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AFLP evaluation of genetic similarity among laurel populations (*Laurus L.*)

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Summary

Two species have traditionally been considered within the genus *Laurus: L. nobilis* L. and *L. azorica* (Seub.) Franco. The first is characterized by the presence of glabrous twig leaves and is located in the Mediterranean region. It can be found as cultivated or naturalized, and has been reported in Spain, France, Italy and Greece. *L. azorica* is characterized by the presence of densely tomentose to hirsute twig leaves and has been described in the Azores, Madeira and the Canary Islands. We have found that some natural populations of *Laurus* in Northern Spain, which are considered to belong to *L. nobilis,* have hirsute young buds, with wide variation in hair number and density, in contradiction with taxonomical descriptions reported for this species. In order to evaluate the genetic similarity between these *Laurus* populations and the two reported species, we have analyzed 14 populations of *L. nobilis* and *L. azorica* from different geographical areas, including the Iberian peninsula, the Canary and Madeira Islands, France and Italy, using amplified fragment length polymorphisms (AFLPs). UPGMA clustering and principal component analysis (PCA) of the AFLP data revealed a low genetic similarity between the Iberian populations, including populations from Northern Spain, and the rest of the populations analyzed from France and Italy. Moreover, laurel accessions from the Iberian peninsula showed higher genetic similarity to those from the Canary Islands and Madeira, originally identified as *L. azorica*, than to samples from populations along the Mediterranean area, morphologically classified as *L. nobilis*.

Introduction

The Lauraceae family includes between thirty and fifty genera (Cronquist, 1981), with a world-wide distribution, in tropical and subtropical areas (Heywood, 1978). The only genus native to Europe is *Laurus* L., which includes two species, *L. nobilis* L. and *L. azorica* (Seub.) Franco. *L. nobilis* (laurel) was initially described with materials from Italy and Greece by Linnaeus (1753). Currently this species, either wild or cultivated, is present in southern and western Europe (Jalas & Suominen, 1991), including all the Mediterranean area and the Atlantic coast of France and the Iberian peninsula. *Laurus nobilis* populations are always found in regions of warm climate and high rainfall (Sfikas, 1993; Pirone, 1995; Begines, 1996), frequently in more humid microclimates such as tubes, canyons and valley funds. Jalas & Suominen (1991) considered as wild laurel only the populations present in the center and east of the Mediterranean basin and estimated that populations in the Iberian Peninsula and western France could be the result of different introduction events. *L. azorica* (Canary island laurel) is native of the Canary islands, Madeira and the Azores (Bramwell, 1983, Jalas & Suominen, 1991) and has also been reported in the Atlas mountains of Northern Africa (Barbero et al., 1981). Populations of *L. azorica*

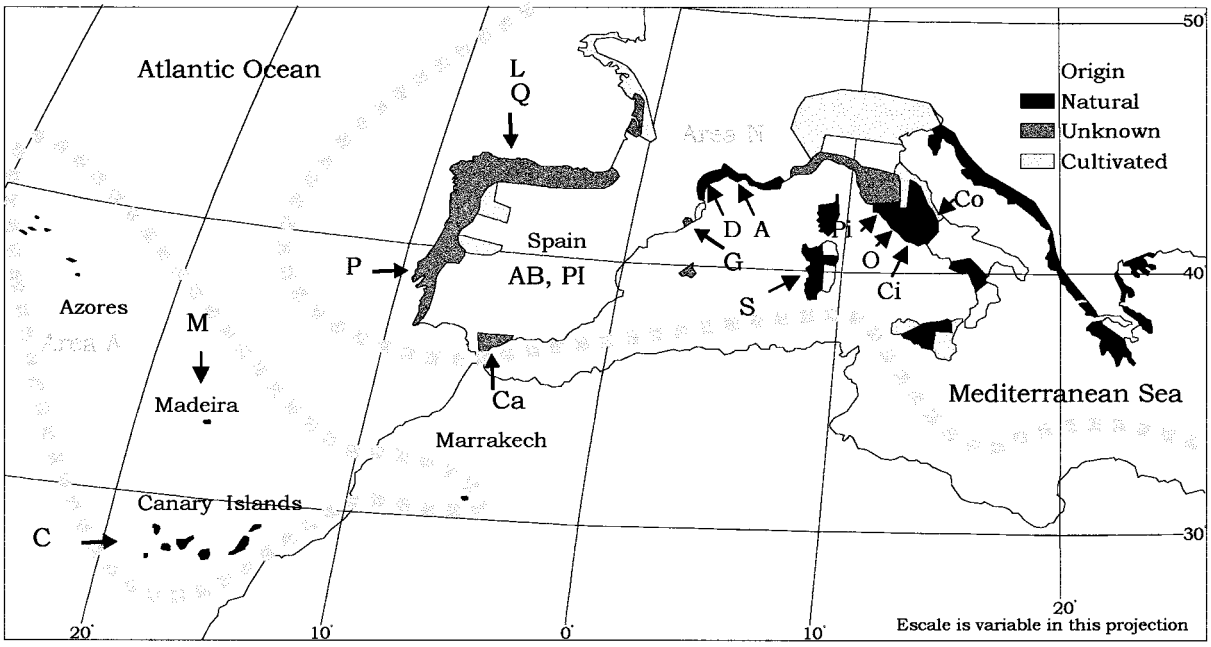

Figure 1. Map displaying the geographically separated areas from which the 62 laurel genotypes were sampled.

are found in the Atlantic islands, in humid mountain areas with abundant fog (Ceballos & Ortuño, 1976; Santos, 1983; Bramwell, 1983; Rivas-Martínez, 1987). Populations in Morocco (classified as *L. azorica*) are also found in similar habitats (Barbero et al., 1981) (Figure 1).

The morphological traits used to differentiate both species are scarce. Young branches and leaves are glabrous in *L. nobilis*, while they are smoothly pubescent in *L. azorica*. Moreover, leaves are wider and have a softer lemon scent in *L. azorica* than in *L. nobilis* (Tutin, 1964; Ceballos & Ortuño, 1976; Moro, 1988; Nees, 1989). Paleobotany considers that both *L. nobilis* and *L. azorica* derive from a common ancestor broadly distributed in Europe from the Miocene till the Pleistocene (Barbero et al., 1981). This ancestor was a component of the lauroid leaf forests (laurisilva) that occupied Southern Europe and Northern Africa about 20 million years ago (Morales et al., 1996; Jiménez et al., 1996). Climatic changes taking place during the quaternary era resulted in a gradual decrease of these populations all over their distribution area.

The Atlantic coast populations of *Laurus* in the Iberian Peninsula live in habitats similar to those for *L. azorica*. In the Cantabric coast (Northern Iberian Peninsula), laurel is an abundant tree in different environments forming bushes on the coast cliffs, riverside forests, groves, etc. (Díaz & Fernández-Prieto, 1994; Loidi et al., 1997). The trees in this area are classified as *L. nobilis,* in spite of the fact that populations show ferruginous apparel of variable density in leaves and young shoots. The characteristic morphology of these Cantabric laurel populations and their uncertain origin led us to compare them with other *Laurus* populations. Since morphological traits have the disadvantage of being influenced by both environmental and genetic factors and may not provide an accurate estimate of genetic similarity, we decided to use a high-multiplex PCR-based method for DNA profiling known as AFLP^{TM} or amplified fragment length polymorphisms (Vos et al., 1995). This assay has the potential to generate a large number of polymorphic markers to analyze genetic similarity without requiring any sequence information of the genome under study. In this work we have used the AFLP data to establish genetic similarities among populations of *Laurus* trees from different locations along the distribution area of the genus, and have compared them with their geographic distribution and with the two species classification based on morphological traits.

Materials and methods

Plant material

Plants classified as belonging to *L. nobilis* and *L. azorica*, and plants belonging to two reference Lauraceae

Table 1. Laurel populations used for AFLP analysis including localities of origin and bibliographical references.

Code	Region origin	Locality	References
A	Alpes Maritimes (France)	Auribeau	Barbero & Loisel, 1983
AB*	Madrid (Spain)	Botanical Garden of Madrid	
C	Canary Islands (Spain)	Anaga mountain	Rivas-Martinez et al., 1993
Ca	Cadiz (Spain)	Alcornocales Park	Begines, 1996
Ci	Lazio (Italy)	Civitavecchia	Corvetta et al., 1998
Co	Marche (Italy)	Conero mountain	Biondi & Baldoni, 1996
D	Var (France)	Dardennes	Barbero & Loisel, 1983
G	Gerona (Spain)	Gerona	Ballesteros, 1981
L	Asturias (Spain)	Luarca (sea cliff)	Díaz & Fernández Prieto, 1984
M	Madeira (Portugal)	Botanical garden of Funchal Curral das Freiras Francisco's House	
O	Toscana (Italy)	Orbetello	Corvetta et al., 1998
P	Sintra (Portugal)	Sintra mountain	Da Silva, 1989
Pi	Toscana (Italy)	Pisa	Corvetta et al., 1998
PI*	Madrid (Spain)	Botanical Garden of Madrid	
Q	Asturias (Spain)	Queruas (sea cliff)	Díaz & Fernández Prieto, 1984
S	Sardegna (Italy)	Uta	

* - non laurel genotypes: AB (*Apollonias barbujana*); PI (*Persea indica*)

species, *Apollonias barbujana* L. and *Persea indica* (L.) Spreng., were used in this study. The samples represented the three types of populations found in the *L. nobilis* group: wild, unknown and cultivated (Figure 1). Populations of cultivated *Laurus* sp., as described by Jalas & Suominen (1991), were not considered in order to avoid possible interpretative errors. Localities where other authors had described the presence of *Laurus* sp. in natural communities were sampled. Populations classified as wild (*nobilis* group) were sampled in the following localities: Dardennes and Auribeau in France (Barbero & Loisel, 1983) and Pisa, Orbetello, Civitavecchia, Conero and Sardinia Island in Italy (Corbetta et al., 1998; Biondi & Baldoni, 1996) (Table 1; Figure 1). Populations of unknown origin (*nobilis* group) were sampled in different regions of the Iberian Peninsula (Blanco et al., 1997): Luarca, Queruas, Cadiz and Gerona (Ballesteros, 1981; Díaz & Fernández-Prieto, 1994; Begines, 1996) in Spain and Sintra (Pinto da Silva, 1989) in Portugal (Table 1; Figure 1). The remaining species in the genus, *L. azorica*, is native of the area where the materials were collected: Tenerife (Rivas-Martínez et al., 1993) in the Canary Islands and Madeira Island (Table 1; Figure 1). Samples of *Apollonias barbujana* and *Persea indica*, were obtained from the collections of the Botanical Gardens of Madrid.

Table 2. Sequences of primers and adaptors

EcoRI adaptor	5′-CTCGTAGACTGCGTACC-3′
	3′-CTGACGCATGGTTAA-5′
*Mse*I adaptor	5′-GACGATGAGTCCTGAG-3′
	3′-TACTCAGGACTCAT-5′
*Eco*RI + 1 primer	E11 5′-GACTGCGTACCAATTC**A**-3′
*Mse*I + 1 primer	M20 5′-GATGAGTCCTGAGTAA**C**-3′
*Mse*I + 1 primer	M21 5′-GATGAGTCCTGAGTAA**T**-3′
*Eco*RI + 3 primers	E 36 5′-GACTGCGTACCAATTC**ACC**-3′
*Mse*I + 3 primers	M31 5′-GATGAGTCCTGAGTAA**CTT**-3′

DNA extraction and AFLP protocol

DNA was extracted from freeze-dried young leaves using the kit Dneasy^{TM} (QIAGEN, Inc., Chatworth, CA, USA) according to the manufacturer's protocol. AFLP analysis was performed according to Vos et al. (1995) with the modifications described by Cervera et al. (1998). DNA was digested with *Mse*I (New England Biolabs) and *Eco*RI (Pharmacia). After ligation adapter, preamplification of purified DNA tem-

plates was performed with primers complementary to the adapter sequences with an additional selective 3′-nucleotide. PCR reactions were performed in a 20μl volume with 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 30 ng of each primer (GENSET) *Eco*RI+A (E11), *Mse*I+C (M20) and *Mse*I+T (M21) (Table 2), 0.4U of *Taq* DNA polymerase (Boehringer) and 5μl of diluted digested-ligated DNA fragments. Selective amplifications were performed using two combinations of primers E36/M31 ($E_{ACC}M_{CTT}$) and E36/M39 ($E_{ACC}M_{TCC}$) (Table 2) with (^{33}P) labelled *Eco*RI primers. We used 5μl of the preamplification template for each PCR reaction. Two replicates of the PCR reactions from independent DNA extractions were performed to determine the accuracy of the analysis. Samples amplified with different primer combinations were loaded onto 4.5% denaturing polyacrilamide gels and electrophoresed for 2 h. Gels were later dried onto chromatography paper, and exposed to autoradiographic film.

Data analysis

Scorable polymorphic amplified bands were scored as 1 when present in an individual and their absence was scored as 0. Estimates of genetic similarity (GS) between pairs of individuals were based on the number of shared amplification products according to Dice (Sneath & Sokal, 1973). GS(ij) = 2a/(2a+b+c), where GS(ij) is the genetic similarity between individuals i and j, a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. Genetic similarities among samples were represented with a dendrogram based on the unweighted pair-group method of arithmetic averages (UPGMA). This analysis was performed using the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System) software package version 2 (Rohlf, 1993). An additional multivariate analysis, was performed using Principal Component Analysis (PCA) with program Statistica (5.0).

Results

To obtain an optimum number of scorable polymorphic bands per primer combination, we tested different numbers of selective nucleotides on a few selected laurel samples. The best results were produced by the combination of three selective nucleotides in both the *Eco*RI and the *Mse*I primers. These combinations yielded an average of 80 bands per amplification reaction (Table 3). Two combinations, E+ACC/M+TCC and E+ACC/M+CTT, were selected for the analyses of the 62 samples yielding a total of 167 amplified products. Among them, 100 (59.8%) were polymorphic and were unambiguously scored. Figure 2 shows a fingerprint of some of the samples after PCR amplification with primers E_{ACC} and M_{CTT}, and autoradiography. The score of the presence or absence for each amplified fragment produced a specific profile for every sample.

Table 3. Number of polymorphic amplified bands scored with the primer combinations used in this study

Primer combination	Total bands	Polymorphic bands	% Polymorphic bands
E+ACC/M+TCC	89	59	66.3
E+ACC/M+CTT	78	41	52.5
Total	167	100	59.8

The presence/absence data was used to generate a matrix of genetic similarities calculated by the Dice coefficient for every possible pair of samples (data not shown). This similarity matrix was used to construct the dendrogram represented in Figure 3 using the UPGMA method, this analysis showed a good fit to the genetic similarity data, as reflected by a product-moment correlation (r) of 0.97. Two samples belonging to *Apollonias barbujana* and *Persea indica* (codes AB and PI respectively), were used as reference outgroups in the cluster and PCA analysis. As shown in Figures 3 and 4, these samples were placed outside of the rest of the samples, with genetic similarities values in the range of 0.3 to 0.4.

Individuals within populations showed genetic similarities ranging between 0.75 and 1. Samples from Italy and France originally classified as *L. nobilis* showed lower genetic variation and some samples showed identical banding patterns. In general, individual samples showed higher similarity within populations than between different populations. The UPGMA analysis grouped the populations in two major clusters, separated at the 0.50 genetic similarity level (Figure 3). One of them included all the populations from France (codes A and D) and Italy (codes S, Pi, O, Ci and Co), considered to be representative of the species *L. nobilis*. Among them, samples from Sardinia

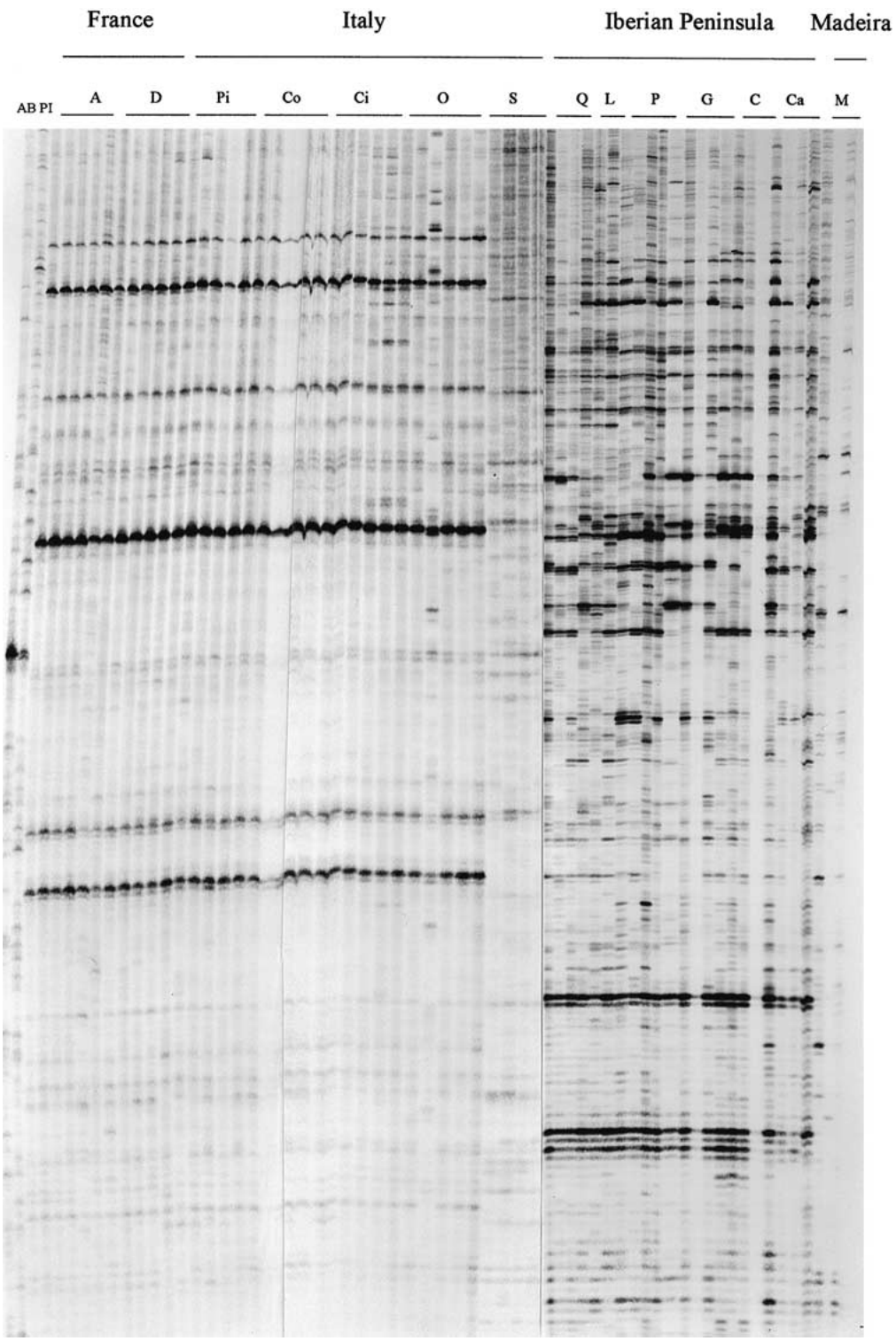

Figure 2. Amplified fragment length polymorphism (AFLP) profiles of laurel individuals generated using the primer combination EcoRI+ACC/MseI+CTT.

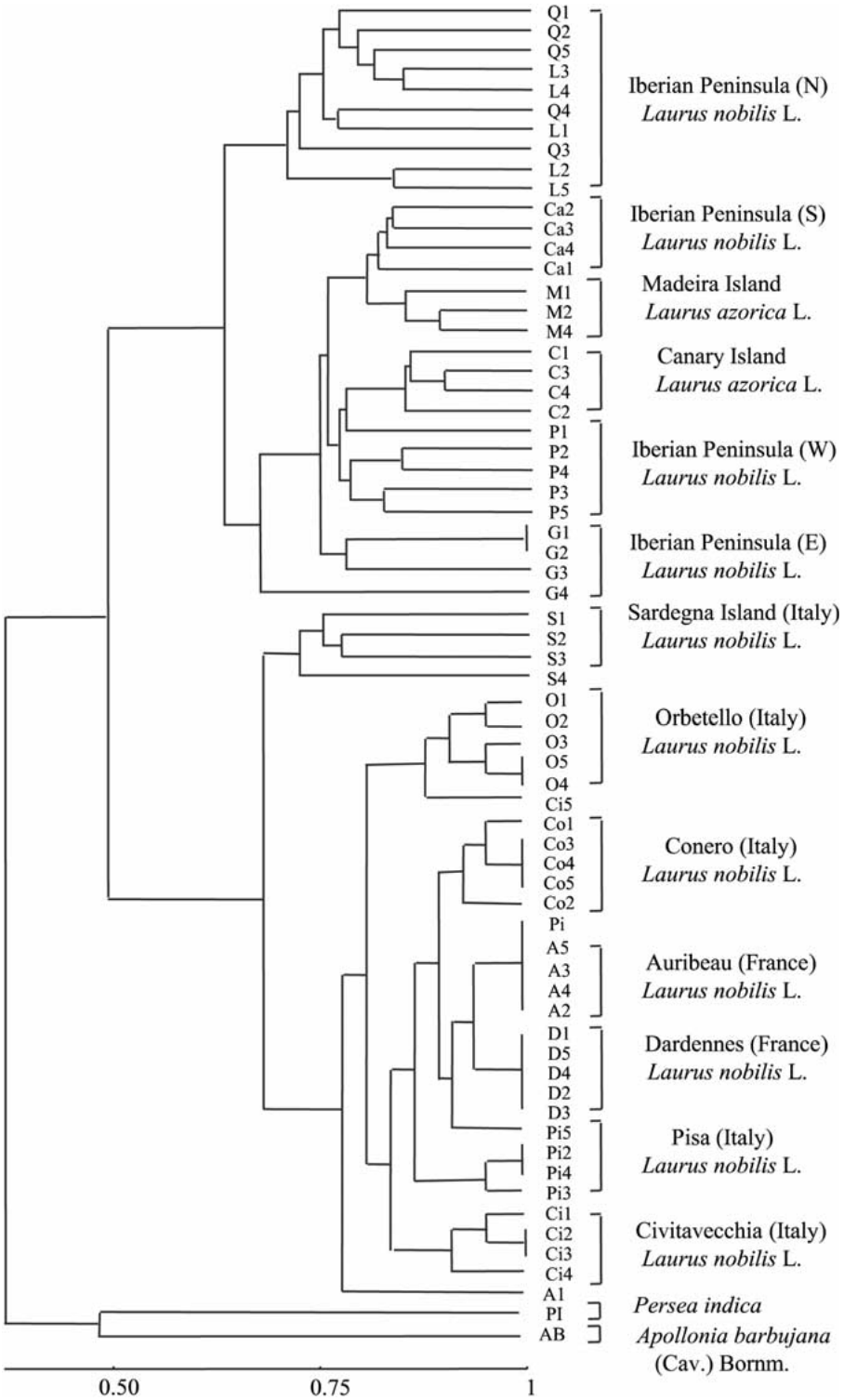

Figure 3. Dendrogram of laurel genotypes derived by average linkage cluster analysis.

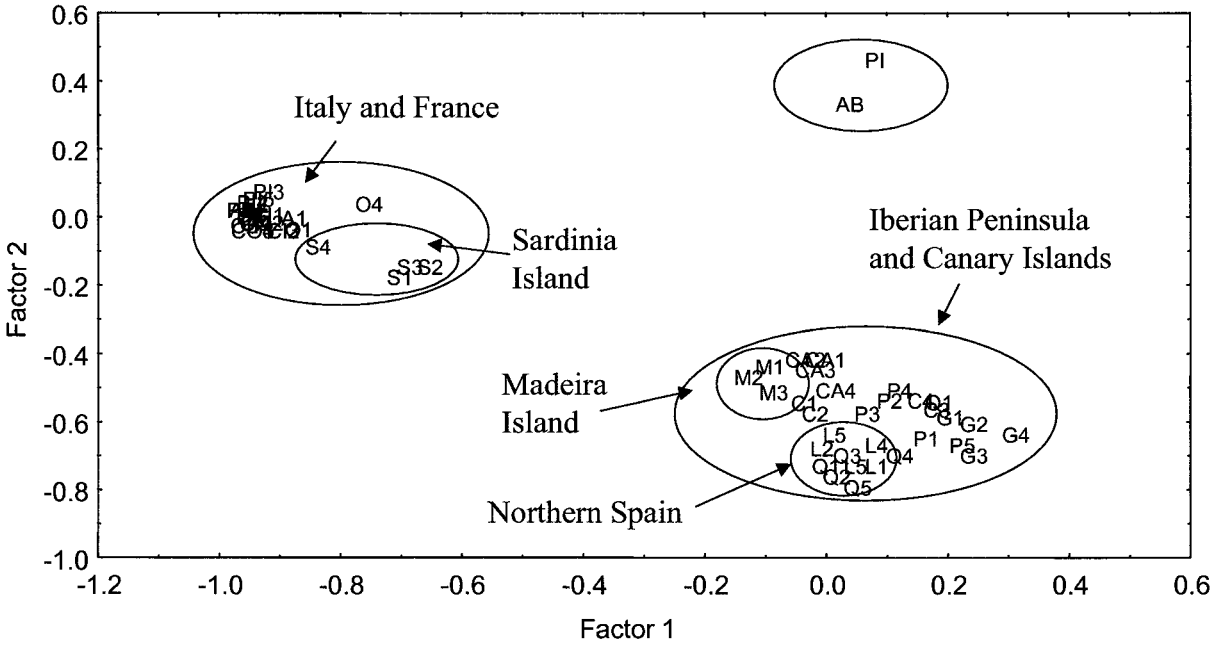

Figure 4. Plot of second (vertical) vs. first (horizontal) principal component analysis.

Island (code S) were all clustered in a more divergent subgroup at the 0.70 similarity level (Figure 3). The other major cluster included populations from the Iberian Peninsula and the Atlantic islands and was divided in two main groups at the level of 0.65 genetic similarity (Figure 3). One of them included all the samples collected in the Cantabric coast (codes L and Q) initially considered as *L. nobilis*. The other included samples classified as *L. nobilis* (codes P, Ca and G) and samples classified as *L. azorica* (codes C and M), located on the Iberian Peninsula and the Atlantic islands respectively (Figure 3).

Principal Component Analysis performed on the matrix of presence or absence of amplified bands produced a very similar result (Figure 4). In this analysis, the first two principal components accounted for more than half of the existent variation (51% in total, corresponding to 36% for axis 1 and 15% for axis 2), with the third axis accounting only for 3.6% of the total variation. Based on these major effects of the first two components, a two-dimensional plot of the sample scores gave a reasonable representation of the intersample similarities. In this plot (Figure 4), the first component was enough to separate in a single dimension the two main clusters. These results also demonstrated higher genetic similarity among Iberian peninsula and Canary islands populations than would be expected from their separate locations. The PCA plot, clearly distinguished Iberian and Italian populations. The Northern Spain and Sardinia samples formed sub-groups within the Iberian and Italian groups respectively, suggesting the possible existence of geographical sub-differentiation.

Discussion

We have used molecular markers (AFLP) for the analysis of genetic similarities among different populations of laurel. UPGMA and PCA analyses of AFLP data revealed two main groups of laurel samples (Figures 3 and 4). One of them, grouped samples from Italy and France (*L. nobilis*). The other one, grouped samples from the Iberian peninsula (initially classified as *L. nobilis*) with samples from Madeira and the Canary Islands, considered to belong to *L. azorica*.

Laurel accessions from the cliffs of Northern Iberian Peninsula (codes L and Q) (Figures 3 and 4), which showed hirsute leaves like those of the *L. azorica* samples from the Atlantic islands, were also included among the Iberian peninsula group. This could be a reflection of wider genetic variation of the *Laurus* species within the Iberian peninsula and suggests that laurels from the cliffs of Northern Iberian peninsula could represent ancestral ones which have been genetically isolated during and after glaciation. The

Cantabrian coast has acted as a refuge area for plants during the last glaciation (Franquinho & Da Costa, 1989; Blanco et al.,. 1997; Schaal et al., 1998), and populations genetically isolated for a long period of time in this area have also been described in other species (Templeton, 1994). Although the isolation of the Cantabrian coastal populations has resulted in enough divergence as to be included in a different subcluster from the remaining Peninsula, Madeira and Canary Islands populations, they still show higher similarity to these populations than to populations from Italy and France.

The high genetic similarity shown by UPGMA and PCA between the laurel samples from the Canary Islands, Madeira Islands and the Iberian Peninsula (codes S, P and G) is surprising and could be consistent with a rapid expansion of laurel in those regions. Current climatic conditions in the Canary Islands are benign for laurel, but in the past, higher temperatures might have not been ideal for laurel. Thus the actual presence of this species in the Canary Islands could be more recent than previously thought. Although the samples from the Canary Islands, Madeira and the Iberian Peninsula were described as two different species, they behave on both analyses as belonging to the same taxonomic group.

Samples from Italy and France show a very low similarity (0.50) to Iberian samples believed to belong to the same species (Figure 3). Also, in the PCA plot, Iberian, Italian and French populations are clearly distinguished (Figure 4). To determine whether the Italian and French populations really correspond to a different *Laurus* species or whether they just represent far related populations from the same species, requires further research. The higher genetic similarity observed between samples collected from France and Italy could be the result of reforestation in the area. Although it has been reported (Paul et al., 1997) that these high similarities can be obtained when selection is applied on the same natural population, we think that this is not the case because high similarity level was found even between materials collected from different geographic regions. In other species (Travis et al., 1996) the low genetic dispersion was attributed to population crashes affecting populations located in areas with stressful conditions due to human impact. Additional characterization of these populations will be required to elucidate the high similarity observed in some cases. Laurel samples from Sardinia show more genetic dispersion within the population and with respect to Italy and France. This situation is parallel to the relationship of Cantabrian populations with other population from the Iberian peninsula, and could also be due to increased genetic isolation.

The lower genetic similarity found between samples from the Iberian peninsula and samples from France and Italy could suggest the existence of two groups of populations which, separated by the natural barrier of the Pyrinees, have evolved independently. Trying to use additional markers to confirm this divergence, we have analysed in *Laurus* the existence of polymorphisms at ten chloroplast microsatellite loci developed by Weising & Gardner (1999). All the primer pairs amplified specific DNA fragments in all the samples, including *A. barbujana* and *P. indica*. The size of the amplification products was different in *A. barbujana*, *P. indica* and *Laurus*. However, no polymorphisms could be detected among *Laurus* samples from different populations. Thus, this result suggests a close similarity between the chloroplast genomes of species belonging to the two different clusters. Additional molecular and biological studies are required to determine whether they represent two different species.

Plant taxonomy has traditionally been based on morphological traits. However, botanists have long recognized that significant morphological variation can be the result of the function of a relatively small number of genes (Schaal et al., 1998). This situation has important implications for evolutionary studies because the morphological divergence relevant for adaptation and evolution of the species may have little relationship to the degree of genetic differentiation between lineages. Thus, it can be difficult to predict the genetic cohesiveness of a group based only on its morphological differences. Furthermore, morphological traits have the additional disadvantage of being influenced by environment as well as genotype enviromental interactions what difficults the estimation of accurate measurements of genetic similarity. Molecular markers like AFLPs can provide an independent set of markers for genotype comparisons, all over the genome, that are not affected by environmental conditions. The AFLP fingerprinting technique allows the visualization of DNA polymorphisms suitable for biosystematic purposes at the species and genus range. The technique can be applied to study genetic similarity at the population and at higher taxonomic levels. In this way, AFLPs have been shown to be very useful to analyse the genetic similarity among natural populations of *Laurus* and have pointed out the

need to review the taxonomy of this genus around the Mediterranean area.

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