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# Genetic Diversity of the Endemic Canary Island Pine Tree, *Pinus canariensis*

Miguel Navascués

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Miguel Navascués. Genetic Diversity of the Endemic Canary Island Pine Tree, *Pinus canariensis*. Ecology, environment. University of East Anglia, Norwich, 2005. English. NNT : . tel-00250082

**HAL Id: tel-00250082**

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# Genetic Diversity of the Endemic Canary Island Pine Tree, *Pinus canariensis*

Miguel de Navascués Melero

Thesis submitted for the degree of Doctor of Philosophy

School of Biological Sciences  
University of East Anglia



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September 2005

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*A Concetta*

*A mis padres y a mis hermanos*

## ABSTRACT

The Canary Island pine, *Pinus canariensis*, is an endemic tree that forms one of the main forest ecosystems within the archipelago, and whose distribution has been reduced in the last five centuries by clear cutting for the extraction of timber and tar. It was in the XX<sup>th</sup> century that exploitation declined and reforestation programs were brought forward for the restoration of an ecosystem that harbours a number of endangered endemic species of plants and animals. In addition to reforestation efforts, an understanding of population genetic processes is also necessary for the successful conservation management of the Canarian pine forest, particularly in light of gathering evidence for local adaptation.

In this thesis historical and contemporary gene flow within *P. canariensis* was studied with nuclear and chloroplast microsatellite markers. High immigration rates (0.68–0.75) were estimated as expected for an outcrossing wind-pollinated tree. Nevertheless, significant population differentiation ( $\theta = 0.019$ ,  $R_{ST} = 0.044$ ) was detectable for sites separated by only a few kilometres. Within the context of reforestation programs the high levels of gene flow detected would appear to have a positive effect, by facilitating the immigration of local alleles from natural stands into potentially genetically depauperate first generation gene pools of reforested stands.

Historical population growth was revealed with chloroplast microsatellites for most populations of *P. canariensis*. Population expansions for the pine parasite weevil *Brachyderes rugatus* were also detected, broadly coinciding with the population expansions within the Canary Island pine forests. Given the estimated times of expansion, these population demographic increases would seem likely related to the process of colonisation of newly emerged islands or local patches after volcanic disturbance. Detection and dating of these expansions from chloroplast microsatellites was, to some degree, negatively affected by homoplasy (i.e. parallel and back mutations).

Coalescent simulations of the evolution of chloroplast microsatellites were ap-

plied to study the effects of homoplasy in the statistical analysis of population structuring. Measures of genetic diversity based on number of haplotypes and measures based on genetic distances were differently affected. Genetic distances were underestimated but were proportional to the actual value. These effects help to explain the lower performance of statistical analyses for the detection and dating of population expansions. Further research on the effects of homoplasy in the analysis of population differentiation using chloroplast microsatellites is essential.

## RESUMEN

El pino canario, *Pinus canariensis*, es un endemismo que forma uno de los principales ecosistemas forestales de las Islas Canarias y cuya distribución se ha reducido progresivamente en los últimos cinco siglos a causa de las actividades extractoras de madera y pez. A partir del siglo XX la explotación de los pinares canarios entra en declive y comienzan los programas de reforestación de este ecosistema, que alberga un cierto número de endemismos de plantas y animales, algunos de ellos amenazados. Además de las actividades de reforestación, el conocimiento de los procesos genéticos poblacionales es fundamental para el desarrollo de planes de gestión y conservación de los pinares canarios; especialmente importante es la investigación concerniente al estudio de adaptaciones locales.

En la presente tesis se han utilizado microsatélites nucleares y cloroplásticos como marcadores moleculares para el estudio del flujo genético histórico y contemporáneo en *P. canariensis*. Como era de esperar en una especie anemófila y alógama, se estimaron altas tasas de inmigración (0.68–0.75). Sin embargo, la diferenciación genética entre poblaciones separadas por pocos kilómetros resultó significativa ( $\theta = 0.019$ ,  $R_{ST} = 0.044$ ). Dentro del contexto de las programas de reforestación, estos altos niveles de flujo genético podrían tener un efecto positivo, al favorecer la inmigración de alelos locales desde las masas naturales, incrementándose así el acervo genético de las repoblaciones, potencialmente empobrecidas en su primera generación.

Mediante el análisis de microsatélites de cloroplasto, se han detectado expansiones demográficas en el tiempos geológicos de la mayoría de las poblaciones de *P. canariensis*. También se han detectado crecimientos poblacionales del gorgojo parásito del pino canario, *Brachyderes rugatus*, aproximadamente coincidentes con las expansiones demográficas del pinar. Considerando las dataciones de los momentos de expansión, estas explosiones demográficas parecen relacionadas con la colonización de islas tras su emersión y la colonización de áreas más restringidas tras los efectos destructores de erupciones volcánicas. La detección y datación

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de estas expansiones con microsatélites de cloroplasto fueron, en cierta medida, afectadas negativamente por homoplasia (es decir, por mutaciones que revierten al estado ancestral o por mutaciones iguales adquiridas independientemente).

Para estudiar los efectos de la homoplasia en los análisis de genética de poblaciones, se han empleado simulaciones (basadas en la coalescencia) de la evolución de los microsatélites de cloroplasto. Las medidas de diversidad genética basadas en el número de haplotipos y las basadas en estimaciones de distancias genéticas se vieron afectadas de forma distinta. Aunque las distancias genéticas fueron subestimadas tenían valores proporcionales a las distancias genéticas verdaderas. Estos efectos ayudan a explicar la reducción de precisión de los análisis de detección y datación de expansiones demográficas. Investigaciones adicionales serían esenciales para revelar los efectos de la homoplasia en el análisis de la diferenciación genética entre poblaciones mediante microsatélites de cloroplasto.

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## LIST OF ABBREVIATIONS

$A$	Number of alleles
$A_e$	Effective number of alleles
AFLP	Amplification fragment length polymorphism
$A_{n_{min}}$	Allelic richness, for a sample size of $n_{min}$ individuals
AMOVA	Analysis of molecular variance
bp	Base pairs (size of DNA fragments)
cDNA	complementary DNA (reverse transcription of RNA)
CI	Confidence interval (95% CI)
cpSSR	Chloroplast simple sequence repeat, chloroplast microsatellite
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
$h_0$	Null hypothesis
$H_e$	Genetic diversity and, for diploid genes, expected heterozygosity
$H_o$	Observed heterozygosity
IAM	Infinite allele model
IUCN	World Conservation Union or International Union for the Conservation of Nature
masl	Metres above sea level
mtDNA	Mitochondrial DNA
mya	Million years ago
NCBI	National Center for Biotechnology Information
$N_e$	Effective population size
$n_e$	Effective number of haplotypes
$n_h$	Number of haplotypes
nSSR	Nuclear simple sequence repeat, nuclear microsatellite
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SMM	Stepwise mutation model
spp.	Species (plural)
subsp.	Subspecies
SSR	Simple sequence repeat, microsatellite
UEA	University of East Anglia
ybp	Years before present



## GLOSSARY

*anemophily* dispersal of pollen by wind

*anemochory* dispersal of fruits or seeds by wind

*coalescence* merging of two lineages into their most recent common ancestor

*endozoochory* dispersal of fruits or seeds inside animal bodies by ingestion

*epicormic (bud)* dormant bud which only elongate after a particular stimulus (fire, air exposure, etc)

*epizoochory* dispersal of fruits or seeds attached on the surface of animal bodies

*hydrochory* dispersal of fruits or seeds through water

*Monte Carlo (methods)* methods using randomly generated numbers which include simulation of stochastic processes and permutations of data to obtain null distributions

*zoochory* dispersal of fruits or seeds by animals

## ACKNOWLEDGEMENTS

The work presented here wouldn't have been possible without the collaboration of many people. First I want to thank Brent Emerson who has been an excellent supervisor. I am in debt to all the people at the UEA 'population genetics corridor' for making the lab run smoothly and in a friendly environment; this is extensive to all CEEC. Specially I want to mention Martin Taylor, Allison Surridge, Shaun Forgie, Daniel Hernández-Velázquez, Ricardo Pereyra and Lorenza Legarreta for specific help on laboratory procedures and Godfrey Hewitt, Jo Ridley and Sara Goodacre for comments on manuscripts.

I must also mention the help and collaboration given by other institutions: Luis Gil (Universidad Politécnica de Madrid, UPM), Roula Vaxevanidou (UPM), Santiago González-Martínez (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, INIA) and José Climent (INIA) shared priceless information and discussion on *Pinus canariensis* genetics; Beppe Vendramin (Istituto Miglioramento Genetico Piante Forestali) screened for cpSSR variation in *P. canariensis*; Pedro Oromí (Universidad de La Laguna, ULL) sent *P. canariensis* samples for early screening of diversity; Bee Bowles (John Innes Center) shared with us lab equipment for DNA extractions, and Guillermo de Navascués (Universidad Autónoma de Madrid) helped in the development of the simulation program.

I am grateful to Carlos Morla Juaristi (UPM), María Soledad Jiménez (ULL), M<sup>a</sup> Victoria Marzol Jaén (ULL), Raimundo Real (Universidad de Málaga, UM), J. Mario Vargas (UM) and Henry Hooghiemstra (Universiteit van Amsterdam) for sending me reprints of their publications otherwise unavailable to me. I thank Vicente Araña (Museo Nacional de Ciencias Naturales), Peter Schönfelder (Universität Regensburg) and Ruth Álvarez for permission to reproduce their photos. María Inés Melero helped me with translations from German papers.

Also I am grateful to the Cabildo Insular of Tenerife for collecting permit. The University of East Anglia provided me a three-year scholarship for my four-year PhD studies.

Part I

INTRODUCTION

## FOREWORD

### *On forestry, forest genetics and pines*

Forestry is the science and technology of the management of forest ecosystems and the resources associated with them. These resources refer both to the extraction of products (timber, resins, etc) and to the conservation of soil, water and wildlife (Chaney et al., 1994). Forest genetics (i.e. genetics of forest trees) play a fundamental role in forestry for tree breeding programs, conservation of biodiversity and research on forest ecosystem processes.

In artificial regeneration of forests, control of the genetic quality of the reproductive material ensures the success of plantations. For instance, the transference of seed from ecologically different areas (e.g. Ribeiro et al., 2002) or the use of seedlots with low genetic diversity (Brown and Hardner, 2000) may produce plantations with low viability. In the cases where there is an economic interest, selecting seeds from ‘superior’ trees is of great importance in ensuring profitable production. The use of progeny tests is the basis for studying the quality of genotypes (Wright, 1976); and genetic markers are also very useful for characterising provenances, production of genetic maps, marker-assisted selection and genetic engineering (Ahuja, 2001).

The understanding of ecological and evolutionary processes of forest trees has significantly profited from the use of genetic tools. The dispersal of propagules (pollen and seeds) is of key relevance because it determines gene exchange among populations and colonisation processes. Contemporary movements of pollen and seed have been studied by means of paternity assignments (e.g. Robledo-Arnuncio and Gil, 2004) and the recent ‘TwoGener’ approach (Smouse et al., 2001). Population differentiation and phylogeographic analysis have revealed the biogeographic processes of forest trees in continents (e.g. Ferris et al., 1998) and islands (e.g. López-de Heredia et al., 2005). Characterisation of mating systems (outcrossing and inbreeding rates), quantification of genetic introgression and phylogenetic

analysis are among the applications of molecular genetic tools that are being used to study key tree species in forest ecosystems.

Pines are among the most important forest trees both ecologically and economically. The genus *Pinus* is the largest of the family Pinaceae. Ecologically, pines are the most important conifer in the Northern Hemisphere to which their natural distribution is restricted (only *Pinus merkussi* crosses the equator with some populations in Sumatra). They occupy habitats as diverse as the boreal taiga, temperate forest and tropical savannah, and grow from Mediterranean-climate coastal habitats to the alpine timberlines (Richardson and Rundel, 1998). Economically, the importance of pines also extends to the Southern Hemisphere where they have been extensively planted (Richardson and Higgins, 1998). The uses of pines include extraction of edible pine-nuts, wood for construction and carpentry, paper fibre and diverse chemicals (including drugs), protection against erosion for soil and watersheds, control of dunes and ornamental gardening (Le Maitre, 1998). The strong interaction of humans with pines has produced (directly or indirectly) significant distribution contractions for many pine species (approximately a third of *Pinus* species are threatened) which has produced an erosion of their genetic diversity (Richardson and Rundel, 1998). The conservation of the genetic diversity of pines is a priority to preserve their capacity to adapt to global change and their gene pools for future breeding programs.

### *Aims and organisation of this work*

The Canary Island pine tree (*Pinus canariensis*) is an endemic species of the Canary archipelago; and it is one of the pines with the most restricted distribution in the world. In the Canarian archipelago *P. canariensis* forests form one of the main ecosystems. Its ecology, importance for conservation and current knowledge on the species is reviewed in Chapter 2. The biology and ecology of the organisms of the Canarian archipelago are greatly influenced by their isolation and contrasting habitats. In Chapter 1 the geological origin of these oceanic islands, their climate and the influence of these factors on their biota is summarised in order to place the Canarian pine into context.

The main objective of this work is to study the geographic variation of the

genetic diversity of the Canary Island pine. Our first target was the development of new polymorphic genetic markers in the species. Chapter 3 reviews the previous knowledge of genetic variation in *P. canariensis* and the markers available to date. In Chapter 4 we present the characteristics of the microsatellite markers transferred from related pine species to *P. canariensis* that have been used in the present work (see also Appendix B.2). The high polymorphism and mutation mechanism in the chloroplast microsatellites employed revealed homoplasy as a factor of concern for the data analysis. Coalescent simulations were used to quantify the effects of homoplasy in the statistical analysis of chloroplast microsatellites and results are presented in Chapters 5 and 6.

For this thesis natural populations and artificial plantations of *P. canariensis* have been studied with three goals. First, historical demography has been inferred from chloroplast microsatellite data to study the influence of the geological events in the demographic dynamics and colonisation process of the Canary Island pine (Chapter 6). Secondly, the study of patterns of gene flow has been addressed in Chapter 7 (potential effect of altitude to produce seasonal isolation) and Chapter 8 (detection of immigrating seedlings by a genotype assignment method). And thirdly, the genetic diversity of artificial plantations was compared to that of natural populations to assess negative impact of silviculture (Chapter 8).

Finally, Chapter 9 summarises the results and conclusions of the present work.

## 1. CANARY ISLANDS

### 1.1 Geography

The Canary Archipelago is a chain of seven major islands and a few smaller islets in the Atlantic Ocean. It is located between 27–29°N and 14–18°W with the closest island to the continent approximately 95 km from the Cape Juby on the African coast (figure 1.1). The total area of the archipelago is 7446 km<sup>2</sup> (see table 1.1). Its topography is very heterogeneous, due to the differences in age, area and altitude among the islands. The geological processes of volcanism and erosion determine whether the topography is rough, as in the heavily eroded older areas (north of La Palma, Anaga and Teno in Tenerife, La Gomera; see Criado, 2001), or more smooth. Also, the high altitudes of the western islands contrast with the low altitudes of Lanzarote and Fuerteventura. El Teide, in Tenerife, with 3718 metres is the highest mountain in all Spanish territory. Water is a scarce resource (the only semi-permanent water streams are on the island of La Palma) and, as a consequence of this, exploitation of the underground waters is very important in the archipelago (Parsons, 1981).

The archipelago is inhabited by almost two million people (1 915 540 in 2004), with 80% of this population concentrated in the islands of Tenerife and Gran Canaria. Travel among islands is by ferry and plane and communication with mainland Spain and Europe is mainly through the airports of Tenerife and Gran Canaria. The archipelago constitutes one of the autonomous regions (*Comunidad Autónoma*) of Spain and is divided into two provinces (*Provincia*) with capitals in Las Palmas de Gran Canaria and Santa Cruz de Tenerife. Each of the seven major islands has a local administration named *Cabildo Insular*. Most natural areas in the Canary Islands are under some degree of protection and collecting plants, animals or rocks is not allowed without official permits, issued by the environment section of the *Cabildos Insulares* (a separate permit is necessary for each island) and by the environmental section of the Canaries government. Restrictions are

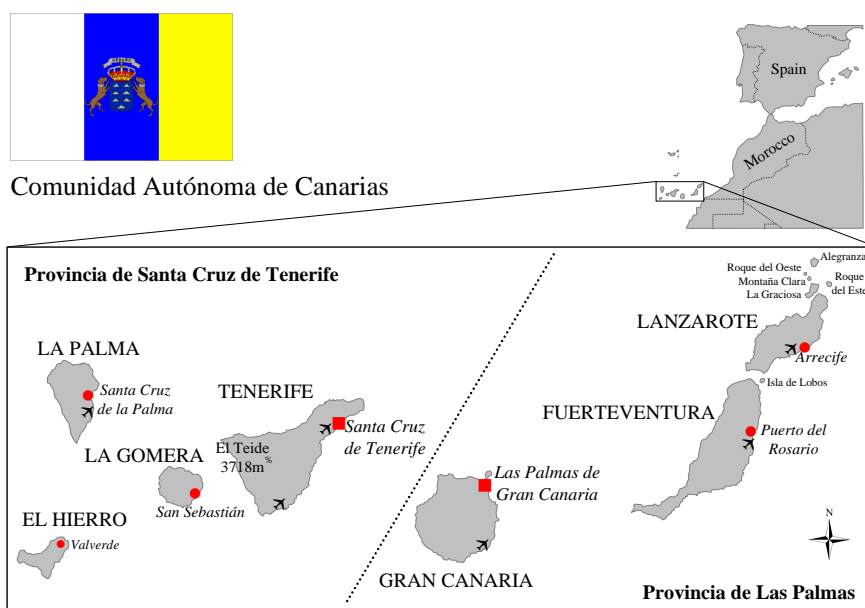


Fig. 1.1: Canary Islands geography and flag. The seven major islands are politically subdivided into two provinces. The highest peak in the archipelago is El Teide (3718 masl), which is also the highest mountain of Spain.

higher in the four National Parks of the archipelago, where visitors cannot leave the marked paths and roads and additional permits are necessary.

## 1.2 Geology

### 1.2.1 Origin

Different explanations for the origin of the Canary Islands have been proposed throughout history (see García Cruz, 2001, for a review). For many centuries they were considered remnants of a greater landmass “the sunken continent of Atlantis” and this scenario was still considered likely by some until the XX<sup>th</sup> century (Bannerman, 1922).

In the XIX<sup>th</sup> century, the first scientific hypotheses were brought forward linking the origin of the archipelago with its volcanic character (figure 1.2). Von Buch (1825) explained the formation of the Canaries with his elevation craters theory. He proposed that high-pressure gases from the volcanic activity deformed a former horizontal ground into mountain-volcanoes. Opposed to this hypothesis, Lyell (1830, vol. 1, ch. XXII) proposed the formation of the Canary Islands by the



Island	Area (km <sup>2</sup> )	Altitude (m)	Coast longi- tude (km)	Distance to Africa (km)	Habitat diversity <sup>a</sup>
Tenerife	2034	3718	269	284	5
Fuerteventura	1655	807	255	95	1
Gran Canaria	1560	1948	197	196	4
Lanzarote	807	670	203	125	1
La Palma	708	2426	126	416	5
La Gomera	370	1487	87	333	3
El Hierro	269	1501	95	383	4
Archipelago	7447	3718	1291	95	5

<sup>a</sup> Number of vegetation zones (see section 1.3.2)

Tab. 1.1: Geographic characteristics of the Canary Islands (after Fernández-Palacios and Martín Esquivel, 2001a; Aguilera Klink et al., 1993). Islands ordered by size.

accumulation of volcanic material and supported that they were never connected to mainland Africa; this view is similar to the scenarios currently accepted.

The developments of modern Earth sciences have established that the Canary Islands are true oceanic islands (i.e. islands built over oceanic crust, never in contact with the continent) originated by accumulation of volcanic materials. However the mechanistic causes for this volcanism are still debated. Currently there are four main hypotheses to explain the Canarian volcanism: the hotspot, propagating fracture, uplifted blocks and unifying models.

### *Hotspot model*

Probably the most popular hypothesis is the hotspot or mantle plume model (Wilson, 1963; Morgan, 1971). In this model, a column of hot material from the mantle (a thermic plume) produces the volcanic activity. With the movement of the oceanic plate the hotspot produces a chain of islands, and only the younger one, situated on the hotspot, would have ongoing volcanic activity (figure 1.3a). However, the composition of the volcanic rocks and the fact that most of the Canary Islands have experienced recent volcanic activity have lead to the proposal of modified versions such as the blob model (Hoernle and Schmincke, 1993). This model suggests a leaning plume within the width of the whole archipelago; only



Fig. 1.2: The Canary Islands are a volcanic archipelago. All the islands have had recent volcanic activity with the exception of La Gomera. The most recent eruption was the Teneguía volcano (La Palma) in 1971 (photos: Vicente Araña).

‘fertile’ blobs within the plume would be able to produce magma (figure 1.3b). For Carracedo et al. (1998), the similarities in the geological structures and evolution with other accepted hotspot-origin archipelagos (Hawaii, Cape Verde and Réunion) support the hotspot model for the Canary Islands and the differences are explained by the close proximity of the continental crust.

#### *Propagating fracture model*

Alternative models, based on plate tectonics, have been put forward to explain different geological features of the Canary Islands. The propagating fracture model (Anguita and Hernán, 1975) proposes a connection between the Canary Islands and the South Atlas fault. It would explain the periods of volcanism and inactivity with processes of decompression and compression of the fault.

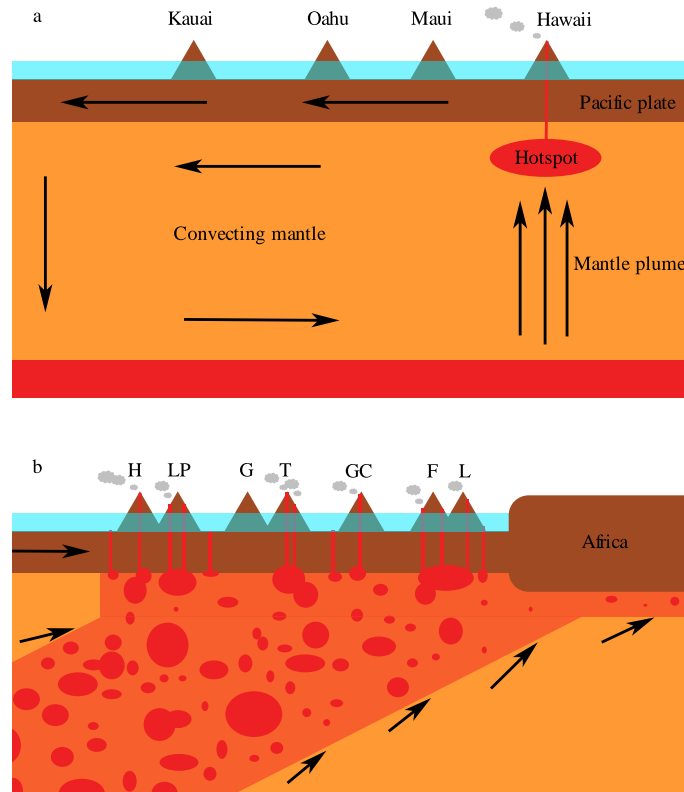


Fig. 1.3: Hotspot hypothesis: a. for the formation of the Hawaiian Archipelago (Wilson, 1963) and b. its modified version, the blob model, for the Canary Islands (Hoernle and Schmincke, 1993). After Anguita and Hernán (2000, figure 3).

### *Uplifted blocks model*

The uplift of tectonic blocks (Araña and Ortiz, 1986) describes each island in a separate block delimited by faults originating from the Mid-Atlantic ridge by compression. This hypothesis places the origin of the volcanism back to the formation of the Atlantic rift (pre-Tertiary) and, later on, in deep faults in the oceanic plate.

### *Unifying model*

Anguita and Hernán (2000, 2003) affirm that none of the above mentioned models fully explain the geology of the archipelago and they propose a unifying or synthetic model that borrows elements from the preceding models. This model is based on a better knowledge of the Atlas geology and on the discovery of a

mantle thermal anomaly under North Africa and the Canary Islands (Hoernle et al., 1995).

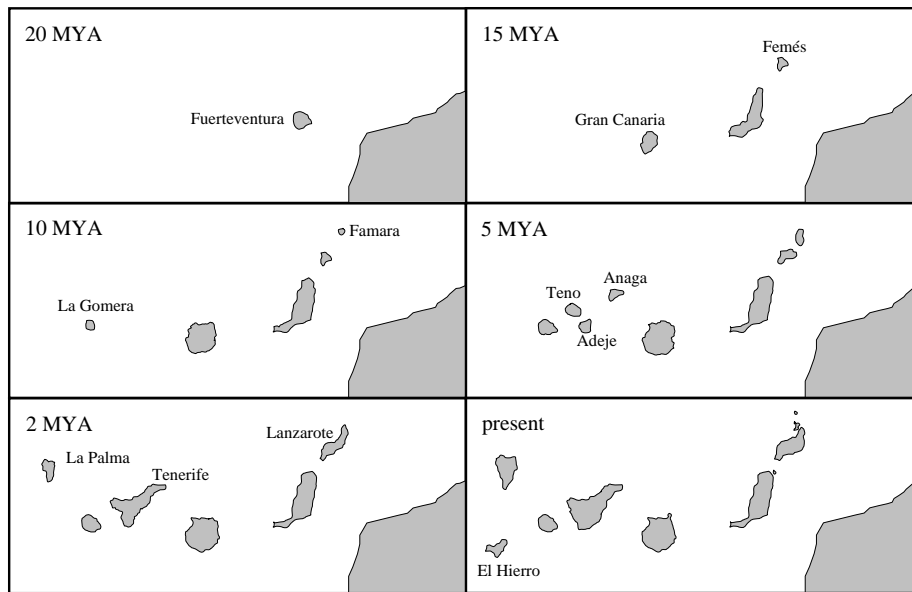
The Anguita and Hernán (2000) model suggests that this thermal anomaly is a fossil plume (the remains of the Triassic plume that produced the opening of the Atlantic) and that it is the origin of the Canary and Atlas volcanism. This helps to explain the chemical properties of the volcanic rocks and the contemporary activity in the whole archipelago.

In this model the magma would outcrop from an extensive fault system. This hypothetical fault system also could explain the periods of volcanic activity and the differential elevation of the islands in a similar way that the propagating fracture and uplifted blocks models did.

### 1.2.2 *Evolution*

The geological evolution of the islands follows four stages: submarine, shield-building, erosional and rejuvenation stages. In the submarine stage the first volcanic activity forms a seamount. After it emerges, there is a period of island formation characterised by shield volcanoes with particularly steep slopes up to 20° or more (Carracedo and Day, 2002). This period lasts for several million years and is followed by a period of volcanic inactivity. Erosion is the main factor shaping the morphology of the islands during this stage. Finally the island may go through a second volcanic stage, when the island rejuvenates with the accumulation of new volcanic material. The evolution of the archipelago depends on the different time of emergence of the islands as shown in figure 1.4.

Other features shaping Canarian topology are volcano collapses (Carracedo and Day, 2002). Volcanic edifices are unstable structures, with steep slopes built with weak unconsolidated rock. Central collapses in the magma chambers produce *calderas* (e.g. Tejada *caldera* on Gran Canaria and younger *calderas* in Las Cañadas volcano on Tenerife). A second type of collapse are the landslides of volcano flanks, involving enormous volumes of loose material. Eleven giant landslide scars and three other structures, possibly related to landslides, have been identified in the Canary Islands (see Carracedo et al., 1999; Masson et al., 2002). These destructive processes had an important impact in the morphology of the Canary Islands and they could also have generated tsunamis that could have reached as far as the American coastline (Ward and Day, 2001).



*Fig. 1.4:* Geological evolution of the Canary archipelago (after Marrero and Francisco-Ortega, 2001, figure 14.1). The time of emergence of the different islands increases from east to west. The island of Tenerife and Lanzarote are the product of the union of previous smaller islands with new accumulation of volcanic materials.

### *Fuerteventura*

Fuerteventura is the oldest island of the archipelago and has gone through all four evolutionary stages of the volcanic islands: as a seamount (48 to 22 mya); shield-stage (22 to 12 mya), erosional stage (12 to five mya) and currently being in the rejuvenation phase (five mya to present). Submarine deposits suggest the occurrence of two giant landslides during its shield-building stage (Stillman, 1999). Also, during periods of low sea level in the Quaternary, it came into contact with Lanzarote (Carracedo and Day, 2002) forming a single island (figure 1.9).

### *Lanzarote*

As part of the same volcanic edifice, the geological history of Lanzarote is similar to that of Fuerteventura. Its shield-building stage lasts from 15.5 to five mya and it began with two separate islands (Los Ajaches and Famara volcanoes). Post-erosional volcanic activity goes from 3.7 mya till present (Carracedo and Day, 2002).

*Gran Canaria*

Like Fuerteventura and Lanzarote, Gran Canaria has gone through two periods of volcanic activity: the shield stage from 14.4 to nine mya and the post-erosional stage from 5.5 mya to the present (Carracedo and Day, 2002). Particularly explosive, in the more recent volcanic period, is the Roque Nublo Cycle (5.5 to three mya) which covered extensive areas of the island with volcanic products (Pérez-Torrado et al., 1995). The scars of two gravitational landslides have also been identified.

*Tenerife*

The evolution of the island of Tenerife is of particular interest. Three independent islands (corresponding to the present areas of Teno, 7.4 mya; Anaga, 6.5 mya, and Roque del Conde, 11.6 mya) were the result of the shield-building stage for Tenerife (from 12 to four mya; Ancochea et al., 1990). It was the second post-erosional volcanic period, occurring in three cycles 3.5–2.7 mya, 2.5–1.4 mya and 1.1–0.2 mya, which built the central part of the island (Las Cañadas) and the ridge of La Esperanza connecting the three older massifs at the beginning of the first cycle (Ancochea et al., 1999). Five giant lateral collapses have also been identified.

*La Gomera*

La Gomera has had only one period of volcanic activity, which finished around four mya, currently being in the erosional stage. Its surface is characterised by the erosion which has left an abrupt orography.

*La Palma*

In this island we can distinguish two volcanic areas: the northern shield (Taburiente and Cumbre Nueva) and the southern Cumbre Vieja. The northern shield emerged around 1.7 mya and became extinct around 0.4 mya. Two volcano collapses have occurred in the northern shield leaving clear scars in its topology. The younger Cumbre Vieja Rift is currently the most active volcanic area at the archipelago (see figure 1.2) and started its activity around 120 thousand years ago. This island is in its shield stage.

### *El Hierro*

The youngest of the Canary Islands, El Hierro, has gone through three volcanic periods (Tiñor volcano, El Golfo volcano and the ongoing rift volcanism; Guillou et al., 1996) separated by two giant lateral collapses. A third lateral collapse (El Julán) has been identified in addition to those which ended Tiñor and El Golfo volcanoes. This island is currently in its shield stage.

### *Las Hijas*

Las Hijas seamounts, located 70 km Southwest to El Hierro, represent the submarine stage in the evolution of the Canary Islands and eventually may emerge to form new islands (Rihm et al., 1998).

## 1.3 *Climate and vegetation*

### 1.3.1 *Climate*

The Canary Islands have a Mediterranean type climate which is influenced by the following factors: the trade winds (figure 1.5), the cold oceanic current (figure 1.6), the eastern winds from the Sahara, the altitude of the islands, the proximity to the continent and the slope exposure (Marzol, 2001). The different influences of these factors among islands and among areas within islands produce very contrasting local climates.

In general, precipitation occurs mainly in winter while summer is hot and dry. The altitude of the islands and their proximity to Africa influence the amount of precipitation. Humid trade winds bring rains to the north-eastern slopes of the higher western islands. As the moist air ascends it cools, forming orographic clouds and rain. The condensation ceases once the air circumvents the peaks, leaving south-western slopes dry (Carracedo and Day, 2002). Lanzarote and Fuerteventura are extremely arid because of their low altitude and the hot and dry harmattan winds from the Sahara, which also affect the south of Gran Canaria (Bramwell and Bramwell, 1990). Forest vegetation has an important role in the hydrologic cycle of the archipelago as it significantly increases the amount of atmospheric water that reaches the soils by fog condensation (Ceballos and Ortuño, 1952; Aboal et al., 1999, 2000a,b).

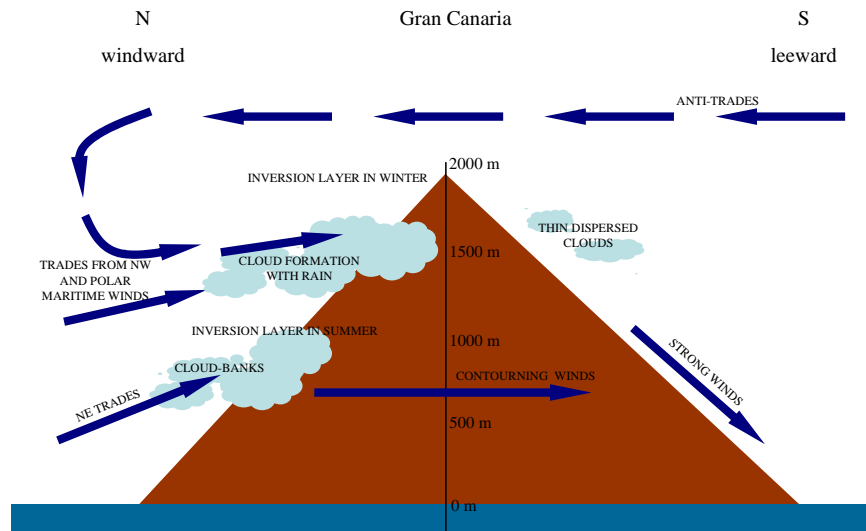


Fig. 1.5: Canary Island wind system. The main dominant winds are the trades coming from the northeast. The location of the archipelago is in between the temperate and tropical zones, which produces an altitudinal change of the trades direction being from northeast in the lower altitudes and from northwest in the higher altitudes (after Fernandopullé, 1976, figure 7).

The temperatures are warm throughout the year, usually within the range of  $18^{\circ}\text{C}$  in the winter and  $26^{\circ}\text{C}$  in the summer (August is the hottest month). These are lower temperatures than expected for their latitudinal position due to the cool ocean currents (Fernandopullé, 1976). Higher temperatures are registered in Lanzarote and Fuerteventura in summer. Also, snow persists for several months in winter above 2000 m on Tenerife and La Palma (mean annual temperature at Pico del Teide is  $1^{\circ}\text{C}$ ; Fernández-Palacios and Martín Esquivel, 2001a).

Trade winds are the cause of the phenomena of temperature inversion and cloud banks (*mar de nubes*). The lower stratum of the trades, with a northeast direction, cools down and acquires humidity from the ocean. The upper stratum is hot and dry, blowing from the northwest. These differences produce a temperature inversion and the formation of stratocumulus clouds in the low layer (usually around 1000 m) when the winds meet the islands with higher elevations and the upper NW trades prevent the ascension of wet and cold northeast trades (Fernandopullé, 1976).

Regarding climatic conditions in the geological past, we can say that glaciations did not affect the archipelago directly but this global change did produce



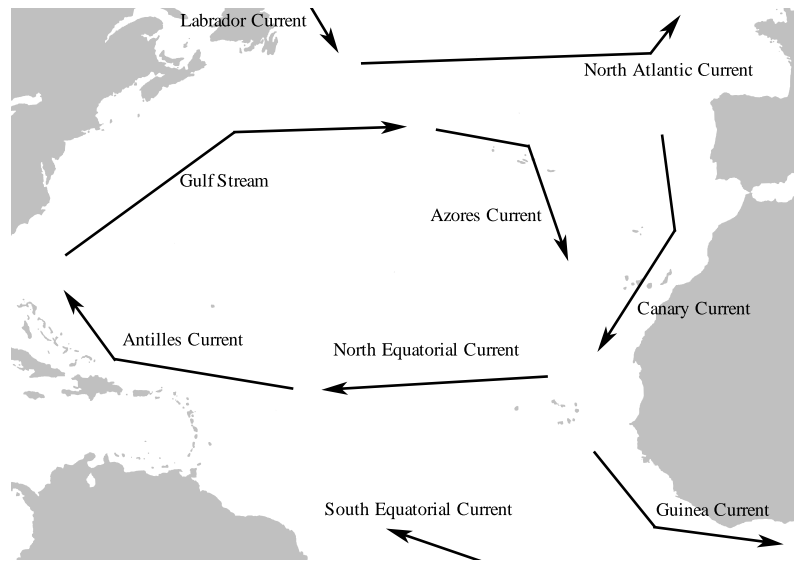


Fig. 1.6: North Atlantic current system. The Canary Current flows from northeast bringing cold waters to the archipelago and making trade wind cold and wet. This oceanic current also favours the dispersal of propagules from the continental landmasses (Iberian Peninsula and Africa).

an increase in precipitation and some other minor changes (Damnati et al., 1996; Criado, 2001). The biota of the Canary Islands was probably little affected, migrating altitudinally to adapt to those changes in palaeoclimates.

### 1.3.2 Vegetation

Five vegetation zones, determined by the climatic differences produced by altitude and exposure (figure 1.7), can be described (Bramwell and Bramwell, 1990; Santos, 2001).

#### *Arid to semiarid scrub*

Xerophyte scrubs (*tabaibal*) grow in the coastal areas of all islands. They are dominated by succulent endemic spurge species (mainly candelabra-like *Euphorbia canariensis* but also *E. balsamifera*, *E. obtusifolia*, *E. regis-jubae*, etc) with endemic *Aeonium* species. In the lower eastern islands (Lanzarote and Fuerteventura) this vegetation occupies most of the territory (Fernández-Palacios et al., 2001). Degraded areas have been transformed with the introduction of *Opuntia* and *Agave* species (Aguilera Klink et al., 1993).

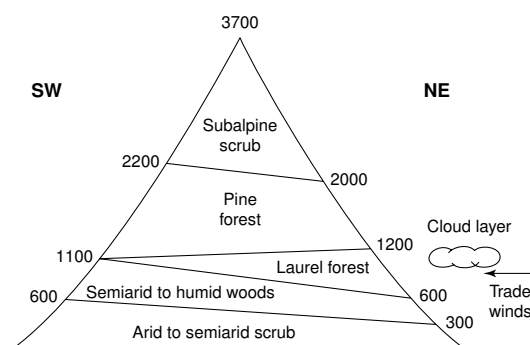


Fig. 1.7: Vegetation belts in the island of Tenerife (from Juan et al., 2000, box 2). Vegetation zones are determined by the altitude and exposure of the slopes, factors that determine the temperature and precipitation of the local climates.

### *Semiarid to humid woods*

This zone is the transition to the proper forest ecosystems (laurel and pine forests). It does not constitute a uniform formation but a heterogeneous wood with different dominating species at each location. The bush species found in this belt are generally of Mediterranean character (*Juniperus canariensis*, *Olea europaea* subsp. *cerasiformis*, *Pistacia* spp.) but there are also species such as *Dracaena draco* and *Phoenix canariensis* with more tropical or subtropical character. This zone was originally present in all islands, but is only nowadays preserved in La Gomera and El Hierro. Most of it has disappeared because it occupied the best areas for agriculture and urbanization.

### *Humid forest (monteverde)*

This forest is formed by hygrophilous species and is restricted to the northern slopes between 600 and 1200 m, where the trade winds create the *mar de nubes* (cloud banks). Four Lauraceae species dominate the *laurisilva*: *Laurus azorica*, *Apollonias barbujana*, *Ocotea foetens* and *Persea indica*. Other genera associated with this vegetation are *Arbutus*, *Ilex*, *Rhamnus*, *Notelaea* and *Visnea* (Ciferri, 1961). In drier areas and in transition to the pine forest, the *fayal-brezal* with heather (*Erica arborea*) and fayatree (*Myrica faya*) occurs. Most of the flora from this zone is thought to be composed of Tertiary palaeoendemisms that have survived in the Macaronesian islands while different climatic changes (glaciations,

Sahara desertification, Messinian salinity crisis) resulted in their extinction in the continental areas.

### *Pine forest*

This zone is an open formation, poor in species diversity and dominated by *Pinus canariensis*, growing in more arid condition than the laurel forest. Pines are usually accompanied by *jaras* (such as *Cistus symphytifolius* or *C. monspeliensis*) or legumes (such as *Adenocarpus viscosus*, *A. foliosus* or *Spartocytisus supranubius*). In the ecotone with the humid forest, pine forest contains also *Erica arborea* and *Myrica faya*.

### *Subalpine scrub*

Only present in the higher islands of Tenerife, La Palma and Gran Canaria, the subalpine scrub is formed mainly by brooms (legumes) and some endemic species such as *Echium wildpreti* and *Viola cheiranthifolia*.

## 1.4 Biogeography<sup>1</sup>

Together with other North Atlantic volcanic archipelagos (Madeira, Azores, Selvagens and Cape Verde)<sup>2</sup> the Canary Islands form the biogeographic region of Macaronesia (from Greek *makaros*, happiness, and *nesos*, islands), recognised both in phytogeography and zoogeography. The isolation and habitat diversity condition the biogeographical processes in the Macaronesia.

### 1.4.1 Colonisation and dispersion

Biogeographically, islands can be divided into either continental or oceanic. Continental islands are situated on the continental platform and were connected to a mainland at some point in the past. Oceanic islands, such as the Canaries, emerge

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<sup>1</sup> Only terrestrial biogeography will be discussed in this section. Aguilera Klink et al. (1993) and Fernández-Palacios and Martín Esquivel (2001b) present descriptions of the Canary Islands marine ecosystems and biogeography.

<sup>2</sup> Some authors also include an area of southwest Morocco (the Argean area) as the 'continental Macaronesia' which presents a similar flora with Tertiary palaeoendemisms (Médail and Quézel, 1999).

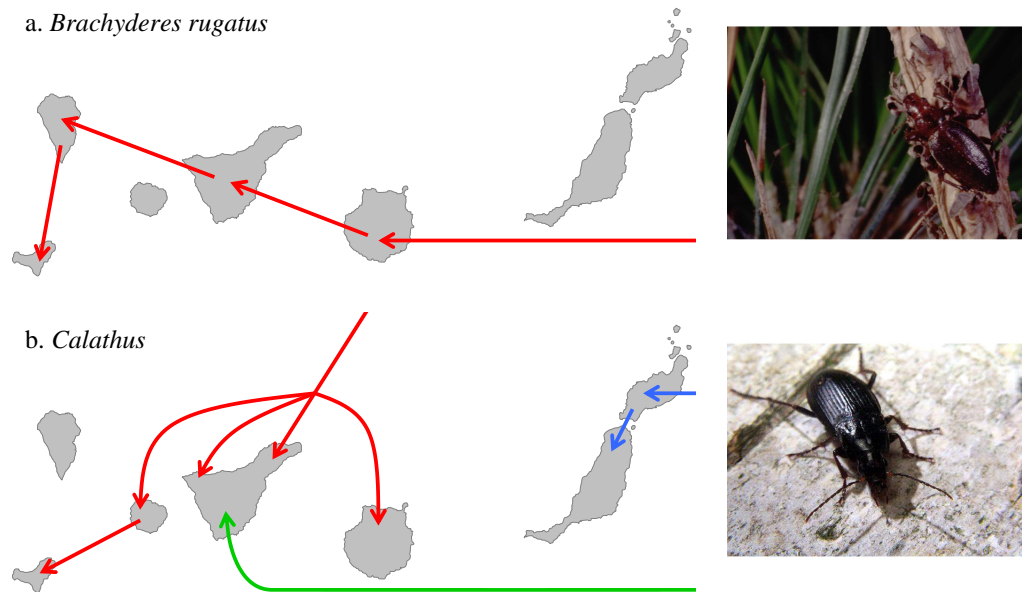


Fig. 1.8: Colonisation patterns of the Canary Islands. a. Stepping stone colonisation by *Brachyderes rugatus* (Emerson et al., 2000a) and b. complex colonisation pattern by three different lineages of genus *Calathus* (Emerson et al., 2000b).

from the oceanic floor. All the flora and fauna of oceanic islands must come via trans-oceanic dispersal from other land masses (see Gillespie and Roderick, 2002, for a review).

The transport of propagules to the Canary Islands can be attributed mainly to the trade winds and Canary sea current (both from northeast, see figure 1.6) and animals (mainly birds). Bramwell (1986), based on the dispersal mechanism of the present Canarian flora, proposed that the colonisation of plants was dominated by zoochory (34% endozoochory, 20% epizoochory) followed by anemochory (26%) and hydrochory (4%), leaving a 16% undetermined (probably endozoochory or anemochory).

The origin of the colonisers is mainly North Africa and Iberian Peninsula (or other Macaronesian islands; Juan et al., 2000), making the Canary Islands biogeographically related to the Mediterranean region (but see de Bolòs, 1996). Canary Island plants with affinities to other floras different to the Mediterranean are usually palaeoendemisms (see section 1.4.4).

In an idealised process of colonisation, a single lineage arrives to the archipelago and hops from island to island (arriving first to the ones older or closer to the con-



Fig. 1.9: Macaronesia, 18 000 ybp. Because of a decrease in the sea level Lanzarote and Fuerteventura formed a single island (Mahan) and numerous seamounts (in *italics*) emerged as islets between the Iberian Peninsula and the Macaronesian Islands (after García-Talavera, 2003, figure 2).

tinental landmass) in a stepping stone fashion (figure 1.8a). Diverse seamounts, situated between the continental landmass and the Macaronesian archipelagos, could have further favoured this stepping stone process at the times of low sea level such as the glaciations (figure 1.9; Fernández-Palacios and Dias, 2001; Aguilera Klink et al., 1993). Textbook examples for this stepping stone pattern have been put forward for *Gallotia* lizards (González et al., 1996), *Hegeter* beetles (Juan et al., 1996), *Gonepteryx* Brimstone butterflies (Brunton and Hurst, 1998) and the olive tree (*Olea*, Hess et al., 2000). The single colonisation of organisms seems to be more frequent than recurrent colonisations, despite the close distance to the continent (95 km between Fuerteventura and Africa); later arrivals of the same organism might have been prevented by competition with the first colonising lineage (Silvertown, 2004). Less frequent patterns of island colonisation involve multiple lineages —*Calathus* beetles (figure 1.8b, Emerson et al., 2000b), *Tarentola* geckos (Carranza et al., 2002), *Lavatera* (Fuertes-Aguilar et al., 2002) and *Hedera* (Vargas et al., 1999)— or back colonisation to the continent —*Aeonium* (Jorgensen and Frydenberg, 1999), *Lotus* (Allan et al., 2004) and *Androcymbium* (Caujapé-Castells, 2004).

### 1.4.2 Disharmony or attenuation

Island ecosystems tend to be similar to those on continents, where the colonisers came from. However, due to the differences in dispersal capacity, insular ecosystems lack many species present on mainland areas. These ecosystems with empty niches are described as disharmonic (Carlquist, 1974).

Disharmonic ecosystems are prone to the process of speciation. The relaxation of the selective pressures because of the empty niches favours the transformation of the coloniser species (Fernández-Palacios and Martín Esquivel, 2001a; Aguilera Klink et al., 1993). The decreased resource availability and reduction in predation pressure can also change ecological relations and life histories leading to body size shifts (Palkovacs, 2003). Examples of this process in the Canary Islands are the fossil species of giant *Gallotia* lizards (Barahona et al., 2000; Maca-Meyer et al., 2003), giant *Canariomys* rat (Michaux et al., 1996; López Martínez and López Jurado, 1987), giant *Geochelone* tortoise (Izquierdo et al., 2001) and giant birds (García-Talavera, 1990)<sup>3</sup>. A similarly interesting process is found in plants that acquire a woody habit, such as *Echium* (Böhle et al., 1996), *Sonchus* (Kim et al., 1996a,b), *Bencomia* (Helfgott et al., 2000) and *Pericallis* (Panero et al., 1999). Although woodiness was first considered as a relict character for some authors (Meusel, 1953, 1965; Bramwell, 1976), recent phylogenetic analyses have demonstrated it is a derived character for several genera (see Emerson, 2002, and references therein) as had been proposed by Carlquist (1974).

Disharmony is also what makes island ecosystems particularly vulnerable to the introduction of species by mankind. The high rate of alien species introduction increases the probability for the displacement of the native species by the newcomers (Aguilera Klink et al., 1993).

### 1.4.3 Speciation

As consequence of disharmony, species change their ‘traditional’ niches to the new opportunities that the islands offer. These speciation processes are often associated with adaptive radiations that can be observed in plants —*Echium* (Carlquist, 1974), *Crambe* (Francisco-Ortega et al., 1999), *Aeonium* (Carlquist,

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<sup>3</sup> Rothe (1964) and Sauer and Rothe (1972) first interpreted these fossil eggs as from Ratites. These fossils were used to support the continental origin of Lanzarote and Fuerteventura (Rothe and Schmincke, 1968; Rothe, 1974).

1974; Jorgensen and Olesen, 2001)— and animals —*Hegeter* beetles (Juan et al., 1996), *Dysdera* spiders (Arnedo and Ribera, 1999; Arnedo et al., 2001). Adaptive radiations are also favoured by the high diversity of habitats that can be found in relatively small geographic areas.

Another cause of speciation is isolation among islands. Isolation produces genetic divergence among populations (see, for instance, *Drosophila subobscura* in Afonso et al., 1990). For species of low dispersal capacity, isolation eventually leads to speciation (like in *Hemicycla* snails; Aguilera Klink et al., 1993). Other vicariant processes could result from habitat fragmentation by volcanic eruptions<sup>4</sup>, landslides or range shifts caused by climatic change such as glacial cycling (Báez et al., 2001).

Other evolutionary changes might be due to selective pressures particular to island environments. For instance, many insect species become flightless as high dispersal capacity leads to higher mortality because of dispersal into the sea (Ashmole and Ashmole, 1988).

#### 1.4.4 Endemicity

The Canary Islands harbour an elevated number of endemic species (table 1.2), which makes the archipelago one of the hotspots of biodiversity in the Mediterranean area (Médail and Quézel, 1999). The isolation and ecological disharmony that results in the speciation and radiation processes have given rise to many neoendemisms. Another type of endemic species are the so called palaeoendemics. These are relict species that were present in Europe and North Africa in the past. In the Palaeocene these regions were more humid (e.g. the current Sahara area was covered with rainforest). The climate became drier in the Miocene [at some point the Tethys ( $\approx$  Mediterranean) Sea desiccated, an event known as the Messinian salinity crisis], starting the desertification of North Africa and the disappearance of laurel-type forest in Europe (Quézel, 1978; Mai, 1989). The climate buffer action of the ocean and the possibility for the species to migrate altitudinally made the Macaronesian islands a refugium for many species that

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<sup>4</sup> Also, it must be noted the role of volcanism as ecological disturbance in the archipelago which is followed by a process of succession with characteristic ‘lavicolous’ hypogean (inhabiting lava tubes) fauna (Ashmole et al., 1992). Particularly explosive eruptions may destroy entire ecosystems in an island (Marrero and Francisco-Ortega, 2001; Emerson, 2003).

	Taxonomic group	Number of species	Endemisms	Percentage of endemisms
FAUNA	Arthropoda	6843	2704	40%
	Mollusca	246	192	78%
	Annelida	62	0	0%
	Chordata	123	22	18%
FLORA	Lichens	1294	26	2%
	Bryophyta	464	10	15%
	Vascular	1995	511	26%
FUNGI	Fungi	1634	107	7%
TOTAL		12661	3572	28%

Tab. 1.2: Terrestrial biota inventory of the Canary Islands, including exotic species (Martín et al., 2001).

became extinct in the continental areas, or migrated to far regions and diverged (Fernández-Palacios and Martín Esquivel, 2001a). Species belonging to this group include many plants from the laurel forest that have Tertiary fossils in Europe (Mai, 1989), genera and species with disjunct distributions in America and the Canary Islands (vicariant divergence associated to the opening of the Atlantic Ocean; Sunding, 1979) or species belonging to the Rand flora (Quézel, 1978) for which closer relatives are found in eastern and southern Africa (figure 1.10).

#### 1.4.5 Species number

The classic biogeographic theory by MacArthur and Wilson (1963, 1967) proposes distance to continent and island size as the two factors determining the number of species that an island maintains with immigration and extinction rates balanced. This simple theory has been the basis for understanding the distribution of biodiversity in islands. Further studies revealed other physical factors determining species diversity in islands: habitat diversity, climate, island age, extreme isolation and equilibrium (see Gillespie and Roderick, 2002, review). Recently, Emerson and Kolm (2005) also demonstrated the influence of the species diversity itself in the rates of speciation in islands, adding a new factor to the dynamics of the distribution of diversity in islands.



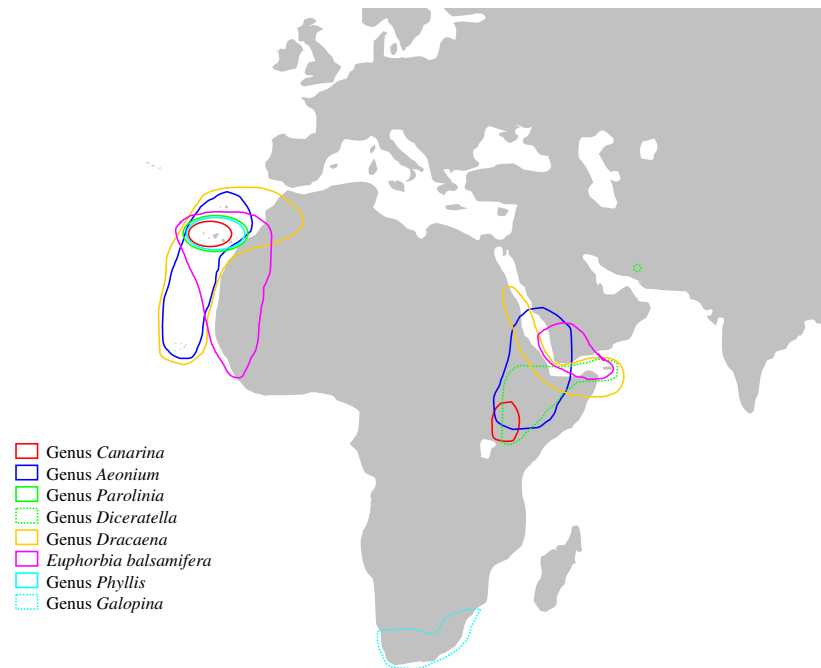


Fig. 1.10: Distribution of some of the Canarian plants and their closer relatives in southern and eastern Africa (after Bramwell, 1986, figures 8, 9, 10, 12, 14 and 19). These species belong to the Rand flora, a group of plants that migrates south and east after the climatic changes in Europe and desertification of North Africa (Bramwell, 1986), but also found a refugium on the Macaronesian islands (Bramwell, 1986).

In the Canary Islands we can broadly see the main factors in play: the older and larger island of Tenerife holds higher species diversity than La Palma, but the big island of Fuerteventura, closer to the continent than La Palma and Tenerife, is poorer in biodiversity because of its lower altitude and number of habitats (see table 1.1). Correlations of species diversity with the above mentioned factors have been done, in the Canary Islands, for birds (Carrascal and Palomino, 2002; Donázar et al., 2005), Heteroptera and Coleoptera (Becker, 1992), spiders (Real et al., 1999), freshwater Ostracoda (Malmqvist et al., 1997), arthropods and plants (Emerson and Kolm, 2005).

### *1.5 Summary*

Canary Islands are oceanic islands created by complex hotspot volcanism that is still active throughout the archipelago. Due to their location, their altitude and the influence of different atmospheric and oceanic currents, the islands present very contrasting climates even at geographically close areas within the same island. The biota of the archipelago is strongly influenced by these physical factors. The isolation among islands and from the continent and the high diversity of habitats favour the processes of genetic differentiation and speciation. Therefore, understanding the geology, climate and biogeography of the archipelago is fundamental for endeavouring the study of all Canarian species.

## 2. CANARY ISLAND PINE *PINUS CANARIENSIS* CHR. SMITH 1828

### 2.1 *Description*

The Canary Island pine, like other pines, has a monopodial growth. Younger individuals have a marked pyramidal crown that rounds with age. Adult trees easily reach more than 30 m high and exceptionally old trees are recorded at up to 60 m. The bark is very thick, scaly and of a reddish brown colour. Buds are broad, ovoid-cylindrical and covered with red-brown scales which are curved and with whitish hairs at the tip. Dwarf shoots (fascicles) hold three long (20–30 cm) needles, retained for two or three years. Needles are green to yellow-green, 1 mm thick, with several lines of stomata in their three faces, often hanging downwards (weeping habit). Juvenile leaves are whitish green, growing directly on the branches of seedlings and on epicormic shoots of mature trees. Seed cones grow solitary and mature in two years when they reach 10–20 cm. Pollen cones are small, grouped in yellowish to orange clusters of 5–10 cm (figure 2.1). Seeds are 10–12 mm long with an 18–20 mm wing that does not easily detach (adnate). References: Page (1974); Delgado González (1986); Blanco Andray et al. (1989); Climent et al. (1996) and Earle and Frankis (1999).

### 2.2 *Ecology*

#### 2.2.1 *Distribution*

The natural distribution of the Canary Island pine is restricted to the western islands of the archipelago: Gran Canaria, Tenerife, La Gomera, El Hierro and La Palma (figure 2.2). *P. canariensis* grows in a wide range of climatic conditions. In the southwestern slopes (500–2500 masl; Fernández-Palacios and de Nicolás, 1995) it grows in xeric conditions and can even exist in localities that barely



Fig. 2.1: In *Pinus canariensis* yellowish orange pollen cones are grouped in clusters while red-brown seed cones are solitary (photos: Peter Schönfelder).

attain 300 mm of annual rain (Blanco Andray et al., 1989). In the north-eastern slopes it benefits from the humid trade winds that form the *mar de nubes* (cloud banks). There, the condensation of the fog in the foliage (throughfall) increases the amount of water that reaches the soil twofold (Ceballos and Ortuño, 1952; Aboal et al., 2000b; Tejedor et al., 2004). In these areas competition with the *monteverde* restricts the distribution of the pine forest to higher altitudes (600–2100 masl; Fernández-Palacios and de Nicolás, 1995).

### 2.2.2 Phytosociology

Several works (see references in section 2.5) have described the different associations of plant species in the Canarian pine forest. Here I will present only a summary (based on that of Climent et al., 1996) to illustrate the diversity of habitats that *P. canariensis* occupies (compared to the Canary Islands vegetation types in section 1.3.2).

#### *Pine forest*

This is typical pine forest. It is an oligospecific community dominated by pines with scarce presence of shrubs and grasses. *Chamaecytisus proliferus* subsp. *angustifolius* and *Cistus symphytifolius* are the characteristic shrub species in this formation.

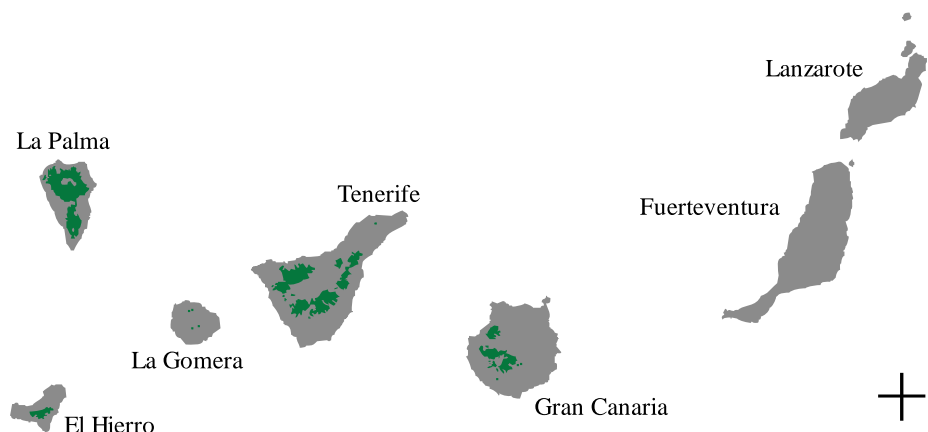


Fig. 2.2: Distribution of the Canary Island pine (after Climent et al., 1996). Natural population are restricted to the western islands which present higher altitudes appropriate for the development of this species.

#### *Pine forest with savins*

This is the pine forest in its more xeric form. It is present in the southwestern (leeward) areas of the island and represents the ecotone with the semiarid woods of savins (*Juniperus turbinata* subsp. *canariensis*). In more degraded areas these pine forests are characterised by shrubs such as *Cistus monspeliensis*, *Micromeria* spp. (thymes), *Euphorbia obtusifolia* (*tabaiba*) and *Salvia canariensis*.

#### *Pine forest with monteverde*

In contrast with the previous association, this is the more humid pine forest. It is found in the ecotone with the *fayal-brezal* in the northeastern (windward) areas of the islands. It is characterised by the presence of heather (*Erica arborea*).

#### *Pine forest with subalpine scrub*

At the higher altitudes conditions become more severe. There is a wide variance in daily temperature with winters characterised by frost and snow. Levels of solar radiation are higher because cloud-banks do not surpass 2000 masl (see effects of trade winds in section 1.3.1). Also, conditions are drier and hydric stress has been suggested to be the factor determining the altitude for the *P. canariensis* timberline (Gieger and Leuschner, 2004). In this formation pines grow with an

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understory of subalpine shrubs with abundance of *Adenocarpus viscosus* (codeso).

### *Pine forest with cedros*

This is a peculiar rupicolous formation where pines grow with the endemic *cedros* (*Juniperus cedrus*).

### 2.2.3 *Reproductive system*

Pines are monoecious trees with sexual reproduction (vegetative reproduction is present in very few species and is related to fire resistance as described in the next section; Ledig, 1998). Their mating system is predominantly outcrossing (see references within Ledig, 1998; Boshier, 2000), which seems to be a consequence of strong inbreeding depression (Ledig, 1998). In the Canary Island pine, flowering occurs from the end of February to the end of March (–May; Delgado González, 1986) varying with the particular climatic conditions (Climent et al., 1996). Pollen is dispersed by wind and can potentially reach great distances (see figure 2.3). After fertilisation seed cones mature for two years and seed release is generally initiated in the third summer (Climent et al., 1996). A proportion of seed cones remain closed for several years; these are serotinous cones and correspond to an adaptation to fire discussed in the next section. Seeds are winged and dispersed by wind (Lanner, 1998). Climent et al. (1996) observed that fructification is produced in trees from 25–30 years old (but Krugman and Jenkinson, 1974, give 15–20 years for maturity in *P. canariensis*). Regeneration is usually abundant but seedlings generally die unless there is a low density canopy (Climent et al., 1996).

### 2.2.4 *Fire adaptation*

Pines are species linked to fire-prone environments and follow two strategies to face wildfires: individual survival and stand resilience (seeds stored in the canopy and released after fire; Agee, 1998). Canary Island pine fits with the first strategy: adult trees have a thick protective bark and the capacity to resprout due to epicormic buds<sup>1</sup> (Climent et al., 2004) and seeds are large and heat resistant

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<sup>1</sup> Epicormic buds only sprout after fire or frost damage, however sprouting occurs spontaneously in some *Pinus canariensis* cultivars of California, probably because of artificial selection

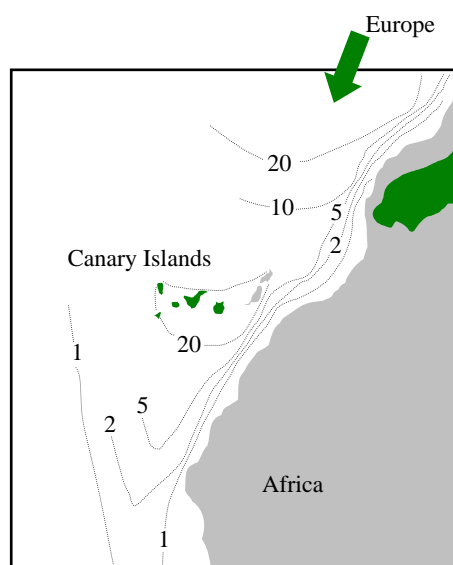


Fig. 2.3: Percentage representation of *Pinus* fossil pollen grains in marine surface sediments at the ocean floor off northwest Africa. Green areas represent current sources of *Pinus* pollen. Isopercentage lines coincide with main flow patterns of wind transport (after Hooghiemstra et al., 1986, figure 10).

(Escudero et al., 2000). Nevertheless, *P. canariensis* also presents serotinous cones corresponding to the strategy of stand resilience. The proportion of serotinous cones is very variable among populations and seems to be linked to the occurrence of recent fires (Climent et al., 2004). Some of these fire-adaptation traits are very common in other species of pine. In contrast, resprouting capacity is quite uncommon among other species of pine (Keeley and Zedler, 1998). Climent et al. (2004) interpreted this adaptation of *P. canariensis* to fire to the result of selective pressure from volcanism.

### 2.3 Evolution and biogeography

Gymnosperms were the first flowering plants to evolve around the middle Devonian (see table 2.1). For the family Pinaceae, fossils are known from the Jurassic (160 mya), including the genus *Pinus*. From the Cretaceous (130 mya) two subgenera are distinguished: *Strobus*, Haploxyton or ‘soft pines’ and *Pinus*, Diploxyton or ‘hard pines’ (Willis and McElwain, 2002). Climatic changes be-

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due to propagation by cuttings (Earle and Frankis, 1999).



Fig. 2.4: *Pinus canariensis* fossil deposit map and photo of fossil cone from Alicante (southeastern Iberian Peninsula). Map based on information from Page (1974); Kasapligil (1977); Gregor (1980); Klaus (1989); García-Talavera et al. (1995); Morla Juaristi et al. (2003).

ginning in the late Mesozoic left the distribution of pines fragmented during the Eocene and led to the differentiation of the major *Pinus* groups (Millar, 1993, 1998).

*Pinus canariensis* belongs to a group of Mediterranean<sup>2</sup> pines (*Pinus pinaster*, *P. heldreichii*, *P. halepensis*, *P. brutia*, *P. pinea* and Himalayan *P. roxburghii*) that can be defined both by morphological (Klaus, 1989; Frankis, 1999) and DNA affinities (Geada López et al., 2002; Gernandt et al., 2005). The closest relative to the Canary Island pine seems to be the Himalayan Chir pine (*P. roxburghii*) with which *P. canariensis* can hybridise (Keng and Little Jr, 1961) and with which it shares a very similar morphology (Page, 1974).

Tertiary fossils (see figure 2.4) attributed to *P. canariensis* or its ancestor species have been found in the Iberian Peninsula (Morla Juaristi et al., 2003), France (Saporta, 1865, 1868, 1873; Depape, 1922), Austria (Klaus, 1982, 1984) and Turkey (Kasapligil, 1977), and it has been suggested that a *canariensis*-

<sup>2</sup> In older references (e.g. Cook, 1898; Hutchinson, 1918) a closer relation with Mexican pines is attributed to *P. canariensis* that is not longer considered. Also Klaus (1989) consider this group of Mediterranean pines “closely related to Caribbean and C. American taxa” (Haploxyylon and Diploxyylon American pines), instead to the Eurasian Diploxyylon pines as is suggested in DNA phylogenies (e.g. Gernandt et al., 2005).



Era	Period	Epoch	Life & <i>Pinus canariensis</i>	Geology and climate	Age( <i>mya</i> )	
Cenozoic (Quaternary)	Neogene	Holocene		Last Glacial Maximum	0.02	
			Fossils in Tenerife		0.6	
Pleistocene		Fossil in La Palma Extinction in Europe ?	Sahara desertification El Hierro emerges Glaciation cycles start	1.2 1.8		
		Fossils in Iberian Peninsula	La Palma emerges	2.0		
Pliocene		Fossils in Turkey		5.3		
				Messinian salinity crisis La Gomera emerges Tenerife emerges	6 11.0 12.0	
Cenozoic (Tertiary)	Palaeogene	Miocene	Fossils in Gran Canaria Fossils in S. France Fossils in Austria	Gran Canaria emerges Lanzarote emerges Fuerteventura emerges	13 14.5 15.5 22 23.8	
			Oligocene			33.7
			Eocene			54.8
		Palaeocene	K/T extinction		65	
Mesozoic	Cretaceous		Subgenera <i>Pinus</i> & <i>Strobus</i>	Canaries as seamounts Atlantic Ocean opens	75 130 144 160	
	Jurassic		<i>Pinus</i> Pinaceae	Pangea breaks	206	
	Triassic			Tethys Ocean opens	248	
Palaeozoic	Permian				290	
	Pennsylvanian		Conifers	Pangea formed	323	
	Mississippian				354	
	Devonian		Gymnosperms		360	
					417	
	Silurian		Vascular (land) plants		443	
	Ordovician				490	
	Cambrian			Gondwana formed	543	

Tab. 2.1: The geological timescale and major events in the evolution of *Pinus canariensis* and the development of the Canary Islands. Note that dating of fossils in this table is approximate. Based on information from: Willis and McElwain (2002) (evolution of plant kingdom), Page (1974); Kasapligil (1977); Gregor (1980); García-Talavera et al. (1995); Morla Juaristi et al. (2003) (*Pinus canariensis* fossils).

*roxburghii* ancestor was distributed throughout the shores of the Tethys Sea. The Alpine Orogeny and climatic changes at the end of the Tertiary are thought to have fragmented this distribution into the extant populations of *P. canariensis* in the Canary Islands and *P. roxburghii* in the Himalayas (Morla Juaristi et al., 2003).

Less is known about the palaeobiogeography of *P. canariensis* in the Canarian archipelago. Schenck (1907) attributed the arrival of *P. canariensis* to the Canary Islands in the Tertiary by bird dispersal, although there is little evidence for this. The oldest pine fossils in the archipelago are bark impressions (13 mya; García-Talavera et al., 1995) that suggest the presence of a pine species in the island of Gran Canaria soon after its emergence (14 mya; Carracedo and Day, 2002). However these fossils could belong to another pine species that could have become extinct with explosive volcanic events such as the Roque Nublo cycle (Pérez-Torrado et al., 1995). A fossil cone identified as *P. canariensis* was found in La Palma (Gregor, 1980) also suggesting a quick colonisation of the island after emergence. However, this fossil offers little information on the earlier presence of *P. canariensis* in the archipelago.

## 2.4 Canarian pine and humans

### 2.4.1 Uses of the Canary Island pine forest

Colonisation of the Canary Islands by humans is dated in the first millennium B.C. (González Antón et al., 1998). However, little is known about the early human settlements and their relationship with the forest, but more is known about the *Guanches* (the indigenous people inhabiting the Canary Islands) at the time Europeans arrived at the archipelago (end of XV<sup>th</sup> century). The *Guanches* used pine wood as fuel and for the construction of implements (weapons, staffs, coffins, shovels, etc) and huts. The resin was used in their medicine and pine nuts were eaten. The agriculture, livestock and human-origin fires of the *Guanches* probably resulted in some regression of pine forest (del Arco Aguilar et al., 1992; Pérez de Paz et al., 1994a).

The decline of the pine forest was mainly produced after the Spanish colonisation. Wood was employed in the production of charcoal and as fuel, construction of buildings and ships, and production of tools (figure 2.5). Especially appreci-



Fig. 2.5: Column and capital made from *Pinus canariensis* wood, La Oratava (from Fototeca de la de la Consejería de Medio Ambiente y Ordenación Territorial del Gobierno de Canarias, [www.gobcan.es/medioambiente/](http://www.gobcan.es/medioambiente/)).

ated was the resinous heartwood (*tea*) because of its beauty and its resistance to water and insects (Parsons, 1981). However, the usage which most affected the pine forest was the extraction of pitch for the caulking of ships (Parsons, 1981). The overexploitation was so great that soon laws regulating this industry were proclaimed. Nevertheless those laws were never respected and clandestine exploitation was always present until the XX<sup>th</sup> century (del Arco Aguilar et al., 1992; Pérez de Paz et al., 1994a).

In the XX<sup>th</sup> century the exploitation of the pines was greatly reduced. Wood uses were restricted to the production of charcoal, fuel and agricultural tools. A new use appears with the extraction of *pinocha* (dead pine needles collected from the ground in the pine forest). *Pinocha* was used as a shock-absorber in the packing of bananas and as fertiliser or mulch (Parsons, 1981).

Nowadays the exploitation of pine forest in the Canary Islands is in decline. Wood extraction from *P. canariensis* has been very low with a tendency towards completely abandoning its use in the last decade. Current wood production from *P. canariensis* and *Pinus radiata* is mainly a by-product of forest management and the policy of substitution of the introduced *P. radiata* for autochthonous

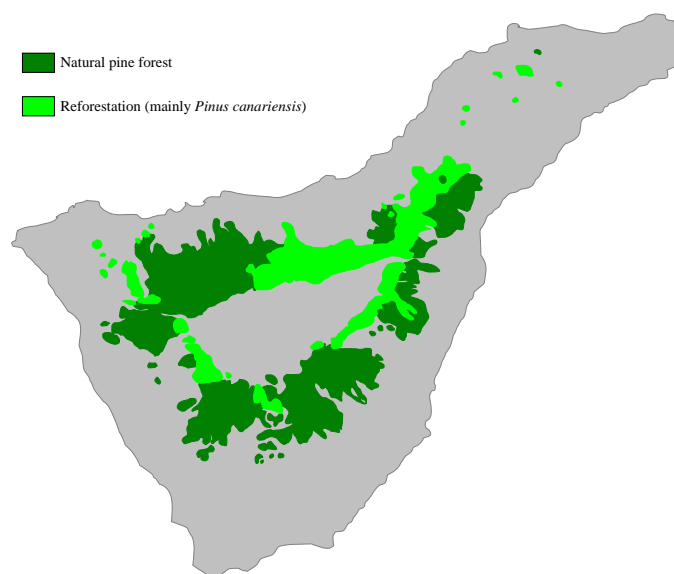


Fig. 2.6: Tenerife *Pinus canariensis* forest, natural and reforested stands (after del Arco Aguilar et al., 1992). The current distribution of pine forest in the archipelago is the product of several centuries of deforestation that left a fragmented forest plus the recent (from 1950's) reforestation activity that connect the old growth forest patches.

species (Viceconsejería de Medio Ambiente, 2002). *Pinocha* is currently the only important resource from the pine forest to be still utilised.

#### 2.4.2 Reforestation and conservation

The interest and public awareness about the restoration of pine forest in the islands started at the beginning of the XX<sup>th</sup> century, but it was not until the 1950's that serious reforestation plans were developed (del Arco Aguilar et al., 1992; Pérez de Paz et al., 1994a). Some of the reforestation was initially done with exotic species (mainly *P. radiata* but also *P. halepensis*, *P. pinaster* and *P. sylvestris*). At present there are 17 500 ha of pine reforestation (see figure 2.6 for their distribution in Tenerife) with 86% being dominated by *P. canariensis*, 11% *P. radiata* and 3% comprised of other introduced conifers (Viceconsejería de Medio Ambiente, 2002).

Most of these plantations have been left unmanaged until the 1990's. Thinning management of high density reforestations is of importance to promote a diversity of tree sizes and natural regeneration (Blanco Andray et al., 1989). Pine



Fig. 2.7: Elimination of *Pinus radiata* and reforestation with *monteverde* in Lomo Gordo (La Esperanza). From Fototeca de la Consejería de Medio Ambiente y Ordenación Territorial del Gobierno de Canarias, [www.gobcan.es/medioambiente/](http://www.gobcan.es/medioambiente/).

plantations in areas that potentially correspond to *monteverde* or Mediterranean woods have also been under active management for the restoration of the original habitat (Naranjo Borges, 2001; Fernández, 2001). Plantations of the introduced *P. radiata* are also being transformed to *P. canariensis* forest or *monteverde* (figure 2.7). Although these plantations of foreign species are unpopular they have the merit of favouring forest succession of the native species. This has acted to prevent soil erosion and fire disturbances, and the current recommended management strategy is the gradual removal of *P. radiata* and replacement planting of native species (Arévalo and Fernández-Palacios, 2005).

The abundance of pine forest, projects of reforestation actions (Viceconsejería de Medio Ambiente, 2002) and the lack of timber exploitation were taken into account by the IUCN to re-classify the threat status of *P. canariensis* as of “Least Concern” (Farjon, 2003). Although the species itself might be not threatened, Vaxevanidou et al. (2005) highlighted the importance of preserving the genetic diversity of some small isolated populations of *P. canariensis*. The preservation of natural forest combined with reforestation is not only important for the protection of the Canary pine but also to (a) protect the soil from erosion and (b) facilitate water capture for the subterranean aquifers (Viceconsejería de Medio Ambiente, 2002; Tejedor et al., 2004).

The conservation-oriented management of the current pine forest is also very necessary to protect the endemic and threatened species of this ecosystem which include several rare bird species (figure 2.8). For instance, the endemic great spot-



Fig. 2.8: Threatened birds linked to the pine forest (Bacallado, 1976): a. Canary Island blue chaffinch (*Fringilla teydea*) and b. great spotted woodpecker (*Dendrocopos major* subsp. *canariensis* and subsp. *thanneri*). From The Gateway to the European Union Web (europa.eu.int) © European Communities, 1995-2003). Management of pine forest can affect these species as they are strongly linked to the pine.

ted woodpecker subspecies (*Dendrocopos major* subsp. *thanneri* and subsp. *canariensis*), which are restricted to the pine forest, require mature stands with some abundance of dead wood (González, 2002b,a). The gradual elimination of *P. radiata* by tree ringing in mixed stands favours the development of *P. canariensis* but also provisions resources of dead wood for these endangered woodpeckers (Velázquez Padrón, 2001). Another emblematic and threatened bird of the pine forest is the endemic Canary Island blue chaffinch (*Fringilla teydea*) which can be affected by collection of pine seeds (Viceconsejería de Medio Ambiente, 2002).

Finally, the extraction of *pinocha* also requires some control. This practice can be detrimental to the ecosystem as it may affect the arthropod fauna (Viceconsejería de Medio Ambiente, 2002). Removing these dry leaves is defended as a means to prevent wildfires, however pine forest communities are adapted to fires and fires can also have a beneficial effect in the transformation of artificial plantations into more natural ecosystems (Arévalo et al., 2001).

#### 2.4.3 *Pinus canariensis* outside the archipelago

The Canary Island pine has been planted all around the world: including the Mediterranean area, Africa (North, central and South), Near and Middle East, India, Australia, New Zealand, California and South America (Chapman, 1948;

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FAO, 1952; Din, 1958; Castilla Lattke, 1999; Gravano, 2002; Nyoka, 2002). However, its slow growth can be a problem for establishing commercially viable plantations (Spencer, 2001). In the Mediterranean area (Roussis et al., 1995), Australia (Richardson and Higgins, 1998), New Zealand (Heenan et al., 2002) and South Africa (Richardson et al., 1994; Richardson and Higgins, 1998; Moran et al., 2000) it has escaped cultivation and has naturalised, sometimes invading endangered ecosystems.

### 2.5 *Summary and further reading*

In this chapter the biological features of *P. canariensis* that can affect the geographic distribution of genetic diversity have been discussed. The reproductive system (outcrossing) and propagule dispersal (anemophilous and anemochorous) are fundamental to our understanding of gene exchange among individuals and populations. The historical biogeography and the influence of disturbances (i.e. fire and volcanism) can also shape the genetic structure of populations by means of founder effects, bottlenecks and differentiation by isolation. Similarly recent human disturbances and reforestation processes might change the distribution of genes by means of seed transference, artificial selection and forest fragmentation. Finally, the diversity of climatic conditions can promote the appearance of local adaptations and differentiation. Nevertheless, I have considered that the readers of a monograph on *P. canariensis* might have a wider interest in this species and a compilation of references on diverse topics is offered to them:

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Field	references
General bibliography	Nogales Ventoso (1917); del Arco Aguilar et al. (1990, 1992); Pérez de Paz et al. (1994a) and Pérez de Paz et al. (1994b)
Phylogeny/Taxonomy	Page (1974); Klaus (1989); Strauss and Doerksen (1990); Krupkin et al. (1996); Frankis (1999); Liston et al. (1999); Wang et al. (1999); Gonzalez-Andrés et al. (1999); Geada López et al. (2002) and Gernandt et al. (2005)
Fossils	Saporta (1865, 1868, 1873); Depape (1922); Schmincke (1967, 1968); Kasapligil (1977, 1978); Gregor (1980); Klaus (1982, 1984); Alonso Blanco (1989) and Morla Juaristi et al. (2003)
Ecology	Blanco Andray et al. (1989); Nogales et al. (1990); Arévalo et al. (2001); Srutek et al. (2002); Jonsson et al. (2002); Grotkopp et al. (2002) and Tapias et al. (2004)
Phytosociology	Ceballos and Ortuño (1976); Esteve (1969); Fernández-Pello Martín (1987); del Arco Aguilar et al. (1987); Rivas-Martínez (1987); Rivas-Martínez et al. (1993a) and Rivas-Martínez et al. (1993b)
Ecophysiology	Jiménez et al. (1997); Morales et al. (1999); Jiménez and Morales (2001); Tausz et al. (1998a,b, 2001, 2004); Gasulla et al. (2001); Wieser et al. (2002); Zelling et al. (2002); Peters et al. (2003a,b); Gieger and Leuschner (2004); Grill et al. (2004) and Stabentheiner et al. (2004)
Fire adaptation	Escudero et al. (2000) and Climent et al. (2004)
Hydrology	Aboal et al. (2000b) and Tejedor et al. (2004)
Cone morphology	Franco (1943); Climent et al. (2001a) and Gil et al. (2002)
Population genetics	Schiller et al. (1999); Korol et al. (1999); Gómez et al. (2003); Vaxevanidou et al. (2005) and this work
Provenance tests	Bellefontaine and Raggabi (1979); Climent et al. (2001b, 2002b) and Climent et al. (2005)
Silviculture	Delgado González (1986); Martínez et al. (1990); Khat-tabi (1991); Pita et al. (1998); Spencer (2001) and Gravano (2002)
Wood	Peraza and López de Roma (1967); Coetzee (1978); Climent et al. (1993, 1998, 2002a) and Climent et al. (2003)
Essential oils	Roussis et al. (1995) and Pfeifhofer (2000)
Conservation	Farjon (2003) and Arévalo and Fernández-Palacios (2005)

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Part II

DETECTION OF GENETIC DIVERSITY IN  
*PINUS CANARIENSIS*

### 3. MEASURING GENETIC VARIATION IN *PINUS CANARIENSIS*

Genetic diversity in forest trees is essentially studied by two contrasting methods: progeny tests and genetic markers. Progeny tests consist of growing progenies from different origin (from different individuals, families or provenances) under the same environmental conditions to study the differences in the traits of interest (Wright, 1976). Genetic markers are phenotypic features from which the genotypes of the individuals can be deduced. Glaubitz and Moran (2000) distinguish three types of genetic markers: morphological, biochemical and DNA-based markers. In this chapter I will review the application of these different methods to the study of *Pinus canariensis* genetic diversity (but see Glaubitz and Moran, 2000, for more information on genetic markers for forest trees).

#### 3.1 *Provenance tests*

It has been shown in several studies that molecular markers can be poor indicators of population differentiation in adaptive traits (Yang et al., 1996; Karhu et al., 1996; Bekessy et al., 2003). To study adaptation to local conditions common garden tests of populations is the definitive tool (Mátyás, 1996). In these experiments seeds from different origins are germinated and grown under the same environmental conditions. The phenological differences observed in response to the environment are interpreted as genetic adaptive variation (additional details in design and analysis of provenance tests can be found in Wright, 1976; Mátyás, 1996; Hansen et al., 2003).

Performance trials for *P. canariensis* in numerous countries are mentioned in the literature (Hutchinson, 1918; Chapman, 1948; FAO, 1952; Din, 1958; Krugman and Jenkinson, 1974; Le Maitre, 1998; Spencer, 2001; Gravano, 2002) but results are seldom published (only Bellefontaine and Raggabi, 1979). Recently, provenance tests have been set for *P. canariensis* by Climent et al. (2001b) to

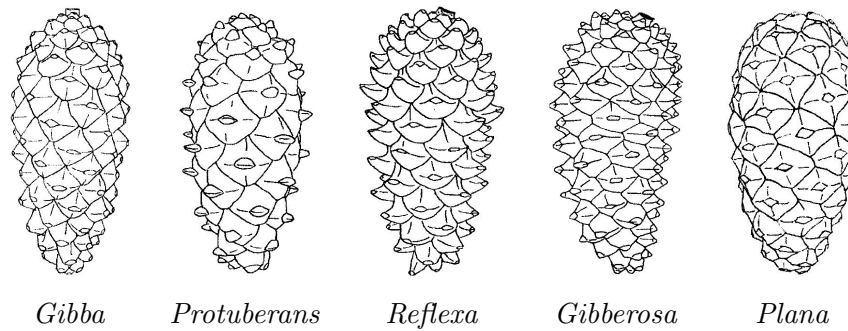


Fig. 3.1: Cone morphology variation in *Pinus canariensis*. *Gibba* cones are the most common in the Canary Island pine (from Gil et al., 2002).

test the genetic differences in growth and morphology among ecological regions (defined in Climent et al., 1996). First results of these tests have revealed geographic variation: drier provenances have higher survival rate under drought stress (Climent et al., 2002b) and a prolonged juvenile stage (interpreted as a consequence of sclerophyllous growth; Climent et al., 2005).

### 3.2 Morphological variation

The use of morphological markers is rare in trees because few characters present Mendelian inheritance (e.g. chlorophyll deficiency; Glaubitz and Moran, 2000). In conifers, seed-cone morphology is genotypically determined (Khalil, 1984) and has been used to detect population differentiation (e.g. *Picea abies* and *Pinus strobus*; Borghetti et al., 1988; Beaulieu and Simon, 1995). Cone morphology in *P. canariensis* is extremely variable (figure 3.1), and Klaus (1989) attributed this variation to “climatic races”; however Gil et al. (2002) found no clear geographical distribution of the morphological traits.

### 3.3 Biochemical markers

#### 3.3.1 Secondary metabolites

The production of terpenes and phenols exhibits variation among individuals that is due to genetic differences. Chromatography analysis can be employed to determine the terpene composition of essential oils and resins of trees. However the difficulty of assigning exact genotypes, the complex treatment of the data

(different proportions of different terpene compound; Birks and Kanowski, 1988) and the influence of environment on the expression of a compound (e.g. Supuka et al., 1997) make the use of terpenes as genetic markers overly complex (but see Hanover, 1992; Bojovic et al., 2005).

Chemical components (mainly terpenes) of essential oils in *Pinus canariensis* have been described by Roussis et al. (1995) and Pfeifhofer (2000). Pfeifhofer (2000) was only interested in the chemical composition of the essential oils; nevertheless Pfeifhofer (2000) found high variation among individuals in the concentration of several compounds which was attributed to genetic variability. Roussis et al. (1995) studied the interspecific variation of these terpenes with other Mediterranean pines but intraspecific variation has never been studied.

### 3.3.2 Protein markers

#### *Isozymes*

Isozymes or allozymes (allelic variants of an enzyme)<sup>1</sup> are one of the most extensively used molecular markers. Polymorphism is studied with starch or polyacrylamide gel electrophoresis where isozymes (extracted from plant tissue with special buffers) are separated with an electric current due to their differences in size, shape and charge. The enzymatic properties of the isozymes are employed to visualise their position in the gel by coupling the enzymatic reaction to staining reaction. Usually the gel is sliced to stain for different enzyme reactions on the same run. The genotypes of the individual plants can be deduced from banding pattern developed. Usually isozymes are codominant markers and there is a constant number of loci for each enzyme system within a genus.

Isozymes have been used by Schiller et al. (1999) and Korol et al. (1999) in *P. canariensis* for the study of population differentiation ( $G_{ST} = 0.091$ ;  $N_e m = 2.52$ ). Schiller et al. (1999) describe electrophoresis methodology for 16 enzyme systems containing 32 loci (28 of them were found to be polymorphic) from the seed megagametophyte (haploid tissue derived from the female gametophyte). There is also an isozyme study for the discrimination of pine species that includes *P. canariensis* (Gonzalez-Andrés et al., 1999).

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<sup>1</sup> Strictly speaking isozymes refer to variants of an enzyme system (different loci and different alleles within a locus) detectable by electrophoresis while allozymes refer to allelic variants of an enzyme locus (Gillet, 1999).

### *Seed storage proteins*

Less usual than isozymes is the study of polymorphism in proteins without enzymatic activity. These are separated by electrophoresis and visualised by silver staining or other methodologies. In pines, seed storage proteins from the megagametophyte have been used to study genetic diversity of populations (Al-lona et al., 1996; Álvarez et al., 2004) but these markers have not been utilised in *P. canariensis*.

### 3.4 DNA markers

While previous genetic markers examined the protein products of genes, DNA-based markers allow for the examination of the genetic material itself. Plants have three different genomes (chloroplast, mitochondrial and nuclear) with contrasting modes of inheritance. Characteristically within pines, chloroplast DNA is paternally inherited, mitochondrial DNA is maternally inherited and nuclear genes are biparentally inherited (Neale and Sederoff, 1989; Wagner, 1992). This difference is fundamental for the understanding that loci from different genomes will provide different information about the movement of genes (figure 3.2).

The analysis of DNA polymorphism is based mainly on two techniques: the use of restriction enzymes (enzymes that cut DNA at specific nucleotide sequences) and the polymerase chain reaction (PCR; amplification of specific DNA sequences). The use of these techniques separately (PCR: SSRs, RAPDs; restriction enzymes: RFLPs) or combined (AFLPs, PCR-RFLPs) define different types of marker. Several reviews discuss the different DNA markers (Bachmann, 1994; Karp et al., 1997; Parker et al., 1998; Sunnucks, 2000; Avise, 2004) and their use in forest trees (Gillet, 1999; Glaubitz and Moran, 2000; Jiménez and Collada, 2000; Namkoong and Koshy, 2001; Wang and Szmidt, 2001; Li and Gu, 2003) and will not be considered further here.

To my knowledge, among the various DNA markers only chloroplast microsatellite markers have been used for *P. canariensis* in previous population genetic studies (Gómez et al., 2003; Vaxevanidou et al., 2005). Gómez et al. (2003) found significant population differentiation among populations ( $\Phi_{ST} \approx 0.19$ ) but no differentiation among islands. Gómez et al. (2003) discussed their results in relation to the processes of colonisation, gene flow and isolation by distance; how-

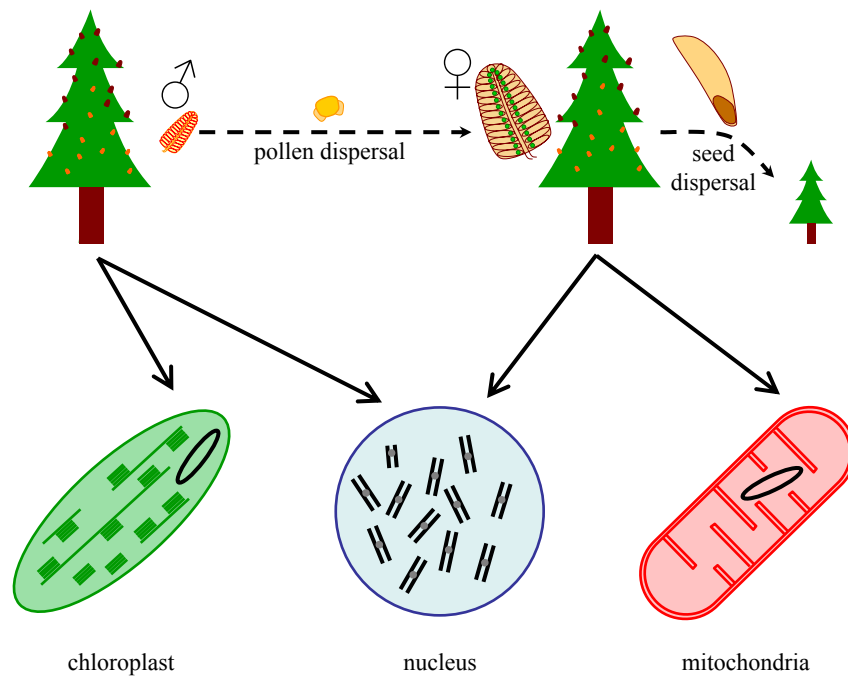


Fig. 3.2: Paternal inheritance of chloroplasts and maternal inheritance of mitochondria in Pinaceae (Neale and Sederoff, 1989; Wagner, 1992). The contrasting inheritance of the organelle genomes has implications for the analysis of gene flow with nuclear, chloroplast and mitochondrial loci (Hu and Ennos, 1999). The influence on measures of population differentiation of pollen dispersal is higher for chloroplast loci than for nuclear loci while for mitochondria only the seed dispersal has an influence.

ever no definite conclusions were reached. Vaxevanidou et al. (2005) extended the previous study to small isolated populations and revealed that some of them contained distinct and highly diverse gene pools and emphasized the importance of preserving the genetic diversity within these populations. Additional to chloroplast microsatellites, primers for the amplification of nuclear microsatellites in pine species have been tested for *P. canariensis* (González-Martínez et al., 2004; Chagné et al., 2004, and this work; results presented in Chapter 4) but have not been used in previous analyses.

### 3.5 Summary

There is a wide range of genetic markers available for *P. canariensis* though some of them, such as the morphological and terpene markers, would probably have little relevance in future research. Protein markers may still be useful for future studies where a large number of markers are required (similar to the one described in Chapter 8) and are still widely used in population genetics of pine species (e.g. Sáenz-Romero and Tapia-Olivares, 2003; Mitton and Duran, 2004; Slavov and Zhelev, 2004; Korshikov et al., 2004, 2005; Nijensohn et al., 2005). It is remarkable the lack of mtDNA markers analysed in *P. canariensis*. This is attributable to the low genetic variation found in the plant mitochondrial genome, however, newly developed mtDNA markers for pines (e.g. Godbout et al., 2005) offer new opportunities to find variation for a genome that can provide valuable information on seed dispersal. Finally, information on local climatic adaptations of the Canary Island pine will be soon available from provenance tests (Climent et al., 2001b).

## 4. MICROSATELLITES AVAILABLE FOR *PINUS CANARIENSIS*

### 4.1 *Nuclear microsatellites*

Microsatellites (or simple sequence repeats; SSR) are sequences of repetitive DNA where a single motif consisting of one to six base pairs is repeated in tandem a number of times (see Li et al., 2002, for a review). Polymorphism in these sequences is mainly due to variation in the number of repeats of the motif. Two mutational mechanisms have been proposed to explain this type of variation: replication slippage (Tachida and Iizuka, 1992) and recombination with out-of-phase aligning (Harding et al., 1992). Both processes result in changes in the number of repeat units that are compatible with the observed size polymorphism of microsatellites.

Nuclear microsatellites (nSSR) are among the most used molecular markers for population genetics studies (Jarne and Lagoda, 1996; Sunnucks, 2000) and offer several advantages as genetic markers. First, they can be isolated for species with no previous knowledge of their genetic structure (see Toonen, 1997; Zane et al., 2002, for details on methods) relatively quickly and cheaply (Squirrell et al., 2003). Second, they are assayed by simple PCR. Third, they provide highly polymorphic, single-locus, codominant data and information on genealogical relationships among alleles (Sunnucks, 2000). The main drawback of the use of nSSR as genetic markers is homoplasy, as a consequence of the dominant stepwise mutational mechanisms of SSRs (Estoup et al., 2002).

In conifers, the development of microsatellites presents some technical difficulties due to the large size of the genome and the high density of repetitive sequences that acts to reduce the probability of obtaining single-locus markers (Hicks et al., 1998; Echt et al., 1999). A strategy to improve the yield of SSR markers has been to target the low-copy portion of the genome by different methods (see Echt et al., 1996; Elsik et al., 2000; Zhou et al., 2002). Another approach



is to avoid *de novo* isolation and transfer already described microsatellite markers from related species (Echt et al., 1999). Transference of SSR markers among pine species seems to work well within the same subgenus (Echt et al., 1999) and can also be successful across subgenera (Kutil and Williams, 2001). The large number of microsatellite markers already isolated for several pine species (e.g. Smith and Devey, 1994; Kostia et al., 1995; Hicks et al., 1998; Fisher et al., 1998; Echt et al., 1999; Mariette et al., 2001; Liewlaksaneeyanawin et al., 2004) makes this last option our choice for obtaining nSSR markers for *Pinus canariensis*.

#### 4.1.1 Transference of nuclear microsatellite markers

Primer pairs for the amplification of nSSR in *Pinus densiflora* (Lian et al., 2000), *P. halepensis* (Keys et al., 2000), *P. sylvestris* (Soranzo et al., 1998) and *P. taeda* (Chagné et al., 2004) were tested on *P. canariensis*. Amplification was tried using the PCR conditions described in the original references, but when amplification was poor or null, different conditions were tried varying annealing temperature and magnesium concentration (optimised values are reported in Appendix B.2). For those primer pairs showing specific amplification, polymorphism was assessed in 96 individuals by high resolution electrophoresis (ABI PRISM 3700 DNA Analyzer — Applied Biosystems). Polymorphic loci were then used to genotype seven natural populations and two artificial stands of Canary Island pine from Tenerife (Arico 1000, Arico 1500, Arico 2000, Güímar, Vilaflor 1000, Vilaflor 1500, Vilaflor 2000, Arico reforested and Fasnía, locations described in Appendix A). Population data from the seven natural sites were analysed with MICRO-CHECKER to assess the presence of null alleles and scoring errors due to stuttering and large allele dropout (Van Oosterhout et al., 2004).

#### 4.1.2 Results

The results of the transference are summarised in table 4.1. From 25 primer pairs tested, six polymorphic nSSR markers (SPAC 11.5, SPAC 11.8, SPAG 7.14, *ssrPt\_ctg4363*, *ssrPt\_ctg4698* and *ssrPt\_ctg7731*) were found for the Canary Island pine. From these six loci, SPAG 7.14 was already transferred by González-Martínez et al. (2004), who also found PtTX3116 and PtTX4001 (from Auckland et al., 2002) to be polymorphic for *P. canariensis*. Allele frequencies found in the natural populations for those eight loci are presented in figure 4.1. Further test-

ing of loci PHAF02, PHAF09, PHAF10 and SPAC 11.4 might also provide some additional markers in *P. canariensis*. Locus SPAC 11.6 was confirmed to be polymorphic (120–146bp) as described in González-Martínez et al. (2004) but had to be excluded because of the presence of non-specific bands. Locus RPtest9, which was also polymorphic (316–342bp), was also excluded because of the presence of three bands in most of the samples.

Two previous studies have tested the amplification of nSSR primer pairs from pine species in the Canarian pine (Chagné et al., 2004; González-Martínez et al., 2004) but only González-Martínez et al. (2004) checked for polymorphism. When considering the results from Chagné et al. (2004), González-Martínez et al. (2004) and this study together, we can estimate the attrition of loci at each stage in the transference of nSSR markers to *P. canariensis*: 54 out of 82 (66%) primer pairs amplified DNA; 24 out of 33 (73%) primer pairs that amplified did so for a single locus; 10 out of 20 (50%) nSSRs loci were polymorphic, and eight out of 10 (80%) polymorphic nSSRs were scorable. Therefore, approximately 20% of the primer pairs tested became useful markers in the transference. This is however a rough estimate and successful transference depends on both relatedness between species (Echt et al., 1999) and the location of the loci (for nSSR isolated from cDNA successful transference is higher; Chagné et al., 2004).

#### *Description of successfully transferred markers<sup>1</sup>*

*SPAC 11.5* Dinucleotide repeat. Allele size (PCR product size) ranged between 98 and 198bp. MICRO-CHECKER (Van Oosterhout et al., 2004) analysis suggested null alleles in two populations (Arico 1000 and Vilaflor 2000) due to a general (but not significant) excess of homozygotes for most allele size classes.

*SPAC 11.8* Dinucleotide repeat. Allele size ranged between 142 and 319bp. Allele sizes were not compatible with a simple stepwise mutation model (SMM). In the range 142–182bp, alleles were mostly found to have an even number of base pairs but with some having an odd number, while in the range 183–319bp all alleles had an odd number of base pairs. Larger alleles had weaker amplification and were harder to detect thus repeated genotyping was done for dubious samples (i.e. apparent homozygosity for small alleles). MICRO-CHECKER (Van Ooster-

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<sup>1</sup> Additional details on laboratory procedures are presented in Appendix B.2.

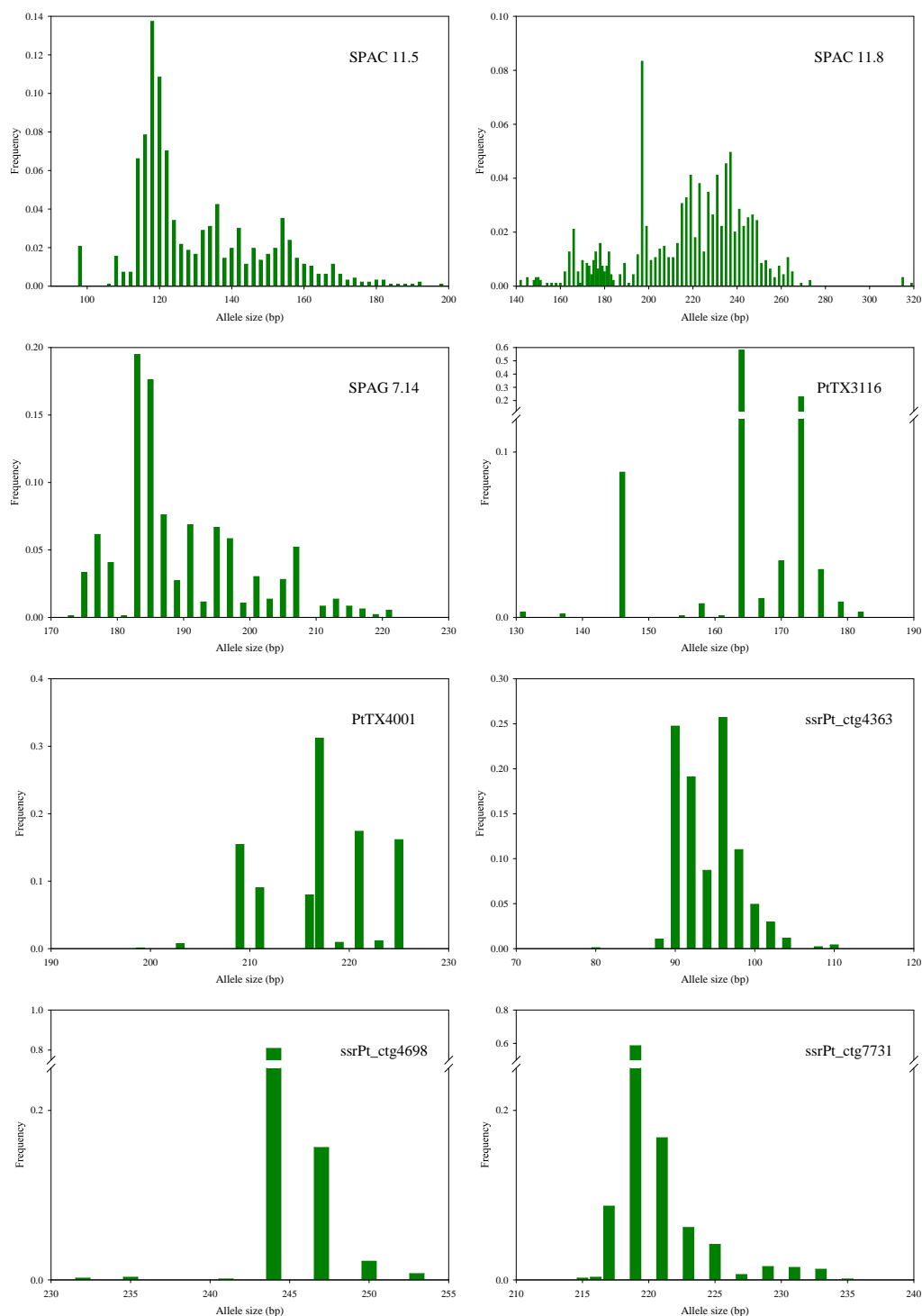


Fig. 4.1: Allele frequencies for the eight nuclear microsatellites markers found in the six natural populations studied (Arico 1000, Arico 1500, Arico 2000, Güímar, Vilaflor 1000, Vilaflor 1500 and Vilaflor 2000, locations described in Appendix A). Alleles ordered by size (bp). For loci PtTX3116, ssrPt\_ctg4698 and ssrPt\_ctg7731, scale was amplified for the lower frequencies to make visible the presence of rare alleles.

Locus	Amplification	Multi-locus amplification <sup>a</sup>	Polymorphism	Original species	Original reference
Pde14	✓	✓	n.a.	<i>P. densiflora</i>	Lian et al. (2000)
PHAF01	✓	✓	n.a.	<i>P. halepensis</i>	Keys et al. (2000)
PHAF02	✓	×	n.a. <sup>b</sup>	<i>P. halepensis</i>	Keys et al. (2000)
PHAF05	✓	✓	n.a.	<i>P. halepensis</i>	Keys et al. (2000)
PHAF07	✓	✓	n.a.	<i>P. halepensis</i>	Keys et al. (2000)
PHAF08	✓	✓	n.a.	<i>P. halepensis</i>	Keys et al. (2000)
PHAF09	✓	×	n.a. <sup>b</sup>	<i>P. halepensis</i>	Keys et al. (2000)
PHAF10	✓	×	n.a. <sup>b</sup>	<i>P. halepensis</i>	Keys et al. (2000)
PtTX3116	✓	×	✓	<i>P. taeda</i>	Auckland et al. (2002)
PtTX4001	✓	×	✓	<i>P. taeda</i>	Auckland et al. (2002)
RPtest9	✓	✓	✓	<i>P. taeda</i>	Chagné et al. (2004)
SPAC 11.4	✓	×	n.a. <sup>b</sup>	<i>P. sylvestris</i>	Soranzo et al. (1998)
SPAC 11.5	✓	×	✓	<i>P. sylvestris</i>	Soranzo et al. (1998)
SPAC 11.6	✓	✓	✓	<i>P. sylvestris</i>	Soranzo et al. (1998)
SPAC 11.8	✓	×	✓	<i>P. sylvestris</i>	Soranzo et al. (1998)
SPAC 12.5	✓	✓	n.a.	<i>P. sylvestris</i>	Soranzo et al. (1998)
SPAG 3.7	×	n.a.	n.a.	<i>P. sylvestris</i>	Soranzo et al. (1998)
SPAG 7.14	✓	×	✓	<i>P. sylvestris</i>	Soranzo et al. (1998)
ssrPt_ctg865	✓	✓	n.a.	<i>P. taeda</i>	Chagné et al. (2004)
ssrPt_ctg3021	✓	×	× <sup>c</sup>	<i>P. taeda</i>	Chagné et al. (2004)
ssrPt_ctg3089	✓	✓	n.a.	<i>P. taeda</i>	Chagné et al. (2004)
ssrPt_ctg4363	✓	×	✓	<i>P. taeda</i>	Chagné et al. (2004)
ssrPt_ctg4698	✓	×	✓	<i>P. taeda</i>	Chagné et al. (2004)
ssrPt_ctg7444	✓	×	×	<i>P. taeda</i>	Chagné et al. (2004)
ssrPt_ctg7731	✓	×	✓	<i>P. taeda</i>	Chagné et al. (2004)
ssrPt_ctg16811	✓	×	×	<i>P. taeda</i>	Chagné et al. (2004)
ssrPt_ctg18103	✓	×	×	<i>P. taeda</i>	Chagné et al. (2004)

n.a. not applicable

<sup>a</sup> Non-specific amplification or complex banding patterns

<sup>b</sup> Polymorphism not tested

<sup>c</sup> Polymorphism tested with only eight individuals

Tab. 4.1: Nuclear microsatellite transference.

hout et al., 2004) analysis did not find evidence for large allele dropout, nor stuttering mistyping. Null alleles were suggested in only one population (Güímar) due to a general (but not significant) excess of homozygotes for most allele size classes.

*SPAG 7.14* Dinucleotide repeat. Allele size ranged between 173 and 245bp. In my experience, primers for this locus degenerate easily and special care is advisable to prolong their mean life.

*PtTX3116* Trinucleotide repeat. Allele size ranged between 131 and 182bp.

*PtTX4001* Dinucleotide repeat. Allele size ranged between 199 and 225bp. It presents one allele (216bp) incongruent with the SMM, the presence of this allele was confirmed by repeated genotyping.

*ssrPt\_ctg4363* Dinucleotide repeat. Allele size ranged between 80 and 110bp.

*ssrPt\_ctg4698* Trinucleotide repeat. Allele size ranged between 232 and 253bp.

*ssrPt\_ctg7731* Dinucleotide repeat. Allele size ranged between 215 and 235bp. It presents one allele (216bp) incongruent with the SMM, the presence of this allele was confirmed by repeated genotyping.

## 4.2 *Chloroplast microsatellites*

Chloroplast microsatellites (cpSSRs) are an important molecular marker within studies of plants because they provide a way of measuring polymorphisms within a genome characterised by low levels of variation at other loci (see Provan et al., 1999b, 2001, reviews). Unlike nuclear microsatellites which are usually isolated from genomic libraries, identification of cpSSRs has been done from DNA sequence databases. Vendramin et al. (1996) identified 20 mononucleotide repeats in the chloroplast genome of *Pinus thunbergii* (figure 4.2). The use of these microsatellites as genetic markers for population genetic studies has been successful not only in several pine species (e.g. Bucci et al., 1998; Echt et al., 1998; Vendramin et al., 1998; Cuenca et al., 2003) but also in other genera of conifers (e.g. *Abies*, *Cedrus* and *Picea*; Vendramin and Ziegenhagen, 1997; Fady et al., 2003; Bucci and Vendramin, 2000).

### 4.2.1 *Transference of chloroplast microsatellite markers*

Eleven primer pairs (Pt1254, Pt15169, Pt26081, Pt30204, Pt36480, Pt41093, Pt63718, Pt71936, Pt79951, Pt87268 and Pt110048) from Vendramin et al. (1996) were tested in *P. canariensis*. In an initial screening for diversity 33 individuals were amplified for these 11 loci following Vendramin et al. (1996) and their PCR product size scored. Polymorphic cpSSRs markers were genotyped for seven natural populations and two artificial stands of the Canary Island pine from

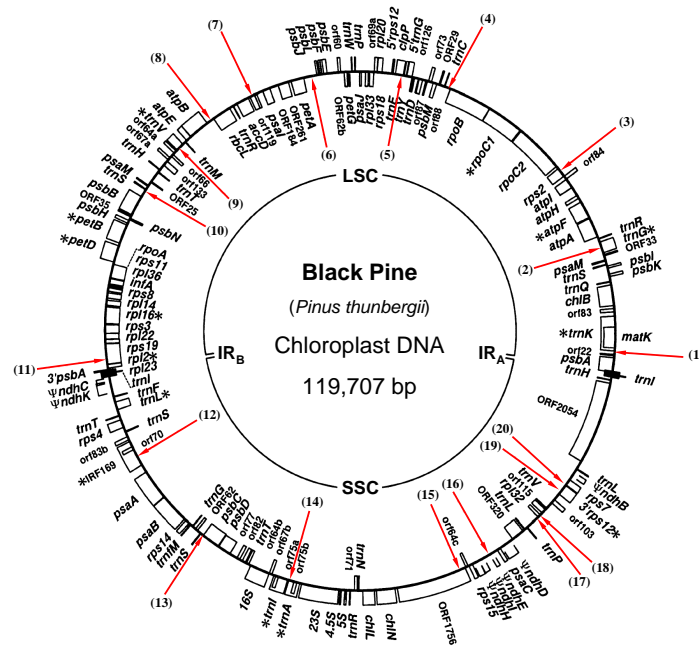


Fig. 4.2: Chloroplast microsatellite loci on the *Pinus thunbergii* chloroplast chromosome described in Vendramin et al. (1996). Pt1254 (1), Pt15169 (3), Pt26081 (4), Pt30204 (5), Pt36480 (6), Pt41093 (7), Pt63718 (11), Pt71936 (12), Pt79951 (13), Pt87268 (14) and Pt110048 (20). Chloroplast gene map from Wakasugi et al. (1994).

Tenerife. Additionally, seven individuals from a previous study (Gómez et al., 2003) were genotyped to ensure compatibility between both datasets.

#### 4.2.2 Results

In the initial screening only seven loci (Pt1254, Pt15169, Pt30204, Pt41093, Pt71936, Pt87268 and Pt110048) showed variation. Nevertheless, loci Pt26081 and Pt36480 were included in the analysis because they had presented polymorphism for *P. canariensis* in Gómez et al. (2003). Locus Pt41093 had to eventually be excluded because it presented amplification of two bands for several individuals.

All cpSSR loci but one (Pt36480) were polymorphic with an average of five alleles per locus (figure 4.3), yielding 117 different chloroplast haplotypes. Haplotype H002 (see table C.2) was the most abundant and widespread haplotype

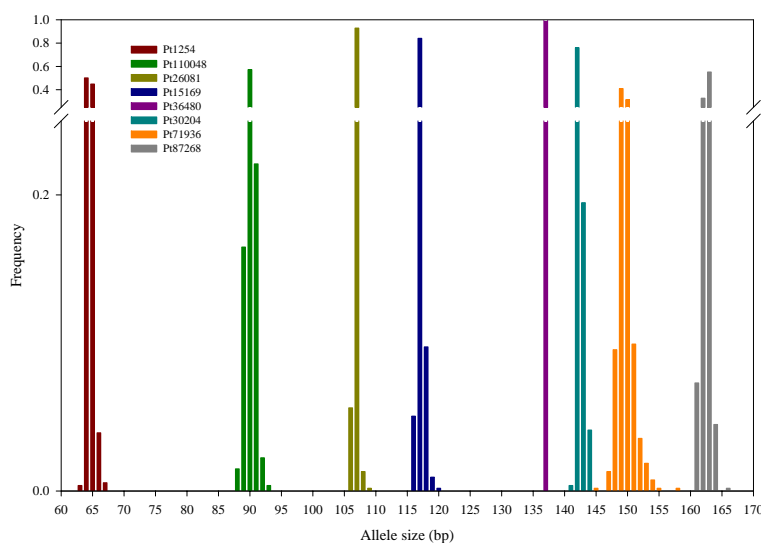


Fig. 4.3: Allele frequencies, for the eight chloroplast microsatellites, markers found in the seven natural populations studied (Arico 1000, Arico 1500, Arico 2000, Güímar, Vilaflor 1000, Vilaflor 1500, Vilaflor 2000 and Chinyero, locations described in Appendix A). Alleles ordered by size (bp).

across all populations. Gómez et al. (2003) also found a high frequency, widespread haplotype (named H23 in their study); and when we compare the alleles (of those loci that are shared in both studies) between these two haplotypes we can see that not all of them coincide (see table 4.2 for equivalences between studies). This is most probably due to the two additional loci used in our study that allow us to discern several additional haplotypes within those defined in Gómez et al. (2003), and their H23 was probably composed of several low frequency haplotypes. In the absence of homoplasy these differences should not be a problem for the interpretation of results because a monophyletic group of haplotypes would have a biological meaning. However, due to the mutational model of microsatellites, high levels of homoplasy are expected (recurrent parallel and back stepwise mutations) and haplotype H23 may be a paraphyletic grouping of many different haplotypes.

The presence of homoplasy in cpSSR data can be best illustrated by a bidimensional network of two-locus haplotypes (figure 4.4; representing the two most variable cpSSR loci in *P. canariensis*). There, each dimension (axis) represents one locus and nodes represent theoretical combinations of alleles; haplotypes

sample	Pt1254	Pt15169		Pt26081		Pt30204		Pt36480		Pt71936		Pt87268		Pt110048
	UEA	INIA	UEA	INIA	UEA	INIA	UEA	INIA	UEA	INIA	UEA	INIA	UEA	UEA
INIA_B	64	118	117	111	107	143	142	138	137	149	149	165	163	90
INIA_C	65	118	117	111	107	143	142	138	137	149		164	162	90
INIA_D	64	118	117	111	107	143	142	138	137	149	149	165	163	90
INIA_E	64	118	117	111	107	143	142	138	137	148	148	165	163	90
INIA_F	65	118	117	111	107	143	142	138	137	150	150	165	163	88
INIA_G	65	118	117	111	107	143	142	138	137	150	150	165	163	91
INIA_H	64	118	117	111	107	143	142	138	137	149	149	165	163	90

Tab. 4.2: Equivalent size scoring values with Gómez et al. (2003). Seven haplotypes were genotyped at the CIFOR-INIA and UEA labs for establishing the reading differences between both teams and ensure the compatibility of the data.

found in the Canary Island pine are marked with a circle. At least one homoplastic mutation is necessary to explain any conformation of haplotypes occupying the four nodes which delimit a square of the network [for example, haplotypes H(90, 147), H(90, 148), H(91, 147) and H(91, 148)]. If we focus on one particular stepwise change, for instance 90–91 in locus Pt110048, we observed seven haplotype pairs connected by that mutational change which can only be explained by at least six homoplastic mutations. Following the same reasoning we have to invoke the occurrence of at least 14 homoplastic mutations in the network, and more would be detected if all seven polymorphic cpSSRs were considered. This highlights the significance of homoplasmy in cpSSR polymorphisms, and this can have important negative effects for data analysis and interpretation (Estoup et al., 2002). The effects of homoplasmy on the statistical analysis of cpSSRs are explored in Chapter 5 (and partially in Chapter 6).

### 4.3 Summary

Eight nuclear and eight chloroplast microsatellite markers are available for genetic studies of *P. canariensis*. Transference of nuclear microsatellites described for related pine species (subgenus *Pinus*) to the Canarian pine can lead to a recovery of 20% of markers from the total number of trialed primer pairs. Therefore, this transference approach seems a viable strategy for acquiring more nSSR markers for the species for future studies. Chloroplast microsatellite markers were highly polymorphic (117 different haplotypes found). Our results strongly indicate the presence of extensive homoplastic mutations in the evolution of chloroplast haplotypes, as a consequence of the high polymorphism and predominant stepwise mutational changes. Results obtained from the analysis of cpSSR must consider



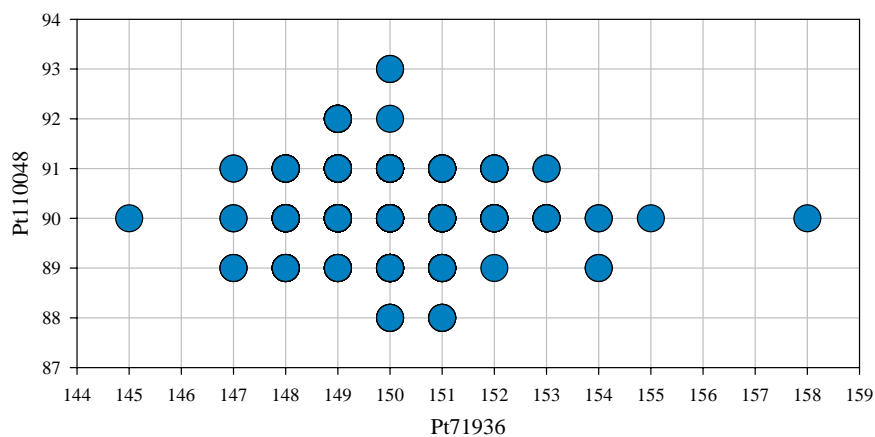


Fig. 4.4: Chloroplast microsatellite bidimensional network. Graphical representation of the variability of just two of the microsatellite loci as in Doyle et al. (1998). Nodes with blue circles represent the haplotypes found in *P. canariensis*. The presence of such conformation of haplotypes is only possible by recurrent back or parallel mutation.

these levels of homoplasy and further research on the effect of homoplasy should be undertaken.

## 5. CHLOROPLAST MICROSATELLITES: MEASURES OF GENETIC DIVERSITY AND THE EFFECT OF HOMOPLASY<sup>1</sup>

### *Abstract*

Chloroplast microsatellites have been widely used in population genetic studies of conifers in recent years. However, their haplotype configurations suggest that they could have high levels of homoplasy, thus limiting the power of these molecular markers. A coalescent-based computer simulation was used to explore the influence of homoplasy on measures of genetic diversity based on chloroplast microsatellites. The conditions of the simulation were defined to fit isolated populations originating from the colonisation of one single haplotype into an area left available after a glacial retreat. Simulated data were compared with empirical data available from the literature for a species of *Pinus* that has expanded north after the last glacial maximum. In the evaluation of genetic diversity, homoplasy was found to have little influence on Nei's unbiased haplotype diversity ( $H_e$ ) while Goldstein et al.'s genetic distance estimates ( $D_{sh}^2$ ) were much more affected. The effect of the number of chloroplast microsatellite loci for evaluation of genetic diversity is also discussed.

### 5.1 *Introduction*

Microsatellites, or simple sequence repeats (SSRs), are sequences of repetitive DNA where a single motif consisting of one to six base pairs is repeated tandemly a number of times. Microsatellite sequences have been identified in the three eukaryote genomes: nucleus, chloroplast (Powell et al., 1995) and mitochondrion (Soranzo et al., 1999). Nuclear microsatellite loci are usually highly polymorphic with alleles varying in the number of repeat units; they are codominant and

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<sup>1</sup> Published in Navascués and Emerson (2005).

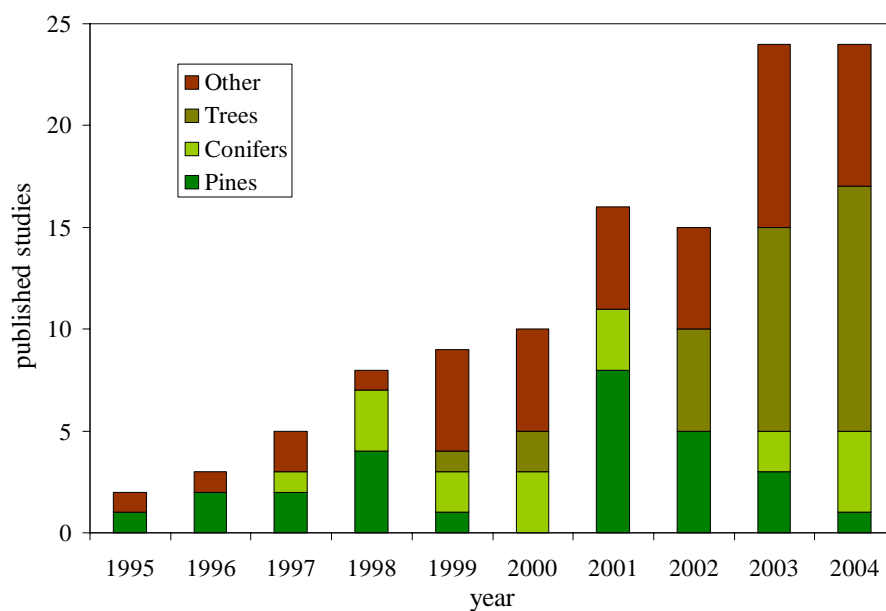


Fig. 5.1: Number of studies using chloroplast microsatellites published in ten years since their discovery. Based on GOOGLE SCHOLAR and NCBI PUBMED searches.

inherited in a Mendelian mode. These characteristics, plus being considered selectively neutral, have made them a popular marker for population genetic studies (Sunnucks, 2000). With respect to the organelle genomes, mitochondrial microsatellites have had little impact so far, but chloroplast microsatellites have been increasingly used in population genetics since their discovery (figure 5.1). Conserved primers for the amplification of chloroplast microsatellites (cpSSRs) have been reported for conifers (Vendramin et al., 1996), gramineae (Provan et al., 2004) and dicotyledons (Weising and Gardner, 1999), but it is among conifers, for studies of population genetics, that chloroplast microsatellite markers have mainly been used (e.g. Cuenca et al., 2003; Fady et al., 2003; Gómez et al., 2003).

Chloroplast microsatellites typically consist of mononucleotide motifs that are repeated eight to fifteen times. Levels of polymorphism in cpSSRs are quite variable across loci and across species, and some loci have been found to be monomorphic in all species studied. There are two important features that differentiate chloroplast from nuclear microsatellites. First, chloroplasts are uni-

parentally inherited. Some species have maternal inheritance of the chloroplast, others paternal. This means that cpSSRs provide information for the lineages of only one of the sexes. Also, the chloroplast chromosome is a non-recombinant molecule and, therefore, all cpSSR loci are linked. The genotyping of cpSSRs will result in haplotypes that will be composed of the combination of alleles found at each cpSSR locus.

Mutation rates for length variation in microsatellites have been found to be higher ( $10^{-2}$  to  $10^{-6}$ ) than point mutation rates (Li et al., 2002). In order to explain this difference two kinds of mutational mechanism have been proposed: replication slippage (Tachida and Iizuka, 1992) and recombination with out-of-phase aligning (Harding et al., 1992). Both processes result in changes in the number of repeat units which are compatible with the observed size polymorphism of microsatellites. One consequence of these mutational mechanisms is that the same genetic state (i.e. number of repeats) may evolve in two different microsatellite lineages through independent mutational events, a phenomenon known as homoplasia.

Homoplasia may cause problems in the analysis of population genetics as it can affect measures of genetic diversity, gene flow, genetic distances (both between individuals and populations), neighborhood size, assignment methods and phylogenetic analysis (see Estoup et al., 2002, for a review). Homoplasia within cpSSRs is considered to be a potential limitation for its use as a genetic marker (Provan et al., 2001), however the problem has only been addressed at the genus level (Doyle et al., 1998; Hale et al., 2004). Researchers have generally considered homoplasia levels low enough to allow population genetic analysis, and even when homoplasia has been evident (i.e. a haplotype network with up to nine loops) it has been considered as “moderate” and its potential for confounding results disregarded (Cuenca et al., 2003).

In the present study, simulation analysis was used to investigate the evolution of cpSSRs in conifers and how homoplasia may influence the informativeness of these markers. Instead of a more traditional simulation approach where the whole population is considered, only the genetic makeup of a sample of individuals was studied, following a coalescence-based approach. The strategy consists of: first, generating a genealogy for a sample of individuals from the last generation; and second, placing random mutations on the genealogy to generate the genetic state

of the sample (Hudson, 1990). This approach has proved useful for the study of levels of homoplasy in nuclear microsatellites under different mutational models (Estoup et al., 2002).

## 5.2 Modelling chloroplast microsatellite evolution<sup>2</sup>

### 5.2.1 Simulation of coalescent events

The probability of a coalescent event for any two lineages in a given generation depends on the population size, the population structure and the mating system. Thus, it became necessary to establish the biological scenario of the simulation, which would determine the shape of the genealogy.

After the last glacial maximum most conifer distributions shifted northward leaving their refugia (Jackson et al., 2000). When populations were established, they had a period of expansion followed by a period of approximately constant population density until the present time (MacDonald and Cwynar, 1991). Our simulations reproduced populations under such conditions. A range of population ages (i.e. coalescence times) were investigated with population origins at 50, 100, 150, 200 or 250 generations before the present to simulate colonisation events at different stages of the glacial retreat [assuming: 1 generation = 100 years (as in Provan et al., 1999a) and the last glacial maximum = 20 000 ybp (Hewitt, 1996)]. Population growth was determined by the logistic equation:

$$N_{t+1} = N_t e^{r(1 - \frac{N_t}{K})} \quad (5.1)$$

where  $N_t$  is the population size in the generation  $t$  ( $N_0 = 1$ ),  $r$  is the population growth rate and  $K$  is the carrying capacity of the population. This model has been widely employed to describe population growth following colonisation events (Shigesada and Kawasaki, 1997). The growth rate was set at  $r = 0.7$ , producing a period of expansion with a duration of 22 generations ( $\approx 2200$  years), which is within the range observed for *Pinus* (MacDonald and Cwynar, 1991). Then, population size remained constant at carrying capacity for the remaining 28, 78, 128, 178 or 228 generations. Because effective population sizes are considered to be ‘large’ in forest trees (Muona and Harju, 1989), carrying capacity was

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<sup>2</sup> Program source code (in FORTRAN) is available from the author on request (m.navascues@gmail.com).

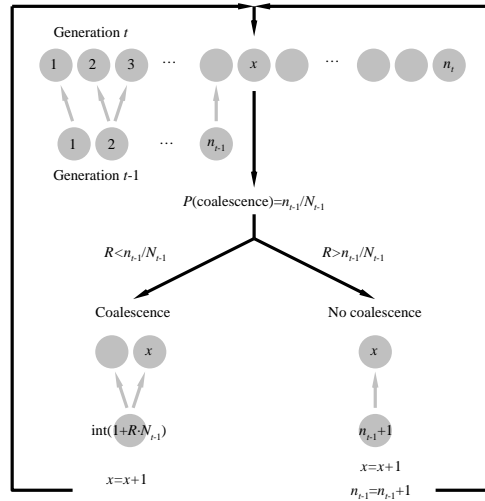


Fig. 5.2: Coalescence events were simulated with a generation by generation algorithm. This algorithm assigned to every individual,  $x$ , of the generation  $t$ , its ancestor in the immediately previous generation,  $t - 1$ . The probability for this ancestor to be shared with another individual was calculated from the population size in the previous generation,  $N_{t-1}$ , and the number of ancestors already assigned,  $n_{t-1}$  (see text for details).

arbitrarily set at  $K = 10\,000$ ; a size big enough to avoid the effects of genetic drift (Savolainen and Kuittinen, 2000).

Generally, chloroplasts are considered paternally inherited in conifers, although there is a possibility that low levels of maternal leakage and heteroplasmy may be present (Cato and Richardson, 1996). Thus, the coalescent process influencing the genealogy of chloroplast haplotypes was considered to be the simplest case possible: a neutral, haploid, non-recombinant genome with every individual having the same probability of being the parent of any individual in the following generation. With the demographic history determined, the genealogy was constructed using a generation-by-generation algorithm. This type of algorithm allows the simulation of complex demographic and dispersal models, contrasting with other coalescence algorithms which are faster for computational time (Leblois et al., 2003).

The algorithm worked backward in time, starting with the last generation and finishing in the first one ( $t = 0$ ). In every generation the coalescent events were

generated by an algorithm that assigned to every individual,  $x$ , from the sample ( $x \in [1, \dots, nt]$ ) in generation  $t$ , its ancestor,  $y$ , in the previous generation  $t - 1$  (Figure 5.2). Every individual  $x$  from the sample in generation  $t$  had a probability  $P = n_{t-1}/N_{t-1}$  (where  $n_{t-1}$  is the number of ancestors already assigned and  $N_{t-1}$  is the population size in generation  $t - 1$ ) to share its ancestor  $y$  with any of the individuals from the sample which ancestors had already been assigned. A random number,  $0 < R < 1$ , drawn with a uniform probability distribution function, is used to determine the occurrence of a coalescent event. When  $R < n_{t-1}/N_{t-1}$ , the ancestor  $y$  of the individual  $x$  is within the  $n_{t-1}$  previously assigned ancestors ( $y \in [1, \dots, n_{t-1}]$ ). In order to determine which ancestor,  $y$ , took the value of the integer part of  $1 + RN_{t-1}$  ( $|1 + RN_{t-1}| \in [1, \dots, n_{t-1}]$  when  $R < n_{t-1}/N_{t-1}$ ). The generation and lineages involved in this coalescent event were recorded to construct the genealogy of the sample. When  $R > n_{t-1}/N_{t-1}$ , the ancestor  $y$  was a new individual and  $y$  took the value  $n_{t-1} + 1$ ; for the next individual  $x + 1$  the value of  $n_{t-1}$  increased by one unit.

Coalescent events were simulated using this algorithm on every generation until all lineages converged to a single lineage. Because the population size in the first generation ( $t = 0$ ) is only one individual ( $N_0 = 1$ ) the probability for any number of individuals to share their ancestors was  $P = n_{t-1}/N_{t-1} = 1$ , i.e. all the lineages coalesced at least at the first generation. This allowed controlling the coalescence time within the range of time for the phenomenon simulated (i.e. colonisation after glacial retreat). It is important to note that, due to the non-recombinant nature of the chloroplast genome, all cpSSR loci were linked and shared the same genealogical history.

### 5.2.2 Simulation of mutational events

Several theoretical mutational models have been proposed to describe microsatellite evolution, each one of them with recognized weaknesses and strengths (Estoup and Cornuet, 1999). All of them refer to and have been tested against nuclear microsatellites, where mutational events are believed to occur by two mechanisms: replication slippage and recombination (Li et al., 2002). To our knowledge, no specific model has been developed for cpSSRs (where no recombination occurs) so the stepwise mutation model (SMM) was chosen since it is the simplest realistic model for microsatellites. Mutation rate estimates for cpSSRs are scarce and

	Coalescence time <sup>a</sup>	Mutation rate, $\mu$	Homoplasmy index, $P^b$
Simulation 01	50	$10^{-5}$	0.00
Simulation 02	100	$10^{-5}$	0.00
Simulation 03	150	$10^{-5}$	0.00
Simulation 04	200	$10^{-5}$	0.00
Simulation 05	250	$10^{-5}$	0.00
Simulation 06	50	$10^{-4}$	0.00
Simulation 07	100	$10^{-4}$	0.00
Simulation 08	150	$10^{-4}$	0.00
Simulation 09	200	$10^{-4}$	0.00
Simulation 10	250	$10^{-4}$	0.00
Simulation 11	50	$5 \times 10^{-4}$	0.01
Simulation 12	100	$5 \times 10^{-4}$	0.03
Simulation 13	150	$5 \times 10^{-4}$	0.03
Simulation 14	200	$5 \times 10^{-4}$	0.07
Simulation 15	250	$5 \times 10^{-4}$	0.17
Simulation 16	50	$10^{-3}$	0.02
Simulation 17	100	$10^{-3}$	0.06
Simulation 18	150	$10^{-3}$	0.11
Simulation 19	200	$10^{-3}$	0.33
Simulation 20	250	$10^{-3}$	0.43

<sup>a</sup> coalescence time in number of generations

<sup>b</sup> mean from 20 replicates

Tab. 5.1: Combinations of parameters for coalescence time and mutation rate used in different simulations, and the index of homoplasmy found for each.

vary from  $10^{-3}$  (Marshall et al., 2002) to  $10^{-5}$  (Provan et al., 1999a) per locus per generation, so simulations were run under four different mutation rates:  $10^{-3}$ ,  $5 \times 10^{-4}$ ,  $10^{-4}$ ,  $10^{-5}$  per locus, per generation.

The genetic state of nine cpSSR loci in samples of 25 individuals was simulated under 20 different combinations of population ages and mutation rates (Table 5.1). For each of these simulations 20 replicates were run. The output of every replicate consisted of the genotypic information of all the individuals from the sample and the genealogical tree that describe their relationships, with information on the number of mutations and number of generations for every branch.



The raw data obtained were analysed as described in the following section.

### 5.3 Genetic diversity analysis

The effect of homoplasy was studied on a number of standard measures of genetic diversity for cpSSRs: total number of haplotypes ( $n_h$ , direct count of different haplotypes), effective number of haplotypes ( $n_e$ , Kimura and Crow, 1964), unbiased haplotype diversity ( $H_e$ , Nei, 1978) and average genetic distances among individuals ( $D_{sh}^2$ , Goldstein et al., 1995) applied to cpSSRs by Morgante et al. (1997):

$$n_e = 1 / \sum_{h=1}^{n_h} p_h^2 \quad (5.2)$$

$$H_e = \frac{n}{n-1} \left( 1 - \sum_{h=1}^{n_h} p_h^2 \right) \quad (5.3)$$

$$D_{sh}^2 = \frac{2}{n(n-1)} \frac{1}{L} \sum_{i=1}^n \sum_{j=i+1}^n d_{ij}^2 \quad (5.4)$$

$$d_{ij} = \sum_{k=1}^L |a_{ik} - a_{jk}| \quad (5.5)$$

where  $n$  is the number of individuals in the simulated sample,  $p_h$  is the relative frequency of the  $h^{\text{th}}$  haplotype,  $n_h$  is the number of different haplotypes in the simulated sample,  $L$  is the number of loci simulated,  $a_{ik}$  is the size (measured in repeat units) of the allele for the  $i^{\text{th}}$  individual and at the  $k^{\text{th}}$  locus, and  $a_{jk}$  is the size of the allele for the  $j^{\text{th}}$  individual and at the  $k^{\text{th}}$  locus.

Indices  $n_h$ ,  $n_e$  and  $H_e$  were calculated both for the stepwise mutation model (SMM; where haplotypes were defined by their genetic state) and for the infinite allele model (IAM; where every mutation defined a new haplotype, even if the haplotype state produced was already present in the sample). The difference between the SMM and IAM values represents information about genetic diversity that is lost due to homoplasy.

Estoup et al. (2002) defined an index of homoplasy,  $P$ , to quantify theoretically the effects of mutational and population variables on homoplasy. This index of homoplasy is the probability that two haplotypes sharing the same genetic state

are not identical by descent. We have calculated a similar index generated from Nei's genetic diversity for the SMM and IAM:

$$P = 1 - \left( \frac{1 - H_{eIAM}}{1 - H_{eSMM}} \right) \quad (5.6)$$

The number of mutations occurring between every pair of lineages was scored and the average genetic distance, based on number of mutations ( $D_M^2$ ), was calculated following equation 5.4 where  $d_{ij}$  is substituted for the number of mutations scored between the individual  $i$  and  $j$ . Due to the possibility of recurrent mutation and back mutations under the SMM, the genetic distance estimate is expected to be incongruent to some degree with the actual number of mutations between lineages. The differences between the absolute values  $D_{sh}^2$  and  $D_M^2$  were compared. In addition, the correlation of the matrices of actual and estimated genetic distances was analysed with a Mantel test (Mantel, 1967) when the difference between  $D_{sh}^2$  and  $D_M^2$  was large.

#### 5.4 Results and discussion

The results for all the simulations are presented in figures 5.3 and 5.4. Each of the 20 replicates performed for each simulation is equivalent to an independent random sampling from the same population. Hence, for any of the genetic diversity indices, the mean value for the 20 replicates is interpreted as an estimate for the actual population value of that statistic and the standard deviation as the error associated to the sample size used. The effect of sample size was assessed by performing some simulations with larger sample sizes. Not surprisingly this resulted in a reduction of the variance for the different measures; for instance, gene diversity in simulation 16 ( $H_{eIAM} = 0.552 \pm 0.178$ ;  $H_{eSMM} = 0.542 \pm 0.178$  for 25 individuals, as shown in figure 5.4G;  $H_{eIAM} = 0.561 \pm 0.037$ ;  $H_{eSMM} = 0.553 \pm 0.036$  for 1000 individuals) and average genetic distance in simulation 20 ( $D_{sh}^2 = 1.665 \pm 0.382$ ;  $D_M^2 = 2.746 \pm 0.706$  for 25 individuals, as shown in figure 5.4H;  $D_{sh}^2 = 1.696 \pm 0.185$ ;  $D_M^2 = 2.589 \pm 0.292$  for 1000 individuals). The effect of the sample size could be eliminated by simulating the coalescent history for the whole population. However that would require excessive computational time, and it does not appear to be a problem warranting this.

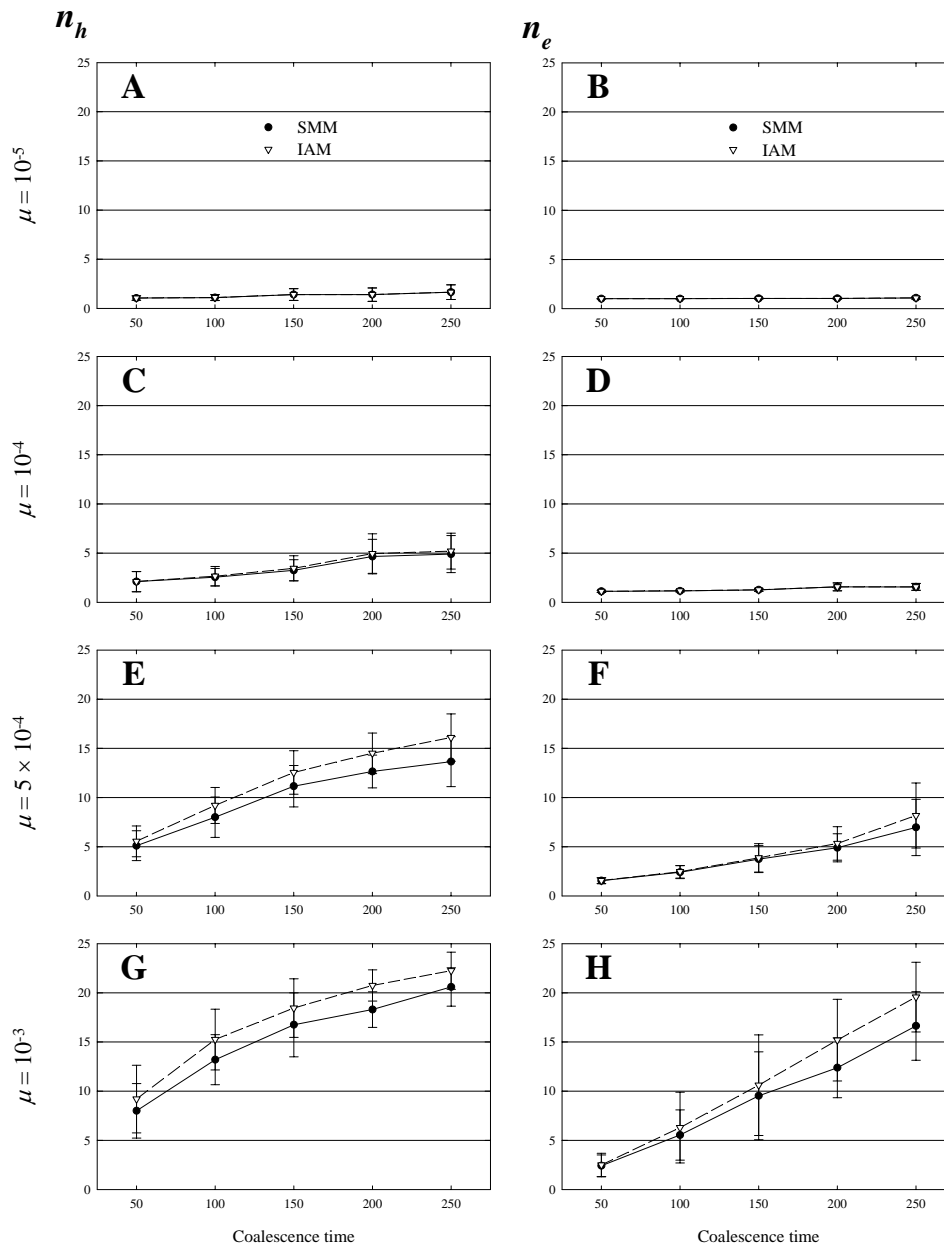


Fig. 5.3: Number of haplotypes,  $n_h$ , and effective number of haplotypes,  $n_e$ , for the 20 simulations. Each graph represents the values for one of the indices,  $n_h$  or  $n_e$ , for the five simulations with the mutation rate,  $\mu$ , shown on the left and the coalescence time (in number of generations) shown on the abscissa axis. The mean and standard deviation (from 20 replicates) is shown for each simulation. Both indices were calculated under the stepwise mutation model (SMM) and the infinite allele model (IAM). The difference between both values represents the extent to which information is lost due to homoplasy.

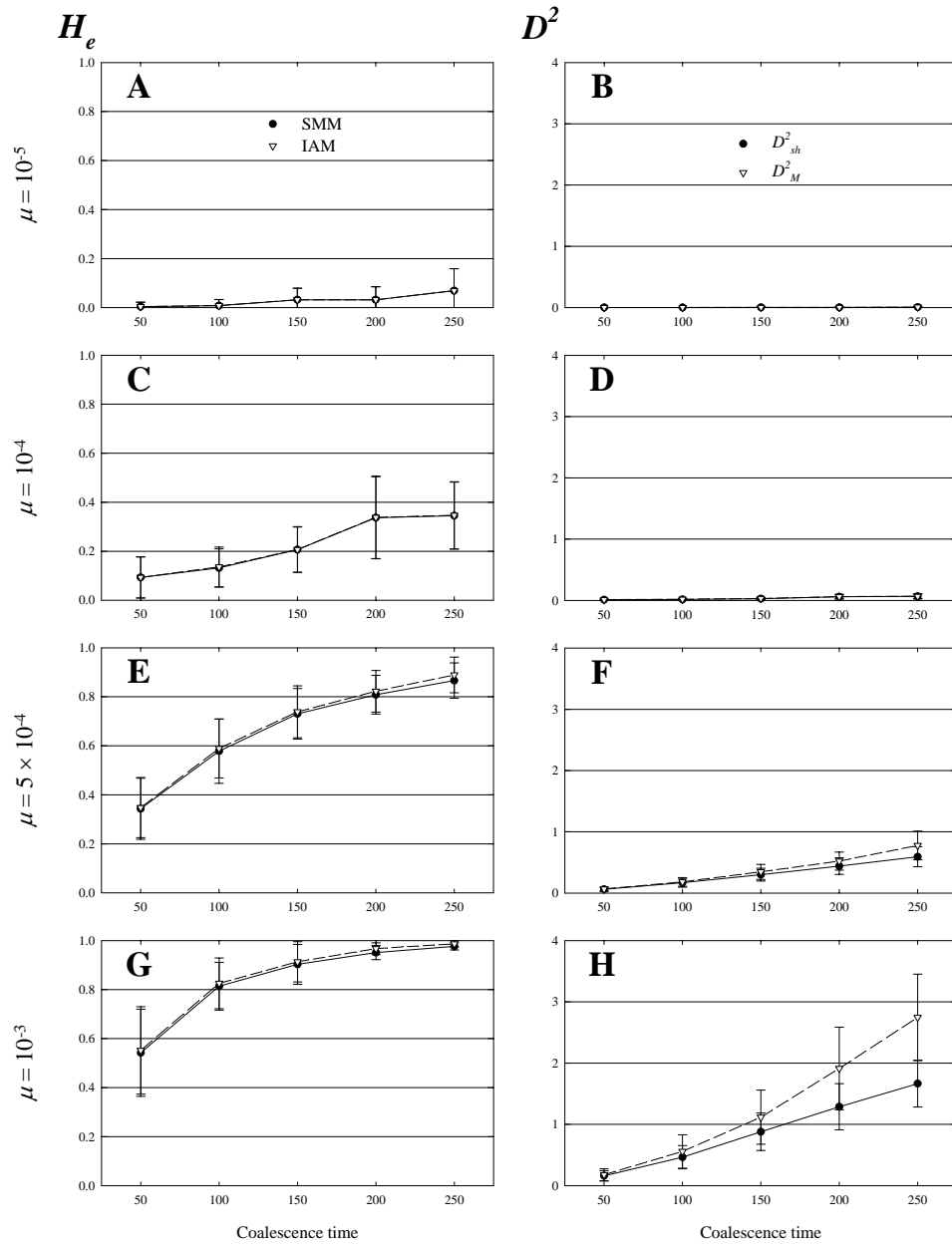


Fig. 5.4: Unbiased haplotype diversity,  $H_e$ , and average genetic distances among individuals,  $D^2$ , for the 20 simulations. Each graph represents the values for one of the indices,  $H_e$  or  $D^2$ , for the five simulations with the mutation rate,  $\mu$ , shown on the left and the coalescence time (in number of generations) shown on the abscissa axis. The mean and standard deviation (from 20 replicates) is represented for each simulation.  $H_e$  was calculated for the stepwise mutation model (SMM) and the infinite allele model (IAM).  $D^2$  was calculated for estimated distances ( $D_{sh}^2$ ) based on the number of observed mutations and for the true distances based on the actual number of mutations ( $D_M^2$ ). The difference between both values represents the extent to which information is lost due to homoplasy.

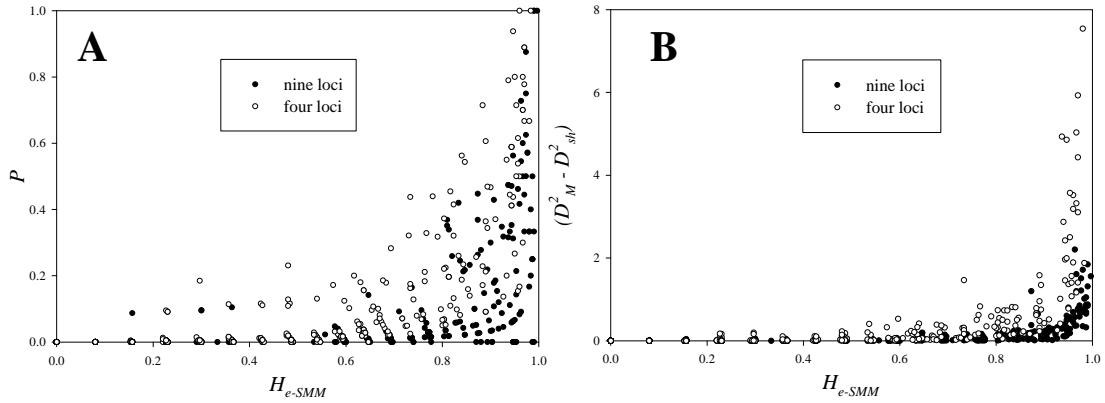


Fig. 5.5: Levels of homoplasy plotted against genetic diversity for every replicate of the 20 simulations (see table 5.1 for simulation conditions). Levels of homoplasy are represented: (A) with the homoplasy index,  $P$ , and (B) with the difference between the actual average genetic distance ( $D_M^2$ ) and the estimated genetic distance ( $D_{sh}^2$ ). Filled circles represent simulations with nine loci and empty circles represent equivalent simulations with only four loci.

As was expected, the simulations with parameters that produced higher genetic diversity also showed higher levels of homoplasy (see table 5.1 and figure 5.5). For simulations with mutation rates higher than  $10^{-4}$ , homoplasy caused an underestimation, to different degrees, for the four diversity indices. However, Nei's haplotypic diversity values for SMM and IAM were very close in all simulations.

In order to understand the effect of homoplasy on Goldstein et al.'s genetic distance estimates, a more complex approach is necessary. A difference between the values of  $D_{sh}^2$  and  $D_M^2$  will lead to an underestimation of the absolute time of coalescence for that sample. However, the actual and estimated distances between individuals could be correlated, and if that were the case, then the distance estimates could be used, or even corrected, to study relative genetic distances. In order to test that correlation, simulation 20 was chosen as it presents the biggest differences between estimated and actual distances. The Mantel test performed for the correlation of the actual and estimated genetic distance resulted in a significant correlation ( $p$ -value  $< 0.025$ ) for all the replicates, with the correlation coefficient,  $r$ , ranging from 0.52 to 0.82. Since the correlation with the actual

genetic distances is significant but not predictable, we conclude that, for populations with high genetic diversity, the Goldstein et al.'s genetic distance estimates can be misleading, and any attempt to apply a correction to these estimates would be prone to error.

The different effect of homoplasy on the various indices is explained by the nature of these indices. The three indices based on the number of haplotypes ( $n_h$ ,  $n_e$  and  $H_e$ ) seem to perform better for assessing levels of genetic diversity than the one based on distance estimates ( $D_{sh}^2$ ). Estimated distances are influenced by every parallel mutation and back mutation; however, these mutations may have no influence in the number of haplotypes in the sample. For example, two lineages with unique and parallel mutations will have two distinct haplotypes at the final generation, thus the number of haplotypes will not be reduced. However, the genetic distance will be underestimated because of the parallel mutations. Within the indices based on number of haplotypes, the indices that consider their frequencies ( $n_e$  and  $H_e$ ) were less affected by homoplasy. This result is due to the low frequency that most of the homoplastic haplotypes had within the simulated populations.

In the present work, the use of cpSSRs for phylogenetic reconstruction has not been assessed, but our results would serve to discourage this practise. For instance, in simulation 20, the high occurrence of homoplastic mutations (78.8% parallel mutations and 6.4% back mutations) would lead to highly inaccurate results using a maximum parsimony approach. Distance-based methods would also fail to recover an accurate phylogeny due to the influence of homoplasy. However, further work will be necessary to quantify these error rates.

The number of cpSSR loci studied also influenced levels of homoplasy. A set of simulations performed for four loci produced higher values for the homoplasy index,  $P$ , and higher differences between expected and actual average distances than the equivalent simulations performed for nine loci (figure 5.5). Thus, the linkage of cpSSRs can be seen as beneficial for the analysis of genetic diversity. With a greater number of loci analysed one has more power to distinguish haplotypes with homoplastic alleles at a given cpSSR locus through the polymorphism of linked loci.

Mutation rate and time of coalescence are the two factors influencing the levels of genetic diversity in our simulations. The different combinations of these

parameters produced a broad range of genetic diversity, from simulation 01 with null diversity to simulation 20 with the highest diversity (see figures 5.3 and 5.4). This set of simulations reproduces the simplest scenario that could describe the recent history of a conifer population: an isolated population originating from a single colonisation event, followed by a population expansion. The recent population history of *Pinus resinosa* would seem to be consistent with such conditions. During the last glaciation *P. resinosa* was restricted to southern refugial populations and has colonised northern areas after the glacial retreat (Fowler and Morris, 1977). A study of the population genetics of *P. resinosa* with cpSSRs also supports a metapopulation with restricted gene flow between populations (Echt et al., 1998). This case provides us with an empirical study of a conifer with isolated populations and with different colonisation ages (either because of the metapopulation dynamics or colonisation after glacial retreat). Also, the number of cpSSR loci and sample sizes (nine loci, 21–24 individuals) were similar to the simulations presented here, providing an appropriate combination of conditions for comparison.

The patterns of genetic diversity found in the empirical study of *P. resinosa* with cpSSRs (Echt et al., 1998) can be best compared with simulations 11–15. In both cases the populations are composed of one high frequency haplotype (the ancestral haplotype in the simulations) plus several low frequency haplotypes. The diversity levels for the different indices,  $n_h$ ,  $n_e$ ,  $H_e$  and  $D_{sh}^2$ , are also comparable and consistent. Therefore, we can argue that the genetic diversity and distances were unlikely to have been underestimated within *P. resinosa* (see figures 5.3E, 5.3F, 5.4E and 5.4F).

Any further comparison of our simulations with other conifer species studied with cpSSRs has to be done with caution. The simulations we have performed do not take into account a number of additional factors that may influence homoplasy. Thus the current set of simulations will represent the minimum amount of homoplasy that could be present within a given population. Demographic scenarios including migration or more ancient coalescent events (where colonisation events were produced by more than one haplotype), would result in increased levels of homoplasy. Higher mutation rates and size constraints in the mutational model will also increase the levels of homoplasy (Estoup et al., 2002).

## 5.5 *Conclusions*

To conclude, further simulation studies would be beneficial for the understanding of the homoplasy in the analysis of cpSSRs. In particular, we are now working on the implementation of the generation-by-generation algorithm for the simulation of multi-population scenarios with dispersal that will allow us to understand the effects of homoplasy in the measurement of gene flow and genetic distances among populations. Regarding future empirical studies with cpSSRs, it is strongly recommended that studies use as many cpSSR loci as are available in order to reduce the negative consequences of homoplasy on estimations of genetic diversity. In order to assess levels of genetic diversity Nei's index seems to perform the best, being least affected by homoplasy. In contrast conclusions made with Goldstein et al.'s genetic distances should be regarded with caution, as these can underestimate absolute distances.



Part III

POPULATION GENETICS OF *PINUS CANARIENSIS*

## 6. CHLOROPLAST MICROSATELLITES REVEAL COLONISATION AND METAPOPOPULATION DYNAMICS IN THE CANARY ISLAND PINE<sup>1</sup>

### *Abstract*

Chloroplast microsatellites are becoming increasingly popular markers for population genetic studies in plants, but there has been little focus on their potential for demographic inference. In this work the utility of chloroplast microsatellites for the study of population expansions was explored. First, we investigated the power of mismatch distribution analysis and the  $F_S$  test with coalescent simulations of different demographic scenarios. We then applied those methods to empirical data obtained for the Canary Island pine (*Pinus canariensis*). The results of the simulations showed that chloroplast microsatellites are sensitive to sudden population growth. The power of the  $F_S$  test and accuracy of demographic parameter estimates, such as the time of expansion, were reduced proportionally to the level of homoplasmy within the data. The analysis of Canary Island pine chloroplast microsatellite data indicated population expansions for almost all sample localities. Demographic expansions at the island level can be explained by the colonisation of the archipelago by the pine, while population expansions of different ages in different localities within an island appear to be the result of local extinctions and recolonisation dynamics. Comparable mitochondrial DNA sequence data from a parasite of *P. canariensis*, the weevil *Brachyderes rugatus*, supports this scenario, suggesting a key role for volcanism in the evolution of pine forest communities in the Canary Islands.

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<sup>1</sup> Article in preparation by M. Navascués, Z. Vaxevanidou, S.C. González-Martínez, J. Clement, L. Gil and B.C. Emerson

## 6.1 Introduction

In plants the chloroplast genome is used extensively for evolutionary genetic studies within species in the same way as the mitochondrial genome is used within animal studies. However, finding enough sequence variation is a challenge due to the low mutation rates that characterize the chloroplast genome. In contrast, chloroplast microsatellites, or simple sequence repeats (cpSSRs), present higher levels of polymorphism and are easily genotyped. This has made them useful and popular markers for population genetic studies (Provan et al., 2001). Although used extensively for studying population structure and gene flow, the potential of cpSSRs to study population demographic history has received little attention. In this study we investigate in the utility of cpSSR data for the detection of population expansions.

The study of historical demography by means of genetic information is based on coalescent theory (see Emerson et al., 2001, for a review). In a stable population coalescent events are scarcer towards the past giving a genealogy dominated by an ancient bifurcation with mutations mainly distributed in inter-node branches (Reich and Goldstein, 1998; King et al., 2000). Contrastingly, in the case of sudden population growth, coalescent events occur mainly during the expansion, leaving a ‘comblike’ genealogy; and mutations are more abundant along the terminal branches (singleton mutations) than in inter-node branches (figure 6.1 shows the main differences of the two opposing scenarios). As a consequence, population expansions can be detected because of an excess of singletons (Tajima, 1989; Fu and Li, 1993) or an excess of haplotypes (as a consequence of the excess of singletons, Fu, 1997). Also, the divergence between most lineages dates from the time of expansion, producing unimodal distributions of pairwise genetic distances (Slatkin and Hudson, 1991). The study of such distributions also allows for the estimation of the time and magnitude of the population increase (Rogers, 1995; Schneider and Excoffier, 1999).

The methods for studying population expansions are fairly robust for a genetic marker evolving under the unrealistic infinite sites model, where singletons and genetic distances are identified without error. However, in the evolution of sequences under a finite sites model, parallel and back mutations (i.e. homoplasic mutations) will erase part of the genetic information producing inaccurate estimates of singletons and genetic distances. This affects the power of the statis-

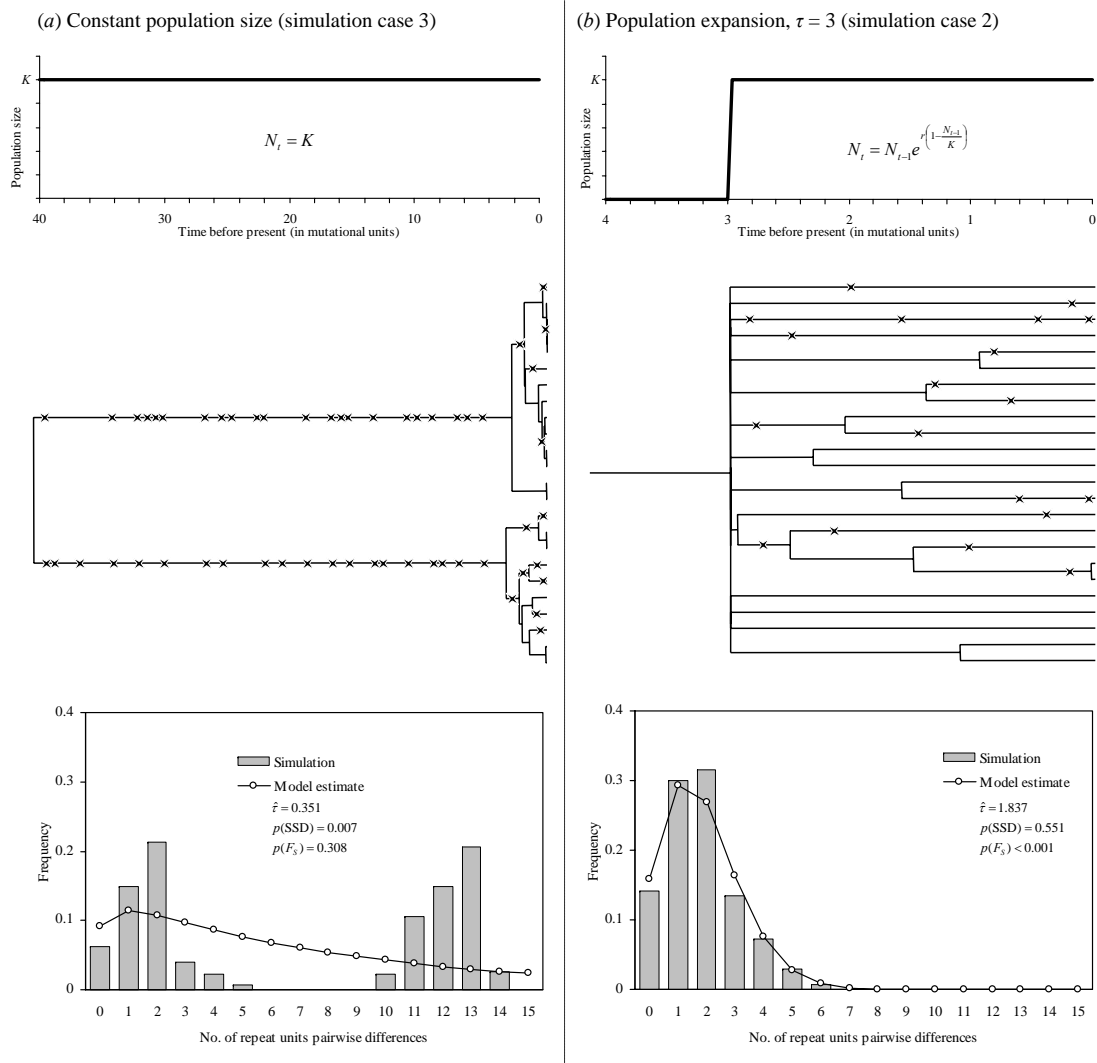


Fig. 6.1: Coalescent process under two contrasting scenarios: constant population size and sudden population expansion. For each case, the demographic history and, in the same timescale (measured in mutational units =  $\frac{1}{2\mu}$  generations), the simulated genealogy of a random sample of genes is represented, with stars representing mutational events. Below, mismatch distribution and results of the  $F_S$  test and demographic parameter estimates are shown.

tical tests and the estimates of time and magnitude of the demographic growth (Bertorelle and Slatkin, 1995; Aris-Brosou and Excoffier, 1996). In nucleotide sequence data, the usual markers for studying population expansions, the effect of homoplasy is small (Rogers et al., 1996) and can be accounted for in more sophisticated analyses (Schneider and Excoffier, 1999). In cpSSRs, which evolve in a stepwise fashion, higher levels of homoplasy are expected in comparison with sequence data and therefore statistical analyses developed for DNA sequence data may prove unreliable.

In the present work we simulated the evolution of cpSSRs under constant population size and under population expansion to test the usefulness of these markers for the study of demographic expansions. These theoretical results were then compared with empirical results from the Canary Island pine (*Pinus canariensis*). The presence of *P. canariensis* on each of the five volcanic islands on which it occurs must be through colonisation after the emergence of each island, followed by population expansion.

## 6.2 Materials and methods

### 6.2.1 Simulations

Demographic histories of population expansions (recent and old) and stable population size were modelled with coalescent simulations to obtain theoretical expectations of the behaviour of cpSSRs. The coalescent simulation (described in Navascués and Emerson, 2005, and Chapter 5) consists of the generation of a genealogy for a sample of individuals under a particular demographic history followed by the distribution of mutations randomly onto those lineages. For the population expansions the demographic history was modelled with the logistic equation setting the initial population size ( $N_0$ ) as one individual (coloniser) at the time of expansion ( $\tau$ , in mutational units). Microsatellite evolution was simulated following a symmetrical single-step mutation model where mutation rates were either heterogeneous (two-rates model) or uniform (one-rate model) across loci. Heterogeneous mutation rates can be considered a more realistic scenario taking into account the differences in polymorphism among cpSSR loci (see, for example, Gómez et al., 2003). As well as being more realistic, heterogeneous mutation rates will also produce higher levels of homoplasy by concentrating the

Case	Expansion time, $\tau$	Mutation rate, $\mu$ , loci 1–2	Mutation rate, $\mu$ , loci 3–6	Proportion of non-significant $F_S$ test	Proportion of significant SSD	Homoplasmy index, $P$
1	1 (recent)		$5.5 \times 10^{-5}$	0.038	0.052	0.049
2	3 (old)		$5.5 \times 10^{-5}$	0.002	0.051	0.297
3	no expansion		$5.5 \times 10^{-5}$	0.893	0.144	0.065
4	1 (recent)	$1.65 \times 10^{-4}$	$10^{-7}$	0.553	0.095	0.122
5	3 (old)	$1.65 \times 10^{-4}$	$10^{-7}$	0.240	0.060	0.606
6	no expansion	$1.65 \times 10^{-4}$	$10^{-7}$	0.931	0.080	0.263

Tab. 6.1: Population expansion signal on the  $F_S$  neutrality test, mismatch distribution analysis and homoplasmy level in the six simulated cases.

mutations onto particular loci, thus providing a more rigorous assessment of the demographic utility of cpSSRs. The three different demographic histories and the two mutation models gave a combination of six different cases considered (table 6.1). Simulations were performed for a sample size of 24 individuals and six cpSSR loci. For each case, 1000 replicates were run and their output (genetic state of a sample of individuals in the present generation) was analysed as described in section 6.2.3. For each simulated case the level of homoplasmy was quantified as the probability that two haplotypes identical in state are not identical by descent (homoplasmy index, equation 5.6 after Estoup et al., 2002).

### 6.2.2 Plant material and molecular markers

Empirical data were obtained from two previous studies of *P. canariensis* (Gómez et al., 2003; Vaxevanidou et al., 2005). Additionally, three populations from Tenerife (nine, 12 and 13 in table 6.2 and figure 6.2) were also genotyped for the present analysis and the compatibility of the data was assured by repeated genotyping of four haplotypes from the previous studies. All individuals were genotyped for six cpSSR loci: Pt15169, Pt30204, Pt71936, Pt87268, Pt26081 and Pt36480 (Vendramin et al., 1996).

### 6.2.3 Data analysis

In order to use ARLEQUIN 2.0 (Schneider et al., 1999) for the analyses, microsatellite data were binary coded: the number of repeats were coded with ‘1’ and shorter alleles were coded filling the difference in repeats with ‘0’ (Pereira et al., 2002). Analyses for the empirical samples were carried out at two levels: (1) sample sites as the unit of analysis, (2) islands as the unit of analysis with

Population	$n$	Fu (1997)		Schneider and Excoffier (1999)			<i>B. rugatus</i> (mya)	
		$F_S$	$p(F_S)$	$\tau$ (95% CI)	$t$ (mya)	SSD		$p(SSD)$
<b>Gran Canaria</b>	<b>145</b>	<b>-26.260</b>	<b>&lt; 0.001*</b>	<b>2.544 (1.372–5.010)</b>	<b>1.970</b>	<b>0.002</b>	<b>0.569</b>	<b>&gt; 2.56</b>
1 Arguineguín	30	-21.890	< 0.001*	3.703 (2.138–5.636)	2.868	0.003	0.469	
2 Galdar	19	-4.076	0.017*	2.722 (1.128–3.892)	2.108	0.004	0.554	
3 Mogán	24	-0.849	0.275	0.969 (0.000–1.558)	0.750	0.020	0.077	
4 Tamadaba	24 (23)	-1.304	0.142	<i>0.871 (0.000–1.489)</i>	<i>0.675</i>	<i>0.014</i>	<i>0.138</i>	
5 Tirajana	24	-5.052	0.014*	1.743 (0.413–5.224)	1.350	0.008	0.399	
6 Tirma	24	-6.845	0.001*	2.438 (0.779–3.342)	1.888	0.002	0.783	
<b>Tenerife</b>	<b>280</b>	<b>-26.710</b>	<b>&lt; 0.001*</b>	<b>2.374 (1.330–3.144)</b>	<b>1.839</b>	<b>0.000</b>	<b>0.910</b>	<b>1.89–2.56</b>
7 Anaga	24	-0.192	0.185	3.000 (0.523–3.000)	2.323	0.010	0.062	
8 Arico	24	-8.983	< 0.001*	2.314 (0.675–3.185)	1.792	0.003	0.542	
9 Chinyero	50 (49)	-15.290	< 0.001*	<i>2.294 (0.978–2.900)</i>	<i>1.777</i>	<i>0.001</i>	<i>0.636</i>	
10 La Esperanza	24	-3.040	0.022	2.722 (0.483–6.413)	2.108	0.015	0.567	
11 La Guancha	24	-3.545	0.024	2.036 (0.501–2.875)	1.577	0.003	0.623	
12 Güímar	47	-22.280	< 0.001*	3.280 (1.795–4.143)	2.540	0.001	0.702	
13 Ifonche	39	-8.615	< 0.001*	2.482 (1.035–3.172)	1.922	0.007	0.130	
14 Oratava	24	-6.456	0.002*	2.730 (0.993–6.926)	2.114	0.010	0.285	
15 Vilaflor	24	-0.165	0.437	1.081 (0.000–1.766)	0.837	0.009	0.192	
<b>La Gomera</b>	<b>36</b>	<b>-0.424</b>	<b>0.427</b>	<b>1.558 (0.380–3.379)</b>	<b>1.207</b>	<b>0.008</b>	<b>0.431</b>	
16 Garabato	12	-	-	-	-	-	-	
17 Imada	24	-0.879	0.294	1.307 (0.182–2.064)	1.012	0.002	0.711	
<b>La Palma</b>	<b>48</b>	<b>-2.826</b>	<b>0.072</b>	<b>1.244 (0.311–1.726)</b>	<b>0.963</b>	<b>0.004</b>	<b>0.285</b>	<b>1.58–2.00</b>
18 Fuencaliente	24	-1.063	0.224	1.289 (0.048–2.002)	0.998	0.008	0.305	
19 Garafía	24	-1.279	0.184	1.206 (0.053–1.904)	0.934	0.002	0.720	
<b>El Hierro</b>	<b>24 (23)</b>	<b>-3.513</b>	<b>0.008*</b>	<b>1.291 (0.040–2.035)</b>	<b>1.000</b>	<b>0.008</b>	<b>0.301</b>	<b>1.00</b>

\* Significant at  $\alpha = 0.05$  ( $p$ -value < 0.02)

Tab. 6.2: Results for the  $F_S$  neutrality test for population expansion (Fu, 1997) and population expansion parameters following Schneider and Excoffier (1999). Estimates are presented in *italics* when the algorithm did not converge (see section 6.3 for details). The time of expansion expressed in million of years before present (mya) is calculated using mutation rates in the range  $1.076 \times 10^{-5}$  per generation per locus. The results for the islands (pooling samples from the same island) are presented in **bold**. Garabato is a monomorphic population and tests could not be performed. For comparison, time for the colonisation of *Brachyderes rugatus* is also presented from Emerson et al. (2000a).

sample sites within an island pooled together.

A general description of diversity indices and population structure found within *P. canariensis* using cpSSRs is presented in Gómez et al. (2003); thus here we focus on the assessment of demographic history, using two different but complementary approaches. Firstly, we performed the  $F_S$  neutrality test for population expansion (Fu, 1997). This test is based on different expectations for the number of haplotypes when comparing a stationary with an expansion demography. The  $F_S$  statistic takes a large negative value within a population affected by expansion due to an excess of rare haplotypes (recent mutations). Significance of the test was calculated with 10 000 data bootstraps (Schneider et al., 1999). An

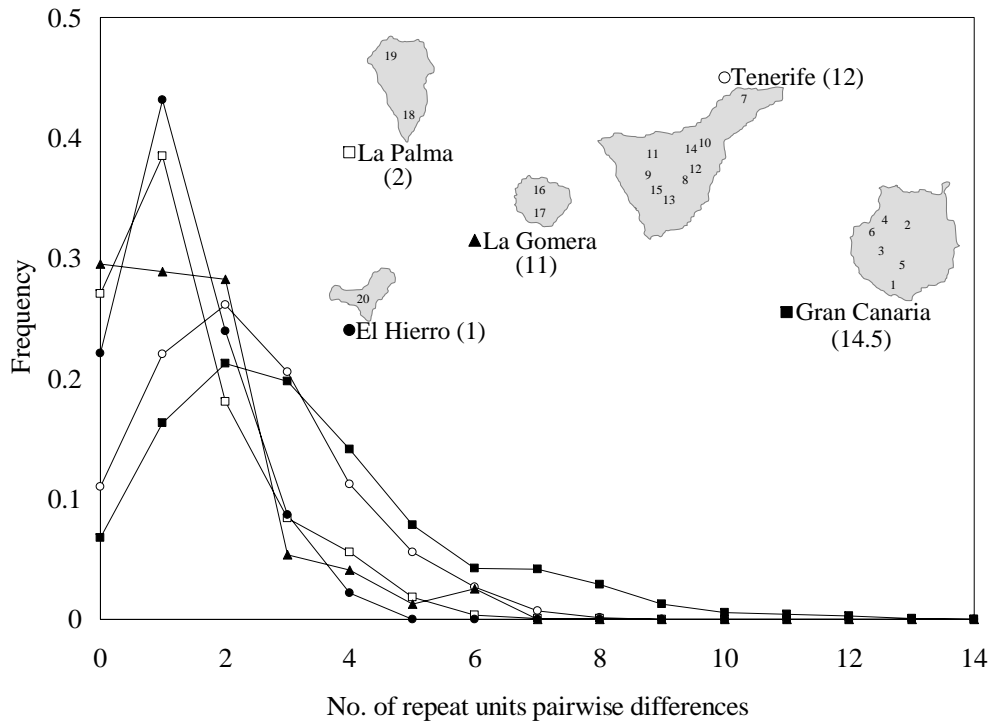


Fig. 6.2: Mismatch distribution for the islands and map of the western Canary Islands. Age of the islands (in million of years) are shown in parenthesis. Sampling localities marked with numbers corresponding to populations in table. 6.2

$F_S$  statistic with  $p(F_S) < 0.02$  ( $\alpha = 0.05$ , due to a particular behaviour of this statistic, Fu, 1997) was considered evidence of population expansion.

The second analysis consists of the estimation of the demographic model of Rogers and Harpending (1992) described with the parameters:  $\tau = 2\mu t$ ,  $\theta_0 = 2\mu N_0$  and  $\theta_1 = 2\mu N_1$  (where  $\mu$  is the mutation rate,  $t$  is the number of generations since expansion and  $N_0$  and  $N_1$  are the population sizes before and after expansion). Parameters are estimated from the distribution of pairwise differences (difference in number of repeats) between individuals within a sample. Although, in our case, the pairwise differences calculated cannot be strictly called mismatches, we will refer to their distribution as ‘mismatch distribution’ as it is the most common term used throughout the literature (Harpending et al., 1993). This distribution is affected by the demography of the sample; sudden growth produces unimodal distributions while within stationary populations dis-



tributions are ragged and multimodal (Slatkin and Hudson, 1991). An algorithm, which minimizes the sum of squared differences (SSD) between model and data, can be used to estimate the combination of parameters with the best fit to the empirical data (Schneider and Excoffier, 1999). The strength of the estimated model is then evaluated from the SSD distribution which is obtained from 10 000 data bootstraps (1000 for the simulation output), making  $p(\text{SSD})$  the proportion of bootstraps with the SSD larger than the original (Schneider and Excoffier, 1999). A significant SSD value,  $p(\text{SSD}) < 0.05$ , implies the rejection of the estimated demographic model. The confidence interval (95% CI) for the estimated parameter is also calculated from the bootstrap process (Schneider and Excoffier, 1999). Confidence intervals for parameters related to the magnitude of expansion ( $\theta_0$  and  $\theta_1$ ) will not be discussed as they are usually too wide and are of less interest for the interpretation of the results (Excoffier and Schneider, 1999). Dating the population expansions was done from the parameter using its relationship with time and mutation rate:  $\tau = 2l\mu t$  (where  $l$  is the number of cpSSR loci and  $\mu$  is the mutation rate per locus).

## 6.3 Results and discussion

### 6.3.1 Simulations

The results from the simulations are summarised in table 6.1. In the two analyses performed, cpSSR polymorphism was sensitive to population growth; however the results were not as precise as would be desirable.

#### *F<sub>S</sub> neutrality test*

In the cases of uniform mutation rate across loci the performance of the  $F_S$  test to detect population expansion was acceptable. Type II error for the  $F_S$  test (no evidence of population expansion in cases 1 and 2) was very low, and type I error (rejection of stationary population size in case 3) was low (11% of the replicates of case 3), although greater than expected at the given confidence level (expected 5% for  $\alpha = 0.05$ ).

In the cases evolving under the two-rate model (cases 4–6), the power of the  $F_S$  test decreased dramatically, and this was accompanied by an increase in homoplasy. Detection of recent expansions was especially affected and the reason

for this relates to the estimates of genetic distance and the number of haplotypes used in the test. First, the test uses the average genetic distance among individuals to calculate the expected number of haplotypes under a stationary demography scenario. The effect of homoplasy in this calculation is proportional to the time of expansion, with an average reduction of 19% in the distance estimates of recent expansions (case 4) and 41% in the older expansions (case 5). The expected number of haplotypes is then compared to the observed number of haplotypes. While the effect of homoplasy in genetic distance estimates was proportional to the time of expansion, homoplasy decreases the detectable number of haplotypes by approximately 40% both in the recent and older expansions. It seems that the power of the test varies with the time of expansions because the error in the estimates of genetic distances and number of haplotypes is more unbalanced for recent expansions.

#### *Demographic model estimation*

For cpSSRs evolving under the one-rate mutation model, estimates of the time of expansion were fairly accurate, although older expansion times were slightly underestimated. The average estimated time of expansion ( $\hat{\tau}$ ) for the recent expansions (case 1,  $\tau = 1.0$ ) was 1.1 and the true value was always within the 95% CI, while for older expansions (case 2,  $\tau = 3.0$ ) average  $\hat{\tau}$  was 2.5 and the true value falls outside the 95% CI in 15% of the replicates. In the simulations using the two-rate mutation model the estimates for recent expansion (case 5,  $\tau = 1.0$ ) were accurate, with average  $\hat{\tau} = 1.0$  and the true value fell outside the 95% CI in only 2% of the replicates. However in older expansions (case 6,  $\tau = 3.0$ ) the expansion time was largely underestimated for the two-rate mutation model with the average value of  $\hat{\tau}$  being 1.8 and the true value falling outside the 95% CI in 77% of the replicates. Although these results appear discouraging it is important to note that the relative times of expansion are still discernible, and that it may be possible to develop new statistical analyses to improve the estimates as has been done for heterogeneous mutation rates within sequence data (Schneider and Excoffier, 1999).

### 6.3.2 The empirical case: *Pinus canariensis*

The results for the detection of population expansions in the *P. canariensis* samples are reported in table 6.2. For the estimation of the demographic model the algorithm was unable to find a combination of parameters with a minimum SSD in three samples (Tamadaba, Chinyero and El Hierro). This inability of the algorithm to converge sometimes has been observed in previous works (e.g. Stamatis et al., 2004) and in our simulations. A simple solution is to obtain the estimation from a reduced sample obtained by randomly removing one individual. This reduction of the sample size changes the shape of the mismatch distribution slightly enough for the algorithm to converge while still maintaining a very similar shape to the mismatch distribution from the original data set. The mismatch distributions from the reduced samples were used to produce the parameter estimations presented in *italics* in table 6.2.

The demographic expansion model estimated for different sampling sites (including the grouping of sampling sites at the island level) was, in general, fairly robust [ $p(\text{SSD}) \gg 0.05$ ] and mismatch distributions were clearly unimodal (figure 6.2, opposite to the ragged distribution expected with a stable population). The results of the  $F_S$  test yielded evidence of population expansion for nearly half of the samples. It is interesting to note that the samples for which the  $F_S$  test could not reject a stable population scenario [ $p(F_S) > 0.02$ ] were the ones with the lowest  $\hat{\tau}$  values. In the light of our simulation results it is expected that the  $F_S$  test will have lower power to detect very recent population expansions, especially under the more realistic scenario of heterogeneous mutation rates across loci. Thus we could consider that most of the *P. canariensis* populations are likely to have been subject to demographic growth and the lack of statistical evidence is due to the low power of the  $F_S$  test for the most recent expansions. Probable exceptions to this are the Anaga and La Gomera samples, tiny isolated populations that are most likely either recent colonisations or relict populations subject to high genetic drift for a long period (Vaxevanidou et al., 2005).

#### *Island level: colonisation*

Compared to continental areas, oceanic island populations are typically established by only one or a few individual founders which successfully reproduce, leading to demographic expansions. Whether the population expansions detected



Fig. 6.3: *Brachyderes rugatus* on a pine branch (photos: Brent Emerson).

for *P. canariensis* at the island level reflect the initial colonisation of the islands or subsequent demographic events is difficult to know. However, times of expansion in relation to the geological history of the archipelago can supply the necessary clues to discern between both possibilities.

Potential maximum times for expansion are bound to the emergence times of the islands. The maximum subaerial geological age of El Hierro, the youngest island, is approximately one million years (Carracedo and Day, 2002). If we consider that the time of the population expansion in El Hierro is  $\hat{\tau} = 1.291$  and the relationship  $\tau = 2l\mu t$  we obtain a mutation rate estimate of  $1.076 \times 10^{-5}$  per locus per generation (considering generation time to be 100 years as in Provan et al., 1999a). Using this mutation rate estimate we calculated the maximum age of population expansion for each sample, reported in table 6.2.

In order to establish a minimum time of expansion we have analysed mtDNA COII sequence data for *Brachyderes rugatus* (figure 6.3) from Emerson et al. (2000a, 2005, and unpublished data). Because the niche of this species is the pine tree, its demographic expansions must have occurred either during or after the establishment of the pine forest on each island. Population expansions have been detected (significant  $F_S$  test) for the islands of La Palma and Tenerife (138 and 182 individuals respectively, sampled throughout the islands). The times of expansion for *B. rugatus* were estimated from the mismatch distributions to be approximately 0.72 million years ago (mya) for Tenerife and 1.11 mya for La Palma [considering divergence rate to be between 2%, DeSalle et al. (1987), and 2.3%, Brower (1994), per million years]. These dates strengthen the age estimates for the expansion of the pine forest obtained with the geological age calibration.

These age estimates suggest expansions of the pine tree increasing in age from west to east, and coinciding broadly with the colonisation ages estimated for *B. rugatus* (Emerson et al., 2000a), as shown in table 6.2. We interpret the expansions at the island level as a result of the colonisation process and linked to the volcanic history of the archipelago. The creation of new emerged landmass by recent (up to 2 mya) volcanic activity in the younger islands (La Palma and El Hierro) opened new territories for *P. canariensis* to colonise. Note that the age of Tenerife presented in figure 6.2 refers to its older massifs which are the remains of two or three smaller precursor islands. However, the majority of the landmass of Tenerife was mainly formed by the activity of Las Cañadas volcano starting around 2 mya (Ancochea et al., 1990) and it is this event which would appear to be causally related to the pine forest expansion. In the island of Gran Canaria, an episode of heavy volcanic activity (Roque Nublo volcano, Pérez-Torrado et al., 1995) is believed to have destroyed almost all terrestrial ecosystems within the island, with perhaps the exclusion of some coastal regions, between 5.5 and 3 mya (Marrero and Francisco-Ortega, 2001), and this hypothesis has gained recent support from a meta-analysis by Emerson (2003). The expansion of the pine forest in Gran Canaria after that event can be explained either by colonisation of *P. canariensis* to the island or by a bottleneck if a small pocket of pine forest survived through the Roque Nublo eruptive period.

#### *Sample level: metapopulation dynamics*

The islands of the Canary archipelago have a geological history marked by recent dramatic volcanic activity and giant landslides (Carracedo and Day, 2002). These destructive events would have produced local elimination of pine forest, as has been recorded for historical volcanic eruptions (del Arco Aguilar et al., 1992; Pérez de Paz et al., 1994a). Also, the Canary Island pine is renowned for its capacity colonising lava flows (figure 6.4, del Arco Aguilar et al., 1992; Pérez de Paz et al., 1994a), which suggest that a metapopulation dynamic occurs within the pine forest. One of the genetic signals expected in the local recolonisations after volcanic disturbances are those of the demographic expansions, as it has been shown in other organisms subject to similar metapopulation dynamics in other volcanic archipelagos (Beheregaray et al., 2003; Vandergast et al., 2004).

It seems very likely that local expansions detected for *P. canariensis* are the



Fig. 6.4: San Antonio volcano crater (eruption in 1677), colonised by *Pinus canariensis*. Volcanic terrain, such as lava flows, are first colonised by lichens and ‘livi-colous’ arthropods that live on biological fall-out (Ashmole et al., 1992). After few hundred years soil is formed and first plants (including *P. canariensis*) colonise the lavas (Juan et al., 2000). Photo: Ruth Álvarez Asenjo.

product of metapopulation dynamics. When we consider different samples within the same island (in Tenerife and Gran Canaria) we observe that the expansion of pine forest at some areas is younger than the main demographic expansion affecting the island. We hypothesize that the apparently more recent expansions may be areas recolonised after geological disturbance. The role of volcanism and giant landslides in the reduction of genetic diversity has also been proposed to explain the pattern of diversification of *Brachyderes rugatus* in La Palma, El Hierro, Tenerife and Gran Canaria (Emerson et al., 2000a).

## 6.4 Conclusions

This study demonstrates the utility of cpSSRs for the detection of demographic expansions and the estimation of their relative ages. The application of population genetic demographic methodology to cpSSR data for *P. canariensis* populations revealed new insights into the population history of this species. The

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volcanic activity of the archipelago appears to be a disturbance agent in the pine forest ecosystem, conditioning the areas available for the pine tree. Mitochondrial DNA data may further complement data from cpSSRs to elucidate the colonisation and population dynamic history of *P. canariensis* on the Canary Islands. An mtDNA phylogeographic analysis would reflect the historical seed movements of *P. canariensis*, which are limited relative to pollen and may contain more fine scale phylogeographic information. Additionally, a sampling design including historical and isotope-dated lava flows within the pine forest may provide a good test for the hypothesis of a metapopulation dynamic.

Our simulations have revealed homoplasy as a problem for the analyses (mainly in the detection of younger expansions) because it reduces the power of the  $F_S$  test and accuracy of absolute expansion time estimates. The development of statistics taking into account the effects of homoplasy would further improve the usefulness of cpSSRs as well as other linked microsatellite markers, such as Y-chromosome microsatellites, for demographic studies.

## 7. EFFECT OF ALTITUDE ON THE PATTERNS OF GENE FLOW<sup>1</sup>

### *Abstract*

*Pinus canariensis* is endemic to the western Canary Islands, where it forms forest spanning an altitude from 500 to 2500m. The dramatic changes in environmental conditions (temperature, moisture and solar radiation) over short distances due to this elevation gradient suggest the possible existence of altitudinal ecotypes for the Canary Island pine. In order to maintain local races in a continuous forest some barriers to gene flow should be present. Altitudinal differences in temperature may lead to asynchronous flowering times at different elevations. In this study we used nuclear and chloroplast microsatellites to characterise the genetic structure of two altitudinal transects on the southern slopes of Tenerife to test for genetic isolation among altitudes. Only nuclear microsatellites presented significant differentiation among populations but this differentiation was not related to differences in altitude. Nevertheless, altitude seems to be playing some role in the patterns of gene flow among sites because an increase in levels of gene flow and genetic diversity with elevation was detected.

### 7.1 *Introduction*

Canary Island pine, *Pinus canariensis*, grows in the western Canary Islands where it occupies a wide altitudinal range (from 500 to 2500m in the leeward mountainside, Fernández-Palacios and de Nicolás, 1995). Because of the steep slopes of the islands, dramatic differences in altitude —and, therefore, in environmental conditions— are found over short linear distances. The capacity of this species to live under such different conditions raises the following questions: are there local

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<sup>1</sup> Article in preparation by M. Navascués, G.G. Vendramin and B.C. Emerson



races adapted to the different altitudes or does the species have the plasticity to acclimate to different altitudes? If there are altitudinal ecotypes, how can they be maintained in a continuous forest where high levels of gene flow are expected?

Many other tree species with wide altitudinal ranges of distribution have given rise to the same questions to scientists. For *Pinus flexilis*, seasonal isolation among altitudes was suggested, because non-overlapping pollination times were found for elevations separated by more than 400m (Schuster et al., 1989). However, very low population differentiation among altitudinal samples was detected with different molecular markers (Schuster et al., 1989; Latta and Mitton, 1997). Later, Schoettle and Rochelle (2000) considered the effects of the altitudinal conditions on the growth of *P. flexilis* finding no significant differences among elevational sites. Based on these results, and the low genetic differentiation found in the above mentioned studies, Schoettle and Rochelle (2000) concluded that phenotypic plasticity enables this pine to occupy such a broad altitudinal range.

However, a low level of genetic differentiation in neutral markers does not rule out the possibility of differentiation in adaptive traits (Karhu et al., 1996). A recent study of an elevation transect of *Pinus oocarpa* did not show  $F_{ST}$  values significantly different from zero for isozyme analysis (Sáenz-Romero and Tapia-Olivares, 2003), however a common garden experiment on seeds from the very same transect revealed altitudinal patterns in growth and number of cotyledons (Sáenz-Romero, 2003; Sáenz-Romero et al., 2003). Therefore, common garden experiments are fundamental to distinguish between altitudinal adaptation (e.g. Oleksyn et al., 1998) and altitudinal acclimation (e.g. Cordell et al., 1998).

In the case of the Canary Island pine we know that the altitudinal gradient entails changes in water availability (Gieger and Leuschner, 2004), environmental temperature ( $\sim 0.45^{\circ}\text{C}/100\text{m}$ , Marzol, 1981, 2001) and degree of insolation (Jiménez and Morales, 2001). Water stress at timberline altitude seems to be the cause of reduced tree growth for *P. canariensis* (Jonsson et al., 2002; Srutek et al., 2002; Gieger and Leuschner, 2004). Some evidence on local drought adaptation has been found for *P. canariensis* in a common garden trial (Climent et al., 2002b); however, the experiment did not include different altitude provenances and acclimation to drought has also been described (Grill et al., 2004). Temperature adaptation is also possible as seedlings from higher altitudes in Tenerife

were more resistant to cold stress than the ones from lower altitudes in an unpublished provenance test (Krugman and Jenkinson, 1974). On the other hand, the capacity of *P. canariensis* to live under different levels of solar radiation is due to its plasticity for regulating the proportion of photosynthetic pigments allowing it to protect itself against excessive insolation (Jiménez et al., 1997; Tausz et al., 1998b; Peters et al., 1999).

Confirmation of altitudinal ecotypes for *P. canariensis* might only be expected from provenance tests, like the ones recently initiated by Climent et al. (2001b). However, these are long-term experiments and may take several years before there are significant results. Meanwhile, the use of neutral molecular markers can answer questions on the patterns of gene flow among altitudes. In a continuous cline there must be strong selection or the presence of barriers to gene flow for the development of local races (Slatkin, 1973). Barriers, in an altitudinal gradient, can be the product of out-of-phase pollination times, like the situation described by Schuster et al. (1989) in *P. flexilis*.

In the present work we study the possible influence of altitude on the patterns of gene flow in *P. canariensis*. It is important from the outset to be aware that these patterns may be difficult to detect, because previous studies of altitudinal transects suggest very low levels of genetic differentiation among sites for pine species (Kara et al., 1997; Sáenz-Romero and Tapia-Olivares, 2003, both using isozymes). In order to overcome that difficulty we have resorted to microsatellite markers, which are expected to have higher resolving power than isozymes (see, for instance, Estoup et al., 1998) and a sampling design that includes two parallel elevation transects that allow genetic differentiation comparisons within altitudes and among altitudes.

## 7.2 *Materials and methods*

### 7.2.1 *Plant material and sampling design*

Two altitudinal transects were studied in Arico<sup>2</sup> and Vilaflor (Tenerife, Canary Islands). Both transects were located in the southern (leeward) slopes of the island to minimize the effects of climatic differences (figure 7.1). Sampling points

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<sup>2</sup> It must be noted that site ‘Arico 2000’ corresponds to the same sample used in Chapter 8 as ‘Arico natural’.

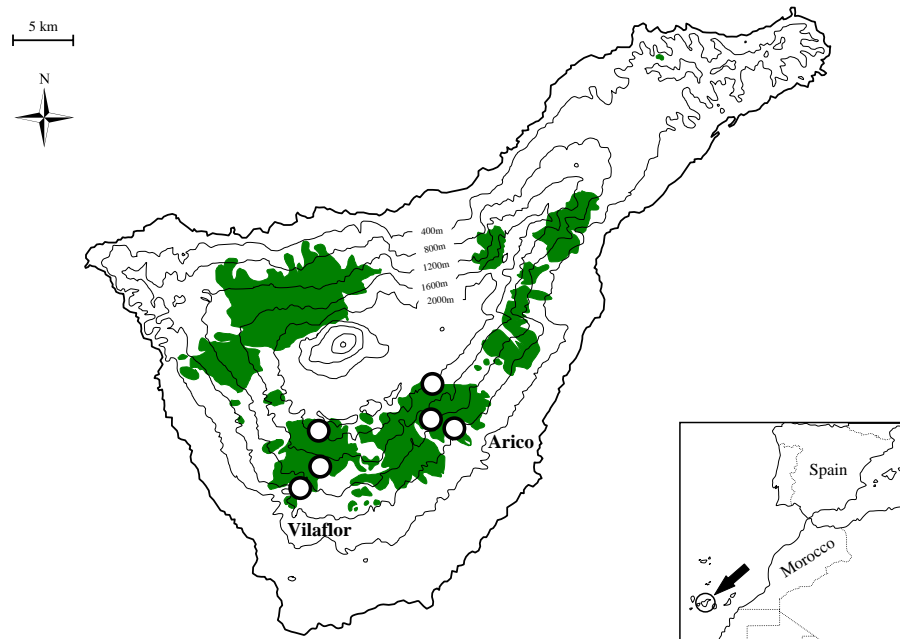


Fig. 7.1: The two altitudinal transects studied are located at the southern slope of Tenerife. Each transect was sampled at three points. White circles mark the location of the sampling points; distribution of natural pine forest is shown in green.

were selected within natural stands of *Pinus canariensis* forest (del Arco Aguilar et al., 1992) at approximately 1000, 1500 and 2000m of altitude (table 7.1). At each sampling point 38–50 old trees, with an average separation of 10 m among them, were selected randomly. From each tree, needles were collected and preserved in silica gel in the summer of 2002.

### 7.2.2 Molecular markers

Genomic DNA was purified using a CTAB protocol based on the Doyle and Doyle (1987) method (see appendix B.1). Samples were genotyped for eight chloroplast microsatellites (Pt1254, Pt15169, Pt26081, Pt30204, Pt36480, Pt71936, Pt87268 and Pt110048) transferred from *Pinus thunbergii* (Vendramin et al., 1996) and eight nuclear microsatellites (SPAC 11.5, SPAC 11.8, SPAG 7.14, PtTX3116, PtTX4001, ssrPt\_ctg4363, ssrPt\_ctg4698 and ssrPt\_ctg7731; Soranzo et al., 1998; Auckland et al., 2002; Chagné et al., 2004) transferred from *Pinus sylvestris* and *Pinus taeda* (González-Martínez et al., 2004, and this study). PCR amplifica-

ID	latitude	longitude	altitude	sample size, $n$
Arico 1000	28° 11.87' N	16° 31.84' W	1061m	40
Arico 1500	28° 12.19' N	16° 32.92' W	1513m	40
Arico 2000	28° 14.39' N	16° 32.90' W	2135m	50
Vilaflo 1000	28° 07.97' N	16° 41.28' W	1035m	39
Vilaflo 1500	28° 10.04' N	16° 38.15' W	1553m	38
Vilaflo 2000	28° 11.39' N	16° 39.96' W	2108m	42

Tab. 7.1: Sampling sites geographic information of the two altitudinal transects. The transects were selected in the leeward slope of El Teide mountain to minimize climatic differences between transects.

tions were performed in a Perkin-Elmer 9700 thermal cycler (see details for PCR conditions in appendix B.2). PCR products were sized in an ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

### 7.2.3 Data analysis

#### *Chloroplast microsatellites, cpSSR*

Total number of haplotypes ( $n_h$ , direct count), effective number of haplotypes ( $n_e$ , equation 5.2), unbiased haplotype diversity ( $H_e$ , equation 5.3) and average genetic distances among individuals ( $D_{sh}^2$ , equation 5.4) were calculated for each population.

Genetic isolation between altitudinal localities was tested by performing a hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992) which takes into account the genetic distance between haplotypes. Total variance in cpSSR distances is partitioned into: 1) within-population covariance, 2) among-altitudes covariance (three altitudinal groups) and 3) among-samples-within-altitude covariance. Genetic distances between haplotypes are estimated as the sum across loci of squared differences in number of repeats:

$$\delta_{ij}^2 = \sum_{k=1}^L |a_{ik} - a_{jk}|^2 \quad (7.1)$$

where  $L$  is the number of loci,  $a_{ik}$  is the size (measured in repeat units) of the allele for the  $i^{\text{th}}$  individual and at the  $k^{\text{th}}$  locus, and  $a_{jk}$  is the size of the allele for the  $j^{\text{th}}$  individual and at the  $k^{\text{th}}$  locus. Genetic distance estimates based on

differences in allele size underestimate actual genetic distances as a consequence of homoplasy (see Navascués and Emerson, 2005, and Chapter 5). However, it seems reasonable to assume that, because the estimates are proportional to actual distances (Navascués and Emerson, 2005, and Chapter 5), the variance of distances and, thus, AMOVA will be little affected by homoplasy<sup>3</sup>. The significance of obtained  $\Phi$ -statistics was computed using a permutation procedure (10 000 permutations) with ARLEQUIN 2.0 (Schneider et al., 1999).

Minimum sample size necessary for the analysis was assessed by a permutation method. Haplotypes from the empirical data were resampled (with replacement) 1000 times per batch, in batches of increasing sample size (from two individuals to 38) and the AMOVA was performed for each iteration. Haplotypes were resampled among populations to estimate the parameter distributions under the null (no differentiation) hypothesis. Additionally, resampling within populations was done to estimate the parameter distribution under the alternative hypothesis (level of differentiation described by the  $\Phi$ -statistics obtained empirically). For each sample size the distribution of the  $\Phi$ -statistics was extracted, and the mean and 95% confidence interval (percentile method) calculated. Dyer and Sork (2001), using a similar bootstrap approach, considered an appropriate sample size the one “beyond which the mean and variance stabilize” in the alternative hypothesis, which we think requires a rather subjective decision on whether they stabilize. As an alternative, we propose to calculate a Monte Carlo estimate of the statistical power of the AMOVA (i.e. the probability of accepting the alternative hypothesis when it is true). The power was calculated as the proportion of iterations not significantly different to the null distribution (i.e. iterations with  $\Phi$ -statistic values within the 95% CI of the null hypothesis).

#### *Nuclear microsatellites, nSSR*

Number of alleles ( $A$ , direct count of alleles), effective number of alleles ( $A_e$ , Kimura and Crow, 1964), allelic richness ( $A_{n_{min}}$ , El Mousadik and Petit, 1996) and unbiased gene diversity ( $H_e$ , Nei, 1987, equation 7.39) were calculated for each locus and site with FSTAT 2.9.3.2 (Goudet, 1995) and GENEPOP 3.4 (Ray-

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<sup>3</sup> However, further simulation work on this topic may be necessary to confirm this.

mond and Rousset, 1995b):

$$A_e = 1 / \sum_{i=1}^A p_i^2 \quad (7.2)$$

$$A_{n_{min}} = \sum_{i=1}^A \left[ 1 - \frac{\binom{2n - n_i}{2n_{min}}}{\binom{2n}{2n_{min}}} \right] \quad (7.3)$$

$$H_e = \frac{n}{n-1} \left( 1 - \sum_{i=1}^A p_i^2 - \frac{H_o}{2n} \right) \quad (7.4)$$

where  $n$  is the number of individuals in the sample (i.e. sample size),  $n_{min}$  is the sample size of the smallest sample in the study ( $n_{min} = 38$ , i.e.  $A_{n_{min}} = A_{38}$ ),  $p_i$  is the relative frequency of the  $i^{\text{th}}$  allele in the sample,  $n_i$  is the number of times the  $i^{\text{th}}$  allele presents in the sample,  $A$  is the total number of different alleles in the sample and  $H_o$  is the observed number of heterozygotes in the sample.

ARLEQUIN 2.0 was used to perform a hierarchical AMOVA to calculate population differentiation statistics based on SMM genetic distances ( $R_{ST}$ ; Slatkin, 1995) and allele frequencies ( $\theta$ ; Weir and Cockerham, 1984). The variance was partitioned in the same altitudinal groups as the AMOVA on cpSSRs described above.

Correlations of genetic differentiation with geographical distance and with altitudinal difference were analysed with Mantel tests (Mantel, 1967). Partial Mantel tests (Legendre and Legendre, 1998) were used to check combined effects of distance and altitude. Calculations were performed with ISOLATION BY DISTANCE web service (Bohonak, 2002; Jensen et al., 2005). The significance of the correlations between genetic distances, expressed as  $\hat{M}$  (Slatkin, 1993) or  $F_{ST}/(1 - F_{ST})$  (Rousset, 1997), with the geography was estimated with 30 000 permutations.

In order to detect patterns of directionally-biased gene flow two approaches were followed. First, departure from the island model of migration (Wright, 1931) was tested with ISLANDMODELTEST (Porter, 2003). This program tests individual locus  $F_{ST}$  scores for the null (island model) distribution obtained by a parametric bootstrap method from the  $F_{ST}$  averaged over loci (Porter, 2003). Significant results may indicate directional bias in the movement of alleles, but

may also be due to violations of other assumptions of the island model (selection, non-equilibrium conditions, etc). Secondly, gene flow between pairs of populations was studied. Population pairwise genetic differentiation ( $\theta$ ; Weir and Cockerham, 1984) was calculated with FSTAT 2.9.3.2 and the significance tested by 10 000 randomization of genotypes among samples. In addition, number of migrants ( $N_e m$ ) exchanged between pairs of populations was calculated using the private allele method (Slatkin, 1985; Barton and Slatkin, 1986) with GENEPOP 3.4.

## 7.3 Results and discussion

### 7.3.1 Genetic diversity

All cpSSR loci but one (Pt36480) were polymorphic with an average of five alleles per locus, yielding 86 different chloroplast haplotypes (in a total of 249 individuals). Three haplotypes (H002, H005 and H026; see table C.2 for haplotype definitions) were shared among all populations, and one of them (H002) had the highest frequency among all haplotypes (present in 12.45% of individuals). Nuclear microsatellite loci had heterogeneous levels of polymorphism with the number of alleles ranging from seven (ssrPt\_ctg4698) to 56 (SPAC 11.8). Levels of genetic diversity were similar across populations (table 7.2A and table 7.3) for both types of SSR.

Several studies on genetic diversity in altitudinal transects of pine species have focused on correlations of genetic diversity levels (Isik and Kara, 1997; Klumpp and Stefsky, 2001) or allele frequencies (Mitton et al., 1980; Kara et al., 1997) with elevation. In our study there is no apparent relationship of genetic diversity and altitude, as different indices and loci follow different trends. Also, the discussion of correlations between the frequencies of particular alleles with altitude does not seem relevant in the present study. The correlations found in isozyme alleles with altitude in *Pinus ponderosa* by Mitton et al. (1980) were given an interpretation based on natural selection. In our study, which uses microsatellites, invoking natural selection would be unreasonable.

CpSSR diversity in *Pinus canariensis* was previously study by Gómez et al. (2003). Their study did not include loci Pt1254 and Pt110048. In order to establish a comparison with Gómez et al. (2003), genetic diversity indices were

	$n_h$	$n_e$	$H_e$	$D_{sh}^2$
A				
Arico 1000	27	21.622	0.978	3.217
Arico 1500	23	10.000	0.923	2.259
Arico 2000	27	16.667	0.959	3.193
Vilaflor 1000	26	17.483	0.968	2.151
Vilaflor 1500	27	15.696	0.962	2.560
Vilaflor 2000	27	18.375	0.969	3.350
B				
Arico 1000	21	12.308	0.942	2.039
Arico 1500	18	9.757	0.922	1.512
Arico 2000	21	7.812	0.890	2.273
Vilaflor 1000	16	9.447	0.918	1.162
Vilaflor 1500	18	12.237	0.942	2.039
Vilaflor 2000	19	10.756	0.929	2.317

Tab. 7.2: Chloroplast microsatellite diversity indices (number of haplotypes,  $n_h$ ; effective number of haplotypes,  $n_e$ ; unbiased haplotype diversity,  $H_e$  and average genetic distances among individuals,  $D_{sh}^2$ ) calculated among the altitudinal transects. A: using the eight loci, and B: removing loci Pt1254 and Pt110048 from the analysis for comparison with Gómez et al. (2003) data.

also calculated after removing those loci (table 7.2B), resulting in higher genetic diversity levels than any of the populations analysed by Gómez et al. (2003). Those differences might be a product of the larger sample sizes used in our study (38–50 against 24 used by Gómez et al., 2003) that might have allowed us to detect a higher number of haplotypes. Because of the high polymorphism of these markers small sample sizes might leave a big proportion of haplotypes unsampled and the result might not be accurate or representative of the true genetic diversity of the populations. To test this hypothesis we have pooled the data from the six populations (to have a better representation of the cpSSR diversity) and we have subsampled (sampling with replacement) for increasing sample sizes to study the behaviour of the diversity indices. From that analysis (figure 7.2) we conclude that the lower diversity found for  $n_h$ ,  $n_e$  and  $H_e$  may be a consequence of the lower sample size but the differences in the  $D_{sh}^2$  have probably been produced by chance.



		SPAC	SPAC	SPAG	PtTX	PtTX	ssrPt	ssrPt	ssrPt
		11.5	11.8	7.14	3116	4001	_ctg	_ctg	_ctg
							4363	4698	7731
Arico	$A$	19	25	15	6	8	9	4	7
1000	$A_e$	11.636	11.808	10.159	2.550	4.255	7.619	1.773	2.008
	$A_{38}$	18.742	24.539	14.942	5.994	7.900	9.000	3.950	6.900
	$H_e$	0.927	0.927	0.913	0.616	0.776	0.879	0.441	0.509
Arico	$A$	20	26	17	7	7	7	3	8
1500	$A_e$	9.357	16.842	10.224	2.678	4.665	4.931	1.497	2.397
	$A_{38}$	19.787	25.637	16.696	6.850	6.996	6.950	3.000	7.850
	$H_e$	0.904	0.953	0.913	0.635	0.796	0.806	0.336	0.589
Arico	$A$	27	31	14	8	6	8	4	8
2000	$A_e$	18.315	18.657	7.163	2.750	4.897	4.521	1.432	3.083
	$A_{38}$	25.039	27.854	13.274	7.679	6.000	7.759	3.520	7.462
	$H_e$	0.955	0.957	0.870	0.643	0.804	0.786	0.304	0.683
Vilaflor	$A$	22	23	12	6	6	7	3	6
1000	$A_e$	10.864	11.184	8.497	2.510	4.105	4.315	1.700	2.565
	$A_{38}$	21.819	22.768	12.000	5.923	6.000	7.000	2.974	5.974
	$H_e$	0.920	0.923	0.894	0.610	0.766	0.778	0.418	0.617
Vilaflor	$A$	28	29	16	6	7	7	6	7
1500	$A_e$	12.951	14.660	6.238	2.028	5.470	4.167	1.658	3.815
	$A_{38}$	28	29	16	6	7	7	6	7
	$H_e$	0.936	0.945	0.852	0.514	0.828	0.771	0.402	0.747
Vilaflor	$A$	25	30	13	6	6	6	3	7
2000	$A_e$	12.082	18.568	7.056	2.143	4.600	4.494	1.334	2.243
	$A_{38}$	23.998	28.987	12.903	5.991	5.992	5.999	2.992	6.889
	$H_e$	0.929	0.958	0.869	0.540	0.792	0.786	0.254	0.560

Tab. 7.3: Nuclear microsatellite diversity indices (number of alleles,  $A$ ; effective number of alleles,  $A_e$ ; allelic richness,  $A_{38}$ , and unbiased gene diversity,  $H_e$ ) within the altitudinal transects.

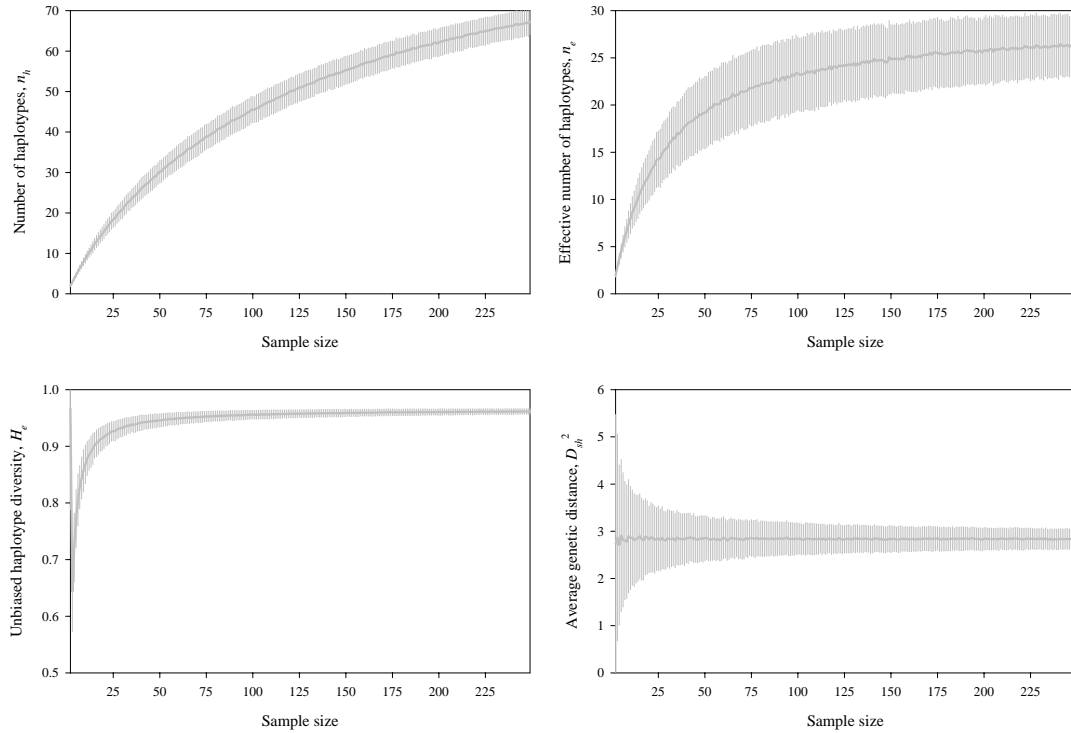


Fig. 7.2: Effect of sample size on the estimation of the number of haplotypes,  $n_h$ ; effective number of haplotypes,  $n_e$ ; unbiased haplotype diversity,  $H_e$ , and average genetic distance,  $D_{sh}^2$ . The data from the six populations studied were pooled and random subsamples (sampling with replacement) were extracted of increasing sizes. For each size 1000 iteration were performed and the means (lines) and standard deviations (bars) for each index were calculated.

### 7.3.2 Genetic differentiation among populations and among altitudes

No significant differentiation among altitudes was detected in any of the three AMOVAs performed (table 7.4). Significant genetic differentiation among populations—overall and within altitudes—was detected for nSSRs using either allele frequencies ( $\theta$ ) or SMM genetic distances ( $R_{ST}$ ). The level of genetic differentiation among populations obtained from cpSSR data was higher than the differentiation among altitudes—following the same pattern observed with nSSR data—but not significantly different from zero. Variance decomposition showed that the majority of the variation was contained within sampling sites (> 95%) with little variation among altitudes (< 1.5%).

cpSSR		nSSR			
$\Phi$ -statistics		F-statistics		R-statistics	
A					
$\Phi_{ST} = 0.007$	$p = 0.165$	$\theta = 0.019$	$p < 0.0001$	$R_{ST} = 0.044$	$p < 0.0001$
B					
$\Phi_{CT} = -0.003$	$p = 0.163$	$\theta_{CT} = 0.001$	$p = 0.336$	$R_{CT} = 0.015$	$p = 0.195$
$\Phi_{SC} = 0.009$	$p = 0.081$	$\theta_{SC} = 0.018$	$p < 0.0001$	$R_{SC} = 0.032$	$p < 0.0001$
$\Phi_{ST} = 0.007$	$p = 0.470$	$\theta_{ST} = 0.019$	$p < 0.0001$	$R_{ST} = 0.046$	$p < 0.0001$

Tab. 7.4: AMOVA genetic differentiation results for the altitudinal transects. Analysis on cpSSRs (using SMM genetic distances,  $\Phi$ -statistics) and nSSRs (using allele frequencies, F-statistics, and SMM genetic distances, R-statistics). Subindices indicate differentiation among altitudes (*CT*), among populations within altitudes (*SC*) and among populations (*ST*). A: standard analysis (populations not grouped) and B: hierarchical analysis (populations grouped by altitude).

The difference between nuclear and chloroplast SSR in the significance of the AMOVA has two possible explanations. On the one hand, the nature of the markers (levels of variation and linkage among loci) influences the statistical power of genetic differentiation analyses (see, for example, Estoup et al., 1998). On the other hand, the different inheritance modes of chloroplast (paternal inheritance) and nuclear (biparental inheritance) genomes influence the genetic differentiation statistics. The weight of the seed migration rate in the F-statistics is twice as big for biparentally inherited loci than for paternally inherited loci (Hu and Ennos, 1999, equations 12b and 13b). We must also consider that haploid genomes have half the effective population size of diploid genomes and, therefore, differentiation by means of genetic drift can be quicker. Thus, it is complex to discern the different effects of these elements in the calculation of differentiation statistics from the two different types of marker. Studies analysing population structure with markers from the mitochondrial genome (maternally inherited, structure only influenced by seed dispersal) and the chloroplast genome (paternally inherited, structure influenced by seed and pollen dispersal) show big differences in the differentiation statistics (see, for instance, Latta et al., 1998); however, differences between nuclear and chloroplast markers are probably more subtle, as both are influenced by seed and pollen dispersal.

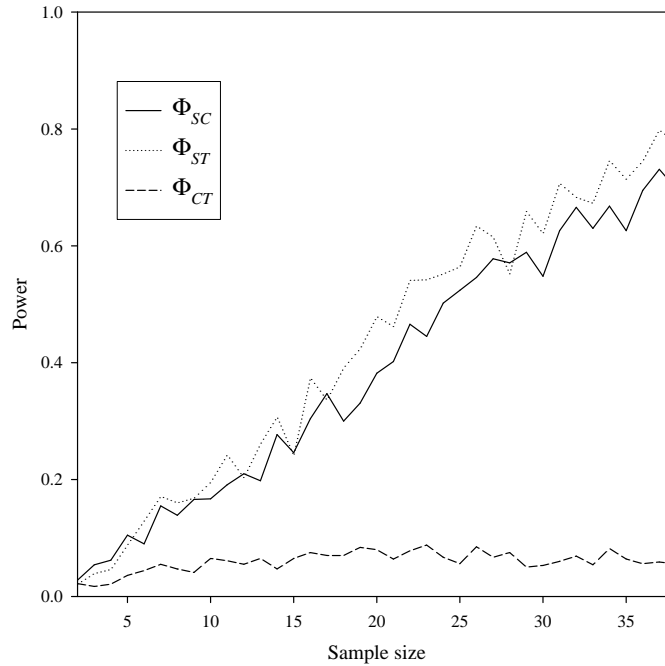


Fig. 7.3: Effect of sample size on the statistical power of the AMOVA of cpSSR data.

The power of the test was estimated with a data bootstrap method (resampling individuals within samples) as the proportion of iterations not significantly different to the null distribution. Subindices of the  $\Phi$ -statistics indicate differentiation among altitudes ( $CT$ ), among populations within altitudes ( $SC$ ) and among populations ( $ST$ ). Power to detect genetic differentiation among populations ( $\Phi_{ST}$ ) increases with sample size but does not reach 100% for the sizes used in this study.

Permutations of the empirical cpSSR data show that for our sample sizes the AMOVA statistical power is lower than 100% (figure 7.3). Extrapolation of our permutation analysis suggests that sample sizes well over 50 individuals would have been necessary to obtain an acceptable statistical power. This contrasts with the results obtained by Dyer and Sork (2001) where they propose that seven individuals per site were enough for describing the cpSSR variation among the populations of their study. However, differences in the levels of diversity in the populations, the genetic distances used for the AMOVA (they use  $\delta_{ij}^2$  as the square of the number of loci for which individuals  $i$  and  $j$  are different) and the criteria to choose the minimum appropriate sample size make comparisons

between their study and ours difficult. Nevertheless we can argue about the applicability of the Dyer and Sork (2001) criteria in comparison to the calculation of the statistical power. In figure 7.4 we can observe that the  $\Phi_{ST}$  mean and 95% CI of the alternative hypothesis ‘stabilize’ beyond 25–30 individuals and we could have chosen any of these numbers as the minimum appropriate sample size for the AMOVA. However the statistical power obtained for those samples sizes is hardly higher than 0.6 (i.e. approximately 40% of the cases of differentiated populations would not be detected). The statistical significance ( $p$ -value) of the AMOVA is standardly calculated by permutations of the data among populations (Schneider et al., 1999); in this study we demonstrate the informativeness of permutations within populations to obtain statistical power estimates of the analysis. The application of this method might be useful in future studies for deciding whether increasing the sample size is worthwhile or which markers are more useful (i.e. provide higher statistical power).

### 7.3.3 Population pairwise genetic differentiation and patterns of gene flow

Although genetic differentiation among altitudes was not significant the genetic differentiation among sites might be related to geographic factors (including altitude) that could be revealed using population pairwise comparisons. The most simple geographic pattern to test is the reduction of mating probability with physical distance (i.e. isolation by distance; Wright, 1938, 1940). Using Mantel tests on matrices of genetic and geographic distances is a standard method to test this pattern (Slatkin, 1993; Rousset, 1997). Using a third matrix we can test for the effects of an additional geographic feature (altitude). However, for none of these tests significant correlations were found (table 7.5). It is possible that, for the geographic scale used in this study, isolation by distance is not detectable, but it could be for smaller scales (Castric and Bernatchez, 2003, found correlation between genetic and geographic distances decreasing with the spatial scale in brook charr). Studying isolation by distance within populations (using distances among individuals, Epperson and Li, 1996; Hardy and Vekemans, 1999), could be a more successful approach for future investigations on *P. canariensis*.

Another basic model to describe gene flow among populations is the island model (Wright, 1931). We used the program ISLANDMODELTEST (Porter, 2003) to test departures from this model but the results were not informative as the

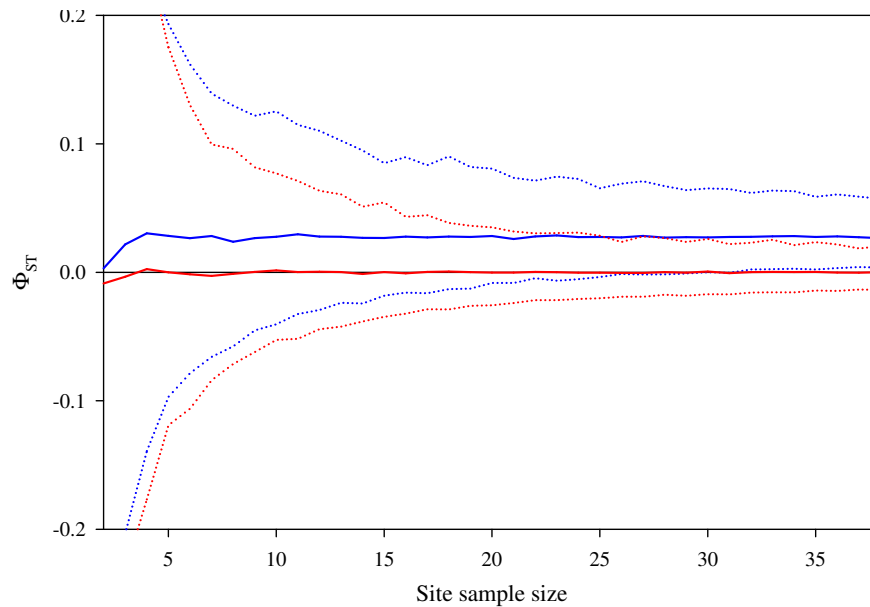


Fig. 7.4: Distribution of  $\Phi_{ST}$  under the null and alternative hypotheses for increasing sample size. Mean (solid line) and 95% CI (dotted lines) of the null (no differentiation, red lines) and alternative (differentiation with  $\Phi_{ST} = 0.007$ , blue lines) distributions obtained by resampling the data (among and within sites, respectively) for different sample sizes. The power of the test is illustrated graphically by the overlap of the distributions (see figure 7.3).

model could not be rejected (table 7.6). No simple model seems to fully explain the differences in genetic differentiation between pairs of populations which are very similar among pairs of sites (table 7.7). However, the level of differentiation between the sites at the higher altitude, Arico 2000-Vilaflor 2000, seems to be specially low (see  $\theta$  and  $N_e m$  values in table 7.7), despite being separated by a longer distance than pairs of sites within transects. The actual mechanism promoting this higher gene exchange at higher altitudes would be difficult to identify. Pollen dispersal by the local wind system could produce a biased gene flow (Klumpp and Stefsky, 2001). However, the local wind system in the Canary Islands is dominated by the northeast trades (see figure 1.5) and seems unrelated to the patterns found. High temperature, increased sunshine and moisture stress favour the initiation and amount of flowering (Bonner, 2003) and are conditions which vary with altitude. Other factors characterising timberline altitudes of the Canary Islands pine forest and favoring wind dispersal of propagules are low

Genetic distance	1st geographic matrix	Indicator matrix	$Z$	$r$	$p$ -value
$\hat{M}$	geographic distance	none	2302.240	-0.012	0.501
$\hat{M}$	altitudinal difference	none	119390.443	-0.319	0.902
$\hat{M}$	geographic distance	altitudinal difference		-0.051	0.564
$\hat{M}$	altitudinal difference	geographic distance		-0.322	0.899
$F_{ST}/(1 - F_{ST})$	log (geographic distance)	none	0.277	0.258	0.177
$F_{ST}/(1 - F_{ST})$	log (altitudinal difference)	none	169.923	0.025	0.365
$F_{ST}/(1 - F_{ST})$	log (geographic distance)	log (altitudinal difference)		0.261	0.179
$F_{ST}/(1 - F_{ST})$	log (altitudinal difference)	log (geographic distance)		0.052	0.326

Tab. 7.5: Mantel and partial Mantel test results for isolation by distance and altitude analysis. No significant correlations between genetic differentiation and geographic distance or altitudinal difference were found.  $Z$ , Mantel test statistic ( $Z = \sum_{i,j} A_{i,j}B_{i,j}$  for matrices  $A$  and  $B$ ) and  $r$ , correlation coefficient.

Locus	$F_{ST}$	$p$
SPAC 11.5	0.021	0.046*
SPAC 11.8	0.033	0.796
SPAG 7.14	0.026	0.316
PtTX3116	0.029	0.368
PtTX4001	0.023	0.318
ssrPt_ctg4363	0.023	0.374
ssrPt_ctg4698	0.014	0.096
ssrPt_ctg7731	0.024	0.174
Multilocus	0.025	0.188

Tab. 7.6: Results of the test for the island model of migration using ISLANDMODEL-TEST. Only one locus showed a significant departure from the model.

canopy density (Nathan and Katul, 2005) and dry weather (Bonner, 2003). The study of the flowering phenology along an altitudinal gradient in Tenerife might throw some light on the problem as times and duration of pollen shedding and female receptivity are fundamental to understand the exchange of genes among sites.

## 7.4 Conclusions

To conclude, altitude seems to be of little importance for the development of isolation among localities. Neither significant genetic differentiation among alti-

	Arico 1000	Arico 1500	Arico 2000	Vilaflor 1000	Vilaflor 1500	Vilaflor 2000
Arico 1000		3.405	4.063	2.947	4.274	4.734
Arico 1500	0.014*		3.799	3.252	3.387	4.286
Arico 2000	0.021*	0.020*		3.117	5.025	5.013
Vilaflor 1000	0.024*	0.022*	0.019*		4.029	4.003
Vilaflor 1500	0.035*	0.026*	0.011*	0.015*		5.611
Vilaflor 2000	0.022*	0.021*	0.005*	0.017*	0.014*	

\* Significant at  $\alpha = 0.05$  ( $p$ -value  $< 0.0033$ )

Tab. 7.7: Population pairwise genetic differentiation ( $\theta$ ; lower triangle) and number of migrants calculated from private alleles ( $N_e m$ ; upper triangle). Differentiation is similar among pairs but it is interesting to note that pairs involving at least one population from the 2000m altitude have, in general, lower genetic differentiation.

tudinal groups or correlation between genetic distances and altitudinal differences were found. The hypothetical seasonal isolation due to altitudinal differences in temperature is probably counterbalanced by stepping-stone gene flow along the continuous elevation gradient (Schuster et al., 1989). Therefore, if altitudinal ecotypes are confirmed for *P. canariensis*, they must be driven by strong selective forces. Nevertheless, some environmental factors, linked to altitude, seem to be promoting higher gene exchange between the upper elevation sites. Further investigations in *P. canariensis* mating system and flowering phenology in elevation transects might offer new answers to the higher genetic connectivity at high altitudes.

Regarding the genetic markers used, this study revealed higher statistical power in nuclear microsatellites than in chloroplast microsatellites for population differentiation analysis. This difference could be because the variation and linkage of chloroplast loci diminished the statistical power or because paternally inherited genes present slightly lower population differentiation. In any case we propose a resampling procedure to estimate the statistical power of the analysis. The use of this method will allow the researchers to assess whether they are using appropriate sample sizes and which type of marker offers higher resolution.



## 8. RESTORATION OF GENETIC DIVERSITY IN REFORESTED AREAS<sup>1</sup>

### *Abstract*

The endemic pine, *Pinus canariensis*, forms one of the main forest ecosystems in the Canary Islands. In this archipelago, pine forest is a mosaic of natural stands (remnants of past forest overexploitation) and artificial stands planted from the 1940's. The genetic makeup of the artificially regenerated forest is of some concern. The use of reproductive material with uncontrolled origin or from a reduced number of parental trees may produce stands ill adapted to local conditions or unable to adapt in response to environmental change. The genetic diversity in a transect of reforested stands connecting two natural forest fragments has been studied with nuclear and chloroplast microsatellites. Little genetic differentiation and similar levels of genetic diversity with the surrounding natural stands was found for nuclear markers. However, chloroplast microsatellites presented lower haplotype diversity. Because of the lower effective population size of the chloroplast genome, chloroplast markers could have a higher sensitivity to bottlenecks. Also, the understory natural regeneration within the reforestation was analysed to study gene flow from natural forest into artificial stands. Immigration rate into artificially regenerated forest was high (0.68–0.75), producing a significant increase of genetic diversity (both in chloroplast and nuclear microsatellites) which indicates the capacity for genetic recovery for *P. canariensis* reforestations surrounded by larger natural stands.

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<sup>1</sup> Article in preparation by M. Navascués and B.C. Emerson

## 8.1 *Introduction*

Silvicultural practices have high potential to affect the genetic structure of forests and this problem is of concern for forest geneticists (see Rajora and Mosseler, 2001; Finkeldey and Ziehe, 2004; Lefèvre, 2004, for reviews). In particular, artificial regeneration of forest (plantations or sowing) may produce a loss of genetic diversity through the processes of selection and genetic drift. Selection in silviculture is present in the tree breeding programs (El-Kassaby, 2000). Unplanned artificial selection is also present in the handling and storage of seeds, germination and growth in the nursery, and seedling handling and plantation processes (Ledig, 1992). There is little evidence for loss of genetic diversity due to artificial selection in studies using molecular markers (El-Kassaby, 2000) but, by definition, the presence of selection implies some decrease in diversity. Detection of a reduction in genetic diversity with neutral markers because of artificial selection is unlikely because only a few genes might be involved (Lefèvre, 2004) but the detection of the loss of adaptive variation because of genetic drift can be deduced from neutral molecular markers as it is a process that affects the whole genome (Glaubitz et al., 2003b). Bottleneck effects may be present within forest regeneration practises because of the use of a limited number of seed trees and the unevenness of the number of seeds collected or produced per tree (Glaubitz et al., 2003a; Burgarella, 2004). The extent to which a reduction of genetic diversity is manifest within the reforestation process depends on the particular circumstances of each species and location. Some studies have detected a reduction of genetic diversity in plantations (Raja et al., 1998; Macdonald et al., 2001; Rajora, 1999; Glaubitz et al., 2003a; Li et al., 2005) while others found no reduction or even higher diversity levels in artificial stands (Savolainen and Yazdani, 1991; Thomas et al., 1999; Rajora and Pluhar, 2003; Glaubitz et al., 2003b). Although some general patterns can be deduced from the different cases, each species needs an independent study to assess the genetic consequences of artificial regeneration.

An additional concern for artificial populations is the extent of genetic exchange with natural populations. Potential negative effects of gene flow from artificial plantations into natural forest ('genetic pollution') have been considered (Lenormand, 2002) and have gained recent public awareness with the use of transgenic crops (including tree species; DiFazio et al., 2004). Gene flow from natural populations into seed orchards (i.e. seed production plantations for refor-

estation) is also considered a negative effect ('pollen contamination') because it reduces the genetic gain obtained from the breeding programs and may increase maladaptation (El-Kassaby, 2000). However, under other circumstances, gene exchange between artificial and natural populations can also be positive. For small declining populations gene flow from surrounding reforestation may increase their effective population size ('demographic rescue'; Lefèvre, 2004). Also, gene flow from natural forest into reforested stands may be a natural way to recover the genetic variation lost in the reforestation process.

*Pinus canariensis* is endemic to the western Canary Islands, where it forms one of the main forest ecosystems of the archipelago. Its area of distribution has been diminished by five centuries of overexploitation (decreasing from covering 25% of the territory to now covering 12%). A great reforestation effort has been made since the 1940's, resulting in a mosaic of reforested and natural pine forest (del Arco Aguilar et al., 1990, 1992; Pérez de Paz et al., 1994a,b). In the present study we analyse the genetic diversity and immigration rates within a transect of reforested stands connecting two natural forest fragments in Tenerife. Hence, we are assessing the genetic integrity of the artificial stands and their potential to genetically recover to the levels of diversity of the surrounding natural forest.

## 8.2 Materials and methods

### 8.2.1 Plant material and sampling design

El Teide mountain (Tenerife, Spain) is crowned by a pine forest belt (the Corona Forestal Natural Park). A proportion of these pine woods are artificial stands planted from the 1940's to repair the past overexploitation (Parsons, 1981; del Arco Aguilar et al., 1992). We have chosen as our study site an area where there is still a small interruption of the forest (Fernández, 2001) with only a narrow strip of planted trees connecting two natural forest fragments (figure 8.1). Artificial *Pinus canariensis* stands were planted in Fasnía between 1956 and 1965 and in Arico between 1981 and 1985 (figure 8.2). The current vegetation cover for those areas is 30–60% for Fasnía and < 30% for Arico (del Arco Aguilar et al., 1992). The provenance origin of the plants is unknown but most probably seed were collected from different locations in Tenerife island (Climent et al., 1996). Nine sites were sampled in these plantations (sites 2–6 in Arico and 7–10 in Fasnía,

figure 8.1); in each of the nine sampling stations 10 planted adult trees and 10 understory naturally regenerated seedlings were sampled (except at site eight where 11 planted trees and 10 seedlings were sampled). From the natural forest in Arico<sup>2</sup> (site 1) and Güímar (site 11) we have sampled 50 and 47 mature trees respectively. Both old trees and seedlings were selected randomly and with an average separation of 10 m among them. From each individual needles were collected and preserved in silica gel in the summer of 2002.

### 8.2.2 Molecular markers

Genomic DNA was purified using a CTAB protocol based on the Doyle and Doyle (1987) method (see appendix B.1). Samples were genotyped for eight chloroplast microsatellites (Pt1254, Pt15169, Pt26081, Pt30204, Pt36480, Pt71936, Pt87268 and Pt110048; Vendramin et al., 1996) transferred from *Pinus thunbergii* and eight nuclear microsatellites (SPAC 11.5, SPAC 11.8, SPAG 7.14, PtTX3116, PtTX4001, ssrPt\_ctg4363, ssrPt\_ctg4698 and ssrPt\_ctg7731; Soranzo et al., 1998; Auckland et al., 2002; Chagné et al., 2004) transferred from *Pinus sylvestris* and *Pinus taeda* (González-Martínez et al., 2004, and this study). PCR amplifications were performed in a Perkin-Elmer 9700 thermal cycler (see details for PCR conditions in appendix B.2). PCR products were sized in an ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

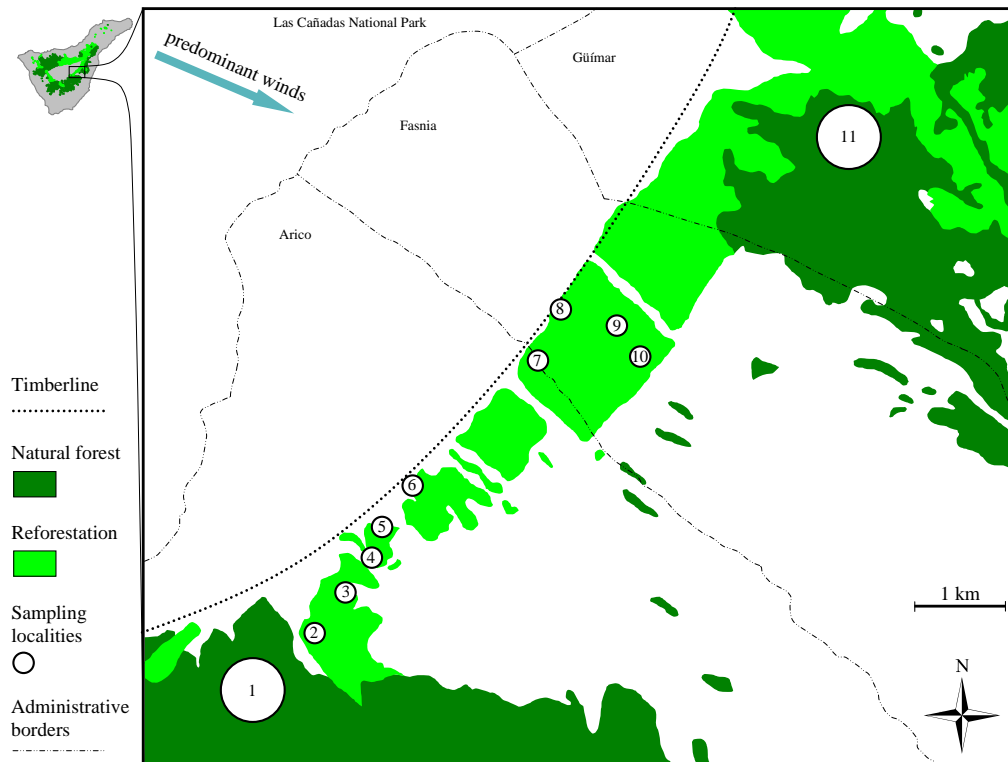
### 8.2.3 Data analysis

#### *Genetic diversity indices*

For chloroplast microsatellites, total number of haplotypes ( $n_h$ , direct count), effective number of haplotypes ( $n_e$ , equation 5.2), unbiased haplotype diversity ( $H_e$ , equation 5.3) and average genetic distances among individuals ( $D_{sh}^2$ , equation 5.4) were calculated for each sample. For nuclear microsatellites, number of alleles ( $A$ , direct count), effective number of alleles ( $A_e$ , equation 7.2), allelic richness ( $A_{n_{min}}$ , equation 7.3) and unbiased genetic diversity ( $H_e$ ; Nei, 1978) were

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<sup>2</sup> It must be noted that this site corresponds to the same sample used in Chapter 7 as ‘Arico 2000’.



*Fig. 8.1:* Natural and reforested stands studied where located in the southern slope of Teide mountain. The reforestation strip in Arico (1981–1985) and Fasnía (1956–1965) —sites 2–10— and the two fragments of natural forest in Arico and Güímar —sites 1 and 11— where studied. A patch of artificial forest in Arico planted in the 1990s (between sites 6 and 7) was not sampled as it lacked of understory natural regeneration. Predominant wind direction in the area is determined by the trade winds that blow from the northwest at that altitude (around 2000m). Administrative borders delimit the Las Cañadas National Park and the City Council territories of Arico, Fasnía and Güímar.



Fig. 8.2: Reforested stand in Arico. The trees were planted between 1981 and 1985. Arico plantations are formed by scattered even-aged trees, contrasting with the higher densities of the reforested Fasnía stands (not shown).

calculated for each locus and sample:

$$H_e = \frac{2n}{2n - 1} \left( 1 - \sum_{i=1}^A p_i^2 \right) \quad (8.1)$$

where  $n$  is the sample size,  $A$  is the total number of alleles and  $p_i$  is the relative frequency of the  $i^{\text{th}}$  allele in the sample.

Significant differences in the genetic diversity indices between pairs of samples were tested by a Monte Carlo approach<sup>3</sup> (see Degen et al., 1999; Glaubitz et al., 2003a, for similar analyses). The null distribution (both samples hold the same genetic diversity) of differences for each index was constructed by resampling (with replacement) individuals among samples. The result is a mixture of individuals from both populations distributed in two arbitrary samples (with the original sample sizes). The genetic diversity parameters of both samples and their differences are computed (samples are compared always in the same order and sign of differences is kept). This process is repeated 10 000 times to build the null distribution of the genetic diversity differences. The empirical difference value is then compared with the null distribution. Since we are interested in testing for the reduction of genetic diversity (i.e. null hypothesis,  $h_0$ : diversity in

<sup>3</sup> Program source code (in FORTRAN) is available from the author on request (m.navascues@gmail.com).

natural forest  $\leq$  diversity in artificial regeneration) we perform a one-tailed test<sup>4</sup>. The  $p$ -value for rejecting the null hypothesis is estimated as the proportion of iterations with differences greater than or equal to the observed difference value. This process is used to test chloroplast haplotype diversity, individual nuclear locus diversity and average (among loci) nuclear diversity.

Significant differences in allele frequencies (nSSR) between pairs of samples were tested with a Fisher exact test (Raymond and Rousset, 1995a) using the program GENEPOP 3.4 (genic differentiation option of the program; Raymond and Rousset, 1995b).

#### *Temporal change in allelic frequencies*

Allele frequencies change through time through the influences of gene flow and genetic drift (assuming neutral variation). the change in allele frequencies in samples of different generations has been used mainly for estimating effective population sizes (Waples, 1989; Anderson et al., 2000; Wang, 2001). Recently Wang and Whitlock (2003) have developed a new maximum likelihood method that estimates immigration rates and effective population sizes simultaneously. This method, implemented in the MLNE program, has been used on the nSSR data of the reforested samples using the planted trees as the parental generation and the natural regeneration as the following generation. To estimate the gene flow, the method also uses the allele frequencies of the potential external source of genes, which in our case were taken from the surrounding natural stands.

#### *Pollen pool heterogeneity*

A hierarchical AMOVA on cpSSRs was used to study the pollen pool heterogeneity from the sampled seedlings, as in Dyer and Sork (2001). The chloroplast is paternally inherited in conifers; therefore, cpSSR variation in the seedlings will reflect the genetic diversity of the pollen clouds of the area. The analysis of the heterogeneity of the pollen pool potentially allows the estimation of the distance of pollen dispersal (see TwoGener method: Smouse et al., 2001; Austerlitz and Smouse, 2001; Dyer et al., 2004); however, in our case, having sampled seedlings on the ground, we cannot ignore the influence of seed dispersal in our

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<sup>4</sup> In order to perform a two-tailed test ( $h_0$ : diversity in sample A = diversity in sample B) the same procedure is followed but  $p$ -value is estimated from the absolute value of differences.

data. Canopy density can influence the dispersal of propagules diminishing wind-speed (see Nathan and Katul, 2005). Thus, sampling sites were nested into two AMOVA groups, Arico (low vegetation cover) and Fasnía (high vegetation cover, del Arco Aguilar et al., 1992).

#### *Origin of reforestation understory regeneration*

The genotype assignment method developed by Rannala and Mountain (1997), implemented in their program IMMANC 5, was used on the nSSR data to detect immigrants among the seedlings. This method allows performing the test for different levels of immigrant ancestry considering the probabilities of external origin of: A) the whole genotype (first generation immigrant, i.e. the individual has two foreign parents), B) half of the genotype (second generation immigrant, i.e. the individual has one foreign parent), etc. The program calculates the relative probability for each individual to be born from parents with no recent immigrant ancestry rather than from both foreign parents (denoted  $\Lambda_{d=0}$ ) or from one foreign parent (denoted  $\Lambda_{d=1}$ ). These calculations are based on the allele frequencies of the studied population and the potential source populations using a Bayesian approach. The null distribution (individual with no immigrant ancestry) of the statistic  $\ln \Lambda$  is built by a Monte Carlo simulation and  $p$ -values and statistical power (for  $\alpha = 0.05$ ) are estimated for each test.

In our case the interpretation of the Rannala and Mountain (1997) assignment tests are slightly different, because we have effectively only one generation of possible immigrants. ‘First generation’ immigrants (whole genotype of external origin) will correspond to seeds developed and fertilised outside the stand. ‘Second generation’ immigrants (half genotype of external origin) will correspond to seeds from the stand fertilised with pollen from outside (the alternative possibility, seed from outside fertilised with pollen from the stand and dispersed into the stand, will be considered highly improbable and will be ignored in the interpretation). The proportion of ‘seed-immigrants’ ( $d = 0$ ) and ‘pollen-immigrants’ ( $d = 1$ ) in the analysed seedlings will be estimates of seed and pollen immigration rates. To check the accuracy of the method we examined the results of these tests on the planted trees, which we consider by definition ‘non-immigrants’. The proportion of planted trees detected as immigrants will give us the error rate of the method.



### 8.3 Results and discussion

#### 8.3.1 Genetic differences between samples

Significant differentiation ( $\chi^2 = 50.438$ , *d.f.* = 16,  $p < 0.001$ ) in the nSSR allele frequencies were detected between planted and natural forest. However these differences cannot be interpreted as introduction of foreign material as significant differentiation ( $\chi^2 = 54.787$ , *d.f.* = 16,  $p < 0.001$ ) was also detected between the two spatially close natural stands. Also, there were significant differences in the allele frequencies between planted trees and understory natural regeneration for both reforested stands (Arico:  $\chi^2 = 27.282$ , *d.f.* = 16,  $p = 0.038$  and Fasnja:  $\chi^2 = 28.005$ , *d.f.* = 16,  $p = 0.032$ ). These changes may be the result of the effects of drift and gene flow in the reforested stands (see next section 8.3.2).

Levels of nSSR genetic diversity in the reforested stands are, in general, similar to those found in the surrounding natural forest (table 8.1, results for individual nSSR loci not shown). Mean expected heterozygosity presented a significantly lower value in the plantations but none of the diversity indices based on allele number, which are more sensitive to population bottlenecks (Nei et al., 1975; Spencer et al., 2000), showed significantly lower values and in some cases allele diversity was even higher. Therefore, it is unclear that the lower mean  $H_e$  value indicates a genetic impoverishment of the reforestation due to genetic drift. The higher proportion of rare alleles (alleles with frequency  $< 0.01$ ) in the reforested stands (27.45%) in comparison to the natural forest (19.42%) suggests to us that a possible mixed origin of the seedling stock (or different seedling origins for the different reforestation phases) could produce an accumulation of alleles from different areas (explaining high levels of allelic diversity) but with lower frequencies in the mixture (resulting in low  $H_e$  values).

For cpSSRs, there is a significantly lower effective number of haplotypes in both artificial stands (table 8.1). Bottlenecks associated with the reforestation process could have had a stronger effect on cpSSR diversity than in nSSRs because the effective population size for the chloroplast genome is half the nuclear effective population size.

In the comparison of the reforested areas with their natural regeneration we found a significant increase in the effective number of haplotypes and in the mean effective number of alleles (table 8.1). Although the increase on mean  $A_e$  was

	Natural vs. Planted			Planted vs. Regeneration		
			<i>p</i>			<i>p</i>
$n_h$	49	46	0.312	46	49	0.219
$n_e$	25.778	15.956	0.010*	15.956	25.962	0.009*
$H_e$	0.971	0.948	0.142	0.948	0.972	0.193
$D_{sh}^2$	3.120	2.739	0.261	2.739	3.033	0.322
mean $A$	17.735	19.125	0.990	19.125	19.375	0.342
mean $A_e$	8.825	8.533	0.350	8.533	9.682	0.022*
mean $A_{n_{min}}$	$A_{91} = 17.158$	$A_{91} = 19.096$	0.989	$A_{85} = 18.766$	$A_{85} = 19.351$	0.295
mean $H_e$	0.753	0.729	0.032*	0.729	0.760	0.027*
	Natural vs. Arico planted			Arico planted vs. Arico regeneration		
			<i>p</i>			<i>p</i>
$n_h$	49	32	< 0.001*	32	36	0.106
$n_e$	25.778	17.606	0.027*	17.606	25.000	0.021*
$H_e$	0.971	0.962	0.287	0.962	0.980	0.211
$D_{sh}^2$	3.120	2.584	0.156	2.584	3.194	0.163
mean $A$	17.735	15.875	0.895	15.875	14.250	0.949
mean $A_e$	8.825	8.737	0.776	8.737	8.479	0.596
mean $A_{n_{min}}$	$A_{50} = 14.877$	$A_{50} = 15.822$	0.943	$A_{46} = 15.440$	$A_{46} = 14.212$	0.922
mean $H_e$	0.753	0.726	0.038*	0.726	0.738	0.213
	Natural vs. Fasnja planted			Fasnja planted vs. Fasnja regeneration		
			<i>p</i>			<i>p</i>
$n_h$	49	25	< 0.001*	25	24	0.689
$n_e$	25.778	10.066	< 0.001*	10.066	14.815	0.065
$H_e$	0.971	0.923	0.115	0.923	0.956	0.228
$D_{sh}^2$	3.120	2.984	0.461	2.984	2.771	0.573
mean $A$	17.735	13.875	0.340	13.875	13.750	0.498
mean $A_e$	8.825	7.298	0.099	7.298	7.830	0.189
mean $A_{n_{min}}$	$A_{41} = 14.038$	$A_{41} = 13.817$	0.467	$A_{39} = 13.638$	$A_{39} = 13.684$	0.441
mean $H_e$	0.753	0.733	0.119	0.733	0.736	0.438

\* Significant at  $\alpha = 0.05$

Tab. 8.1: Diversity indices in the natural forest, reforestation and understory regeneration. Differences in the levels of genetic diversity were statistically tested by resampling the data among samples ( $h_0$ : natural forest diversity  $\leq$  planted trees diversity; or  $h_0$ : natural regeneration diversity  $\leq$  planted tree diversity).

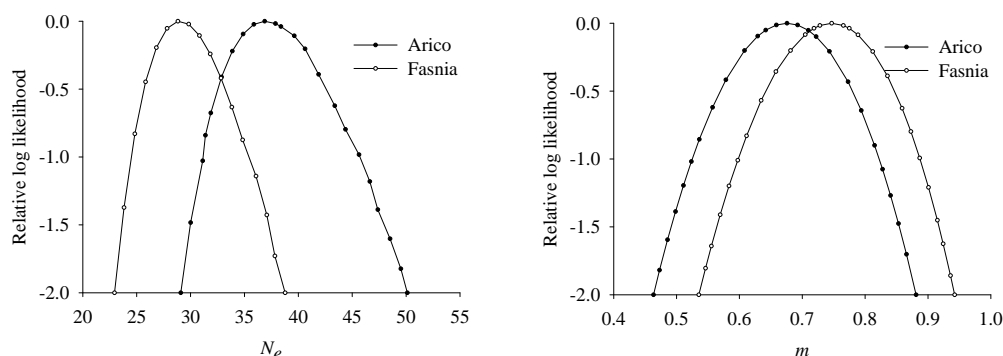


Fig. 8.3: Relative profile log-likelihood curves for the effective population size ( $N_e$ ) and immigration rate ( $m$ ) for reforested stands of Arico and Fasnja. Intersections of the curves with the value of log-likelihood =  $-2.0$  correspond to the 95% CI.

not apparent when the two reforested stands were studied separately, the whole reforestation area had a significant gain in alleles for loci SPAC 11.8, PtTX3116, PtTX4001, ssrPt\_ctg4698 and ssrPt\_ctg7731 (data not shown). This increase of the allelic diversity in the reforestation might be attributable to gene flow from the natural stands.

### 8.3.2 Gene flow into the artificial stands

Three different methods have been used to explore the immigration dynamics within the reforested stands. First, we examined the temporal changes in allele frequencies between the planted trees and the following generation, represented by the understory natural regeneration. We applied the Wang and Whitlock (2003) maximum likelihood method using the allele frequencies in the natural forest as the potential external source of immigrants. The estimates of effective population size ( $N_e$ ) and immigration rate ( $m$ ) obtained from this method are  $N_e = 36.88$  and  $m = 0.68$  for Arico and  $N_e = 28.83$  and  $m = 0.75$  for Fasnja (figure 8.3).

A second method to estimate immigration rates was the identification of immigrants among the natural regenerated seedlings with the Rannala and Mountain (1997) assignment test. Individuals that gave a positive result in the assignment tests are shown in table 8.2. The immigration rates into the reforested patches, calculated as the proportion of identified immigrants over the sample

size, are  $m_{seed} = 0.07$  and  $m_{pollen} = 0.12$ . However, these estimates are unreliable as when the same assignment test was applied to the planted trees (considered ‘non-immigrants’) 15 individual were identified as second generation immigrants (pollen dispersal) and 6 as first generation immigrants (seed dispersal). These error rates are in the same order of magnitude as the estimated immigration rates obtained. This poor performance of the test is due to the low genetic differentiation among the samples which resulted in very low statistical power (see table 8.2). In order to obtain enough statistical power a large number of genetic markers would be needed (Rannala and Mountain, 1997). The approach we have taken, although unsuccessful in our study, might be of use for other studies where a higher number of molecular markers are available and a higher differentiation is found among samples. For instance, the case of *Pinus pinaster* plantations in southern France with seed of Portuguese provenance (Ribeiro et al., 2002) could be a good case study as there is significant differentiation between provenances (Derory et al., 2002) and a large number of markers developed (Mariette et al., 2001; Chagné et al., 2004).

Finally, the genetic heterogeneity of the pollen cloud was studied by analysing the genetic diversity of cpSSR for the natural recruitment. The results of the AMOVA test gave non-significant differentiation of the pollen diversity for both among sites and among stands ( $\Phi$ -statistics  $< 0.033$ ,  $p > 0.17$ ). This result suggests a high pollen dispersal capacity. However, it must be noted that the statistical power to detect genetic differentiation in cpSSRs for the sample size used might be very low (see Chapter 7).

The high levels of gene flow suggested by these results are not surprising as pines are predominantly outcrossing and wind pollinated (Ledig, 1998). Previous estimates of immigration rates in artificial stands are available from pollen contamination studies in seed orchards. Among the factors influencing the pollen contamination levels in seed orchards are the distance to the nearest stands of the same species and the relative pollen production of the seed orchard to the surrounding forest (Adams and Burczyk, 2000). Wind direction can also increase pollen contamination locally (Yazdani and Lindgren, 1991). The range of pollen immigration rates found for pines in such studies are high, ranging from 0.26 to 0.75 (both estimates for *Pinus sylvestris* seed orchards; Harju and Muona, 1989; Yazdani and Lindgren, 1991) depending on the particulars of each case. Immi-

ID	potential source	$d = 0$			$d = 1$			immigrant propagule
		$\ln \Lambda$	$p$	power	$\ln \Lambda$	$p$	power	
CAI10	Güímar	-2.876	0.007*	0.931	-2.086	0.011*	0.536	pollen
CAI11	Güímar	-0.801	0.034*	0.936	-0.840	0.074	0.536	seed
CAI12	Fasnia	-3.394	0.003*	0.798	-1.704	0.014*	0.418	pollen
CAI16	Güímar	-2.848	0.007*	0.925	-2.003	0.015*	0.536	pollen
CAI17	Arico natural	-2.376	0.013*	0.913	-0.851	0.081	0.467	seed
CAI19	Fasnia	-0.990	0.049*	0.801	-0.461	0.125	0.407	seed
CAII12	Güímar	-0.503	0.044*	0.928	-0.890	0.077	0.516	seed
CAII13	Arico natural	-2.094	0.017*	0.907	-1.631	0.026*	0.462	pollen
CAII18	Fasnia	-0.897	0.055	0.796	-1.243	0.034*	0.402	pollen
CAII20	Arico natural	-0.030	0.075	0.908	-1.198	0.050*	0.464	pollen
CAIV11	Arico natural	-2.772	0.009*	0.918	-1.980	0.017*	0.444	pollen
CAIV19	Fasnia	-2.087	0.016*	0.801	-0.360	0.143	0.431	seed
CAV12	Arico natural	-1.014	0.040*	0.911	0.416	0.337	0.452	seed
CFI15	Güímar	-2.044	0.011*	0.938	-2.105	0.010*	0.578	pollen
CFII18	Güímar	0.984	0.103	0.938	-1.330	0.027*	0.603	pollen
CFIII13	Güímar	2.533	0.208	0.941	-1.692	0.020*	0.576	pollen
CFIV11	Güímar	-1.041	0.027*	0.940	-1.044	0.048*	0.581	pollen

Tab. 8.2: Results of the genotype assignment tests (only significant tests shown). Immigrant origin was detected for 17 seedlings. However, because of the low statistical power of the method these assignments are not reliable and results have not been taken in consideration (see text for more details).

gration rates calculated in natural stands from paternity analysis are also high 0.30–0.31 (minimum estimates; González-Martínez et al., 2003; Lian et al., 2001) but decrease to 0.05–0.07 in isolated populations (Robledo-Arnuncio and Gil, 2004; Schuster and Mitton, 2000). Therefore, the close distance to big natural forest and the small size of the reforested stands studied offers some explanation for the high levels of immigration into the reforested area.

#### 8.4 Conclusions

The effect of the reforestation process was detected in the chloroplast genome with a reduction in the effective number of haplotypes while the effects on the nuclear genome were uncertain. The difference in the effective population size between the two genomes could explain a different sensitivity to bottlenecks.

Despite the lower haplotype diversity found for the plantations the richness of their future genetic pool does not seem jeopardized because both chloroplast and nuclear genetic diversities increase in the understory natural regeneration. High levels of gene flow from the adjacent and larger natural stands explain this rise in diversity. These results highlight the importance of the presence and abundance of natural forest for the regeneration of genetically rich forest (see, for example, Glaubitz et al., 2003a,b); therefore, special care must be taken in reforestation when there is scarce or no presence of natural populations of planted species, because there will be low rates of gene flow ‘naturalising’ the gene pool of artificial stands. Finally, it is important to remark that the genetic diversity and gene flow levels detected for the studied stands might not be representative for other *P. canariensis* plantations on the Canary Islands. Artificial stands more isolated or larger than their surrounding natural forest might have lower immigration rates and thus changes in their gene pools would be slower.

Part IV

GENERAL DISCUSSION AND CONCLUSIONS

## 9. GENERAL DISCUSSION AND CONCLUSIONS

### 9.1 *Canary Island pine population genetics*

*Pinus canariensis* is an endemic species of the western Canary Islands, constituting the largest forest ecosystem of the archipelago. Several centuries of over-exploitation have reduced its distribution to half of what it was before European colonists arrived (now around 55 000 ha). Conservation of the natural forest and recovery of deforested areas with new reforestations are necessary to maintain this ecosystem which contains a number of endangered endemic plant and animal species. Although the pine itself is considered out of danger, disappearance of marginal populations could diminish the gene pool of the species (Vaxevanidou et al., 2005).

The original target for this project was the study of the population structure of *P. canariensis* throughout the archipelago by means of DNA markers of contrasting inheritance (paternal inherited of chloroplast DNA, maternal inherited of mitochondrial DNA and biparental inheritance of nuclear DNA, see Chapter 3). However, the publication of Gómez et al. (2003) work with chloroplast microsatellites on *P. canariensis* and technical problems on the detection and development of mitochondrial polymorphic markers led us to focus in more detailed aspects of *P. canariensis* population structure.

For the study of processes of population genetics affecting the whole genome, such as gene flow and effective population size changes, neutral markers distributed throughout the genome are the appropriate ones. Microsatellites can be considered, in general, neutral and are distributed randomly on the genome (Schlötterer, 2000). In addition they are codominant markers, allowing their use for the population assignment test used in Chapter 8, and thus they were chosen over other molecular markers, such as AFLPs or RAPDs. The already developed chloroplast microsatellites were also included in the study to complement the information from the nuclear markers.



### 9.1.1 Gene flow

Forest scientists have been interested in the study of gene flow as one of the main factors affecting the genetic structure of forest because understanding the gene exchange among populations has implications for forest management and conservation (Burczyk et al., 2004). Two different approaches can be taken to estimate levels of gene flow with molecular markers: inferences from historical effects of gene exchange (i.e. population structure) and contemporary detection of gene flow (Sork et al., 1999), and both methods have been applied in the present study.

Analysis of population structure in natural populations (Chapter 7) suggests high levels of gene flow ( $\theta = 0.019$ ,  $R_{ST} = 0.044$ ) among populations. The fact that this differentiation is significantly different from zero may indicate some limiting factor for gene flow at a small scale (5–15 km). However pairwise population differences, which were heterogeneous, could not be associated with the influence of isolation by altitudinal differences or geographical distance.

Contemporary gene flow was inferred from the genetical constitution of understory natural regeneration in comparison to the gene pools of the adult stand (of reforestation origin) and the surrounding natural stands which were potential sources of immigrating genes (Chapter 8). A maximum likelihood estimate of immigration rates ( $0.68 \lesssim m \lesssim 0.75$ ) confirms the high level of gene flow among populations of *P. canariensis*, and these immigration rates are similar to those found in other wind-pollinated trees (Burczyk et al., 2004). The application of a promising assignment test that could potentially distinguish pollen and seed immigrants among the seedlings was unfruitful. However, this last approach could be useful in other species where a considerable number of highly polymorphic markers are available or genetic differentiation among the populations analysed is large.

To sum up, low (but significant) genetic differentiation is found among populations of *P. canariensis* as a consequence of high migration rates characteristic of outcrossing, anemophilous trees. Further research will be necessary to understand the factors determining the heterogeneity of genetic differentiation across the landscape. In particular, detailed knowledge of the flowering phenology of populations growing under contrasting climatic conditions (see Chapters 1 and 2) may enlighten us about their different opportunities for gene exchange. The lower

genetic differentiation found between the populations at higher altitudes (Chapter 7) could be related to higher insolation, lower canopy density or higher hydric stress as these factors may increase pollen shedding and dispersal capacity. Future population genetic research should focus on the use of mitochondrial DNA polymorphic markers that can reveal patterns of seed movements and historical colonisation processes. It will be also of great interest the study of quantitative trait loci, expressed genes and other techniques which address adaptive variation to understand the genetic differences among pine forest growing in contrasting climatic conditions.

### 9.1.2 Demographic dynamics

Volcanism was identified as a factor playing an important role in the shaping of the genetic structure of the pine forest (Chapter 6). The analysis of chloroplast microsatellites revealed historical population expansions of *P. canariensis*. The heterogeneity of estimated ages for different samples within and among islands suggests that these expansions could be associated with the process of succession after strong local disturbances relating to volcanic eruptions and landslides. Population expansions broadly coinciding with those found in the Canary pine were also revealed in its parasite weevil *Brachyderes rugatus* by mtDNA COII analysis. This has implications for the understanding of the ecological dynamics of forest species within the archipelago.

Volcanism has a substantial influence in the Canary Islands biota (Chapter 1) and its impact on the pine forest is yet to fully understand. The use of mitochondrial DNA markers to study *P. canariensis* populations are expected to reveal more clear patterns of the historical colonisation process as they are only influenced by the limited dispersal of the seeds. Also, further genetic studies *B. rugatus* and other taxa linked to the pine forest ecosystem, sampled on historical or isotope-dated lavas, could validate and further develop the hypothesis of metapopulation dynamics.

### 9.1.3 Afforestation and conservation genetics

Restoration of the former distribution of the Canary Island pine forest has been undertaken in a gradual process that began around six decades ago. Therefore, there is heterogeneity not only in the ages and forestry practices but also in

the suitability of the reproductive material used (indeed some of the early reforestations were done with foreign *P. radiata* or on areas potentially belonging to *monteverde*). The genetic diversity of artificial stands studied in the present work (Chapter 8) suggests that silvicultural practices resulted in a loss of genetic diversity in comparison to natural areas (although seed origin stands should have been known and characterized in order to obtain a definite conclusion; e.g. Burgarella, 2004). Under the particular circumstances of the artificial stands studied (relatively small and flanked by natural forest) this diminished gene pool is of minor concern as high levels of gene flow produced a significant increase of genetic diversity in the understory natural regeneration. However the situation may be very different in other areas of the pine forest. Vaxevanidou et al. (2005) describe a situation —opposite to our study case— where a reforested stand of unknown seed origin surrounds a tiny natural population of pines in Galdar (Gran Canaria) where the gene pool may be threatened by potential contamination from the reforested gene pool. For future monitoring of silvicultural practices regarding (either on *P. canariensis* or other forest species) the loss of genetic variability the use of cytoplasmic DNA (i.e. chloroplast and mitochondrial DNA) markers is recommended as they might be more sensitive to bottleneck effects because of its lower effective population size.

## 9.2 Statistical analysis of chloroplast microsatellites

The discovery of chloroplast microsatellites (cpSSRs) is an important contribution to the population genetic analysis of plants by providing polymorphic markers for a genome characterised by its low variation (see Provan et al., 1999b, 2001, for reviews). The importance of cpSSRs in this thesis is twofold. First, I have analysed the sensitivity of cpSSR to recent population expansions (Chapter 6), an application of these markers little explored until now (but see Vendramin et al., 1998; Cuenca et al., 2003) and which has been revealed to have great potential. Contrastingly, the second aspect of cpSSR analysis that has been explored in this work is its limitations. I found very high levels of genetic diversity in cpSSRs that produced two types of problem: difficulty for obtaining a representative sample of the population genetic diversity and high levels of homoplasy because of recurrent parallel and back mutations.

### 9.2.1 Homoplasy

The issue of homoplasy had little relevance to population geneticists until the extensive usage of microsatellites as genetic markers (Estoup et al., 2002). Since microsatellites evolve (mainly) in a stepwise fashion, alleles identical in state are recurrently created by back and parallel mutations. The presence of these homoplastic alleles (or haplotypes in the case of the chloroplast genome) in the analysis of populations can cause problems for measures of genetic diversity, gene flow, genetic distances, assignment methods and phylogenetic reconstruction (Estoup et al., 2002).

In this work we have employed coalescent simulations of chloroplast lineages to assess the importance of homoplasy for different genetical analyses (Chapters 5 and 6). Our results revealed very different effects of homoplasy on measures based on the number of haplotypes ( $n_e$ ,  $H_e$ ) in comparison to measures based on averaged genetic distance estimates ( $D_{sh}^2$ ). This is because every homoplastic mutation affects the estimation of the genetic distances while the number of haplotypes might not be changed (for instance two parallel mutations in one locus may occur in haplotypes that already differ by singleton mutations in another locus). The consequence of this impaired effect on different genetic diversity estimates is reflected in the lower statistical power of the  $F_S$  test for very recent expansions.

For the estimation of genetic distances we found that actual distances were underestimated. Due to that inaccuracy the analysis of mismatch distributions produced an underestimation in the dating of population expansions. Nevertheless, the high correlation between the estimated and the actual genetic distances might allow the application of the estimates as relative rather than absolute distances. With this respect, further research about the effects of homoplasy on the AMOVA will need to be addressed.

Finally, it is interesting to note that these conclusions on the effects of homoplasy are applicable to the analysis of other fully-linked microsatellites such as those found in the Y-chromosome. For the attenuation of the negative effects of homoplasy on the analysis of linked microsatellites I found it is highly recommendable to use as many microsatellite loci as possible. Because of the linkage of loci homoplastic mutations occurring at one locus can be ‘compensated’ by independent mutations occurring at different loci. Another way to reduce the

effects of homoplasy will be the development of new statistical analysis taking into account the stepwise mutation model to account for homoplastic mutations. It is, therefore, very necessary to continue the research on the presence of homoplasy in microsatellite data. Simulations have been proved to be a powerful tool for this purpose (Estoup et al., 2002, and Chapters 5 and 6) and its use should be expanded to nuclear microsatellites and to other types of analysis (population structure, assignment test, etc) not addressed in this work. It would be also very useful the development of empirical indices of homoplasy that could be related to theoretical index described by Estoup et al. (2002) as they will allow to interpret the results according to the levels of homoplasy affecting them.

### 9.2.2 Sample size

The high level of haplotypic diversity found for cpSSRs implies that only really big sample sizes will be representative of the full diversity of populations and the values of genetic diversity indices will be dependent on the sample size used (Chapter 7). This is a problem for comparing the genetic diversity among samples of different sizes. This difficulty can be overcome when resampling methods are used for comparisons between samples as these methods take into account the stochasticity of sampling (Chapter 8). When the raw data are not available (i.e. summary data extracted from publications) the problem is difficult to solve, therefore, it would be highly desirable to report genetic diversity by means of indices based on rarefaction (e.g. El Mousadik and Petit, 1996; Kalinowski, 2004).

Big sample sizes were also necessary to distinguish genetically similar populations by means of cpSSRs. A resampling procedure was developed to assess the population differentiation statistical power of the genetic markers for different sample sizes (Chapter 7). This procedure can be useful in the early stages of research to determine whether it is worthwhile to increase sample sizes, or to determine which molecular markers offer greater statistical power for a smaller sample size.

## 9.3 Conclusions

Our results confirm the genetic differentiation of *P. canariensis* at the population level found in previous studies (Schiller et al., 1999; Gómez et al., 2003).

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Volcanism, as a disturbance agent, seems to be a significant factor shaping the genetic structure of pine forest. Nevertheless, estimations of gene flow among populations are high. These high levels of gene flow have different conservation implications when the gene flow is from natural to artificial stands (naturalisation of the reforestation) than when the gene flow is from artificial to natural stands (genetic contamination), which depends on the particular local conditions of each stand.

Regarding the use of chloroplast microsatellites, homoplasmy was found to be a major influence on the accuracy of statistical analysis. The use of a large number of loci, application of simulations to assess the effects of homoplasmy in the particular analysis performed and caution in the interpretation of the results are strongly suggested for future studies using chloroplast microsatellites. Further research on the effects of homoplasmy in the analysis of population differentiation and in the development of statistical analyses accounting for homoplasmy should be undertaken.

## APPENDICES

## A. SAMPLING SITES

### A.1 *Natural forest*

*Arico 1000*: Montaña de los Albarderos, Arico (Tenerife).

*Arico 1500*: Montaña de los Albarderos, Arico (Tenerife).

*Arico 2000 = Arico natural*: Low density pine forest at the timberline. Pino de la Pelota, Arico (Tenerife).

*Chinyero*: Monte U.P. nº 12, Pinar, Santiago del Teide (Tenerife). Sampled near TF-38 road, km 15.

*Güímar*: Monte TF-3011, Comunal de Güímar, Güímar (Tenerife).

*Vilafior 1000 = Ifonche*: Ifonche, Vilafior (Tenerife). Sampled near TF-567 road, km 2.5.

*Vilafior 1500*: Monte U.P. nº 6, Lomo Gordo y Agua Agria, Vilafior (Tenerife). Sampled near TF-21 road, km 66.

*Vilafior 2000*: Monte U.P. nº 7, Vica y Lajas, Montaña de Las Lajas, Vilafior (Tenerife). Sampled near TF-21 road, km 58.5.

### A.2 *Reforestation*

*Arico reforested*: Area reforested between 1974 and 1985 (del Arco Aguilar et al., 1992). Samples were collected at five points at Cumbres de Arico, Arico (Tenerife). Ten adult (planted) trees and ten seedlings (natural regeneration) were sampled in each point.



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*Fasnia*: Area reforested between 1956 and 1965 (del Arco Aguilar et al., 1992). Samples were collected at four points at Lomo de Las Arorias, Fasnia (Tenerife). Ten adult (planted) trees and ten seedlings (natural regeneration) were sampled in each point.

Site	ID	latitude	longitude	altitude	date
Arico1000	MAI	28° 11.87' N	16° 31.84' W	1061m	29 <sup>th</sup> June 2002
Arico1500	MAII	28° 12.19' N	16° 32.92' W	1513m	29 <sup>th</sup> June 2002
Arico2000	PP	28° 14.39' N	16° 32.90' W	2135m	28 <sup>th</sup> June 2002
Chinyero	CH	28° 17.06' N	16° 45.96' W	1461m	27 <sup>th</sup> June 2002
Güímar	CG	28° 17.77' N	16° 28.58' W	1788-1840m	27 <sup>th</sup> June 2002
Vilaflor1000	IF	28° 07.97' N	16° 41.28' W	1035m	29 <sup>th</sup> June 2002
Vilaflor1500	LG	28° 10.04' N	16° 38.15' W	1553m	29 <sup>th</sup> June 2002
Vilaflor2000	VL	28° 11.39' N	16° 39.96' W	2108m	29 <sup>th</sup> June 2002
Arico reforested	CAI	28° 14.42' N	16° 32.16' W	1925m	28 <sup>th</sup> June 2002
	CAII	28° 14.17' N	16° 31.96' W	1955m	28 <sup>th</sup> June 2002
	CAIII	28° 14.88' N	16° 31.81' W	1897m	28 <sup>th</sup> June 2002
	CAIV	28° 15.06' N	16° 31.74' W	1987m	28 <sup>th</sup> June 2002
	CAV	28° 15.35' N	16° 31.49' W	2009m	28 <sup>th</sup> June 2002
Fasnia	CFI	28° 16.16' N	16° 29.87' W	1814m	26 <sup>th</sup> June 2002
	CFII	28° 16.31' N	16° 29.99' W	1890m	26 <sup>th</sup> June 2002
	CFIII	28° 16.32' N	16° 30.57' W	2081m	28 <sup>th</sup> June 2002
	CFIV	28° 16.04' N	16° 30.80' W	2076m	28 <sup>th</sup> June 2002

Tab. A.1: Sampling sites geographic location.



Fig. A.1: Sampling sites (base maps from del Arco Aguilar et al., 1992).



Fig. A.1: Continued from previous page.

## B. LABORATORY PROTOCOLS

### *B.1 DNA extraction protocol*

CTAB protocol for DNA extraction from dried pine needles tissue, based on the method of Doyle and Doyle (1987).

1. Harvest, into a 1.5 ml Eppendorf tube, 5–15 approximately 1 cm long pieces of needle tissue using the tube cap as a cutter. Add one 3 mm stainless steel bead and grind the tissue using the TissueLyser<sup>®</sup> (QIAGEN<sup>®</sup>) at 30 Hz for 5 minutes. (Alternatively use a plastic pestle, adding about 10 mg of fine sand to improve the grinding).
2. Add 1 ml of 2 × CTAB extraction buffer, remove the metal bead and mix contents gently. Incubate at 65°C for 30 minutes. (When using manual grinding: add 100  $\mu$ l of 2 × CTAB extraction buffer and continue grinding until the tissue forms a more-or-less homogeneous slurry. Add the additional 900  $\mu$ l of extraction buffer and mix contents gently, followed by incubation as above).
3. Add 10  $\mu$ l of 2 mg/ml DNase free RNase. Mix gently and incubate at 37°C for 1 hour.
4. Remove the tube from the water bath and allow to cool to ambient temperature before adding 200  $\mu$ l ‘wet’ chloroform. Mix gently to obtain a momentary single phase. Centrifuge for 2 minutes at 13 000 rpm.
5. Remove the aqueous (upper) phase to a clean tube. Re-extract with 200  $\mu$ l ‘wet’ chloroform. Mix gently to obtain a momentary single phase. Centrifuge for 2 minutes at 13 000 rpm.
6. Remove the aqueous (upper) phase to a clean tube. Add 600  $\mu$ l cold (–20°C) propan-2-ol to the aqueous layer and mix gently to precipitate

the nucleic acids. Leave for 30–60 minutes in ice. Pellet the nucleic acid precipitate by centrifuging for 2 minutes at 13 000 rpm.

7. Remove the supernatant and add 1 ml of wash buffer. Vigorously agitate to release the pellet from the bottom of the tube and leave for at least 60 minutes.
8. Pellet the nucleic acids by centrifuging for 2 minutes. Remove the supernatant and place the tubes in an incubator to dry. Once the pellets have dried dissolve in 100  $\mu$ l of TE and store at 4°C (–20°C for long term storage).

Reagents:

- 2  $\times$  CTAB extraction buffer
  - 2% CTAB
  - 1.4 M sodium chloride
  - 20 mM EDTA, disodium
  - 100 mM tris-HCl (ph 8.0)
  - 1% PVP-40T
- ‘Wet’ chloroform
  - 24:1 chloroform:octan-1-ol
- Wash buffer
  - 76% ethanol
  - 10 mM ammonium acetate
- TE
  - 10 mM Tris-HCl (pH 7.4)
  - 1 mM EDTA, disodium

## B.2 PCR protocols

Amplification of microsatellite loci by polymerase chain reaction for the genotyping of individual by ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

1. Remove the DNA dilutions for the samples to be PCR'd from freezer and allow them to defrost.
2. Put on a fresh pair of gloves and for each sample number the PCR tubes so that you will remember the sample order (do this in the PCR area). Also label a 1.5 ml Eppendorf tube for the PCR master mix.
3. Remove the PCR reaction components from the freezer and allow to thaw. Keep the *taq* polymerase in the freezer till the last moment and keep it in an ice bucket while in use.
4. Prepare the basic reaction cocktail for 10  $\mu$ l microsatellite PCR reaction as:

	Pt1254 Pt15169 Pt26081 Pt30204 Pt36480 Pt71936 Pt87268 Pt110048	SPAC 11.5	SPAC 11.8	SPAG 7.14	PtTX3116 PtTX4001 ssrPt_ctg4363 ssrPt_ctg4698 ssrPt_ctg7731
H <sub>2</sub> O, sterile	6.75 $\mu$ l	6.35 $\mu$ l	5.75 $\mu$ l	5.95 $\mu$ l	5.85 $\mu$ l
NH <sub>4</sub> <sup>+</sup> buffer (10 $\times$ ) <sup>a</sup>	1.00 $\mu$ l	1.00 $\mu$ l	1.00 $\mu$ l	1.00 $\mu$ l	1.00 $\mu$ l
MgCl <sub>2</sub> (50 mM) <sup>a</sup>	0.50 $\mu$ l	0.50 $\mu$ l	0.60 $\mu$ l	0.40 $\mu$ l	0.50 $\mu$ l
dNTPs (10mM)	0.20 $\mu$ l	0.40 $\mu$ l	0.40 $\mu$ l	0.40 $\mu$ l	0.40 $\mu$ l
BSA (1 mg/ml)	0.10 $\mu$ l	0.10 $\mu$ l	0.10 $\mu$ l	0.10 $\mu$ l	0.10 $\mu$ l
Primer F (10 $\mu$ M)	0.20 $\mu$ l	0.25 $\mu$ l	0.50 $\mu$ l	0.50 $\mu$ l	0.50 $\mu$ l
Primer R (10 $\mu$ M)	0.20 $\mu$ l	0.25 $\mu$ l	0.50 $\mu$ l	0.50 $\mu$ l	0.50 $\mu$ l
<i>taq</i> <sup>a</sup>	0.05 $\mu$ l	0.15 $\mu$ l	0.15 $\mu$ l	0.15 $\mu$ l	0.15 $\mu$ l

<sup>a</sup> Biotaq<sup>TM</sup> DNA polymerase kit (Bioline<sup>®</sup>)

Make up enough master mix for all your samples plus one control, allowing 5–10% of additional volume for pipetting error (Toonen, 1997).

Add the components in the order given above. Add the *taq* last, minimising the time it is out of the freezer. After adding the *taq*, mix the cocktail well

with the pipette otherwise the *taq* will remain at the bottom because it is in a glycerol solution.

5. Aliquot 9  $\mu\text{l}$  of the master mix to each of the reaction tubes and the control (the control may not get the full amount but this does not matter).
6. Take the PCR tubes to your work bench.
7. Using filtered P10 tips, add 1  $\mu\text{l}$  of the DNA dilution to the reaction tubes.
8. Start the appropriate cycling profile for PCR in the PE 9700. Put samples at the PCR machine after the block has reached 80°C:
  - (a) Pt1254, Pt15169, Pt26081, Pt30204, Pt36480, Pt71936, Pt87268 and Pt110048:
    - i. 95°C for 5 minutes.
    - ii. 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute ( $\times 25$ ).
    - iii. 72°C for 30 minutes, 20°C for 5 minutes then shut off.
  - (b) SPAC 11.5:
    - i. 94°C for 3 minutes.
    - ii. 94°C for 45 seconds, 61°C for 45 seconds, 72°C for 1 minute ( $\times 30$ ).
    - iii. 72°C for 30 minutes, 20°C for 5 minutes then shut off.
  - (c) SPAC 11.8:
    - i. 94°C for 3 minutes.
    - ii. 94°C for 45 seconds, 57°C for 45 seconds, 72°C for 1 minute ( $\times 30$ ).
    - iii. 72°C for 30 minutes, 20°C for 5 minutes then shut off.
  - (d) SPAG 7.14:
    - i. 94°C for 3 minutes.
    - ii. 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 1 minute ( $\times 30$ ).
    - iii. 72°C for 30 minutes, 20°C for 5 minutes then shut off.
  - (e) PtTX3116:
    - i. 94°C for 10 minutes.



- ii. 94°C for 1 minute, 55↓45°C for 1 minute, 72°C for 1 minute (×20).  
(drop 0.5°C each cycle)
- iii. 94°C for 1 minute, 45°C for 1 minute, 72°C for 1 minute (×20).
- iv. 72°C for 30 minutes, 20°C for 5 minutes then shut off.

(f) PtTX4001 and *ssrPt\_ctg4363*:

- i. 94°C for 10 minutes.
- ii. 94°C for 1 minute, 60↓50°C for 1 minute, 72°C for 1 minute (×20).  
(drop 0.5°C each cycle)
- iii. 94°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute (×20).
- iv. 72°C for 30 minutes, 20°C for 5 minutes then shut off.

(g) *ssrPt\_ctg4698*:

- i. 94°C for 3 minutes.
- ii. 94°C for 1 minute, 49°C for 1 minute, 72°C for 1 minute (×30).
- iii. 72°C for 30 minutes, 20°C for 5 minutes then shut off.

(h) *ssrPt\_ctg7731*:

- i. 94°C for 3 minutes.
- ii. 94°C for 1 minute, 51°C for 1 minute, 72°C for 1 minute (×30).
- iii. 72°C for 30 minutes, 20°C for 5 minutes then shut off.

9. Preparation of PCR products for their analysis in the ABI PRISM 3700 DNA Analyzer (Applied Biosystems); using the four-dye system (set D: ROX, NED, HEX, FAM) and GeneScan-400HD [ROX] size standard.

Prepare the mixture of size standard (0.20  $\mu$ l per sample) and formamide (8.80  $\mu$ l per sample). Aliquot 9  $\mu$ l of the mix into a 96 well PCR plate and add 1  $\mu$ l of pooled PCR products.

Multiplexing:

NED	SPAC 11.5 and PtTX4001
HEX	Pt15169, Pt36480, Pt71936, SPAG 7.14 and <i>ssrPt_ctg4698</i>
FAM	Pt1254, Pt26081, Pt30204, Pt87268, Pt110048, SPAC 11.8, PtTX3116, <i>ssrPt_ctg4363</i> and <i>ssrPt_ctg7731</i>

- 
- (a) Pt1254, Pt15169, Pt26081, Pt30204, Pt36480, Pt71936, Pt87268 and Pt110048:
- i. Dilute Pt1254, Pt15169, Pt26081 and Pt36480 PCR products to 1:10.
  - ii. Dilute Pt30204 PCR product to 1:5.
  - iii. Aliquot 5  $\mu$ l from each locus PCR product (pure or diluted) into a new tube and use pooled products for analysis.
- (b) SPAC 11.5 and SPAC 11.8:
- i. Aliquot 5  $\mu$ l from each locus PCR product (pure) into a new tube and use pooled products for analysis.
- (c) SPAG 7.14, PtTX3116 and PtTX4001:
- i. Add 1  $\mu$ l PCR product (pure) from loci PtTX3116 and PtTX4001 into the undiluted SPAG 7.14 PCR product and use pooled products for analysis.
- (d) *ssrPt\_ctg4363*, *ssrPt\_ctg4698* and *ssrPt\_ctg7731*:
- i. Dilute *ssrPt\_ctg4363* PCR product to 1:2.
  - ii. Aliquot 5  $\mu$ l from each locus PCR product (pure or diluted) into a new tube
  - iii. Dilute pooled products to 1:2 and use for analysis.

Locus	Repeat motif	Forward primer	Reverse primer
<b>nSSR</b>			
SPAC 11.5	(AT) <sub>8</sub> (GT) <sub>19</sub> ... (TA) <sub>11</sub>	TGGAGTGGAAAGTTTGAGAAGC	TTGGTTACGATACAGACGATG
SPAC 11.8	(TG) <sub>16</sub>	AGGGAGATCAATAGATCATGG	CAGCCAAGACATCAAAAATG
SPAG 7.14	(TG) <sub>17</sub> (AG) <sub>21</sub>	TTCGTAGGACTAAAAATGTGTG	CAAAGTGGATTTGACCG
PtTX3116	(TTG) <sub>7</sub> ... (TTG) <sub>5</sub>	CCTCCCAAAGCCTAAAGAAT	CATACAAGGCCTTATCTTACAGAA
PtTX4001	(GT) <sub>15</sub>	CTATTTGAGTTAAGAGGGAGTC	CTGTGGGTAGCATCATC
ssrPt_ctg4363	(AT) <sub>10</sub>	TAATAATTCAAGCCACCCCG	AGCAGGCTAATAACAACACGC
ssrPt_ctg4698	(ATC) <sub>10</sub>	CGAAAAGGTGGTTCTGATGG	TTTTCCGCTGGATTTACCAC
ssrPt_ctg7731	(AT) <sub>12</sub>	AGTGGTGAAGGGTCCATCTG	GCATAACACAAAAGCCAGCA
<b>cpSSR</b>			
Pt1254	T <sub>17</sub>	CAATTGGAATGAGAACAGATAGG	TGCGTTGCACTTCGTTATAG
Pt15169	C <sub>8</sub> T <sub>8</sub> AT <sub>8</sub>	CTTGGATGGAATAGCAGCC	GGAAGGGCATTAAAGGTCATTA
Pt26081	T <sub>14</sub>	CCCGTATCCAGATATACTTCCA	TGGTTTGATTTCATTCGTTTCAT
Pt30204	A <sub>12</sub> G <sub>10</sub>	TCATAGCGGAAGATCCTCTTT	CGGATTGATCCTAACCATAACC
Pt36480	T <sub>11</sub>	TTTTGGCTTACAAAATAAAAAGAGG	AAATTCCTAAAGAAGGAAGAGCA
Pt71936	T <sub>16</sub>	TTCATTGGAATACACTAGCCC	AAAACCGTACATGAGATTCCC
Pt87268	T <sub>14</sub>	GCCAGGGAAAATCGTAGG	AGACGATTAGACATCCAACCC
Pt110048	T <sub>10</sub>	TAAGGGGACTAGAGCAGGCTA	TTCGATATTGAACCTTGGACA

Tab. B.1: Microsatellite primers used (Auckland et al., 2002; Soranzo et al., 1998; Chagné et al., 2004; Vendramin et al., 1996). Repeat motifs correspond to the original species.

## C. GENOTYPE TABLES

The following tables contain the genotypic information of all the individuals analysed in this work. Each individual is identified with a code composed by a series of letters identifying the sampling locality and a number that identifies the individual within the sampling locality. The codes ‘CA’ and ‘CF’ refer to the reforestations of Arico and Fasnía respectively, and are followed by roman numerals which identify the sampling locality within the stands (see table A.1 and figure A.1). In these localities, individuals with numbers from 1 to 10 are planted trees, while numbers 11–20 refer to the understorey natural regeneration (with the exception of CFI11 which is planted and CFI21 which is regenerated). ‘CG’, ‘CH’, ‘IF’, ‘LG’, ‘MAI’, ‘MAII’, ‘PP’ and ‘VL’ stand for Güímar, Chinyero, Ifonche, Vilaflor 1500, Arico 1000, Arico 1500, Arico 2000 and Vilaflor 2000 respectively. Three individuals (CG14bis, VL30bis and VL35bis) were assigned in the field with an already assigned code by mistake and were renamed adding the word ‘bis’ to the code. ‘CSA’ (Cruz de San Antonio, Gran Canaria), ‘LLA’ (Llanos de Aridane, La Palma), ‘MZ’ (Mazo, La Palma), ‘RA’ (Raíces, Tenerife) and ‘RM’ (Roque de los Muchachos, La Palma) correspond to localities from which only a few individuals were sampled for the first screening of genetic diversity in chloroplast microsatellites.

### *C.1 Chloroplast microsatellite haplotypes*

Tab. C.1: Genotyping: cpSSR.

ID	Pt1254	Pt15169	Pt26081	Pt30204	Pt36480	Pt71936	Pt87268	Pt110048
CAI01	65	117	107	142	137	150	163	88
CAI02	64	117	107	142	137	149	163	89
CAI03	65	117	107	142	137	150	163	89
CAI04	65	117	107	143	137	148	162	91
CAI05	64	118	107	142	137	149	163	90
CAI06	65	117	107	142	137	151	162	90
CAI07	64	117	107	143	137	150	163	90
CAI08	64	117	107	142	137	149	162	90
CAI09	64	117	107	142	137	149	163	90

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Table C.1: *continued from previous page*

ID	Pt1254	Pt15169	Pt26081	Pt30204	Pt36480	Pt71936	Pt87268	Pt110048
CAI10	64	117	107	142	137	150	163	90
CAI11	65	117	107	142	137	150	163	89
CAI12	65	117	107	142	137	150	163	89
CAI13	64	117	107	143	137	149	162	90
CAI14	65	117	107	143	137	149	162	90
CAI15	65	117	107	142	137	150	163	89
CAI16	64	116	107	142	137	149	163	90
CAI17	64	117	107	141	137	149	163	89
CAI18	64	117	107	143	137	148	163	90
CAI19	64	117	107	142	137	149	163	90
CAI20	65	117	107	142	137	150	163	89
CAII01	65	117	107	142	137	148	162	91
CAII02	64	117	107	142	137	149	163	90
CAII03	64	117	107	142	137	150	163	90
CAII04	65	117	107	142	137	150	163	89
CAII05	64	117	107	142	137	150	164	89
CAII06	64	117	107	142	137	149	163	90
CAII07	65	117	107	142	137	148	162	91
CAII08	64	117	107	142	137	150	163	90
CAII09	64	117	107	143	137	149	163	90
CAII10	64	117	107	142	137	149	163	90
CAII11	65	117	107	142	137	152	162	90
CAII12	65	117	107	142	137	150	163	89
CAII13	64	117	107	142	137	148	162	90
CAII14	64	117	107	142	137	148	163	90
CAII15	64	117	107	142	137	149	163	90
CAII16	64	117	107	142	137	150	163	91
CAII17	65	118	107	143	137	154	163	89
CAII18	64	117	107	142	137	149	162	90
CAII19	65	118	107	143	137	152	163	90
CAII20	64	117	107	142	137	150	162	90
CAIII01	64	117	107	142	137	149	163	89
CAIII02	64	117	107	143	137	150	163	90
CAIII03	64	117	107	143	137	149	162	90
CAIII04	65	116	107	142	137	149	161	91
CAIII05	65	117	106	142	137	150	161	89
CAIII06	64	117	107	142	137	149	163	90
CAIII07	65	116	107	143	137	150	163	89
CAIII08	65	117	107	143	137	149	162	91
CAIII09	66	117	108	143	137	147	164	90
CAIII10	65	116	107	142	137	150	162	91
CAIII11	65	119	107	143	137	150	163	90
CAIII12	64	117	107	143	137	150	163	90
CAIII13	65	118	107	142	137	149	164	90
CAIII14	64	117	107	142	137	150	164	89
CAIII15	65	117	107	143	137	149	162	90
CAIII16	65	118	107	143	137	151	162	90
CAIII17	65	118	107	144	137	153	163	90
CAIII18	65	117	107	142	137	150	163	89
CAIII19	64	117	107	142	137	149	161	90
CAIII20	65	117	107	142	137	149	162	91
CAIV01	65	117	107	143	137	149	162	90
CAIV02	64	117	107	142	137	150	163	90
CAIV03	65	118	107	143	137	150	163	90
CAIV04	65	119	107	143	137	150	163	90
CAIV05	64	117	107	142	137	149	162	90
CAIV06	65	117	107	142	137	151	162	91
CAIV07	65	117	107	142	137	150	163	93
CAIV08	65	117	107	142	137	152	162	90
CAIV09	65	118	107	144	137	154	163	90
CAIV10	64	117	107	142	137	150	162	90
CAIV11	64	117	107	142	137	151	162	90
CAIV12	65	117	107	142	137	151	162	91
CAIV13	65	119	107	143	137	151	163	90
CAIV14	65	117	107	142	137	149	162	91

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Table C.1: *continued from previous page*

ID	Pt1254	Pt15169	Pt26081	Pt30204	Pt36480	Pt71936	Pt87268	Pt110048
CAIV15	65	116	107	143	137	151	162	90
CAIV16	64	117	107	142	137	149	162	90
CAIV17	65	117	107	143	137	151	163	90
CAIV18	64	117	107	142	137	151	164	89
CAIV19	66	117	107	142	137	149	162	91
CAIV20	65	118	107	144	137	153	163	90
CAV01	64	116	107	142	137	150	163	90
CAV02	64	117	107	142	137	148	163	89
CAV03	64	117	107	142	137	150	163	90
CAV04	64	117	107	142	137	149	163	90
CAV05	64	117	107	142	137	149	163	90
CAV06	64	117	107	142	137	149	163	90
CAV07	64	117	107	142	137	150	163	91
CAV08	65	117	107	142	137	151	161	91
CAV09	65	117	107	142	137	151	161	91
CAV10	64	117	107	143	137	150	163	90
CAV11	64	117	107	142	137	149	162	90
CAV12	64	117	107	142	137	148	163	90
CAV13	65	117	107	142	137	151	162	91
CAV14	65	118	107	142	137	149	162	90
CAV15	65	117	108	142	137	150	163	89
CAV16	67	117	107	142	137	151	161	91
CAV17	65	117	106	142	137	150	162	89
CAV18	64	117	106	142	137	150	162	90
CAV19	65	118	107	143	137	152	163	90
CAV20	65	117	107	142	137	151	161	91
CFI01	65	117	107	143	137	150	161	89
CFI02	64	116	107	142	137	150	163	90
CFI03	64	117	107	142	137	149	163	90
CFI04	65	117	107	142	137	149	163	91
CFI05	65	117	107	142	137	151	162	91
CFI06	65	117	107	142	137	151	162	90
CFI07	65	118	107	143	137	150	163	90
CFI08	64	117	107	142	137	149	162	90
CFI09	64	117	107	142	137	149	163	89
CFI10	64	117	107	142	137	149	163	90
CFI11	65	117	107	143	137	149	162	90
CFI12	64	117	107	142	137	149	161	90
CFI13	64	117	107	142	137	149	163	90
CFI14	65	118	107	142	137	150	163	89
CFI15	64	117	107	142	137	150	164	89
CFI16	65	117	107	142	137	149	163	91
CFI17	65	118	107	144	137	154	163	90
CFI18	64	117	107	143	137	150	163	90
CFI19	64	117	107	142	137	148	163	90
CFI20	64	117	107	142	137	149	162	90
CFI21	65	117	107	142	137	151	162	91
CFII01	65	117	107	142	137	150	163	92
CFII02	64	117	107	142	137	148	163	90
CFII03	65	117	107	142	137	149	162	91
CFII04	64	117	107	142	137	149	163	90
CFII05	65	118	107	143	137	158	163	90
CFII06	66	118	107	144	137	148	162	89
CFII07	64	117	107	142	137	149	163	90
CFII08	64	117	107	142	137	151	163	90
CFII09	64	118	107	144	137	151	162	90
CFII10	64	117	107	142	137	149	163	90
CFII11	65	117	106	142	137	150	162	89
CFII12	65	117	107	142	137	152	162	91
CFII13	65	117	107	142	137	149	162	91
CFII14	65	117	107	142	137	150	163	89
CFII15	65	117	107	142	137	149	162	91
CFII16	65	117	107	143	137	149	162	90
CFII17	64	117	107	142	137	149	163	90
CFII18	65	117	107	142	137	153	162	90

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Table C.1: *continued from previous page*

ID	Pt1254	Pt15169	Pt26081	Pt30204	Pt36480	Pt71936	Pt87268	Pt110048
CFIII19	65	117	107	143	137	149	162	90
CFIII20	65	117	107	142	137	151	162	90
CFIII01	64	117	107	143	137	150	163	90
CFIII02	65	117	107	142	137	150	163	91
CFIII03	64	117	107	142	137	148	163	90
CFIII04	64	117	107	142	137	149	163	90
CFIII05	65	117	107	142	137	147	163	89
CFIII06	65	117	107	142	137	149	163	91
CFIII07	64	117	107	142	137	149	163	90
CFIII08	65	118	107	144	137	151	162	89
CFIII09	64	117	107	142	137	149	163	90
CFIII10	64	117	107	142	137	149	163	90
CFIII11	65	117	106	142	137	150	162	89
CFIII12	64	117	107	142	137	149	163	90
CFIII13	65	117	106	142	137	150	162	89
CFIII14	64	117	107	142	137	149	163	90
CFIII15	65	117	107	142	137	150	162	91
CFIII16	64	117	107	142	137	149	161	90
CFIII17	64	117	107	142	137	149	163	90
CFIII18	65	117	107	143	137	149	162	90
CFIII19	64	117	107	142	137	149	163	90
CFIII20	65	117	106	142	137	149	162	89
CFIV01	65	117	107	142	137	151	163	89
CFIV02	65	117	107	142	137	149	162	91
CFIV03	64	117	107	142	137	149	163	90
CFIV04	65	117	107	142	137	149	163	91
CFIV05	65	117	107	142	137	150	163	89
CFIV06	65	117	107	143	137	149	162	90
CFIV07	65	117	107	142	137	151	166	90
CFIV08	64	118	107	142	137	149	163	90
CFIV09	64	117	107	142	137	149	163	90
CFIV10	65	117	107	143	137	149	162	90
CFIV11	64	117	107	142	137	145	163	90
CFIV12	64	117	107	143	137	150	163	90
CFIV13	65	117	106	142	137	150	162	89
CFIV14	65	117	106	142	137	150	162	89
CFIV15	64	117	107	143	137	148	163	89
CFIV16	65	117	107	142	137	150	163	89
CFIV17	65	117	107	142	137	149	162	92
CFIV18	65	116	107	142	137	149	161	91
CFIV19	65	117	106	142	137	150	162	88
CFIV20	64	117	107	143	137	150	163	90
CG01	64	117	107	142	137	149	163	89
CG02	65	117	107	143	137	151	163	90
CG03	64	117	107	142	137	149	163	90
CG04	65	117	106	142	137	151	162	89
CG05	65	117	107	142	137	149	162	92
CG06	65	117	107	142	137	151	163	88
CG07	64	117	107	142	137	149	163	90
CG08	65	117	107	142	137	149	162	90
CG09	65	117	106	142	137	151	162	89
CG10	64	117	107	142	137	149	163	90
CG11	65	117	107	142	137	153	162	90
CG12	64	117	107	142	137	148	163	90
CG13	65	117	107	142	137	151	161	91
CG14	65	117	107	142	137	151	163	88
CG14bis	64	117	107	142	137	149	161	90
CG15	65	117	107	142	137	149	162	91
CG16	65	117	107	142	137	151	161	91
CG17	65	116	107	142	137	150	162	91
CG18	64	117	107	143	137	149	163	90
CG19	64	118	107	143	137	152	163	90
CG20	65	117	107	142	137	150	164	90
CG21	65	118	107	143	137	151	162	90
CG22	65	117	107	142	137	149	162	91

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Table C.1: *continued from previous page*

ID	Pt1254	Pt15169	Pt26081	Pt30204	Pt36480	Pt71936	Pt87268	Pt110048
CG23	65	117	107	142	137	152	161	91
CG24	64	117	107	142	137	148	163	89
CG25	64	117	107	142	137	148	163	90
CG26	65	119	107	143	137	150	163	90
CG27	65	118	107	144	137	152	162	90
CG28	65	118	107	142	137	149	164	90
CG29	63	117	107	142	137	153	161	91
CG30	64	117	107	142	137	148	163	90
CG31	64	117	107	142	137	149	163	90
CG32	65	117	107	142	137	150	163	91
CG33	65	117	107	142	137	149	162	92
CG34	65	117	107	143	137	149	162	90
CG35	64	117	107	143	137	150	163	91
CG36	64	117	107	142	137	150	163	90
CG37	64	117	107	143	137	149	163	90
CG38	65	117	107	142	137	151	161	91
CG39	65	118	107	142	137	151	162	90
CG40	64	117	107	142	137	149	163	90
CG41	64	117	107	142	137	149	163	90
CG42	65	117	107	142	137	151	162	91
CG43	65	116	107	143	137	150	162	90
CG44	65	117	106	142	137	150	162	89
CG45	65	117	107	142	137	148	162	91
CG46	65	117	107	143	137	149	162	90
CH01	65	117	107	142	137	149	162	91
CH02	64	117	107	142	137	149	163	90
CH03	64	117	107	142	137	149	163	90
CH04	65	117	107	143	137	150	161	89
CH05	64	117	107	142	137	148	162	90
CH06	64	117	107	143	137	150	163	90
CH07	65	117	107	143	137	148	162	91
CH08	64	117	107	142	137	149	162	90
CH09	65	117	107	142	137	150	163	91
CH10	64	117	107	142	137	149	163	90
CH11	64	117	107	142	137	149	163	91
CH12	65	117	107	142	137	149	162	92
CH13	65	117	106	142	137	151	162	88
CH14	64	117	107	142	137	148	163	90
CH15	64	116	107	142	137	149	163	90
CH16	64	116	107	142	137	149	163	90
CH17	64	116	107	144	137	150	163	90
CH18	64	117	107	142	137	149	162	90
CH19	65	117	106	142	137	149	162	92
CH20	64	117	107	142	137	149	163	90
CH21	64	117	107	142	137	149	162	90
CH22	64	117	108	143	137	150	163	90
CH23	64	117	107	143	137	149	163	90
CH24	65	117	107	142	137	149	162	90
CH25	64	117	107	142	137	149	163	90
CH26	65	117	107	142	137	150	163	91
CH27	65	117	107	142	137	149	162	92
CH28	65	117	107	142	137	152	163	89
CH29	65	117	107	142	137	150	163	89
CH30	64	117	107	143	137	150	163	90
CH31	64	117	107	142	137	149	163	90
CH32	65	117	107	142	137	150	161	89
CH33	65	117	107	142	137	149	162	90
CH34	64	117	107	142	137	149	162	90
CH35	64	117	107	142	137	149	163	90
CH36	65	117	107	142	137	150	163	88
CH37	64	117	107	142	137	148	163	90
CH38	64	116	107	142	137	149	163	90
CH39	65	117	107	142	137	151	163	89
CH40	65	117	106	142	137	150	162	89
CH41	66	118	107	144	137	149	162	89

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Table C.1: *continued from previous page*

ID	Pt1254	Pt15169	Pt26081	Pt30204	Pt36480	Pt71936	Pt87268	Pt110048
CH42	65	117	107	143	137	148	162	91
CH43	64	117	107	143	137	150	163	90
CH44	64	117	107	142	137	149	163	90
CH45	64	117	107	142	137	149	163	90
CH46	64	117	107	142	137	150	163	90
CH47	64	117	107	142	137	149	164	90
CH48	64	117	107	142	137	150	163	90
CH49	65	118	107	142	137	150	163	93
CH50	64	117	107	142	137	149	164	90
CSA01	65	118	107	144	137	152	162	90
CSA03	66	117	108	143	137	147	164	90
IF01	64	117	107	143	137	150	163	91
IF02	64	117	107	142	137	150	163	91
IF03	64	117	107	142	137	149	164	90
IF04	64	117	107	142	137	150	163	91
IF05	65	117	107	142	137	153	162	90
IF06	64	117	107	143	137	150	163	90
IF07	65	116	107	142	137	149	162	91
IF08	64	117	107	142	137	150	163	91
IF09	64	117	107	142	137	149	163	91
IF10	64	117	107	143	137	149	163	90
IF11	64	117	107	142	137	150	163	91
IF12	64	117	107	142	137	149	163	90
IF13	64	117	107	142	137	150	163	91
IF14	65	117	107	142	137	149	162	91
IF15	64	117	107	142	137	149	164	90
IF16	65	117	107	142	137	150	163	91
IF17	67	117	107	142	137	151	161	91
IF18	65	117	107	142	137	148	162	91
IF19	65	117	107	142	137	152	162	90
IF20	65	117	107	142	137	149	162	92
IF21	64	117	107	142	137	149	164	90
IF22	65	116	107	142	137	149	162	91
IF23	64	117	107	142	137	149	162	90
IF24	64	117	107	142	137	150	163	90
IF25	64	117	107	142	137	149	163	90
IF26	64	117	107	143	137	149	162	90
IF27	64	117	107	143	137	150	163	90
IF28	64	117	107	142	137	149	162	90
IF29	65	117	107	143	137	149	162	90
IF30	64	117	107	143	137	149	162	90
IF31	64	116	107	142	137	148	163	90
IF32	65	117	107	142	137	151	163	88
IF33	65	117	107	142	137	150	163	89
IF34	64	117	107	142	137	149	162	91
IF36	65	117	106	142	137	150	162	89
IF37	65	117	107	142	137	151	163	89
IF38	65	117	107	143	137	151	163	90
IF39	65	117	106	142	137	150	162	89
IF40	64	117	107	142	137	150	163	91
LG01	65	117	107	142	137	149	162	92
LG02	64	117	107	142	137	149	161	90
LG03	65	117	107	143	137	149	162	90
LG04	64	117	107	142	137	149	163	90
LG05	65	117	107	143	137	149	162	91
LG06	64	117	107	142	137	148	163	90
LG07	64	117	107	142	137	149	161	90
LG08	65	116	106	142	137	151	162	89
LG09	64	117	107	142	137	150	163	90
LG10	64	117	107	141	137	149	162	89
LG11	65	117	107	142	137	152	161	91
LG12	64	117	107	142	137	150	163	91
LG15	65	117	107	142	137	149	162	90
LG16	64	117	107	142	137	149	163	90

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Table C.1: *continued from previous page*

ID	Pt1254	Pt15169	Pt26081	Pt30204	Pt36480	Pt71936	Pt87268	Pt110048
LG17	64	117	107	142	137	149	163	90
LG18	64	117	107	142	137	148	163	90
LG19	64	117	107	142	137	149	163	90
LG20	65	117	107	142	137	150	163	91
LG21	64	117	107	142	137	149	161	90
LG22	64	117	107	142	137	150	163	90
LG23	65	116	107	142	137	149	162	91
LG24	64	117	107	143	137	151	163	90
LG25	64	117	107	142	137	150	162	90
LG26	64	116	107	142	137	149	163	90
LG27	64	117	107	143	137	149	163	90
LG28	65	117	107	142	137	151	162	90
LG29	64	117	107	142	137	148	163	91
LG30	64	117	107	142	137	149	162	90
LG31	66	118	107	144	137	148	162	89
LG32	65	117	107	142	137	149	162	92
LG33	64	117	107	142	137	149	163	90
LG34	65	117	107	142	137	151	163	89
LG35	65	118	107	143	137	152	163	90
LG36	64	117	107	142	137	149	163	90
LG37	67	117	107	142	137	151	161	91
LG38	64	117	107	142	137	149	163	90
LG39	64	117	107	143	137	150	163	90
LG40	64	117	107	143	137	150	164	90
LLA03	64	116	107	142	137	149	162	91
MAI01	65	117	107	142	137	150	163	89
MAI02	64	117	107	143	137	150	163	90
MAI03	64	117	107	142	137	150	164	90
MAI04	64	117	107	143	137	150	163	90
MAI05	65	117	107	142	137	147	161	91
MAI06	64	117	106	143	137	151	163	89
MAI07	66	118	107	144	137	149	162	89
MAI08	66	117	107	142	137	149	163	90
MAI09	65	118	107	144	137	152	162	90
MAI10	65	117	107	142	137	150	163	90
MAI11	64	117	107	142	137	150	163	90
MAI12	64	117	107	142	137	148	163	90
MAI13	64	117	107	143	137	150	163	89
MAI14	65	117	109	142	137	151	163	89
MAI15	64	117	107	142	137	150	163	90
MAI16	64	118	107	142	137	149	163	90
MAI17	65	117	107	142	137	149	162	91
MAI18	64	117	107	142	137	150	163	91
MAI19	66	118	107	144	137	149	162	89
MAI20	64	117	107	142	137	149	161	90
MAI21	65	117	107	142	137	150	163	91
MAI22	66	118	107	144	137	149	162	89
MAI23	65	117	107	143	137	149	162	91
MAI24	64	117	107	142	137	149	163	90
MAI25	66	117	107	142	137	149	163	90
MAI26	64	117	107	143	137	150	163	90
MAI27	65	118	107	142	137	150	162	91
MAI28	65	117	108	142	137	150	163	89
MAI29	65	117	106	142	137	151	162	89
MAI30	65	117	106	142	137	150	162	88
MAI31	65	117	107	142	137	150	163	91
MAI32	65	118	107	144	137	152	162	90
MAI33	64	117	107	143	137	149	163	90
MAI34	65	118	107	142	137	150	162	91
MAI35	64	117	107	142	137	149	163	90
MAI36	66	117	107	142	137	149	163	90
MAI37	64	117	106	142	137	149	163	90
MAI38	65	118	107	142	137	150	162	91
MAI39	65	117	107	142	137	151	162	91

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Table C.1: *continued from previous page*

ID	Pt1254	Pt15169	Pt26081	Pt30204	Pt36480	Pt71936	Pt87268	Pt110048
MAI40	64	117	107	143	137	149	163	90
MAII01	64	117	107	142	137	149	163	90
MAII02	63	117	107	142	137	150	164	90
MAII03	64	117	107	143	137	150	163	90
MAII04	64	117	107	143	137	150	163	90
MAII05	64	117	107	142	137	148	162	90
MAII06	64	117	107	143	137	150	163	90
MAII07	64	117	107	142	137	150	163	91
MAII08	64	117	107	142	137	150	163	90
MAII09	64	117	107	142	137	152	161	91
MAII10	64	117	107	142	137	151	161	91
MAII11	65	117	107	142	137	150	163	90
MAII12	64	117	107	142	137	149	163	90
MAII13	65	117	107	142	137	150	162	91
MAII14	64	117	107	142	137	149	163	90
MAII15	64	117	107	142	137	149	163	90
MAII16	65	117	107	142	137	150	164	91
MAII17	64	117	107	142	137	152	161	91
MAII18	65	117	106	142	137	148	162	91
MAII19	65	117	106	142	137	149	162	92
MAII20	64	117	107	142	137	148	163	91
MAII21	65	117	107	142	137	148	162	90
MAII22	64	117	107	142	137	149	163	90
MAII23	64	117	107	142	137	149	163	90
MAII24	64	117	107	142	137	149	164	90
MAII25	65	117	107	143	137	149	162	90
MAII26	65	117	106	142	137	151	162	89
MAII27	64	117	107	142	137	150	163	90
MAII28	64	117	107	142	137	149	163	90
MAII29	65	117	107	143	137	150	161	89
MAII30	65	117	106	142	137	150	162	89
MAII31	64	117	107	142	137	148	163	90
MAII32	65	118	107	142	137	150	162	91
MAII33	65	117	107	142	137	149	162	91
MAII34	64	117	107	143	137	150	163	90
MAII35	64	117	107	142	137	152	161	91
MAII36	65	117	107	143	137	150	161	89
MAII37	64	117	107	142	137	149	163	90
MAII38	64	117	107	142	137	149	163	90
MAII39	64	117	107	143	137	150	163	90
MAII40	64	117	107	142	137	149	163	90
MZ03	64	116	107	142	137	149	163	90
MZ04	65	117	107	142	137	149	162	91
PP01	65	117	107	142	137	150	163	89
PP02	64	117	107	142	137	148	163	90
PP03	64	116	107	142	137	149	163	90
PP04	65	117	107	143	137	149	162	90
PP05	64	117	107	142	137	149	162	91
PP06	64	117	107	142	137	150	163	90
PP07	64	117	107	143	137	149	163	90
PP08	65	117	107	142	137	150	163	90
PP09	64	117	107	142	137	149	162	91
PP10	66	117	107	142	137	149	163	90
PP11	64	117	107	143	137	150	163	90
PP12	65	118	107	142	137	153	162	90
PP13	65	117	107	143	137	149	162	90
PP14	64	117	107	142	137	153	161	91
PP15	64	117	107	142	137	149	163	90
PP16	64	117	107	142	137	148	163	90
PP17	65	117	107	142	137	150	163	91
PP18	65	119	107	143	137	150	163	90
PP19	66	118	107	144	137	148	162	89
PP20	65	118	107	142	137	153	162	90
PP21	64	117	107	142	137	148	163	90

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Table C.1: *continued from previous page*

ID	Pt1254	Pt15169	Pt26081	Pt30204	Pt36480	Pt71936	Pt87268	Pt110048
PP22	65	116	107	142	137	150	162	91
PP23	64	117	107	142	137	148	161	91
PP24	65	118	107	142	137	153	162	90
PP25	65	117	107	142	137	150	163	89
PP26	64	117	107	142	137	150	163	90
PP27	64	117	107	142	137	149	163	90
PP28	64	117	107	142	137	150	163	90
PP29	64	117	107	142	137	149	163	90
PP30	66	117	108	143	137	147	164	91
PP31	64	117	107	142	137	150	163	90
PP32	64	117	107	142	137	150	163	90
PP33	65	117	107	142	137	152	162	90
PP34	64	117	107	142	137	149	162	91
PP35	65	117	107	142	137	148	162	91
PP36	65	117	107	142	137	150	163	91
PP37	65	117	107	142	137	148	162	91
PP38	65	118	107	143	137	154	163	89
PP39	65	117	107	142	137	150	163	91
PP40	65	117	107	142	137	150	163	91
PP41	64	117	107	142	137	149	163	90
PP42	64	117	107	142	137	148	161	91
PP43	64	117	107	142	137	150	163	90
PP44	64	117	107	142	137	149	163	91
PP45	65	118	107	142	137	150	163	89
PP46	65	117	107	142	137	149	162	92
PP47	64	117	107	142	137	150	163	90
PP48	65	117	107	142	137	150	163	91
PP49	65	117	107	144	137	150	162	90
PP50	66	117	106	143	137	151	163	89
RA02	64	117	107	142	137	149	163	90
RM02	65	117	106	142	137	147	162	89
VL01	64	117	107	142	137	148	163	90
VL02	66	118	107	143	137	155	163	90
VL03	65	120	107	143	137	150	163	90
VL04	64	117	107	142	137	149	164	90
VL05	64	117	107	142	137	149	163	90
VL06	64	117	107	142	137	148	163	89
VL07	64	117	107	142	137	150	163	90
VL08	65	117	107	143	137	150	161	89
VL09	64	117	107	142	137	150	163	90
VL10	64	116	107	142	137	149	163	90
VL11	65	117	107	142	137	150	163	91
VL12	64	116	107	142	137	149	163	90
VL13	65	117	107	143	137	150	161	89
VL14	64	117	107	142	137	149	163	90
VL15	65	117	107	142	137	150	162	91
VL16	64	117	107	142	137	149	163	90
VL17	64	117	107	142	137	149	163	90
VL18	65	117	107	142	137	150	164	90
VL19	64	117	107	142	137	149	163	90
VL20	64	117	107	142	137	149	163	90
VL21	65	117	107	142	137	150	163	89
VL22	65	117	107	142	137	147	163	89
VL23	66	118	107	144	137	148	162	89
VL24	64	116	107	142	137	149	163	90
VL25	66	118	107	144	137	148	162	89
VL26	66	118	107	144	137	148	162	89
VL27	65	117	107	142	137	149	162	91
VL28	64	117	107	142	137	148	163	90
VL29	65	117	107	142	137	150	163	91
VL30	65	117	107	142	137	151	162	90
VL30bis	65	117	107	142	137	149	162	90
VL31	64	117	107	143	137	150	163	90
VL32	65	118	107	142	137	150	163	90

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Table C.1: *continued from previous page*

ID	Pt1254	Pt15169	Pt26081	Pt30204	Pt36480	Pt71936	Pt87268	Pt110048
VL33	64	117	107	142	137	149	162	90
VL34	66	118	108	143	137	148	164	90
VL35	64	117	107	142	137	148	163	89
VL35bis	64	117	107	142	137	149	163	91
VL36	65	118	107	142	137	149	164	90
VL37	65	117	107	142	137	149	162	91
VL38	65	117	107	142	137	150	163	90
VL39	65	117	106	143	137	150	161	89
VL40	64	117	107	142	137	150	163	91

Tab. C.2: Chloroplast SSR haplotype definition.

Haplotype	Frequency	Pt1254	Pt110048	Pt26081	Pt30204	Pt87268	Pt15169	Pt36480	Pt71936
H001	10	65	91	107	142	162	117	137	149
H002	65	64	90	107	142	163	117	137	149
H003	6	65	89	107	143	161	117	137	150
H004	2	64	90	107	142	162	117	137	148
H005	20	64	90	107	143	163	117	137	150
H006	3	65	91	107	143	162	117	137	148
H007	11	64	90	107	142	162	117	137	149
H008	15	65	91	107	142	163	117	137	150
H009	4	64	91	107	142	163	117	137	149
H010	8	65	92	107	142	162	117	137	149
H011	1	65	88	106	142	162	117	137	151
H012	16	64	90	107	142	163	117	137	148
H013	8	64	90	107	142	163	116	137	149
H014	1	64	90	107	144	163	116	137	150
H015	2	65	92	106	142	162	117	137	149
H016	1	64	90	108	143	163	117	137	150
H017	9	64	90	107	143	163	117	137	149
H018	5	65	90	107	142	162	117	137	149
H019	1	65	89	107	142	163	117	137	152
H020	9	65	89	107	142	163	117	137	150
H021	1	65	89	107	142	161	117	137	150
H022	2	65	88	107	142	163	117	137	150
H023	4	65	89	107	142	163	117	137	151
H024	5	65	89	106	142	162	117	137	150
H025	4	66	89	107	144	162	118	137	149
H026	24	64	90	107	142	163	117	137	150
H027	7	64	90	107	142	164	117	137	149
H028	1	65	93	107	142	163	118	137	150
H029	4	64	89	107	142	163	117	137	149
H030	2	65	90	107	143	163	117	137	151
H031	4	65	89	106	142	162	117	137	151
H032	3	65	88	107	142	163	117	137	151
H033	2	65	90	107	142	162	117	137	153
H034	5	65	91	107	142	161	117	137	151
H035	5	64	90	107	142	161	117	137	149
H036	3	65	91	107	142	162	116	137	150
H037	1	64	90	107	143	163	118	137	152
H038	2	65	90	107	142	164	117	137	150
H039	1	65	90	107	143	162	118	137	151
H040	2	65	91	107	142	161	117	137	152
H041	4	64	89	107	142	163	117	137	148
H042	3	65	90	107	143	163	119	137	150
H043	3	65	90	107	144	162	118	137	152
H044	2	65	90	107	142	164	118	137	149
H045	1	63	91	107	142	161	117	137	153
H046	11	65	90	107	143	162	117	137	149
H047	2	64	91	107	143	163	117	137	150
H048	1	65	90	107	142	162	118	137	151

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Table C.2: *continued from previous page*

Haplotype	Frequency	Pt1254	Pt110048	Pt26081	Pt30204	Pt87268	Pt15169	Pt36480	Pt71936
H049	4	65	91	107	142	162	117	137	151
H050	1	65	90	107	143	162	116	137	150
H051	6	65	91	107	142	162	117	137	148
H052	3	64	90	107	142	163	118	137	149
H053	4	65	90	107	142	162	117	137	151
H054	1	64	89	107	142	164	117	137	150
H055	3	64	90	107	143	162	117	137	149
H056	1	65	91	107	142	161	116	137	149
H057	1	65	89	106	142	161	117	137	150
H058	1	65	89	107	143	163	116	137	150
H059	3	65	91	107	143	162	117	137	149
H060	1	66	90	108	143	164	117	137	147
H061	2	65	90	107	143	163	118	137	150
H062	1	65	93	107	142	163	117	137	150
H063	3	65	90	107	142	162	117	137	152
H064	1	65	90	107	144	163	118	137	154
H065	2	64	90	107	142	162	117	137	150
H066	2	64	90	107	142	163	116	137	150
H067	11	64	91	107	142	163	117	137	150
H068	3	65	91	107	142	163	117	137	149
H069	1	65	92	107	142	163	117	137	150
H070	1	65	90	107	143	163	118	137	158
H071	6	66	89	107	144	162	118	137	148
H072	1	64	90	107	142	163	117	137	151
H073	1	64	90	107	144	162	118	137	151
H074	2	65	89	107	142	163	117	137	147
H075	1	65	89	107	144	162	118	137	151
H076	1	65	90	107	142	166	117	137	151
H077	3	65	91	107	142	162	116	137	149
H078	2	67	91	107	142	161	117	137	151
H079	1	64	90	107	142	163	116	137	148
H080	4	64	91	107	142	162	117	137	149
H081	1	65	89	106	142	162	116	137	151
H082	1	64	89	107	141	162	117	137	149
H083	1	64	90	107	143	163	117	137	151
H084	2	64	91	107	142	163	117	137	148
H085	1	65	90	107	143	163	118	137	152
H086	1	64	90	107	143	164	117	137	150
H087	1	64	90	107	142	164	117	137	150
H088	1	65	91	107	142	161	117	137	147
H089	1	64	89	106	143	163	117	137	151
H090	4	66	90	107	142	163	117	137	149
H091	4	65	90	107	142	163	117	137	150
H092	1	64	89	107	143	163	117	137	150
H093	1	65	89	109	142	163	117	137	151
H094	4	65	91	107	142	162	118	137	150
H095	1	65	89	108	142	163	117	137	150
H096	1	65	88	106	142	162	117	137	150
H097	1	64	90	106	142	163	117	137	149
H098	1	63	90	107	142	164	117	137	150
H099	3	64	91	107	142	161	117	137	152
H100	1	64	91	107	142	161	117	137	151
H101	2	65	91	107	142	162	117	137	150
H102	1	65	91	107	142	164	117	137	150
H103	1	65	91	106	142	162	117	137	148
H104	1	65	90	107	142	162	117	137	148
H105	3	65	90	107	142	162	118	137	153
H106	1	64	91	107	142	161	117	137	153
H107	2	64	91	107	142	161	117	137	148
H108	1	66	91	108	143	164	117	137	147
H109	1	65	89	107	143	163	118	137	154
H110	1	65	89	107	142	163	118	137	150
H111	1	65	90	107	144	162	117	137	150
H112	1	66	89	106	143	163	117	137	151
H113	1	66	90	107	143	163	118	137	155

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Table C.2: continued from previous page

Haplotype	Frequency	Pt1254	Pt110048	Pt26081	Pt30204	Pt87268	Pt15169	Pt36480	Pt71936
H114	1	65	90	107	143	163	120	137	150
H115	1	66	90	108	143	164	118	137	148
H116	1	65	89	106	143	161	117	137	150
H117	1	65	90	107	142	163	118	137	150

## C.2 Nuclear microsatellite genotypes

Tab. C.3: Genotyping: nSSR.

ID	SPAC 11.5		SPAC 11.8		SPAG 7.14		PtTX3116		PtTX4001		ctg4363		ctg4698		ctg7731	
CAI01	180	180	197	247	183	187	164	173	217	221	94	96	244	244	219	219
CAI02	122	132	176	231	183	197	164	176	217	217	92	98	244	244	219	221
CAI03	116	122	176	249	183	187	164	176	209	225	92	92	244	244	219	221
CAI04	118	136	199	227	185	203	164	164	223	225	90	98	244	244	219	219
CAI05	134	134	217	219	183	191	164	173	221	225	92	96	244	244	221	221
CAI06	154	154	239	241	183	191	164	173	209	217	96	100	244	244	219	221
CAI07	122	168	172	178	197	197	164	173	217	221	92	96	244	244	219	221
CAI08	118	120	170	176	187	205	164	164	216	216	92	100	244	244	219	219
CAI09	150	150	217	223	183	183	146	164	217	221	92	96	244	244	219	221
CAI10	142	156	217	235	183	183	164	173	217	221	90	96	244	244	219	219
CAI11	120	136	227	227	185	185	164	164	209	225	96	98	244	244	221	221
CAI12	122	122	199	199	183	183	164	176	217	221	92	98	244	247	217	219
CAI13	98	98	176	247	183	185	146	164	217	225	90	94	244	244	219	219
CAI14	114	148	181	229	193	195	146	146	225	225	94	96	244	244	219	223
CAI15	124	124	249	249	183	183	176	176	209	217	92	98	244	244	219	219
CAI16	136	158	231	253	175	215	164	164	209	225	92	94	232	244	219	219
CAI17	134	134	237	237	183	183	164	170	209	209	90	92	244	244	219	219
CAI18	122	126	199	223	187	197	164	173	217	221	90	92	244	244	219	219
CAI19	150	150	223	237	185	185	170	170	209	209	90	92	244	247	219	221
CAI20	116	124	249	265	187	187	176	176	217	217	92	98	244	244	221	221
CAII01	150	160	263	263	185	201	164	173	211	216	90	92	244	244	219	219
CAII02	124	128	162	237	183	187	164	173	211	211	90	100	244	244	221	221
CAII03	142	162	164	215	175	185	164	173	209	211	92	92	244	244	219	219
CAII04	132	152	219	243	183	211	164	164	209	221	90	102	244	244	217	219
CAII05	116	120	197	219	185	185	164	164	211	217	96	98	244	244	219	219
CAII06	164	180	223	227	185	185	146	146	217	217	90	96	244	247	219	219
CAII07	118	122	233	233	177	185	164	173	209	221	92	96	244	244	219	219
CAII08	118	160	199	225	187	201	164	164	216	217	90	96	244	244	217	221
CAII09	126	152	221	247	181	185	164	164	217	217	88	90	244	247	219	221
CAII10	114	162	223	237	183	191	164	164	209	217	98	98	244	253	219	219
CAII11	162	162	229	237	185	191	164	164	217	217	96	96	244	247	219	219
CAII12	142	148	164	253	191	201	164	173	216	217	90	96	244	244	219	221
CAII13	98	140	164	197	185	207	164	176	211	217	90	90	244	244	219	225
CAII14	116	116	231	231	183	203	164	164	209	209	90	92	244	244	221	221
CAII15	168	168	249	249	183	193	164	170	211	221	94	96	244	244	221	221
CAII16	116	124	148	233	179	201	164	164	217	225	94	94	244	244	219	219
CAII17	170	170	261	261	183	185	164	164	221	221	90	90	244	244	219	219
CAII18	112	124	229	243	183	207	164	164	217	217	92	100	244	244	219	225
CAII19	130	132	221	245	177	185	164	164	203	216	96	96	244	244	219	225
CAII20	98	124	229	241	175	185	164	164	216	221	90	92	247	247	219	219
CAIII01	166	166	207	217	195	215	164	164	211	216	96	100	244	244	219	219
CAIII02	120	168	156	243	183	187	164	164	217	217	90	90	244	244	221	225
CAIII03	130	132	231	243	191	211	164	173	217	221	92	100	244	247	217	219
CAIII04	120	140	197	247	185	217	164	173	211	221	90	100	244	247	219	223
CAIII05	134	134	193	257	185	201	164	164	217	217	90	90	244	244	219	221
CAIII06	118	118	197	227	191	211	164	173	217	225	90	92	247	247	219	219
CAIII07	118	148	241	241	195	213	164	164	223	225	94	104	244	244	219	221
CAIII08	128	162	164	207	179	187	164	164	217	221	96	100	244	247	219	219
CAIII09	120	128	197	197	245	245	167	167	217	217	96	98	244	244	221	221

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Table C.3: *continued from previous page*

ID	SPAC 11.5		SPAC 11.8		SPAG 7.14		PtTX3116		PtTX4001		ctg4363		ctg4698		ctg7731	
CAIII10	132	156	249	249	185	185	164	164	217	221	94	96	244	244	217	219
CAIII11	116	116	173	173	183	183	146	164	217	221	94	96	244	244	221	221
CAIII12	118	120	233	261	183	195	173	173	221	223	92	96	244	244	221	221
CAIII13	114	118			197	221	164	164	221	225	94	96	244	244	219	223
CAIII14	116	124			183	185	164	164	219	225	90	96	244	244	219	233
CAIII15	114	122			183	183	164	164	211	225	96	108	244	244	221	229
CAIII16	120	120	173	225	185	205	164	173	219	225	94	100	244	244	219	223
CAIII17	114	118	231	231	183	205	164	164	211	223	90	98	244	244	221	221
CAIII18	118	120	177	213	183	221	164	164	217	221	94	98	244	244	219	221
CAIII19	118	118	181	181	197	197	164	164	217	219	94	94	244	244	219	219
CAIII20	114	114	241	241	183	183	146	146	217	217	88	88	244	244	229	229
CAIV01	114	120	213	229	195	195	164	164	217	225	96	96	244	244	219	223
CAIV02	114	124	241	241	187	197	146	164	225	225	94	98	244	244	219	221
CAIV03	118	120	219	247	183	195	164	173	216	217	94	94	244	244	223	223
CAIV04	120	120	237	237	197	219	164	164	217	217	90	94	244	244	221	221
CAIV05	120	120	221	221	183	185	164	173	211	225	90	108	244	244	219	231
CAIV06	116	122	191	245	197	221	164	164	217	223	90	96	244	244	219	223
CAIV07	120	172	259	261	183	197	131	164	223	225	94	98	244	244	221	221
CAIV08	118	120	177	177	197	197	164	173	216	217	88	98	244	244	217	219
CAIV09	118	118	180	180	195	245	164	164	219	219	94	96	244	244	219	219
CAIV10	122	140	223	223	175	195	164	173	217	225	90	92	244	247	219	229
CAIV11	116	146	197	219	195	195	146	173	225	225	90	90	244	244	225	231
CAIV12	122	124	235	235	187	195	164	164	217	225	88	92	244	250	219	219
CAIV13	122	122	233	241	195	221	146	164	223	225	92	96	244	244	219	223
CAIV14	118	120	173	184	185	187	173	179	225	225	90	94	244	247	219	219
CAIV15	116	120	265	265	197	207	164	173	217	221	90	96	244	244	219	221
CAIV16	132	132	175	175	197	197	164	173	209	225	90	98	244	244	223	223
CAIV17	116	130	145	235	195	197	164	164	217	225	98	98	244	244	219	223
CAIV18	118	148	162	162	185	205	164	164	217	217	88	88	244	244	223	223
CAIV19	118	118	183	183	199	199	164	164	217	225	90	90	244	247	219	229
CAIV20	122	130	223	223	205	207	164	170	219	225	96	96	244	247	219	225
CAV01	154	154	176	233	177	195	164	173	216	217	90	98	244	247	219	219
CAV02	170	170	197	197	189	189	164	173	217	225	90	90	244	244	221	221
CAV03	122	124	211	227	185	195	146	164	221	225	90	90	244	244	221	221
CAV04	154	198	199	199	177	195	164	173	211	217	92	96	244	244	221	221
CAV05	138	170	207	229	185	201	146	164	211	217	90	92	244	244	221	221
CAV06	116	116	197	209	177	185	164	164	211	216	90	96	244	244	219	219
CAV07	142	162	173	245	185	187	164	164	211	217	94	98	244	244	219	225
CAV08	116	128	197	239	185	197	164	173	217	221	94	102	244	244	219	219
CAV09	116	122	182	182	185	193	173	173	221	221	92	94	244	247	219	225
CAV10	124	152	243	255	183	187	146	146	209	209	94	96	244	247	219	219
CAV11	124	164	176	233	177	183	164	164	209	225	90	90	244	244	219	219
CAV12	140	140	215	215	177	177	146	164	216	216	96	96	244	244	219	219
CAV13	114	118	182	182	185	195	170	173	217	225	90	92	244	244	219	219
CAV14	114	118	148	225	183	211	164	164	217	221	96	96	244	244	219	229
CAV15	116	116	187	217	183	197	173	173	225	225	92	92	244	247	219	219
CAV16	120	120	199	251	185	205	173	173	217	217	96	96	244	244	217	219
CAV17	116	122	227	255	187	187	164	173	225	225	90	92	244	247	219	219
CAV18	148	148	142	142			164	164	225	225	94	96	244	244	217	219
CAV19	132	164	223	223	177	183	164	164	209	216	96	102	244	244	219	225
CAV20	120	162	170	243	177	183	146	164	209	221	96	96	244	244	219	219
CFI01	150	154	197	269	179	185	173	176	217	221	90	90	247	247	219	219
CFI02	118	142	223	263	183	205	158	164	217	217	92	98	244	244	219	225
CFI03	98	120	189	219	185	185	146	164	217	217	90	90	244	247	219	221
CFI04	136	152	197	243	185	207	164	173	217	217	90	92	244	244	219	221
CFI05	118	120	149	149	183	183	164	164	211	225	92	96	244	244	219	219
CFI06	118	120	247	247	175	183	164	164	225	225	90	98	244	244	219	223
CFI07	118	118	227	227	207	207	164	164	217	225	94	98	244	244	219	219
CFI08	124	174	154	253	183	185	164	164	217	221	90	94	244	247	219	219
CFI09	136	152	227	229	183	195	164	164	199	217	96	96	247	250	219	221
CFI10	112	122	172	178	179	213	146	164	209	217	90	96	244	244	219	219
CFI11	124	124	247	247	183	197	164	173	216	217	92	94	244	244	219	219
CFI12	118	120	158	239	197	197	164	173	216	216	90	90	244	244	219	219
CFI13	114	116	203	203	195	197	164	164	211	225	90	98	244	244	219	231
CFI14	118	136	197	243	185	199	164	164	221	225	90	90	244	244	219	219

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Table C.3: *continued from previous page*

ID	SPAC 11.5		SPAC 11.8		SPAG 7.14		PtTX3116		PtTX4001		ctg4363		ctg4698		ctg7731	
CFI15	136	146	183	219	185	215	146	173	209	225	88	98	244	247	221	223
CFI16	118	168	193	193	183	191	164	164	217	225	96	98	244	247	217	219
CFI17	114	114	235	235	193	193	164	164	217	219	90	90	244	244	219	219
CFI18	138	156	241	253	179	199	146	164	209	209	90	92	244	244	219	219
CFI19	118	118	178	181	195	195	164	164	216	217	96	96	244	244	217	217
CFI20	118	122	227	227	183	195	164	164	217	225	94	98	244	244	219	231
CFI21	118	120	175	175	183	183	173	179	225	225	90	92	244	247	219	233
CFII01	114	116	219	233	183	195	164	167	217	221	90	90	244	244	217	225
CFII02	118	120	247	247	183	195	164	164	209	225	90	96	244	244	219	219
CFII03	114	134	237	237	183	193	164	164	216	225	90	98	244	247	217	219
CFII04	122	128	217	251	183	205	167	176	217	221	92	94	244	247	219	221
CFII05	118	118	181	239	197	205	164	164	217	225	92	94	244	247	219	223
CFII06	120	126	178	178	185	195	164	164	216	217	90	92	244	244	219	223
CFII07	120	142	223	227	189	195	146	164	209	217	92	92	244	244	221	227
CFII08	114	122	177	177	199	199	164	164	221	225	90	96	244	244	221	221
CFII09	120	142	227	255	189	195	146	164	217	221	92	92	244	244	221	227
CFII10	98	122	197	221	173	185	146	173	209	221	92	94	244	247	219	219
CFII11	112	112	195	243	185	185	164	164	217	225	96	96	244	244	217	219
CFII12	116	120	237	247	183	195	164	170	209	209	96	98	244	244	217	219
CFII13	120	122	149	197	183	197	164	164	221	225	90	96	244	244	219	219
CFII14	112	146	217	263	195	197	146	164	223	225	90	90	244	247	219	219
CFII15	118	120	173	173	183	195	173	173	225	225	92	92	244	244	219	231
CFII16	120	156	219	219	175	183	164	173	219	225	90	90	244	244	219	223
CFII17	124	130	239	239	177	177	164	164	217	217	96	96	244	244	217	219
CFII18	120	156	221	227	179	211	164	173	209	211	98	100	244	244	217	219
CFII19	114	116	195	241	175	183	164	170			90	90	244	244	217	219
CFII20	114	116	164	219	185	199	164	164	216	225	94	98	244	247	219	219
CFIII01	142	150	227	243	179	203	164	164	209	216	90	92	244	247	219	223
CFIII02	146	152	176	199	179	183	164	164	217	221	92	96	244	244	219	223
CFIII03	116	166	203	229	177	195	167	176	217	225	90	92	244	247	221	221
CFIII04	136	142	237	237	195	195	164	170	217	221	90	96	244	244	219	221
CFIII05	120	122	178	213	185	187	164	164	225	225	96	98	244	244	219	219
CFIII06	122	144	217	241	191	207	146	170	203	221	96	100	244	244	219	219
CFIII07	134	134	233	233	177	203	164	173	217	221	94	100	247	247	219	221
CFIII08	148	186	164	239	199	203	164	164	221	221	96	102	244	244	217	219
CFIII09	120	138	197	265	191	195	146	146	209	217	90	96	244	244	219	219
CFIII10	114	114	227	231	177	195	146	164	209	217	90	92	244	247	219	223
CFIII11	136	142	237	237	183	185	164	170	217	221	90	92	244	247	219	219
CFIII12	122	158	199	199	185	207	170	173	217	217	90	96	244	244	219	219
CFIII13	118	132	172	199	179	183	164	164	217	221	100	100	244	247	219	219
CFIII14	114	114	203	231	177	195	146	164	209	209	92	96	244	247	219	223
CFIII15	126	152	203	273	183	185	173	173	221	221	100	100	244	244	217	219
CFIII16	154	154	203	263	185	187	164	173	221	221	94	96	244	244	219	221
CFIII17	122	122	199	199	185	207	170	173	217	221	90	96	244	244	219	219
CFIII18	128	142	213	243	189	203	164	164	216	216	92	92	247	247	219	225
CFIII19	122	122	176	217	183	207	164	173	217	221	90	92	244	247	219	219
CFIII20	118	118	229	255	193	193	173	173	221	221	90	90	247	247	219	219
CFIV01	122	158	217	241	183	185	173	173	217	221	90	90	244	244	219	219
CFIV02	118	122	182	199	197	207	164	173	216	217	90	92	244	250	219	221
CFIV03	114	136	241	243	187	205	170	173	211	217	90	98	244	244	219	219
CFIV04	116	122	168	181	185	187	146	164	203	217	90	96	244	244	219	223
CFIV05	116	122	217	249	187	207	173	176	217	217	90	92	244	244	219	223
CFIV06	116	118	178	203	187	197	164	164	217	221	90	96	244	244	219	221
CFIV07	158	168	197	209	183	207	146	164	216	221	96	96	244	244	219	221
CFIV08	140	142	197	197	185	185	164	170	225	225	92	98	244	244	217	219
CFIV09	116	122	197	215	177	187	164	164	217	217	92	98	244	244	219	219
CFIV10	118	122	197	197	175	183	164	164	217	225	94	96	244	244	219	219
CFIV11	118	156	223	263	185	187	146	173	211	211	96	96	244	244	219	219
CFIV12	150	156	199	225	179	197	164	176	221	221	90	92	244	247	219	219
CFIV13	116	122	178	235	177	177	164	176	209	209	92	92	244	244	221	229
CFIV14	106	132	237	237	183	185	164	164	211	211	90	90	244	244	219	219
CFIV15	122	152	205	205	197	197	164	164	209	209	96	96	244	244	219	219
CFIV16	124	152	239	249	183	183	176	176	209	221	90	98	244	244	219	219
CFIV17	118	124	205	231	185	185	170	173	209	217	90	90	244	244	219	223
CFIV18	124	142	217	219	201	201	155	170	217	225	92	102	244	244	219	221

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Table C.3: continued from previous page

ID	SPAC 11.5		SPAC 11.8		SPAG 7.14		PtTX3116		PtTX4001		ctg4363		ctg4698		ctg7731	
CFIV19	116	122	178	235	177	185	176	176	221	221	92	92	244	244	221	229
CFIV20	126	126	160	315	183	183	164	164	217	221	90	90	244	244	217	219
CG01	116	136	178	182	175	185	164	164	221	225	98	98	244	244	219	219
CG02	118	120	187	263	183	197	164	164	223	225	96	98	244	244	219	219
CG03	116	134	179	235	175	177	164	164	209	209	92	96	244	244	217	225
CG04	98	116	172	231	187	201	164	164	217	221	92	100	247	250	217	219
CG05	118	118	164	253	203	207	173	173	217	223	90	102	244	253	219	223
CG06	98	98	189	235	185	187	164	173	209	217	96	96	244	244	219	219
CG07	108	146	207	247	177	183	173	173	211	221	98	100	244	244	217	219
CG08	154	192	211	231	183	217	164	164	211	217	96	98	244	244	219	219
CG09	116	118	179	233	177	185	173	173	217	217	90	92	244	244	219	221
CG10	138	152	197	249	183	183	164	173	209	211	90	90	244	244	219	221
CG11	120	156	227	237	179	211	164	164	211	217	90	98	244	247	217	219
CG12	124	152	225	247	183	185	164	173	217	225	90	98	244	247	219	223
CG13	128	154	172	197	183	183	164	173	216	221	96	98	244	244	217	219
CG14	98	136	197	235	185	191	164	173	211	211	96	96	244	247	219	221
CG14bis	126	136	197	223	177	191	164	164	209	211	96	96	244	247	219	221
CG15	152	152	170	229	177	199	164	173	216	217	96	96	244	247	215	219
CG16	108	142	229	235	175	185	164	173	216	217	96	96	244	244	219	225
CG17	116	122	176	179	177	177	164	173	221	225	92	96	244	244	217	219
CG18	118	168	179	251	183	201	164	164	217	225	90	90	244	244	219	221
CG19	122	148	217	241	191	205	164	164	221	225	90	100	244	247	217	219
CG20	136	136	197	225	183	191	164	173	211	217	96	98	247	253	221	221
CG21	118	120	175	175	207	221	164	164	225	225	90	96	244	247	221	225
CG22	120	122	177	219	183	183	164	164	209	225	98	98	244	244	219	219
CG23	118	136	197	215	177	201	146	173	217	221	90	98	244	244	219	219
CG24	116	118	180	197	201	201	164	173	217	225	92	98	244	244	219	219
CG25	150	178	172	265	177	201	164	164	209	221	88	90	247	247	219	219
CG26	134	136	176	179	183	187	137	137	216	217	90	90	244	247	219	221
CG27	124	156	211	235	179	191	164	164	209	216	98	100	244	244	217	219
CG28	132	158	178	253	183	199	164	179	221	221	90	98	250	253	219	219
CG29	112	160	195	217	185	207	164	164	217	221	92	96	244	244	217	219
CG30	132	156	227	263	175	179	170	173	211	217	98	100	244	247	217	219
CG31	120	154	211	245	177	183	170	173	217	225	96	100	244	244	217	231
CG32	140	168	219	219	183	195	164	164	209	225	90	92	244	244	219	219
CG33	144	156	211	227	175	191	164	173	211	211	90	92	244	244	216	219
CG34	114	152	197	219	185	191	164	164	211	211	90	96	244	247	221	223
CG35	98	132	162	211	185	191	164	173	216	217	92	98	244	244	216	219
CG36	114	116	197	209	191	205	164	164	209	217	92	100	244	244	219	219
CG37	132	132	235	235	179	185	164	170	221	225	96	100	244	244	217	219
CG38	122	136	235	235	177	185	146	164	217	221	94	96	244	244	219	219
CG39	116	122	182	182	185	193	173	173	221	221	92	94	244	247	219	225
CG40	98	158	174	253	185	187	164	164	217	221	96	98	244	247	219	221
CG41	116	136	227	235	177	187	164	176	217	217	98	100	244	244	217	219
CG42	120	122	145	145	215	215	173	173	217	225	90	92	244	244	219	221
CG43	114	122	175	183	183	205	173	173	219	225	88	100	244	244	219	219
CG44	112	116	195	243	185	185	164	164	217	225	90	90	244	244	219	219
CG45	108	142	207	263	177	185	164	173	211	221	92	100	244	244	217	219
CG46	118	118	169	184	185	207	164	173	217	225	94	94	244	244	221	225
CH01	118	132			177	195	146	164	209	216						
CH02	132	142			195	205	164	173	209	221						
CH03	114	182			179	197	164	167	209	209						
CH04	118	150			187	187	164	164	221	225						
CH05	120	184					170	182	217	221						
CH06	114	134					146	164	217	217						
CH07	98	114					146	173	209	217						
IF01	140	158	197	237	185	185	173	173	217	225	94	96	244	244	219	225
IF02	108	114	180	231	179	187	164	173	217	225	94	96	244	244	219	219
IF03	108	132	221	221	185	191	164	164	209	211	92	96	244	244	217	219
IF04	110	118	207	231	191	201	164	164	217	221	90	102	244	244	219	221
IF05	114	118	168	221	183	185	146	164	211	217	96	100	244	244	219	219
IF06	108	126	231	231	175	183	164	164	221	221	94	102	244	244	217	219
IF07	118	126	201	231	183	207	173	173	211	217	90	92	244	247	219	219
IF08	108	110	168	237	185	201	146	173	209	221	92	96	244	247	219	221

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Table C.3: *continued from previous page*

ID	SPAC 11.5		SPAC 11.8		SPAG 7.14		PtTX3116		PtTX4001		ctg4363		ctg4698		ctg7731	
IF09	108	156	168	221	185	195	173	173	211	217	96	96	247	247	219	219
IF10	108	152	168	235	191	195	164	164	217	221	92	96	244	247	219	219
IF11	118	128	231	237	185	201	164	164	209	221	96	102	244	247	219	221
IF12	114	142	197	237	185	185	164	173	209	217	92	100	244	247	219	225
IF13	108	128	207	231	191	201	164	164	217	221	90	102	244	244	219	221
IF14	108	114	231	239	179	197	173	173	209	211	96	96	244	244	219	219
IF15	118	132	231	231	185	185	164	164	211	217	90	96	244	247	217	219
IF16	108	120	201	231	179	189	164	173	217	225	92	96	244	247	219	231
IF17	120	122	201	245	179	185	146	173	211	217	92	96	244	247	217	231
IF18	120	132	201	231	189	201	164	173	216	225	92	96	244	247	219	231
IF19	108	126	201	249	175	189	164	173	217	225	90	96	247	247	219	231
IF20	118	178	233	233	179	191	164	173	217	225	96	96	244	247	219	223
IF21	120	122	221	231	189	191	164	173	217	217	90	96	244	244	219	219
IF22	108	120	201	219	187	189	164	164	217	217	92	96	247	247	219	231
IF23	108	134	205	231	183	189	164	173	209	217	92	98	244	247	219	221
IF24	114	156	174	181	185	197	164	173	217	217	90	94	244	247	219	225
IF25	118	146	215	227	183	189	170	173	211	217	90	96	244	244	219	225
IF26	118	118	219	237	191	207	146	173	216	225	90	90	244	244	219	219
IF27	116	130	215	229	175	183	164	173	217	221	90	92	244	244	217	219
IF28	118	130	215	231	179	183	146	146	209	216	90	90	244	244	217	221
IF29	124	130	215	249	177	177	164	164	217	217	94	102	244	244	219	221
IF30	118	146	219	231	191	207	164	173	216	225	90	90	244	253	219	221
IF31	118	120	219	239	197	207	164	176	216	217	96	96	247	247	219	221
IF32	130	146	215	215	183	189	146	173	209	217	90	98	244	244	217	225
IF33	118	146	211	215	189	207	146	164	209	225	92	98	244	244	219	225
IF34	134	142	197	229	191	201	164	179	217	225	96	98	244	244	219	219
IF36	120	150	233	235	185	187	164	164	225	225	96	100	244	244	217	219
IF37	120	120	225	237	183	185	164	164	211	217	90	90	244	244	219	219
IF38	122	150	237	237	187	195	164	173	225	225	90	96	244	244	219	219
IF39	120	120	221	233	185	185	164	164	217	217	90	96	244	244	217	219
IF40	114	160	195	237	177	185	164	173	216	217	96	100	244	247	217	225
LG01	124	136	229	237	185	189	164	164	216	217	96	96	244	244	217	221
LG02	124	164	251	251	191	191	164	173	217	225	92	96	235	244	219	221
LG03	114	118	197	259	183	183	164	164	211	216	96	96	244	244	225	233
LG04	118	122	197	197	183	185	164	164	209	211	90	96	244	247	221	225
LG05	118	142	197	259	183	183	164	164	211	225	96	96	244	253	219	233
LG06	114	164	164	241	183	191	164	164	209	225	92	102	244	247	217	223
LG07	120	136	219	237	185	211	146	164	211	217	92	92	235	244	221	225
LG08	120	120	223	237	185	191	146	164	217	217	92	96	244	244	217	221
LG09	120	124	243	247	185	191	164	164	209	209	96	102	247	247	217	219
LG10	120	120	237	237	191	191	146	146	211	217	92	92	244	244	221	221
LG11	120	162	189	241	183	191	164	173	211	221	90	92	244	250	217	233
LG12	120	150	189	197	185	185	146	164	211	221	90	92	244	244	217	219
LG15	120	174	189	189	191	201	146	173	217	217	90	92	244	244	217	219
LG16	116	118	170	231	185	187	164	164	221	225	90	96	244	247	219	219
LG17	114	136	197	241	183	185	164	164	209	225	90	90	244	247	219	227
LG18	136	142	197	239	185	207	164	164	209	216	90	96	244	247	219	221
LG19	120	146	235	237	185	185	146	146	217	225	92	94	244	244	221	225
LG20	120	158	174	189	185	191	146	164	211	217	92	102	244	244	217	219
LG21	120	136	219	237	185	185	164	173	217	225	90	92	235	244	221	225
LG22	124	158	219	237	185	195	164	164	216	225	96	96	244	244	219	221
LG23	118	136	237	273	185	185	164	173	216	217	92	92	244	244	219	221
LG24	118	136	237	259	205	205	164	164	209	216	90	92	244	244	221	233
LG25	126	140	189	247	191	197	164	170	211	225	92	96	244	244	217	219
LG26	118	152	245	259	183	185	164	164	209	221	90	92	244	244	219	233
LG27	166	166	197	213	187	189	164	164	217	217	96	102	244	244	219	225
LG28	118	134	245	259	183	191	164	179	221	225	96	96	244	244	219	233
LG29	118	156	164	172	185	205	164	164	211	217	90	90	244	244	217	219
LG30	122	152	197	239	187	191	164	173	211	221	96	100	244	244	219	219
LG31	154	154	215	223	183	185	164	173	217	217	90	96	244	250	219	219
LG32	124	160	151	237	187	191	164	164	211	225	90	98	244	247	219	223
LG33	116	130	233	237	191	203	164	164	203	221	96	100	244	244	219	221
LG34	114	118	209	245	187	207	164	173	211	217	96	98	244	244	217	219
LG35	132	140	215	231	177	205	146	164	211	221	90	90	232	244	219	221
LG36	148	182	219	219	217	217	167	173	209	225	90	96	247	250	217	221

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Table C.3: *continued from previous page*

ID	SPAC 11.5		SPAC 11.8		SPAG 7.14		PtTX3116		PtTX4001		ctg4363		ctg4698		ctg7731	
LG37	138	176	211	217	185	187	164	173	225	225	90	98	244	244	219	219
LG38	122	130	193	263	175	201	164	164	217	225	94	98	244	244	219	219
LG39	118	150	239	259	187	207	164	173	211	225	90	96	244	247	219	233
LG40	120	154	174	217	195	215	164	173	209	216	90	94	244	244	219	219
MAI01	114	118	197	245	183	205	164	164	217	221	94	96	244	244	217	221
MAI02	120	182	201	229	183	185	164	164	209	216	90	102	244	244	219	219
MAI03	118	122	187	221	183	205	164	164	209	217	90	94	244	244	219	219
MAI04	114	118	187	223	197	205	164	173	209	211	94	100	244	247	219	219
MAI05	156	156	170	235	177	185	164	173	211	221	90	96	244	247	219	219
MAI06	120	136	207	207	183	191	164	173	209	225	98	104	244	244	219	219
MAI07	122	138	151	245	187	205	164	164	221	225	90	98	244	244	219	233
MAI08	118	138	205	245	183	205	146	164	209	225	92	104	244	244	223	235
MAI09	138	154	197	249	175	185	164	164	203	217	98	100	244	247	217	221
MAI10	126	136	179	179	183	207	164	173	221	221	90	98	244	247	219	221
MAI11	116	118	166	170	175	185	164	164	209	217	96	102	244	247	219	219
MAI12	116	120	207	237	177	179	164	173	216	217	92	102	244	244	219	219
MAI13	116	118	166	205	177	195	164	164	216	217	92	96	244	244	219	219
MAI14	116	142	166	207	175	177	173	173	216	217	92	102	244	247	219	219
MAI15	116	116	166	205	175	185	131	173	217	225	92	96	244	244	219	219
MAI16	116	116	166	170	177	201	164	173	217	221	98	102	244	247	219	219
MAI17	116	116	166	235	177	187	170	170	217	217	92	102	241	247	219	219
MAI18	118	118	164	213	179	195	164	173	211	217	92	96	244	244	219	219
MAI19	120	142	207	249	175	187	164	164	216	221	90	92	244	247	219	221
MAI20	116	116	166	229	183	183	173	173	209	217	92	102	244	244	219	219
MAI21	120	142	223	249	183	187	164	173	209	221	90	98	244	244	219	233
MAI22	120	122	245	249	187	207	164	164	221	221	90	96	244	244	221	233
MAI23	120	134	197	225	193	207	146	164	217	217	90	96	244	247	219	219
MAI24	110	140	166	215	187	189	164	173	209	209	98	104	244	244	219	219
MAI25	110	118	166	257	183	189	164	173	209	223	92	104	244	250	219	221
MAI26	110	128	197	235	185	217	131	173	209	209	96	104	244	250	219	223
MAI27	118	140	166	255	185	187	164	164	209	221	98	98	244	244	217	219
MAI28	122	134	197	215	189	193	146	173	217	217	96	104	247	250	219	223
MAI29	134	172	197	203	189	197	173	173	209	217	90	104	244	250	219	221
MAI30	120	138	197	197	185	197	173	173	209	217	92	92	244	244	219	221
MAI31	134	140	166	247	183	189	164	173	217	217	100	110	244	244	219	219
MAI32	134	138	197	197	185	189	173	173	221	221	90	104	244	244	217	223
MAI33	122	128	235	245	177	217	164	173	209	209	94	96	244	244	219	219
MAI34	126	134	166	255	177	189	173	176	217	225	98	104	244	250	219	219
MAI35	110	140	166	215	185	187	164	173	209	209	98	110	244	244	219	219
MAI36	114	134	166	257	183	189	146	173	221	221	92	104	244	250	219	221
MAI37	134	176	166	178	185	195	164	173	209	217	98	110	244	250	219	225
MAI38	142	142	197	229	185	185	173	176	221	221	90	90	244	244	223	223
MAI39	110	150	166	215	189	191	164	173	209	217	94	110	244	247	219	219
MAI40	118	122	213	229	195	207	164	164	209	217	92	94	247	247	219	221
MAII01	114	148	221	245	179	207	164	164	221	221	92	96	244	250	219	221
MAII02	130	154	207	219	187	191	158	164	209	209	92	98	244	244	219	229
MAII03	114	142	207	219	185	191	158	173	209	221	92	96	244	244	221	229
MAII04	116	154	219	235	179	187	164	164	209	217	92	96	247	250	219	229
MAII05	116	124	195	209	175	185	173	173	216	217	94	98	244	247	219	221
MAII06	114	162	219	225	179	187	158	164	216	216	92	98	244	244	221	221
MAII07	116	130	195	247	185	215	164	167	216	221	90	98	244	247	219	221
MAII08	160	160	223	227	183	187	146	164	209	209	90	92	244	247	219	219
MAII09	144	154	219	245	187	191	158	164	216	217	92	96	244	244	217	229
MAII10	118	122	217	219	183	195	164	164	225	225	90	98	244	247	219	221
MAII11	154	174	219	241	179	179	146	158	217	221	92	96	244	244	219	229
MAII12	118	132	223	231	177	213	164	173	217	225	92	94	244	247	217	219
MAII13	114	116	178	245	185	191	164	164	209	217	92	98	244	244	219	219
MAII14	126	132	209	235	185	191	164	164	209	216	90	98	244	244	219	221
MAII15	116	120	221	235	185	207	164	173	203	221	98	100	244	244	219	219
MAII16	120	146	233	243	197	203	164	164	217	217	96	102	244	244	219	225
MAII17	118	120	223	245	187	213	164	173	209	217	90	92	244	244	217	219
MAII18	116	124	197	229	175	201	173	182	209	216	92	98	244	244	219	225
MAII19	156	156	219	245	179	183	158	173	209	217	92	96	247	250	225	229
MAII20	116	118	195	217	183	185	164	173	216	217	92	96	244	247	219	219

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Table C.3: *continued from previous page*

ID	SPAC 11.5		SPAC 11.8		SPAG 7.14		PtTX3116		PtTX4001		ctg4363		ctg4698		ctg7731	
MAH21	116	126	201	201	187	191	164	164	217	217	90	98	244	247	219	219
MAH22	114	160	170	245	179	243	158	173	209	216	90	92	244	244	219	221
MAH23	124	154	180	217	187	213	164	164	221	225	90	90	244	247	217	219
MAH24	118	120	166	197	183	211	164	173	217	217	90	92	244	244	219	221
MAH25	118	120	209	247	185	213	146	173	216	216	92	96	244	244	219	231
MAH26	118	158	219	221	191	213	164	173	211	221	92	98	244	247	219	219
MAH27	126	142	197	229	183	201	164	164	216	216	94	94	244	244	219	221
MAH28	118	132	223	227	207	213	173	173	209	225	92	100	244	244	219	219
MAH29	118	154	209	209	187	207	164	164	211	217	90	96	244	244	219	221
MAH30	118	120	215	229	185	187	164	164	209	217	94	96	244	244	219	219
MAH31	118	118	223	247	213	245	164	173	209	221	92	100	244	244	219	227
MAH32	116	126	227	235	195	213	173	173	203	209	90	94	244	244	219	219
MAH33	118	118	223	229	179	195	164	173	209	217	90	98	244	244	219	223
MAH34	118	146	231	315	197	213	173	173	217	221	94	98	244	247	219	221
MAH35	118	144	233	315	195	207	164	173	209	216	90	92	244	244	219	221
MAH36	118	152	223	235	183	213	173	173	209	217	90	98	244	244	219	219
MAH37	118	162	215	223	187	213	161	173	217	221	92	96	244	247	219	219
MAH38	120	160	217	245	187	207	164	164	216	217	96	96	244	244	217	219
MAH39	118	174	197	241	185	197	164	173	209	221	92	94	244	244	219	219
MAH40	114	118	199	223	183	195	164	173	209	217	90	92	244	244	219	219
PP01	138	146	205	219	177	185	164	179	209	225	90	102	244	250	219	221
PP02	118	146	213	247	187	195	164	164	217	221	90	98	244	244	219	219
PP03	122	134	209	227	183	191	164	164	221	225	92	96	244	244	219	221
PP04	114	116	213	227	183	197	164	170	209	217	96	100	244	247	219	223
PP05	118	144	251	253	195	195	173	179	221	221	96	96	244	244	216	217
PP06	116	168	213	239	197	205	170	173	209	217	92	96	244	247	219	219
PP07	114	148	213	213	197	197	164	170	209	217	90	100	244	247	217	223
PP08	134	160	217	217	183	183	164	164	225	225	90	98	244	244	219	219
PP09	116	144	213	251	183	183	170	173	217	221	96	100	244	247	217	223
PP10	114	116	227	227	185	197	146	164	211	217	90	96	244	247	219	223
PP11	118	126	237	239	177	207	146	173	209	217	92	92	244	247	219	219
PP12	120	146	203	239	183	185	164	173	217	221	96	102	244	247	217	219
PP13	118	150	197	231	191	207	164	164	217	221	90	96	244	244	219	219
PP14	126	140	237	249	207	207	146	164	211	217	92	94	244	247	219	219
PP15	132	156	197	319	175	177	164	164	209	217	90	100	244	244	219	219
PP16	136	192	197	241	185	187	173	182	209	217	94	96	244	244	215	221
PP17	136	136	211	223	177	185	164	167	209	217	94	96	244	247	219	221
PP18	138	160	175	197	183	185	146	164	216	216	90	96	244	244	219	219
PP19	116	118	233	233	185	187	164	164	217	225	92	96	244	247	221	221
PP20	116	118	235	243	183	191	164	173	217	217	90	96	244	247	221	225
PP21	98	98	197	249	183	205	164	164	221	221	90	90	244	244	219	219
PP22	98	158	233	249	183	185	164	173	221	221	90	98	244	247	219	219
PP23	98	154	197	229	183	185	146	164	211	221	90	98	244	244	219	221
PP24	98	128	197	243	183	191	164	176	217	221	96	96	244	244	219	221
PP25	98	146	235	249	179	183	164	164	221	225	90	96	244	247	219	223
PP26	124	158	231	243	177	207	146	167	217	225	90	100	244	247	217	217
PP27	118	128	217	243	177	195	164	173	217	221	90	92	244	244	219	219
PP28	114	140	217	219	183	195	164	164	216	225	96	96	244	244	219	223
PP29	144	166	219	237	191	195	146	176	216	221	90	96	244	244	223	225
PP30	138	168	217	239	177	197	164	173	221	225	92	102	244	244	217	231
PP31	116	136	164	231	179	185	173	173	221	225	96	98	244	244	219	219
PP32	116	144	227	249	179	185	164	173	209	225	92	96	244	244	219	219
PP33	98	120	219	235	183	187	164	164	216	221	92	96	244	253	217	223
PP34	116	158	231	247	179	187	173	176	211	225	92	92	244	244	219	219
PP35	144	156	197	217	185	185	164	164	209	216	80	92	244	244	219	219
PP36	118	120	197	241	185	207	164	164	217	217	92	102	244	244	219	219
PP37	126	146	213	241	175	187	146	164	216	217	90	92	244	244	221	225
PP38	118	122	221	227	207	219	146	164	211	217	92	98	244	247	219	219
PP39	126	148	213	241	175	183	164	173	216	225	92	96	244	244	219	225
PP40	120	122	197	237	205	215	170	173	209	217	90	92	244	244	219	221
PP41	136	154	182	223	183	183	164	164	211	221	90	96	244	244	221	223
PP42	136	152	197	223	183	191	146	164	221	221	90	96	244	244	219	221
PP43	114	154	164	223	183	203	146	164	209	211	90	96	244	244	219	221
PP44	136	154	223	223	183	183	164	164	211	221	90	96	244	244	221	223
PP45	130	154	223	241	183	195	146	164	221	221	96	102	244	244	219	221

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Table C.3: *continued from previous page*

ID	SPAC 11.5		SPAC 11.8		SPAG 7.14		PtTX3116		PtTX4001		ctg4363		ctg4698		ctg7731	
PP46	128	136	182	215	183	183	164	164	209	221	90	96	244	244	219	223
PP47	118	134	197	245	179	205	146	167	217	217	90	96	244	247	221	221
PP48	138	156	211	223	177	183	146	164	209	221	90	94	244	244	219	221
PP49	134	134	166	182	175	183	164	164	211	217	94	96	244	244	223	231
PP50	120	154	178	182	183	185	164	164	211	217	94	96	244	244	223	223
VL01	118	140	150	239	201	203	164	176	209	225	96	96	244	244	219	219
VL02	132	136	199	237	179	191	146	146	209	221	92	94	244	244	219	223
VL03	130	156	203	217	177	183	164	164	217	217	92	96	244	247	219	221
VL04	118	128	215	225	185	197	164	164	209	221	90	94	244	244	219	219
VL05	122	132	176	237	185	201	146	164	209	209	94	98	244	244	221	223
VL06	120	120	195	235	183	207	164	164	209	217	94	96	244	247	219	219
VL07	122	144	215	217	185	185	173	173	216	221	94	100	244	244	219	221
VL08	128	128	199	215	183	197	164	164	217	225	90	94	244	244	219	221
VL09	120	120	215	237	183	187	164	170	209	217	94	96	244	247	219	221
VL10	132	154	215	231	183	183	164	164	216	225	94	96	244	244	219	221
VL11	114	136	235	235	183	207	164	164	211	217	96	98	244	244	217	219
VL12	114	114	217	235	183	187	164	173	211	221	96	98	244	244	219	219
VL13	114	128	199	199	183	207	164	164	217	217	90	98	244	244	217	219
VL14	142	154	235	247	191	203	164	179	209	221	96	96	244	244	217	219
VL15	154	190	231	235	183	191	164	164	211	225	90	94	244	244	219	219
VL16	136	136	229	239	183	191	164	173	217	225	90	90	244	247	221	223
VL17	124	170	197	247	179	179	164	164	217	225	90	94	244	247	217	219
VL18	118	122	195	225	185	207	164	173	211	217	94	96	244	250	219	219
VL19	120	134	195	227	175	197	164	179	211	217	92	96	247	247	219	219
VL20	120	120	225	235	183	183	164	164	211	217	92	92	244	244	219	221
VL21	136	146	162	170	185	195	164	164	221	225	96	98	244	244	223	229
VL22	120	156	197	205	177	195	146	164	217	225	90	96	244	244	219	221
VL23	114	120	205	229	183	201	164	164	217	221	98	100	244	244	219	219
VL24	120	120	215	215	185	187	164	164	221	225	92	96	244	247	219	221
VL25	120	154	205	243	183	197	164	176	209	225	96	98	244	244	219	221
VL26	114	134	178	205	183	183	164	170	211	217	96	98	244	247	219	219
VL27	120	144	172	175	197	207	164	164	217	217	92	96	244	247	217	223
VL28	114	118	150	241	203	207	164	164	209	225	96	96	244	244	219	219
VL29	148	172	197	241	177	207	164	164	217	225	92	96	244	244	219	219
VL30	114	164	205	217	183	183	164	170	209	217	92	96	244	250	219	225
VL30bis	122	130	197	227	183	185	164	173	217	217	90	100	244	244	217	219
VL31	118	140	150	253	183	187	164	164	209	209	90	96	244	244	219	219
VL32	114	154	166	235	183	201	173	173	221	225	90	96	244	244	217	219
VL33	142	168	176	231	191	195	173	176	225	225	96	96	244	244	219	219
VL34	118	154	197	235	177	183	146	164	217	221	92	96	244	244	219	219
VL35	120	122	243	245	183	185	164	173	209	217	90	92	244	244	219	219
VL35bis	118	122	217	245	185	197	164	173	209	217	94	98	244	244	219	223
VL36	114	118	197	197	183	185	146	164	211	217	90	92	244	244	219	219
VL37	116	146	219	249	191	197	164	173	211	221	94	96	244	244	217	219
VL38	154	158	215	251	175	191	164	173	217	221	90	96	244	244	219	227
VL39	140	188	245	247	189	191	164	164	217	221	92	98	244	244	219	225
VL40	118	120	166	235	175	175	146	173	211	221	92	96	244	244	219	227

## D. SOFTWARE

ARLEQUIN: Program created by Schneider et al. (1999) to perform several genetic analysis on different type of genetic data.

Download at <http://lgb.unige.ch/arlequin/>

CONVERT: Program created by Glaubitz (2004) to produce input files for a several common programs for population genetic analysis.

Download at [http://www.agriculture.purdue.edu/fnr/html/faculty/Rhodes/Students and Staff/glaubitz/software.htm](http://www.agriculture.purdue.edu/fnr/html/faculty/Rhodes/Students_and_Staff/glaubitz/software.htm)

FSTAT: Program created by Goudet (1995) for computation of genetic diversity and genetic differentiation indices.

Download at <http://www2.unil.ch/izea/software/fstat.html>

GENEPOP: Program created by Raymond and Rousset (1995b) for computation of exact tests (for Hardy-Weinberg equilibrium, etc), estimates of genetic parameters and conversion of input files to formats for other genetic analysis software.

Download at <http://wbiomed.curtin.edu.au/genepop/>

ISOLATION BY DISTANCE: Program created by Bohonak (2002) and Jensen et al. (2005) to study isolation by distance. It performs  $F_{ST}$  calculations and Mantel test.

Web service available at <http://phage.sdsu.edu/~jensen/>

IMMANC: Program created by Rannala and Mountain (1997) to detect migrants by means of assignment methods.

Download at <http://www.rannala.org/labpages/software.html>

ISLANDMODELTEST: Program created by Porter (2003) to test departure from the island model of gene flow.

Download at <http://www-unix.oit.umass.edu/~aporter/software/>

MICRO-CHECKER: Program created by Van Oosterhout et al. (2004) to detect genotyping errors due to null alleles, large allele dropout and stuttering in microsatellite data.

Download at <http://www.microchecker.hull.ac.uk/>

MICROSATELLITE TOOLKIT: Microsoft Excel<sup>®</sup> add-in created by Park (2001) to produce input files for several population genetic analysis programs and to perform basic checks on data and genetic diversity calculations.

Download at <http://oscar.gen.tcd.ie/~sdepark/ms-toolkit/index.php>

MLNE: Program created by Wang and Whitlock (2003) to estimate effective population size and immigration rate from differences in allele frequency in temporal samples of the same population.

Download at <http://www.zoo.cam.ac.uk/ioz/software.htm>



## BIBLIOGRAPHY

- J. R. Aboal, M. S. Jiménez, D. Morales, and J. M. Hernández. Rainfall interception in laurel forest in the Canary Islands. *Agricultural and Forest Meteorology*, 97(2):73–86, 1999. URL [http://dx.doi.org/10.1016/S0168-1923\(99\)00083-0](http://dx.doi.org/10.1016/S0168-1923(99)00083-0).
- J. R. Aboal, M. S. Jiménez, and D. Morales. Evaluación de la precipitación de niebla a través de la precipitación penetrante. *Vieraea*, 28:15–29, 2000a.
- J. R. Aboal, M. S. Jiménez, D. Morales, and P. Gil. Effects of thinning on throughfall in Canary Islands pine forest — The role of fog. *Journal of Hydrology*, 238(3–4):218–230, 2000b. URL [http://dx.doi.org/10.1016/S0022-1694\(00\)00329-2](http://dx.doi.org/10.1016/S0022-1694(00)00329-2).
- W. T. Adams and J. Burczyk. Magnitude and implications of gene flow in gene conservation reserves. In A. G. Young, D. H. Boshier, and T. J. Boyle, editors, *Forest Conservation Genetics. Principles and Practice*, pages 215–224. CSIRO Publishing & CABI Publishing, Oxon, United Kingdom, 2000.
- J. M. Afonso, A. Volz, M. Hernández, H. Ruttkay, M. González, J. M. Larruga, V. M. Cabrera, and D. Sperlicht. Mitochondrial DNA variation and genetic structure in Old-World populations of *Drosophila subobscura*. *Molecular Biology and Evolution*, 7(2):123–142, 1990. URL <http://mbe.oxfordjournals.org/cgi/content/abstract/7/2/123>.
- J. K. Agee. Fire and pine ecosystems. In D. M. Richardson, editor, *Ecology and Biogeography of Pinus*, pages 193–218. Cambridge University Press, Cambridge, United Kingdom, 1998.
- F. Aguilera Klink, A. Brito Hernández, C. Castilla Gutiérrez, A. Díaz Hernández, J. M. Fernández-Palacios, A. Rodríguez Rodríguez, F. Sabaté Bel, and

- J. Sánchez García. *Canarias: Economía, Ecología y Medio Ambiente*. Francisco Lemus Editor S.L., La Laguna, Tenerife, Spain, 1993.
- M. R. Ahuja. Recent advances in molecular genetics of forest trees. *Euphytica*, 121(2):173–195, 2001. URL <http://dx.doi.org/10.1023/A:1012226319449>.
- G. J. Allan, J. Francisco-Ortega, A. Santos-Guerra, E. Boerner, and E. A. Zimmer. Molecular phylogenetic evidence for the geographic origin and classification of Canary Island *Lotus* (Fabaceae: Loteae). *Molecular Phylogenetics and Evolution*, 32(1):123–138, 2004. URL <http://dx.doi.org/10.1016/j.ympev.2003.11.018>.
- I. Allona, J. A. Saiz-Omenaca, R. Casado, and C. Aragoncillo. Megagametophyte salt-soluble proteins as genetic markers in *Pinus pinaster* Ait. *Silvae Genetica*, 45(1):21–24, 1996. URL <http://www.bfafh.de/inst2/sg45pdf.htm>.
- J. J. Alonso Blanco. *Estudio Volcanoestratigráfico y Volcanológico de los Piroclastos Sálidos del Sur de Tenerife*. Secretariado de Publicaciones. Universidad de La Laguna, La Laguna, Tenerife, Spain, 1989.
- J. B. Álvarez, M. J. Toledo, B. Abellanas, and L. M. Martín. Use of megagametophyte storage proteins as markers of the genetic diversity in stone pine (*Pinus pinea* L.) in Andalucía, Spain. *Genetic Resources and Crop Evolution*, 51(6):621–627, 2004. URL <http://dx.doi.org/10.1023/B:GRES.0000024647.74194.4e>.
- E. Ancochea, J. M. Fúster, E. Ibarrola, A. Cendrero, J. Coello, F. Hernán, J. M. Cantagrel, and C. Jamond. Volcanic evolution of the island of Tenerife (Canary Islands) in the light of new K–Ar data. *Journal of Volcanology and Geothermal Research*, 44(3–4):231–249, 1990. URL [http://dx.doi.org/10.1016/0377-0273\(90\)90019-C](http://dx.doi.org/10.1016/0377-0273(90)90019-C).
- E. Ancochea, M. J. Huertas, J. M. Cantagrel, J. Coello, J. M. Fúster, N. Arnaud, and E. Ibarrola. Evolution of the Cañadas edifice and its implications for the origin of the Cañadas Caldera (Tenerife, Canary Islands). *Journal of Volcanology and Geothermal Research*, 88(3):177–199, 1999. URL [http://dx.doi.org/10.1016/S0377-0273\(98\)00106-1](http://dx.doi.org/10.1016/S0377-0273(98)00106-1).

- E. C. Anderson, E. G. Williamson, and E. A. Thomson. Monte Carlo evaluation of the likelihood of  $N_e$  from temporally spaced samples. *Genetics*, 156(4): 2109–2118, 2000. URL <http://www.genetics.org/cgi/content/abstract/156/4/2109>.
- F. Anguita and F. Hernán. A propagating fracture model versus a hot spot origin for the Canary Islands. *Earth and Planetary Science Letters*, 27(1):11–19, 1975. URL [http://dx.doi.org/10.1016/0012-821X\(75\)90155-7](http://dx.doi.org/10.1016/0012-821X(75)90155-7).
- F. Anguita and F. Hernán. The Canary Islands origin: a unifying model. *Journal of Volcanology and Geothermal Research*, 103(1–4):1–26, 2000. URL [http://dx.doi.org/10.1016/S0377-0273\(00\)00195-5](http://dx.doi.org/10.1016/S0377-0273(00)00195-5).
- F. Anguita and F. Hernán. El origen de las Islas Canarias: un modelo de síntesis. *Enseñanza de las Ciencias de la Tierra*, 7(3):254–261, 2003. URL [http://usuarios.lycos.es/aepect/astenosfera/documentos/D0C\\_9.htm](http://usuarios.lycos.es/aepect/astenosfera/documentos/D0C_9.htm).
- V. Araña and R. Ortiz. Marco geodinámico del volcanismo canario. *Anales de Física*, 82:202–231, 1986. Special number.
- J. R. Arévalo and J. M. Fernández-Palacios. Gradient analysis of exotic *Pinus radiata* plantations and potential restoration of natural vegetation in Tenerife, Canary Islands (Spain). *Acta Oecologica*, 27(1):1–8, 2005. URL <http://dx.doi.org/10.1016/j.actao.2004.08.003>.
- J. R. Arévalo, J. M. Fernández-Palacios, M. J. Jiménez, and P. Gil. The effect of fire intensity on the understorey species composition of two *Pinus canariensis* reforested stands in Tenerife (Canary Islands). *Forest Ecology and Management*, 148(1–3):21–29, 2001. URL [http://dx.doi.org/10.1016/S0378-1127\(00\)00478-3](http://dx.doi.org/10.1016/S0378-1127(00)00478-3).
- S. Aris-Brosou and L. Excoffier. The impact of population expansion and mutation rate heterogeneity on DNA sequence polymorphism. *Molecular Biology and Evolution*, 13(3):494–504, 1996. URL <http://mbe.oupjournals.org/cgi/content/abstract/13/3/494>.
- M. A. Arnedo and C. Ribera. Radiation of the genus *Dysdera* (Araneae, Dysderidae) in the Canary Islands: the island of Tenerife. *The Journal of*

- Arachnology*, 27(3):604–662, 1999. URL [http://www.americanarachnology.org/JoA\\_tocs/JOA\\_v27n3.html#604](http://www.americanarachnology.org/JoA_tocs/JOA_v27n3.html#604).
- M. A. Arnedo, P. Oromí, and C. Ribera. Radiation of the spider genus *Dysdera* (Araneae, Dysderidae) in the Canary Islands: cladistic assessment based on multiple data sets. *Cladistics*, 17(4):313–353, 2001. URL <http://dx.doi.org/10.1006/clad.2001.0168>.
- N. P. Ashmole and M. J. Ashmole. Insect dispersal on Tenerife, Canary Islands: high altitude fallout and seaward drift. *Arctic and Alpine Research*, 20(1):1–12, 1988. URL <http://www.csa.com/partners/viewrecord.php?requester=gs&collection=ENV&recid=1716210>.
- N. P. Ashmole, P. Oromí, M. J. Ashmole, and J. L. Martin. Primary faunal succession in volcanic terrain: lava and cave studies on the Canary Islands. *Biological Journal of the Linnean Society*, 46(1–2):207–234, 1992. URL <http://md1.csa.com/partners/viewrecord.php?requester=gs&collection=ENV&recid=2815181>.
- L. Auckland, T. Bui, Y. Zhou, M. Shepherd, and C. Williams. *Conifer Microsatellite Handbook*. Texas A&M University, College Station, Texas, USA, 2002.
- F. Austerlitz and P. E. Smouse. Two-generation analysis of pollen flow across a landscape. II. Relation between  $\Phi_{FT}$ , pollen dispersal and inter-female distance. *Genetics*, 157(2):851–857, 2001. URL <http://www.genetics.org/cgi/content/abstract/157/2/851>.
- J. C. Avise. *Molecular Markers, Natural History, and Evolution*. Sinauer Associates, Sunderland, United States of America, 2<sup>nd</sup> edition, 2004.
- J. J. Bacallado. Notas sobre la distribución y evolución de la avifauna canaria. In G. Kunkel, editor, *Biogeography and Ecology in the Canary Islands*, volume 30 of *Monographiae Biologicae*, pages 413–431. Junk Publishers, The Hage, Netherlands, 1976.
- K. Bachmann. Molecular markers in plant ecology. *New Phytologist*, 126(3):403–418, 1994. URL [http://links.jstor.org/sici?sici=0028-646X%](http://links.jstor.org/sici?sici=0028-646X%2F126(3)403-418)

- 28199403%29126%3A3%3C403%3ATR6MM%3E2.0.CO%3B2-U. Tansley Review No. 63.
- M. Báez, J. L. Martín Esquivel, and P. Oromí. Diversidad taxonómica terrestre. In J. M. Fernández-Palacios and J. L. Martín Esquivel, editors, *Naturaleza de las Islas Canarias. Ecología y Conservación*, pages 119–125. Ediciones Turquesa, Santa Cruz de Tenerife, Spain, 2001.
- D. A. Bannerman. *The Canary Islands: Their History, Natural History and Scenery*. Gurney and Jackson, London, United Kingdom, 1922. URL <http://bdigital.ulpgc.es/mdc/visualizar/propiedad.php?accion=Texto&id=56&vol=no&td>.
- F. Barahona, S. E. Evans, J. A. Mateo, M. García-Márquez, and L. F. López-Jurado. Endemism, gigantism and extinction in island lizards: the genus *Galotia* on the Canary Islands. *Journal of Zoology*, 250(3):373–388, 2000. URL <http://dx.doi.org/10.1017/S0952836900003101>.
- N. H. Barton and M. Slatkin. A quasi-equilibrium theory of the distribution of rare alleles in a subdivided population. *Heredity*, 56(3):409–415, 1986. URL [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=3733460](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3733460).
- J. Beaulieu and J.-P. Simon. Variation in cone morphology and seed characters in *Pinus strobus* in Quebec. *Canadian Journal of Botany*, 73(2):262–271, 1995. URL <http://md1.csa.com/partners/viewrecord.php?requester=gs&collection=ENV&recid=377090406/07/2004>.
- P. Becker. Colonization of islands by carnivorous and herbivorous Heteroptera and Coleoptera: effects of island area, plant species richness, and “extinction” rates. *Journal of Biogeography*, 19(2):163–171, 1992. URL <http://links.jstor.org/sici?sici=0305-0270%28199203%2919%3A2%3C163%3AC0IBCA%3E2.0.CO%3B2-D>.
- L. B. Beheregaray, C. Ciofi, D. Geist, J. P. Gibbs, A. Caccone, and J. R. Powell. Genes record a prehistoric volcano eruption in the Galápagos. *Science*, 302(5642):75, 2003. URL <http://dx.doi.org/10.1126/science.1087486>.

- S. A. Bekessy, R. A. Ennos, M. A. Burgman, A. C. Newton, and P. K. Ades. Neutral DNA markers fail to detect genetic divergence in an ecologically important trait. *Biological Conservation*, 110(2):267–275, 2003. URL [http://dx.doi.org/10.1016/S0006-3207\(02\)00225-2](http://dx.doi.org/10.1016/S0006-3207(02)00225-2).
- R. Bellefontaine and M. Raggabi. Provenances et origines de *Pinus canariensis*. Résultats des essais à court terme installés au Maroc depuis 1972. *Annales de la Recherche Forestière au Maroc*, 19:309–326, 1979.
- G. Bertorelle and M. Slatkin. The number of segregating sites in expanding human populations, with implications for estimates of demographic parameters. *Molecular Biology and Evolution*, 12(5):887–892, 1995. URL <http://mbe.oupjournals.org/cgi/content/abstract/12/5/887>.
- U.-R. Böhle, H. H. Hilger, and W. F. Martin. Island colonization and evolution of the insular woody habit in *Echium* L. (Boraginaceae). *Proceedings of the National Academy of Sciences of the United States of America*, 93(21):11740–11745, 1996. URL <http://www.pnas.org/cgi/content/abstract/93/21/11740>.
- J. S. Birks and P. J. Kanowski. Interpretation of the composition in coniferous resin. *Silvae Genetica*, 37(1):29–39, 1988. URL <http://www.bfafh.de/inst2/sg37pdf.htm>.
- A. Blanco Andray, M. Castroviejo Bolibar, J. L. Fraile Sánchez, J. M. Gandullo Guitiérrez, L. A. Muñoz de la Fuente, and O. Sánchez Palomares. *Estudio Ecológico del Pino Canario*, volume 6 of *Serie Técnica*. ICONA, Madrid, Spain, 1989.
- A. J. Bohonak. IBD (ISOLATION BY DISTANCE): a program for analyses of isolation by distance. *Journal of Heredity*, 93(2):153–154, 2002. URL <http://jhered.oxfordjournals.org/cgi/content/extract/93/2/153>.
- S. Bojovic, M. Jurc, D. Drazic, P. Pavlovic, M. Mitrovic, L. Djurdjevic, R. S. Dodd, Z. Afzal-Rafii, and M. Barbero. Origin identification of *Pinus nigra* populations in southwestern Europe using terpene composition variations. *Trees — Structure and Function*, 19(5):531–538, 2005. URL <http://dx.doi.org/10.1007/s00468-005-0411-x>.

- F. T. Bonner. Seed biology. In R. G. Nisley, editor, *Woody Plant Seed Manual*. USDA Forest Service, 2003. URL <http://www.nsl.fs.fed.us/wpsm/>.
- M. Borghetti, R. Glannini, and P. Menozzi. Geographic variation in cones of Norway spruce [*Picea abies* (L.) Karst.]. *Silvae Genetica*, 37(5–6):178–184, 1988. URL <http://www.bfafh.de/inst2/sg37pdf.htm>.
- D. H. Boshier. Mating systems. In A. G. Young, D. H. Boshier, and T. J. Boyle, editors, *Forest Conservation Genetics. Principles and Practice*, pages 63–79. CSIRO Publishing & CABI Publishing, Oxon, United Kingdom, 2000.
- D. Bramwell. Contribución a la biogeografía de las Islas Canarias. *Botánica Macaronésica*, 14(1985):3–34, 1986.
- D. Bramwell. The endemic flora of the Canary Islands; distribution, relationships and phytogeography. In G. Kunkel, editor, *Biogeography and Ecology in the Canary Islands*, volume 30 of *Monographiae Biologicae*, pages 207–240. Junk Publishers, The Hage, Netherlands, 1976.
- D. Bramwell and Z. I. Bramwell. *Flores Silvestres de las Islas Canarias*. Editorial Rueda, Madrid, Spain, 1990.
- A. Brower. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proceedings of the National Academy of Sciences of the United States of America*, 91(14):6491–6495, 1994. URL <http://www.pnas.org/cgi/content/abstract/91/14/6491>.
- A. H. D. Brown and C. M. Hardner. Sampling the gene pools of forest trees for *ex situ* conservation. In A. G. Young, D. H. Boshier, and T. J. Boyle, editors, *Forest Conservation Genetics. Principles and Practice*, pages 185–196. CSIRO Publishing & CABI Publishing, Oxon, UK, 2000.
- C. F. A. Brunton and G. D. D. Hurst. Mitochondrial DNA phylogeny of Brimstone butterflies (genus *Gonepteryx*) from the Canary Islands and Madeira. *Biological Journal of the Linnean Society*, 63(1):69–79, 1998. URL <http://dx.doi.org/10.1006/bijl.1997.0180>.

- G. Bucci and G. G. Vendramin. Delineation of genetic zones in the European Norway spruce natural range: preliminary evidence. *Molecular Ecology*, 9(7):923–934, 2000. URL <http://dx.doi.org/10.1046/j.1365-294x.2000.00946.x>.
- G. Bucci, M. Anzidei, A. Madaghiele, and G. G. Vendramin. Detection of haplotypic variation and natural hybridization in *halepensis*-complex pine species using chloroplast simple sequence repeat (SSR) markers. *Molecular Ecology*, 7(12):1633–1643, 1998. URL <http://dx.doi.org/10.1046/j.1365-294x.1998.00466.x>.
- J. Burczyk, S. P. DiFazio, and W. T. Adams. Gene flow in forest trees: how far do genes really travel? *Forest Genetics*, 11(2–3):1–14, 2004. URL [http://www.esd.ornl.gov/PGG/burczyk\\_04.pdf](http://www.esd.ornl.gov/PGG/burczyk_04.pdf).
- C. Burgarella. *Genetica di Popolazione Applicata alla Conservazione e Gestione del Patrimonio Forestale di Quercus ilex L. in Sicilia e in Andalusia*. PhD, Università degli Studi di Palermo, 2004.
- S. H. Carlquist. *Island Biology*. Columbia University Press, New York, USA, 1974.
- J. C. Carracedo and S. J. Day. *Canary Islands*, volume 4 of *Classic Geology in Europe*. Terra Publishing, Harpenden, United Kingdom, 2002. URL <http://www.rjpc.demon.co.uk/terratitles.htm#anchor104171>.
- J. C. Carracedo, S. J. Day, H. Guillou, E. Rodríguez Badiola, J. A. Canas, and F. J. Pérez Torrado. Hotspot volcanism close to a passive continental margin: the Canary Islands. *Geological Magazine*, 135(5):591–604, 1998. URL <http://dx.doi.org/10.1017/S0016756898001447>.
- J. C. Carracedo, S. J. Day, H. Guillou, and F. J. Pérez Torrado. Giant Quaternary landslides in the evolution of La Palma and El Hierro, Canary Islands. *Journal of Volcanology and Geothermal Research*, 94(1–4):169–190, 1999. URL [http://dx.doi.org/10.1016/S0377-0273\(99\)00102-X](http://dx.doi.org/10.1016/S0377-0273(99)00102-X).
- S. Carranza, E. N. Arnold, J. A. Mateo, and P. Geniez. Relationships and evolution of the North African geckos, *Geckonia* and *Tarentola* (Reptilia:



- Gekkonidae), based on mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution*, 23(2):244–256, 2002. URL [http://dx.doi.org/10.1016/S1055-7903\(02\)00024-6](http://dx.doi.org/10.1016/S1055-7903(02)00024-6).
- L. M. Carrascal and D. Palomino. Determinantes de la riqueza de especies de aves en las islas Selvagem y Canarias. *Ardeola*, 49(2):211–221, 2002. URL [http://www.ardeola.org/files/ardeola\\_501.pdf](http://www.ardeola.org/files/ardeola_501.pdf).
- F. Castilla Lattke. *Árboles del Arboreto Luis Ceballos*. Consejería de Medio Ambiente de Madrid (C.A.M.), Madrid, Spain, 1999.
- V. Castric and L. Bernatchez. The rise and fall of isolation by distance in the anadromous brook charr (*Salvelinus fontinalis* Mitchill). *Genetics*, 163(3):983–996, 2003. URL <http://www.genetics.org/cgi/content/abstract/163/3/983>.
- S. A. Cato and T. E. Richardson. Inter- and intraspecific polymorphism at chloroplast SSR loci and the inheritance of plastids in *Pinus radiata* D. Don. *Theoretical and Applied Genetics*, 93(4):587–592, 1996. URL <http://dx.doi.org/10.1007/s001220050319>.
- J. Caujapé-Castells. Boomerangs of biodiversity?: the interchange of biodiversity between mainland north Africa and the Canary Islands as inferred from cpDNA RFLPs in genus *Androcymbium*. *Botánica Macaronésica*, 25:53–69, 2004. URL <http://www.step.es/jardcan/BOTANICA%20MACARONESICA25.HTML>.
- L. Ceballos and F. Ortuño. El bosque y el agua en Canarias. *Montes*, 8(48):418–423, 1952.
- L. Ceballos and F. Ortuño. *Vegetación y Flora Forestal de las Canarias Occidentales*. Cabildo Insular de Tenerife, Madrid, Spain, 2<sup>nd</sup> edition, 1976.
- D. Chagné, P. Chaumeil, A. Ramboer, C. Collada, A. Guevara, M. T. Cervera, G. G. Vendramin, V. Garcia, J.-M. Frigerio, C. Echt, T. Richardson, and C. Plomion. Cross-species transferability and mapping of genomic and cDNA SSRs in pines. *Theoretical and Applied Genetics*, 109(6):1204–1214, 2004. URL <http://dx.doi.org/10.1007/s00122-004-1683-z>.

- W. R. Chaney, P. E. Pope, H. L. Edlin, and G. T. Tsoumis. Forestry and wood production. In R. McHenry and Y. C. Hori, editors, *The New Encyclopædia Britannica — Macropædia*, volume 19, pages 410–426. Encyclopædia Britannica, Inc., Chicago, United States of America, 15<sup>th</sup> edition, 1994.
- G. W. Chapman. Forestry in Iraq. *Unasylva*, 2(5), 1948. URL [http://www.fao.org/documents/show\\_cdr.asp?url\\_file=///docrep/x5346e/x5346e06.htm](http://www.fao.org/documents/show_cdr.asp?url_file=///docrep/x5346e/x5346e06.htm).
- R. Ciferri. La laurisilva canaria: una paleoflora vivente. *La Ricerca Scientifica*, 32(1):111–134, 1961.
- J. Climent, L. Gil, and J. A. Pardos. Heartwood and sapwood development and its relationship to growth and environment in *Pinus canariensis* Chr. Sm. ex DC. *Forest Ecology and Management*, 59(1–2):165–174, 1993. URL <http://md1.csa.com/partners/viewrecord.php?requester=gs&collection=ENV&recid=3030034>.
- J. Climent, L. Gil, and M. de Tuero. *Regiones de Procedencia de Pinus canariensis Chr. Sm. ex DC*. ICONA, Madrid, Spain, 1996.
- J. Climent, L. Gil, and J. A. Pardos. Xylem anatomical traits related to resinous heartwood formation in *Pinus canariensis* Sm. *Trees — Structure and Function*, 12(3):139–145, 1998. URL <http://www.springerlink.com/link.asp?id=dfc64dfxqgu30tfk>.
- J. Climent, I. Ortiz, and L. Gil. Variabilidad morfológica de las piñas en *Pinus canariensis* Chr. Sm. In *III Congreso Forestal Español. Montes para la Sociedad del Nuevo Milenio*, pages 195–201, Granada, Spain, 2001a. Junta de Andalucía. URL <http://www.juntadeandalucia.es/medioambiente/ponencias/1269.htm>.
- J. Climent, E. Pérez, L. Gil, and J. A. Pardos. Ensayos de procedencias de *Pinus canariensis* Sm.: diseño, instalación y primeros resultados. In *III Congreso Forestal Español. Montes para la Sociedad del Nuevo Milenio*, pages 903–909, Granada, Spain, 2001b. Junta de Andalucía. URL <http://www.juntadeandalucia.es/medioambiente/ponencias/1466.htm>.

- J. Climent, M. R. Chambel, E. Pérez, L. Gil, and J. A. Pardos. Relationship between heartwood radius and early radial growth, tree age, and climate in *Pinus canariensis*. *Canadian Journal of Forest Research*, 32(1):103–111, 2002a. URL <http://pubs.nrc-cnrc.gc.ca/sample/x01-178.pdf>.
- J. Climent, L. Gil, E. Pérez, and J. A. Pardos. Efecto de la procedencia en la supervivencia de plántulas de *Pinus canariensis* Sm. en medio árido. *Investigación Agraria. Sistemas y Recursos Forestales*, 11(1):171–180, 2002b. URL <http://www.inia.es/gcont/publicaciones/index.jsp?idcategoria=1382&regini=10>.
- J. Climent, M. R. Chambel, L. Gil, and J. A. Pardos. Vertical heartwood variation patterns and prediction of heartwood volume in *Pinus canariensis* Sm. *Forest Ecology and Management*, 174(1–3):203–211, 2003. URL [http://dx.doi.org/10.1016/S0378-1127\(02\)00023-3](http://dx.doi.org/10.1016/S0378-1127(02)00023-3).
- J. Climent, R. Tapias, J. A. Pardos, and L. Gil. Fire adaptations in the Canary Islands pine (*Pinus canariensis*). *Plant Ecology*, 171(1–2):185–196, 2004. URL <http://dx.doi.org/10.1023/B:VEGE.0000029374.64778.68>.
- J. Climent, M. R. Chambel, R. López, S. Mutke, R. Alía, and L. Gil. The adaptive significance of heteroblasty in the Canary Island pine (*Pinus canariensis*, Pinaceae). *AJB*, 2005. In press.
- P. F. Coetzee. The influence of different factors on bending strenght of *Pinus canariensis* and *Pinus pinaster* poles. *Southern African Forestry Journal*, 105: 18–25, 1978.
- A. C. Cook. A sketch of the flora of the Canary Islands. *Bulletin of the Torrey Botanical Club*, 25(7):351–358, 1898. URL <http://links.jstor.org/sici?sici=0040-9618%28189807%2925%3A7%3C351%3AAS0TF0%3E2.0.CO%3B2-H>.
- S. Cordell, G. Goldstein, D. Mueller-Dombois, D. Webb, and P. M. Vitousek. Physiological and morphological variation in *Metrosideros polymorpha*, a dominant Hawaiian tree species, along an altitudinal gradient: the role of phenotypic plasticity. *Oecologia*, 113(2):188–196, 1998. URL <http://dx.doi.org/10.1007/s004420050367>.

- C. Criado. Las formas de modelado. In J. M. Fernández-Palacios and J. L. Martín Esquivel, editors, *Naturaleza de las Islas Canarias. Ecología y Conservación*, pages 81–85. Ediciones Turquesa, Santa Cruz de Tenerife, Spain, 2001.
- A. Cuenca, A. E. Escalante, and D. Piñero. Long-distance colonization, isolation by distance, and historical demography in a relictual Mexican pinyon pine (*Pinus nelsonii* Shaw) as revealed by paternally inherited genetic markers (cpSSRs). *Molecular Ecology*, 12(8):2087–2097, 2003. URL <http://dx.doi.org/10.1046/j.1365-294X.2003.01890.x>.
- B. Damnati, N. Petit-Maire, M. Fontugne, J. Meco, and D. Williamson. Quaternary palaeoclimates in the eastern Canary Islands. *Quaternary International*, 31:37–46, 1996. URL [http://dx.doi.org/10.1016/1040-6182\(95\)00019-F](http://dx.doi.org/10.1016/1040-6182(95)00019-F).
- O. de Bolòs. Acerca de la flora macaranésica. *Anales del Jardín Botánico de Madrid*, 54:457–461, 1996.
- B. Degen, R. Streiff, and B. Ziegenhagen. Comparative study of genetic variation and differentiation of two pedunculate oak (*Quercus robur*) stands using microsatellite and allozyme loci. *Heredity*, 83(5):597–603, 1999. URL <http://dx.doi.org/10.1046/j.1365-2540.1999.00622.x>.
- M. J. del Arco Aguilar, P. L. Pérez de Paz, and W. Wildpret. Contribución al conocimiento de los pinares de la isla de Tenerife. *Lazaroa*, 7:67–84, 1987.
- M. J. del Arco Aguilar, P. L. Pérez de Paz, W. Wildpret, V. L. Sauquillo, and M. Salas. *Atlas Cartográfico de los Pinares Canarios I: La Gomera y El Hierro*. Gobierno de Canarias, Consejería de Política Territorial, Santa Cruz de Tenerife, Spain, 1990.
- M. J. del Arco Aguilar, P. L. Pérez de Paz, O. Rodríguez Delgado, M. Salas, and W. Wildpret. *Atlas Cartográfico de los Pinares Canarios II: Tenerife*. Gobierno de Canarias, Consejería de Política Territorial, Santa Cruz de Tenerife, Spain, 1992.
- J. C. Delgado González. *Propagación de árboles canarios*. Cabildo Insular de Gran Canaria, Las Palmas, Gran Canaria, Spain,

1986. URL <http://bdigital.ulpgc.es/mdc/visualizar/propiedad.php?accion=Texto&id=1205&vol=no>.
- G. Depape. Recherches sur la flore pliocène de la Vallée du Rhône. Flores de St-Marcel (Ardèche) et de Théziers (Gard). *Annales des Sciences Naturelles, Botanique*, 10(4):73–265, 1922.
- J. Derory, S. Mariette, S. C. González-Martínez, D. Chagné, D. Madur, S. Gerber, J. Brach, F. Persyn, M. M. Ribeiro, and C. Plomion. What can nuclear microsatellites tell us about maritime pine genetic resources conservation and provenance certification strategies? *Annals of Forest Science*, 59(5–6):699–708, 2002. URL <http://dx.doi.org/10.1051/forest:2002058>.
- R. DeSalle, T. Freedman, E. M. Prager, and A. C. Wilson. Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. *Journal of Molecular Evolution*, 26(1–2):157–64, 1987. URL [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=3125333](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3125333).
- S. P. DiFazio, G. T. Slavov, J. Burczyk, S. Leonardi, and S. H. Strauss. Gene flow from tree plantations and implications for transgenic risk assessment. In C. Walter and M. Carson, editors, *Forest Biotechnology for the 21<sup>st</sup> Century*, pages 405–422. Research Signpost, Kerala, India, 2004. URL <http://wwdata.forestry.oregonstate.edu/tgbb/publications/RS%20Walter%2023.pdf>.
- U. A. Din. Pines for tropical areas. *Unasylva*, 12(3), 1958. URL [http://www.fao.org/documents/show\\_cdr.asp?url\\_file=/docrep/x5388e/x5388e03.htm](http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/x5388e/x5388e03.htm).
- J. A. Donázar, L. Gangoso, M. G. Forero, and J. Juste. Presence, richness and extinction of birds of prey in the Mediterranean and Macaronesian islands. *Journal of Biogeography*, 32(10):1701–1713, 2005. URL <http://dx.doi.org/10.1111/j.1365-2699.2005.01294.x>.
- J. J. Doyle and L. J. Doyle. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, 19(1):11–15, 1987.
- J. J. Doyle, M. Morgante, S. V. Tingey, and W. Powell. Size homoplasy in chloroplast microsatellites of wild perennial relatives of soybean (*Glycine* sub-

- genus *Glycine*). *Molecular Biology and Evolution*, 15(2):215–218, 1998. URL <http://mbe.oupjournals.org/content/vol15/issue2/index.shtml>.
- R. J. Dyer and V. L. Sork. Pollen pool heterogeneity in shortleaf pine, *Pinus echinata* Mill. *Molecular Ecology*, 10(4):859–866, 2001. URL <http://dx.doi.org/10.1046/j.1365-294X.2001.01251.x>.
- R. J. Dyer, R. D. Westfall, V. L. Sork, and P. E. Smouse. Two-generation analysis of pollen flow across a landscape. V. A stepwise approach for extracting factors contributing to pollen structure. *Heredity*, 92(3):204–211, 2004. URL <http://dx.doi.org/10.1038/sj.hdy.6800397>.
- C. J. Earle and M. P. Frankis. *Pinus canariensis* C. Smith 1828. In C. J. Earle, editor, *Gymnosperm Database*. Department of Botany, Rheinische Friedrich-Wilhelms-Universität Bonn, Germany, 1999. URL <http://www.botanik.uni-bonn.de/conifers/pi/pin/canariensis.htm>. Updated: 14 March 2004.
- C. S. Echt, P. May-Marquardt, M. Hseih, and R. Zahorchak. Characterization of microsatellite markers in eastern white pine. *Genome*, 39(6):1102–1108, 1996. URL [http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2\\_desc\\_e?gen](http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2_desc_e?gen).
- C. S. Echt, L. L. DeVerno, M. Anzidei, and G. G. Vendramin. Chloroplast microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait. *Molecular Ecology*, 7(3):307–316, 1998. URL <http://dx.doi.org/10.1046/j.1365-294X.1998.00350.x>.
- C. S. Echt, G. G. Vendramin, C. D. Nelson, and P. Marquardt. Microsatellite dna as shared genetic markers among conifer species. *Canadian Journal of Forest Research*, 29(3):365–371, 1999. URL [http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2\\_abst\\_e?cjfr\\_x99-009\\_29\\_ns\\_nf\\_cjfr3-99](http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2_abst_e?cjfr_x99-009_29_ns_nf_cjfr3-99).
- Y. A. El-Kassaby. Effect of forest tree domestication on gene pools. In A. G. Young, D. H. Boshier, and T. J. Boyle, editors, *Forest Conservation Genetics. Principles and Practice*, pages 197–213. CSIRO Publishing & CABI Publishing, Oxon, United Kingdom, 2000.
- A. El Mousadik and R. J. Petit. High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L.) Skeels]

- endemic to Morocco. *Theoretical and Applied Genetics*, 92(7):832–839, 1996. URL <http://dx.doi.org/10.1007/s001220050200>.
- C. G. Elsik, V. T. Minihan, S. E. Hall, A. M. Scarpa, and C. G. Williams. Low-copy microsatellite markers for *Pinus taeda* L. *Genome*, 43(3):550–555, 2000. URL [http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2\\_abst\\_e?gen\\_g00-002-43\\_ns\\_nf\\_gen3-00](http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2_abst_e?gen_g00-002-43_ns_nf_gen3-00).
- B. C. Emerson. Evolution on oceanic islands: molecular phylogenetic approaches to understanding pattern and process. *Molecular Ecology*, 11(6):951–966, 2002. URL <http://dx.doi.org/10.1046/j.1365-294X.2002.01507.x>.
- B. C. Emerson. Genes, geology and biodiversity: faunal and floral diversity on the island of Gran Canaria. *Animal Biodiversity and Conservation*, 26(1):9–20, 2003. URL [http://bcnweb13.bcn.es:81/NASApp/wprmuseuciencies/Museu.GeneradorPagines?idioma=1&seccio=3\\_1\\_3\\_1.765&visible=1](http://bcnweb13.bcn.es:81/NASApp/wprmuseuciencies/Museu.GeneradorPagines?idioma=1&seccio=3_1_3_1.765&visible=1).
- B. C. Emerson and N. Kolm. Species diversity can drive speciation. *Nature*, 434(7036):1015–1017, 2005. URL <http://dx.doi.org/10.1038/nature03450>.
- B. C. Emerson, P. Oromí, and G. M. Hewitt. Colonization and diversification of the species *Brachyderes rugatus* (Coleoptera) on the Canary Islands: evidence from mitochondrial DNA COII gene sequences. *Evolution*, 54(3):911–923, 2000a. URL <http://evol.allenpress.com/evolonline/?request=get-abstract&issn=0014-3820&volume=054&issue=03&page=0911>.
- B. C. Emerson, P. Oromí, and G. M. Hewitt. Interpreting colonization of the *Calathus* (Coleoptera: Carabidae) on the Canary Islands and Madeira through the application of the parametric bootstrap. *Evolution*, 54(6):2081–2090, 2000b. URL <http://evol.allenpress.com/evolonline/?request=get-abstract&issn=0014-3820&volume=054&issue=06&page=2081>.
- B. C. Emerson, E. Paradis, and C. Thebaud. Revealing the demographic histories of species using DNA sequences. *Trends in Ecology & Evolution*, 16(12):707–716, 2001. URL [http://dx.doi.org/10.1016/S0169-5347\(01\)02305-9](http://dx.doi.org/10.1016/S0169-5347(01)02305-9).
- B. C. Emerson, S. Forgie, S. Goodacre, and P. Oromí. Testing phylogeographic predictions on an active volcanic island: *Brachyderes rugatus* (Coleoptera:

- Curculionidae) on La Palma (Canary Islands). *Molecular Ecology*, 2005. In press.
- B. K. Epperson and T. Li. Measurement of genetic structure within populations using Moran's spatial autocorrelation statistics. *Proceedings of the National Academy of Sciences of the United States of America*, 93(19):10528–10532, 1996. URL <http://www.pnas.org/cgi/content/abstract/93/19/10528>.
- A. Escudero, Y. Nuñez, and F. Pérez-García. Is fire a selective force of seed size in pine species? *Acta Oecologica*, 21(4–5):245–256, 2000. URL [http://dx.doi.org/10.1016/S1146-609X\(00\)01083-3](http://dx.doi.org/10.1016/S1146-609X(00)01083-3).
- C. F. Esteve. Estudio de las alianzas y asociaciones del orden *Cytiso-Pinetalia* en las Canarias Orientales. *Boletín de la Real Sociedad Española de Historia Natural (Biología)*, 67:77–104, 1969.
- A. Estoup and J. M. Cornuet. Microsatellite evolution: inferences from population data. In D. B. Goldstein and C. Schlötterer, editors, *Microsatellites. Evolution and Applications*, pages 49–65. Oxford University Press, Oxford, United Kingdom, 1999.
- A. Estoup, F. Rousset, Y. Michalakis, J.-M. Cornuet, M. Adriamanga, and R. Guyomard. Comparative analysis of microsatellite and allozyme markers: a case study investigating microgeographic differentiation in brown trout (*Salmo trutta*). *Molecular Ecology*, 7(3):339–353, 1998. URL <http://dx.doi.org/10.1046/j.1365-294X.1998.00362.x>.
- A. Estoup, P. Jarne, and J. M. Cornuet. Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. *Molecular Ecology*, 11(9):1591–1604, 2002. URL <http://dx.doi.org/10.1046/j.1365-294X.2002.01576.x>.
- L. Excoffier and S. Schneider. Why hunter-gatherer populations do not show signs of Pleistocene demographic expansions. *Proceedings of the National Academy of Sciences of the United States of America*, 96(19):10597–10602, 1999. URL <http://www.pnas.org/cgi/content/abstract/96/19/10597>.
- L. Excoffier, P. E. Smouse, and J. M. Quattro. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human



- mitochondrial DNA restriction data. *Genetics*, 131(2):479–491, 1992. URL <http://www.genetics.org/cgi/content/abstract/131/2/479>.
- B. Fady, F. Lefèvre, M. Reynaud, G. G. Vendramin, M. Bou Dagher-Kharrat, M. Anzidei, R. Pastorelli, A. Savouré, and M. Bariteau. Gene flow among different taxonomic units: evidence from nuclear and cytoplasmic markers in *Cedrus* plantation forests. *Theoretical and Applied Genetics*, 107(6):1132 – 1138, 2003. URL <http://dx.doi.org/10.1007/s00122-003-1323-z>.
- Anonymous. FAO. Forestry in the Middle East. *Unasylva*, 6(3), 1952. URL [http://www.fao.org/documents/show\\_cdr.asp?url\\_file=//docrep/x5364e/x5364e03.htm](http://www.fao.org/documents/show_cdr.asp?url_file=//docrep/x5364e/x5364e03.htm).
- A. Farjon. *Pinus canariensis*. In *2004 IUCN Red List of Threatened Species*. IUCN, 2003. URL <http://www.redlist.org>.
- Á. B. Fernández. Conservación y restauración ecológica de los bosques. In J. M. Fernández-Palacios and J. L. Martín Esquivel, editors, *Naturaleza de las Islas Canarias. Ecología y Conservación*, pages 375–382. Ediciones Turquesa, Santa Cruz de Tenerife, Spain, 2001.
- J. M. Fernández-Palacios and J. P. de Nicolás. Altitudinal pattern of vegetation variation on Tenerife. *Journal of Vegetation Science*, 6(2):183–190, 1995. URL [http://www.opuluspress.se/index.php?page=shop/article\\_abstract&product\\_id=8&Itemid=56&option=com\\_phpshop&article=16768&nr=-1](http://www.opuluspress.se/index.php?page=shop/article_abstract&product_id=8&Itemid=56&option=com_phpshop&article=16768&nr=-1).
- J. M. Fernández-Palacios and E. Dias. Marco biogeográfico macaronésico. In J. M. Fernández-Palacios and J. L. Martín Esquivel, editors, *Naturaleza de las Islas Canarias. Ecología y Conservación*, pages 45–52. Ediciones Turquesa, Santa Cruz de Tenerife, Spain, 2001.
- J. M. Fernández-Palacios and J. L. Martín Esquivel. Las islas como experimento de laboratorio. In J. M. Fernández-Palacios and J. L. Martín Esquivel, editors, *Naturaleza de las Islas Canarias. Ecología y Conservación*, pages 39–43. Ediciones Turquesa, Santa Cruz de Tenerife, Spain, 2001a.

- J. M. Fernández-Palacios and J. L. Martín Esquivel, editors. *Naturaleza de las Islas Canarias. Ecología y Conservación*. Ediciones Turquesa, Santa Cruz de Tenerife, Spain, 2001b.
- J. M. Fernández-Palacios, Á. Vera, and A. Brito. Los ecosistemas. In J. M. Fernández-Palacios and J. L. Martín Esquivel, editors, *Naturaleza de las Islas Canarias. Ecología y Conservación*, pages 157–165. Ediciones Turquesa, Santa Cruz de Tenerife, Spain, 2001.
- L. Fernández-Pello Martín. Configuración espacial del pinar de El Hierro. *Revista de Geografía Canaria*, 2:87–104, 1987.
- D. Fernandopullé. Climatic characteristics of the Canary Islands. In G. Kunkel, editor, *Biogeography and Ecology in the Canary Islands*, volume 30 of *Monographiae Biologicae*, pages 185–206. Junk Publishers, The Hage, Netherlands, 1976.
- C. Ferris, R. A. King, R. Väinölä, and G. M. Hewitt. Chloroplast DNA recognizes three refugial sources of European oaks and suggests independent eastern and western immigrations to Finland. *Heredity*, 80(5):584–593, 1998. URL <http://www.nature.com/hdy/journal/v80/n5/abs/6883420a.html>.
- R. Finkeldey and M. Ziehe. Genetic implications of silvicultural regimes. *Forest Ecology and Management*, 197(1–3):231–244, 2004. URL <http://dx.doi.org/10.1016/j.foreco.2004.05.036>.
- P. J. Fisher, T. E. Richardson, and R. C. Gardner. Characteristics of single- and multi-copy microsatellites from *Pinus radiata*. *Theoretical and Applied Genetics*, 96(6–7):969–979, 1998. URL <http://dx.doi.org/10.1007/s001220050828>.
- D. P. Fowler and R. W. Morris. Genetic diversity in red pine: evidence of low genic heterozygosity. *Canadian Journal of Forest Research*, 7:343–347, 1977.
- J. Francisco-Ortega, J. Fuertes-Aguilar, C. Gómez-Campo, A. Santos-Guerra, and R. K. Jansen. Internal transcribed spacer sequence phylogeny of *Crambe* L. (Brassicaceae): molecular data reveal two old world disjunctions. *Molecular Phylogenetics and Evolution*, 11(3):361–380, 1999. URL <http://dx.doi.org/10.1006/mpev.1998.0592>.

- J. M. Franco. Una nova variedade do pinheiro das Canárias. *Anais do Instituto Superior de Agronomia*, 14:159–163, 1943.
- M. Frankis. *Pinus brutia*. *Curtis's Botanical Magazine*, 16(3):173–184, 1999. URL <http://dx.doi.org/10.1111/1467-8748.00216>. Plate 367.
- Y. X. Fu. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, 147(2):915–925, 1997. URL <http://www.genetics.org/cgi/content/abstract/147/2/915>.
- Y. X. Fu and W. H. Li. Statistical tests of neutrality of mutations. *Genetics*, 133(3):693–709, 1993. URL <http://www.genetics.org/cgi/content/abstract/133/3/693>.
- J. Fuertes-Aguilar, M. F. Ray, J. Francisco-Ortega, A. Santos-Guerra, and R. K. Jansen. Molecular evidence from chloroplast and nuclear markers for multiple colonizations of *Lavatera* (Malvaceae) in the Canary Islands. *Systematic Botany*, 27(1):74–83, 2002. URL <http://www.bioone.org/bioone/?request=get-document&issn=0363-6445&volume=027&issue=01&page=0074>.
- C. M. García Cruz. *The Origin of the Canary Islands. A Chronology of Ideas and Related Concepts, from the Antiquity to the end of 20<sup>th</sup> Century*. IN-HIGEO, Sidney, Australia, 2001. URL <http://www.aepect.org/antigua/hemeroteca/Garcia-Cruz01.htm>.
- F. García-Talavera. La Macaronesia — Consideraciones geológicas, biogeográficas y paleoecológicas, 2003. URL <http://elguanche.net/macaronesiav2003.htm>. El Guanche. La voz de Canarias Libre.
- F. García-Talavera. Aves gigantes en el Mioceno de Famara (Lanzarote). *Revista de la Academia Canaria de Ciencias*, 2:71–79, 1990.
- F. García-Talavera, L. Sánchez-Pinto, and S. Socorro. Vegetales fósiles en el complejo traquítico-sienítico de Gran Canaria. *Revista de la Academia Canaria de Ciencias*, 7(2–4):77–91, 1995.
- F. Gasulla, S. Fos, and E. Barreno. Comportamiento fenológico y variabilidad fenotípica de *Pinus canariensis* (Chr. Sm. ex DC.). In *III Congreso Forestal Español. Montes para la Sociedad del Nuevo Milenio*, Granada,

- Spain, 2001. Junta de Andalucía. URL <http://www.juntadeandalucia.es/medioambiente/ponencias/120.htm>.
- G. Geada López, K. Kamiya, and K. Harada. Phylogenetic relationships of Diploxyton pines (subgenus *Pinus*) based on plastid sequence data. *International Journal of Plant Sciences*, 163(5):737–747, 2002. URL <http://www.journals.uchicago.edu/IJPS/journal/issues/v163n5/020079/brief/020079.abstract.html>.
- D. S. Gernandt, G. Gaeda López, S. Ortiz García, and A. Liston. Phylogeny and classification of *Pinus*. *Taxon*, 54(1):29–42, 2005. URL <http://www.ingentaconnect.com/content/iapt/tax/2005/00000054/00000001/art00005>.
- T. Gieger and C. Leuschner. Altitudinal change in needle water relations of *Pinus canariensis* and possible evidence of a drought-induced alpine timberline on Mt. Teide, Tenerife. *Flora*, 199(2):100–109, 2004. URL <http://dx.doi.org/10.1078/0367-2530-00139>.
- L. Gil, J. Climent, N. Nanos, S. Mutke, I. Ortiz, and G. Schiller. Cone morphology variation in *Pinus canariensis* Sm. *Plant Systematics and Evolution*, 235(1–4): 35–51, 2002. URL <http://dx.doi.org/10.1007/s00606-002-0218-9>.
- R. G. Gillespie and G. K. Roderick. Arthropods on islands: colonization, speciation, and conservation. *Annual Review of Entomology*, 47(1):595–632, 2002. URL <http://dx.doi.org/10.1146/annurev.ento.47.091201.145244>.
- E. M. Gillet, editor. *Which DNA Marker for Which Purpose?* Institut für Forstgenetik und Forstpflanzenzüchtung, Universität Göttingen, Göttingen, Germany, 1999. URL <http://webdoc.sub.gwdg.de/ebook/y/1999/whichmarker/index.htm>. Final Compendium of the Research Project *Development, Optimisation and Validation of Molecular Tools for Assessment of Biodiversity in Forest Trees* in the European Union DGXII Biotechnology FW IV Research Programme *Molecular Tools for Biodiversity*.
- J. C. Glaubitz. CONVERT: a user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. *Molecu-*

- lar Ecology Notes*, 4(2):309–310, 2004. URL <http://dx.doi.org/10.1111/j.1471-8286.2004.00597.x>.
- J. C. Glaubitz and G. F. Moran. Genetic tools: the use of biochemical and molecular markers. In A. G. Young, D. H. Boshier, and T. J. Boyle, editors, *Forest Conservation Genetics. Principles and Practice*, pages 39–59. CSIRO Publishing & CABI Publishing, Oxon, United Kingdom, 2000.
- J. C. Glaubitz, J. C. Murrell, and G. F. Moran. Effects of native forest regeneration practices on genetic diversity in *Eucalyptus consideniana*. *Theoretical and Applied Genetics*, 107(3):422–431, 2003a. URL <http://dx.doi.org/10.1007/s00122-003-1262-8>.
- J. C. Glaubitz, H. X. Wu, and G. F. Moran. Impacts of silviculture on genetic diversity in the native forest species *Eucalyptus sieberi*. *Conservation Genetics*, 4(3):275–287, 2003b. URL <http://dx.doi.org/10.1023/A:1024025331750>.
- J. Godbout, J. P. Jaramillo-Correa, J. Beaulieu, and J. Bousquet. A mitochondrial DNA minisatellite reveals the postglacial history of jack pine (*Pinus banksiana*), a broad-range North American conifer. *Molecular Ecology*, 14(11):3497–3512, 2005. URL <http://dx.doi.org/10.1111/j.1365-294X.2005.02674.x>.
- D. B. Goldstein, A. R. Linares, L. L. Cavalli-Sforza, and M. W. Feldman. An evaluation of genetic distances for use with microsatellite loci. *Genetics*, 139(1):463–471, 1995. URL <http://www.genetics.org/cgi/content/abstract/139/1/463>.
- A. Gómez, S. C. González-Martínez, C. Collada, L. Gil, and J. Climent. Complex population genetic structure in an endemic Canary Island pine using chloroplast microsatellite markers. *Theoretical and Applied Genetics*, 107(6):1123–1131, 2003. URL <http://dx.doi.org/10.1007/s00122-003-1320-2>.
- P. González, F. Pinto, M. Nogales, J. Jiménez-Asensio, M. Hernández, and V. M. Cabrera. Phylogenetic relationships of the Canary Islands endemic lizard genus *Gallotia* (Sauria: Lacertidae), inferred from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, 6(1):63–71, 1996. URL <http://dx.doi.org/10.1006/mpev.1996.0058>.

- F. Gonzalez-Andrés, J. M. Pita, and J. M. Ortiz. Identification of Iberian and Canarian species of the genus *Pinus* with four isoenzyme systems. *Biochemical Systematics and Ecology*, 27(3):235–242, 1999. URL <http://www.sciencedirect.com/science/article/B6T4R-3W5SNV3-2/2/fe6a5565366717f9230ab4ba36819703>.
- S. C. González-Martínez, S. Gerber, M.-T. Cervera, J.-M. Martínez-Zapater, R. Alía, and L. Gil. Selfing and sibship structure in a two-cohort stand of maritime pine (*Pinus pinaster* Ait.) using nuclear SSR markers. *Annals of Forest Science*, 60(2):115–121, 2003. URL <http://dx.doi.org/10.1051/forest:2003003>.
- S. C. González-Martínez, J. J. Robledo, C. Collada, A. Díaz, C. G. Williams, R. Alía, and M. T. Cervera. Cross-amplification and sequence variation of microsatellite loci in Eurasian hard pines. *Theoretical and Applied Genetics*, 109(1):103–111, 2004. URL <http://dx.doi.org/10.1007/s00122-004-1596-x>.
- C. González. Management statement for the Tenerife great spotted woodpecker (*Dendrocopos major canariensis*). Technical report, BirdLife International, 25 June 2002a. URL [http://www.coe.int/T/E/Cultural\\_Co-operation/Environment/Nature\\_and\\_biological\\_diversity/Nature\\_protection/sc22\\_inf10e.pdf](http://www.coe.int/T/E/Cultural_Co-operation/Environment/Nature_and_biological_diversity/Nature_protection/sc22_inf10e.pdf).
- C. González. Management statement for the Gran Canaria great spotted woodpecker (*Dendrocopos major thanneri*). Technical report, BirdLife International, 25 June 2002b. URL [http://www.coe.int/T/E/Cultural\\_Co-operation/Environment/Nature\\_and\\_biological\\_diversity/Nature\\_protection/sc22\\_inf11e.pdf](http://www.coe.int/T/E/Cultural_Co-operation/Environment/Nature_and_biological_diversity/Nature_protection/sc22_inf11e.pdf).
- R. González Antón, M. C. del Arco Aguilar, R. de Balbín Berhmann, and P. Bueno Ramírez. El poblamiento de un archipiélago Atlántico: Canarias en el proceso colonizador del primer milenio a.C. *Eres (Arqueología)*, 8(1):43–100, 1998. URL [http://www.museosdetenerife.com/museos/m\\_publicaciones.asp?al\\_idioma=0&al\\_id\\_mus=3](http://www.museosdetenerife.com/museos/m_publicaciones.asp?al_idioma=0&al_id_mus=3).
- J. Goudet. FSTAT version 1.2: a computer program to calculate F-statistics. *Journal of Heredity*, 86(6):485–486, 1995.

- E. Gravano. *Pinus canariensis*. In *Pines of Silvicultural Importance*, pages 35–38. CABI Publishing, Oxon, United Kingdom, 2002. URL <http://www.cabi-publishing.org/bookshop/BookDisplay.asp?SubjectArea=&Subject=&PID=1543>.
- H.-J. Gregor. Funde von *Pinus canariensis* Ch. Smith fossilis aus den Neogen von La Palma (Kanarische Inseln). *Vieraea*, 9(1–2):57–64, 1980.
- D. Grill, M. Tausz, U. Pöllinger, M. S. Jiménez, and D. Morales. Effects of drought on needle anatomy of *Pinus canariensis*. *Flora*, 199(2):85–89, 2004. URL <http://dx.doi.org/10.1078/0367-2530-00137>.
- E. Grotkopp, M. Rejmánek, and T. L. Rost. Toward a causal explanation of plant invasiveness: seedling growth and life-history strategies of 29 pine (*Pinus*) species. *American Naturalist*, 159(4):396–419, 2002. URL <http://www.journals.uchicago.edu/AN/journal/issues/v159n4/010049/010049.html>.
- H. Guillou, J. C. Carracedo, F. Pérez Torrado, and E. R. Rodríguez Badiola. K–Ar ages and magnetic stratigraphy of a hotspot-induced, fast grown oceanic island: El Hierro, Canary Islands. *Journal of Volcanology and Geothermal Research*, 73(1–2):141–155, 1996. URL [http://dx.doi.org/10.1016/0377-0273\(96\)00021-2](http://dx.doi.org/10.1016/0377-0273(96)00021-2).
- M. L. Hale, A. M. Borland, M. H. Gustafsson, and K. Wolff. Causes of size homoplasy among chloroplast microsatellites in closely related *Clusia* species. *Journal of Molecular Evolution*, 58(2):182–190, 2004. URL <http://dx.doi.org/10.1007/s00239-003-2540-4>.
- J. W. Hanover. Applications of terpene analysis in forest genetics. *New Forests*, 6(1–4):159–178, 1992. URL <http://dx.doi.org/10.1007/BF00120643>.
- C. P. Hansen, A. Pedersen, and L. Graudal. *International Series of Provenance Trials of Pinus kesiya. Field Assessment Manual*, volume 16 of *Results and Documentation*. Danida Forest Seed Centre, Humlebaek, Denmark, 2003. URL <http://www.dfsc.dk/Technicalnotes.htm>.

- R. M. Harding, A. J. Boyce, and J. B. Clegg. The evolution of tandemly repetitive DNA: recombination rules. *Genetics*, 132(3):847–859, 1992. URL <http://www.genetics.org/cgi/content/abstract/132/3/847>.
- O. J. Hardy and X. Vekemans. Isolation by distance in a continuous population: reconciliation between spatial autocorrelation analysis and population genetics models. *Heredity*, 83(2):145–154, 1999. URL <http://dx.doi.org/10.1038/sj.hdy.6885580>.
- A. Harju and O. Muona. Background pollination in *Pinus sylvestris* seed orchards. *Scandinavian Journal of Forest Research*, 4:513–520, 1989.
- H. C. Harpending, S. T. Sherry, A. R. Rogers, and M. Stoneking. The genetic structure of ancient human populations. *Current Anthropology*, 34(4):483–496, 1993. URL <http://links.jstor.org/sici?sici=0011-3204%28199308%2F10%2934%3A4%3C483%3ATGSOAH%3E2.0.CO%3B2-3>.
- P. B. Heenan, P. J. de Lange, E. K. Cameron, and P. D. Champion. Checklist of dicotyledons, gymnosperms, and pteridophytes naturalised or casual in New Zealand: additional records 1999–2000. *New Zealand Journal of Botany*, 40(2):155–174, 2002. URL <http://www.rsnz.org/publish/nzjb/2002/014.php>.
- D. M. Helfgott, J. Francisco-Ortega, A. Santos-Guerra, R. K. Jansen, and B. B. Simpson. Biogeography and breeding system evolution of the woody *Bencomia* alliance (Rosaceae) in Macaronesia based on ITS sequence data. *Systematic Botany*, 25(1):82–97, 2000. URL <http://www.bioone.org/bioone/?request=get-abstract&issn=0363-6445&volume=025&issue=1&page=0082>.
- J. Hess, J. W. Kadereit, and P. Vargas. The colonization history of *Olea europaea* L. in Macaronesia based on internal transcribed spacer 1 (ITS-1) sequences, randomly amplified polymorphic DNAs (RAPD), and intersimple sequence repeats (ISSR). *Molecular Ecology*, 9(7):857–868, 2000. URL <http://dx.doi.org/10.1046/j.1365-294x.2000.00942.x>.
- G. M. Hewitt. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society*, 58(3):247–276, 1996. URL <http://dx.doi.org/10.1006/bijl.1996.0035>.



- M. Hicks, D. Adams, S. O'Keefe, E. Macdonald, and R. Hodgetts. The development of RAPD and microsatellite markers in lodgepole pine (*Pinus contorta* var. *latifolia*). *Genome*, 41(6):797–805, 1998. URL [http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2\\_abst\\_e?gen\\_g98-084\\_41\\_ns\\_nf\\_gen6-98](http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2_abst_e?gen_g98-084_41_ns_nf_gen6-98).
- K. Hoernle and H. U. Schmincke. The role of partial melting in the 15-Ma geochemical evolution of Gran Canaria: a blob model for the Canary hotspot. *Journal of Petrology*, 34:599–626, 1993.
- K. Hoernle, Y. S. Zhang, and D. Graham. Seismic and geochemical evidence for large-scale mantle upwelling beneath the eastern Atlantic and western and central Europe. *Nature*, 374:34–39, 1995. URL <http://dx.doi.org/10.1038/374034a0>.
- H. Hooghiemstra, C. O. C. Agwu, and H.-J. Beug. Pollen and spore distribution in recent marine sediments: a record of NW-African seasonal wind patterns and vegetation belts. *Meteor Forschungs Ergebnisse*, C(40):87–135, 1986.
- X. S. Hu and R. A. Ennos. Impacts of seed and pollen flow on population genetic structure for plant genomes with three contrasting modes of inheritance. *Genetics*, 152(1):441–450, 1999. URL <http://www.genetics.org/cgi/content/abstract/152/1/441>.
- R. R. Hudson. Gene genealogies and the coalescent process. In D. Futuyma and J. Antonovics, editors, *Oxford Surveys in Evolutionary Biology*, volume 7, pages 1–44. Oxford University Press, Oxford, United Kingdom, 1990.
- J. Hutchinson. *Pinus canariensis*. *Kew Bulletin*, 1918(1):1–3, 1918.
- K. Isik and N. Kara. Altitudinal variation in *Pinus brutia* Ten. and its implication in genetic conservation and seed transfers in southern Turkey. *Silvae Genetica*, 46(2–3):113–120, 1997. URL <http://www.bfafh.de/inst2/sg46pdf.htm>.
- I. Izquierdo, J. L. Martín, N. Zurita, and M. Arechavaleta, editors. *Lista de Especies Silvestres de Canarias (Hongos, Plantas y Animales Terrestres)*. Banco de Datos de Biodiversidad de Canarias. Consejería de Política Territorial y Medio Ambiente Gobierno de Canarias, La Laguna, Tenerife, Spain, 2001. URL <http://www.gobcan.es/medioambiente/biodiversidad/ceplam/bancodatos/libro.html>.

- S. T. Jackson, R. S. Webb, K. H. Anderson, J. T. Overpeck, T. Webb III, J. W. Williams, and B. C. S. Hansen. Vegetation and environment in eastern North America during the last glacial maximum. *Quaternary Science Reviews*, 19(6): 489–508, 2000. URL [http://dx.doi.org/10.1016/S0277-3791\(99\)00093-1](http://dx.doi.org/10.1016/S0277-3791(99)00093-1).
- P. Jarne and P. J. L. Lagoda. Microsatellites, from molecules to populations and back. *Trends in Ecology & Evolution*, 11(10):424–429, 1996. URL [http://dx.doi.org/10.1016/0169-5347\(96\)10049-5](http://dx.doi.org/10.1016/0169-5347(96)10049-5).
- J. L. Jensen, A. J. Bohonak, and S. T. Kelley. ISOLATION BY DISTANCE, web service. *BMC Genetics*, 6(13), 2005. URL <http://dx.doi.org/10.1186/1471-2156-6-13>.
- M. S. Jiménez and D. Morales. Pino canario — Ejemplo de adaptación. *Investigación y Ciencia*, 302:23–24, November 2001.
- M. S. Jiménez, M. Tausz, G. Zelling, J. Peters, D. Grill, and D. Morales. Environmental stresses and antioxidative responses of *Pinus canariensis* at different field stands in Tenerife. *Phyton*, 37(3):109–114, 1997.
- P. Jiménez and C. Collada. Técnicas para la evaluación de la diversidad genética y su uso en los programas de conservación. *Investigación Agraria. Sistemas y Recursos Forestales*, 2:237–248, 2000. URL <http://www.inia.es/gcont/publicaciones/index.jsp?idcategoria=1390&regini=10>. Fuera de serie.
- S. Jonsson, B. Gunnarson, and C. Criado. Drought is the major limiting factor for tree-ring growth of high-altitude Canary Island pines on Tenerife. *Geografiska Annaler Series A — Physical Geography*, 84(1):51–71, 2002. URL <http://dx.doi.org/10.1111/j.0435-3676.2002.00161.x>.
- T. H. Jorgensen and J. Frydenberg. Diversification in insular plants: inferring the phylogenetic relationship in *Aeonium* (Crassulaceae) using ITS sequences of nuclear ribosomal DNA. *Nordic Journal of Botany*, 19:613–621, 1999.
- T. H. Jorgensen and J. M. Olesen. Adaptive radiation of island plants: evidence from *Aeonium* (Crassulaceae) of the Canary Islands. *Perspectives in Plant Ecology, Evolution and Systematics*, 4(1):29–42, 2001. URL <http://dx.doi.org/10.1078/1433-8319-00013>.

- C. Juan, P. Oromí, and G. M. Hewitt. Phylogeny of the genus *Hegeter* (Tenebrionidae, Coleoptera) and its colonization of the Canary Islands deduced from cytochrome oxidase I mitochondrial DNA sequences. *Heredity*, 76(4):392–403, 1996. URL <http://www.nature.com/hdy/journal/v76/n4/abs/6888140a.html>.
- C. Juan, B. C. Emerson, P. Oromí, and G. M. Hewitt. Colonization and diversification: towards a phylogeographic synthesis for the Canary Islands. *Trends in Ecology & Evolution*, 15(3):104–109, 2000. URL [http://dx.doi.org/10.1016/S0169-5347\(99\)01776-0](http://dx.doi.org/10.1016/S0169-5347(99)01776-0).
- S. T. Kalinowski. Counting alleles with rarefaction: private alleles and hierarchical sampling designs. *Conservation Genetics*, 5(4):539–543, 2004. URL <http://dx.doi.org/10.1023/B:COGE.0000041021.91777.1a>.
- N. Kara, L. Korol, K. Isik, and G. Schiller. Genetic diversity in *Pinus brutia* Ten.: altitudinal variation. *Silvae Genetica*, 46(2–3):155–161, 1997. URL <http://www.bfafh.de/inst2/sg46pdf.htm>.
- A. Karhu, P. Hurme, M. Karjalainen, P. Karvonen, K. Kärkkäinen, D. Neale, and O. Savolainen. Do molecular markers reflect patterns of differentiation in adaptive traits of conifers? *Theoretical and Applied Genetics*, 93(1–2):215–221, 1996. URL <http://dx.doi.org/10.1007/s001220050268>.
- A. Karp, S. Kresovich, K. V. Bhat, W. G. Ayad, and T. Hodgkin. *Molecular Tools in Plant Genetic Resources Conservation: A Guide to the Technologies*, volume 2 of *IPGRI Technical Bulletin*. International Plant Genetic Resources Institute, Rome, Italy, 1997. URL <http://www.ipgri.cgiar.org/publications/pubfile.asp?ID.PUB=138>.
- B. Kasapligil. A Late-Tertiary conifer-hardwood forest from the vicinity of Güvem village, near Kızılcahamam, Ankara. *Bulletin of the Mineral Research and Exploration Institute of Turkey*, 88:25–33, 1977. URL <http://www.mta.gov.tr/english/dergi/derg88.asp>.
- B. Kasapligil. Past and present pines of Turkey. *Phytologia*, 40:99–199, 1978.

- J. E. Keeley and P. H. Zedler. Evolution of life histories in *Pinus*. In D. M. Richardson, editor, *Ecology and Biogeography of Pinus*, pages 219–251. Cambridge University Press, Cambridge, United Kingdom, 1998.
- H. Keng and E. L. Little Jr. Needle characteristics of hybrid pines. *Silvae Genetica*, 10:131–146, 1961.
- R. N. Keys, A. Autino, K. J. Edwards, B. Fady, C. Pichot, and G. G. Vendramin. Characterization of nuclear microsatellites in *Pinus halepensis* Mill. and their inheritance in *P. halepensis* and *Pinus brutia* Ten. *Molecular Ecology*, 9(12): 2157–2159, 2000. URL <http://dx.doi.org/10.1046/j.1365-294X.2000.10532.x>.
- M. A. K. Khalil. Genetics of cone morphology of black spruce [*Picea mariana* (Mill.) B.S.P.] in Newfoundland, Canada. *Silvae Genetica*, 33(4–5):101–109, 1984. URL <http://www.bfafh.de/inst2/sg33pdf.htm>.
- A. Khattabi. Contribution a la connaissance de la qualité technologique du bois de *Pinus canariensis*. *Annales de la Recherche Forestière au Maroc*, 25:54–61, 1991.
- S.-C. Kim, D. J. Crawford, J. Francisco-Ortega, and A. Santos-Guerra. A common origin for woody *Sonchus* and five related genera in the Macaronesian islands: molecular evidence for extensive radiation. *Proceedings of the National Academy of Sciences of the United States of America*, 93(15):7743–7748, 1996a. URL <http://www.pnas.org/cgi/content/abstract/93/15/7743>.
- S.-C. Kim, D. J. Crawford, and R. K. Jansen. Phylogenetic relationships among the genera of the subtribe Sonchinae (Asteraceae): evidence from ITS sequences. *Systematic Botany*, 21(3):417–432, 1996b.
- M. Kimura and J. F. Crow. The number of alleles that can be maintained in a finite population. *Genetics*, 49(4):725–738, 1964. URL <http://www.genetics.org/content/vol49/issue4/index.shtml>.
- J. P. King, M. Kimmel, and R. Chakraborty. A power analysis of microsatellite-based statistics for inferring past population growth. *Molecular Biology and Evolution*, 17(12):1859–1868, 2000. URL <http://mbe.oupjournals.org/cgi/content/abstract/17/12/1859>.

- W. Klaus. Ein *Pinus canariensis* Smith-zapfenfund aus dem ober-Miozän pannon des Wiener Beckens. *Annalen des Naturhistorischen Museums in Wien*, 84:79–84, 1982.
- W. Klaus. Mediterranean pines and their history. *Plant Systematics and Evolution*, 162:133–163, 1989.
- W. Klaus. Zur mikroflora des Unter-Sarmat am Alpen-Südostrand. *Beiträge zur Paläontologie von Österreich*, 11:291–437, 1984.
- R. T. Klumpp and M. Stefsky. Genetic variation of *Pinus cembra* along an elevational transect in Austria. In R. A. Snieszko, S. Samman, S. E. Schlarbaum, and H. B. Kriebel, editors, *Breeding and Genetic Resources of Five-Needle Pines: Growth, Adaptability and Pest Resistance*, Medford, Oregon, USA, 2001. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. URL [http://www.fs.fed.us/rm/pubs/rmrs\\_p032.html](http://www.fs.fed.us/rm/pubs/rmrs_p032.html).
- L. Korol, L. Gil, J. Climent, A. Zehavi, and G. Schiller. Canary Islands pine (*Pinus canariensis* Chr. Sm. ex DC.) 2. Gene flow among native populations. *Forest Genetics*, 6(4):277–282, 1999. URL <http://www.tuzvo.sk/~paule/FG99/cover64.htm>.
- I. I. Korshikov, F. Ducci, N. S. Terliga, S. A. Bychkov, and E. M. Gorlova. Allozyme variation of *Pinus pallasiana* D. Don in natural Crimean populations and in plantations in technogenously-polluted areas of the Ukraine steppes. *Annals of Forest Science*, 61(5):389–396, 2004. URL <http://dx.doi.org/10.1051/forest:2004032>.
- I. I. Korshikov, L. A. Kalafat, Y. V. Pirko, and T. I. Velicoridko. Population-genetic variation in Scots pine *Pinus sylvestris* L. from the main forest regions of Ukraine. *Russian Journal of Genetics*, 41(2):155–166, 2005. URL <http://dx.doi.org/10.1007/s11177-005-0039-3>.
- S. Kostia, S. L. Varvio, P. Vakkari, and P. Pulkkinen. Microsatellite sequences in a conifer, *Pinus sylvestris*. *Genome*, 38(6):1244–1248, 1995. URL [http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2\\_desc\\_e?gen](http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2_desc_e?gen).

- S. L. Krugman and J. L. Jenkinson. *Pinus* L. In S. C. Schopmeyer, editor, *Seeds of Woody Plants in the United States*, pages 598–638. USDA Forest Service, Washington DC, USA, 1974. URL <http://www.nsl.fs.fed.us/wpsm/>.
- A. B. Krupkin, A. Liston, and S. H. Strauss. Phylogenetic analysis of the hard pines (*Pinus* subgenus *Pinus*, Pinaceae) from chloroplast DNA restriction site analysis. *American Journal of Botany*, 83(4):489–498, 1996. URL <http://links.jstor.org/sici?sici=0002-9122%28199604%2983%3A4%3C489%3APA0THP%3E2.0.CO%3B2-1>.
- B. L. Kutil and C. G. Williams. Triplet-repeat microsatellites shared among hard and soft pines. *Journal of Heredity*, 92(4):327–332, 2001. URL <http://jhered.oupjournals.org/cgi/content/abstract/92/4/327>.
- R. M. Lanner. Seed dispersal in *Pinus*. In D. M. Richardson, editor, *Ecology and Biogeography of Pinus*, pages 281–295. Cambridge University Press, Cambridge, United Kingdom, 1998.
- R. G. Latta and J. B. Mitton. A comparison of population differentiation across four classes of gene marker in limber pine (*Pinus flexilis* James). *Genetics*, 146(3):1153–1163, 1997. URL <http://www.genetics.org/cgi/content/abstract/146/3/1153>.
- R. G. Latta, Y. B. Linhart, D. Fleck, and M. Elliot. Direct and indirect estimates of seed versus pollen movement within a population of ponderosa pine. *Evolution*, 52(1):61–67, 1998. URL <http://links.jstor.org/sici?sici=0014-3820%28199802%2952%3A1%3C61%3ADAIEOS%3E2.0.CO%3B2-V>.
- D. C. Le Maitre. Pines in cultivation: a global view. In D. M. Richardson, editor, *Ecology and Biogeography of Pinus*, pages 407–431. Cambridge University Press, Cambridge, United Kingdom, 1998.
- R. Leblois, A. Estoup, and F. Rousset. Influence of mutational and sampling factors on the estimation of demographic parameters in a “continuous” population under isolation by distance. *Molecular Biology and Evolution*, 20(4):491–502, 2003. URL <http://dx.doi.org/10.1093/molbev/msg034>.

- F. T. Ledig. Genetic variation in *Pinus*. In D. M. Richardson, editor, *Ecology and Biogeography of Pinus*, pages 251–280. Cambridge University Press, Cambridge, United Kingdom, 1998.
- F. T. Ledig. Human impacts on genetic diversity in forest ecosystems. *Oikos*, 63(1):87–108, 1992. URL [md1.csa.com/partners/viewrecord.php?requester=gs&collection=ENV&recid=2698860](http://md1.csa.com/partners/viewrecord.php?requester=gs&collection=ENV&recid=2698860).
- F. Lefèvre. Human impacts on forest genetic resources in the temperate zone: an updated review. *Forest Ecology and Management*, 197(1–3):257–271, 2004. URL <http://dx.doi.org/10.1016/j.foreco.2004.05.017>.
- P. Legendre and L. Legendre. *Numerical Ecology*. Elsevier, New York, USA, 2<sup>nd</sup> edition, 1998.
- T. Lenormand. Gene flow and the limits to natural selection. *Trends in Ecology & Evolution*, 17(4):183–189, 2002. URL [http://dx.doi.org/10.1016/S0169-5347\(02\)02497-7](http://dx.doi.org/10.1016/S0169-5347(02)02497-7).
- B. Li and W. F. Gu. Review on genetic diversity in *Pinus*. *Yi Chuan*, 25(6):740–748, 2003. URL [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=15639973](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15639973).
- Y.-C. Li, A. B. Korol, T. Fahima, A. Beiles, and E. Nevo. Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. *Molecular Ecology*, 11(12):2453–2465, 2002. URL <http://dx.doi.org/10.1046/j.1365-294X.2002.01643.x>.
- Y.-Y. Li, X.-Y. Chen, X. Zhang, T.-Y. Wu, H.-P. Lu, and Y.-W. Cai. Genetic differences between wild and artificial populations of *Metasequoia glyptostroboides*: implications for species recovery. *Conservation Biology*, 19(1):224–231, 2005. URL <http://dx.doi.org/10.1111/j.1523-1739.2005.00025.x>.
- C. Lian, M. Miwa, and T. Hogetsu. Isolation and characterization of microsatellite loci from the Japanese red pine, *Pinus densiflora*. *Molecular Ecology*, 9(8):1186–1188, 2000. URL <http://dx.doi.org/10.1046/j.1365-294x.2000.00954-10.x>.

- C. Lian, M. Miwa, and T. Hogetsu. Outcrossing and paternity analysis of *Pinus densiflora* (Japanese red pine) by microsatellite polymorphism. *Heredity*, 87(1): 88–98, 2001. URL <http://dx.doi.org/10.1046/j.1365-2540.2001.00913.x>.
- C. Liewlaksaneeyanawin, C. E. Ritland, Y. A. El-Kassaby, and K. Ritland. Single-copy, species-transferable microsatellite markers developed from loblolly pine ests. *Theoretical and Applied Genetics*, 109:361–369, 2004. URL <http://dx.doi.org/10.1007/s00122-004-1635-7>.
- A. Liston, W. A. Robinson, D. Piñero, and E. R. Alvarez-Buylla. Phylogenetics of *Pinus* (Pinaceae) based on nuclear ribosomal DNA internal transcribed spacer region sequences. *Molecular Phylogenetics and Evolution*, 11(1):95–109, 1999. URL <http://dx.doi.org/10.1006/mpev.1998.0550>.
- U. López-de Heredia, P. Jiménez, P. Díaz-Fernández, and L. Gil. The Balearic Islands: a reservoir of cpDNA genetic variation for evergreen oaks. *Journal of Biogeography*, 32(6):939–949, 2005. URL <http://dx.doi.org/10.1111/j.1365-2699.2004.01232.x>.
- N. López Martínez and L. F. López Jurado. Un nuevo múrido gigante del Cuaternario de Gran Canaria. *Canariomys tamarani* nov. sp. (Rodentia, Mammalia). Interpretación filogenética y biogeográfica. *Doñana*, 2:1–60, 1987.
- C. Lyell. *Principles of Geology*. John Murray, London, United Kingdom, 1830. URL <http://www.esp.org/books/lyell/principles/facsimile/>.
- N. Maca-Meyer, S. Carranza, J. C. Rando, E. N. Arnold, and V. M. Cabrera. Status and relationships of the extinct giant Canary Island lizard *Gallotia goliath* (Reptilia: Lacertidae), assessed using ancient mtDNA from its mummified remains. *Biological Journal of the Linnean Society*, 80(4):659–670, 2003. URL <http://dx.doi.org/10.1111/j.1095-8312.2003.00265.x>.
- R. H. MacArthur and E. O. Wilson. An equilibrium theory of insular zoogeography. *Evolution*, 17(4):373–387, 1963. URL <http://links.jstor.org/sici?sici=0014-3820%28196312%2917%3A4%3C373%3AAET0IZ%3E2.O.CO%3B2-X>.
- R. H. MacArthur and E. O. Wilson. *The Theory of Island Biogeography*. Princeton University Press, Princeton, USA, 1967.



- G. M. MacDonald and L. C. Cwynar. Post-glacial population growth rates of *Pinus contorta* subsp. *latifolia* in western Canada. *Journal of Ecology*, 79(2):417–429, 1991. URL <http://links.jstor.org/sici?sici=0022-0477%28199106%2979%3A2%3C417%3APPGRP%3E2.0.CO%3B2-X>.
- S. E. Macdonald, B. R. Thomas, D. M. Cherniawsky, and B. G. Purdy. Managing genetic resources of lodgepole pine in west-central Alberta: patterns of isozyme variation in natural populations and effects of forest management. *Forest Ecology and Management*, 152(1–3):45–58, 2001. URL [http://dx.doi.org/10.1016/S0378-1127\(00\)00616-2](http://dx.doi.org/10.1016/S0378-1127(00)00616-2).
- D. H. Mai. Development and regional differentiation of the European vegetation during the Tertiary. *Plant Systematics and Evolution*, 162(1–4):79–91, 1989. URL <http://dx.doi.org/10.1007/BF00936911>.
- B. Malmqvist, C. Meisch, and A. N. Nilsson. Distribution patterns of freshwater Ostracoda (Crustacea) in the Canary Islands with regards to habitat use and biogeography. *Hydrobiologia*, 347(1–3):159–170, 1997. URL <http://dx.doi.org/10.1023/A:1003087806637>.
- N. A. Mantel. The detection of disease clustering and a generalized regression approach. *Cancer Research*, 27:209–220, 1967. URL [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list\\_uids=6018555&dopt=Citation](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=6018555&dopt=Citation).
- S. Mariette, D. Chagné, S. Decroocq, G. G. Vendramin, C. Lalanne, D. Madur, and C. Plomion. Microsatellite markers for *Pinus pinaster* Ait. *Annals of Forest Science*, 58(2):203–206, 2001. URL <http://dx.doi.org/10.1051/forest:2001119>.
- A. Marrero and J. Francisco-Ortega. Evolución en islas: la metáfora espacio-tiempo-forma. In J. M. Fernández-Palacios and J. L. Martín Esquivel, editors, *Naturaleza de las Islas Canarias: Ecología y Conservación*, pages 133–140. Turquesa, Santa Cruz de Tenerife, Spain, 2001.
- H. D. Marshall, C. Newton, and K. Ritland. Chloroplast phylogeography and evolution of highly polymorphic microsatellites in lodgepole pine (*Pinus con-*

- torta). *Theoretical and Applied Genetics*, 104(2–3):367–378, 2002. URL <http://dx.doi.org/10.1007/s001220100687>.
- J. L. Martín, I. Izquierdo, M. Arechavaleta, M. A. Delgado, A. García Ramírez, M. d. C. Marrero, E. Martín, L. Rodríguez, S. Rodríguez Núñez, and N. Zurita. Las cifras de la biodiversidad taxonómica terrestre de Canarias. In I. Izquierdo, J. L. Martín, N. Zurita, and M. Arechavaleta, editors, *Lista de Especies Silvestres de Canarias (Hongos, Plantas y Animales Terrestres)*, pages 17–26. Consejería de Política Territorial y Medio Ambiente Gobierno de Canarias, La Laguna, Tenerife, Spain, 2001. URL <http://www.gobcan.es/medioambiente/biodiversidad/ceplam/bancodatos/libro.html>.
- C. Martínez, I. S. Harry, and T. A. Thorpe. In vitro regeneration of plantlets of Canary Island pine (*Pinus canariensis*). *Canadian Journal of Forest Research*, 20:1200–1211, 1990.
- M. V. Marzol. El clima de montaña de la Isla de Tenerife. Variaciones en el gradiente térmico. In *Actas del VII Coloquio de Geografía*, volume 1, pages 163–168, Pamplona, Spain, 1981. Asociación de Geógrafos Españoles.
- M. V. Marzol. El clima. In J. M. Fernández-Palacios and J. L. Martín Esquivel, editors, *Naturaleza de las Islas Canarias: Ecología y Conservación*, pages 87–93. Publicaciones Turquesa S.L., Santa Cruz de Tenerife, Spain, 2001.
- D. G. Masson, A. B. Watts, M. J. R. Gee, R. Urgeles, N. C. Mitchell, T. P. Le Bas, and M. Canals. Slope failures on the flanks of the western Canary Islands. *Earth-Science Reviews*, 57(1–2):1–35, 2002. URL [http://dx.doi.org/10.1016/S0012-8252\(01\)00069-1](http://dx.doi.org/10.1016/S0012-8252(01)00069-1).
- F. Médail and P. Quézel. Biodiversity hotspots in the Mediterranean basin: setting global conservation priorities. *Conservation Biology*, 13(6):1510–1513, 1999. URL <http://dx.doi.org/10.1046/j.1523-1739.1999.98467.x>.
- F. Médail and P. Quézel. The phytogeographical significance of S.W. Morocco compared to the Canary Islands. *Plant Ecology*, 140(2):221–244, 1999. URL <http://dx.doi.org/10.1023/A:1009775327616>.
- H. Meusel. Über wuchsformen, verbreitung und phylogenie einiger mediterraneanmitteleuropäischer angiospermen-gattungen. *Flora*, 139:333–393, 1953.

- H. Meusel. Die reliktovegetation der Kanarischen Inseln in ihren Beziehungen zur süd- und mitteleuropäischen Flora. In M. Gersch, editor, *Gesammelte Vorträge über Moderne Probleme der Abstammungslehre*, volume 1, pages 117–136. Jena, Germany, 1965.
- J. Michaux, N. López-Martínez, and J. L. Hernández-Pacheco. A 14C dating of *Canariomys bravoii* (Mammalia, Rodentia), the extinct giant rat from Tenerife (Canary Islands) and the recent history of the endemic species in the archipelago. *Vie Milieu*, 46(3–4):261–266, 1996. URL <http://md1.csa.com/partners/viewrecord.php?requester=gs&collection=ENV&recid=4076673>.
- C. I. Millar. Impact of the Eocene on the evolution of *Pinus* L. *Annals of the Missouri Botanical Garden*, 8(2):471–498, 1993. URL <http://links.jstor.org/sici?sici=0026-6493%281993%2980%3A2%3C471%3AIOTEOT%3E2.O.CO%3B2-V>.
- C. I. Millar. Early evolution of pines. In D. M. Richardson, editor, *Ecology and Biogeography of Pinus*, pages 69–91. Cambridge University Press, Cambridge, United Kingdom, 1998.
- J. B. Mitton and K. L. Duran. Genetic variation in piñon pine, *Pinus edulis*, associated with summer precipitation. *Molecular Ecology*, 13(5):1259–1264, 2004. URL <http://dx.doi.org/10.1111/j.1365-294X.2004.02122.x>.
- J. B. Mitton, K. B. Sturgeon, and M. L. Davis. Genetic differentiation in ponderosa pine along a steep elevational transect. *Silvae Genetica*, 29(3–4):100–103, 1980. URL <http://www.bfafh.de/SEARCH/ELFIST/DDW?W%3DPUBLIKATIONSJAHR+INC+%271980%27+AND+QUELLE+PH+WORDS+%27Silvae+Genetica%27+ORDER+BY+TRNN/Ascend%26M%3D31%26K%3DT3+04+189%26R%3DY%26U%3D1>.
- D. Morales, J. Peters, M. S. Jiménez, M. Tausz, A. Wonisch, and D. Grill. Gas exchange of irrigated and non-irrigated *Pinus canariensis* seedlings growing outdoors in La Laguna, Canary Islands, Spain. *Zeitschrift für Naturforschung C*, 54c(9/10):693–697, 1999.

- V. C. Moran, J. H. Hoffmann, D. Donnelly, B. W. Van Wilgen, and H. G. Zimmermann. Biological control of alien, invasive pine trees (*Pinus* species) in South Africa. In N. R. Spencer, editor, *X International Symposium on Biological Control of Weeds*, pages 941–953, Bozeman, Montana, USA, 2000. Montana State University,. URL <http://www.invasive.org/publications/xsymposium/Session13.html>.
- W. J. Morgan. Convection plumes in the lower mantle. *Nature*, 230:42–43, 1971.
- M. Morgante, N. Felice, and G. G. Vendramin. Analysis of hypervariable chloroplast microsatellites in *Pinus halepensis* reveals a dramatic genetic bottleneck. In A. Karp, P. G. Isaac, and D. S. Ingram, editors, *Molecular Tools for Screening Biodiversity. Plants and Animals*, pages 407–412. Chapman and Hall, London, United Kingdom, 1997.
- C. Morla Juaristi, C. Alcalde Olivares, J. M. Postigo Mijarra, and E. Barrón. Paleobiogeografía de *Pinus canariensis*: estróbilos y semillas fósiles del Plioceno ibérico (Cuenca del Bajo Segura, Alicante, España). In M. E. Arozena Concepción, E. Beltrán Yanes, and P. Dorta Antequera, editors, *La Biogeografía: Ciencia Geográfica y Ciencia Biológica*, pages 311–323. Universidad de La Laguna, La Laguna, Tenerife, Spain, 2003.
- C. Mátyás. Climatic adaptation of trees: rediscovering provenance tests. *Euphytica*, 92(1–2):45–54, 1996. URL <http://dx.doi.org/10.1007/BF00022827>.
- O. Muona and A. Harju. Effective population sizes, genetic variability, and mating system in natural stands and seed orchards of *Pinus sylvestris*. *Silvae Genetica*, 38(5–6):221–228, 1989. URL <http://www.bfafh.de/inst2/sg38pdf.htm>.
- G. Namkoong and M. P. Koshy. Application of genetic markers to forest tree species. Technical report, International Plant Genetic Resources Institute, 2001. URL [http://www.ipgri.cgiar.org/publications/pubfile+.asp?id\\_pub=737](http://www.ipgri.cgiar.org/publications/pubfile+.asp?id_pub=737). Draft report to IPGRI of the project *Developing Decision-making Strategies on Priorities for Conservation and Use of Forest Genetic Resources*.
- J. Naranjo Borges. Los aprovechamientos forestales. In J. M. Fernández-Palacios and J. L. Martín Esquivel, editors, *Naturaleza de las Islas Canarias. Ecología*

- y Conservación*, pages 269–274. Ediciones Turquesa, Santa Cruz de Tenerife, Spain, 2001.
- R. Nathan and G. G. Katul. Foliage shedding in deciduous forests lifts up long-distance seed dispersal by wind. *Proceedings of the National Academy of Sciences of the United States of America*, 102(23):8251–8256, 2005. URL <http://dx.doi.org/10.1073/pnas.0503048102>.
- M. Navascués and B. C. Emerson. Chloroplast microsatellites: measures of genetic diversity and the effect of homoplasmy. *Molecular Ecology*, 14(5):1333–1341, 2005. URL <http://dx.doi.org/10.1111/j.1365-294X.2005.02504.x>.
- D. B. Neale and R. R. Sederoff. Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. *Theoretical and Applied Genetics*, 77(2):212–216, 1989.
- M. Nei. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 89(3):583–590, 1978. URL <http://intl.genetics.org/cgi/content/abstract/89/3/583>.
- M. Nei. *Molecular Evolutionary Genetics*. Columbia University Press, New York, USA, 1987.
- M. Nei, T. Maruyama, and R. Chakraborty. The bottleneck effect and genetic variability in populations. *Evolution*, 29(1):1–10, 1975. URL <http://links.jstor.org/sici?sici=0014-3820%28197503%2929%3A1%3C1%3ATBEAGV%3E2.0.CO%3B2-Z>.
- S. E. Nijensohn, P. G. Schaberg, G. J. Hawley, and D. H. DeHayes. Genetic subpopulation structuring and its implications in a mature eastern white pine stand. *Canadian Journal of Forest Research*, 35(5):1041–1052, 2005. URL [http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2\\_abst\\_e?cjfr\\_x05-029\\_35\\_ns\\_nf\\_cjfr5-05](http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2_abst_e?cjfr_x05-029_35_ns_nf_cjfr5-05).
- M. Nogales, P. Oromí, J. M. Peraza, and M. Marrero. Datos sobre la fauna corticícola del tronco del pino canario (*Pinus canariensis* Chr. Sm. ex DC.). *Vieraea*, 18:143–147, 1990.

- J. V. Nogales Ventoso. *Pinus canariensis* Chr. Sm. *Bulletin de la Société Nationale d'Acclimatation de France*, 1917:322–325, 1917.
- B. I. Nyoka. The status of invasive alien forest trees species in southern Africa. *Forest Genetic Resources*, 30:11–13, 2002. URL <http://www.fao.org/DOCREP/005/Y4341E/Y4341E00.htm>.
- J. Oleksyn, J. Modrzynski, M. G. Tjoelker, R. Z. ytkowiak, P. B. Reich, and P. Karolewski. Growth and physiology of *Picea abies* populations from elevational transects: common garden evidence for altitudinal ecotypes and cold adaptation. *Functional Ecology*, 12(4):573–590, 1998. URL <http://dx.doi.org/10.1046/j.1365-2435.1998.00236.x>.
- N. C. Page. Morphology and affinities of *Pinus canariensis*. *Notes of the Royal Botanical Garden Edinburgh*, 33(2):317–323, 1974.
- E. P. Palkovacs. Explaining adaptive shifts in body size on islands: a life history approach. *Oikos*, 103(1):37–44, 2003. URL <http://dx.doi.org/10.1034/j.1600-0706.2003.12502.x>.
- J. L. Panero, J. Francisco-Ortega, R. K. Jansen, and A. Santos-Guerra. Molecular evidence for multiple origins of woodiness and a New World biogeographic connection of the Macaronesian island endemic *Pericallis* (Asteraceae: Senecioneae). *Proceedings of the National Academy of Sciences of the United States of America*, 96(24):13886–13891, 1999. URL <http://www.pnas.org/cgi/content/abstract/96/24/13886>.
- S. D. E. Park. *Trypanotolerance in West African Cattle and the Population Genetic Effects of Selection*. PhD, University of Dublin, 2001.
- P. G. Parker, A. A. Snow, M. D. Schug, G. C. Booton, and P. A. Fuerst. What molecules can tell us about populations: choosing and using a molecular marker. *Ecology*, 79(2):361–381, 1998. URL <http://www.esajournals.org/esaonline/?request=get-abstract&issn=0012-9658&volume=079&issue=02&page=0361>.
- J. J. Parsons. Human influences on the pine and laurel forests of the Canary Islands. *Geographical Review*, 71(3):253–271, 1981. URL

<http://links.jstor.org/sici?sici=0016-7428%28198107%2971%3A3%3C253%3AHIOTPA%3E2.0.CO%3B2-%23>.

- C. Peraza and A. López de Roma. *Estudio de las Principales Maderas de Canarias*. IFIE, Madrid, 1967.
- L. Pereira, M. J. Prata, and A. Amorim. Mismatch distribution analysis of Y-STR haplotypes as a tool for the evaluation of identity-by-state proportions and significance of matches — the European picture. *Forensic Science International*, 130(2–3):147–155, 2002. URL [http://dx.doi.org/10.1016/S0379-0738\(02\)00371-7](http://dx.doi.org/10.1016/S0379-0738(02)00371-7).
- P. L. Pérez de Paz, M. J. del Arco, O. Rodríguez, J. R. Acebes, M. Marrero, and W. Wildpret. *Atlas Cartográfico de los Pinares Canarios III: La Palma*. Gobierno de Canarias, Consejería de Política Territorial, Santa Cruz de Tenerife, Spain, 1994a.
- P. L. Pérez de Paz, M. Salas, O. Rodríguez, J. R. Acebes, M. J. del Arco, and W. Wildpret. *Atlas Cartográfico de los Pinares Canarios IV: Gran Canaria y Plantaciones de Fuerteventura y Lanzarote*. Gobierno de Canarias, Consejería de Política Territorial, Santa Cruz de Tenerife, Spain, 1994b.
- F. J. Pérez-Torrado, J. C. Carracedo, and J. Mangas. Geochronology and stratigraphy of the Roque Nublo Cycle, Gran Canaria, Canary Islands. *Journal of the Geological Society*, 152(5):807–818, 1995. URL <http://jgs.geoscienceworld.org/cgi/content/abstract/152/5/807>.
- J. Peters, M. S. Jiménez, and D. Morales. Effect of extreme temperature on the photosynthetic apparatus of the Canarian endemic pine (*Pinus canariensis*). *Zeitschrift für Naturforschung C*, 54(9–10):681–687, 1999.
- J. Peters, M. S. Jiménez, and D. Morales. Variaciones en el intercambio gaseoso de *Pinus canariensis* en función de la estacionalidad, edad y posición de las acículas en el dosel. In *XV Reunión de la Sociedad Española & VIII Congreso Hispano-Luso de Fisiología Vegetal*, Palma de Mallorca, Spain, 2003a.
- J. Peters, D. Morales, and M. S. Jiménez. Gas exchange characteristics of *Pinus canariensis* needles in a forest stand on Tenerife, Canary Islands. *Trees* —

- Structure and Function*, 17(6):492–500, 2003b. URL <http://dx.doi.org/10.1007/s00468-003-0261-3>.
- H. W. Pfeifhofer. Composition of the essential oil of *Pinus canariensis* Sweet ex Sprengel. *Flavour and Fragrance Journal*, 15(4):266–270, 2000. URL [http://dx.doi.org/10.1002/1099-1026\(200007/08\)15:4<266::AID-FFJ908>3.0.CO;2-E](http://dx.doi.org/10.1002/1099-1026(200007/08)15:4<266::AID-FFJ908>3.0.CO;2-E).
- J. M. Pita, V. Sanz, and A. Escudero. Seed cryopreservation of seven Spanish native pine species. *Silvae Genetica*, 47(4):220–223, 1998. URL <http://www.bfafh.de/inst2/sg47pdf.htm>.
- A. H. Porter. A test for deviation from island-model population structure. *Molecular Ecology*, 12(4):903–915, 2003. URL <http://dx.doi.org/10.1046/j.1365-294X.2003.01783.x>.
- W. Powell, M. Morgante, R. McDevitt, G. G. Vendramin, and J. A. Rafalski. Polymorphic simple sequence repeat regions in chloroplast genomes: applications to the population genetics of pines. *Proceedings of the National Academy of Sciences of the United States of America*, 92(17):7759–7763, 1995. URL <http://www.pnas.org/cgi/content/abstract/92/17/7759>.
- J. Provan, N. Soranzo, N. J. Wilson, D. B. Goldstein, and W. Powell. A low mutation rate for chloroplast microsatellites. *Genetics*, 153(2):943–947, 1999a. URL <http://www.genetics.org/cgi/content/abstract/153/2/943>.
- J. Provan, N. Soranzo, N. J. Wilson, J. W. McNicol, M. Morgante, and W. Powell. The use of uniparentally inherited simple sequence repeat markers in plant population studies and systematics. In P. M. Hollingsworth, R. M. Bateman, and R. J. Gornall, editors, *Molecular Systematics and Plant Evolution*, pages 35–50. Taylor & Francis, London, United Kingdom, 1999b.
- J. Provan, W. Powell, and P. M. Hollingsworth. Chloroplast microsatellites: new tools for studies in plant ecology and evolution. *Trends in Ecology & Evolution*, 16(3):142–147, 2001. URL [http://dx.doi.org/10.1016/S0169-5347\(00\)02097-8](http://dx.doi.org/10.1016/S0169-5347(00)02097-8).
- J. Provan, P. M. Biss, D. McMeel, and S. Mathews. Universal primers for the amplification of chloroplast microsatellites in grasses (Poaceae). *Molecular Ecology*



- Notes*, 4(2):262–264, 2004. URL <http://dx.doi.org/10.1111/j.1471-8286.2004.00636.x>.
- P. Quézel. Analysis of the flora of Mediterranean and Saharan Africa. *Annals of the Missouri Botanical Garden*, 65(2):479–534, 1978. URL <http://links.jstor.org/sici?sici=0026-6493%281978%2965%3A2%3C479%3AAOTFOM%3E2.O.CO%3B2-0>.
- R. G. Raja, C. G. Tauer, R. F. Wittwer, and Y. Huang. Regeneration methods affect genetic variation and structure in shortleaf pine (*Pinus echinata* Mill.). *Forest Genetics*, 5(3):171–178, 1998. URL [http://www.srs.fs.usda.gov/pubs/ja/ja\\_raja001.pdf](http://www.srs.fs.usda.gov/pubs/ja/ja_raja001.pdf).
- O. P. Rajora. Genetic biodiversity impacts of silvicultural practices and phenotypic selection in white spruce. *Theoretical and Applied Genetics*, 99(6):954–961, 1999. URL <http://dx.doi.org/10.1007/s001220051402>.
- O. P. Rajora and A. Mosseler. Challenges and opportunities for conservation of forest genetic resources. *Euphytica*, 118(2):197–212, 2001. URL <http://dx.doi.org/10.1023/A:1004150525384>.
- O. P. Rajora and S. A. Pluhar. Genetic diversity impacts of forest fires, forest harvesting, and alternative reforestation practices in black spruce (*Picea mariana*). *Theoretical and Applied Genetics*, 106(7):1203–1212, 2003. URL <http://dx.doi.org/10.1007/s00122-002-1169-9>.
- B. Rannala and J. L. Mountain. Detecting immigration by using multilocus genotypes. *Proceedings of the National Academy of Sciences of the United States of America*, 94(17):9197–9201, 1997. URL <http://www.pnas.org/cgi/content/abstract/94/17/9197>.
- M. Raymond and F. Rousset. An exact test for population differentiation. *Evolution*, 49(6):1280–1283, 1995a. URL <http://links.jstor.org/sici?sici=0014-3820%28199512%2949%3A6%3C1280%3AAETFPD%3E2.O.CO%3B2-7>.
- M. Raymond and F. Rousset. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, 86:248–249, 1995b.

- R. Real, J. Olivero, J. C. Guerrero, J. M. Vargas, and A. L. Márquez. Contrastación de hipótesis explicativas de la distribución de la diversidad específica de arañas (Arachnida, Araneae) en las Islas Canarias. *Boletín Sociedad Entomológica Aragonesa*, 26:573–581, 1999. URL <http://entomologia.rediris.es/sea/bol/vol26/s4/>. Monographic Volume: *Evolución y Filogenia de Arthropoda*.
- D. E. Reich and D. B. Goldstein. Genetic evidence for a Paleolithic human population expansion in Africa. *Proceedings of the National Academy of Sciences of the United States of America*, 95(14):8119–8123, 1998. URL <http://www.pnas.org/cgi/content/abstract/95/14/8119>.
- M. M. Ribeiro, G. LeProvost, S. Gerber, G. G. Vendramin, M. Anzidei, S. Decroocq, A. Marpeau, S. Mariette, and C. Plomion. Origin identification of maritime pine stands in France using chloroplast simple-sequence repeats. *Annals of Forest Science*, 59(1):53–62, 2002. URL <http://dx.doi.org/10.1051/forest:2001005>.
- D. M. Richardson and S. I. Higgins. Pines as invaders in the Southern Hemisphere. In D. M. Richardson, editor, *Ecology and Biogeography of Pinus*, pages 450–473. Cambridge University Press, Cambridge, United Kingdom, 1998.
- D. M. Richardson and P. W. Rundel. Ecology and biogeography of *Pinus*: an introduction. In D. M. Richardson, editor, *Ecology and Biogeography of Pinus*, pages 3–46. Cambridge University Press, Cambridge, United Kingdom, 1998.
- D. M. Richardson, P. A. Williams, and R. J. Hobbs. Pine invasions in the Southern Hemisphere: determinants of spread and invadability. *Journal of Biogeography*, 21(5):511–527, 1994. URL <http://links.jstor.org/sici?sici=0305-0270%28199409%2921%3A5%3C511%3APIITSH%3E2.0.CO%3B2-T>.
- R. Rihm, C. L. Jacobs, S. Krastel, H.-U. Schmincke, and B. Alibes. Las Hijas seamounts — The next Canary island? *Terra Nova*, 10(3):121–125, 1998. URL <http://dx.doi.org/10.1046/j.1365-3121.1998.00176.x>.
- S. Rivas-Martínez. *Memoria del Mapa de Series de Vegetación de España*. ICONA, Madrid, 1987.

- S. Rivas-Martínez, W. Wildpret, T. E. Díaz, P. L. Pérez de Paz, M. J. del Arco, and O. Rodríguez Delgado. Excursion guide: outline vegetation of Tenerife island (Canary Islands). *Itinera Geobotánica*, 7:5–169, 1993a.
- S. Rivas-Martínez, W. Wildpret, M. J. del Arco Aguilar, O. Rodríguez Delgado, P. L. Pérez de Paz, A. García-Gallo, J. Acebes, T. E. Díaz, and F. Fernández-González. Las comunidades vegetales de la isla de Tenerife (Islas Canarias). *Itinera Geobotánica*, 7:169–374, 1993b.
- J. J. Robledo-Arnuncio and L. Gil. Patterns of pollen dispersal in a small population of *Pinus sylvestris* L. revealed by total-exclusion paternity analysis. *Heredity*, 94:13–22, 2004. URL <http://dx.doi.org/10.1038/sj.hdy.6800542>.
- A. R. Rogers. Genetic evidence for a Pleistocene population explosion. *Evolution*, 49(4):608–615, 1995. URL <http://links.jstor.org/sici?sici=0014-3820%28199508%2949%3A4%3C608%3AGEFAPP%3E2.0.CO%3B2-8>.
- A. R. Rogers and H. Harpending. Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, 9(3):552–569, 1992. URL <http://mbe.oupjournals.org/cgi/content/abstract/9/3/552>.
- A. R. Rogers, A. E. Fraley, M. J. Bamshad, W. S. Watkins, and L. B. Jorde. Mitochondrial mismatch analysis is insensitive to the mutational process. *Molecular Biology and Evolution*, 13(7):895–902, 1996. URL <http://mbe.oupjournals.org/cgi/content/abstract/13/7/895>.
- P. Rothe. Fossile straußeneier auf Lanzarote. *Natur und Museum*, 94(5):175–218, 1964.
- P. Rothe. Canary Islands — Origin and evolution. *Naturwissenschaften*, 61(12):526–533, 1974. URL <http://dx.doi.org/10.1007/BF00606512>.
- P. Rothe and H.-U. Schmincke. Contrasting origins of the eastern and western islands of the Canarian Archipelago. *Nature*, 218:1152–1154, 1968.
- F. Rousset. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, 145(4):1219–1228, 1997. URL <http://www.genetics.org/cgi/content/abstract/145/4/1219>.

- V. Roussis, P. V. Petrakis, A. Oritz, and B. E. Mazomenos. Volatile constituents of needles of five *Pinus* species grown in Greece. *Phytochemistry*, 39(2):357–361, 1995. URL [http://dx.doi.org/10.1016/0031-9422\(94\)00885-W](http://dx.doi.org/10.1016/0031-9422(94)00885-W).
- C. Sáenz-Romero. Planning forest genetic resource Conservation Units for non-endangered Mexican pines. In J. Bealieu, editor, *Symposium of the North American Forest Commission, Forest Genetic Resources Study Group, and the International Union of Forest Research (IUFRO)*, Silviculture and the Conservation of Genetic Resources for Sustainable Forest Management. Information Report LAU-X-128, pages 37–42, Quebec City, Canada, 2003. Laurentian Forestry Centre, Canadian Forest Service, Natural Resources Canada.
- C. Sáenz-Romero and B. L. Tapia-Olivares. *Pinus oocarpa* isoenzymatic variation along an altitudinal gradient in Michoacán, México. *Silvae Genetica*, 52(5–6): 237–240, 2003. URL <http://www.bfafh.de/inst2/sg52pdf.htm>.
- C. Sáenz-Romero, H. Viveros-Viveros, and R. R. Guzmán-Reyna. Altitudinal genetic variation among *Pinus oocarpa* populations on Michoacán, western México. Preliminary results from a nursery test. *Forest Genetics*, 2003. Submitted.
- A. Santos. Flora vascular nativa. In J. M. Fernández-Palacios and J. L. Martín Esquivel, editors, *Naturaleza de las Islas Canarias. Ecología y Conservación*, pages 185–192. Ediciones Turquesa, Santa Cruz de Tenerife, Spain, 2001.
- G. Saporta. Etudes sur la végétation du Sud-Est de la France à l'époque Tertiaire. *Annales des Sciences Naturelles, Botanique*, 5(3):5–152, 1865.
- G. Saporta. Etudes sur la végétation du Sud-Est de la France à l'époque Tertiaire. *Annales des Sciences Naturelles, Botanique*, 5(9):5–62, 1868.
- G. Saporta. Etudes sur la végétation du Sud-Est de la France à l'époque Tertiaire — Supplément II. Révision de la flore des gypses d'Aix. *Annales des Sciences Naturelles, Botanique*, 5(17):5–44, 1873. URL <http://visualiseur.bnf.fr/CadresFenetre?O=NUMM-98619&M=notice&Y=Image>.
- E. G. F. Sauer and P. Rothe. Ratite eggshells from Lanzarote, Canary Islands. *Science*, 176:43–45, 1972.

- O. Savolainen and H. Kuittinen. Small population processes. In A. G. Young, D. H. Boshier, and T. J. Boyle, editors, *Forest Conservation Genetics. Principles and Practice*, pages 91–100. CSIRO Publishing & CABI Publishing, Oxon, United Kingdom, 2000.
- O. Savolainen and R. Yazdani. Genetic comparison of natural and artificial populations of *Pinus sylvestris*. In G. Müller-Starck and M. Ziehe, editors, *Genetic Variation in European Populations of Forest Trees*, pages 228–234. Sauerländer's Verlag, Frankfurt am Main, Germany, 1991.
- H. Schenck. *Beiträge zur Kenntnis der Vegetation der Canarischen Inseln*, volume III of *Wissenschaftliche Ergebnisse der Deutschen Tiefsee — Expedition auf dem Dapfer "Valdivia" 1898–1899*. Verlag von Gustav Fischer, Jena, Germany, 1907. URL <http://humboldt.mpiwg-berlin.mpg.de/05.sources.htm>.
- G. Schiller, L. Korol, E. D. Ungar, A. Zehavi, L. Gil, and J. Climent. Canary Islands pine (*Pinus canariensis* Chr. Sm. ex DC.) 1. Differentiation among native populations in their isoenzymes. *Forest Genetics*, 6(4):257–276, 1999. URL <http://www.tuzvo.sk/~pau1e/FG99/cover64.htm>.
- C. Schlötterer. Evolutionary dynamics of microsatellite DNA. *Chromosoma*, 109(6):365–371, 2000. URL <http://dx.doi.org/10.1007/s004120000089>.
- H. U. Schmincke. Mid-Pliocene fossil wood from Gran Canaria — Preliminary note. *Cuadernos de Botánica*, 2:19–20, 1967.
- H. U. Schmincke. Pliozäne subtropische vegetation auf Gran Canaria. *Die Naturwiss*, 55(4):185–186, 1968.
- S. Schneider and L. Excoffier. Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. *Genetics*, 152(3):1079–1089, 1999. URL <http://www.genetics.org/cgi/content/abstract/152/3/1079>.
- S. Schneider, D. Roessli, and L. Excoffier. ARLEQUIN: a software for population genetics data analysis, 1999. URL <http://lgb.unige.ch/arlequin/>.

- A. W. Schoettle and S. G. Rochelle. Morphological variation of *Pinus flexilis* (Pinaceae), a bird-dispersed pine, across a range of elevations. *American Journal of Botany*, 87(12):1797–1806, 2000. URL <http://www.amjbot.org/cgi/content/abstract/87/12/1797>.
- W. S. Schuster and J. B. Mitton. Paternity and gene dispersal in limber pine (*Pinus flexilis* James). *Heredity*, 84(3):348–361, 2000. URL <http://dx.doi.org/10.1046/j.1365-2540.2000.00684.x>.
- W. S. Schuster, D. L. Alles, and J. B. Mitton. Gene flow in limber pine: evidence from pollination phenology and genetic differentiation along an elevational transect. *American Journal of Botany*, 76(9):1395–1403, 1989. URL <http://links.jstor.org/sici?sici=0002-9122%28198909%2976%3A9%3C1395%3AGFILPE%3E2.O.CO%3B2-M>.
- N. Shigesada and K. Kawasaki. *Biological Invasions: Theory and Practice*. Oxford Series in Ecology and Evolution. Oxford University Press, Oxford, United Kingdom, 1997.
- J. Silvertown. The ghost of competition past in the phylogeny of island endemic plants. *Journal of Ecology*, 92(1):168–173, 2004. URL <http://dx.doi.org/10.1111/j.1365-2745.2004.00853.x>.
- M. Slatkin. Rare alleles as indicators of gene flow. *Evolution*, 39(1):53–65, 1985. URL <http://links.jstor.org/sici?sici=0014-3820%28198501%2939%3A1%3C53%3ARAAI0G%3E2.O.CO%3B2-1>.
- M. Slatkin. Isolation by distance in equilibrium and non-equilibrium populations. *Evolution*, 47(1):264–279, 1993. URL <http://links.jstor.org/sici?sici=0014-3820%28199302%2947%3A1%3C264%3AIBDIEA%3E2.O.CO%3B2-V>.
- M. Slatkin. Gene flow and selection in a cline. *Genetics*, 75(4):733–756, 1973. URL <http://www.genetics.org/cgi/content/abstract/75/4/733>.
- M. Slatkin. A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, 139(1):457–462, 1995. URL <http://intl.genetics.org/content/vol139/issue1/index.shtml>.

- M. Slatkin and R. R. Hudson. Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics*, 129(2):555–562, 1991. URL <http://www.genetics.org/cgi/content/abstract/129/2/555>.
- G. T. Slavov and P. Zhelev. Allozyme variation, differentiation, and inbreeding in populations of *Pinus mugo* in Bulgaria. *Canadian Journal of Forest Research*, 34(12):2611–2617, 2004. URL [http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2\\_abst\\_f?cjfr\\_x04-127\\_34\\_ns\\_nf\\_cjfr](http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2_abst_f?cjfr_x04-127_34_ns_nf_cjfr).
- D. Smith and M. E. Devey. Occurrence and inheritance of microsatellites in *Pinus radiata*. *Genome*, 37(6):977–983, 1994. URL [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=7828844](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7828844).
- P. E. Smouse, R. J. Dyer, R. D. Westfall, and V. L. Sork. Two-generation analysis of pollen flow across a landscape. I. Male gamete heterogeneity among females. *Evolution*, 55(2):260–271, 2001. URL <http://evol.allenpress.com/evolonline/?request=get-abstract&issn=0014-3820&volume=055&issue=02&page=0260>.
- N. Soranzo, J. Provan, and W. Powell. Characterization of microsatellite loci in *Pinus sylvestris* L. *Molecular Ecology*, 7(9):1260–1261, 1998. URL <http://dx.doi.org/10.1046/j.1365-294x.1998.00406.x>.
- N. Soranzo, J. Provan, and W. Powell. An example of microsatellite length variation in the mitochondrial genome of conifers. *Genome*, 42(1):158–161, 1999. URL [http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2\\_abst\\_e?gen\\_g98-111\\_42\\_ns\\_nf\\_gen1-99](http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2_abst_e?gen_g98-111_42_ns_nf_gen1-99).
- V. L. Sork, J. Nason, D. R. Campbell, and J. F. Fernández. Landscape approaches to historical and contemporary gene flow in plants. *Trends in Ecology & Evolution*, 14(6):219–224, 1999. URL [http://dx.doi.org/10.1016/S0169-5347\(98\)01585-7](http://dx.doi.org/10.1016/S0169-5347(98)01585-7).
- C. C. Spencer, J. E. Neigel, and P. L. Leberg. Experimental evaluation of the usefulness of microsatellite DNA for detecting demographic bottlenecks. *Mole-*

- cular Ecology*, 9(10):1517–1528, 2000. URL <http://dx.doi.org/10.1046/j.1365-294x.2000.01031.x>.
- D. Spencer. *Conifers in the Dry Country*. Rural Industries Research & Development Corporation, Barton, Australia, 2001. URL <http://www.rirdc.gov.au/reports/AFT/01-146sum.html>.
- J. Squirrell, P. M. Hollingsworth, M. Woodhead, J. Russell, A. J. Lowe, M. Gibby, and W. Powell. How much effort is required to isolate nuclear microsatellites from plants? *Molecular Ecology*, 12(6):1339–1348, 2003. URL <http://dx.doi.org/10.1046/j.1365-294X.2003.01825.x>.
- M. Srutek, J. Dolezal, and T. Hara. Spatial structure and associations in a *Pinus canariensis* population at the treeline, Pico del Teide, Tenerife, Canary Islands. *Arctic, Antarctic, and Alpine Research*, 34(2):201–210, 2002. URL <http://www.colorado.edu/INSTAAR/arcticalpine/volume34/34-2abs.html>.
- E. Stabentheiner, H. W. Pfeifhofer, J. Peters, M. S. Jiménez, D. Morales, and D. Grill. Different surface characteristics of primary and secondary needles of *Pinus canariensis*. *Flora*, 199(2):90–99, 2004. URL <http://doi:10.1078/0367-2530-00138>.
- C. Stamatis, A. Triantafyllidis, K. A. Moutou, and Z. Mamuris. Mitochondrial DNA variation in Northeast Atlantic and Mediterranean populations of Norway lobster, *Nephrops norvegicus*. *Molecular Ecology*, 13(6):1377–1390, 2004. URL <http://dx.doi.org/10.1111/j.1365-294X.2004.02165.x>.
- C. J. Stillman. Giant Miocene landslides and the evolution of Fuerteventura, Canary Islands. *Journal of Volcanology and Geothermal Research*, 94(1–4):89–104, 1999. URL [http://dx.doi.org/10.1016/S0377-0273\(99\)00099-2](http://dx.doi.org/10.1016/S0377-0273(99)00099-2).
- S. H. Strauss and A. H. Doerksen. Restriction fragment analysis of pine phylogeny. *Evolution*, 44(4):1081–1096, 1990. URL <http://links.jstor.org/sici?sici=0014-3820%28199007%2944%3A4%3C1081%3ARFAOPP%3E2.0.CO%3B2-5>.
- P. Sunding. Origins of the Macaronesian flora. In D. Bramwell, editor, *Plants and Islands*, pages 13–40. Academic Press, London, United Kingdom, 1979.



- P. Sunnucks. Efficient genetic markers for population biology. *Trends in Ecology & Evolution*, 15(5):199–203, 2000. URL [http://dx.doi.org/10.1016/S0169-5347\(00\)01825-5](http://dx.doi.org/10.1016/S0169-5347(00)01825-5).
- J. Supuka, F. Berta, and A. Chladná. The influence of the urban environment on the composition of terpenes in the needles of black pine (*Pinus nigra* Arnold). *Trees — Structure and Function*, 11(3):176–182, 1997. URL <http://www.springerlink.com/openurl.asp?genre=article&id=6TUFK81T17LL7JDN>.
- H. Tachida and M. Iizuka. Persistence of repeated sequences that evolve by replication slippage. *Genetics*, 131(2):471–478, 1992. URL <http://www.genetics.org/cgi/content/abstract/131/2/471>.
- F. Tajima. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123(3):585–595, 1989. URL <http://www.genetics.org/cgi/content/abstract/123/3/585>.
- R. Tapias, J. Climent, J. A. Pardos, and L. Gil. Life histories of Mediterranean pines. *Plant Ecology*, 171(1–2):53–68, 2004. URL <http://dx.doi.org/10.1023/B:VEGE.0000029383.72609.f0>.
- M. Tausz, M. S. Jiménez, and D. Grille. Antioxidative defence and photoprotection in pine needles under field conditions. A multivariate approach to evaluate patterns of physiological responses at natural sites. *Physiologia Plantarum*, 104(4):760–764, 1998a. URL <http://dx.doi.org/10.1034/j.1399-3054.1998.1040435.x>.
- M. Tausz, J. Peters, M. S. Jiménez, D. Morales, and D. Grill. Element contents and stress-physiological characterization of *Pinus canariensis* needles in Mediterranean type field stands in Tenerife. *Chemosphere*, 36(4–5):1019–1023, 1998b. URL [http://dx.doi.org/10.1016/S0045-6535\(97\)10165-5](http://dx.doi.org/10.1016/S0045-6535(97)10165-5).
- M. Tausz, A. Wonisch, J. Peters, M. S. Jiménez, D. Morales, and D. Grill. Short-term changes in chloroplast pigment and free-radical scavengers in *Pinus canariensis* needles as affected by mild drought stress. *Journal of Plant Physiology*, 158(2):213–319, 2001. URL <http://dx.doi.org/10.1078/0176-1617-00178>.

- M. Tausz, W. Trummer, A. Wonisch, W. Goessler, D. Grill, M. S. Jiménez, and D. Morales. A survey of foliar mineral nutrient concentrations of *Pinus canariensis* at field plots in Tenerife. *Forest Ecology and Management*, 189(1–3):49–55, 2004. URL <http://dx.doi.org/10.1016/j.foreco.2003.07.034>.
- M. Tejedor, C. Jiménez, C. Monteverde, and J. Parra. The influence of deforestation on soil water conservation in a pine forest in Tenerife (Canary Islands, Spain). In S. R. Raine, A. J. W. Biggs, N. W. Menzies, D. M. Freebairn, and P. E. Tolmie, editors, *Conserving Soil and Water for Society: Sharing Solutions. Proceedings 13th International Soil Conservation Organisation Conference*, Brisbane, Australia, 2004. Australian Society of Soil Science Incorporated & International Erosion Control Association. URL <http://www.tucson.ars.ag.gov/isco/isco13/PAPERS%20R-Z/TEJEDOR%201.pdf>. Paper No. 760.
- B. R. Thomas, S. E. Macdonald, M. Hicks, D. L. Adams, and R. B. Hodgetts. Effects of reforestation methods on genetic diversity of lodgepole pine: an assessment using microsatellite and randomly amplified polymorphic DNA markers. *Theoretical and Applied Genetics*, 98(5):793–801, 1999. URL <http://dx.doi.org/10.1007/s001220051136>.
- R. J. Toonen. Microsatellites for ecologists: non-radioactive isolation and amplification protocols for microsatellite markers, 1997. URL <http://biogeek.ucdavis.edu/Msats/>.
- C. Van Oosterhout, W. F. Hutchinson, D. P. M. Wills, and P. Shipley. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, 4(3):535–538, 2004. URL <http://dx.doi.org/10.1111/j.1471-8286.2004.00684.x>.
- A. G. Vandergast, R. G. Gillespie, and G. K. Roderick. Influence of volcanic activity on the population genetic structure of Hawaiian *Tetragnatha* spiders: fragmentation, rapid population growth and the potential for accelerated evolution. *Molecular Ecology*, 13(7):1729–1743, 2004. URL <http://dx.doi.org/10.1111/j.1365-294X.2004.02179.x>.
- P. Vargas, H. A. McAllister, C. Morton, S. L. Jury, and M. J. Wilkinson. Polyploid speciation in *Hedera* (Araliaceae): phylogenetic and biogeographic

- insights based on chromosome count and ITS sequence. *Plant Systematics and Evolution*, 219(3–4):165–179, 1999. URL <http://dx.doi.org/10.1007/BF00985577>.
- Z. Vaxevanidou, S.C. González-Martínez, J. Climent, and L. Gil. Tree populations bordering on extinction: a case study in the endemic Canary Island pine. *Biological Conservation*, 2005. in press.
- C. Velázquez Padrón. Selvicultura extensiva: el anillamiento como técnica selvícola. *Revista de la Consejería de Política Territorial y Medio Ambiente*, 21, 2001. URL <http://www.gobcan.es/medioambiente/revista/2001/21/267/>.
- G. G. Vendramin and B. Ziegenhagen. Characterisation and inheritance of polymorphic plastid microsatellites in *Abies*. *Genome*, 40(6):857–864, 1997. URL [http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2\\_desc\\_e?gen](http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2_desc_e?gen).
- G. G. Vendramin, L. Lelli, P. Rossi, and M. Morgante. A set of primers for the amplification of 20 chloroplast microsatellites in Pinaceae. *Molecular Ecology*, 5(4):595–598, 1996. URL <http://dx.doi.org/10.1046/j.1365-294X.1996.00111.x>.
- G. G. Vendramin, M. Anzidei, A. Madaghiele, and G. Bucci. Distribution of genetic diversity in *Pinus pinaster* Ait. as revealed by chloroplast microsatellites. *Theoretical and Applied Genetics*, 97(3):456–463, 1998. URL <http://dx.doi.org/10.1007/s001220050917>.
- Anonymous. Viceconsejería de Medio Ambiente. Plan forestal de Canarias. Technical report, Gobierno de Canarias, 2002. URL <http://www.gobiernodecanarias.org/medioambiente/biodiversidad/planforestal/>.
- L. von Buch. *Physikalische Beschreibung der Canarischen Inseln*. Berlin, Germany, 1825.
- D. B. Wagner. Nuclear, chloroplast, and mitochondrial DNA polymorphisms as biochemical markers in population genetic analyses of forest trees. *New Forests*, 6(1–4):373–390, 1992. URL <http://dx.doi.org/10.1007/BF00120653>.
- T. Wakasugi, J. Tsudzuki, S. Ito, K. Nakashima, T. Tsudzuki, and M. Sugiura. Loss of all *ndh* genes as determined by sequencing the entire chloroplast genome

- of the black pine *Pinus thunbergii*. *Proceedings of the National Academy of Sciences of the United States of America*, 91(21):9794–9798, 1994. URL <http://www.pnas.org/cgi/content/abstract/91/21/9794>.
- J. Wang. A pseudo-likelihood method for estimating effective population size from temporally spaced samples. *Genetical Research*, 78(3):243–57, 2001. URL <http://journals.cambridge.org/action/displayAbstract?fromPage=online&aid=94763>.
- J. Wang and M. C. Whitlock. Estimating effective population size and migration rates from genetic samples over space and time. *Genetics*, 163(1):429–446, 2003. URL <http://www.genetics.org/cgi/content/abstract/163/1/429>.
- X.-R. Wang and A. E. Szmidt. Molecular markers in population genetics of forest trees. *Scandinavian Journal of Forest Research*, 16(3):199–220, 2001. URL <http://dx.doi.org/10.1080/02827580118146>.
- X.-R. Wang, Y. Tsumura, H. Yoshimaru, K. Nagasaka, and A. E. Szmidt. Phylogenetic relationships of Eurasian pines (*Pinus*, Pinaceae) based on chloroplast *rbcL*, *matK*, *rpl20-rps18* spacer, and *trnV* intron sequences. *American Journal of Botany*, 86(12):1742–1753, 1999. URL <http://intl.amjbot.org/cgi/content/abstract/86/12/1742>.
- R. S. Waples. A generalized approach for estimating effective population size from temporal changes in allele frequency. *Genetics*, 121(2):379–391, 1989. URL <http://www.genetics.org/cgi/content/abstract/121/2/379>.
- S. N. Ward and S. J. Day. Cumbre Vieja volcano — Potential collapse and tsunami at La Palma, Canary Islands. *Geophysical Research Letters*, 28(17):3397–3400, 2001. URL <http://dx.doi.org/10.1029/2001GL013110>.
- B. S. Weir and C. C. Cockerham. Estimating F-statistics for the analysis of population structure. *Evolution*, 38(6):1358–1370, 1984. URL <http://links.jstor.org/sici?sici=0014-3820%28198411%2938%3A6%3C1358%3AEFFTA0%3E2.0.CO%3B2-0>.
- K. Weising and R. C. Gardner. A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledo-

- nous angiosperms. *Genome*, 42(1):9–19, 1999. URL [http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2\\_abst\\_e?gen\\_g98-104\\_42\\_ns\\_nf\\_gen1-99](http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2_abst_e?gen_g98-104_42_ns_nf_gen1-99).
- G. Wieser, J. Peters, V. C. Luis, D. Morales, and M. S. Jiménez. Ecophysiological studies on the water relations in a *Pinus canariensis* stand, Tenerife, Canary Islands. *Phyton*, 42(2):291–304, 2002.
- K. J. Willis and J. C. McElwain. *The Evolution of Plants*. Oxford University Press, Oxford, United Kingdom, 2002.
- M. Wilson. A possible origin for the Hawaiian Islands. *Canadian Journal of Physics*, 41:863–870, 1963.
- J. W. Wright. *Introduction to Forest Genetics*. Academic Press, New York, United States of America, 1976.
- S. Wright. Evolution in Mendelian populations. *Genetics*, 16(2):97–159, 1931. URL <http://www.genetics.org/content/vol16/issue2/index.shtml>.
- S. Wright. Size of population and breeding structure in relation to evolution. *Science*, 87:430–431, 1938.
- S. Wright. Breeding structure of populations in relation to speciation. *American Naturalist*, 74(752):232–248, 1940. URL <http://links.jstor.org/sici?sici=0003-0147%28194005%2F06%2974%3A752%3C232%3ABSOPIR%3E2.O.CO%3B2-6>.
- R.-C. Yang, F. C. Yeh, and A. D. Yanchuk. A comparison of isozyme and quantitative genetic variation in *Pinus contorta* subsp. *latifolia* by  $F_{ST}$ . *Genetics*, 142(3):1045–1052, 1996. URL <http://www.genetics.org/cgi/content/abstract/142/3/1045>.
- R. Yazdani and D. Lindgren. Variation of pollen contamination in a Scots pine seed orchard. *Silvae Genetica*, 40(5–6):243–246, 1991. URL <http://www.bfafh.de/inst2/sg40pdf.htm>.
- L. Zane, L. Bargelloni, and T. Patarnello. Strategies for microsatellite isolation: a review. *Molecular Ecology*, 11(1):1–16, 2002. URL <http://dx.doi.org/10.1046/j.0962-1083.2001.01418.x>.

- 
- G. Zelling, J. Peters, M. S. Jiménez, D. Morales, D. Grill, and A. Perktold. Three-dimensional reconstruction of the stomatal complex in *Pinus canariensis* needles using serial sections. *Plant Biology*, 4(1):70–76, 2002. URL <http://dx.doi.org/10.1055/s-2002-20438>.
- Y. Zhou, T. Bui, L. D. Auckland, and C. G. Williams. Undermethylated DNA as a source of microsatellites from a conifer genome. *Genome*, 45(1):91–9, 2002. URL <http://pubs.nrc-cnrc.gc.ca/rp/rppdf/g01-119.pdf>.