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The Effect of Altitude on the Pattern of Gene Flow in the Endemic Canary Island Pine, *Pinus canariensis*

By M. Navascués[1),*)], G. G. Vendramin[2)] and B. C. Emerson[1),3)]

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Abstract

Pinus canariensis is endemic to the western Canary Islands (NW coast of Africa), where it forms forest spanning an altitude from 500 to 2500 m. There are dramatic changes in environmental conditions (temperature, moisture and solar radiation) over short distances due to this elevation gradient in the Canary Island pine forest. Those differences in environmental conditions may lead to asynchronous flowering times among elevations. In this study we used nuclear and chloroplast microsatellites to characterize the genetic structure of two altitudinal transects on the southern slopes of Tenerife Island to test for genetic isolation among altitudes. Although significant differentiation among sites was detected, this differentiation was very low ($F_{ST} = 0.013$ with chloroplast markers, $F_{ST} = 0.019$ with nuclear markers) and appeared to be unrelated to altitude. The contrasting results between nuclear and chloroplast markers are also discussed in terms of statistical accuracy of markers and genome inheritance.

Key words: chloroplast microsatellites, nuclear microsatellites, population differentiation, isolation by altitude.

1) Centre for Ecology, Evolution and Conservation, School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom.

2) Istituto di Genetica Vegetale, Consiglio Nazionale delle Ricerche, Via Madonna del Piano 10, 50019 Sesto Fiorentino (Firenze), Italy.

3) Correspondence: Brent Emerson, University of East Anglia, Norwich NR4 7TJ, UK. Telephone: (44) 01603 592237. Fax: (44) 01603 592250. E-Mail: b.emerson@uea.ac.uk

*) Current address: Équipe Éco-évolution mathématique, CNRS UMR 7625 Écologie et Évolution, Université Pierre et Marie Curie, École Normale Supérieure, 46 rue d'Ulm, 75230 Paris, France.

Introduction

The Canary Islands are a volcanic archipelago situated 100 km off the NW coast of Africa (*Figure 1*). Pine forests in the Canary Islands are oligospecific communities dominated by the endemic *Pinus canariensis* Chr. Sm. ex DC., the only autochthonous pine of the archipelago. These forests are found in the higher altitudes (from 500 to 2500 m, Fernández-Palacios and de Nicolás, 1995) of the islands forming a more or less continuous belt around their volcanic edifices. Its original natural distribution has been fragmented because of human exploitation (del Arco Aguilar *et al.,* 1992; see *Figure 1*). Because of the steep slopes of the islands, dramatic differences in altitude, and therefore environmental conditions, are found over short linear distances. High temperature, increased sunshine and moisture stress favour the initiation and amount of

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
Vilaflor 1000	28° 07.97' N	16° 41.28' W	1035m	39
Vilaflor 1500	28° 10.04' N	16° 38.15' W	1553m	38
Vilaflor 2000	28° 11.39' N	16° 39.96' W	2108m	42

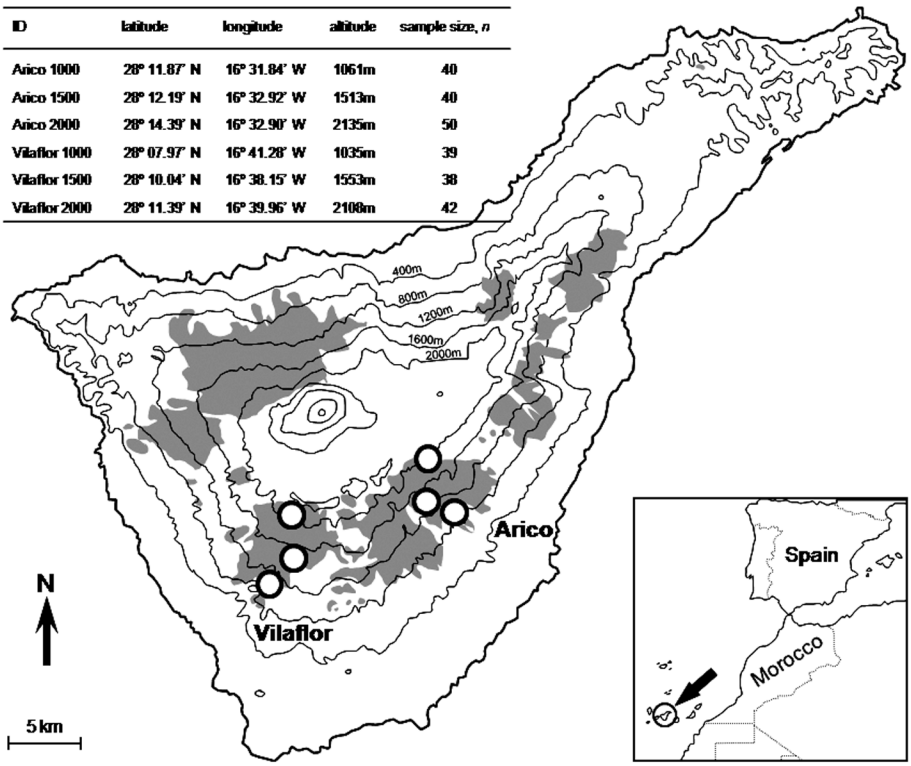

Figure 1. – The island of Tenerife showing the two studied altitudinal transects (in the municipalities of Arico and Vilaflor) of *Pinus canariensis*. Each transect was sampled at three altitudes. White circles mark the location of the sampling sites; distribution of natural pine forest is shown in grey. Sampling site coordinates are presented in the table within the figure.

flowering (BONNER, 2003) and differences in flowering phenology are expected among elevations. Asynchrony in flowering time along elevational transects has been previously described in conifers (SCHUSTER *et al.,* 1989; SILEN, 1963) and has been suggested as a potential barrier to gene flow between altitudes (NEALE and ADAMS, 1985).

Previous population genetic studies of *P. canariensis* found some genetic differentiation between populations (within the same island) for chloroplast ($F_{ST} = 0.19$; GÓMEZ *et al.,* 2003) and nuclear ($F_{ST} = 0.09$; SCHILLER *et al.,* 1999) markers, which suggest that despite the high levels of gene flow expected (because of close distance and wind pollination) some level of genetic differentiation can be maintained. In the present work we study the possible influence of altitude on the patterns of gene flow in *P. canariensis*. Several previous isozyme studies of genetic differentiation in conifers among stands at different altitudes (ETTL and PETERSON, 2001; KARA *et al.,* 1997; NEALE and ADAMS, 1985; SÁENZ-ROMERO and TAPIA-OLIVARES, 2003; SCHUSTER *et al.,* 1989) have found low ($F_{ST} < 0.06$) or non-significant levels of genetic differentiation among altitudes. This lack of differentiation can be explained by stepping stone gene flow along the continuous elevation gradient connecting stands with non-overlapping pollination periods (SCHUSTER *et al.,* 1989). Therefore, in order to detect an altitudinal effect on gene flow, an experimental design should contrast within-altitude and between-altitude genetic differentiation. Most studies are based on a single elevational transect (KLUMPP and STEFSKY, 2004; MITTON *et al.,* 1980; NEALE and ADAMS, 1985; SÁENZ-ROMERO and TAPIA-OLIVARES, 2003; SCHUSTER *et al.,* 1989) and thus fail to present such a comparison. Additionally, differences between among-altitude and within-altitude comparisons of genetic differentiation might be subtle, in the light of previous studies suggesting high levels of gene flow among sites (e.g. ETTL and PETERSON, 2001; SÁENZ-ROMERO and TAPIA-OLIVARES, 2003; e.g. SCHUSTER *et al.,* 1989). In order to be able to detect small differences we have employed microsatellite markers, which are expected to have higher resolving power than isozymes (see, for instance, ESTOUP *et al.,* 1998).

Materials and Methods

Plant material and sampling design

Two altitudinal transects were studied in the municipalities of Arico and Vilaflor (Tenerife, Canary Islands). Both transects were located in the southern (leeward) slopes of the island to minimize the effects of climatic differences. Sampling points were selected within natural stands of *Pinus canariensis* forest (DEL ARCO AGUILAR *et al.,* 1992) at approximately 1000, 1500 and 2000m of altitude (*Figure 1*). That is, two transects and three altitudes were studied, giving us six sampling sites. At each sampling site needles from 38–50 mature trees (randomly selected, leaving a separation of at least 10 metres between each tree) were collected and preserved in silica gel in the summer of 2002. Distances between sampling sites ranged from four to 18 km.

Molecular markers

Genomic DNA was purified using a CTAB protocol based on the DOYLE and DOYLE (1987) method. Samples were genotyped for eight chloroplast microsatellites (Pt1254, Pt15169, Pt26081, Pt30204, Pt36480, Pt71936, Pt87268 and Pt110048; VENDRAMIN *et al.,* 1996) and eight nuclear microsatellites (ssrPt_ctg4363, ssrPt_ctg4698 and ssrPt_ctg7731, CHAGNÉ *et al.,* 2004; SPAC 11.5, SPAC 11.8 and SPAG 7.14, SORANZO *et al.,* 1998; PtTX3116 and PtTX4001, ZHOU *et al.,* 2002). PCR amplifications were performed in a Perkin-Elmer 9700 thermal cycler. Standard protocols for microsatellite amplification can be found elsewhere (e.g. AUCKLAND *et al.,* 2002) and annealing temperatures are contained within original publications (CHAGNÉ *et al.,* 2004; SORANZO *et al.,* 1998; VENDRAMIN *et al.,* 1996; ZHOU *et al.,* 2002). Particular modifications used in this study were: a) for all reactions a final extension step (72°C) of 30 minutes was used (for the reduction of plus-A double peaks) and b) loci PtTX3116, PtTX4001 and ssrPt_ctg4363 were amplified using a touchdown procedure starting ten degrees above annealing temperature and decreasing 0.5°C at each cycle. PCR products were sized in an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) using GeneScan-400D[ROX] as a size standard. Primers were labelled with a four dye system allowing multiplexing in the sizing of PCR products. Electrophoretograms were analysed with GeneScan® and Genotyper® (Applied Biosystems) software. Genotyping data obtained for nuclear microsatellites was analysed for each population with MICRO-CHECKER to assess the presence of scoring errors due to null alleles, stuttering or large allele dropout (VAN OOSTERHOUT *et al.,* 2004).

Data analysis

Chloroplast microsatellites (*cpSSR*). Total number of haplotypes (n_h, direct count), unbiased effective number of haplotypes (n_e; NIELSEN *et al.,* 2003; equation 16), unbiased haplotype diversity (H_E; NEI, 1978) and average genetic distances among individuals (D^2_{sh}; GOLDSTEIN *et al.,* 1995, as modified by MORGANTE *et al.,* 1997) were calculated for each population. Genetic isolation among altitudes was tested by performing a hierarchical analysis of molecular variance (AMOVA, EXCOFFIER *et al.,* 1992) based on haplotype frequencies (WEIR and COCKERHAM, 1984). Total variance (σ^2_T) in cpSSR haplotype frequencies is partitioned into: (1) among-individuals-within-sites covariance (σ^2_s), (2) among-individuals-within-altitudes covariance (σ^2_a) and (3) within-sites-within-altitudes covariance (σ^2_{sa}). F-statistics are calculated from these variances as follows: differentiation among altitudes, $F_{AT} = \sigma^2_{sa}/\sigma^2_T$; differentiation between sites within altitudes, $F_{SA} = \sigma^2_a/(\sigma^2_a+\sigma^2_s)$, and differentiation among sites, $F_{ST} = (\sigma^2_{sa} + \sigma^2_a)/\sigma^2_T$. Calculation and significance of F-statistics was computed using ARLEQUIN 3.01 (EXCOFFIER *et al.,* 2005) with a randomization procedure for the test of 10 000 permutations of the data.

Nuclear microsatellites (*nSSR*). Number of alleles (A, direct count of alleles), unbiased effective number of alleles (A_e; NIELSEN *et al.,* 2003; equation 16) and unbiased gene diversity (H_E; NEI, 1987) were calculated for each locus and site. ARLEQUIN 3.01 was used to perform a hierarchical AMOVA to calculate population differentiation statistics based on allele frequencies (WEIR and COCKERHAM, 1984). The total variance was partitioned as described above, for the cpSSR analysis, for the calculation of F-statistics. Correlations of genetic differentiation with geographical distance and with altitudinal difference were analysed with Mantel tests (MANTEL, 1967) and partial Mantel tests (LEGENDRE and LEGENDRE, 1998) were used to check combined effects of distance and altitude. For Mantel tests, the significance of the correlation r between two matrices is assessed by permutation of rows and columns of the second matrix. This analysis was performed with ISOLATION BY DISTANCE web service (BOHONAK, 2002; JENSEN *et al.,* 2005). The significance of the correlations between genetic distances, expressed as $F_{ST}/(1\text{-}F_{ST})$ (ROUSSET, 1997), and geographic distance was estimated with 30 000 permutations. In order to detect other spatial patterns of gene flow, pairwise genetic differentiation (WEIR and COCKERHAM, 1984) between sampling sites was calculated with FSTAT 2.9.3.2 (GOUDET, 1995) and the significance tested by 10 000 randomizations of genotypes

among samples. Pairwise genetic differentiation statistics were standardized by their maximum value following MEIRMANS (2006) for quantitative comparison. Linkage disequilibrium between pairs of loci was tested with FSTAT 2.9.3.2 using G-statistics and randomization of data for the whole dataset (GOUDET, 1995).

Results and Discussion

Genetic diversity

Seven out of eight cpSSR loci were polymorphic with an average of five alleles per locus (Pt36480 was monomorphic among samples from this study), yielding 86 different chloroplast haplotypes (in a total of 249 individuals). Three haplotypes were shared among the six sites, and one of them had the highest frequency among all haplotypes (present in 12.5% 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). No evidence for scoring errors was found with the MICRO-CHECKER analysis and only SPAC 11.5 presented a general excess of homozygotes, suggesting a possible presence of null alleles, in only two populations (Arico 1000 and Vilaflor 2000). Amplification of larger alleles in SPAC 11.8 was very low (i.e. producing weaker bands) for heterozygotes with small-size alleles. Double genotyping for apparent homozygotes for small-size alleles, high sensitivity of ABI PRISM 3700 DNA Analyzer and a lack of signal for large allele dropout in the MICRO-CHECKER analysis give us a high confidence that the error rate in the genotyping of locus SPAC 11.8 due to large allele dropout is insignificant. Only two pairs of loci gave a significant (at 5% nominal level) result for the linkage disequilibrium test: SPAC 11.8-ssrPt_ctg7731 and SPAC 11.5-ssrPt_ctg7731. Levels of genetic diversity were similar across populations (*Table 1A* and *Table 2*) for both types of microsatellites.

Several studies of genetic diversity in altitudinal transects of pine species have focused on correlations of genetic diversity levels (ISIK and KARA, 1997; KLUMPP and STEFSKY, 2004) or allele frequencies (KARA *et al.,* 1997; MITTON *et al.,* 1980) with elevation. In our study there is no apparent relationship between genetic diversity and altitude, as different indices and loci follow different trends. To discuss correlations between the frequencies of particular alleles with altitude does not seem relevant in the present study. The correlations found for isozyme alleles with altitude in *Pinus ponderosa* by MITTON *et al.* (1980) were given an interpretation based on natural selection for particular isozyme alleles. In our study, which uses microsatellites, arguments invoking natural selection are very unlikely and no testable with the current data.

CpSSR diversity in *Pinus canariensis* has previously been studied 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 also calculated after removing these loci (*Table 1B*), resulting in higher genetic diversity levels than any of the populations analysed by GÓMEZ *et al.* (2003). These differences may be a product of the larger sample sizes used in our study (38–50 against 24 used by GÓMEZ *et al.,* 2003) that have allowed us to detect a higher

Table 1. – Chloroplast microsatellite diversity indices within sampling sites for two altitudinal transects of *Pinus canariensis* on the island of Tenerife, both with (A) and without (B) loci Pt1254 and Pt110048.

	Number of haplotypes (n_h)	Effective number of haplotypes (n_e)	Haplotype diversity (H_E)	Average genetic distance (D^2_{sh})
A: eight loci				
Arico 1000	27	43.39	0.978	3.22
Arico 1500	23	12.80	0.923	2.26
Arico 2000	27	24.04	0.959	3.19
Vilaflor 1000	26	29.68	0.968	2.15
Vilaflor 1500	27	25.14	0.962	2.56
Vilaflor 2000	27	30.78	0.969	3.35
B: six loci (Pt1254 and Pt110048 removed)				
Arico 1000	21	16.98	0.942	2.04
Arico 1500	18	11.32	0.922	1.51
Arico 2000	21	9.01	0.890	2.27
Vilaflor 1000	16	11.97	0.918	1.16
Vilaflor 1500	18	17.17	0.942	2.04
Vilaflor 2000	19	13.90	0.929	2.32

Table 2. – Nuclear microsatellite diversity indices (A, number of alleles; A_e, effective number of alleles, and H_E, unbiased gene diversity) within two altitudinal transects of *Pinus canariensis* on the island of Tenerife.

	Arico 1000			Arico 1500			Arico 2000			Vilaflor 1000			Vilaflor 1500			Vilaflor 2000		
	A	A_e	H_E	A	A_e	H_E	A	A_e	H_E	A	A_e	H_E	A	A_e	H_E	A	A_e	H_E
SPAC 11.5	19	13.2	0.927	20	10.28	0.904	27	21.83	0.955	22	12.23	0.92	28	15.08	0.936	25	13.7	0.929
SPAC 11.8	25	13.42	0.927	26	20.6	0.953	31	22.33	0.957	23	12.64	0.923	29	17.53	0.945	30	23.04	0.958
SPAG 7.14	15	11.29	0.913	17	11.37	0.913	14	7.55	0.87	12	9.25	0.894	16	6.6	0.852	13	7.5	0.869
PtTX3116	6	2.57	0.616	7	2.7	0.635	8	2.77	0.643	6	2.52	0.61	6	2.03	0.514	6	2.15	0.54
PtTX4001	8	4.37	0.776	7	4.82	0.796	6	5.04	0.804	6	4.21	0.766	7	5.72	0.828	6	4.74	0.792
ssrPt_ctg4363	9	8.18	0.879	7	5.11	0.806	8	4.63	0.786	7	4.44	0.778	7	4.28	0.771	6	4.63	0.786
ssrPt_ctg4698	4	1.77	0.441	3	1.49	0.336	4	1.42	0.304	3	1.69	0.418	6	1.65	0.402	3	1.32	0.254
ssrPt_ctg7731	7	2.01	0.509	8	2.41	0.589	8	3.11	0.683	6	2.58	0.617	7	3.9	0.747	7	2.25	0.56

number of haplotypes. Because of the high polymorphism of these markers small sample sizes may leave a large proportion of haplotypes unsampled resulting in inaccurate or unrepresentative estimates of genetic diversity within populations. Additionally, it must be noted that we have used a less biased estimator (NIELSEN *et al.,* 2003) for the effective number of alleles, which we expect to yield higher values for small samples, compared to the estimator used by GÓMEZ *et al.* (2003).

Genetic differentiation among populations and among altitudes

No significant differentiation among altitudes was detected in any of the AMOVAs performed on nSSR and cpSSR data (*Table 3*). Significant genetic differentiation among sites (both overall and within altitudes) was detected for both nSSR and cpSSR data, but with very low values, thus significance might be an indication of the high resolving power of microsatellites rather than biological meaningful differentiation (HEDRICK, 2001). Variance decomposition revealed that the majority of the variation was contained within sampling sites (> 95%) with little variation among altitudes (< 1.5%).

Theoretical expectations for F-statistics are that paternally inherited genomes should present higher differentiation than biparentally inherited genomes ($F_{ST(p)}>F_{ST(b)}$) since the weight of seed migration rate and effective number of adult plants in $F_{ST(b)}$ is higher than for $F_{ST(p)}$ (see HU and ENNOS, 1999, equations 12b and 13b). However, in our empirical results, $F_{ST(b)}$ is lower than $F_{ST(p)}$. This might not be considered unusual in the light of results from other studies (LATTA and MITTON, 1997; see also PETIT *et al.,* 2005 meta-analysis). PETIT *et al.* (1993) suggested that cases where $F_{ST(p)}$ is higher than $F_{ST(b)}$ can be produced during transient periods because chloroplast differentiation will reach a steady state faster than nuclear variation (due to the lower effective population size of the former). This scenario would require an initial situation in which the genetic differentiation among populations is higher than at the steady state. In the case of *P. canariensis* this could be produced within the hypothesized metapopulation scenario described by NAVASCUÉS *et al.* (2006) where volcanic activity will produce local extinctions of the pine forest followed by recolonisation (with an associated founder effect that would increase its genetic differentiation compared to unaffected stable areas). However there is little geological evidence to postulate such an explanation for the current set of populations studied. In fact, NAVASCUÉS *et al.* (2006) neglected to consider that the differences of expansion times among sites (within island) found in their study can be attributed to the error of the estimator, thus, the metapopulation hypothesis for *P. canariensis* is mostly speculative. On the other hand, at a steady state the values for $F_{ST(p)}$ and $F_{ST(b)}$ are expected to be similar in value (PETIT *et al.,* 2005), as indeed they are in the present study. Therefore, it is reasonable to assume that errors associated with the estimates of F-statistics could be responsible for a slightly higher $F_{ST(b)}$ without biological meaning. If we consider that: a) actual genetic diversity of the chloroplast was incompletely sampled (higher effective numbers of haplotypes estimates than actual sampled numbers of haplotypes strongly suggest this); b) estimates from the chloroplast are made from linked loci (while unlinked nuclear loci offer independent replicates); and c) sam-

Table 3. – AMOVA genetic differentiation results for the two altitudinal transects of *Pinus canariensis* on the island of Tenerife*).

cpSSRs		nSSRs	
F_{AT} = 0.006	p = 0.068	F_{AT} = 0.001	p = 0.336
F_{SA} = 0.007	p = 0.002	F_{SA} = 0.018	p < 0.0001
F_{ST} = 0.013	p < 0.001	F_{ST} = 0.019	p < 0.0001

*) F-statistics sub-indexes denote: AT-differentiation among altitudes, SA-differentiation among sampling sites within altitudes, ST-differentiation among sampling sites.

Table 4. – Mantel and partial Mantel test results for isolation by distance and altitude analyses for altitudinal transects of *Pinus canariensis* on the island of Tenerife.

1st geographic matrix	Indicator matrix	*r*	*p-value*
log (geographic distance)	none	0.258	0.177
log (altitudinal difference)	none	0.025	0.365
log (geographic distance)	log (altitudinal difference)	0.261	0.179
log (altitudinal difference)	log (geographic distance)	0.052	0.326

Table 5. – Pairwise population genetic differentiation (lower tringle) between sampling sites of *Pinus canariensis* on the island of Tenerife. Standardized population genetic differentiation (upper triangle) (MEIRMANS, 2006).

	Arico 1000	Arico 1500	Arico 2000	Vilaflor 1000	Vilaflor 1500	Vilaflor 2000
Arico 1000		0.053	0.085	0.096	0.138	0.082
Arico 1500	0.014*		0.077	0.085	0.104	0.078
Arico 2000	0.021*	0.020*		0.074	0.044	0.020
Vilaflor 1000	0.024*	0.022*	0.019*		0.058	0.060
Vilaflor 1500	0.035*	0.026*	0.011*	0.015*		0.052
Vilaflor 2000	0.022*	0.021*	0.005*	0.017*	0.014*	

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

ple size for the chloroplast data is half the size of nuclear data; we can expect lower accuracy in the F-statistic estimates from the cpSSRs that could be responsible for the $F_{ST(p)} < F_{ST(b)}$ result. From simulations performed by HAMILTON and MILLER (2002) it can be seen that stochasticity can produce lower fixation index estimates in the organelle genome than in the nuclear genome.

Pairwise genetic differentiation between sites and patterns of gene flow

Although genetic differentiation among altitudes was not significant the genetic differentiation among sites may be related to geographic factors (including altitude) that may 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) and using Mantel tests on matrices of genetic and geographic distances is a standard method to test this pattern (ROUSSET, 1997). Using a third matrix we tested for the effects of an additional geographic feature, altitude, which revealed no significant correlation (*Table 4*). It is possible that, for the geographic scale used in this study, isolation by distance is not detectable, but it may occur for smaller spatial scales [CASTRIC and BERNATCHEZ (2003) found correlation between genetic and geographic distances decreasing with the spatial scale in brook charr].

Pairwise differentiation between sites was calculated to check possible directional patterns of gene flow. Results indicated that there are no striking differences among pairs (*Table 5*) with the exception of the pair containing the sites at the highest altitude (Arico 2000-Vilaflor 2000, which are close to the timberline) for which genetic differentiation is rather lower than any other pair. Canary Island pine forest is subject to higher solar radiation (JIMÉNEZ and MORALES MÉNDEZ, 2001) and water stress (GIEGER and LEUSCHNER, 2004; JONSSON *et al.,* 2002) at the timberline, both of which are factors that can increase time and amount of pollen shedding (BONNER, 2003). The study of the flowering phenology along an altitudinal gradient in Tenerife might throw some light on the significance of this result as times and duration of pollen shedding and female receptivity are fundamental to understand the exchange of genes among sites. Nevertheless, the lower differentiation observed for Vilaflor 2000-Arico 2000 is less striking when standardized genetic differentiation (*Table 5* upper triangle) is considered rendering this result less relevant.

Conclusions

Altitude seems to be of little importance for the development of genetic isolation among sites. Neither significant genetic differentiation among altitudinal groups nor correlation between genetic distances and altitudinal distances was found. Any seasonal isolation due to altitudinal differences in environmental conditions is probably counterbalanced by stepping-stone gene flow along the continuous elevation gradient, similar to the finding of SCHUSTER *et al.* (1989). Further investigations of the mating system of *P. canariensis* and flowering phenology in relation to altitude should provide new insights into genetic exchange among sites and the role of altitude.

Despite the fact that chloroplast microsatellites presented higher diversity than nuclear microsatellites, population differentiation estimates based on these markers were lower, opposite to the theoretical expectations for paternally inherited genes (HU and ENNOS, 1999). This is probably because of the inherent properties of chloroplast markers (single locus, half sample size of nuclear markers) that could limit the accuracy on F-statistic estimates.

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