were separated by capillary electrophoresis, with a 400 bp size standard, using a megabace (GE Healthcare) automatic sequencer. Alleles were sized using fragment profiler version 1.2 (GE

Healthcare). Amplified fragments of the polymorphic microsatellite regions were sequenced to verify variation in the SSR stretches.

2.4 Data analysis

Once obtained the DNA sequence, it is necessary to study the functional and structural information encoded in the sequence. One method for sequence comparison, which allows evaluating relationships between homologous sequences has important implications in phylogenetic analysis, is sequence alignment. "Sequence alignment is the procedure of comparing two (pairwise) or more (multiple sequence alignment) sequences by searching for a series of individual characters or character patterns that are the same in order in the sequences" (W.-C. Liew et al., 2005). In sum, it allows to determine if two sequences display sufficient similarity such that two genes share a common evolutionary history (Schuler, 2001). In this study, nucleotide sequences were aligned using Clustal W (Thompson et al., 1994) and manually corrected with BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). *Trnh-psbA* and *rbcL* sequenced regions were analyzed separately and then combined to increase the number of informative characters. Phylogenetic trees for the two regions and the combined set were estimated using PAUP* beta version 4.0b10 (Swofford, 2002). The evolution models, for single and combined plastid regions, were selected by ModelTest v3.7 (Posada and Crandall, 1998) based on Hierarchical Likelihood Ratio Test (HLTR). The selected model was F81+I+G (Felsenstein, 1981). Model parameters were then imported into PAUP, and the heuristic searches were executed to construct a Maximum Likelihood tree. Bootstrap resampling was conducted using ML with 100 replicates. We also performed, for the trnH-psbA fragment, a likelihood ratio test (LRT) for comparing two hypothesis (clock model versus no-clock model) using PAUP. The statistical test [Likelihood ratio = 2 * (lnL1– lnL0)] with L0 representing the probability of the null hypothesis and number of degree of freedom n-2 (with n being the number of taxa), was calculated with LRT calculator Mode (option – l) implemented in ModelTest v3.7 (Ratio= 30.497070, this is not significant at the alpha level of 0.0100). On the basis of the hypothesis of a molecular clock, the divergence time (T) between two lineages

was estimated using the sequence divergence and the average rate of nucleotide substitutions by calculating T=DXY/2r where r= 0.8 ± 0.04 x 10–9 mutations per site per year in chloroplast intergenic regions (Yamane et al., 2006).

A matrix with the PCR-RFLPs bands was obtained based on the band dimensions and their presence/absense, which was related to each individual/population with each combination primer/enzyme. PCR-RFLPs and size scores for the polymorphic cpSSR fragments were combined in order to derive the chloroplast haplotype for each individual: chloroplast DNA does not experience recombination and consequently can be considered as a single locus.

Populations analyzed were then regrouped in haplotypes based on the uniformity of the polymorphisms compared, distinguished and assigned different colors in a map, with the support of the GPS coordinates. The software Permut (Pons and Petit, 1996) was used to compute genetic diversity (h_S, h_T, v_S, v_T) and differentiation among populations (G_{ST}, N_{ST}). The software GENALEX 6 (Peakall and Smouse, 2006) was used to measure the molecular analysis of variance (AMOVA), and hierarchical testing of population structure, which estimates the distribution of genetic diversity within and among populations. SAMOVA analysis (Spatial Analysis of MOlecular VAriance; Dupanloup et al., 2002) was used to identify groups of populations that are geographically homogenous and maximally differentiated from each other. SAMOVA searches for K groups of adjacent populations to maximize among-group genetic variation (F_{ct}). The underlying assumption is that maximum genetic differentiation among K groups best circumscribes spatially homogeneous population clusters. We tested for K = 2–10, selecting 100 independent simulated annealing processes (Dupanloup et al., 2002). Statistical parsimony was used to reconstruct phylogenetic relationships between haplotypes (TCS, version 1.06; Clement et al., 2000) by combining PCR-RFLP and microsatellite data.

2.5 Germination trials

Picconia azorica fruit is a drupe, with a fleshy outer layer surrounding the oblong seed enclosed by a woody endocarp. This species seeds are orthodox dormant. It presents an exogenous dormancy type, namely a mechanical dormancy that is imposed by the seed-enclosing structure that is too strong to allow the dormant embryo to expand during germination. This type of primary dormancy can be broken with the removal of the fruit endocarp and with warm or cold stratification. Due to the seeds dormancy, pre-treatments to break it are a pre-requisite to allow a more complete and homogeneous germination to begin.

No germination protocol is known for *P. azorica*, reason for which pre-treatments used were the ones that commonly are applied to *Oleaceae* species with a drupe, namely the *Olea europaea* L. *subps. europaea* described by Piotto and Di Noi (2001).

Fruits were collected from three populations: Varadouro (Faial Island), S.ta Luzia (Pico Island) and Serreta (Terceira Island) in the summer of 2008.

The research laboratories of the University of the Azores at the Agricultural Sciences Department in Terceira Island were used to accomplish these experiments within a scientific collaboration with the University of Tuscia. Each seed lot was properly identified and subsequently characterized. Eight replicates, each of 100 seeds were counted randomly and weighted to determine the parameters weight of 1000 fruits and weight of 1000 seeds. Additionally, seed lots were individually characterized for the number of pure fruits per 100 grams (g). Finally, for 100 fruits of each provenance it was measured the length and diameter of the fruit and its respective seed.

Fruits prior to be subjected to any pre-treatment had to be clean, and so it was necessary to remove the fruit pulp. With that purpose, they were soaked in boiling water and left there as the water-cooled for a maximum of 48 h. The fruit tissue became easier to remove, nevertheless to completely achieve an adequate depulping, fruits had to be manually pressed and rubbed in a net surface. It is necessary to remove all fragments of the pulp from around the stone, as it can be the source for fungi development. This step with *P. azorica* fruits was particularly labour intensive. The following step was to air dry the seeds, by leaving them in an aerated place at environmental conditions.

A biochemical test to ascertain the seeds viability – the topographical tetrazolium test, was done according to the ISTA guidelines (ISTA, 2004) to Pico, Faial and Terceira islands provenances. The test was carried out on a sub-sample of 100 seeds.

Finally, seeds from Terceira provenance were prepared, disinfected and allocated to the diverse pre-treatments to break their dormancy. The disinfection procedure consisted in soaking the seeds 10 m in a 30% sodium hypochlorite solution. Subsequently seeds were washed abundantly with distilled water. Pre-treatments consisted in chemical scarification as referred to other *Oleaceae* species, namely *Olea cuspidata* and *Olea europaea* (Piotto and Di Noi, 2001).

Five treatments were applied: (1) soaking for 10 m in sulphuric acid at 98%; (2) soaking for 15 m in sulphuric acid at 98%; (3) soaking for 10 m in sodium hydroxide at 1%; (4) soaking for 20 m in sodium hydroxide at 1% and (5) soaking in a 3% solution of Na_2CO_3 for 5 h, followed by immersion in a 0.5% solution of KOH for 6 h. Subsequently, all seeds were washed under running tap water for 2h and finally washed abundantly with distilled water four times.

Germination tests were carried out with four replicates of 100 seeds per treatment. Seeds were placed in autoclaved Petri dishes having filter paper as growth medium. As Petri dishes were parafilm sealed. Seeds from each treatment and control were moistened with distilled water, sprayed with Benlate ($1gL^{-1}$ - active principle benomyl) and transferred to a growth chamber. Observations were made every 2/3 days to detect fungal activity.

Environmental conditions in the growth chambers tend to be optimal. Petri dishes were allocated in a growth chamber with an eighteen hours photoperiod and 20±2ºC (20.000 LX light intensity) and 75% humidity.

2.5.1 Statistical analysis

Data was analysed with ANOVA two-way with the Statistical package StatPlus from Analyst Soft, version 5.6.0. Means were compared using Scheffé test as described in Steel and Torrie (1980). All comparisons between means were made for $P>0.05$.

2.6 Wood characterization (anatomical and technological)

2.6.1 Material collection

At the moment it is impossible to harvest *Picconia azorica* trees as the species is legally protected. Therefore, wood samples (three discs and boards from each plant) were collected from three individuals, which were harvested some years ago in Terceira Island. The plants were growing in a stand with 15 trees/ha (the typical *P. azorica* population density ranges from 4-5 to 30 trees/ha). The sampled trees diameter varied between 10 to 32 cm; trees height ranged from 4 to 7 meters. The site was characterized by a very rocky/stony soil. Samples examined were in a very good conservation state.

2.6.2 Macroscopic observations

First observations were made at the naked eye to describe the most evident macroscopic characteristics, namely colour, differentiation between sapwood and heartwood and the presence of visible rays and growth rings.

The wood colour was objectively characterised with a Chroma meter Minolta CR-100. For each point, three measurements were taken along the radial direction of the disks and along the tangential surface of the boards. The average value was assumed as the point value.

The parameters considered for all the measurements were:

- L * a * b * (L indicates brightness, a * and b *, the chromaticity space - CIElab colorimetric reference). The values a * and b * can be positive or negative: the parameter a * indicates the direction of the colour ranging from red (positive a *) to green (negative a *) and b * indicates the direction of the colour ranging from yellow (b * positive) to blue (negative b *);

- lD: reflectance curve of the dominant wavelength of the measured point (dominant wavelength of the reflectance curve of the measured point);
- P%: purity percentage (having white as reference).

2.6.3. Microscopic observations

All wood samples were investigated using both light microscope and scanning electronic microscope (SEM).

For light microscopy observations thin sections (from cubic samples wide 5–15 mm) were prepared using a sliding microtome (Reichert Yung). Microtome sections, thick 20-25 μm, in the three anatomical directions were dissected from specimens of mature wood in different cubes in the direction from the pit to bark. After which were stained with safranine (1% in alcohol 50%) to highlight the presence of lignin. Subsequently to staining, sections were washed with distilled water, dehydrated with ethanol and mounted on slides with Canada balsam.

Observations were carried out first by means of a stereomicroscope Wild M420, afterwards with a microscope Reichert–Jung Polivar 100. Photographs were taken with a Moticam 2500 – 5,0 MPixel digital camera and analyzed by the software Motic Image PLUS 2.0 ML.

Additional samples were fixed with 4% paraformaldehyde + 5% glutaraldehyde, pH 7.2 in 0.1 M cacodylate buffer for 1h at 4°C (Karnovsky, 1965) to be scanned with electron microscope (SEM). After rinsing overnight in the same buffer, they were post-fixed in cacodylate-buffered 1% osmium tetroxide for 1 h. Specimens were dehydrated in a graded aceton series. They were then dried by the critical point method using CO2 in a Balzers Union CPD 020, sputter-coated with gold in a Balzers MED 010 unit, and observed with a JEOL JSM 5200 electron microscope (Fig. 2.4).

Figure 2.4 - *Picconia azorica* wood samples sputter-coated with gold.

2.6.4 Maceration analysis

A maceration technique was applied to separate which individual cell element, according to the protocol of Chaffey (2002) and also with some knowledge of the *Olea europea* wood. Hence, to analyse each single cellular element, the wood samples were prepared in the longitudinal direction, two centimetres long and just about some millimetres wide. Subsequently were immersed in a 50 – 50% solution of oxygen peroxide and acetic acid 30 volume in a closed tube. They were left at room temperature for about 48 h to reach bleaching. Afterwards were water-bathed at 60ºC for another 48h period. Once the wood bleaching was reached, the maceration solution was decanted carefully to avoid damaging the samples and washed several times with distilled water; washing out any remaining macerating solution. Then, a saturated solution of sodium chloride (NaCl) was added to buffer the acidity excess. After this, it was again washed with distilled water and the tube was filled up to half with distilled water. It was then agitated vigorously to disaggregate the xylem. Some drops of the wood-pulp mixture were collocated in a quartz microscope slide covered with a quartz cover slip.

The identification of the cellular elements was conducted at a Zeiss Axioskop microscope. Measurements were made with the system Axio Vision AC, in the "Michele Cordaro" Diagnose Laboratory from the "Facolta di Conservazione dei Beni Culturali".

The anatomical descriptions have been conducted following the major protocols of IAWA (Wheeler et al., 1989) on this field of research.

2.6.5 Technological analyses

Following an air-equalizing process in an unheated room to reach 12% moisture content, clear specimens were cut from boards and disks according to the Italian standards (Berti, 1979). The parameters considered are as follows:

1 – Wood density ($g.cm^{-3}$, at 12 % moisture content and at dry conditions) was determined on a

set of 20x20x30 mm specimens, representing both heartwood and sapwood, according to the UNI ISO 3131 standard (1985).

2 – Shrinkage: Radial, tangential and volumetric total shrinkage was ascertained on a set of 20x20x30 mm specimens, which were dissected according to the UNI ISO 4469 (1985) and the UNI ISO 4858 (1988).

3 – High heating value and ash content: the Higher Heating Value (HHV) was assessed on random samples with 0% moisture content, by means of an adiabatic calorimeter (Parr, Model 6200) (Canagaratna and Witt 1988). Ashes were estimated according to Miller (1998).

4 – Compression and bending strength: the compression strength was determined on a set of 20x20x30 mm specimens (12 % moisture content) according to UNI ISO 3787 (1985), bending strength was evaluated on an identical set of samples according to UNI ISO 3133 (1985).

5 – Quality Value, the coefficient or static quality factor is a coefficient that allows understanding the wood quality of a given species, relating the compression resistance characteristics and the volumic mass. This coefficient was obtained calculating the relation between the resistance and the axial compression (expressed in $kg.cm^{-2}$) and the volumic mass ($g.cm^{-3}$ multiplied by 100) at 12% moisture content.

2.6.6 Statistical analysis

The various samples measurements taken have been subjected to a statistical analysis with the S-plus package v.11.0.

Taxus baccata L.

2.7 Field trips

Two field trips were made to Pico Island, in late summer 2007 and mid-spring 2008. Altitude and GPS coordinates of the exact location of each yew were obtained using a Garmin GPSMAP® 76S. Slope and aspect were obtained from Digital Elevation Model (DEM) (CLIMAAT, 2009) using ESRI ArcMap 9.1 software.

Stem diameter (Diameter at Breast Height – DBH) was measured with a caliper at 1.30 m, and height was assessed with an electronic clinometer (Haglöf, Sweden) for each tree. Test areas of 10 meters radius were set to search for natural regeneration around each individual. Morphology and sex were visually assessed, as well as a phytosanitary visual survey to ascertain tree health status.

2.8 Historical research and site description

Historical *Taxus baccata* occurrence in the Azores was assessed by a critical review of the literature. The first investigations were restricted to Pico Island, more specifically to the Mountain Plateau (Dias, 1996; Carqueijeiro et al., 2005). Pico Island stretches from 38°23‘ to 38°34‘ north latitude and from 28°01‘ to 28°33‘ west longitude, lying roughly WNW to ESE; its climate characterization was determined using the CIELO Model (Azevedo, 1996 and 1999; Arruda, 2004). Its parameters of 761 m altitude, annual mean values for temperature of roughly 12.5°C, rainfall of 2465 mm, wind of 45 km h^{-1} and 96% relative humidity, characterize it as a humid type climate (Oro-Oceanic bioclimatic unit). Azorean soils are mainly classified as Andisols (Pinheiro, 2007), as a consequence of the islands volcanic origin. High permeability, high organic matter, and high potassium content due to the basaltic bedrock characterize these soils. High infiltration capacity and good percentage of organic matter contribute to a low risk of erosion. Natural vegetation is mainly found on Incipient or Organic Soils, as the most developed ones are used for agricultural production (SRAM, 2005). Pico Mountain is a majestic, 2351m high, stratovolcano (Carqueijeiro et al., 2005) and Portugal‘s

highest peak. The presence of yew was assessed in a Site of Community Interest (SCI) area within Pico Mountain Plateau, where the main soils are only suitable for permanent natural vegetation (SRAM, 2005). The last eruption occurred in 1718. Some areas are classified as very-humid ones (> 6000 mm). This SCI includes the largest and finest core of endemic woodlands, with the most developed Azorean Laurisilva formations. The mountain plateau slope ranges between 0 and 10%, with a north and east aspect. Strong winds are quite frequent, with clearly visible effects on the natural vegetation, limiting their growth in addition to the effects of poorly developed soils and waterlogging. The major threats to this area are soil use conversion, exotic species plantations, road construction, tourism and water eutrophization as well as the proliferation of invasive alien species.

2.9 Plant characterization and leaf morphology observations

A first visual assessment of the plants prompted more in-depth leaf morphometric analysis, and comparison with other provenances and data available in literature.

Leaf samples were collected, labelled, placed in plastic bags, and sent by airmail to DAF Laboratory in Italy, where they were stored at -20ºC.

Leaf morphometric analysis was performed using the Digimizer image analysis software (MedCalc, 2009) on three plants from the Azores, and compared with three plants from a *T. baccata* natural population in Carpineto, Central Italy. A total of six trees were scanned (N=100) and for each individual leaf, the length, width, area and the length/width ratio were calculated with the image analyzer. Statistical analysis was performed using S-plus package v.11.0.

Leaf morphology was also observed using a stereomicroscope (Wild M420) at 16 to 20°C resolution. Photographs were taken with a Moticam 2500 – 5.0 MPixel camera, and images were analysed using the Motic Image PLUS 2.0 ML software in order to characterize the leaves' abaxial surfaces of each population, recording stomatal row numbers, stomata numbers and density.

Three yew provenances were also observed by electron microscopy to study stomatal morphology and dimensions: Azores, Carpineto (Latium, Central Italy), and Rosello (Abruzzi, Central Italy).

Leaves from each sample were fixed with 4% paraformaldehyde + 5% glutaraldehyde, pH 7.2 in 0.1 M cacodylate buffer for 1 h at 4°C (Karnovsky, 1965) to be observed using a scanning electron microscope (SEM). After rinsing overnight in the same buffer, they were post-fixed in cacodylate-buffered 1% osmium tetroxide for 1 h. Specimens were dehydrated in a graded acetone series. They were then dried by the critical point method using CO_2 in a Balzers Union CPD 020, sputter-coated with gold in a Balzers MED 010 unit, and observed with a JEOL JSM 5200 electron microscope (see Fig. 2.5).

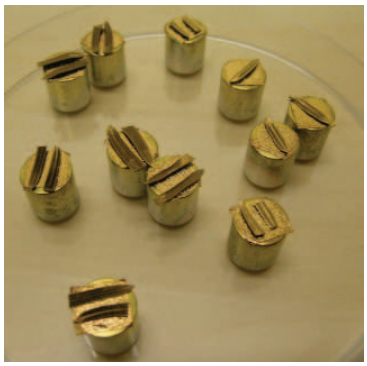


Figure 2.5 - *Taxus baccata* L. leaves sputter-coated with gold.

A total of 50 stomata, randomly chosen from each provenance, were appraised and measured using Digimizer (MedCalc, 2009). The yew's stomatal apparatus is quite different from that typical of other Gymnosperms, and requires a specialized measurement approach, such as the one recently developed by Sweeney (2004). In the present work, only a first characterization of the species was carried out, measuring the length of the "stomatal pore" according to Dempsey and Hook (2000). Finally, for a qualitative study of leaf anatomy, transverse sections of leaves were observed by light microscopy (Reichert–Jung Polivar), after fixation of the material in a Karnovsky solution, embedding in an Epon and Araldite mixture, and staining with Toluidine blue.

2.10 Molecular analysis

A phylogeographical investigation used molecular analysis to estimate the relative affinity of the yews from the Azores with some representatives of adjacent areas (Mediterranean Basin, north and central Europe) (see Table 2.4)

Table 2.4 - Taxon and sample provenance. Note: samples were collected from natural populations and five individuals were sampled in Pico Island.

Taxon	Sample Provenance
Taxus baccata L.	Italy - Lazio
Taxus baccata L.	Italy - Sardinia
Taxus baccata L.	Italy - Apulia
Taxus baccata L.	Austria
Taxus baccata L.	Morocco
Taxus baccata L.	Wales
Taxus baccata L.	Algeria
Taxus baccata L.	Azores Archipelago – Pico Island

All living yew trees from the Azores were sampled; other yew provenances were collected in the wild and used for comparison (Morocco, Central and Southern Italy, Algeria, Wales - UK and Austria). Leaves were carefully chosen to remove damaged tissues, and lyophilized in a FreeZone 2.5 L (Labconco). The lyophilized material was reduced to a powder by using a mortar and pestle and sterile quartz sand (silicon dioxide, Sigma S9887). Total genomic DNAs were extracted individually using the DNeasy Plant minikit (QIAGEN), following the manufacturer's instructions.

Firstly several primers were tried out to evaluate their efficiency. Hence, four primers were screened, one chloroplastic (*trnS–trnQ*) and three nuclear, the taxadiene synthase (TS), the 10-deacetylbaccatin-III-10β-*o*-acetyltransferase (DBAT) and the 18S rDNA. Between the four, the primer that demonstrated to be the most efficient was the chloroplastic one, which was therefore the one used to carry out the genetic investigations.

Genetic variation of the Azorean *T. baccata* provenance was assessed through sequence analysis of a specific plastid DNA (cpDNA) region. Chloroplast *trnS–trnQ* intergenic spacer is located in

the large single-copy region of the plastid genome, between *trnS* and *trnQ* genes. This marker was used for the first time to evaluate relationships within *Taxus* for comparison with morphology-based classifications and to geographical distribution by Hao et al., (2008), they found this fragment able to discriminate among 14 Old World and New World *Taxus* species. The primers used for amplification of the *trnS– trnQ* spacer region were designed after Hao et al., (2008), based on selecting the most variable region in the spacer of a *T. baccata* SQ sequence (GenBank acc. no. EF017309), located between 225 and 901 bp: GGAATAGATCATCAATGTTTGCATC (forward) and TGCCAATTATACCTTTGTTCTTTTT (reverse). The PCR reaction mix contained 50 ng of DNA from each sample, 2 μM of each primer and a puRe Taq Ready-To-Go PCR bead (GE Healthcare) containing enzyme, reaction buffer, nucleotides and 1.5 mM $MgCl_2$, in a final volume of 25 μl. The following PCR conditions were used: 1 min. at 94° (denaturation), 30 sec. at 53° (annealing), and 30 sec. at 72° (polimerization) for 35 cycles.

Distinct single-banded fragments were purified and directly sequenced in both directions by using the amplification primers. Cycle Sequencing and the BigDye Terminator Ready Reaction Kit (Applied Biosystems) were used. Data were collected on an ABI Prism 373A automated gel reader. The resulting sequences were further checked by eye with the CHROMAS 2.3 software (www.technelysium.com.au). A BLAST (Altschul et al., 1997) search was performed to exclude the sequencing of any contaminant organism.

Relationships within *Taxus* were established by use of *trnS–trnQ* DNA sequences available in the NCBI Database, including two additional *T. baccata* sequences one from Canada and another from the U.K. (see Table 2.5). Other species and provenances in this Database were not accessible, precluding any comparison.

Table 2.5 - Taxon, sample provenance and NCBI acession number. Note: Taxus x media = *Taxus media* = *Taxus baccata* x *Taxus cuspidata*; *Taxus x hunnewelliana* = *Taxus canadensis* x *Taxus cuspidata*.

Taxon	Sample Provenance	Accession Number
Taxus baccata L.	U.K	EF017309.1
Taxus baccata L.	Canada	EU107160.1
Taxus fuana Nan Li & R.R. Mill	unknown	EU107158.1
Taxus fuana Nan Li & R.R. Mill	unknown	EU107146.1
Taxus wallichiana var. *chinensis* Zucc.	unknown	EU107157.1
Taxus wallichiana var. *chinensis* Zucc.	unknown	DQ888590.1
Taxus x media	China	DQ888587.1
Taxus x hunnewelliana	Canada	EF017313.1
Taxus cuspidata Siebold & Zucc.	Japan	EU107156.1
Taxus cuspidata Siebold & Zucc	China	DQ888591.1
Taxus brevifolia Nutt.	China	EU107162.1
Taxus sumatrana (Miquel) de Laub.	unknown	EU107148.1
Taxus globosa Schltdl.	Mexico	EU107151.1
Taxus floridiana Nutt. ex Chapm.	U.S.A.	EU107149.1
Taxus canadensis Marshall	Canada	EF017308.1
Torreya nucifera (L.) Siebold & Zucc.	Japan	EU107154

The sequences were aligned using Clustal W (Thompson, 1994) and manually corrected with BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Polymorphism of cpDNA was explored using DnaSP4.5 (Rozas et al., 2003). The distance matrix was built to estimate evolutionary divergence between sequences using the Kimura 2-parameter method in MEGA4 (Kimura, 1980). Phylogenetic trees were reconstructed in Mega4 (Tamura et al., 2007) using the neighbour-joining algorithm with the Kimura 2P model of nucleotide substitutions; 1000 bootstrap replicates were performed to estimate clade robustness.

2.11 Vegetative propagation

Taxus baccata can be multiplied by sexual (seeds) and asexual (cuttings) methods (Martí, 2007). However, in Pico in none of the three field trips was possible to observe fruits.

The best period for yew propagation is during the dormant season, late fall to late winter. Unfortunately it was not possible to collect the plant material to do such trials in this period. As a result, plant material was collected in July's 2009 last week, from two individuals from Pico Island. Properly conditioned in humid conditions the plant material was transported to Terceira Island, where experiments were set, without regarding from which individual were they originated due to plant material scarcity.

Yew is a very slow to root species therefore several specific techniques were employed to "help" the root formation.

Plant material was trimmed to allow cuttings of about 10-15 cm, as it was not possible to obtain bigger cuttings. All cuttings were wounded at their base as it: (1) increases the quantity and quality of roots and; (2) allows an increase in water uptake by the cuttings from the rooting medium (McDonald, 1986). Subsequently, cuttings were immersed in distilled water for 24h. After which all were subjected to a rooting hormone treatment with the auxin IBA (indole-3-butyric acid) in two concentrations 2.000 ppm (Bellarosa, 2003) and 5.000 ppm (Hartman et al., 1996). Each cutting, to a depth of 5-10mm, was dipped for 20 seconds in the IBA and cuttings dipped in distilled water represented the control group. Two growth media were tested, 50% pure peat + 50% perlite and Jiffy-7 Forestry.

Observations were made daily to evaluate the seedlings vitality and survival. Irrigation, always with distilled water, was carried out by capillarity and field capacity was obtained. Irrigation by aspersion was also employed. Cuttings were allocated under a shading mash at field conditions, were climatic conditions were not controlled.

A total of 300 cuttings were equally allocated to the combination of two IBA concentrations, two growth media and controls, with the same volume of 15 c.c. (19 mm diam. x 45 mm h x 18 mm plug hole).

2.11.1 Statistical analysis

Data registered consisted on survival and rooting percentage. Data was analysed with ANOVA one way with the Statistical package StatPlus from Analyst Soft, version 5.6.0. Means were compared using Scheffé test as described in Steel and Torrie (1980). All comparisons between means were made for $P>0.05$.

3. RESULTS

Picconia azorica (Tutin) Knobl.

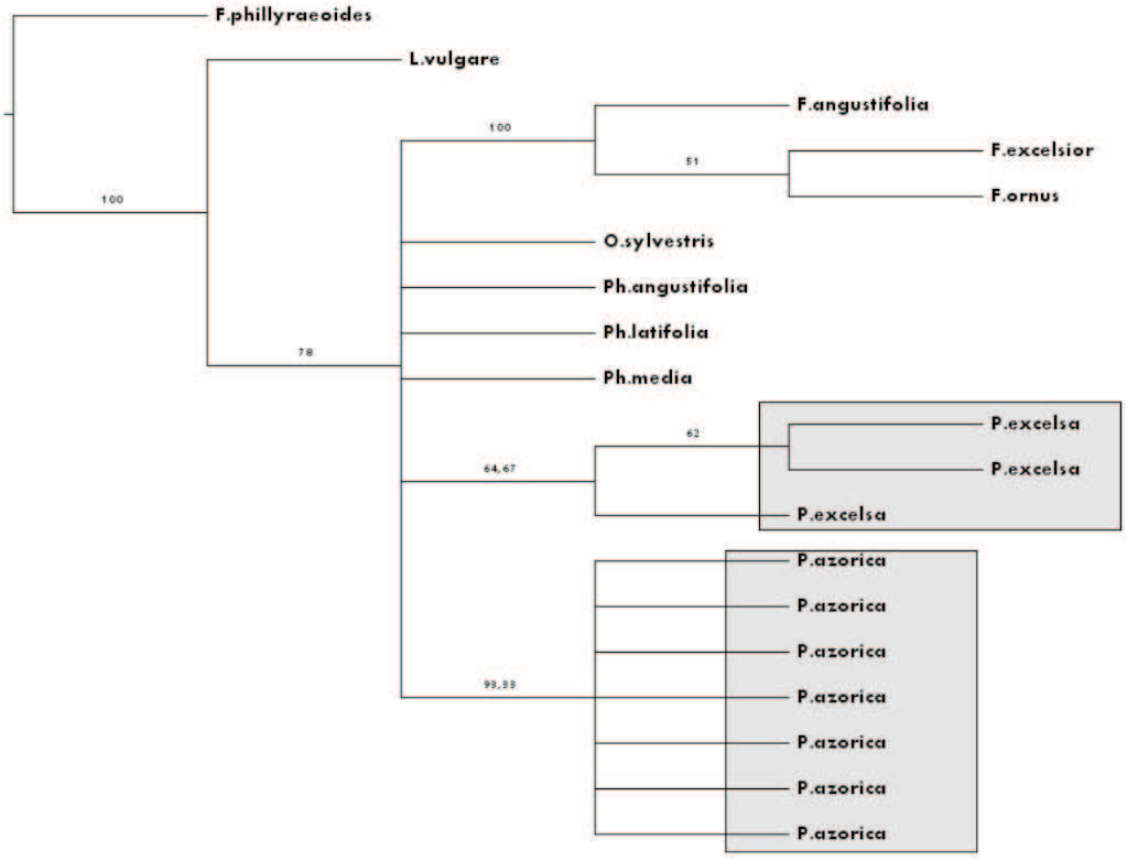
3.1 Phylogenetic analysis

Sequences of *rbcL* and *trnH-psbA* obtained from every sample were deposited in the GenBank under accession numbers FJ862055-FJ862064, EU854410-EU854427, GU120315-GU120325 (see Annex III). Resulting ClustalW multialignment was 670 bp and 600 bp for *rbcL* and *trnH-psbA* regions, respectively, and showed eight segregating sites (four synonymous and four replacement changes) in *rbcL* and 58 polymorphic sites with 12 indels in *trnH-psbA*. The two fragments (*rbcL* and *trnH-psbA*) were then concatenated and the final multialignment was 1270 bp long, with 67 polymorphic sites for a total of 70 mutations.

Molecular data from the *rbcL* gene region showed all identical sequences in *P. azorica* and *P. excelsa*, and suggested a deep relationship with *Phillyrea* spp. and *Ligustrum vulgare*, that are respectively split by one non-synonymous and one synonymous base change. The *trnH-psbA* non-coding region provided a greater level of variation. *Picconia* was resolved in two lineages, a first one grouping *P. azorica* with all identical sequences, and a second one with *P. excelsa* displaying a different sequence for every individual analysed. The net average number of nucleotide differences between *P. azorica* and *P. excelsa* sequences was 3.667. Kimura 2-p distances (Kimura, 1980) ranged from 0.005 (between *Phillyrea* and *P. excelsa*) to 0.87 (between *Fontanesia* and *Fraxinus*), with a value of 0.006 between *P. excelsa* and *P. azorica*. A search in the Genbank for related sequences retrieved only *rbcL* gene regions of *Osmanthus americanus* (DQ673311), *Nestegis sandwicensis* (DQ673305) and *Notelaea ovata* (DQ673306), and displayed *Notelaea ovata* sharing the highest sequence identity with *Phillyrea* spp., along with the same nucleotide polymorphism separating this group of species from *Picconia*. *Osmanthus* and *Nestegis* sequences were differentiated from those of *Picconia* by four and two substitutions, respectively.

No *trnH-psbA* sequences were available for further comparisons. Figure 3.1a,b shows the majority rule consensus tree and the resulting phylogram generated with the two combined fragments. The phylogenetic reconstruction reflects the molecular phylogeny of Oleaceae proposed by Wallambert and Albert (2000) confirming the ancestral divergence of *Fontanesia* (with bootstrap=100%) and *Ligustrum*, and the positions of *Fraxinus* and *Olea* relative to the ingroup (*Picconia* and *Phillyrea*). Phylogram topology suggests monophily of genus *Picconia* with *Phillyrea* as sister taxon. The sister relationship is not strongly supported (bootstrap <50%) as previously evidenced by Wallambert and Albert (2000) by use of two other non-coding chloroplast regions (*rps16* and *trnL*). *P. azorica* and *P. excelsa* were resolved in two distinct clades, to confirm a significant inter-taxa genetic variation and supporting their systematic ranking as separate species.

On the basis of the hypothesis of a molecular clock, the divergence time between *P. excelsa* and *P. azorica* lineages was estimated to be 4.9 ± 0.2 million years (Myr).



a)

b)

Figure 3.1 - a) Maximum-likelihood tree of *Picconia* and Mediterranean Oleaceae based on two cpDNA regions (rbcL and trnH-psbA). One sample of *P. azorica* per each island and three Madeiran and Canarian samples of *P. excelsa* were analysed. *Fontanesia phillyraeoides* was used as outgroup to root the tree; b) Majority-rule consensus tree; bootstrap values are indicated on tree branches (value less than 50% for 100 replicates are not shown).

3.2 Genetic diversity and differentiation

PCR-RFLP analysis yielded 16 polymorphic bands (two alleles each) in *Picconia*, out of 54 total. Combinations TF/HinfI and AS/HinfI produced six polymorphic bands each; SR/HinfI and DT/TaqI produced two polymorphic bands each, whereas FV/TaqI and CD/TaqI resulted monomorphic. Two examples of the PCR-RFLP gels can be seen in Fig.3.2.

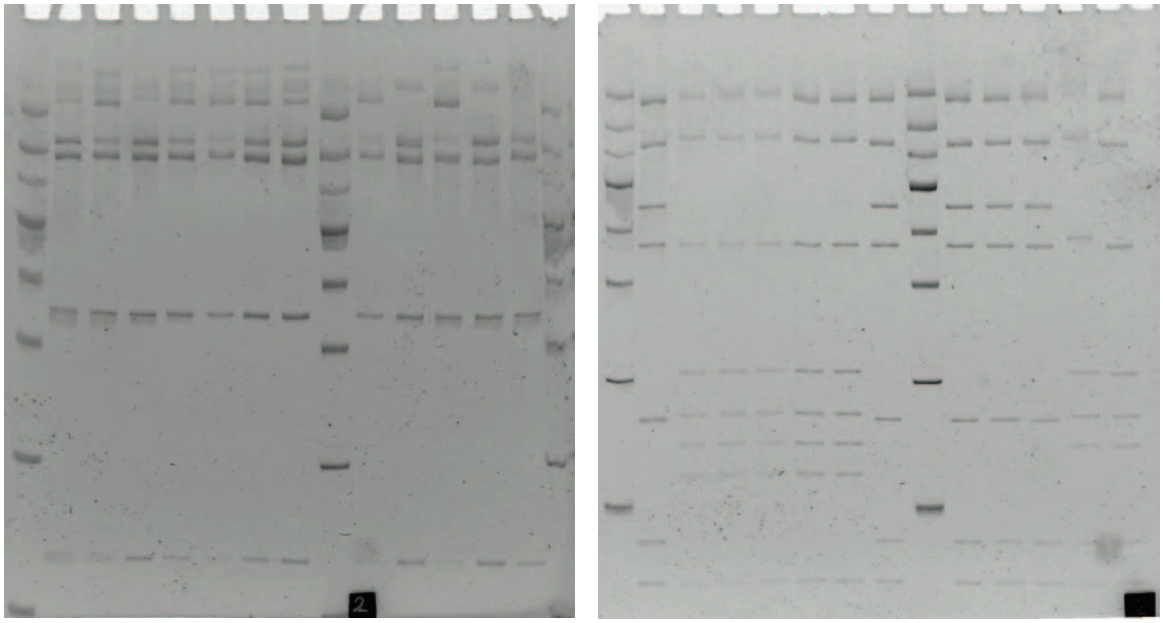
a)  b)

Figure 3.2 - Acrylamide gel ethidium bromide stained with samples from the (a) primer DT, first and seventh positions with the Molecular Marker, 5 positions with samples from S.Jorge, 2 from Terceira, 1 from Flores, 2 from Madeira, 1 from Tenerife and 1 from S.Miguel islands. (b) primer TF, first and eighth positions with the Molecular Marker, 1 sample from Terceira, 3 from Madeira, 2 from Tenerife, 1 from Sta. Maria, 1 from S.Miguel, 2 from Terceira, 1 *Olea sylvestris* from Portugal, 1 *Phillyrea angustifolia* from Portugal.

In Table 3.1 can be seen the list of the different fragments originated from the PCR-RFLP technique and the haplotypes identified. Below each haplotype is described:

H1 – *Picconia azorica* (all islands);

H2 – *Picconia excelsa* (M62, sample from Madeira and all samples from Canary Islands);

H3 – *Picconia excelsa* (M64, sample from Madeira);

H4 – *Picconia excelsa* (M63, sample from Madeira);

H5 – *Phillyrea angustifolia* (Portugal and Spain);

H6 – *Phillyrea angustifolia* (Italy);

H7 – *Olea europaea* var. *sylvestris.*

Table 3.1 - Matrix with the PCR-RFLP results. Polymorphism is identified in red as well as the band dimensions (bp).

Bands	**Haplotypes**						
	H1	*H2*	*H3*	*H4*	*H5*	*H6*	*H7*
TF/Hinf 1	480	480	480	480	480	480	480
2	370	370	370	370	370	370	375
3	275	/	/	/	/	/	/
4	240	240	240	240	240	240	245
5	/	155	155	155	155	155	155
6	140	143	143	143	143	143	143
7	/	130	130	130	130	130	130
8	/	120	120	120	/	/	/
9	90	/	/	90	90	90	90
10	75	75	75	75	75	75	75
AS/Hinf1	600	600	600	600	600	600	600
2	450	450	450	450	450	450	450
3	355	355	355	355	355	355	355
4	/	315	315	315	315	315	315
5	/	/	/	312	/	/	/
6	305	307	307	307	307	307	307
7	290	290	290	290	290	/	/
8	/	/	/	260	/	/	/
9	/	/	/	/	/	253	/
10	250	250	250	250	250	250	250
11	242	/	/	/	/	/	/
12	240	240	240	240	240	240	240
13	/	/	/	/	/	/	175
14	130	/	/	130	/	130	130
15	120	120	120	120	120	120	120

16	90	90	90	90	90	90	90
17	80	80	80	80	80	80	80
18	70	70	70	70	70	70	70
DT/Taq1	650	/	650	/	/	/	/
2	600	600	600	600	/	/	/
3	580	580	580	580	/	/	/
4	/	/	/	/	/	/	550
5	530	/	530	/	530	530	530
6	/	/	/	/	520	520	/
7	440	440	440	440	440	440	440
8	400	400	400	400	400	400	400
9	225	225	225	225	225	225	225
10	115	115	115	115	115	115	115
11	90	90	90	90	90	90	90
CD/Taq 1	780	780	780	780	780	780	/
2	700	700	700	700	700	700	700
3	400	400	400	400	400	400	390
4	370	370	370	370	370	370	/
5	365	365	365	365	365	365	/
6	280	280	280	280	280	275	280
7	225	225	225	225	220	225	/
8	/	/	/	/	215	/	215
9	195	195	195	195	195	195	195
10	190	190	190	190	190	190	190
11	175	175	175	175	175	175	175
12	155	155	155	155	155	155	155
13	/	/	/	/	/	/	120
SR/ Hinf 1	/	/	/	/	/	/	530
2	520	520	520	520	/	/	/

3	/	/	/	/	500	500	/
4	370	370	370	370	/	/	/
5	/	/	/	/	365	365	/
6	/	/	/	/	/	/	360
7	/	185	185	185	/	/	/
8	180	/	/	/	180	180	180
9	170	170	170	170	170	170	170
10	165	165	165	165	165	165	165
11	125	125	125	125	125	125	125

Three polymorphic chloroplast microsatellites were also identified: ccmp1 (two alleles), ccmp5 (three alleles), and cscm8 (two alleles). Sequence data confirmed that the detected variation is due to differences in the number of repeats within the microsatellite stretches; in addition, 1-bp substitution was detected in the microsatellite region ccmp1 of *Phillyrea* (sequences deposited in GenBank under accession numbers GU085250-GU085259 – see Annex III).

Under the assumption of unordered haplotypes (Pons and Petit, 1996), the analysis of within population diversity (h_S), total diversity (h_T) and differentiation (G_{ST}) accounted for 0.449, 0.557 and 0.194, respectively. Taking distances among haplotypes into genetic account, the corresponding values for within population diversity (v_S), total diversity (v_T) and differentiation (N_{ST}) with ordered haplotypes (Pons and Petit, 1996) were 0.081, 0.099 and 0.177 respectively.

The AMOVA analysis showed that 90% of total genetic diversity is represented within populations.

3.3 Phylogeography

Congruent with the sequence diversity data, PCR-RFLP alleles clearly differentiated *P. azorica* from *P. excelsa*. No intra-specific diversity was revealed for *P. azorica*, whereas three haplotypes were identified in *P. excelsa*. From the combination of PCR-RFLPs and the three polymorphic

cpSSR we identified six haplotypes in *P. excelsa*, four haplotypes in *Phillyrea* and five haplotypes in *Olea*.

The three polymorphic cpSSR loci allowed the identification of five different haplotypes in *P.azorica* (H1-H5, Tab.3.2). Haplotypes H1, H4 and H5 resulted population-private, each one occurring on different islands: H1 in S.ta Maria, the south-eastern most island, H4 and H5 in the central part of the archipelago (Pico and S. Jorge, respectively) (Figure 3.3). H2 and H3 were the most common haplotypes (44% and 53%, respectively); the latter was detected in all islands, whereas H2 was not present in the eastern islands S. Miguel and S.ta Maria. Five populations out of seventeen were fixed for either H2 or H3. Of the six haplotypes observed in *P. excelsa*, four where identified in Madeira (H6-H9) and two in Tenerife (H10, H11). No haplotype sharing was detected between *P. azorica* and individuals of *P. excelsa*.

Table 3.2 - Description of *P. azorica* haplotypes detected by fragment analysis in three chloroplast DNA loci; *np* number of populations possessing the haplotypes; *nfp* number of populations fixed on one type. Note: microsatellites dimensions are in bp.

Haplotype	CCMP1	CCMP5	CMCS8	Relative frequency	np	nfp
H1	120	101	180	0.01	1	0
H2	130	101	179	0.44	12	2
H3	130	101	180	0.53	15	3
H4	130	103	180	0.01	1	0
H5	130	104	179	0.01	1	0

The statistical parsimony network of the haplotypes is represented in Figure 3.3. H3 had the largest out-group weight (0.47), suggesting greatest antiquity (Castelloe and Templeton, 1994).

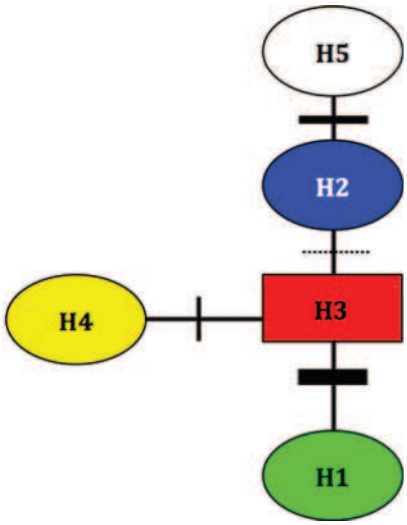


Figure 3.3 – Phylogram (Statistical Parsimony Network) of the chloroplast haplotypes obtained by the TCS analysis at three chloroplast polymorphic loci. Thickness of bars indicates indel of 10, 3, 2 and 1 bp.

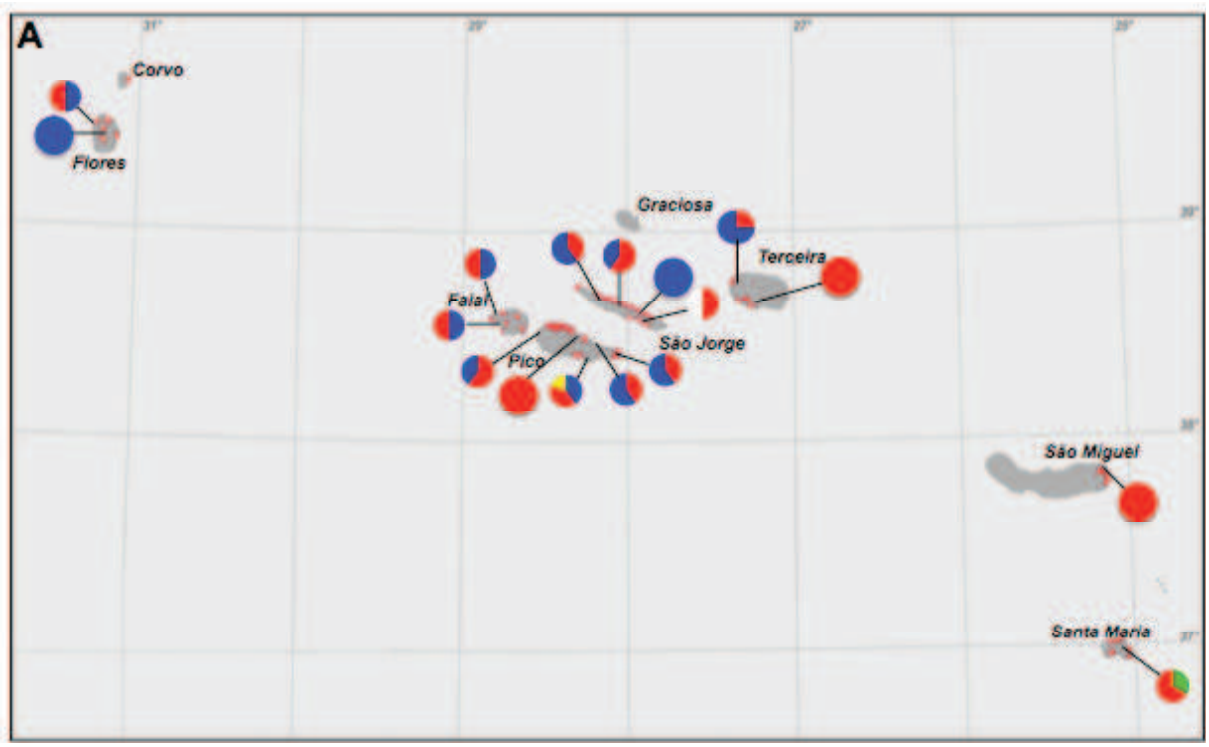


Figure 3.4 - A) Geographic distribution of the chloroplast haplotypes of *Picconia azorica*. Red areas show the species distribution on each island (according to the local forest service the species is now extinct on Corvo).

The coefficient of genetic differentiation for ordered alleles (N_{ST}= 0.18) was not significantly different from G_{ST} (0.19), thus indicating the absence of a phylogeographic structure (Pons and Petit, 1996). The SAMOVA algorithm identified two groups (Figure 3.5) and confirmed the lack of a geographic structure. Most populations, characterised by the presence of H2 and/or H3, were grouped together, with the exception of Valverde (in S.ta Maria island), which formed a separate group.

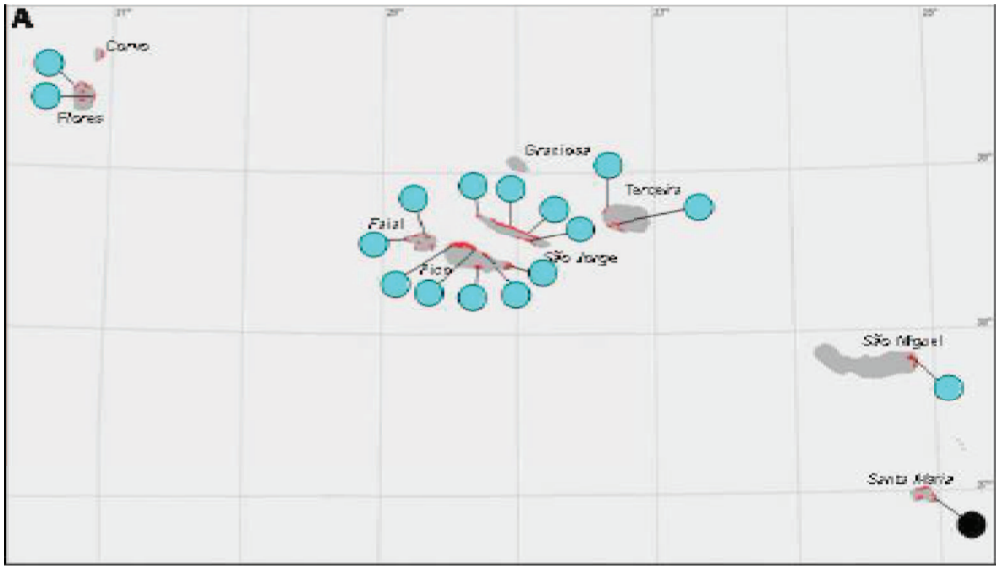


Figure 3.5 - Group structure defined by spatial analysis of molecular variance (SAMOVA). *P. azorica* populations were clustered in order to maximize differentiation among groups. Two groups were defined, clearly separating Valverde population in S.ta Maria to all other populations.

3.4 Germination trials

3.4.1 Seed lots characterization

The number of fruits per kilogram was 1790 for Pico, 1986 for Terceira and 2162 for Faial. Both fruits and seeds from Pico were heavier. The 1000 fruits weight was 565.8 g for Pico, 532.4 for Terceira and 519.8 for Faial. The 100 seeds weight was 163.3 for Pico, 146.6 for Terceira and 137.8 for Faial.

Table 3.3 - Seedlots average values for number of fruits per 1000 and 100 grams, weight of 1000 fruits and 1000 seeds, expressed in grams, for the three Islands (Terceira, Pico and Faial).

Island	No. Fruits /kg	No. Fruits / 100g	Weight 1000 fruits	Weight 1000 seeds
Terceira	1986	182	532.4	146.6
Pico	1790	174	565.8	163.3
Faial	2162	197	519.8	137.8

Table 3.4 – Fruit and seed length and diameter (mm) of the three provenances (Terceira, Pico and Faial islands).

Island	Fruit length	Fruit diameter	Seed length	Seed diameter
Terceira	11.53 [a]	7.89 [a]	7.86 [a]	3.62 [b]
Pico	11.78 [a]	8.00 [a]	7.57 [ab]	3.84 [a]
Faial	11.16 [a]	7.83 [a]	7.27 [b]	3.56 [b]
Standard deviation	0.16	0.08	0.24	0.15

In each column, averages followed by different letters are significantly different ($P<0.05$).

Average fruit and seed length and diameter are summarized in Table 3.4. There is no significant difference in the fruit length and diameter of the three provenances. However, there are significant differences for the seed length between Terceira (7.86 mm) and Faial (7.27 mm). There are differences in diameter between the seeds of Pico (3.84 mm) and the seeds of Terceira and Faial, respectively, 3.62 and 3.56 mm.

3.4.2 Tetrazolium test

After the 24h test duration, a particular aspect recorded was that embryos “escaped” from the seeds and were floating in the solution.

The provenance that presented the higher number of viable seeds was Terceira, followed by Faial and finally by Pico (Table 3.5). The percentage of viable seeds was low in the three provenances.

Table 3.5 - Tetrazolium test results for each seed provenance.

Seed provenance	Viable seeds (%)	Non-viable seeds (%)
Terceira Island	65	35
Faial Island	46	54
Pico Island	40	60

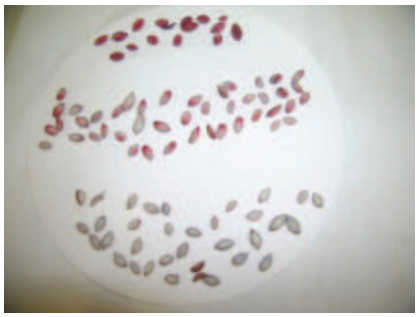


Figure 3.6 - Seeds from Terceira Island after the tetrazolium test. In the upper part of the filter paper can be observed completely stained seeds. In the middle partially stained seeds and in the bottom non-stained seeds and/or having non-essential structures stained.

3.4.3 Seed treatments

Once seed treatments trials were set, fungal activity soon started to show up, despite all the measures taken to avoid contamination (Fig.3.7). During the test period seeds were fungicide treated with fungicide to prevent and also whenever fungus proliferation was higher. To some point, a new autoclaved Petri dish with new filter paper replaced each individual one. Seeds prior to be transferred were washed in a fungicide mix (1% azoxistrobina and 1% miclobutanil). After which seed treatments continued to be rigorously controlled to restrain fungal activity, unfortunately they did proliferate, contaminating the seed germination trials. Consequently, no accurate results were obtained.

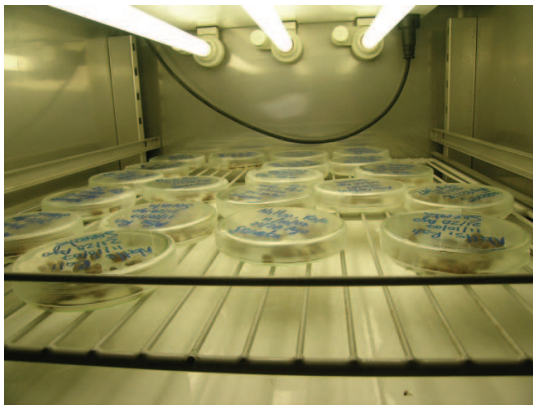
a)   b)

Figure 3.7 – a) Petri dishes parafilm sealed in the growth chamber; b) Detail of the fungi contamination.

3.5 Wood characterization

3.5.1 Wood anatomy

Picconia azorica wood colour ranges from pink to yellowish, the hearthwood being slightly darker than the sapwood. This species wood is characterized as diffuse-porous with aggregate vessels, with oblique to dendrytic pattern as Y or X, normally associated with vascular tracheids and paratracheal parenchyma. Spiral thickenings are very well developed on vascular tracheids (length 42.17 - 565.25 μm). Marginal parenchyma bands mark growth rings. Two-three row small vessels not visible to the naked eye characterize the growth ring limit. In the transition zone, from earlywood to latewood, vessels diameter is practically constant. Vessel member length varies from 103.74 up to 592.47 μm (Fig. 3.8). Vessel perforations are simple in oblique position, however it is common an almost horizontal position. Inter-vessel pits are alternate, sometimes are round others elliptic, nonvestured. Vessel-ray and vessel parenchyma pits are similar even if sometimes fairly different. Uni-seriate rays including heterogeneous and homogeneous type are not visible to the naked eye. Occasionally bi-seriate rays can be observed. Body ray cells procumbent or erect with one row upright of square marginal cells. It could be counted up to 3-6 rays per millimetre. Axial parenchyma cells are squared ranging from 40.86 to 245.29 μm. Libriform fibres length varies in between 422.86 and 1060.18 μm, being very thick-walled. Crystals usually are in ray and axial

parenchyma cells in minute, cubical, diamond-shaped or other forms. Spherical bodies, apparently white-opaque in the SEM analysis (presumably oil's drops or oil cells) are frequent (see Figure 3.9). Fungal presence is frequent in the bark of air-seasoning wood.

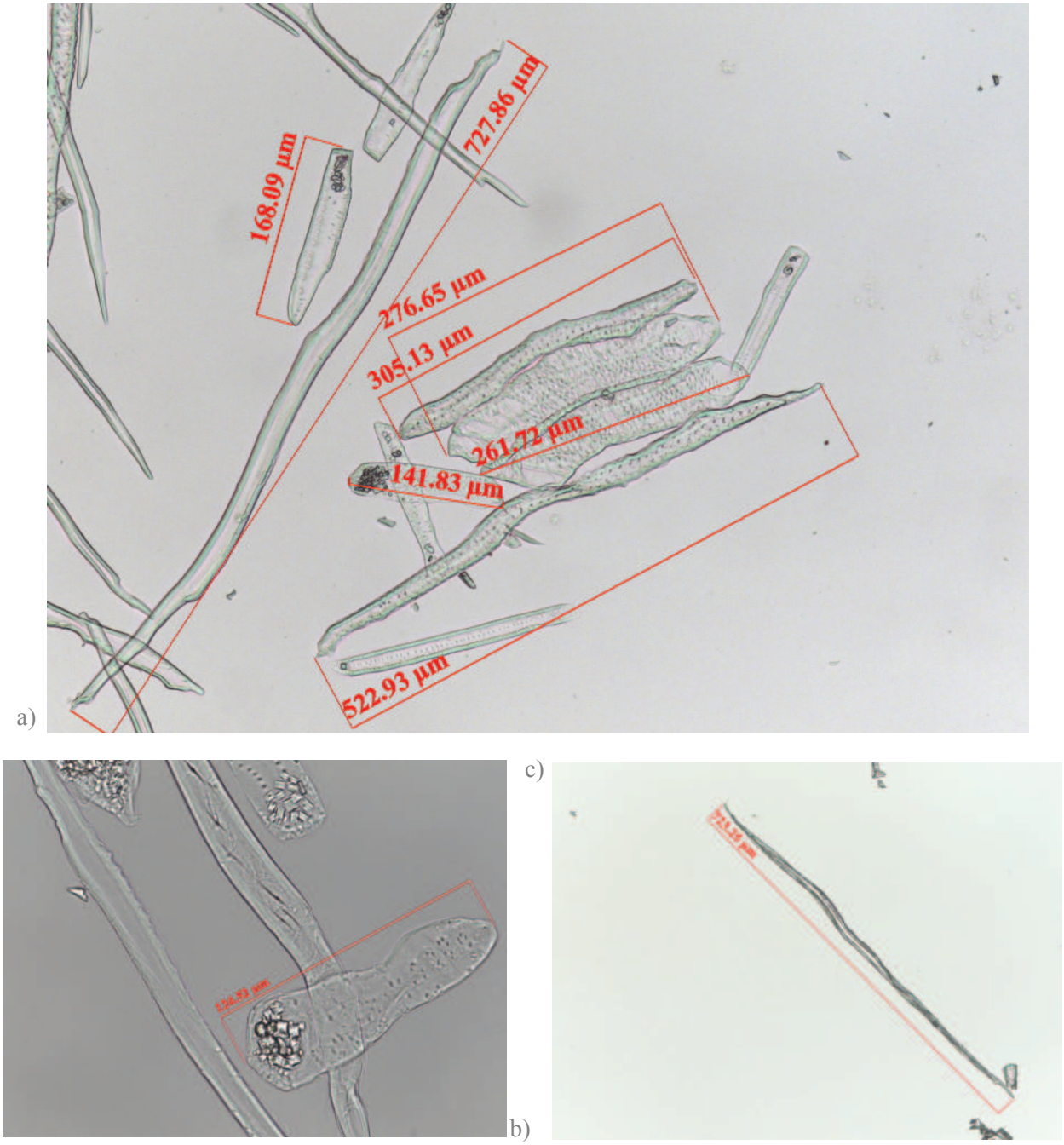


Figure 3.8 - *Picconia azorica* macerato were can be seen: a) vessels 276.65 and 261.72 µm and vascular tracheids vessels-associated 305.13 and 522.93 µm (10x); b) tracheid elements with crystals 124.52 µm (40x) and c) fibre 723.25 µm (10x).

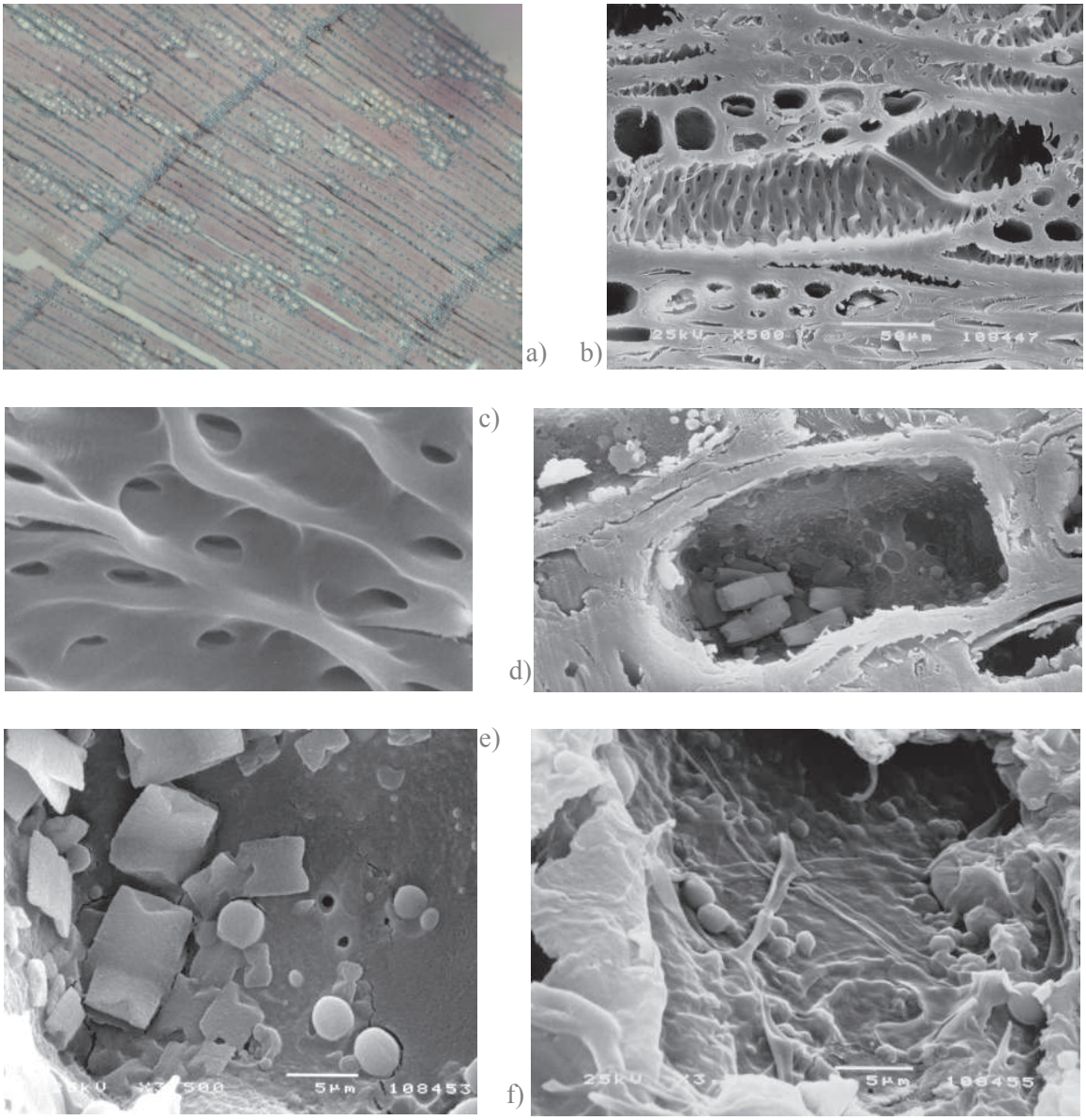


Figure 3.9 - (a) *P.azorica* wood cross section, (b) spiral thickenings in a vascular tracheid, 500x; (c) inter-vessel pits, 1.500x; (d) crystals, 1.500x; (e) crystals and spherical bodies, presumably oil drops or oil cells, 3.500x and (f) bark detail with fungus presence, 3.500x.

3.5.2. Wood technological properties

An interesting aspect to note was when samples were softened in water, for 48 h, prior to technological analyses fungus development was observed. Also extractives coloured the water in a brownish colour and it had an intense odour.

Results obtained for each physical characteristic can be examined in Table 3.6, whereas results for the mechanical characteristics can be observed in Table 3.8. Values for p-level attained by applying an ANOVA non-parametric Kruskal Wallis test are summarized in Table 3.7 (physical characteristics) and Table 3.9 (mechanical characteristics). There are no statistical differences between the wood samples for the parameters analysed.

The static quality coefficient dimensions are given in terms of length – kilometres (km). This coefficient is also known has the rupture length and it indicates the hypothetical length of an element in a constant section that, if put vertically, will break due to the effect of its own weight (Giordano, 1999). This coefficient corroborates the wood classification as resistant.

Table 3.6 - *P. azorica* wood physical characteristics (MC = moisture content; d.m = dry matter; Var = Variance; SD = Standard Deviation; SE = Standard Error).

Parameter	Sample No.	Average	Coeff. confidence -95,00% ; 95,00%		Min.	Max.	Var.	SD	SE
Density (MC 12%) (g/cm^3)	94	0.82	0.81	0.83	0.55	0.92	0	0.05	0.01
Density (MC 0%) (g/cm^3)	94	0.77	0.76	0.78	0.48	0.84	0	0.05	0
Axial shrinkage (%)	26	1.36	1.06	1.66	0.51	3.01	0.56	0.75	0.15
Radial shrinkage (%)	26	6.29	5.08	6.79	4.6	9.99	1.48	1.22	0.24
Tangential shrinkage (%)	26	8.12	7.86	8.34	6.48	9.13	0.43	0.66	0.13
Volumetric shrinkage (%)	26	13.90	13.32	14.49	11.32	18.18	2.11	1.45	0.28
HHV d.m (MJ/kg)	13	18.66	17.93	19.38	16.35	20.01	1.43	1.20	0.33
Ash content (% on weight)	13	1.34	1.19	1.49	1	1.18	0.06	0.25	0.07

Table 3.7 - Statistical difference between samples for each parameter examined for the physical characterisation (ANOVA Kruskal Wallis non-parametric test).

Parameter	Significant Difference	p-level
Density (MC 12%) (g/cm^3)	N	0.1929
Density (MC 0%) (g/cm^3)	N	0.1601
Axial shrinkage (%)	N	2.2314
Radial shrinkage (%)	N	0.5190
Tangential shrinkage (%)	N	0.9431
Volumetric shrinkage (%)	N	0.7190
HHV d.m (MJ/kg)	N	0.8979
Ash content (% on weight)	N	0.7887

Note: N= no significant difference between plants ($P < 0.05$).

Table 3.8 - *P. azorica* wood mechanical characteristics (σ y12% = compression parallel to grain at 12% M.C.; σ b12% = ultimate strength in static bending at 12% M.C.; M.C. = moisture content; Var = Variance; SD = Standard Deviation; SE = Standard Error).

Parameter	Sample No.	Average	Coeff. confidence -95,00%	Coeff. confidence 95,00%	Min.	Max.	Var.	SD	SE
σ y12% (MPa)	94	61.85	60.49	63.22	42.98	79.42	44.57	6.68	0.69
Quality value [km]	94	7.66	7.56	7.75	6.50	8.90	0.21	0.46	0.08
σ b12% (MPa)	31	92.18	84.12	100.24	55.42	120.11	482.56	21.97	3.95

Table 3.9 - Statistical difference between samples, for each parameter examined for the mechanical characterisation (ANOVA Kruskal Wallis non-parametric test).

Parameter	Statistical Difference	p-level
σ y12% (MPa)	N	p=0.2096
Quality value [km]	N	p=0.2072
σ b12% (MPa)	N	p=0.8506

Note: N= no significant difference between plants ($P < 0.05$).

Taxus baccata L.

3.6 *Taxus baccata* L. historical distribution in the Azores

Since 1440, i.e. 20 years after Azores settlement, this Portuguese archipelago forest cover and its native flora were diminishing as a result of the settlement itself and human activities (e.g. opening land for agricultural purposes, cutting timber for human use).

First descriptions of the Azores by Frutuoso (1583) reported *T. baccata* woods on six of the nine Azorean islands (Corvo, Flores, Faial, Pico, S. Jorge and S. Miguel). In one passage the author states: "S. Miguel island, when found (…) had some places hills covered only with yew". In parallel, according to Martins (1981), in the Azores, yew wood exploitation for luxury furniture making in the Azores dated back to 1450, essentially because it was an already known noble species. Yew furniture dating to that period can still be found all over the nine islands in the archipelago, implying that *T. baccata* was originally present all across the Azores. According to data collected by Martins (1981), yew furniture was no longer being produced in Santa Maria, Terceira and Graciosa by as early as 1500 (i.e. before Frutuoso's description), while in Pico it continued until 1760.

Yew rapidly became an important wood for exportation (Drummond, 1859; Webster, 1821), as also did *Frangula azorica* Tutin, *Juniperus brevifolia* (Seub.) Antoine and *Picconia azorica* (Tutin) Knobl. For example, Frutuoso (1583) describes its use in shipyards: "In Flores there is a considerable abundance of fine woods, with which wood needs of Faial island shipyards are sometimes satisfied, with white yew". As a result, *T. baccata*'s fate was its almost extinction in the Azores Islands, despite the fact that, for example, by 1509 in Pico Island, at the time with only 45 inhabitants, a municipal edict banned tree harvesting (Frutuoso, 1583) and placed yew under the Royal House Control, becoming known as the "queen wood". Moreover, it is believed that farmers eradicated the species due to its toxicity, as even young seedlings disappeared completely soon after farming gained more importance in areas of higher altitudes (Dias, 2007).

References to *T. baccata* occurrence and its wood use are common in Azorean history, as well as in botanical records and descriptions by Seubert (1843), Drouet (1866), Watson (1870), Trelease (1897) and Guppy (1917), mainly referring to Pico Island (Figure 3.10).

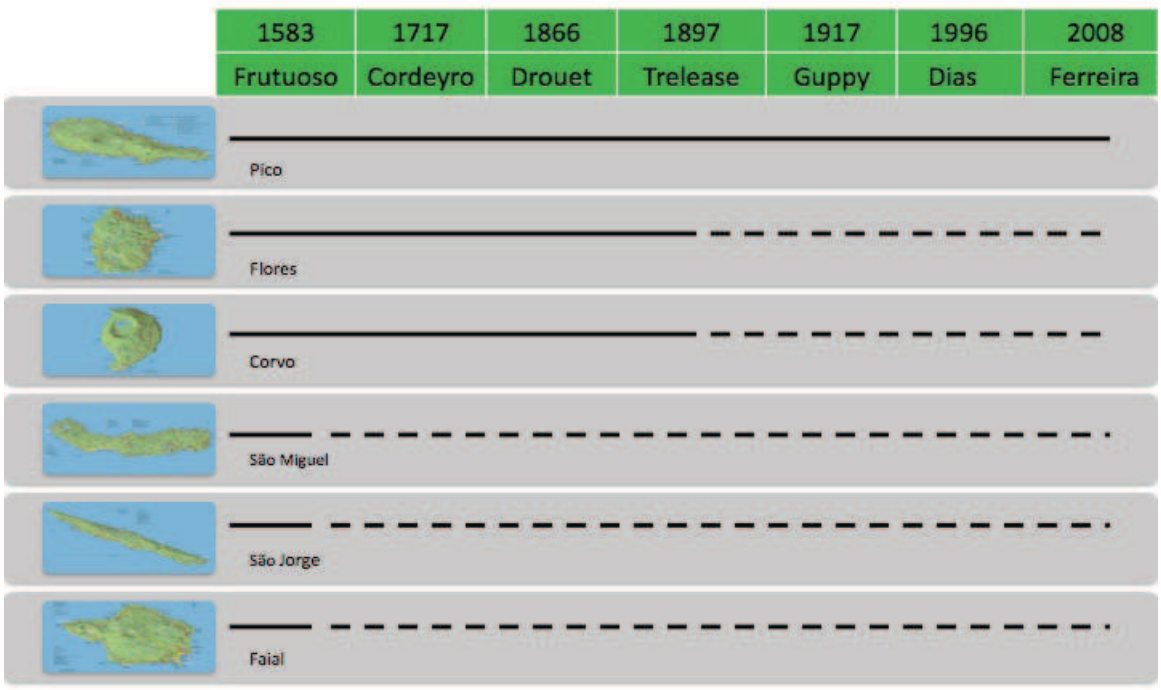


Figure 3.10 - *Taxus baccata* L. presence — /absence - - - according to botanical records and historical descriptions.

About yew wood buried in volcanic ash from earlier eruptions at Pico Island, Frutuoso states "The stems that still now can be found below ground, of about eight to ten palms (160 to 200 cm) long and three palms (60 cm) width, are used for offices and luxury tables"; and that "yew trees in S. Roque village had superficial root systems very prone to windthrow". In 1717, Cordeyro refers to yew in Pico as a "fruit" of great economic value. Two centuries later, Guppy (1917) stated that the species was almost extinct in Pico, with individuals being found between 600 and 750 m above sea level (a.s.l.), where the species flourished in the original forests. This author stated that yew was, at this time, restricted to "surviving in the gulleys, about 600 m a.s.l., on the mountain slopes" behind São Roque village.

Indeed, the past species abundance and distribution in Pico is reflected in the name of several places of the island. Specifically, the lagoon "Lagoa das Teixas" (Figure 3.11), also known as "Lagoa do Capitão" (Guppy, 1917), and a hill "Monte das Teixas" (Pereira, pers. comm.). Teixo or teixa in Portuguese means yew. The last ascertained evidence of a few yew individuals growing in Pico in association with *Erica azorica* Hochs and *Juniperus brevifolia*, was collected by Sjogren in 1995 (in verbis, Spada, 2003).

Figure 3.11 - This map points out the location of "Lagoa das Teixas" in Pico Island. (Adapted after Guppy 1917).

Azorean inhabitants related to Seubert (1843) that yew grew spontaneously in the mountains. Drouet (1866) reported the presence of yew on Flores Island, and drew attention to its increasing scarcity. A few years later, Macedo (1871) mentioned that yew wood was chosen to build the choir chairs of an Azorean church. Trelease (1897) noted that the species "formerly occurred in workable size on Corvo and Flores, whence it was exported as a source of royal revenue. Now seemingly exterminated." A fact recently confirmed for Corvo Island (Pereira et al. 2007).

Dias et al. (2007) recognize the extinction of an endemic formation of the Azores, the yew forests, which were in the past dominant forests at low altitudes. It is currently acknowledged that the species is almost extinct in the Azores (Thomas and Polwart, 2003; Cardoso et al., 2008).

3.7 *Taxus baccata* L. current distribution in the Azores

Despite the results reported by Cardoso et al. (2008) regarding the possible extinction of *T. baccata*, the species still occurs on Pico Island, although only in the form of a very few individuals, as previously reported (Carqueijeiro et al., 2005; Dias, 1996; ETC/BD, 2005). Only five living individuals were found in this study; one additional yew tree was found already dead. All individuals seemed not wind-resistant, as they were noticeably damaged by wind action. Moreover, no natural regeneration was found. All trees were in the mountain plateau area (Figure 3.13); the precise location (GPS coordinates) of these *T. baccata* individuals will not be released to avoid additional pressure.

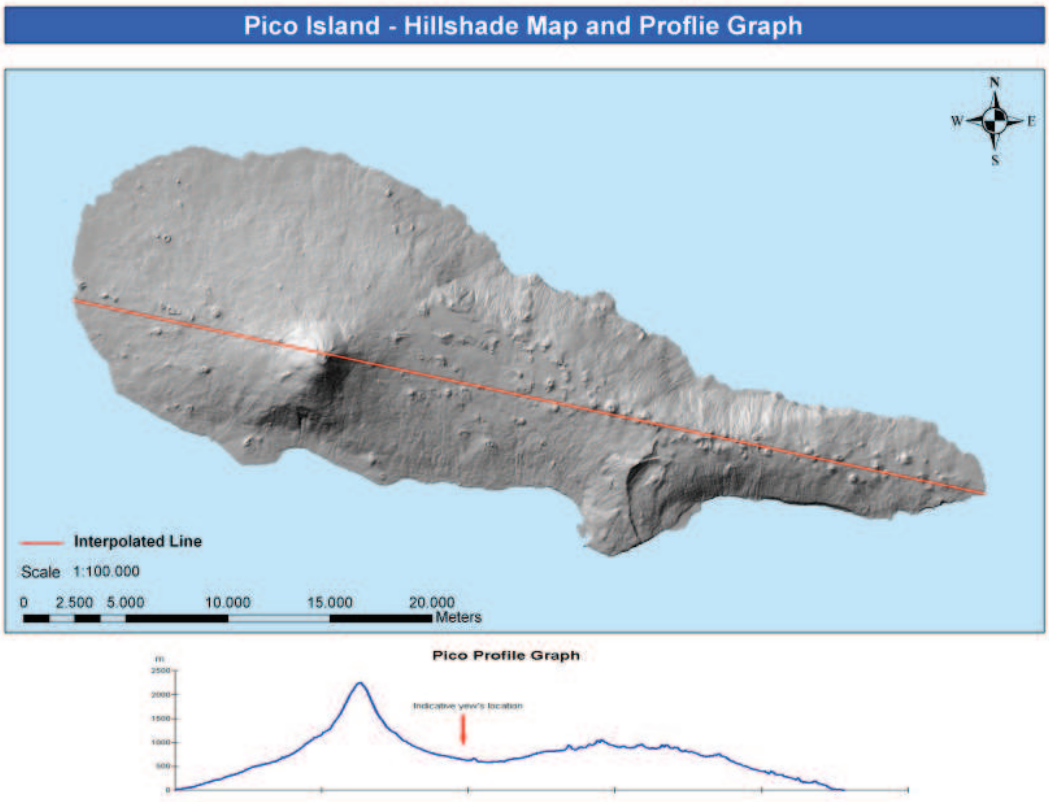


Figure 3.12 - Pico Island physiographic map (Source: Portuguese Army Geographical Institute) and Pico Island profile where the red arrow points out the area where the identified yew trees are located in Pico Mountain Plateau.

Starting from the lowest altitude of Pico Island and going towards the Pico mountain plateau, a first plant was found approximately at 100 m a.s.l., facing NE on a slope of 5.5%. It was a male individual (Fig. 3.13 a), probably about 80 years old (Pereira, pers. comm.), located in a private garden. The stem was monocormic and the crown form was Vshaped. The tree was 5 m high and 38 cm DBH. It had good phytosanitary appearance. A second individual in this garden was windblown some years ago (Pereira, pers. comm.). All the other plants were found on the Pico mountain plateau. One individual was at 800 m a.s.l., 5 m high and 28 cm DBH (Fig. 3.13 b). It was on an area with a slope of about 7%, facing E, where beef cattle were maintained extensively during Spring and Summer. The stem was monocormic and the crown V-shaped, exhibiting a considerable number of dead branches; the plant showed good phytosanitary status.

Three other yews were found at around 1000 m a.s.l. (Fig. 3.13 c-e). Another one was found dead. All trees were within the native forest, in areas with difficult access. Natural vegetation, a transition type between the so-called "Azorean Lowland Heaths" and the "Azorean Upper woods' Heaths" (nos. 31.34 and 31.35 of CORINE Biotope Manual, 1991), this transition area consisted of trees around 2.5 to 7 m high, comprising mainly *Frangula azorica* Tutin, *Ilex perado* Aiton ssp. *azorica* (Loes.) Tutin , *Laurus azoric*a (Seub.) Franco, *Erica azorica* Hochst, *Juniperus brevifolia* (Seub.) Antoine and *Vaccinium cylindraceum* Sm.

a)

b)

Figure 3.13 – a) male inflorescences of the low altitude individual; b) yew individual at 800 a.s.l.; c and d) the light-brownish green tree is a yew mixed with the Azorean endemic vegetation; e) detail of a yew shoot.

One yew was found at 1030 m a.s.l., on a 2.5% slope with a SE aspect. It was 5 m high, with an indistinguishable polycormic stem. It presented a V-shaped crown, also with a considerable number of dead branches at crown and stem level. Excluding wind damage, it appeared to be in good phytosanitary condition. No natural regeneration was found except for a new individual sprouting at the root level.

A second individual was found in an area with 5.2% slope, facing SE at 1050 m a.s.l. It was smaller

than the former, only 2.7 m high. Apparently it was a young individual, with a smaller crown, without dead branches. It was not possible to access the stem, which was not wider than 7 cm.
A third yew was found within the 1050 m a.s.l. belt. The tree was 4 m high, with an indistinguishable polycormic stem. The crown was V-shaped and appeared in good phytosanitary condition.
Sex was established only for one tree. It was not possible to observe male or female inflorescences on the other four individuals, so the possibility that female individuals are absent assumes crucial ecological and conservational relevance.

3.8 Azorean yew characterization

3.8.1 Leaf morphology comparison

Morphometric analyses of Azorean yew leaf dimensions showed that the mean leaf length was 11.10 mm, width was 2.30 mm, area was 21.30 mm^2, and the length/width ratio was 7.7. These values are consistent with the range previously reviewed by Thomas and Polwart (2003), who indicated the limits of *T. baccata* leaf length as being between 1 and 3 (4.5) cm, with an average width of 2 to 3 mm. However, Azorean yew leaf length, width, their relative ratio and leaf area are significantly smaller than those detected in previous studies (Di Sapio et al., 1997; Cope, 1998; Mitchell, 1998; Dempsey and Hook, 2000; Hageneder, 2007; Iszkulo et al., 2009).
The t-Student test on the collected samples shows significant differences between the Italian and the Azorean provenances. *T. baccata* leaf length, width, area and length/width ratio values from Italy are indeed greater than the Azorean values ($P<0.001$) (Table 3.10); at the same time, Azorean yew leaves are among the smallest of all *Taxus* species (cf. Cope, 1998).

Table 3.10 - Comparing means of morphometric parameters for collected data (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) (SD = Standard Deviation).

		Length (mm) ***		Width (mm) ***		Area (mm^2) ***		L/W ratio ***		Stomatal density (n/mm^2) ***	
	N	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Azores	326	11.10	2.92	2.30	0.40	21.30	7.91	7.70	1.99	231.87	2.43
Italy	294	17.68	2.93	2.40	0.24	33.99	8.04	7.20	1.38	140.00	1.34

The number of stomata rows on one-half of the abaxial leaf surface appears of great importance for species classification within *Taxus* (Strobel and Hess, 1996; Hageneder, 2007; Spjut, 2007a; Iszkulo et al., 2009). According to Strobel and Hess (1996), North American yew species are characterized by 3–5 stomata rows, whereas the Eurasian species usually exhibit 7–10 rows. From the three provenances analyzed by electron microscopy (Azores, Carpineto and Rosello in Italy) it was possible to ascertain that each one presented an identical number of stomata rows on one leaf-half of the abaxial side (the Azorean provenance ranging from 7 to 10–11 and Italian provenances ranging from 7 to 9–10), thus confirming the setting of the whole group within *T. baccata* (Figures 3.14 A, B).

Most authors consider the stomata density per mm^2 also to be an important taxonomical and ecological character (Salisbury, 1927; Di Sapio et al., 1997; Mitchell, 1998; Dempsey and Hook, 2000; Iszkulo et al., 2009). In *T. baccata*, an average density of 115 stomata per mm^2 of leaf surface was first described by Salisbury (1927); more recently, Di Sapio et al. (1997) found an average density of 59, whereas Mitchell (1998) described a density of 89.4, for leaves growing in full sunlight. Dempsey and Hook (2000) reported values ranging between 82.28 and 119.53 in different *T. baccata* L. cultivars, with generally higher values in the other yew species; Iszkulo et al. (2009) reported higher densities in female individuals (75.06) than in males (68.64).

Observations, by electron microscopy, showed that Azorean Pico yew possesses a significantly

higher number of stomata per mm^2 ($P<0.001$) than Italian provenances: Pico had 231.87 ±2.43 stomata per mm^2; 138.12 ±1.34 for Carpineto; and 141.87 ±1.37 for Rosello.

Also, the Italian provenances show stomata density values slightly higher than those reported in the literature. A possible explanation could be that the provenances sampled in previous studies always analysed plants from Central Europe or extra-European countries, while this study focused on Italian ones, the first report of Mediterranean provenances analysed for this character.

Yew presents tetracytic sunken stomata disposed in longitudinal rows (Strobel and Hess, 1996; Di Sapio et al., 1997). The stomata show the so-called Florin‘s ring (Figure 3.14 C), formed by 4–6 subsidiary papillose cells that encircle each stoma (Dempsey and Hook, 2000; Spjut, 2008), characteristic of different species of Gymnosperms (Willmer and Fricker, 1996; Di Sapio et al., 1997). The single stoma shape, as well as the epicuticular wax features, does not seem useful for reliable discrimination among *Taxus* species (Strobel and Hess, 1996). Even stomatal dimensions do not constitute a useful taxonomic discriminating character, although this might be dependent on the different morphometric approaches used by many authors.

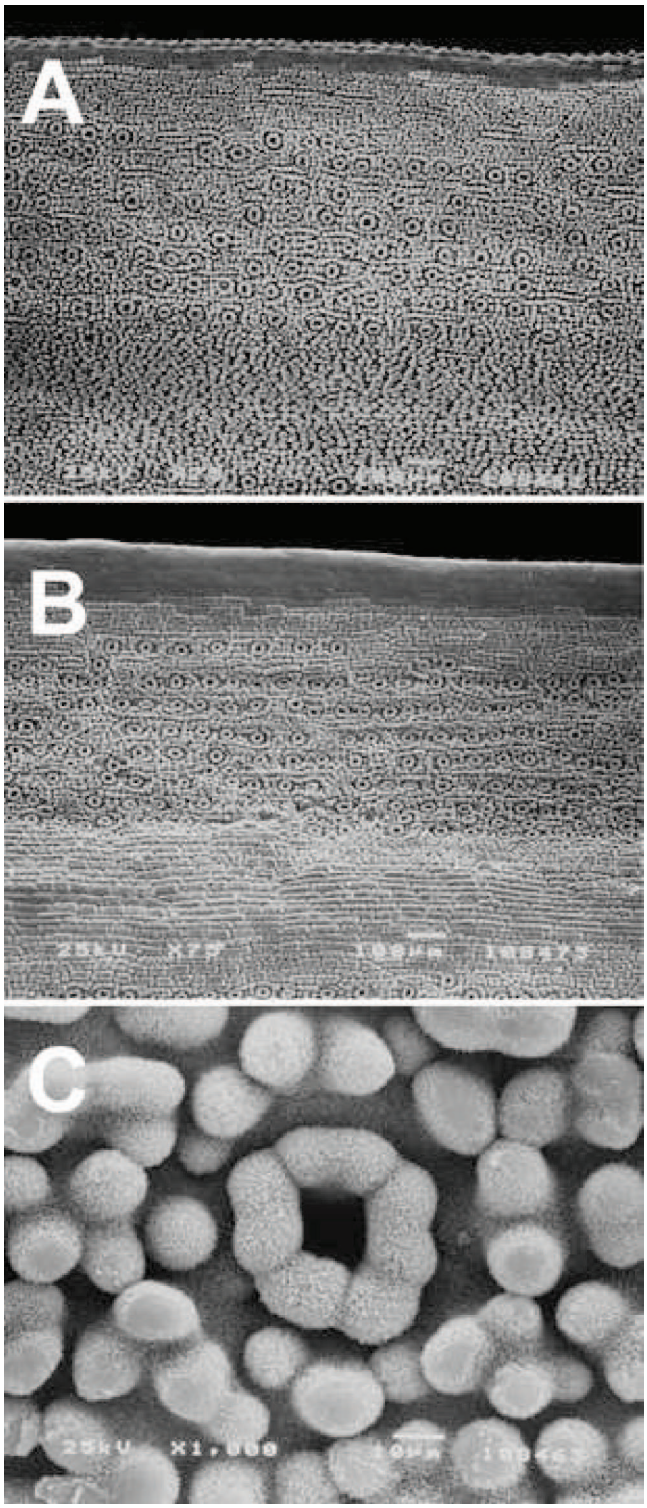


Figure 3.14 - SEM images of abaxial side of the yew leaves from Italy (A) and Azores (B) differing in marginal and mid-rib cells features. Particular of stoma morphology with Florin‘s ring and papillose cells (C) in the Azorean yew.

However, the comparison of stomata dimensions between Azorean and Italian yews shows the latter being significantly bigger than the yews from Azores (14.21 ±2.44 µm vs. 16.57 ± 3.57 µm), even though both are well in line with the range of sizes cited in the literature (cf. Dempsey and Hook, 2000).

Finally, the transverse foliar sections reveal that the Azorean yew belongs to the *Baccata* Group *sensu* Spjut (2007a), recognizable mainly by the elliptical epidermal cells and the anticlinal layers of palisade cells (Figures 3.15 A, B).

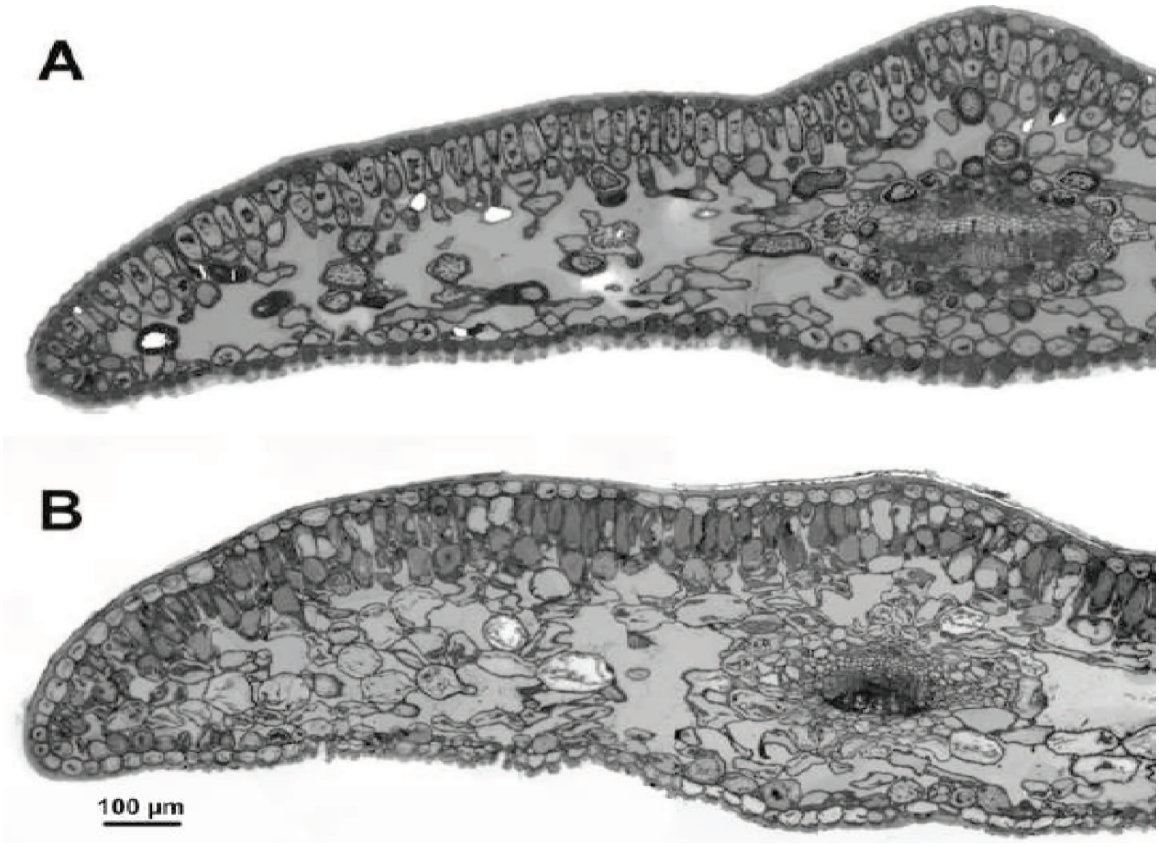


Figure 3.15 - Transverse foliar sections of Italian (a) and Azorean (b) yew observed by light microscope (10 X). Provenances differ by epidermal cells and papillae in the inner surface, and anticlinal layers of palisade cells in the upper one.

3.9 DNA analysis

Sequences of the gene investigated from every sample were deposited at the NCBI GenBank under accession numbers GU320033 – GU320044 (see Annex IV).

The alignment of the 14 *T. baccata* sequences (12 novel and 2 extracted from NCBI) showed two polymorphic sites in position 400 (T/G) and position 504 (A/G). On the basis of this intra-specific variation, two haplotypes were defined: Hap_1 and Hap_2 (Figure 3.16). Hap_1 is widespread in the Euro-Mediterranean area, whereas Hap_2 is restricted to the Azores.

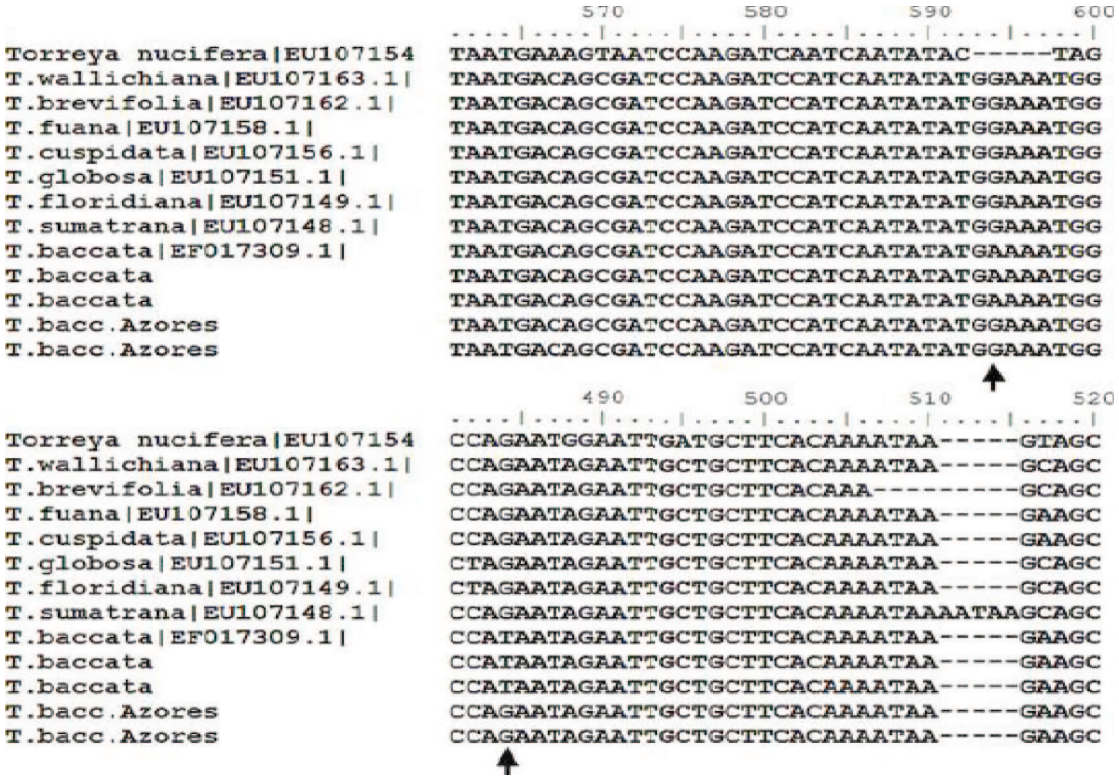


Figure 3.16 - Relevant positions in the alignment of the chloroplast *trnS–trnQ* spacer sequences that differentiate provenances of *T. baccata* and closely related species. Arrows indicate positions discriminating Azorean *T. baccata* haplotype.

The multialignment with nine *Taxus* spp. (13 sequences) and *Torreya nucifera* (L.) Siebold & Zucc. displayed all samples sharing the same nucleotide substitutions with the Azorean population (a gap in position 504 was present in *T. nucifera*). Both substitutions may therefore represent a symplesiomorphy within *Taxus*. Furthermore, the distance data matrix suggests that *T. baccata* samples from continental areas are more divergent (d = 0.186) from the outgroup *T. nucifera* than Azorean individuals (d = 0.184).

The phylogenetic data are well in line with the inter-specific relationships drawn by Hao et al. (2008), with no paraphyletic groupings of sequences, although species are scarcely resolved, and with *Torreya nucifera* (L.) Siebold & Zucc., as an outgroup species.

The inferred tree showed two well-supported clades within *T. baccata* (Figure 3.17). These clades are monophyletic and match with the previously defined haplotypes Hap_1 and Hap_2.

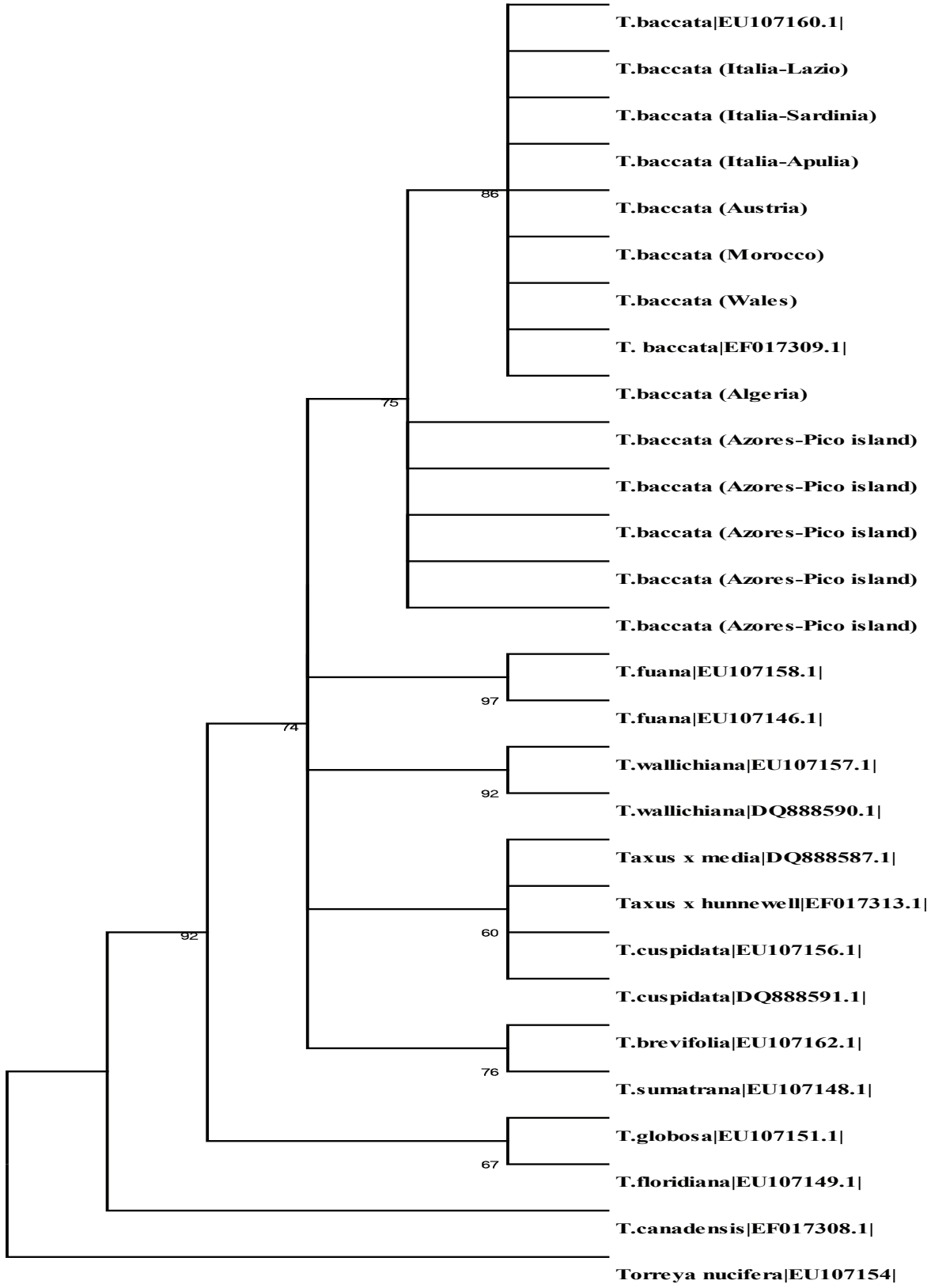


Figure 3.17 - Neighbor-joining inferred tree of chloroplast *trnS–trnQ* spacer sequences in *Taxus* spp.. Bootstrap support is indicated below branches (1000 replications).

3.10. Vegetative propagation

Due to the destructive character of most biological analyses (root and shoot electrolyte leakage – REL and SEL), dry weight and moisture content of root, stem and leaves and due to the scarcity of cuttings to allow statistical significant results, such tests were not performed.

Rooted cuttings were manually transplanted to 1L volume pots, and maintained outdoors at field conditions.

Results for survival and rooting for each of the treatments are summarized in Table 3.11. No statistical difference was observed between treatments, all exhibiting reduced survival and rooting rates.

Table 3.11 – Yew cuttings survival and rooting for each of the six treatments. Treatments are not statistically different.

Treatment	Survival (%)	Rooting (%)
Pure peat (no IBA)	18	6
Pure peat (IBA 2.000 ppm)	26	16
Pure peat (IBA 5.000 ppm)	18	10
Jiffy (no IBA)	16	8
Jiffy (IBA 2.000 ppm)	22	14
Jiffy (IBA 5.000 ppm)	32	18
Mean Standard Deviation	8	7

4. DISCUSSION

***Picconia azorica* (Tutin) Knobl.**

4.1 Biosystematics and phylogeny of *Picconia azorica*

Macaronesian endemics include both groups that have radiated spectacularly in the islands (e.g., Aeonium, Argyranthemum, Crambe, Echium) and groups that show little or no evidence of island radiation (e.g., Androcymbium, Arbutus, Genista, Olea), Carine et al. (2004). Genetic diversity assessed in this work demonstrated that Picconia belongs to the latter group and strongly supports the systematic ranking as separate species of the two archipelago-specific, morphologically similar taxa: *P. excelsa* and *P. azorica*. Conversely, a recent molecular study showed that *Myrica faya* Aiton. and *M. rivas-martinezii* A., a plant group with similar taxonomic, geographical and conservational issues as *Picconia*, share the same genetic pool despite a pronounced morphological differentiation, and suggested that they are probably two morphs of the same species (Gonzalez-Perez et al., 2009).

Carine et al. (2004) estimated 80% of Macaronesian endemics to have a Mediterranean sister group. Our Mediterranean dataset suggest monophily of the genus *Picconia* with *Phillyrea* as a sister taxon, and such a close relationship appears to be supported by the few bio-systematic data currently available (Wallander and Albert, 2000). Nevertheless, even if only few Macaronesian groups are known to have closer relationships to far-off areas, such as east Asia or the New World (Comes, 2004; Carine et al., 2004) a closer inspection of molecular affinities between these two taxa and Asian-Australian groups *Osmanthus*, *Nestegis* and *Notelaea* is certainly required to precisely assess *Picconia* relationships (cf. Rabaey et al., 2008). However, sequence comparisons with available rbcL gene regions from the GenBank provided no evidence for a closer relationship of *Picconia* with this group of species.

In our phylogeny, *Picconia* correspond to a "crown-based" lineage (Vargas, 2007), i.e. the result

of a more recent establishment in Macaronesia relative to the mainland. However, the absence of congenerics outside Macaronesia may affect such age inferences, and the antiquity of *Picconia* lineage(s) may not be easily recognizable.

Owing to the current scarcity of fossil data, little can be definitively stated about the timing and modes of colonization of Macaronesian archipelagos by *Picconia*. If we consider the descriptions of *Picconia* from Pliocene deposits in central Europe given by Depape (1922) and its current distribution, *Picconia* experienced an extraordinary range retreat, followed by extinction on the mainland and survival or colonization of Macaronesian islands as refuge areas; in this case, differentiation of *P. azorica* might have occurred either on the mainland or in the Azores. Alternatively, speciation(s) took place in Macaronesia from a widespread, now extinct ancestor and the two species never colonized the mainland, unless related lineages disappeared in modern times. In either case, and considering the current range reduction of the once more widely distributed *P. azorica* in the Azores, *Picconia* is to be considered a true relict under the taxonomic, genetic and geographic points of view (Vargas, 2007).

We estimated ca. 5 Myr as a probable divergence time for the two species. This value fits with the Pliocene datation of the only *Picconia* fossil currently known (in central Europe; Depape, 1922) and within the relative ages of the three archipelagos: the Canary islands oldest sub-aerial geological formations span from 1.2 to 20.6 Myr (Carracedo et al., 2002), Madeira from 4.6 to 14.3 (Geldmacher et al., 2007), and the Azores from 0.25 to 8.12 Myr (Borges and Brown 1999). Speciation rates are likely to depend on plant groups, and specific ecological factors in oceanic volcanic islands may drive colonists towards radiation or stability (Silvertown, 2004); for instance, the Canary Islands are older than the Azores and their flora comprises substantially more endemics, about 570 vs. 68 (Francisco-Ortega et al., 2000). However, the estimated divergence time is also in line with the rates of diversification that have been inferred for *Aeonium* and *Crambe* in the Canary Islands (Kim et al., 2008), and with the divergence times recently estimated for Azorean endemic *Pericallis malvifolia* with respect to the Canarian and Madeiran sister species (Carine and Schaefer, 2009). Kim et al. (2008) estimated three opportunity windows for the colonization

of Macaronesia: middle Miocene (16-14 Myr ago), late Miocene (8-7 Myr) and early Pliocene (about 3 Myr ago), in coincidence with discrete climatic changes in the western Mediterranean and northern Africa.

Accordingly, we might speculate a late Miocene separation of the two species in the Mediterranean and a Pliocene refugial in Macaronesia, following range retreat during the onset of the first glacial cycle that led to massive extinction and migration of plants (Fauquette et al., 1999). Independent colonization of each of the three Macaronesian archipelagos cannot be excluded. At the same time, an earlier isolation of *Picconia* in the Canary Islands and in Madeira, and a late Miocene diversification/isolation of *P. azorica* in the Azores, during a period of major geological and climatic changes leading to species extinction in the Mediterranean (e.g. the Messinian salinity crisis; Krijgsman et al., 1999) is also possible. In the Azores, colonization and evolutionary processes eventually started on the oldest island (S.ta Maria, 8.12 Myr), which is also located nearer to Madeira and mainland Europe, and was only available for colonization about 5.5 Myr ago, due to an eruptive phase that gave origin to its most recent part (Serralheiro and Madeira, 1993). However, because of the genetic diversity observed between and within *P. excelsa* and *P. azorica*, we can infer the latter as the result of a single colonization event followed by *in situ* diversification (i.e. the SSR haplotype variation), and due to the absence of modern era congenerics on the mainland, we assume back colonization never occurred.

4.2 Genetic diversity and phylogeography

The Azores harbour distinct *P. azorica* cpDNA haplotypes, indicating that these islands have sustained populations long enough to allow their genetic divergence. Apparently, no haplotypes are shared between *P. azorica* (Azores) and *P. excelsa* (Canary Islands and Madeira), indicating a barrier to seed flow between the Azores and the southern archipelagos, at least in recent times, and genetic isolation of the two species. Molecular divergence and the absence of genetic exchange between Canarian and Azorean provenances were recently identified also in *Laurus azorica* (Rodriguez-Sanchez et al., 2009). Within-archipelago genetic structure is weak, a likely

consequence of efficient seed movement among islands and populations. Nevertheless, results of SAMOVA, which defines groups of populations that are geographically homogeneous and maximally differentiated from each other, clearly underlines the presence of a unique group (Valverde in S.ta Maria) that is differentiated from all other populations. Of particular interest is the fact that this population presents flowers with higher dimensions. Further studies are certainly required to assess the evolutionary significance of this group.

Picconia is an outcrossing, woody species displaying wind pollination and zoochory (Arteaga et al., 2006; Dias et al., 2007); such features have been described as the most suitable for successful long-distance dispersal in Macaronesia (Vargas, 2007). The success of *Picconia* seed dispersal would be attested not only by inter-archipelago colonization from the Canary Islands to Madeira (a distance of 400 km) and eventually to the Azores (a distance of 800 km), but also by colonization within the Azorean archipelago (extending 600 km). The existence of now submerged volcanic isles between the archipelagos and mainland (Dias, 1996; Garcıa-Talavera, 1997) might have favored the dispersal processes until isolation took place, and genetic drift due to archipelago isolation could have been counteracted by inter- and intra-island bird-mediated seed dispersal to homogenize the genetic structure.

Haplotype H3 occurs all across the Azores; its wide distribution might be explained either with an ancient origin or with a greater suitability for seed dispersal and/or wider ecological adaptation. The eastern islands seem devoid of haplotype H2, while haplotypes H1, H4, H5 are extremely localized. Identification of haplotype H3 as the most ancient to colonize the archipelago is supported by having the highest outgroup weight; according to Castelloe and Templeton (1994), the most ancient haplotype (i.e. the one with highest outgroup weight) is located at the centre of haplotype network and is the most widespread. In this case, H2 would have originated later, either in the central or in the western sub-archipelagos, where islands spanning from 3.5 (Terceira) to 2.16 Myr (Flores) are located (Borges and Brown, 1999) and colonized younger islands, with eventual retro-colonization events reflecting the different volcanic activities and dispersal possibilities within Azores. Alternatively, H2 was eroded in the eastern islands. However, the geographical distances

that separate the eastern, central, and western Azores subarchipelagos do not seem to have acted as barriers preventing seed flow. Therefore, the identification of rare haplotypes, restricted to three populations on different islands, requires a deeper insight. In the absence of eco-physiological motivations, the limited distribution of haplotypes H1, H4 and H5 is likely to witness genetic erosion due to population reduction/habitat loss. The scarcity of seed dispersers (either in recent times and/or during critical periods of the species history) may have further compromised these haplotypes' ability to expand and/or colonize new territories, and ultimately affected the species' genetic diversity and structure. The absence of a geographic structure and the large occurrence of non-fixed populations speaks in favour of this interpretation, rather than intermediate populations diverging by drift, with rare and geographically restricted haplotypes being the result of barriers preventing diffusion of new lineages and/or a diffuse erosion of genetic diversity that are eventually too recent to be evident in the observed patterns.

The high proportion of the total genetic diversity residing within populations (h_T = 0.557; h_S =0.449) and a very low population differentiation with no geographic clustering (G_{ST} = 0.194; N_{ST} = 0.177) showed a marked genetic uniformity of *P. azorica* all across its current range, where only three rare molecular variants contribute to define three additional haplotypes (H1, H4, H5).

Compared to other Oleaceae, the value of total genetic diversity in *P. azorica* is similar to the value calculated for *Olea europaea* cpDNA (h_T = 0.64), and mtDNA (h_T = 0.60) (Besnard and Bervillé, 2000), and for *Fraxinus ornus*: h_T = 0.552 (Heuertz et al., 2006), whereas total cpDNA diversity is higher in *Fraxinus excelsior*: h_T = 0.717, and in *F. angustifolia*: h_T = 0.812 (Heuertz et al., 2004; Heuertz et al., 2006). The value of cpDNA genetic differentiation is clearly lower in *Picconia*: G_{ST} = 0.194 than in *Olea europaea*: G_{ST} = 0.55, *Fraxinus excelsior*: G_{ST} = 0.888, *F. angustifolia*: G_{ST} = 0.913, and *F. ornus*: G_{ST} = 0.967 (Besnard and Bervillé, 2000; Heuertz et al., 2004; Heuertz et al., 2006).

Unfortunately, there are no published works to compare values of plastid genetic diversity in *P. azorica* with other Azorean endemics, nor there are comparative works on plant genetic diversity in the Azorean and the Canary Islands archipelagos.

In the review by Francisco-Ortega et al. (2000) allozyme variation in 69 endemic Canarian species (belonging to 18 genera and eight families) was measured, showing that the average species-level genetic diversity at allozyme loci is twice as high as the mean reported for endemics of Pacific archipelagos. Moreover, an average of 28% of the genetic diversity resulted to be among populations and absence of a geographical structure was generally observed. Such distinctiveness was tentatively attributed to different causes, including closer proximity to the mainland, multiple colonization events, the prevalently out-crossing breeding system, and small population size; however, most factors contributing to the higher diversity of the Canary Islands compared to the Pacific archipelagos still remain obscure.

In this study, the limited intra-specific genetic diversity of *P. azorica*, as compared with that observed within the two populations of *P. excelsa* and the molecular divergence of the two species seems to agree with the speculations of Carine and Schafer (2009) who infer that inter-island species stability, as opposed to radiation in other Macaronesian archipelagos, is the striking feature of Azorean flora, and it is most likely due to the absence of strong palaeoclimatic oscillations. In *P. azorica*, such species stability would agree with the observed low variation at the cpDNA level.

Substitution rates are known to be particularly low in trees, a likely consequence of their long generation time (Kay et al., 2006; Petit and Hampe, 2006). Other reliable explanations for low cpDNA variation include demographic stability of most populations and isolation of endemic species (Petit and Hampe, 2006; Smith and Donoghue, 2008).

Most probably a combination of low mutation rates, isolation from original source populations, long ecological stability (as exemplified by the enduring persistance of Laurissilva forest patches), and past efficient seed dispersal are the most feasible explanations for the low genetic diversity and structure displayed by *P. azorica*. At the same time, it is possible that additional lineage variants may have gone extinct, or become extremely rare, due to alteration of the ecological equilibria in Azorean native ecosystems.

4.3 Implications for conservation

Island endemics occurring in fragmented populations are more prone to extinction (Whittaker, 2000), as isolation, small overall area and associated population size may severely promote greater reactions to stochastic processes, genetic drift and lower levels of immigration (Frankham, 2005; Whittaker and Fernandez-Palacios, 2007). Thus, the importance of conserving island biotas lies both in their potential uniqueness and in their susceptibility to diversity loss. Indeed, all these issues are of concern with *P. azorica*. The current distribution of *P. azorica* as well as the results presented in this study highlight the weak genetic structure of the species and the actual risk of genetic impoverishment, most likely due to recent disturbances.

The very first references to *P. azorica* in the Azores were made one century after the Azores settlement in 1440 (Frutuoso, 1583). By that time the author described, on all nine islands, the presence of dense and pure high woods of this species. To date, *P. azorica* is extinct in Graciosa, near to extinction in S. Miguel, and despite the fact that the GEVA map lists some populations on Corvo Island, the local Forest Services stated that in the present time it is not possible to find any *P. azorica* individuals. The small size of this island (17 km^2 area) leads to the suggestion that the species is now extinct on Corvo. No pure population was found in the remaining islands. Mixed stands including *P. azorica* are small, ranging from 1 to 125 ha, the largest stand in Varadouro, Faial Island. Populations are very sparse, with densities as low as 4-5 trees/ha to about 35-40 trees/ha in Varadouro. Even though exhibiting an average – good conservation state, most populations are confined to marginal sites and only 18% are considered not threatened (SRAM, 2005).

The decline of *P. azorica* is primarily due to 500 years of over-exploitation and human disturbance, in particular the cutting of the Laurel forest for grazing and grain crops and the introduction of aggressive exotic species that are now widespread. In particular, last century wide plantations with *Cryptomeria japonica* and *Eucalyptus globulus* have eroded large areas of the original Azorean Laurel forest, and invasive *Acacia malanoxylon*, *Pittosporum undulatum*, *Hedychium gardneranum*, *Clethra arborea* are now widely scattered through the whole of the native forest, leading the species to become almost extinct on all islands.

Picconia azorica has several peculiarities that stress the need for urgent actions to decrease such severe erosion. First, the species has an endemic status and is included in the 2009 IUCN Red List of Threatened Species (http://www.iucnredlist.org); second, the species has a strong ecological role, including being part of the diet of birds protected under the Birds Directive (EC, 1979), and third, this taxon is a relevant example of a relict plant on an oceanic archipelago.

Good strategies are needed to assure that the existing species will not become extinct in few decades, and to maintain the potential for adaptation (Eriksson and Ekberg, 2001). Clearly, the best strategy for conservation of endemics is preservation of natural habitats. Due to *P. azorica* endangered status it will be wise to bring together *in situ* and *ex situ* management approaches to conserve and preserve the species on the long-term for future generations. Furthermore, a conservation programme needs to be drawn. Ideally, the programme will take into account the whole species distribution. Moreover, envisage a re-visit to the populations identified under the Natura 2000 network and all other populations, if possible, to update their information. Hence, once gathered as much information as possible and taking into account the genetic diversity here assessed, a conservation strategy should be drafted. Within the *in situ* measures, *P. azorica* populations should be given conditions for continued existence under natural conditions and in vigorous populations, continuing to be exposed to evolutionary processes. The different populations will be subjected to different selection pressures and in time continue to develop in different directions (Eriksoon et al., 2006). Protection of the stands and contiguous landscapes will be imperative, such will also imply the need to remove and control invasive alien species (including their soil seed bank) and so encourage seedling regeneration. It would be extremely relevant and interesting to establish a network of permanently protected areas (in each island could be accomplished through the Island Park recently created), were *P.azorica* populations could be important "natural laboratories" for scientific studies. Therefore, these protected populations could serve as important base populations and allow deepening the knowledge of the species by assessing:

- Seeding (percentage of fruiting individuals, quantity per individual, frequency of mast years and why, number of viable seeds, germination rates, survival rates of new seedlings);

- Soil seed bank (focusing on the target species, invasive alien species and other endemic species);
- Response and susceptibility to pests and diseases and other environmental disturbances as wind and water logging;
- Growth rate (diameter and height).

Monitoring does play an important role as by doing it one can measure the reproductive and ecological success of the *in situ* management actions applied to each population and classify it as an expanding population, population in equilibrium or mature and senescent population (Marrero-Gomèz et al., 2003).

Finally, raise public awareness as well as of landholders of the importance of the species, being alerted to the high biodiversity relevance of natural patches of forests and their high conservation priorities as well as for the ecological and economical benefits that the species can provide. Island authorities should definitely promote control of alien invasive species to reduce competition and habitat subtraction.

Ex situ measures will consist primarily in conserving and managing the range of variability identified within the species, essentially by developing and managing various forms of regeneration. The *ex situ* conservation strategy should consist in an increased use of the genetic resources, allowing both ecological and economic payoffs. Actions can consist on reintroductions into the natural environment, water basins, artificial established plantations, silvopastoral systems and seed orchards. In parallel a forest tree-breeding programme should be developed. Trials would allow ascertaining the best edaphic conditions for the species, evaluate the potential of the species for CO_2 assimilation (wood and root system), evaluate quantitative traits (survival, height, diameter, stem form and fruiting/flowering) determining the existence of symbiosis and co-associations (e.g. mycorriza) and ultimately determine a silvicultural system.

Nevertheless, a fundamental step for preserving a species is to address the conservation of its genetic diversity (Gregorius, 1991). Therefore, information about the history of a species, estimates

of population subdivision, as well as rates of, and barriers to gene flow form an important basis for defining the most appropriate conservation practices (e.g. *in situ* and *ex situ* conservation units, reintroduction programs) for the effective and sustainable management of a threatened species (Eriksson and Ekberg, 2001).

In this work, the first step was to assess the specific ranking of *P. azorica* on a molecular basis, which is an important issue in a conservation concern due to the management policies for monitorization of endangered plant species, stating that the species is the minimum unit for legal protection (IUCN, 2006); the observed genetic divergence with congeneric *P. excelsa* prevents the effectiveness of any germplasm transfer from Canary and Madeira archipelagos. In addition, results revealed a limited intra-species genetic diversity, and found important sources of genetic variation in small isolated populations, which should be preserved to avoid further genetic decline.

Thus, management efforts like *in situ* and *ex situ* conservation strategies, and reintroduction programs, will need to focus on the existence of defined genetic units where biological diversity, the natural processes of evolution and ecological functions are complied in order to preserve all lineages, to reinforce the species demography, and to avoid undesired genetic admixtures. The consequences of gene flow resulting from transplantations on the genetic structure of particular populations are unpredictable, but possible results include decreased fitness through outbreeding or inbreeding depression and reduction of local variation (Ellstrand and Elam, 1993; Barnaud and Houliston, 2009). Therefore, although based on the evaluation of neutral markers, these data will be helpful in management decision-making.

For instance, haplotype detection presented here might prove useful in regulating artificial inter-island germplasm transfer, and in complementing any future assessment of the species performances by provenance trials (e.g. for reforestations, either with or without a commercial purpose; Ferreira and Eriksson 2006) with lineage trials.

At the same time, it is suggested that populations of Valverde (S. Maria), P. Silveira (S. Jorge), and Lajes (Pico), hosting unique and rare haplotypes, to be kept under *in situ* protection regimes in order to safeguard the survival/spreading of their genepool. However, *ex situ* conservation of their

germplasm for exclusive reintroduction programs should be planned as well, and eco-physiological evaluation of the different haplotype-bearing lineages should be performed by means of progeny trials. Several factors, as said above, should be considered, including seed production and year variation of each tree, seed germination rate, the vigor of the young plants, and rate of survival in the first years (Eriksson et al., 1993). On the other hand, populations fixed for one haplotype (H3 in Nordeste S. Miguel, Pomar in Terceira and S.ta Luzia in Pico; H2 in Rocha do Touro in Flores and Meia Altitude in S. Jorge) should be evaluated for the risk of genetic drift.

In light of the results obtained for the haplotype recognition it is possible to state that germplasm transfers can occur or not between the following populations:

- Haplotype H2 can not go to S.Miguel and S.ta Maria islands;
- Germplasm from the population Fajã Grande, Flores) can be used in Faial island;
- Germplasm from the population Rocha do Touro, Flores can be used in the area contiguous to the population Meia altitude, São Jorge;
- Germplasm from populations of Faial and from Fajã Grande, Flores can be used in the south part of S.Jorge island up to the limit of Urzelina village;
- Germplasm from populations from Rosais and Aeroporto, both from S.Jorge island can be used in the contiguous areas of Praínha do Norte, Piedade and Madalena populations in Pico island;
- Germplasm from the population Pomar, Terceira as well as from the population S.ta Luzia, Pico island can be used in S.Miguel island;
- Germplasm from the population Serreta, Terceira can be used in Faial island and in the contiguous area of the population Fajã Grande, Flores island;
- No germplasm transfers/movements should take place within the populations with a population private haplotype, namely, Valverde, Sta. Maria (H1); Lajes do Pico, Pico (H4) and P. Silveira, S.Jorge (H5).

Seeing that when considering germplasm transfer, the issue most relevant is nuclear genetic diversity, the main question that follows is to investigate the nuclear genetic structure of *Picconia azorica*.

Finally, both research and active measures should also improve knowledge and preservation of frugivorous avian populations, which played a fundamental role in the past to allow natural spread of *Picconia* biodiversity in Macaronesia. The demographic crisis involving two bird taxa feeding on *Picconia*, *Columba palumbus azorica* and *Pyrrhula murina* (Ramos et al., 1996), underpins the need for integrating models of biodiversity preservation in island biotas.

4.4 Germination trials

A first characterization of some seed lots was accomplished and enabled to have an idea of the number of fruits per kilogram as well as other parameters analysed, which could be helpful in the future. Additionally, the tetrazolium test results presented a high percentage of unviable seeds; this aspect should be further inspected in the future to understand the impact it has on the species ecology.

Unfortunately, the results of the germination pre-treatments failed, mainly due to fungi proliferation, damaging the seeds. One reason for the strong fungi development could be the presence of oils in *P. azorica* seed. Apparently, the species in such tests is indeed prone to an extraordinary fungal activity, as the same problem occurred in germination trials carried out by the Terceira Island Forest Services (Belerique, persn. comm.) and it was also the case with *P. excelsa* seeds (Pestana, 2001).

Recent research is focusing in evaluating climate data to predict successful germination conditions and dormancy breaking pre-treatments, as climate and dormancy are related. It is defended that the optimal germination temperature is found in the climate data of the species origin. Despite no results have been achieve with the pre-treatments set, one drawback could have been the temperature at the growth chambers, as it did not reflected the average temperature after the fruit is ripen and becomes part of the soil seed bank. Another drawback could have been having filter

paper as growth medium, probably an agar based one could have been a better alternative.

It is recognized that the ability to germinate all the viable seeds in a collection is essential; it will ensure that the full genetic variability and potential of a population seeds is released for use. Hence, it is vital to define an efficient seed treatment to break *P.azorica* dormancy, as trials completed by the local forest entities demonstrate that it takes nearly 12 months for germination. Moreover, the seed germination rate is quite low, around 20% and not homogenous (Belerique, persn. comm.). The need for forest regeneration material to accomplish restoration actions namely reintroductions of *P.azorica* into the natural environment, and hopefully in the near future forest plantations will increase the demand of such species and there is an urgent need to satisfy such demand. Seed propagation is a viable and plausible way to achieve these objectives, not disregarding the genetic variability assessed in this research.

4.5 Wood characterization

4.5.1 Wood anatomy

It was possible to ascertain that *P.azorica* wood in terms of size and distribution of pores presents an identical structure to the one of *P. excelsa*, *Phillyrea* and *Notelaea* described by Baas et al. (1988). As for *P.excelsa* rays in *P. azorica* tend to be wide 1-2 cells, which is also the case for *Nestegis*. Overall, the characteristics that describe this species wood anatomy are in agreement with the group defined by Baas et al. (1988). In this group one can find the Genera *Phillyrea*, *Picconia*, *Nestegis*, *Notelaea*, amongst others, which are characterized by possessing a "nonseptate libriform fibres, marginal parenchyma, vessels in oblique to dendritic pattern, usually associated with vascular tracheids in a vasicentric position, vessels with well developed spiral thickenings, intervessel pits more than 6 μm in diameter" – such characteristics are verified in *P.azorica*, hence can be legitimately included in this group. Additionally, with such Genera it shares the presence of crystals in ray and axial parenchyma.

4.5.2 Wood technology

Picconia azorica wood is heavy. Moreover, the species is within the ones with high density, with an average value of 0.82 gcm^{-3}, therefore the species is classified as hard (Berti, 1985). It presents a low volumetric shrinkage. The wood is stable, suffering limited deformations, being more stable than *Juglans nigra* and *Fraxinus americana* (USDA, 2009).

From a mechanical point of view the wood is resistant to compression and bending. Nevertheless, these results should be considered with caution due to the limited dimension of the samples used (width 18 - 20 cm) and the scarcity of the available material, as the number of samples to ascertain the resistance and bending was reduced. Even so data obtained allows confirming the designation of the wood as hard.

Overall, the wood behaviour is similar to the one by *Juglans regia*, explaining the local use for furniture making. Other applications to this species wood could be explored, namely utilization on cabinets, in interior panelling, veneer, handles, toys, wooden ware. Such novel applications could boost a niche market and encourage local handcraft, with a positive economy impact.

High heating value is decidedly affected by humidity values, which decreases linearly its calorific potential with the increment in the water content. Moreover, this value for wood material depends in the percentage of lignin, cellulose, hemicelluloses and resin.

With *P.azorica* samples, it was possible to obtain an average HHV of 18.66 MJ/kg. This value means that it is necessary to ”burn” 1 kg of *P.azorica* wood to obtain 18.66 MJ (in terms of heat), at atmospheric pressure. *P.azorica* value classifies the species as medium high/good to be used as biomass. For example, coal has an HHV value of 34.1 MJ/kg and natural gas HHV value is 42.5 MJ/kg. A study conducted by Todaro et al. (2007) for several forest species, grown in Italy forests, ascertained that *Quercus pubescens* had an HHV value of 19.9 MJ/kg whereas the lowest value, 16.9 MJ/kg, was verified in *Onopordum illirium* L. In general terms the species has potential to be used for biomass. Hence, in the future one destiny of *P.azorica* forest management residues could be biomass.

***Taxus baccata* L.**

4.6 The taxonomical and phylogenetic importance of the Azorean yew

The taxonomy of genus *Taxus* is rather complex and still a topic of debate among botanists. Around ten species are currently recognized (*T. baccata* L., *T. brevifolia* Nutt., *T. globosa* Schldl., *T. canadensis* Marsh., *T. floridiana* Nutt., *T. wallichiana* Zucc., *T. yunnanenis* Cheng & Fu, *T. sumatrana* (Miq.) Laubenf., *T. chinensis* (Pilg.) Rehder, *T. cuspidata* Siebold & Zucc.), although some authors support the nonexistence of true species within the genus *Taxus* (cf. Cope, 1998). Indeed, it is surprising the large morphological homogenity that can be observed within some *taxa* with an extremely wide range, such as *T. baccata*.

Despite the fact that Spjut (2006, 2007) identifies several subspecies of *Taxus baccata* in Portugal (mainland, Madeira and Pico Island), the fact is that the authors who contributed to descriptions of Azorean yew always classified it as *T. baccata* L. (Drouet, 1866; Watson, 1870; Trelease, 1897; Guppy, 1917; Dias, 1996). In agreement, the ranking of Pico individuals within a well-defined species, based on both traditional analytical keys, e.g. Flora Europaea (Tutin et al., 1972), and specific studies (Cope, 1998), undoubtedly leads to *T. baccata.* However, Pico individuals have never been the objects of a deep taxonomical study. Only recently, Spjut (2007b) carried out an anatomical evaluation on two samples of *T. baccata* collected in 1972 at Quinta de Rosas (Pico Mountain Plateau, 150 m a.s.l.) and conserved in the Herbarium of the British Museum of Natural History (BM). In this work, Spjut classified the Azorean *Taxus* provenance as *T. baccata* var. *variegata*[1] under the *Elegantissima* Complex of *Baccata* Alliance of its revised classification of genus *Taxus* (in Hageneder, 2007). According to Spjut, this variety could be found also in England (UK), Finland and Sweden, and it may have a hybrid origin. Moreover, some specimens are from

1. It has to be pointed out that the Azorean yew specimen described here presents clear differences with a not well circumstantiated var. *variegata*, described by Dempsey and Hook (2000); this latter is probably different from that of Spjut (2007b).

cultivation. Its main characteristics is considered to be an abaxial leaf margin of four cells across without papillae, followed by 10 rows of papillose cells and with 10–12 rows of stomata. The same peculiar feature was found for the leaf margin in the Pico yews, but other morphological characteristics proposed by Spjut seem less supported. Conversely, leaf dimensions, stomatal density and, especially, DNA data suggest a more complex taxonomical ranking.

Morphometric and anatomical data indicate that Azorean yew samples match the limits of the variation range of *T. baccata,* which, according to Spjut's (2007a) interpretation of the evolutionary trends of morphological characters, would suggest a condition of "ancestry". In agreement, genetic data point out the peculiarity of the Azorean lineage; the phylogenetic analyses suggests a basal position for the Azorean sequences compared with the Afro-European ones, supporting an earlier differentiation of the Pico haplotype from Eurasian *Taxus*.

Yews currently occurring on Pico Island seem therefore to retain some sort of "primitive" traits, and we hypothesize that they are the last survivors of an extremely ancient lineage, older than those present in Eurasia and Africa. Can Azorean yews be really considered an ancestral form of modern European yew? And, in this case, what is the origin of its presence in the Azorean archipelago?

Macaronesian flora experts generally assume that the islands were colonized by long-distance dispersal, with winds and marine current systems allowing the arrival of propagules (Whittaker and Fernández-Palacios, 2007).

In particular, Guppy (1917) reflects on how *T. baccata* arrived in the Azores. He considered that frugivorous birds (namely the Missel thrush, a large European bird of the *Turdidae* in the genus *Turdus*) probably disseminated yew, introducing the first seeds in the archipelago, like other flora species.

Indeed, this hypothesis would be satisfactory to explain the mode of colonization, as *Taxus* is a well-known endozoocorous species, but bird-mediated dispersion should be dated back to ancient times, owing to the differences between Azorean and European yews, which exclude introduction, at least in historical times. Indeed, genetic data seem to highlight the Pico yew sequences as an independent, autochtonous, even more ancient lineage rather than the result of a genetic drift

departing from the European (or African) yew. Present Eurasian lineage would have differentiated later than the one in the Azores, where few representatives of an original lineage would have persisted to date.

Vargas (2007) agrees with the hypothesis that the Macaronesian islands be considered refugial areas for older plant lineages, and suggests the mechanisms by which some ancient species of the subtropical Tertiary vegetation would be still preserved on the Macaronesian islands. Such relict vegetation, well established in the Miocene mild and wet climate, would have been eroded by the Pleistocene climatic change all over the continent(s) but it would have come to constitute part of the Macaronesian Laurissilva on those islands, which acted as refugia owing to climate permissiveness (Bramwell and Richardson, 1973; Mai, 1995). Colonization would have occurred by endozoocory, from the mainland to Macaronesian islands while the archipelagos were forming (Vargas, 2007).

Therefore, *Taxus* from Azores should be considered a relict lineage, i.e. the witness of a subtropical sort of *Taxus "protobaccata"* with a wide distribution in Euro-African lands before the Pleistocene, and brought by birds to the Macaronesian islands, where it would have been conserved, as a rightful element of the Laurissilva (Spada, 2003). If such colonization pattern should hold true, temporal congruence between *Taxus* species evolution and the Azorean archipelago genesis should be inspected.

Macaronesian flora and fauna colonization would have moved across long distances in a stepping stone pattern, starting from the western coast of Morocco (Whittaker and Fernández-Palacios, 2007) and other emerged landmasses (cf. Mai, 1989) during three main temporal windows (Kim et al., 2008): the Middle Miocene (upper Serravallian, 14–11.8 Mya), the upper Miocene (upper Tortonian, 9.6–6.5 Mya) and the late Pliocene (late Zanclean, 5.2–4 Mya).

Taxus can be dated back to the Cenozoic (66.4 Mya) (Brande, 2001). Some European fossil species of *Taxus* can be dated back to the middle Oligocene (32 Mya) (Mai, 1995), but the largest species differentiation is assumed to have happened during Miocene. In particular, *T. baccata,* was present

during the upper Miocene (10–15 Mya) (Hageneder, 2007). It is thus possible that *Taxus* started colonizing the archipelagos during the Tortonian window (9.6–6.5 Mya). Such a primaeval yew would have then preserved the original lineage on the Azores, while differentiating the modern legacies on the Euro-African continents.

4.7 Vegetative propagation

The results obtained cannot be said encouraging as mortality was quite high, and an evident explanation was not found, these also occurred in other similar trials (Maden, 2003). However, one reason could be the period (end of July) in which cuttings were collected, has on similar trials material was obtained in the dormant season, late fall to late winter (Hartmann et al., 1996; Martì, 2007). The nature and substrate properties could have also influenced the cuttings survival by not providing enough aeration. The substrate temperature was never controlled, but references indicate that an average bottom heat temperature of around 20ºC (Martí, 2007) to 23º-27ºC (Hartmann et al., 1996) would be helpful as well. Good results have also been reported for cuttings being collected in May and without bottom heat (Bellarosa, 2003). Despite the good rooting percentage, mortality is these trials were quite high (Bellarosa, persn. comm.). Therefore, if the rooting percentage were considered as the rate of rooted cuttings that survived, these values would have been significantly higher. Interesting is that this author was able to anticipate fructification about 70 years, as some cuttings during the rooting period did in fact fructified. In fact vegetative propagation techniques for yew are well established, however the local phenological cycle of the species has to be accurately studied, in order to identify the optimal period for cutting collection.

A propagation protocol is needed to assure the species conservation and re-introduction in the Azores ecosystems. Hence, a detailed study must the conducted concerning this species asexual propagation, mainly because the species numbers are so scanty and apparently the individuals found do not fructify. As a result, asexual propagation is the means to save this species from an eminent extinction and will be an important aspect to consider on a conservation plan. This propagation method will be the basis to create a clonal bank for the species, where each individual

will be represented. It will not only assure to have several replicas of each individual but also to study and characterize this provenance with as much detail as possible. Moreover, seed orchards can be created, allowing obtaining seeds through open pollination and also through controlled pollination based on genetic data, to enhance the species gene pool. Seed orchards can be an advantageous venue for scientific investigation as well as they can be set at different edaphic conditions, enabling to characterized the species requirements, survival, and so forth. Monitoring will be imperative to appreciate successes and failures, permitting to determine the variables that in the end allow creating self-sustaining populations and ultimately remove the Azorean *Taxus baccata* provenance from its critically endangered status.

4.8 Conservation action plans

Here was described the apparently last yew individuals in the Azores. These investigations allow to consider the Azorean yew to be a paleo-endemism, not yet fully recognized and with great phyto- and phylo-geographical potential interest. Although collected data do not allow the definition of their most appropriated taxonomic rank, and the eventual occurrence of the Azorean (haplo)-type in areas other than those here investigated, phylogeographic reconstruction strongly suggests further research (currently ongoing) in order to assess their actual meaning and importance within the complex evolutionary history and bio-ecology of *T. baccata*.

We cannot exclude that additional individuals could be found in remote sites within this area. Nevertheless, at the time of the fieldwork, only five individuals seemed to persist. Such a tiny number would require extremely urgent conservation strategies: tree populations totaling less than 250 individuals are considered “critically endangered” (cf. IUCN criteria, www.iucn.org), and in this case Azorean *T. baccata* would be really facing extinction. Moreover, during the last three decades, no endemic species, either fauna or flora, have experienced an increase in their effective numbers (Cardoso et al., 2008), except for an Azorean endemic Passeriform, *Pyrrhula murina* Godman (Critically Endangered - IUCN, 2009).

Extreme and lucky recoveries of endemic quasi-extinct taxa have been successfully attempted

with other species in situations similar to that of the Azorean yew, and should be noted as useful examples. Sicilian Fir (*Abies nebrodensis* (Lojac.) Mattei) is a fir native to the Nebrodi and Madonie mountains in northern Sicily and it is considered as "extremely endangered" (cf. IUCN criteria). According to recent estimates its only population consists of 30 adult individuals and a fluctuating number of juveniles derived from natural regeneration; in addition, some hundreds of cultivated plants are preserved as *ex situ* collections (Morandini et al. 1994, Parducci et al., 2001). In 1978, following seed collection, the Forest Service cultivated 110 000 young trees in a nursery. Since the survival rate in nature is so low, an adoption programme was set up in parallel. 40 000 young plants have been planted in the Botanical Garden of Palermo (Italy) as well as in summer villas and second homes in the Madonie Mountains, slightly apart from their natural area of distribution. Several mature trees also grow in botanic gardens and arboreta elsewhere in Europe. Additionally, an EU LIFE-financed project is operational to conserve the existing population. The project includes implementing an action plan that would include forest management, conservation and the gradual elimination of non-indigenous fir species. The goal is to stabilize the current population and improve the survival rate based on natural reproduction. Their location within the Madonie Regional Park guarantees some level of protection.

Another good example is the Café Marron tree, *Ramosmania rodriguesii* Tirveng. & Verdc., endemic to the Mauritian island of Rodrigues, where it was thought to be extinct for forty years until a single surviving tree was spotted by a schoolboy in 1980. Cuttings were taken to Kew Gardens, and although the plant regularly flowers, it never produced seed until horticulturists discovered how to pollinate the flowers (WCMC, 1998). In 2001 eleven rooted cuttings were air-lifted to repatriate the species to the islands. In 2003, the Café Marron bore its first fruit with viable seeds. Slow but steady efforts have been made to grow more Café Marron trees and speed up the pollination process (Royal Botanical Gardens, Kew database, 2003).

In the near future, in addition to further field searches and experimental work it is imperative the absolute protection of each individual. In particular, grazing prevention by fencing is most relevant, in order to allow potential gamic reproduction; ascertain the gender of the individuals, and collect

propagules to start vegetative propagation for new individuals to be preserved *ex situ*, in the event that gamic reproduction will not be feasible. As was done for Café Marron, an appropriate number of cuttings have been taken of Azorean yew. And intensify public awareness at different perceptive levels, including encouraging the collaboration of private individuals for seedling diffusion on their properties.

Since the number of yews is so limited, any form of intervention may appear inadequate, insufficient or difficult; in any case, we must start action soon, to save this lineage from imminent extinction.

5. CONCLUSIONS

This project was focused on two species, *Picconia azorica* and *Taxus baccata*. Both species have had a strong social and economical impact in the past, reason for which their present numbers are so low. An attempt must be made to "save" Azorean forest endemic species. So far no specific conservation action has been specifically targeted at them. And during the last three decades, apart from one endemic passeriforme bird species, no other endemic species (fauna or flora) in Azores has experienced an increase of either its numbers or distribution area.

Researchers all over the World argue that the genetic diversity of a species is the key component for its long-term survival, with bigger impact on rare and endangered species. Moreover, many defend the necessity of a forester being acknowledgeable of these endangered species genetic variation, as this genetic information helps prioritising, in the present case, populations to be conserved and is crucial to formulate specific conservation actions.

The study conducted presented a first genetic characterization of *P. azorica* and *T. baccata* in the Azores, representing the first contribution to understand their genetic diversity. Unfortunately no other studies are available for a comparison, but this one could serve as one for future developments regarding these species. In my opinion, future research on *P.azorica* should concentrate on the implementation of data here presented with nuclear markers to more precisely assess population subdivision and levels of gene flow and stand isolation.

A sound conservation strategy for this species should include its increased use and domestication. Meaning that it should be used in restoration actions, in the management of buffer areas of water basins and other conservation actions, but commercial forest plantations should be implemented as well.

Regarding *Taxus baccata* genetic results, although only preliminary, they suggest that Azorean *T. baccata* represents a separate evolutionary line within *Taxus*, with shared polymorphism speaking in favour of an ancient haplotype, suggesting a derivation closer to the yew ancestral line than those examined from Mediterranean and European regions. In agreement morphometric and anatomical

results confirm this condition of "ancestry". All the same, it is necessary to amplify the sampling area within the species geographical distribution area (Portugal mainland populations, namely Serra do Gerês and Serra da Estrela, Spain, France, Greece, Denmark, etc). Concerning the genetic assessment, it is a priority to investigate other genes to consolidate the results obtained.

As a consequence of this species fragile status it is absolutely necessary to protect each of the five individuals, set in motion more fields' trips to search other individuals and outline a conservation programme and re-introduction actions, considering not only the species ecologic value but also its commercial value (taxol and wood), which will in the medium - long term contribute undoubtedly to the enrichment of Azores forest, and ultimately, its ecosystems.

In sum, a multiple approach conservation strategy will be needed to avoid that *P.azorica* and *T.baccata* go extinct, maintain and/or increase their genetic variation, increase their populations numbers and distribution area is a priority. I strongly believe that both these species can have a positive impact in Azores, allowing adding value to the Azorean forests and ecosystems. A strategy focused in their increased use will allow the implementation of conservation measures, but also the implementation of forest plantations. A trade-off is needed between the two. The commercial and ecological aspects need to complement each other.

6. BIBLIOGRAPHY

AGOSTINHO, J. (1941). Clima dos Açores. IV. *Açoreana.*

AGOSTINHO, J. (1966). The topography and natural features of the Azores archipelago and their influence on the bird life. IN BANNERMAN, D. A. & BANNERMAN, W. M. (Eds.). *Birds of the Atlantic Islands III. A History of the Birds of the Azores.* London, Oliver & Boyd.

ALTSCHUL, S. F., MADDEN, T.L., SCHÄFFER, A.A., ZHANG, J., ZHANG, Z., MILLER, W. AND LIPMAN, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs *Nucleic Acids Res.,* 25**,** 3389-3402.

AMARAL, W., THOMSON, L. & YANCHUK, A. (2004). Introduction. IN FAO, F., IPGRI (Ed.). *Forest genetic resources conservation and management. Vol.1: Overview, concepts and some systematic approaches.* Rome, International Plant Genetic Resources.

ANALYSTSOFT (2008). StatPlus:mac - statistical analysis program for Mac OS. http://www.analystsoft.com/en.

ARRUDA, V. (2004). Caracterização climática das Ilhas do Faial e do Pico com base no modelo CIELO. *Departamento de Ciências Agrárias.* Angra do Heroísmo, Universidade dos Açores.

ARTEAGA, M. A., GONZÁLEZ, G., DELGADO, J. D., ARÉVALO, J. R. & FERNÁNDEZ-PALACIOS (2006). Offspring spatial patterns in Picconia excelsa (Oleaceae). in the Canarian laurel forest. *Flora,* 201**,** 642-651.

AVISE, J. C. (2007). Twenty-five key evolutionary insigths from the phylogeographic revolution in population genetics. IN WEISS, S. & FERRAND, N. (Eds.). *Phylogeography of Southern European Refugia.* Springer.

AXELROD, D. I. (1975). Evolution and biogeography of Mandrean-Tethyan sclerophyll vegetation. *Annals of Missouri Botanical Garden,* 62**,** 280-334.

AZEVEDO, E. B. (1996). Modelação do Clima Insular à Escala Local. Modelo CIELO aplicado à Ilha Terceira. *Departamento de Ciências Agrárias.* Angra do Heroísmo, Universidade dos Açores.

AZEVEDO, E. B., PEREIRA, L.S. AND ITIER, B. (1999). Modelling the local climate in Islands Environments: water balance applications. *Agricultural Water Management,* 40**,** 393-403.

BAAS, P., ESSER, P. M., VAN DER WESTEN, M. E. T. & ZANDEE, M. (1988). Wood anatomy of the Oleaceae. *IAWA Bulletin,* 9**,** 103-182.

BARCELOS, P. (1996). *Análise de crescimento da Cryptomeria japonica (L. Fil) D. Don no concelho do Nordeste - São Miguel.* Relatório de Estágio do Curso de Engenharia Agrícola. Dept. de Ciências Agrárias da Universidade dos Açores.

BARNAUD, A. & HOULISTON, G. J. (2009). Population genetics of the threatened tree daisy Olearia gardneri (Asteraceae), conservation of a critically endangered species. *Conservation Genetics*.

BARRETT, S. C. H., AND KOHN, J.R. (1991). Genetic and Evolutionary Consequences of Small Population Size in Plants: Implications for Conservation. IN FALK, D. A. & HOLSINGER, K. E. (Eds.). *Genetics and Conservation of Rare Plants.* Oxford University Press.

BEGON, M., HARPER, J. L. & TOWNSEND, C. R. (1996). *Ecology: individuals, populations and communities*, Blackwell Science Ltd.

BELLAROSA, R. (2003). La propagazione vegetativa del tasso (Taxus baccata L.). IN SCHIRONE, B., BELLAROSA, R. AND PIOVESAN, G. (Ed.). *Il Tasso - un albero da conoscere e conservare.* Viterbo, Cogecstre Edizioni.

BERTI, R. N. (1979). *La struttura anatomica del legno ed il riconoscimento dei legnami italiani di piú corrente impiego* Firenze, Consiglio nazionale delle ricerche - Istituto del Legno.

BESNARD, G. & BERVILLÉ, A. (2000). Multiple origins for Mediterranean olive (Olea europaea L. subsp. europaea) based upon mitochondrial DNA polymorphisms. *C. R. Acad Sci sér III,* 323**,** 173-181.

BOHLE, U.-R., HILGER, H. H. & MARTIN, W. F. (1996). Island colonization and evolution of the insular woody habitat in Echium L. (Boraginaceae). *Proc Nat Acad Sci USA,* 93.

BOID, C. (1832). A description of the Azores. *Boletim do Instituto Histórico da Ilha Terceira (Tradução de 1949)*. Angra do Heroísmo.

BORGES, P. A. V. (1997). Pasture arthropod community structure in Azorean islands of different geological age. *Department of Biology.* London, Imperial College of Science, Technology and Medicine.

BORGES, P. A. V., CUNHA, R., GABRIEL, R., MARTINS, A.F., SILVA, L. AND VIEIRA, V. (EDS) (2005). *A list of the terrestrial fauna (Mollusca and Arthropoda. and flora (Bryophyta, Pteridaphyta and Spermatophyta). from the Azores,* Horta, Angra do Heroísmo and Ponta Delgada, Direcção Regional do Ambiente and Universidade dos Açores.

BORGES, P. A. V. & BROWN, V. K. (1999). Effect of island geological age on the arthropod species richness of Azorean pastures. *Biol J Linn Soc,* 66**,** 373-410.

BRAMWELL, D., AND RICHARDSON, I.B.K. (1973). Floristic Connections Between Macaronesia and the East Mediterranean Region. *Monographiae Biologicae Canarienses,* N. 4**,** 118-125.

BRANDE, A. (2001). Die Eibe in Berlin einst und jetzt. *Der Eibenfreund,* 8**,** 24-43.

BROMHAM, L. & WOOLFIT, M. (2004). Explosive radiations and the reliability of molecular clocks: island endemic radiations as a test case. *Syst Biol,* 53**,** 758-766.

BURNS, K. C. & NEUFELD, C. J. (2009). Plant extinction dynamics in an insular metacommunity. *Oykos,* 118**,** 191-198.

CANAGARATNA, S. G. & WITT, J. (1988). Calculation of temperature rise in calorimetry. *Journal of Chemical Education,* 65**,** 126-129.

CAPELO, J. M. D. S., M.; JARDIM, R. AND MESQUITA, S. (2007). Biologia e ecologia das florestas das ilhas - Madeira. IN SILVA, E. J. S. (Ed.) *Árvores e Florestas de Portugal.* Fundação Luso Americana para o Desenvolvimento, Público e Liga para a Protecção da Natureza.

CARDOSO, P. B., P.A.V.; COSTA, A.C.; CUNHA, R.T.; GABRIEL, R.; FRIAS MARTINS, A.M.; SILVA, L.; HOMEM, N.; MARTINS, M.; RODRIGUES, P.; MARTINS, B. AND MENDONÇA, E. (2008). A perspectiva arquipelágica: Açores. IN MARTIN, J. L. A., M.; BORGES, P.A.V. AND FARIA, B. (Ed.). *As cem espécies ameaçadas prioritárias em termos de gestão na região europeia biogeográfica da Macaronésia.* Consejería de Medio Ambiente y Ordenación Territorial, Gobierno de Canárias.

CARINE, M. A., RUSSELL, S., SANTOS-GUERRA, A. & FRANCISCO-ORTEGA, J. (2004). Relationships of the Macaronesian and Mediterranean floras: Molecular evidence for multiple colonizations into Macaronesia and back-colonization of the continent in Convolvulus (Convolvulaceae).. *American Journal of Botany,* 91**,** 1070-1085.

CARINE, M. A. & SCHAFER, H. (2009). The Azores diversity enigma: why are there so few Azorean endemic flowering plants and why are they so widespread? *J. Biogeography*.

CARQUEIJEIRO, E. M., H.; SANTOS, R. AND ANTUNES, A. (2005). *Áreas protegidas dos Açores*, Secretaria Regional dos Açores.

CARRACEDO, J. C. P. T., F.J.; ANCOCHEA, E.; MECO, J.; HÉRNAN, F.; CUBAS, C.R.; CASILLAS, R.; RODRÍGUEZ BADIOLA, E. AND AHIJADO, A. (2002). Cenozoic volcanism II: the Canary Islands. IN MORENO, W. G. T. (Ed.). *The geology of Spain.* London, The Geological Society

CATARINO, F. (2007). Relíquias em Terras Altas. IN PÚBLICO, C. S., SA (Ed.). *Do Castanheiro ao Teixo, as outras espécies florestais.* Lisbon, Tipografia Peres, SA.

CEBALLOS, L. (1953). Macaronesia - Algunas consideraciones sobre la flora y vegetación forestal. *Anais do Instituto Superior de Agronomia***,** 79-108.

CENTRE, W. C. M. (1998). Ramosmania rodriguesii. In IUCN Red List of Threatened Species.

CHAFFEY, N. (2002). Wood Microscopical Techniques. IN CHAFFEY, N. J. (Ed.). *Wood formation in trees - cell and molecular biology techniques.* London, New York, Taylor & Francis.

CHIANG, Y. C., HUNG, K. H., SCHAAL, B. A., GE, X. J., HSU, T. W. & CHIANG, T. Y. (2006). Contrasting phylogeographical patterns between mainland and island taxa of the Pinus luchuensis complex. *Molecular Ecology,* 15**,** 765-779.

CLEMENT, M. D., POSADA, M. D. & CRANDALL, K. A. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology,* 9**,** 1657-1660.

CLIMAAT (2009). www.climaat.angra.uac.pt. Angra do Heroísmo.

COMES, H. P. (2004). The Mediterranean region - A hotspot for plant biogeography research. *New Phytol.,* 164**,** 11-14.

COPE, E. A. (1998). Taxaceae: The Genera and Cultivated Species. *Bot. Rev.,* 64**,** 291-322.

CORDEYRO, A. P. (1717). *História Insulana das Ilhas a Portugal Sujeytas no Oceano Occidental,* Terceira, Angra do Heroísmo, Secretaria Regional da Educação e Cultura (1981).

CRAGG, G. M. & NEWMAN, D. J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnoparmacology,* 100**,** 72-79.

CRONK, Q. C. B. (1997). Islands: stability, diversity, conservation. *Biodiversity and Conservation,* 6**,** 477-493.

CRUZ, J. V. A., P.; AMARAL, C.; FRANÇA, Z. AND NUNES, J.C. (2006). Volcanic lakes of the Azores archipelago (Portugal): geological setting and geochemical characterization. *Journal of Volcanology and Geothermal Research,* 156**,** 135-157.

DEMESURE, B., SODZI, N. & PETIT, R. J. (1995). A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Mol. Ecol.,* 4**,** 129-131.

DEMPSEY, D. & HOOK, I. (2000). Yew (Taxus) Species - Chemical and Morphological Variations. *Pharmaceutical Biology (Formerly International Journal of Pharmacognosy),* 38**,** 274-280.

DEPAPE, G. (1922). Recherches sur la flore pliocène de la vallée du Rhône. *Annales des Sciences Naturelles - Botanique,* 10**,** 73-265.

DEVILLERS, P., DEVILLERS-TERSCHUREN, J. & LEDANT, J. P. (1991). CORINE biotopes manual. Habitats of the European Community. Data specifications - Part 2. IN EN, Ed. Luxembourg, Commission of the European Communities, Directorate-General Environment, Nuclear Safety and Civil Protection.

DHAR, A., RUPRECHT, H. & VACIK, H. (2008). Population viability risk management (PVRM) for in situ management of endangered tree species - A case study on a Taxus baccata L. population. *Forest Ecology and Management,* 255**,** 2835-2845.

DI SAPIO, O. A., GATTUSO, S.J. AND GATTUSO, M.A. (1997). Morphoanatomical characters of Taxus baccata bark and leaves. *Fitoterapia,* 68**,** 252-260.

DIAS, E. (1996). Vegetação natural dos Açores. Ecologia e sintaxonomia das florestas naturais. *Angra do Heroísmo.* Universidade dos Açores.

DIAS, E. (2001). *Ecologica e classificação da vegetação natural dos Açores - Cadernos de Botânica 3,* Angra do Heroísmo.

DIAS, E. P., D.; MEDEIROS, V.; MENDES, J.F. AND ELIAS, R.B., (2007). Distribuição das principais manchas florestais - Açores. IN PÚBLICO, C. S., SA (Ed.). *Açores e Madeira, a floresta das ilhas.* Lisboa, Tipografia Peres.

DOYLE, J. J. & DOYLE, J. L. (1990). Isolation of plant DNA from fresh tissue. *Focus,* 12**,** 13-15.

DROTRH/INAG (2001). Plano Regional da água. Relatório técnico. Versão para consulta pública.

DROUET, H. (1866). *Catalogue de la Flore des Iles Açores,* Paris.

DRRF (2007). Inventário Florestal da Região Autónoma dos Açores. Direcção Regional dos Recursos Florestais.

DRUMMOND, F. F. (1859). *Annaes da Ilha Terceira,* Angra do Heroísmo.

DUMOLIN-LAPÈGUE, S., PEMONGE, M. H. & PETIT, R. J. (1997). An enlarged set of consensus primers for the study of organelle DNA in plants. *Mol. Ecol.,* 6**,** 189-191.

DUPANLOUP, I., SCHNEIDER, S. & EXCOFFIER, L. (2002). A simulated annealing approach to define the genetic structure of populations. *Mol. Ecol.,* 11**,** 2571-2581.

EC (1979). Council Directive of 2 April 1979 on the Conservation of Wild Birds 79/409/EEC. European Commission.

EC (1992). Council Directive 92/43/ECC, 21 May 1992 on the conservation of natural habitats and on wild fauna and flora. Brussels, European Commission.

ELLSTRAND, N. C. & ELAM, D. R. (1993). Population genetic consequences of small population size: implication for plant conservation. *Annu Rev Ecol Syst,* 24**,** 217-224.

EMERSON, B. C. (2002). Evolution on oceanic islands: molecular phylogenetic approaches to understanding pattern and process. *Mol. Ecol.,* 11**,** 951-966.

ERIKSSON, G. & EKBERG, I. (2001). *An introduction to forest genetics,* Uppsala, Sweden, Swedish University of Agricultural Sciences.

ERIKSSON, G., EKBERG, I. & CLAPHAM, D. (2006). *An introduction to forest genetics,* Uppsala.

ERIKSSON, G., NAMKOONG, G. & ROBERDS, J. H. (1993). Dynamic gene conservation for uncertain futures. *Forest Ecology and Management,* 62**,** 15-37.

ETC/BD (2005). Natura 2000 unified site database (Special areas of conservation and special protected areas). - Taxus baccata factsheet. IN COMMISSION, Ed. European Topic Centre on Biological Diversity - European Environment Agency.

EUROPA, P. (2009). www.plantaeuropa.org.

EUROPE, C. O. (1993). Convention on the conservation of European wildlife and natural habitats - Annex I. Council of Europe.

EUROPE, C. O. (1998). Resolution No. 6 - listing the species requiring specific habitat conservation measures. Council of Europe.

FAGUNDO, M. C. A. & ISIDORO, A. M. (2006). *Propagação das espécies lenhosas endémicas dos Açores,* Ponta Delgada, Direcção Regional dos Recursos Florestais.

FALK, D. A. (1990). Endangered forest resources in the U.S.: Integrated strategies for conservation of rare species and genetic diversity. *Forest Ecology and Management***,** 91-117.

FAUQUETTE, S., SUC, J.-P., GUIOT, J., DINIZ, F. & FEDDI, N. (1999). Climates and biomes in the Western Mediterranean area during the Pliocene. *Palaeogeogr Paleaoclim Palaeoecol,* 152**,** 15-36.

FELSENSTEIN, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.,* 17**,** 368-376.

FERNANDES, J. G. C. (1985). Terceira - Açores. Estudo geográfico. Universidade dos Açores.

FERREIRA, M., AND ERIKSSON, G. (2006). A Programme for the Management of Forest Tree Genetic Resources in the Azores Islands. *Silva Lusitana,* 14**,** 59-73.

FONTAINE & AL., E. (2007). The European Union's 2010 target: Putting rare species in focus. *Biological Conservation,* 139**,** 167-185.

FRANÇA, Z., CRUZ, J. V., NUNES, J. C. & FORJAZ, V. H. (2003). Geologia dos Açores: uma perspectiva actual. *Açoreana.*

FRANCISCO-ORTEGA, J., SANTOS-GUERRA, A., HINES, A. & JANSES, R. K. (1997). Molecular evidence for a Mediterranean origin of the Macaronesian endemic genus Argyranthemum (Asteraceae). *Am J Bot,* 84**,** 1595-1613.

FRANCISCO-ORTEGA, J., SANTOS-GUERRA, A., KIM, S. C. & CRAWFORD, D. J. (2000). Plant genetic diversity in the Canaries: a conservation perspective. *Am J Bot,* 98**,** 909-919.

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. *Conserv Biol,* 12**,** 665-675.

FRANKHAM, R. (2003). Genetics and conservation biology. *Comp Rendus Biol,* 326**,** 22-29.

FRANKHAM, R. (2005). Genetics and extinction. *Biodiversity and Conservation,* 126**,** 131-140.

FRUTUOSO, G. (1583). *Saudades da Terra,* Ponta Delgada.

GARCÍA, D. & OBESO, J. R. (2003). Facilitation by herbivore-mediated nurse plants in a threatened tree, Taxus baccata: local effects and landscape level consistency. *Ecography,* 26**,** 739-750.

GARCIA-TALAVERA, F. (1997). Las Canarias orientales y la vecina costa africana en el Holoceno. *Eres* 7**,** 55-63.

GARCIA-VERDUGO, C., FAY, M. F., GRANADO-YELA, C., RUBIO DE CASAS, R., BALAGUER, L., BESNARD, G. & VARGAS, P. (2009). Genetic diversity and differentiation processes in the ploidy series of Olea europaea L.: a multiscale approach from subspecies to insular populations. *Mol. Ecol.,* 18**,** 454-467.

GASPAR, C. (2007). Arthropod diversity and conservation planning in native forests of the Azores archipelago. *Depart. of Animal and Plant Sciences.* Sheffield, University of Sheffield.

GELDMACHER, J., BOGAARD, P. V. D., HOERNLE, K. & SCHMINCKE, H. U. (2000). New 40Ar/39Ar dating of the Madeira archipelago and hotspot track (eastern North Atlantic). *Geochem Geophys Geosys,* 1.

GEMMILL, C. E. C., RANKER, T. A., RAGONE, D., PERLMAN, S. P. & WOOD, K. R. (1998). Conservation genetics of the endangered endemic Hawaiian genus Brighamia (Campanulaceae).. *American Journal of Botany,* 85**,** 528-539.

GIELLY, L. A. T., P. (1994). The use of chloroplast DNA to resolve plant phylogenies: noncoding versus rbcL sequences. *Mol. Biol. Evol.,* 11**,** 769-777.

GIORDANO, G. (1999). *Tecnica delle costruzioni in legno,* Milano, Hoepli.

GÓMEZ, A., GONZÁLEZ-MARTÍNEZ, S. G., COLLADA, C., CLIMENT, J. & GIL, L. (2003). Complex population genetic structure in the endemic Canary Island pine revealed using chloroplast microsatellite markers. *Theor Appl Genet,* 107**,** 1123-1131.

GONZÁLEZ-PEREZ, M. A., SOSA, P. A., RIVERO, E., GONZÁLEZ-GONZALEZ, E. A. & NARANJO, A. (2009). Molecular markers reveal no genetic differentiation between Myrica rivas-martinezii and M. faya (Myricaceae). *Ann Bot,* 103**,** 79-86.

GOODMAN, J. & WALSH, V. (2001). *The story of Taxol: nature and politics in the pursuit of an anti-cancer drug.,* Cambridge, Cambridge University Press.

GOSLING, P. (2007). *Raising trees and shrubs from seed,* Edinburgh, Forestry Commission.

GREGORIUS, H. R. (1991). Species conservation and the preservation of adaptability. *Theor Pop Biol,* 40**,** 269-283.

GUPPY, H. B. (1917). *Plants, seeds and currents in the West Indies and Azores,* London.

HAGENEDER, F. (2007). *Yew. A History.* Stroud, Sutton Publishing.

HAGGAR, J. P. (1988). The Structure, Composition and Status of the Cloud Forests of Pico Island in the Azores. *Biological Conservation***,** 7-22.

HAMRICK, J. L., GODT, M. J. W., MURAWSKI, D. A. & LOVELESS, M. D. (1991). Correlation between species traits and allozyme diversity: implications for conservation biology. IN FALK, D. A. & HOLSINGER, K. E. (Eds.). *Genetics and conservation of rare plants.* New York, Oxford University Press.

HAO, D. C., HUANG, B. AND YANG, L. (2008). Phylogenetic relationships of the genus Taxus inferred from chloroplast intergenic spacer and nuclear coding DNA. *Biol. Pharm. Bulletin,* 31**,** 260-265.

HARTMANN, H. T., KESTER, D. E., DAVIES, F. T. J. & GENEVE, R. L. (1996). *Plant propagation: principles and practices*, Prentice Hall.

HELFGOTT, D. M., FRANCISCO-ORTEGA, J., SANTOS-GUERRA, A., JANSEN, R. K. & SIMPSON, B. B. (2000). Biogeography and breeding system evolution of the woody Bencomia alliance (Rosaceae). in Macaronesia based on ITS sequence data. *Syst Biol,* 25**,** 82-97.

HEUERTZ, M., CARNEVALE, S., FINESCHI, S., SEBASTIANI, F., HAUSMAN, J. F., PAULE, L. & VENDRAMIN, G. G. (2006). Chloroplast DNA plylogeography of European ashes, Fraxinus sp. (Oleaceae): roles of hybridization and life history traits. *Mol. Ecol.,* 15**,** 2131-2140.

HEUERTZ, M., FINESCHI, S., ANZIDEI, M., PASTORELLI, R., SALVINI, D., PAULE, L., FRASCARIA-LACOSTE, N., HARDY, O. J., VEKEMANS, X. & VENDRAMIN, G. G. (2004). Chloroplast DNA variation and postglacial recolonisation of common ash (Fraxinus excelsior L.). in Europe. *Mol. Ecol.,* 13**,** 3437-3452.

HEWITT, G. M. (2000). The genetic legacy of the quaternary ice ages. *Nature,* 405**,** 907-913.

HOADLEY, R. B. (1991). *Identifying Wood, accurate results with simple tools*, The Taunton Press.

HOBOHM, C. (2000). Plant species diversity and endemism on islands and archipelagos, with special reference to the Macaronesian Islands. *Flora,* 195**,** 9-24.

HUENNEKE, L. F. (1991). Ecological implications of genetic variation in plant populations. IN FALK, D. A. & HOLSINGER, K. E. (Eds.). *Genetics and conservation of rare plants.* New York, Oxford University Press.

HUMPHRIES, C. J. (1979). Endemism and Evolution in Macaronesia. IN BRAMWELL, D. (Ed.). *Plants and Islands.* London.

IM (2008). Instituto de Meteorologia - Perfil Climático dos Açores.

ISTA (2004). *International Rules for Seed Testing*.

ISZKULO, G., JASINSKA, A.K., GIERTYCH, M.J. AND BORATYNSKI, A. (2009). Do secondary sexual dimorphism and female intolerance to drought influence the sex ratio and extinction risk of Taxus baccata? *Plant Ecology***,** 229-240.

ITOKAWA, H. (2003). Taxoids occuring in the genus Taxus. IN ITOKAWA, H. & LEE, K.-H. (Eds.). *Taxus: the genus Taxus, Medicinal and Aromatic Plants - Industrial Profiles.* Taylor & Francis.

IUCN (2009). 2008 IUCN Red List of Threatened Species. IUCN.

JAMIESON, I. G. (2007). Has the debate over genetics and extinction of island endemics truly been resolved? *Anim Conserv,* 10**,** 139-144.

JENNEWEIN, S. & CROTEAU, R. (2001). Taxol: biosynthesis, molecular genetics and biotechnological applications. *Appl. Microbiol. Biotechnology,* 57**,** 13-19.

JIMENEZ, P., LOPEZ-DE-HEREDIA, U., COLLADA, C., LORENZO, Z. & GIL, L. (2004). High variability of chloroplast DNA in three Mediterranean evergreen oaks indicates complex evolutionary history. *Heredity,* 93**,** 510-515.

JONES, N. O., H. AND THOMAS, H. (1997). Markers and mapping: we are all geneticists now. *New Phytol.,* 137**,** 165-177.

JORGENSEN, T. H. & FRYDENBERG, J. (1999). Diversification in insular plants: inferring the phylogenetic relationship in Aeonium (Crassulaceae). using ITS sequences of nuclear ribosomal DNA. *Nord J Bot,* 19**,** 613-621.

JUAN, C., EMERSON, B. C., OROMÍ, P. & HEWITT, G. M. (2000). Colonization and diversification: towards a phylogeographic synthesis for the Canary Islands. *Tr Ecol Evol,* 15**,** 104-109.

KARNOVSKY, M. J. (1965). A formaldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell. Biol.,* 27**,** 137A-138A.

KAY, K. M., WHITTAL, J. B. & HODGES, S. A. (2006). A survey of nuclear ribosomal internal transcribed spacer substitution rates across angiosperms: an approximate molecular clock with life history effects. *BMC Evol Biol 6***,** 36.

KHOSROUSHAHI, A. Y., VALIZADEH, M., GHASEMPOUR, A., KHOSROWSHAHLI, M., NAGHDIBADI, H., DADPOUR, M. R. & OMIDI, Y. (2006). Improved taxol production by combination of inducing factors in suspension cell culture of Taxus baccata L. *Cell Biology International,* 30**,** 262-269.

KIKUCHI, Y. & YATAGAI, M. (2003). Commercial cultivation of Taxus species. IN ITOKAWA, H. & LEE, K.-H. (Eds.). *Taxus: the genus Taxus, Medicinal and Aromatic Plants - Industrial Profiles.* Taylor & Francis.

KIM, S.-C. M., M.R.; LUBINSKY, P.; BARBER, J.C.; MORT, M.E. AND SANTOS-GUERRA, A. (2008). Timing and tempo of early and successive adaptive radiations in Macaronesia. *PloS One,* 3.

KIMURA, M. (1980). A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.,* 16**,** 111-120.

KNOBLAUCH, E. (1895). Oleaceae. *Die Naturlichen Pflanzenfamilien IV, 2: 1-16.* In: Engler A. (ed.).

KRESS, W. J. A. E., D.L. (2007). A Two-Locus Global DNA Barcode for Land Plants: The Coding rbcL Gene Complements the Non-Coding trnH-psbA Spacer Region. *PloS One***,** 1-10.

KRIJGSMAN, W., HILGEN, F. J., RAFFI ISIERRO, F. J. & WILSON, D. S. (1999). Chronology, causes and progression of the Messinian Salinity Crisis. *Nature,* 400**,** 652-655.

LO MONACO, A. & BERNABEI, M. (2003). Il legno di tasso. IN SCHIRONE, B., BELLAROSA, R. AND PIOVESAN, G. (Ed.). *Il Tasso - un albero da conoscere e conservare.* Viterbo, Cogecstre Edizioni.

LOWE, R. T. (1868). *A Manual Flora of Madeira and the Adjacent Islands of Porto Santo and the Desertas,* London.

MABEY, R. (1996). *Flora Britannica: the definitive new guide to Britain's wild flowers, plants and trees,* London, Sinclair-Stevenson Ltd.

MACEDO (1871). *Arquivo dos Açores,* Ponta Delgada.

MADEN, K. (2003). Community trial on the propagation and conservation of Taxus baccata L. *Our Nature,* 1**,** 30-32.

MADRUGA, J. S. (1986). Andossolos dos Açores. Contributo para o seu estudo. Angra do Heroísmo, Universidade dos Açores, Dept. de Ciências Agrárias.

MAI, D. H. (1989). Development and regional differentiation of the European vegetation during the Tertiary. *Pl. Syst. Evol.,* 162**,** 79-91.

MAI, D. H. (1995). *Tertiare Vegetationgeschichte Europas,* Jena, Stuttgart, New York, Gustav Fischer.

MAKI, M. (2001). Genetic differentiation within and among island populations of the endangered plant Aster miyagii (Asteraceae)., an endemic to the Ryukyu Islands. *Am J Bot,* 88**,** 2189-2194.

MALIYAKAL, E. J. (1992). An efficient method for isolation of RNA and DNA from plants containing polyphenolics. *Nucleic Acid Research,* 20.

MANZI, A. (2003). Il tasso nella cultura popolare abruzzese. IN SCHIRONE, B., BELLAROSA, R. AND PIOVESAN, G. (Ed.). *Il Tasso - un albero da conoscere e conservare.* Viterbo, Cogecstre Edizion.

SRAM (2005). *Áreas ambientais dos Açores,* Ponta Delgada, Secretaria Regional do Ambiente e do Mar.

MARLER, P. A. B., D.J. (1952). An analysis of the vegetation of the Northern slopes of Pico - the Azores. *The Journal of Ecology,* 40**,** 143-155.

MARRERO-GÓMEZ, M. V., BAÑARES-BAUDET, A. & CARQUÉ-ALAMO, E. (2003). Plant resource conservation planning in protected natural areas: an example from the Canary Islands, Spain. *Biological Conservation***,** 399-410.

MARTÍ, X. G. (2007). Producción de material forestal de Taxus baccata L. destinado a planes de conservación. IN GENERALITAT VALENCIANA, C. D. T. I. H. (Ed. *El tejo en el Mediterráneo Occidental: Jornadas internacionales sobre el tejo y las tejeras en el Mediterráneo Occidental.*

MARTÍN, J. L., ARECHAVALETA, M., BORGES, P.A.V. AND FARIA, B. (2008). A perspectiva macaronésia. IN J. L MARTÍN, M. A., P.A.V. BORGES & B. FARIA (Ed.). *TOP 100 - As cem espécies ameaçadas prioritárias em termos de gestão na região europeia biogeográfica da Macaronésia.*, Consejería de Medio Ambiente y Ordenación Territorial, Gobierno de Canarias.

MARTÍN, J. L. A., M.; BORGES, P.A.V. AND FARIA, B. (2008). *As cem espécies ameaçadas prioritárias em termos de gestão na região europeia biogeográfica da Macaronésia*, Gotemberg Digital.

MARTINS, A. M. F. (1993). The Azores - Westernmost Europe: where evolution can be caught red-handed. *Bol. Mus. Mun. Funchal,* Sup. Nº 2**,** 181-198.

MARTINS, F. E. O. (1981). *Mobiliário Açoriano, elementos para o seu estudo,* Angra do Heroísmo, Direcção Regional dos Assuntos Culturais.

MARTINS, R., RODRIGUES, A. & CUNHA, R. (2002). *Aves Nativas dos Açores*, João Azevedo Editor.

MCDONALD, B. (1986). *Practical Wood Plant Propagation for Nursery Growers*, Timber Press.

MÉDAIL, F., AND QUÉZEL, P. (1997). Hot-spots analysis for conservation of plant biodiversity in the Mediterranean Basin. *Annals of Missouri Botanical Garden,* 84**,** 112-127.

MEDCALC, S. (2009). Digimizer 3.6.0. 3.6.0 ed. Gent - Belgium.

MELVILLE, R. (1979). Endangered island floras. *In:* BRAMWELL, D. (ed.) *Plant and Islands.* London.

MILLER, R. O. (1998). High-temperature oxidation: dry ashing. IN KALRA & ED., Y. (Eds.). *Handbook and reference methods for plant analysis.* New York, CRC Press.

MIRANDA, S. (1995). *O ciclo da laranja e os "gentlemen farmers" da Ilha de S.Miguel - 1780 a 1880*, Instituto Cultural de Ponta Delgada.

MITCHELL, A. K. (1998). Acclimation of Pacific yew (Taxus brevifolia). foliage to sun and shade. *Tree Physiology,* 18**,** 749-757.

MOHAPATRA, K. P., SEHGAL, R. N., SHARMA, R. K. & MOHAPATRA, T. (2009). Genetic analysis and conservation of endangered medicinal tree species Taxus wallichiana in the Himalayan Region. *New Forests,* 37**,** 109-121.

MOORE, M., FRANCISCO-ORTEGA, J., SANTOS-GUERRA, A. & JANSEN, R. K. (2002). Chloroplast DNA evidence for the roles of island colonization and extinction in Tolpis (Asteraceae: Lactuceae). *Am J Bot,* 89**,** 518-526.

MORANDINI, R., DUCCI, F. & MENGUZZATO, G. (1994). Abies nebrodensis (Lojac.). Mattei. *Inventario 1992. Ann. Ist. Sper. Selv. XXII.*

MYKING, T. (2002). Evaluating genetic resources of forest trees by means of life history traits - a Norwegian example. *Biodiversity and Conservation,* 11**,** 1681-1696.

NEWTON, A. C. A., T.R.; GILLIES, A.C.M.; LOWE, A.J. AND ENNOS, R.A. (1999). Molecular phylogeography, intraspecific variation and the conservation of tree species. *Tree,* 14**,** 140-145.

NIELSEN, L. R. (2004). Molecular differentiation within and among island populations of the endemic plant Scalesia affinis (Asteraceae). from the Galápagos Islands. *Heredity,* 93**,** 434-442.

PALHINHA, R. T. (1966). *Catálogo das plantas vasculares dos Açores,* Lisboa, Sociedade de Estudos Açorianos Afonso Chaves.

PARDUCCI, L., SZMIDT, A. E., MADAGHIELE, A., ANZIDEI, M. & VENDRAMIN, G. G. (2001). Genetic variation at chloroplast microsatellites (cpSSRs). in Abies nebrodensis (Lojac.) Mattei and three neighboring Abies species. *Theor Appl Genet,* 102**,** 773-740.

PARMAR, V. S., JHA, A., BISHT, K. S., TANEJA, P., SINGH, S. K., KUMAR, A., POONAM, J. & OLSEN, C. E. (1999). Constituents of the yew trees. *Phytochemistry* 50**,** 1267-1304.

PEAKALL, R. & SMOUSE, P. E. (2006). GENEALEX 6.0: genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes,* 6**,** 288-295.

PENA, A. & CABRAL, J. (1997). Roteiros da Natureza. *Temas e Debates.* Funchal, Região Autónoma da Madeira.

PEREIRA, J. C., SCHAFER, H. & PAIVA, J. (2002). New records of Veronica dabneyi Hochst. (Scrophulariaceae)., an Azorean endemic plant not collected since 1983. *Botanical Journal of the Linnean Society***,** 311-315.

PEREIRA, M. J. A., R.; FURTADO, D. AND CABRAL, N. (2007). Breve caracterização da flora vascular corvina. *XIII Expedição Científica do Departamento de Biologia - Flores e Corvo 2007, Rel. Com. Dep. Biol. 35:123.* Ponta Delgada, Universidade dos Açores.

PESTANA, M. C. F. (2001). Estudos de germinação de endemismos madeirenses e macaronésicos. Angra do Heroísmo, University of the Azores.

PETIT, R. J. & HAMPE, A. (2006). Some evolutionary consequences of being a tree. *Annu Rev Ecol Syst,* 37**,** 187-214.

PETIT, R. J. & VENDRAMIN, G. G. (2007). Plant plylogeography based on organelle genes: an introduction. *In:* WEISS, S. & FERRAND, N. (eds.). *Phylogeography of Southern European Refugia.* Springer.

PINHEIRO, J. (2007). Génese e características dos Andossolos dos Açores. Angra do Heroísmo, Universidade dos Açores.

PINHEIRO, J. V. F. (1990). Estudo dos principais tipos de solos da Ilha Terceira (Açores). *Dept. Ciências Agrárias.* Angra do Heroísmo, Universidade dos Açores.

PIOTTO, B. & DI NOI, A. (2001). Propagazione per seme di alberi e arbusti della flora mediterranea. Rome, ANPA - Agenzia Nazionale per la Protezione dell'Ambiente.

PIOVESAN, G., PRESUTTI SABA, E., BIONDI, F., ALESSANDRINI, A., DI FILIPPO, A. & SCHIRONE, B. (2009). Population ecology of yew (Taxus baccata L.). in the Central Apennines: spatial patterns and their relevance for conservation strategies. *Plant Ecology,* (in press).

PONS, O. & PETIT, R. J. (1996). Measuring and testing genetic differentiation with ordered versur unordered alleles. *Genetics,* 144**,** 1237-1245.

PONS, O. A. P., R.J. (1995). Estimation, variance and optimal sampling of gene diversity - I. Haploid locus. *Theor Appl Genet,* 90**,** 462-470.

POREBSKI, S. B., L.G. AND BAUM, B.R. (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Molecular Biology Reporter,* 15**,** 8-15.

POSADA, D. & CRANDALL, K. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics,* 14**,** 817-818.

PRENTICE, H. C., MALM, J. U., MATEU-ANDRES, I. & SEGARRA-MORAGUE, J. G. (2003). Allozyme and chloroplast DNA variation in island and mainland populations of the rare Spanish endemic, Silene hifacensis (Caryophyllaceae).. *Conserv Genet,* 4**,** 543-555.

RABAEY, D., HUYSMANS, S., LENS, F., SMETS, E. AND JANSEN, S. (2008). Micromorphology and systematic distribution of pit membrane thickenings in Oleaceae: tori and pseudo-tori. *IAWA Journal,* 29**,** 409-424.

RAJORA, O. P. & MOSSELER, A. (2001). Challenges and opportunities for conservation of forest genetic resources. *Euphytica***,** 197-212.

RAMOS, J. A. (1996). Introduction of exotic tree species as a threat to the Azores bullfinch population. *J. Appl. Ecol.,* 33**,** 710-722.

RODRIGUEZ-SANCHEZ, F., GUZMAN, B., VALIDO, A., VARGAS, P. & ARROJO, J. (2009). Late Neogene history of the laurel tree (Laurus L., Laureaceae). based on phylogeopgraphical analyses of the Mediterranean and Macaronesian populations. *J. Biogeography,* 36**,** 1270-1281.

ROZAS, J., SÁNCHEZ-DELBARRIO, J. C., MESSENGER, X. & ROZAS, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics,* 19**,** 2496-2497.

SALISBURY, E. J. (1927). On the causes and ecological significance of stomatal frequency, with special reference to the woodland flora. *Philosophical Transactions of the Royal Society of London,* B 216**,** 1-65.

SANTOS, A. (1990). *Bosques de Laurisilva en la región macaronésica,* Estrasburgo, Council of Europe.

SAPORTA, G. (1865). Études sur la végétation du sud-est de la France l'époque tertiaire. *Annales des Sciences Naturelles - Botanique*.

SAVILL, P. S. (1991). *The Silviculture of Trees used in British Forestry,* London, CAB International.

SCHAAL, B. A., HAYWORTH, D. A., OLSEN, K. M., RAUSCHER, J. T. & SMITH, W. A. (1998). Phylogeographic studies in plants: problems and prospects. *Molecular Ecology,* 7**,** 465-474.

SCHAAL, B. A., LEVERICH, W. J. & ROGSTAD, S. H. (1991). Comparison of methods for assessing genetic variation in plant conservation biology. IN FALK, D. A. & HOLSINGER, K. E. (Eds.). *Genetics and conservation of rare plants.* New York, Oxford University Press.

SCHAFER, H. (2002). *Flora of the Azores, A Field Guide*, Margraf Verlag.

SCHUELER, S., TUSCH, A., SCHUSTER, M. & ZIEGENHAGEN, B. (2003). Characterization of microsatellites in wild and sweet cherry (Prunus avium L.). - markers for individual identification and reproductive processes. *Genome,* 46**,** 95-102.

SCHULER, G. D. (2001). Sequence aligment and database searching. IN BAXEVANIS, A. D. & OUELLETTE, B. F. (Eds.). *Bioinformatics - a pratical guide to analysis of genes.* second ed., John Wiley & Sons, Inc.

SEBASTIANI, F., CARNEVALE, S. & VENDRAMIN, G. G. (2004). A new set of mono- and dinucleotide chloroplast microsatellites in Fagaceae. *Mol. Ecol. Notes,* 4**,** 259-261.

SERRALHEIRO, A. & MADEIRA, J. (1993). Stratigraphy and geochronology of Santa Maria Island (Azores).. *Açoreana,* 7**,** 575-592.

SEUBERT, M., AND HOCHSTETTER, C. (1844). *Flora Azorica,* Bonn.

SILVA, J. S. (2007). *Açores e Madeira: a floresta das ilhas,* Lisbon, Público.

SILVA, L., LAND OJEDA, E., RODRÍGUEZ LUENGO, J. L., BORGES, P., OLIVEIRA, P. & JARDIM, R. (2008). Invasive alien species in Macaronesia. IN SILVA, L., LAND OJEDA, E. & RODRÍGUEZ LUENGO, J. L. (Eds.). *Invasive Terrestrial Flora & Fauna of Macaronesia. TOP 100 in Azores, Madeira and Canaries.* Ponta Delgada, ARENA.

SILVA, L. & SMITH, C. W. (2004). A characterization of the non-indigenous flora of the Azores Archipelago. *Biological Invasions,* 6**,** 193-204.

SILVA, L. & SMITH, C. W. (2006). A quantitative approach to the study of non-indigenous plants: an example from the Azores Archipelago. *Biodiversity and Conservation,* 15**,** 1661-1679

SIMEONE, M.C. (2007). Phylogeography of the cork oak (Quercus suber L.) in Italy. PhD thesys (In Italian), Department of Forestry and Environment, (D.A.F. Universita' della Tuscia, Viterbo, Italy.

SJOGREN, E. (1990). Bryophyte flora and vegetation on the island of Graciosa (Azores)., with remarks on floristic diversity of the Azorean islands. *Arquipélago.*

SJOGREN, E. (2001). *Plantas e Flores dos Açores*.

SMITH, S. A. & DONOGHUE, M. J. (2008). Rates of molecular evolution linked to life history in flowering plants. *Science,* 322**,** 86-89.

SPADA, F. (2003). Considerazioni sulla fitogeografia e sulla genesi delle comunità a Taxus baccata L. in Europa. IN SCHIRONE, B., BELLAROSA, R. AND PIOVESAN, G. (Ed.). *Il Tasso - un albero da conoscere e conservare.* Viterbo, Cogecstre Edizioni.

SPEA (2008). Recuperação do habitat do Priolo na ZPE Pico da Vara/Ribeira do Guilherme. IN SPEA (Ed. *Relatório de Progresso do Projecto LIFE Nat/P/000013, 1 de Novembro de 2006 a 30 de Setembro de 2007.* Sociedade Portuguesa para o Estudo das Aves.

SPJUT, R. W. (2007a). A phytogeographical analysis of Taxus (Taxaceae). based on leaf anatomical characters. *J. Bot. Res. Inst. Texas,* 1**,** 291-332.

SPJUT, R. W. (2007b). Taxus baccata alliance. *www.worldbotanical.com/index/html#outline*.

SPJUT, R. W. (2008). Introduction to Taxus: Methodology, taxonomic relationships, leaf and seed characters, phytogeographical relationships, cultivation, and chemistry. Section on Nomenclatural Problems. *www.worldbotanical.com/index/html#outline*.

SRAM (2005). Plano Sectorial para a Rede Natura 2000 para a Região Autónoma dos Açores. Horta, Secretaria Regional do Ambiente e do Mar (SRAM).

SRAM (2007). Carta de Ocupação do Solo da Região Autónoma dos Açores. Direcção Regional do Território e dos Recursos Hídricos.

STORFER, A. (1999). Gene flow and endangered species translocations: a topic revised. *Biological Conservation,* 87**,** 173-180.

STROBEL, G. A., AND HESS, W.M (1996). A scanning eletron microscopy study of Taxus leaves as related to taxonomy. *Scanning Microscopy,* 10**,** 1111-1126.

SUNDING, P. (1979). Origins of the Macaronesian Flora. IN BRAMWELL, D. (Ed.). *Plants and Islands.* London.

SVENNING, J.-C., AND MAGARD, E. (1999). Population ecology and conservation status of the last natural population of English yew Taxus baccata in Denmark. *Biological Conservation***,** 173-182.

SWEENEY, C. A. (2004). A key for the identification of stomata of the native conifers of Scandinavia. . *Review of Palaeobotany and Palynology,* 128**,** 281-290.

SWOFFORD, D. L. (2002). *PAUP*, Phylogenetic Analysis Using Parsimony (*and other Methods).. Version 4.10b.*, Sunderland: Sinauer Associates.

TABERLET, P., GIELLY, L., PATOU, G. & BOUVET, J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol Biol,* 17**,** 1105-1109.

TAKHTAJAN, A. (1969). *Flowering plants: origin and dispersal,* Edinburgh, UK, Oliver and Boyd.

TAMURA, K., DUDLEY, J., NEI, M. & KUMAR, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA). software version 4.0. *Molecular Biology and Evolution,* 24**,** 1596-1599.

TEJEDOR & AL, E. (1985). Suelos de la Región Macaronésica. Andosoles y Suelos Ferralíticos de las Islas Azores. IN AÇORIANAS, A. J. A. (Ed.).

TODARO, L., SCOPA, A. & DE FRANCHI, A. S. (2007). Caratterizzazione energetica di biomasse agro-forestali presenti in aree collinari e montane della Basilicata. *Forest@,* 4**,** 42-50.

THOMAS, P. A., AND POLWART, A. (2003). Biological Flora of the British Isles - Taxus baccata L. *Journal of Ecology,* 91**,** 489-524.

THOMÉ, O. W. (1885). *Flora von Deustschland Osterreich und der Schweiz*.

THOMPSON, J. D., HIGGINS, D. J. & GIBSON, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalities and weight matrix choice. *Nucl. Acids Res.,* 22.

TRELEASE, W. (1897). Botanical Observations on the Azores. *Missouri Botanical Garden Eight Annual Report.* St. Louis, Missouri Botanical Garden.

TUROK, J. & GEBUREK, T. (2000). International collaboration on forest genetic resources: The role of Europe. IN TUROK, J. & GEBUREK, T. (Eds.). *Second EUFORGEN Sterring Committee meeting.* 26-28 November 1998, Plant Genetic Resources Institute, Rome, Italy.

TUTIN, T. G. (1933). Notes from the university herbarium, Cambridge. On Ilex perado Ait. and Notelaea excelsa. *J. Bot.,* 71**,** 99-101.

TUTIN, T. G. (1953). The vegetation of the Azores. *The Journal of Ecology,* 41**,** 53-61.

TUTIN, T. G. (1972). *Flora Europeae - Lycopodiaceae to Plataneaceae,* Cambridge, Cambridge University Press.

TUTIN, T. G., HEYWOOD, V. H., BURGES, N. A., VALENTINE, D. H., WALTERS, S. M. & D.A., W. (1972). *Flora Europeae - Lycopodiaceae to Plataneaceae,* Cambridge, Cambridge University Press.

UNI EN 1534 (2002). Determination of resistance to indentation (Brinell).

UNI ISO 3131 (1985). Determination of density for physical and mechanical tests.

UNI ISO 3133 (1985). Determination of ultimate strength in static bending.

UNI ISO 3787 (1985). Determination of ultimate stress in compression parallel to grain.

UNI ISO 4469 (1985). Determination of radial and tangential shrinkage.

UNI ISO 4858 (1988). Determination of volumetric shrinkage.

VAN RIEL, P. J., K.; VAN HOUTTE, N.; MARTINS, A.M.F.; VERHAGEN, R. AND BACKELJAU, T. (2005). Molecular systematics of the endemic Leptaxini (Gastropoda: Pulmonata). on the Azores islands. *Molecular Phylogenetics and Evolution* 37**,** 132-143.

VARGAS, P. (2007). Are Macaronesian islands refugia of relict plant lineages?: a molecular survey. IN WEISS, S., AND FERRAND, N. (Ed.). *Phylogeography of Southern European Refugia: Evolutionary Perspectives on the Origins and Conservation of European Biodiversity.* Springer.

VARMA, A. P., H. AND SHRIVASTAVA, N. (2007). Plant genomic DNA isolation: An art or a science. *Biotechnology Journal,* 2**,** 386-392.

VAXEVANIDOU, Z., GONZÁLEZ-MARTÍNEZ, S. G., CLIMENT, J. & GIL, L. (2006). Tree populations bordering on extinction: a case study in the endemic Canary Island pine. *Biological Conservation,* 129.

W.-C. LIEW, A., H., Y. & YANG, M. (2005). Data Mining for Bioinformatics. IN CHEN, Y.-P. P. (Ed.). *Bioinformatics Technologies.* Berlin, Heidelberg, New York, Springer-Verlag

WALLANDER, E. & ALBERT, V. A. (2000). Phylogeny and classification of Oleaceae based on Rps16 and TrnL-F sequence data. *Am J Bot,* 87**,** 1827-1841.

WATSON, H. C. (1870). Botany of the Azores. *Natural History of the Azores or Western Islands.* London, Godman, F.D.C.

WEBSTER, J. W. (1821). *Description of the Island of St. Michael,* Boston.

WEISING, K. & GARDNER, R. C. (1999). A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. *Genome,* 42**,** 9-19.

WEISS, S. & FERRAND, N. 2007. Current perspectives in phylogeograhpy and the significance of South European refugia in the creation and maintenance of European biodiversity. *In:* WEISS, S. & FERRAND, N. (eds.). *Phylogeography of Southern European Refugia.* Springer.

WESCHE, K., HENSEN, I. & UNDRAKH, R. (2006). Range-wide genetic analysis provides evidence of natural isolation among populations of the Mongolian endemic Potentilla ikonnikovii Juz. (Rosaceae).. *Plant Species Biology***,** 155-163.

WHEELER, E. A., BAAS, P., GASSON, P.E. (1989). IAWA list of microscopic features for hardwood identification by an IAWA Committee. *IAWA Bulletin,* 10**,** 219-332.

WHITE, P. S. & MATISE, T. C. (2001). Genomic mapping and mapping databases. IN BAXEVANIS, A. D. & OUELLETTE, B. F. (Eds.). *Bioinformatics - a practical guide to the analysis of genes and proteins.* Second ed., Wiley - Interscience.

WHITTAKER, R. J. (2000). Scale, succession and complexity in island biogeography: are we asking the right question? *Glob Ecol Biogeogr,* 9**,** 75-85.

WHITTAKER, R. J., AND FERNÁNDEZ-PALACIOS, J.M. (2007). *Island Biogeography - Ecology, Evolution and Conservation,* London, OUP London.

WHITTAKER, R. J., TRIANTIS, K. A. & LADLE, R. J. (2008). A general dynamic theory of oceanic island biogeography. *J Biogeogr,* 35**,** 977-994.

WILMER, C. M. & FRICKER, M. (1996). *Stomata,* London, Chapman & Hall.

WOLF, A. (2001). Conservation of endemic plants in serpentine landscapes. *Biological Conservation***,** 35-44.

WU, W., WELSH, M. J., KAUFMAN, P. B. & ZANG, H. H. (2004). *Gene Biotechnology*, CRC Press.

YAMANE, K., YANO, K. & KAWAHARA, T. (2006). Pattern and rate of indel evolution inferred from whole chloroplast intergenic regions in sugarcane, maize and rice. *DNA Res,* 13**,** 197-204.

ANNEX I

Table A - List of species and populations sampled for this research.

Taxon	Origin	Population	Latitude	Longitude
Picconia azorica Tutin (Knobl)	Faial Island	Varadouro	38º 33’	28º 45’
		Capelo	38º 34’	28º 46’
	Flores Island	Rocha do Touro	39º 29’	31º 15’
		Fajã Grande	39º 25’	31º 13’
	Pico Island	Santa Luzia	38º 32’	28º 23’
		Madalena	38º 32’	28º 30’
		Lajes	38º 25’	28º 15’
		Praínha do Norte	38º 29’	28º 14’
		Piedade	38º 25’	28º 03’
	São Jorge Island	Rosais	38º 43’	28º 15’
		Aeroporto	38º 39’	28º 10’
		Meia altitude	38º 39’	28º 06’
		P. Silveira	38º 36’	28º 00’
	Santa Maria Island	P. Valverde	36° 57’	25° 08’
	São Miguel Island	Nordeste	37° 49’	25° 08’
	Terceira Island	Serreta	38º 45’	27º 22’
		S. Bartolomeu	38º 40’	27º 16’
Picconia excelsa Aiton (DC)	Madeira Island	Portela	32° 44’	16° 52’
	Tenerife Island	Anaga	28° 33’	16° 16’
Olea europaea L. var. *sylvestris* Brot.	Mainland Portugal	A. de São Bento	No data	No data
	Mainland Spain	Aldea del Fresno	No data	No data
	France	Bordeaux	No data	No data
	Mainland Italy	Ansedonia	No data	No data
Phyllirea latifolia L.	Mainland Spain	Torrelodones	No data	No data
	France	Bordeaux	No data	No data
P. angustifolia L.	Mainland Portugal	Évora	No data	No data
	Mainland Italy	Ansedonia	No data	No data
P. media L.	Sardinia Island - Italy	Cagliari	No data	No data
Ligustrum vulgare L.	Mainland Italy	Pisa	No data	No data
Fraxinus excelsior L.	Mainland Italy	Majella	No data	No data
F. angustifolia Tutin	Mainland Italy	Latina	No data	No data
F. ornus L.	Sardinia Island - Italy	Cagliari	No data	No data
Fontanesia phillyraeoides Labill.	Sicily - Italy	Palermo	No data	No data

ANNEX II

Table B - Taxon, sample provenance of the eleven species sequenced.

Taxon	Sample Provenance
Fraxinus angustifolia Tutin	Italy, Siliqua
Fraxinus excelsior L.	Italy, Majella Nat. Park
Fraxinus ornus L.	Italy, Sardinia Island
Ligustrum vulgare L.	Italy, Sardinia Island
Olea europea ssp. *sylvestris*	Portugal, Évora
Phillyrea angustifolia L.	Italy, Ansedonia
Phillyrea latifolia L.	Italy, Sardinia Island
Phillyrea media L.	Italy, Siliqua
Fontanesia phillyreoides	Italy
Picconia azorica Tutin	Azores Archipelago
Picconia excelsa (Aiton) DC	Canaries Archip., Tenerife Island
Picconia excelsa (Aiton) DC	Madeira Archip., Madeira Island

ANNEX III

Table C - The accession numbers of the sequences, for each species, published at the NCBI GenBank.

Taxon	Sample Provenance	Accession Numbers	
		rcbL-a	trnH-psbA
Fraxinus angustifolia Tutin	Italy, Siliqua	FJ862055	GU120315
Fraxinus excelsior L.	Italy, Majella Nat. Park	FJ862056	GU120316
Fraxinus ornus L.	Italy, Sardinia Island	FJ862057	GU120317
Ligustrum vulgare L.	Italy, Sardinia Island	FJ862059	GU120319
Olea europea ssp. *sylvestris*	Portugal, Évora	FJ862060	GU120320
Phillyrea angustifolia L.	Italy, Ansedonia	FJ862062	GU120321
Phillyrea latifolia L.	Italy, Sardinia Island	FJ862063	GU120322
Phillyrea media L.	Italy, Siliqua	FJ862064	GU120323
Fontanesia phillyreoides	Italy	FJ862058	GU120318
Picconia azorica Tutin	Azores Archipelago	EU854419:	EU854410:
		:EU854425	:EU854416
Picconia excelsa (Aiton) DC	Canaries Archip., Tenerife Island	EU854426	EU854418
Picconia excelsa (Aiton) DC	Madeira Archip., Madeira Island	EU854427	EU854417

Table C.1 - The accession numbers of the microsatellites sequences, for each species, published at the NCBI GenBank.

Taxon	Sample Provenance	Accession Number / Microsatellite
Olea europea ssp. *sylvestris*	Spain	GUO85259 / ccmp10
Phillyrea angustifolia L.	Italy	GU085254 / ccmp10
Phillyrea angustifolia L.	France	GU085245 / ccmp5
Phillyrea angustifolia L.	Portugal	GU085244 / ccmp2
		GU085251 / ccmp7
		GU085253 / ccmp10
Phillyrea latifolia L.	Italy	GU085242 / ccmp1
Picconia azorica Tutin	Faial, Azores	GU085241 / ccmp1
		GU085243 / ccmp2
		GU085249 / ccmp6
		GU085250 / ccmp7
		GU085255 / ccmp10
		GU085256 / cmcs8
		GU085258 / cmcs13
Picconia azorica Tutin	Pico, Azores	GU085247 / ccmp5
		GU085257 / cmcs8
Picconia azorica Tutin	S.Jorge, Azores	GU085246 / ccmp5
Picconia azorica Tutin	S.ta Maria, Azores	GU085240 / ccmp1
Picconia excelsa (Aiton) DC	Canaries Archipelago	GU085252 / ccmp10
		GU085248 / ccmp5

ANNEX IV

Table D – Taxon, sample provenance and accession numbers of *Taxus baccata* L. sequences deposited at NCBI GenBank. Note: samples were collected from natural populations and five individuals were sampled in Pico Island.

Taxon	Sample Provenance	Accession Number
Taxus baccata L.	Italy - Lazio	GU320033
Taxus baccata L.	Italy - Sardinia	GU320034
Taxus baccata L.	Italy - Apulia	GU320035
Taxus baccata L.	Austria	GU320036
Taxus baccata L.	Morocco	GU320037
Taxus baccata L.	Wales	GU320038
Taxus baccata L.	Algeria	GU320039
Taxus baccata L.	Azores Ar. – Pico Island	GU320040 - GU320044