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► **To cite this version:**

Miguel Navascues, Brent C. Emerson. Natural recovery of genetic diversity by gene flow in reforested areas of the endemic Canary Island pine, *Pinus canariensis*. *Forest Ecology and Management*, 2007, 244 (1-3), pp.122-128. 10.1016/j.foreco.2007.04.009 . hal-00258587

HAL Id: hal-00258587

<https://hal.science/hal-00258587v1>

Submitted on 31 Oct 2008

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1 **Natural recovery of genetic diversity by gene flow in reforested areas of the**
2 **endemic Canary Island pine, *Pinus canariensis***

3

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5

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14

15 **Abstract**

16 The endemic pine, *Pinus canariensis*, forms one of the main forest ecosystems in the

17 Canary Islands. In this archipelago, pine forest is a mosaic of natural stands (remnants

18 of past forest overexploitation) and artificial stands planted from the 1940's. The

19 genetic makeup of the artificially regenerated forest is of some concern. The use of

20 reproductive material with uncontrolled origin or from a reduced number of parental

21 trees may produce stands ill adapted to local conditions or unable to adapt in response

22 to environmental change. The genetic diversity within a transect of reforested stands

23 connecting two natural forest fragments has been studied with nuclear and chloroplast

24 microsatellites. Little genetic differentiation and similar levels of genetic diversity to

25 the surrounding natural stands was found for nuclear markers. However, chloroplast

26 microsatellites presented lower haplotype diversity in reforested stands, and this may
27 be a consequence of the lower effective population size of the chloroplast genome,
28 meaning chloroplast markers have a higher sensitivity to bottlenecks. Understory
29 natural regeneration within the reforestation was also analysed to study gene flow
30 from natural forest into artificial stands. Estimates of immigration rate into artificially
31 regenerated forest were high (0.68–0.75), producing a significant increase of genetic
32 diversity (both in chloroplast and nuclear microsatellites) which indicates the capacity
33 for genetic recovery for *P. canariensis* reforestations surrounded by larger natural
34 stands.

35

36 *Keywords:* silviculture, chloroplast microsatellites, nuclear microsatellites, assignment
37 test, temporal samples, gene flow

38

39 **1. Introduction**

40 Silvicultural practices can potentially affect the genetic structure of forests and this
41 problem is of concern for forest geneticists (see Lefèvre, 2004, for a review). In
42 particular, artificial regeneration of forest (plantations or sowing) may produce a loss
43 of genetic diversity through the processes of unplanned selection and genetic drift
44 (Ledig, 1992). Detection of a reduction in genetic diversity with neutral markers
45 because of artificial selection is unlikely because only a few genes might be involved
46 (Lefèvre, 2004) but the quantification of genetic drift to assess potential losses of
47 adaptive variation can be done from neutral molecular markers as it is a process that
48 affects the whole genome (Glaubitz et al., 2003b).

49 Bottleneck effects may be present within forest regeneration practises because of the
50 use of a limited number of seed trees and the unevenness of the number of seeds
51 collected or produced per tree (Glaubitz et al., 2003a; Burgarella, 2004).

52

53 An additional concern for artificial populations is the extent of genetic exchange with
54 natural populations. Potential negative effects of gene flow from artificial plantations
55 into natural forests ('genetic pollution') have been considered (Lenormand, 2002) and
56 have gained recent public awareness with the use of transgenic crops (including tree
57 species, DiFazio et al., 2004). Gene flow from natural population into seed orchards
58 (i.e. seed production plantations for reforestation) is also considered a negative effect
59 ('pollen contamination') because it reduces the genetic gain obtained from the
60 breeding programs and may increase maladaptation (El-Kassaby, 2000). However,
61 under other circumstances, gene exchange between artificial and natural populations
62 can also be positive. For small declining populations gene flow from surrounding
63 reforestation may increase their effective population size ('demographic rescue',
64 Lefèvre, 2004). Also, gene flow from natural forest into reforested stands may be a
65 natural way to recover the genetic variation lost in the reforestation process. This
66 could potentially create an admixed landrace well adapted to local conditions, and
67 with high evolutionary potential to respond to environmental changes.

68

69 *Pinus canariensis* is endemic to the western Canary Islands, where it forms one of the
70 main forest ecosystems of the archipelago. Its area of distribution has been diminished
71 by five centuries of overexploitation (decreasing from covering 25% of the territory to
72 now covering 12%). A great reforestation effort has been made since the 1940's,
73 resulting in a mosaic of reforested and natural pine forest (del Arco Aguilar et al.,

74 1992). A high genetic differentiation has been found among populations of *P.*
75 *canariensis* (Gómez et al., 2003) with some endangered marginal populations
76 presenting particular genetic characteristics (Vaxevanidou et al., 2006). In the present
77 study we analyse the genetic diversity and immigration rates within a transect of
78 reforested stands connecting two natural forest fragments in Tenerife. Hence, we are
79 assessing the effects of the reforestation process on the genetic integrity of the
80 artificial stands and their potential to naturally recover to the levels of diversity of the
81 surrounding natural forest.

82

83 **2. Materials and methods**

84

85 *2.1 Plant material and sampling design*

86 El Teide mountain (Tenerife, Spain) is crowned by a pine forest belt (the Corona
87 Forestal Natural Park). A proportion of these pine woods are artificial stands planted
88 from the 1940's to repair the past overexploitation (Parsons, 1981; del Arco Aguilar et
89 al., 1992). We have chosen as our study site an area where there is still a small
90 interruption of the forest with only a narrow strip of planted trees connecting two
91 natural forest fragments (figure 1). Artificial *Pinus canariensis* stands were planted in
92 Fasnía between 1956 and 1965 and in Arico between 1981 and 1985. The current
93 vegetation cover for those areas is 30–60% for Fasnía and < 30% for Arico (del Arco
94 Aguilar et al., 1992). The provenance of the plants is unknown but most probably seed
95 were collected from different locations in Tenerife island (Climent et al., 1996). Nine
96 sites were sampled in these plantations (sites 2–6 in Arico and 7–10 in Fasnía, figure
97 1); in each of the nine sampling stations 10 planted adult trees and 10 understory
98 naturally regenerated seedlings were sampled (except at site eight where 11 planted

99 trees and 10 seedlings were sampled). From the natural forest in Arico (site 1) and
100 Güímar (site 11) we have sampled 50 and 47 mature trees respectively. Both old trees
101 and seedlings were selected randomly and with an average separation of 10 m among
102 them. From each individual needles were collected and preserved in silica gel in the
103 summer of 2002.

104

105 *2.2 Molecular markers*

106 Genomic DNA was purified using a CTAB protocol based on the Doyle and Doyle
107 (1987) method. Samples were genotyped for eight chloroplast microsatellites (Pt1254,
108 Pt15169, Pt26081, Pt30204, Pt36480, Pt71936, Pt87268 and Pt110048; Vendramin et
109 al., 1996) and eight nuclear microsatellites (SPAC 11.5, SPAC 11.8 and SPAG 7.14,
110 Soranzo et al., 1998; PtTX3116 and PtTX4001, Auckland et al., 2002; *ssrPt_ctg4363*,
111 *ssrPt_ctg4698* and *ssrPt_ctg7731*, Chagné et al., 2004). PCR amplifications were
112 performed in a Perkin-Elmer 9700 thermal cycler (details in Navascués, 2005). PCR
113 products were sized using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

114

115 *2.3 Genetic diversity indices*

116 For chloroplast microsatellites, total number of haplotypes (n_h , direct count of
117 haplotypes which are scored as unique combinations of alleles for all cpSSR loci),
118 effective number of haplotypes (n_e , Nielsen et al., 2003), unbiased haplotype diversity
119 (H_e , Nei, 1978) and average genetic distances among individuals (D_{sh}^2 , Goldstein et
120 al., 1995) were calculated for each sample:

$$121 \quad n_e = (n-1)^2 \left/ \sum_{h=1}^{n_h} p_h^2 \right. (n+1)(n-2) + 3 - n \quad [1]$$

$$122 \quad H_e = \frac{n}{n-1} \left(1 - \sum_{h=1}^{n_h} p_h^2 \right) \quad [2]$$

123
$$D_{sh}^2 = \frac{2}{n(n-1)} \frac{1}{L} \sum_{i=1}^n \sum_{j=i+1}^n \left(\sum_{k=1}^L |a_{ik} - a_{jk}| \right)^2 \quad [3]$$

124 where n is the number of individuals in the sample, p_h is the relative frequency of the
 125 h^{th} haplotype, n_h is the number of different haplotypes in the sample, L is the number
 126 of loci, a_{ik} is the size (number of repeats) of the allele for the i^{th} individual and at the
 127 k^{th} locus, and a_{jk} is the size of the allele for the j^{th} individual and at the k^{th} locus.

128

129 For nuclear microsatellites the arithmetic mean among loci was calculated for the
 130 following indices: number of alleles (A , direct count), effective number of alleles (A_e ,
 131 Nielsen et al., 2003), and unbiased gene diversity (H_e , Nei, 1978):

132
$$A_e = (2n-1)^2 / \sum_{i=1}^A p_i^2 (2n+1)(2n-2) + 3 - 2n \quad [4]$$

133
$$H_e = \frac{2n}{2n-1} \left(1 - \sum_{i=1}^A p_i^2 \right) \quad [5]$$

134 where n is the number of individuals in the sample (i.e. sample size), p_i is the relative
 135 frequency of the i^{th} allele in the sample and A is the total number of different alleles in
 136 the sample.

137

138 Significant differences in the genetic diversity indices between pairs of samples were
 139 tested by a Monte Carlo approach (see, for instance, Glaubitz et al., 2003a for similar
 140 analysis) programmed in Fortran language (source code available from the authors on
 141 request). The null distribution (both samples hold the same genetic diversity) of
 142 differences for each index was constructed by resampling (with replacement)
 143 individuals among samples. The result is a mixture of individuals from both samples
 144 distributed in two new samples (with sizes equal to the original sizes). The genetic
 145 diversity parameters of both samples and their differences are computed (samples are

146 compared always in the same order and sign of differences is kept). This process is
147 repeated 10 000 times to build the null distribution of the genetic diversity differences
148 expected for two samples (with the specific sizes of the empirical samples) drawn
149 from populations with the same genetic diversity (i.e. the same population). The
150 empirical difference value is then compared with the null distribution. Since we are
151 interested in testing for the reduction of genetic diversity (i.e. null hypothesis, h_0 :
152 diversity in natural forest \leq diversity in artificial stands) we perform a one-tailed test.
153 The p -value for rejecting the null hypothesis is estimated as the proportion of
154 iterations with differences greater than or equal to the observed difference value. This
155 process is used to test chloroplast haplotype diversity, individual nuclear locus
156 diversity and average (among loci) nuclear diversity. Two types of comparisons were
157 made, 1) samples from the natural forest with samples from the trees planted in the
158 artificial stands and 2) samples from the trees planted in the artificial stands with the
159 natural regeneration in the artificial stands.

160

161 Significant differences in allele frequencies (nSSR) between pairs of samples were
162 tested with a Fisher exact test (Raymond and Rousset, 1995) using the program
163 Genepop 3.4 (available at <http://wbiomed.curtin.edu.au/genepop/>).

164

165 *2.4 Temporal change in allelic frequencies*

166 Allele frequencies change through time through the influences of gene flow and
167 genetic drift (assuming neutral variation). Using the change in allele frequencies in
168 samples of different generations has been mainly used for estimating effective
169 population sizes (e.g. Waples, 1989). Recently Wang and Whitlock (2003) have
170 developed a new maximum likelihood method that estimates immigration rates and

171 effective population sizes simultaneously. This method, implemented in the MLNE
172 program (available at <http://www.zoo.cam.ac.uk/ioz/software.htm>), has been used on
173 the nSSR data of the reforested samples using the planted trees as the parental
174 generation and the natural regeneration as the following generation. To estimate the
175 gene flow, the method also uses the allele frequencies of the potential external source
176 of genes, which in our case were taken from the surrounding natural stands.

177

178 *2.5 Additional analysis of gene flow*

179 A hierarchical AMOVA on cpSSRs was used to study the paternal heterogeneity from
180 the sampled seedlings, as in Dyer and Sork (2001). The chloroplast is paternally
181 inherited in conifers; therefore, cpSSR variation in the seedlings might reflect the
182 genetic diversity of the pollen clouds of the area. However, in our case, having
183 sampled seedlings on the ground, we cannot ignore some influence of seed dispersal
184 in our data. Canopy density can influence the dispersal of propagules diminishing
185 windspeed (see Nathan and Katul, 2005). Thus, sampling sites were nested into two
186 AMOVA groups, Arico (low vegetation cover) and Fasnía (high vegetation cover, del
187 Arco Aguilar et al., 1992).

188

189 The genotype assignment method developed by Rannala and Mountain (1997),
190 implemented in their program Immanc 5 (available at
191 <http://www.rannala.org/labpages/software.html>), was used on the nSSR data to detect
192 immigrants among the seedlings. This method allows performing the test for different
193 levels of immigrant ancestry considering the probabilities of external origin of: A) the
194 whole genotype (first generation immigrant, i.e. the individual has two foreign
195 parents), B) half of the genotype (second generation immigrant, i.e. the individual has

196 one foreign parent), etc. The program calculates the relative probability for each
197 individual to be born from parents with no recent immigrant ancestry rather than from
198 both foreign parents (denoted $\Lambda_{d=0}$) or from one foreign parent (denoted $\Lambda_{d=1}$).
199 These calculations are based on the allele frequencies of the studied population and
200 the potential source populations using a Bayesian approach. The null distribution
201 (individual with no immigrant ancestry) of the statistic $\ln \Lambda$ is built by a Monte Carlo
202 simulation and p -values and statistical power (for $\alpha = 0.05$) are estimated for each test.
203

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

217

218 **3. Results and discussion**

219 *3.1 Genetic differences among samples*

220 Significant differentiation ($\chi^2 = 50.438$, d.f. = 16, $p < 0.001$) in the nSSR allele
221 frequencies was detected between planted and natural forest. However these
222 differences cannot be interpreted as introduction of foreign material as significant
223 differentiation ($\chi^2 = 54.787$, d.f. = 16, $p < 0.001$) was also detected between the two
224 spatially close natural stands. Also, there were significant differences in the allele
225 frequencies between planted trees and understory natural regeneration for both
226 reforested stands (Arico: $\chi^2 = 27.282$, d.f. = 16, $p = 0.038$ and Fasnica: $\chi^2 = 28.005$, d.f.
227 = 16, $p = 0.032$). These changes may be the result of the effects of drift and gene flow
228 in the reforested stands (see next section).

229

230 Levels of nSSR genetic diversity in the reforested stands are, in general, similar to
231 those found in the surrounding natural forest (table 1, results for individual nSSR loci
232 not shown). Mean expected heterozygosity presented a significantly lower value in
233 the plantations but the diversity index based on allele number, which is more sensitive
234 to population bottlenecks (Nei et al., 1975), showed no significantly lower values and
235 in some cases allele diversity was even higher. Therefore, it is unclear what the lower
236 H_e value indicates. The higher proportion of rare alleles (alleles with frequency $<$
237 0.01) in the reforested stands (27.45%) in comparison to the natural forest (19.42%)
238 suggests to us that a possible mixed origin of the seedling stock (or different seedling
239 origins for the different reforestation phases) could produce an accumulation of alleles
240 from different areas (explaining high levels of allelic diversity) but with lower
241 frequencies in the mixture (resulting in low H_e values). In fact, observed
242 heterozygosity (mean $H_o=0.648$) is lower than the expected heterozygosity suggesting
243 the possibility of a Wahlund effect ($F_{IS}=0.112$, p -value = 0.006).

244

245 For cpSSRs, there is a significantly lower effective number of haplotypes in both
246 artificial stands compared to natural forest (table 1). Bottlenecks associated with the
247 reforestation process could have had a stronger effect on cpSSR diversity than in
248 nSSRs because the effective population size for the chloroplast genome is half the
249 nuclear effective population size. However, no significant reduction was found at H_e
250 and D^2_{sh} , probably because these measures are less sensitive to population size
251 changes. Alternatively, the lower haplotype diversity found could be attributed to a
252 low genetic diversity provenance for the seedlings used in the plantation. However,
253 low diversity populations of *P. canariensis* are mainly marginal (Vaxevanidou et al.
254 2006) which are unlikely locations for seed collection by Forest Services.

255

256 In the comparison of the reforested areas with their natural regeneration we found a
257 significant increase in the effective number of haplotypes and in the mean effective
258 number of alleles (table 2). Although the increase on mean A_e was not apparent when
259 the two reforested stands were studied separately, the whole reforestation area had a
260 significant gain in alleles for loci SPAC 11.8, PtTX3116, PtTX4001, ssrPt_ctg4698
261 and ssrPt_ctg7731 (data not shown). This increase of the allelic diversity in the
262 reforestation may be attributable to gene flow from the natural stands.

263

264 *3.2 Gene flow into the artificial stands*

265 Three different methods have been used to explore the immigration dynamics within
266 the reforested stands. First, we examined the temporal changes in allele frequencies
267 between the planted trees and the following generation, represented by the understory
268 natural regeneration. We applied the Wang and Whitlock (2003) maximum likelihood
269 method using the allele frequencies in the natural forest as the potential external

270 source of immigrants. The estimates (and 95% confidence intervals) of effective
271 population size (N_e) and immigration rate (m) obtained from this method are $N_e =$
272 36.88 (29.09–50.12) and $m = 0.68$ (0.46–0.88) for Arico and $N_e = 28.83$ (22.95–
273 38.78) and $m = 0.75$ (0.54–0.94) for Fasnja.

274

275 Two other methods were employed to characterize gene flow in the area but none of
276 them shed further light on the subject. The results of the AMOVA test gave non-
277 significant differentiation of the pollen diversity for both among sites and among
278 stands (Φ -statistics < 0.033 , $p > 0.17$). This result suggests a high pollen dispersal
279 capacity. A second method was the estimation of immigration rates by the
280 identification of immigrants among the natural regenerated seedlings with the Rannala
281 and Mountain (1997) assignment test. The immigration rates into the reforested
282 patches, calculated as the proportion of identified immigrants over the sample size,
283 are $m_{seed} = 0.07$ and $m_{pollen} = 0.12$. However, these estimates are unreliable as when
284 the same assignment test was applied to the planted trees (considered ‘non-
285 immigrants’) 15 individuals were identified as second generation immigrants (pollen
286 dispersal) and 6 as first generation immigrants (seed dispersal). These error rates are
287 in the same order of magnitude as the estimated immigration rates obtained. This poor
288 performance of the test is due to the low genetic differentiation among the samples
289 which resulted in very low statistical power (results not shown).

290

291 The high levels of gene flow suggested by these results are not surprising as pines are
292 predominantly outcrossing and wind pollinated (Ledig, 1998). Previous estimates of
293 immigration rates in artificial stands are available from pollen contamination studies
294 in seed orchards. Among the factors influencing the pollen contamination levels in

295 seed orchards are the distance to the nearest stands of the same species and the
296 relative pollen production of the seed orchard to the surrounding forest (Adams and
297 Burczyk, 2000). Wind direction can also increase pollen contamination locally
298 (Yazdani and Lindgren, 1991). The range of pollen immigration rates found for pines
299 in such studies are high, ranging from 0.26 to 0.75 (both estimates for *Pinus sylvestris*
300 seed orchards, Harju and Muona, 1989; Yazdani and Lindgren, 1991) depending on
301 the particulars of each case. Immigration rates calculated in natural stands from
302 paternity analysis are also high 0.30–0.31 (minimum estimates, Lian et al., 2001;
303 González-Martínez et al., 2003) but decrease to 0.05–0.07 in isolated populations
304 (Schuster and Mitton, 2000; Robledo-Arnuncio and Gil, 2004). Therefore, the close
305 proximity to big natural forest and the small size of the reforested stands studied
306 offers some explanation for the high levels of immigration into the reforested area.

307

308 **4. Conclusions**

309 The effect of the reforestation process was detected in the chloroplast genome with a
310 reduction in the effective number of haplotypes while the effects on the nuclear
311 genome were uncertain. The difference in the effective population size between the
312 two genomes could explain a different sensitivity to bottlenecks. These results suggest
313 that, for the particular case studied, the planted stock originated from a reduced
314 number of trees. Despite the lower haplotype diversity found within the plantations
315 the richness of their future genetic pool does not seem jeopardized because both
316 chloroplast and nuclear genetic diversities appear to increase within the understory
317 natural regeneration. High levels of gene flow from the adjacent and larger natural
318 stands explain this rise in diversity. These results highlight the importance of the
319 presence and abundance of natural forest for the regeneration of genetically rich forest

320 (see, for example, Glaubitz et al., 2003a; Glaubitz et al., 2003b). However, these
321 conclusions should not be extended to other *P. canariensis* plantations, many of
322 which are located far from natural stands (such as those found on north Tenerife, del
323 Arco Aguilar et al., 1992). Current management of these reforested areas is mainly
324 focused on the reduction of the number of trees to facilitate natural regeneration (Gil,
325 2006). This present work suggests the utility of studying the genetic makeup of
326 artificial stands and their present or future natural regeneration. This may allow for
327 the assessment of whether the reforestation process reduces genetic diversity (which
328 will depend on the particular reforestation management done at different times and
329 places) and, for those stands affected, their potential to recover genetic diversity in the
330 following generations. This may identify stands for which the plantation of new
331 individuals, from a controlled provenance and originating from numerous parental
332 trees, may be beneficial for increasing their pool of genetic diversity.

333

334 **Acknowledgements**

335 We thank Cabildo Insular de Tenerife for sampling permit. University of East Anglia
336 provided of a PhD scholarship to MN.

337

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339

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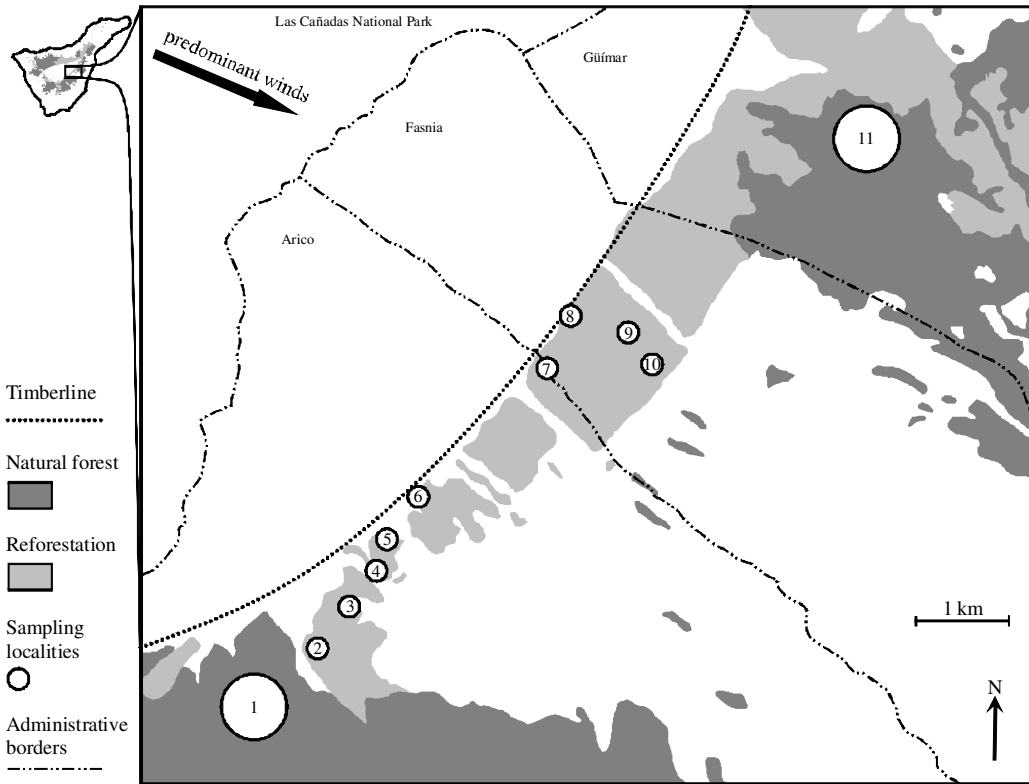
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443

444 *Figure 1.*— Map of the study site on the South-eastern slope of Tenerife (Canary
 445 Islands). Sampling locations are marked with white circles proportional in diameter to
 446 the sampling size.

447

448 *Table 1.*— Diversity indices in the natural forest (pooled Arico and Güímar samples)
 449 and reforested areas (with statistical significance of the difference with natural forest
 450 in parentheses).

		Natural	Planted, both stands (<i>p</i> -value)	Arico, planted trees (<i>p</i> -value)	Fasnia, planted trees (<i>p</i> -value)
	Sample size	97	91	50	41
chloroplast	n_h	49	46 (0.312)	32 (< 0.001*)	25 (< 0.001*)
	n_e	34.496	19.051 (0.006*)	26.085 (0.056)	12.828 (< 0.001*)
	H_e	0.971	0.948 (0.142)	0.962 (0.287)	0.923 (0.115)
	D^2_{sh}	3.120	2.739 (0.261)	2.584 (0.156)	2.984 (0.461)
	mean A	17.735	19.125 (0.990)	15.875 (0.895)	13.875 (0.340)
nuclear	mean A_e	9.618	9.335 (0.360)	10.493 (0.832)	8.583 (0.128)
	mean H_e	0.753	0.729 (0.032*)	0.726 (0.038*)	0.733 (0.119)

451 * Significant at $\alpha = 0.05$

452

453 Table 2.— Diversity indices in the reforested areas and their understory regeneration,
 454 and statistical significance of the differences. Analysis done for the whole reforested
 455 area, reforested area in Arico (planted in 1981-85) and reforested area in Fasnía
 456 (planted in 1956-65). Sample sizes in parentheses.

		Planted (91)	Regeneration (90)	<i>p</i> -value
chloroplast	n_h	46	49	0.219
	n_e	19.051	35.768	0.006*
	H_e	0.948	0.972	0.193
	D^2_{sh}	2.739	3.033	0.322
	mean <i>A</i>	19.125	19.375	0.342
nuclear	mean A_e	9.335	11.100	0.012*
	mean H_e	0.729	0.760	0.027*
		Arico planted (50)	Arico regeneration (50)	<i>p</i> -value
chloroplast	n_h	32	36	0.106
	n_e	26.085	47.152	0.001*
	H_e	0.962	0.980	0.211
	D^2_{sh}	2.584	3.194	0.163
	mean <i>A</i>	15.875	14.250	0.949
nuclear	mean A_e	10.493	10.511	0.489
	mean H_e	0.726	0.738	0.213
		Fasnía planted (41)	Fasnía regeneration (40)	<i>p</i> -value
chloroplast	n_h	25	24	0.689
	n_e	12.828	22.314	0.037*
	H_e	0.923	0.956	0.228
	D^2_{sh}	2.984	2.771	0.573
	mean <i>A</i>	13.875	13.750	0.498
nuclear	mean A_e	8.583	9.723	0.122
	mean H_e	0.733	0.736	0.438

457 * Significant at $\alpha = 0.05$