Genetic Diversity of the Macaronesian Leafy Liverwort *Porella canariensis* Inferred From RAPD Markers

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Plant colonization of the North Atlantic raises the intriguing question of the relationships between extant island species with their continental counterparts (European, African, and American), which may provide clues to past geographic distribution and colonization history. It has been suggested that during past glaciations, many plant species with typical Mediterranean distributions survived in the Atlantic islands that belong to what is today known as Macronesia. We used random amplified polymorphic DNA (RAPD) markers to study 12 populations of the liverwort *Porella canariensis* partly covering its present-day distribution (Azores, Madeira, Canary and Cape Verde Islands, and Iberian Peninsula). Unweighted pair-group (UPGMA) and principal component (PCO) analyses showed a similar geographical pattern that suggested a close relationship between Iberian populations and those from the Canaries and Cape Verde Islands. Populations from Madeira had more genetic variation than those from the Azores, a result from either a richer diversity of habitats in Madeira, which prompted more population diversification, successive colonization waves from different origins, or an older colonization of Madeira. The data show that continuous patches of liverworts are often comprised of more than one individual. Finally, RAPDs can be used to investigate intraspecific diversity within a comparatively large geographic area and, with utmost care, can be used to infer a historic context to explain the patterns observed.

In plant species, the amount of gene flow is a major factor determining genetic variation found throughout the species’ distribution. Gene flow depends on such factors as reproductive mode, breeding system, and selective constraints (Lovell and Hamrick 1984; Mishler 1988). In diploid, outcrossing species it is common that genetic variation among populations is less than that within populations (Lovell and Hamrick 1984). On the other hand, species that are primarily selfed, but short-lived, have most of their genetic diversity among populations (Hamrick and Godt 1996). Species with limited dispersal or that reproduce by selfing present a pattern of geographic genetic variation where drift may play the primary role. Such could be the case of the Macaronesian liverwort *Porella canariensis*.

The Macronesia region consists of the Atlantic islands of the Azores, Madeira, Canary and Cape Verde. It harbors an extremely rich endemic and relict flora which in some places fully characterizes the main elements of the Tertiary European flora (Laurasia). Madeira harbors an extremely well-preserved Tertiary forest called Laurisilva which also exists in a much smaller extension in the Canary Islands. In the Azores, Cape Verde Islands, and Iberia, few elements of this typical forest are present. There is some evidence, however, that Laurisilva also existed on the coast of Morocco (Aubreville 1976). The intermediate position of these archipelagos between three continents makes them extremely interesting for studies of relict taxa of Gondwanalandic or Laurasian origin. Many of these taxa belong to the Macaronesian bryophyte flora, which includes more than 600 species, some of them with worldwide enigmatic distributions. Of these, 9% are exclusively endemic, some with direct relationships to other regions of bryophyte endemics as far away as Australia (Sérgio 1984).

The leafy liverwort genus *Porella* comprises more than 100 species with a worldwide distribution. Most of the taxa are found in southwestern Asia, where the genus is believed to have originated (Hattori 1978). In Europe, only eight species are currently recognized. The genus is known because of its wide phenotypic plasticity and morphologic variation which renders...
Table 1. Location and sample numbers of P. canariensis

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Location</th>
<th>Region</th>
<th>Elevation (m)/plant associations</th>
<th>Poly-morphic sites (%)</th>
<th>Genetic diversity</th>
<th>Genetic diversity</th>
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<td>20</td>
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<td>2.9</td>
<td>.535</td>
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<td></td>
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<td>Cape Verde</td>
<td>760 d</td>
<td>46.2</td>
<td>3.7</td>
<td>.692</td>
</tr>
</tbody>
</table>

a) Ratio of the number of polymorphic RAPD markers within a population over the total number of individuals sampled.
b) Gene diversity as $H_{J}$, the frequency of the $i$th allele at the $i$ locus (Weir 1996), which is identical to $H_{J}$ for random mating populations.
c) Genetically based on morphology problematic (Hattori 1978). Molecular techniques have proved invaluable for detecting genetic differences among taxa (Boisselier-Dubayle and Bischler 1989, 1994; Therrien et al. 1998). P. canariensis is an endemic species present on all Madeiran islands. Eggers (1982) lists it for seven Azorean and five Canarian islands, but not Cape Verde and Deserta Island in Madeira. On the Iberian Peninsula, the species is found in Sintra (Portugal) and Lanzarote (south of Spain), but Jenkyn (1995, in Ros et al. 1999) lists it for North Africa, a distribution that suggests a dual origin for this species: Mediterranean and African. P. canariensis occurs in small isolated colonies or patches, depending on the availability of specific ecological requirements such as atmospheric humidity and shade. Patches grow horizontally on trunks or rocks to 10–50 cm in diameter and can be found from sea level to 1700 m altitude.

The founding and genetic composition of these colonies is a matter of discussion (Bischler and Boisselier-Dubayle 1997 and references herein). They may start with one or several individuals reproducing vegetatively or sexually. It is accepted that these colonies are apparently reproductively isolated (Dewey 1989; Szweykowski 1982; Yamazaki 1982; Zehr 1980). Like most liverworts, P. canariensis has a life cycle characterized by the presence of a dominant haploid gametophyte and a short-lived diploid sporophyte. Spore dispersal is usually limited to short distances (Nissen and Söderström 1988; Levin 1989) and sperm dispersal by water does not go far (from 4 to 65 cm) (Bischler and Boisselier-Dubayle 1997; Wyatt 1985). It is widely accepted that liverworts have limited dispersal abilities, in part because they easily reproduce vegetatively and most of their life cycle is haploid. Vegetative propagation is perhaps more common than sexual reproduction, and isolation of populations should be very effective, even within short distances. Transport of propagules requires the existence of nearby streams, which are not always present. On the other hand, it has been shown that even low rates of sexual recruitment are sufficient to maintain some genetic diversity (Watkinson and Powell 1993). Also, allozyme studies have shown that clonal plants present a remarkable genetic variability, even with low levels of sexual recruitment (Elstand and Rouse 1987; Widén et al. 1994).

If propagation of P. canariensis is mainly vegetative, drift could be the main force determining intraspecific differentiation, and high levels of homogeneity would be expected within populations. Self-fertilization within isolated colonies, especially if colonies are founded by a few individuals, tends to decrease clonal diversity. There is no strong evidence that selection plays a substantial role in determining any genetic variation among colonies of P. canariensis (Bischler and Boisselier-Dubayle 1997). Traditional methods of genetic analysis (isozyme or morphology) applied to these liverworts often fail to reveal variation in most conspecific populations, which implies restricted gene flow (Boisselier-Dubayle et al. 1995; but see a contrary view for other bryophytes in Stoneburner et al. 1991). This is consistent with a pattern of dispersal and founder events (Shaw 1991; Shaw and Schneider 1995). As an alternative, Wyatt et al. (1989b) proposed that intrapopulation differentiation could be due to selection over different genotypes acting because of environmental heterogeneity. Boisselier-Dubayle and Bischler (1994), working with random amplified polymorphic DNA (RAPD) markers, reported the existence of some intraspecific genetic variation in several species of European Porella.

To bring new insights to the debate over the genetic composition and breeding systems of P. canariensis populations, we used RAPD (Williams et al. 1990) banding patterns as markers to analyze intra- and interspecific genetic variation. These markers, presumed to be selectively neutral, should indicate that any pattern of variation found between different populations would be caused by historical effects, drift, and/or migration. This way, variation within populations must be determined by the mating system of the liverwort. The genetic relationships among the populations studied, partly covering the species distribution range, will be addressed which may offer insights into the dispersion and colonization history of P. canariensis in Macaronesia.

Materials and Methods

Plant Material

Any genetic analysis of liverworts like Porella requires a clear definition of “population.” We follow Bischler and Boisselier-Dubayle (1997) and define it as a continuous colony or “carpet” of liverworts.

Ten populations of P. canariensis each represented by a patch 20–30 cm in diameter and belonging to the four Macaronesian archipelagos (Azores, Madeira, Canary Islands, and Cape Verde) and two from Portugal (Sintra) were used in this study (Table 1, Figure 1). The number of individuals (“shoots” or “ramets”) from each population were randomly taken from each patch and are given in Table 1. Locations of the sampling sites are shown in Figure 1.

DNA Extraction

Before DNA extraction took place, all specimens were carefully washed and checked under the microscope to be sure that no possible contaminants (microalgae, fungi) were left. DNA extraction of specimens about 2–3 cm long and weighing just 10 mg required modification of several standard protocols (Doyle and Doyle 1988; Murray and Thompson 1980; Rogers and Bendich 1985). We ground 10 mg of fresh
material (equivalent to one individual of *Porella*) to a fine powder in an Eppendorf tube with a glass rod and liquid nitrogen (tubes are kept with the tissues and filled with nitrogen for only a few s). The powder was then mixed with 500 ml CTAB buffer (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 2% p/v CTAB; 20 mM EDTA(Na)), 1% β-mercaptoethanol, 2% proteinase K (20 mg/ml), and then incubated in a bath at 65°C for 30 min. We added 355 ml of chloroform and 14 ml of 1-octanol to this mixture, followed by centrifuging 15 min at 13,000g. The supernatant was transferred to new Eppendorf tubes, mixed with 2/3 (v/v) isopropanol, and centrifuged for 15 min at 13,000g. Supernatant was discarded and the pellet washed with a solution of 76% propanol, and centrifuged for 15 min at 92°C, the aqueous phase discarded, and the pellet resuspended in 100 ml of ultrapure water. Ten milliliters of AcNa 3 M (pH 5.2) and 250 ml of –20°C absolute ethanol were added, followed by a gentle mix and centrifugation for 15 min at 13,000g. The aqueous phase was removed and the pellet washed again with 70% ethanol and centrifuged as before. Finally, the aqueous phase was discarded and the pellet resuspended in 100 ml of ultrapure water or Tris-EDTA. This procedure recovered at least 0.5 mg of good-quality DNA for RAPD analysis.

**Primers and PCR**

Ten-mer primers from OPERON Technologies (Alameda, CA; series OPA-A,F,G,H,I,J) were screened on individual representatives of the populations under study. Many of the primers produced either complex banding patterns or nonreproducible and inconsistent amplification products. Hence only 10 primers were chosen for the subsequent analysis. For each primer, only bands that could be unambiguously interpreted across all the population samples were chosen. Reproducibility of bands was assessed by replicating amplifications of samples selected at random. Polymerase chain reaction (PCR) was carried out in 20 μl volumes using 2 μl DNA (20 ng/μl), 2 μl dNTP mix (2 mM of each of four nucleotides; Promega), 2 μl 10× Taq DNA buffer (Pharmacia), 2 μl oligonucleotide primer (5 pmol/μl), 1 unit Taq DNA polymerase (Pharmacia), and the remainder of water. PCR conditions were 1 min at 92°C, 1 min at 36°C, and 2 min at 72°C, for 45 cycles, performed in a Biometra thermal cycler. Amplified products were separated in 1.2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet (UV) light. The 100 BPL (Pharmacia) was used as a molecular size marker.

**Data Analysis**

RAPD profiles were scored for each individual as discrete characters (presence or absence of amplified products) across all individuals from all populations and for each primer used. Table 1 shows chosen parameters for genetic diversity within populations: the percentage of polymorphic sites (markers), the genetic diversity calculated as the ratio of the number of polymorphic RAPD markers found in a population over the total number of individuals sampled, and Weir’s D gene diversity. The second parameter may be less influenced by the sample size, but Weir’s D is a good indicator of variability especially for telling species (Weir 1996). An analysis of molecular variance (AMOVA) was performed based on Euclidean distances between all pairs of haplotypes according to Excoffier et al. (1992) and using the Arlequin program (Schneider et al. 1997). The total genetic variation could be divided into three levels of grouping using the five recognizable groups of populations: among regions (archipelagos), among populations within regions, and among individuals within populations. AMOVA components were tested for significance by nonparametric randomization tests using 6000 permutations under the null hypothesis of no population structure. A test for differences in intrapopulation molecular variances was also performed based on Bartlett’s statistics (Excoffier et al. 1992; Stewart and Excoffier 1996). Additional AMOVAs were carried out to investigate the among-group variances allocated for each of the five groups of populations. In this case, only two groups were considered, the particular one being tested and the remaining populations combined. Based on nested variance components, the AMOVA gave Φ values, thus allowing the use of Φ values (an estimate of FST; Excoffier et al. 1992) as interpopulation differentiation measures and the computation of Nm [Nm = 1/(1/ΦST – 1)] as an estimator of gene flow (Slatkin and Barton 1989; Wright 1951). Significance of ΦST values were determined using a nonparametric permutation procedure. Variation among all pairs of samples was evaluated according to the index of genetic similarity of Nei and Li (1979), which is solely based on the shared presence of characters and excludes shared absence as a criterion of similarity. Relationships among populations were evaluated via the unweighted pair-group method (UPGMA) of Sneath and Sokal (1973) using Nei’s (1978) genetic distance, and principal component (PCO) analysis. All analyses were performed using NTSYS (Rohlf 1993).

Correlation of pairwise ΦST values with geographic distances separating the populations (within and among sites) was assessed using a Mantel test. The significance of Mantel’s Z statistics was calculated through a normalized approximation.

**Results**

The 10 primers amplified 80 RAPD bands in the 222 individuals of the 12 popula-
Discussion

A substantial amount of variation in RAPD profiles of *P. canariensis* was found within populations (39.6%) and among populations within each archipelago (41%). Much less variation (19%) was attributed to differences among regions (archipelagos), in spite of the fact that big geographic distances separate them. Similar results were found by Martin et al. (1997) and Sale et al. (1996), but Liao and Hsiao (1998) and Hsiao and Lee (1999) found that variation among regions contributed more than variation among populations within regions to the total variation. Buso et al. (1998) found 77% of variation between regions versus 12% due to variation within regions. Gurgeli et al. (1999) reported 95% of variation within populations and only 1% among regions in the outcrossing and monoclonal *Saxifraga oppositifolia*. No congruence was found by these authors between genetic and geographic distances. In our analysis of *P. canariensis*, variation within populations in RAPD haplotypes (which made every ramet in the patches sampled a different genotype)
contradicts the homogeneity found in morphology, even between populations that are localized in different environments. We have sequenced several individuals from different populations of *P. canariensis* for the ITS region of nuclear rDNA and found no polymorphism (unpublished results).

The high variation within populations revealed by the AMOVAs and the fact that no identical haplotypes existed within any colony indicates that the differences observed might correspond to different individuals sampled from the same patch. An alternative explanation could be that *Porella*, which reproduces vegetatively during most of its long haploid life cycle, accumulates mutations, producing an extremely rich clonal diversity. Individuals sampled in the adult haploid phase would make mutations easily detectable, explaining the high variability found. Waycott (1998), in a study of uniclinal populations of sea grasses, proposed that long-lived individuals characterized by vegetative growth may have accumulated genotypic differences via the accumulation of somatic mutations and recombination, a fact also predicted in theoretical models by Sønne and Watkinson (1979). Gabrielsen et al. (1998) also reported high levels of genetic diversity in the arctic clonal Saxifraga cernua, and Cronberg et al. (1997) explained the high genetic variation found in a clonal bryophyte as due to a perennial stayer life strategy associated with wide wind dispersal of pollen. In contrast, Bauert et al. (1998) found three isolated, relict alpine populations of the same species presented no variation at all. This was explained by postglacial events resulting in bottlenecks or founder events. Also Steinger et al. (1996) reported that patches of slow and clonally growing Carex curvula are genetically uniform. In spite of the fact that the breeding system of *P. canariensis* is poorly understood, it is known that the species reproduces well vegetatively (Sá-Fontinha S, personal observation) and that each individual can live and grow for a long time. In this case, recombination is possible among individuals of different generations during the short period of their diploid and sexual cycle, and vegetative growth preserves these acquired genotypes.

At least in the Madeira archipelago, mature plants in full reproductive phase have been observed in some populations (patches), but no evidence exists that sexual reproduction is the major factor contributing through recombination to the high level of genetic variability. Observations in other populations, like Porto Santo, only rarely show reproductively mature plants (Sá-Fontinha S, personal observation). The fact that RAPD markers were found to be randomly associated is evidence that some sexual reproduction exists within each patch. The widely accepted lack of substantial spore dispersal in *P. canariensis* is supported by 41% of the variation being attributed to differences between populations within archipelagos. Such a pattern suggests that *P. canariensis* is a typical outcrossing species according to Loveless and Hamrick (1984) and Hamrick and Godt (1996). In this case, however, high levels of variation still exist among populations within regions, especially in the case of Madeira, with five populations sampled, which indicates that some reduced gene flow exists. The fact that the species is not widespread, but rather is confined to particular habitat conditions where the colonies can grow, increases variation among populations belonging to the same group. The AMOVA also shows that differences among archipelagos are maximum between the Madeira populations and the others (14%) and minimum between the population of Cape Verde and the others (0.8%). These values may be inflated, however, because the samples from the Canaries are from the same island, and only two islands were screened for the Azores.

RAPD profiles revealed a structuring of populations completely congruent with their geographic location. Variation found within Madeira is high if we compare it to both populations from Tenerife and even to the two Azorean samples on different islands. Both cluster analyses and PCO showed similar grouping of populations and clearly separate the Madeira cluster from the others. In the Madeira group of populations, the two more closely related samples are MA1 on Madeira Island and MA4 on the Desertas Islands. Both populations appear in coastal vegetation which is not the case of MA2, which occurs in...
Laurosilva forest. In the other group of populations, the two trees slightly differ in the clustering of internal nodes. Nevertheless, both agree in placing together the populations from Sintra and Tenerife (Canaries). The populations from the Azores always cluster together and are the two most similar ones. Their place as an outgroup for the Sintra/Tenerife populations may reflect that Cape Verde joins with both the Ten- erife and Sintra populations, and only then forms the two Azores. This has implications for the general pattern of dispersion and colonization history of _P. canariensis_. Mediterranean in origin, the species may have occupied a much broader area than it does today. In fact, the data allow us to speculate that _P. canariensis_ may have existed from Iberia to the coast of Cape Verde when the Sahara was green. Much of its range then was probably associated with the former Tertiary Laussian rain forest. This type of forest is today completely absent from Morocco, but some evidence suggests that it had previously existed (Aubreville 1976). In Cape Verde the last few colonies of _P. canariensis_ are quickly disappearing with desertification. We have only found it on São Vicente Island on a mountain where very few Laurasian elements still exist. Starting from the mainland, colonization of the Cape Verde and Canary Islands would have been possible to the proximity to the coast, probably using other plant species as carriers. The results from the AMOVA analyses, together with the lack of correlation between pairwise _F_{ST}_, values and geographic distances among the 12 samples studied, indicate that habitat differentiation is more important than large-scale geographic differentiation (isolation by distance) to account for variation in these populations of _Porella_. High levels of genetic variation and in bryophytes by Wyatt et al. (1998a) have been explained as genetic adaptations to microhabitats. In fact, the variance among regions is smaller than that among populations within a single region. This result could also be due to the fact that more samples were used in Madeira than in the Canaries or the Azores, but similar results were obtained when we performed the analysis with just the three Madeira Island samples. Large distances between the archipelagos and Sintra 1400 km between Sintra and Canary, 600 km between Sintra and Cape Verde, and 0 km from Sintra to either Madeira or Azores, and the fact that many extant populations are rare and confined to specific areas, make present-day gene flow among regions virtually impossible. The relatedness of populations from Sintra, Can- nary, and Cape Verde as revealed in the dendrograms indicates that part of their RAPD markers are shared and date back to a single large population. Particular habitat conditions on Tenerife probably prevent the two populations analyzed from diverging further. Populations from the Azores may be considered a separate founding event from continental populations. The Azores are the youngest islands and have low habitat diversity. This could explain why the two populations belonging to different islands are separated by a small genetic distance.

Taking into consideration variation within populations of _P. canariensis_, it is clear that colonies are comprised of more than one individual (i.e., multiclonal). Moreover, besides vegetative reproduction, sexual recombination appears to be more common than is usually admitted. The high variation among populations within regions confirms that spore dispersal, even over short distances, is probably only effective within colonies. Ecological constraints may be a strong factor limiting the possibility for colonies to diverge. Finally, the good agreement obtained between the two dendrograms suggests a continuous, large population of _P. canariensis_, at least from Iberia to the coast of Cape Verde in mainland Africa, from which the plant colonized the two southern archipelagos. The close relationship between _P. canariensis_ populations from these Atlantic archipelagos and the samples from Sintra corroborates the hypothesis that some of the _P. canariensis_ polymorphism is not recent, but rather dates back to a preglacial widespread population that found refuge in these habitats that were essentially untouched by climatic changes.

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