Characterization of Quercus suber genomic regions encoding the phenylalanin ammonia-lyase and dirigent proteins

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Abstract.

In the biosynthesis of the lignin and suberin, the main constituents of cork, many enzymes as well as dirigent proteins are involved.

The phenylalanin ammonia-lyase is an initial key regulatory enzyme in the lignin and suberin polymerization, via phenylpropanoids metabolism and the dirigent proteins dictate the type and amount of phenolic monomers that will be incorporated and the type of linkage that will be formed through the specificity and orientation of their binding sites.

In this study, nucleotide polymorphisms in the genes encoding the PAL and the dirigent proteins were assessed.

In order to improve knowledge on the genetic variability of cork oak owing to nucleotide polymorphism and to explain the possibility of the correlation between molecular banding patterns and the qualitative characteristics of the cork tissue, with the use of numerical taxonomy methods, the results thereof and the morphological characteristics of the cork tissue were analysed.

Key words: *Quercus suber*, phenylalanine ammonia–lyase, dirigent proteins, cork quality.

Resumo.

Na biossíntese da lenhina e da suberina, os principais constituintes da cortiça, participam várias enzimas e proteínas dirigentes.

A fenilalanina amónia liase é uma enzima reguladora chave na polimerização da lenhina e da suberina, através do metabolismo dos fenilpropanóides. As proteínas dirigentes determinam o teor e o tipo de monómeros fenólicos que devem ser incorporados e o tipo de ligações que devem ser estabelecidas através da orientação e especificidade dos seus locais de ligação.

Neste estudo avaliaram-se os polimorfismos nucleotídicos resultantes dos genes codificantes da fenilalanina amónia liase (PAL) e das proteínas dirigentes.

Para elucidar a divergência fenotípica da qualidade da cortiça avaliaram-se também as características físicas da cortiça e estabeleceram-se correlações entre sobreiros produtores de boa e má cortiça e a análise molecular com o objectivo de determinar se a PAL e/ ou as proteínas dirigentes poderiam actuar como marcadores moleculares.

Palavras-chave: *Quercus suber*, fenilalanina amónia liase, proteínas dirigentes, qualidade da cortiça.

Introduction

Cork oak, *Quercus suber* L., is the unique Mediterranean evergreen that produces cork, a natural renewable resource of great economical interest, becoming Portugal the world leader on cork production, transformation and trading.

The cork tissue consists mostly of suberin and lignin as well as several others chemical constituents (Natividade, 1950; Cordeiro *et al*., 1997 e 1998).

Lignin is a complex phenolic polymer while suberin is characterized by two domains, namely one aromatic and the other aliphatic. The aromatic domain is predominantly located within the primary cell wall and the aliphatic domain is located between the primary cell wall and the plasmalemma. The two domains are supposed to be cross-linked (Stark & Garbow, 1992; Neto *et al*., 1996; Gil *et al*., 1997; Lopes *et al*., 2000a, b).

Several enzymes have been suggested to be involved in various metabolic steps in the lignin and suberin biosynthesis. The enzyme phenylalanine ammonia-lyase (PAL) is a key enzyme that catalyzes the first step of the phenylpropanoids mechanism.

The first model considered for coupling radicals to produce a functional lignin molecule is based on the principle that lignin formation proceeds through coupling of individual monolignols to the growing lignin polymer in a near random fashion.

According to modern ideas, the biosynthesis of lignin consists of two main stages, the first of which includes the formation of aromatic monomeric precursors of lignin synthesised in the plant from carbon dioxide via carbohydrates and the second involves the construction of the lignin macromolecule from the monomeric precursors. Until recently, has been thought that these precursors would undergo random coupling and no other enzymes or proteins were thought to be required.

Recently, the second model on control of monolignol coupling suggests that lignification is a highly stereo- and region controlled synthesis and must be under strict regulation of specialized proteins, called dirigent proteins, which control the formation of individual bonds (Davin *et al*., 1997; Gang *et al*., 1999; Davin & Lewis, 2000) at specific sites between the citoplasmatic membrane and the cell wall.

The dirigent proteins stereospecifically guide the monomers to specific locations where, in the presence of oxydase and/or peroxydase, those monomers are converted into (+)-pinoresinols, and then, via NADPHdependent (+)–pinoresinol-(+)-lactiresinol reductase, undergo polymerization leading the lignin biosynthesis pathway (Fujita *et al*., 1999). Even though no studies have been published, until now, claiming that dirigent proteins are intermediates in the suberin synthesis pathway, it is important to mention that, regarding the aromatic domain of the suberin synthesis, the oxydase and/or anionic peroxydase participate in the coupling process of phenolic radicals (Bernards, *et al*., 2004).

The occurrence of proteins of different molecular dimensions directly associated with the extracted cork suberin was already demonstraded. These proteins showed a qualitative differential between the two types of cork quality, which seems to reflect the different genomic compositions of each studied cork oak (Nóbrega *et al*., 2000).

In this study, the nucleotide polymorphism of the encoding DNA regions for PAL and dirigent proteins were assessed, as well as the physical characteristics of the cork in order to establish correlations between these encoding regions and the qualitative characteristics of cork.

Material and Methods

Molecular analysis

Young leaves were collected from cork oak trees located in four different geographical populations: Herdade Monte Fava in Ermidas-Sado, Herdade da Palma (Vale Casco) in county of Álcacer do Sal, Companhia das Lezirias in county of Benavente and Herdade da Espirra in county of Vendas Novas.

Genomic DNA was extracted following the protocol of the DNeasy Plant Mini Kit (Qiagen). DNA was quantified and diluted in milliQ sterile water to obtain a final concentration of 100 ng μL^{-1} in order to perform PCR experiments.

Phenylalanine ammonia-lyase (PAL)

For amplification of regions encoding regions of PAL, the primers referred in Table 1 were used, according to nucleotide sequences referenced by several authors (Dvornyk *et al*., 2002; Kao *et al*., 2002; Cochrane *et al.,* 2004) and sequences registered at GenBank [1] database.

Table 1 – Nucleotide sequences of the primers use to amplify the encoding regions of the PAL gene (F) - *primer forward*; (R) - *primer reverse*.

Primers F	Nucleotidic Sequence (5'- 3')	Primers R	Nucleotidic Sequence (5'- 3')
PAL1	TGG GGG TTA GCT GCT GAG GG	PAL2	AAG AGC ACC ACC ATT CTT GG
PAL3	ATG GAT CAA ATC GAA GCA ATG	PAL4	TTA GCA AAT CGG AAT CGG AGC
PAL5	ATG GAG TTT CGT CAA CCA AAC	PAL6	TTA GCA GAT AGA AAT CGG AGC A
PAL7	ATG GAG CTA TGC CAA TCA AAA C	PAL8	TCA ACA GAT TGA AAC CGG AGC
PAL9	GTY ACT ACT GGT TTT GGT GC	PAL10	GCA TYA ATG GAT AGG TWG CAC T
		PAL13	GCA TCA ATG GAT AGG TAG CAC T
PAL11	GTC ACT ACT GGT TTT GGT GC	PAL14	GCA TCA ATG GAT AGG TTG CAC T
PAL12	GTT ACT ACT GGT TTT GGT GC	PAL15	GCA TTA ATG GAT AGG TAG CAC T
		PAL16	GCA TCA ATG GAT AGG TTG CAC T
PAL17	ACA GAC CAC TTG ACA CAT A	PAL18	TTT GAA AAT TGA GCA AAC A
PAL19	ATY GAG GCT GCT GCY ATT ATG	PAL20	ACA TCT TGG TTG TGY TGC TC
PAL21	ATT GAG GCA GCA GCT ATA ATG	PAL22	ACA TCT TGG TTA TGT TGC TC

PCR was carried out on a Hybaid Express thermocycler in a final volume of 25 µl including 100–200 ng genomic DNA and containing a 1x Qiagen PCR Buffer, which provided a reaction mixture of 2,5 mM $MgCl_2$, 200 µM of each dNTP, 0,4 µM of each primer and 2,5 U *Taq* DNA polymerase. The amplification reaction consisted of 35 cycles as referred in Table 2.

Table 2 – PCR conditions.

Steps	Temperature (ºC)	Time (minutes)			Number of cycles
		PAL1 a PAL 8	PAL9 a PAL1 8	PAL21/ PAL22	
Initial denaturation	94	3	3	10	1
Denaturation	94	1,10	0,45	0,30	
Annealing	50	1	0,30	0,30	35
Extension	72	2	2	0,30	
Final extension	72	10	5	10	1

PCR products were separated in a 1 % agarose gel using 0,5X TBE buffer (45 mM Tris-borate, pH 8,0 and 1 mM EDTA) containing 0.5 µg/mL ethidium bromide. Gels images were captured and analysed using Gel VersaDoc and Quantity One software (Bio-Rad). The electrophoretic profiles were analysed using the program NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System), ver. 2.0 ("Applied Biostatistics Inc").

Dirigent proteins

For amplification of regions encoding dirigent proteins were used degenerate oligonucleotide primers, designed from the consensus regions of 18 dirigent homologs obtained from twelve plant species: forward primer PS6F = 5′-KGTGTTYGAYGAYCCYATTACYBTWGACAAC-3′ and reverse primer PS2R = 5′-GAAATAAACATCTCCYTCAWATGMATCRGT-3′, where K=T/G, Y=C/T, B=C/T/G, W=A/T, R=A/G, and M=A/C, S= C/G, and X=A/C/T/G (Xia, 2000).

PCR was carried out on a Hybaid Express thermocycler in a final volume of 25 µl including 20–50 ng genomic DNA and containing a 1x Qiagen PCR Buffer, which provided a reaction mixture of 2,5 mM $MgCl_2$, 200 µM of each dNTP, 0,2 µM of each primer and 2,5 U *Taq* DNA polymerase.

The amplification reaction consisted of an initial denaturation step at 96 ºC for 3 min, 35 cycles of 1 min at 96 ºC, 1 min at 48 ºC and 1 min at 72 ºC, followed by one last extension step of 5 min at 72 ºC.

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and were sent for sequencing using commercial services (StabVida).

Morphological analysis of cork

Cork planks harvested according to Portuguese law, were prepared and submitted to several tests to evaluate seven parameters: calibre (CLA), specific gravity (DEN), rupture strength (FOR) and the corresponding angle (ANG), porosity (POR), number of pores per cm2 (NP/cm2) and humidity (HUM). Considering the values obtained, the cork quality classes were established according to the Portuguese standard NP298 (ISO/DIS 1216), the Portuguese standard NP-2803 and values obtained by several other authors (Fortes *et al.*, 2004 e Pestana da Silva, M., 2003). The results were analysed using the program NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System), ver. 2.0 ("Applied Biostatistics Inc").

Results and Discussion

Molecular analysis

Phenylalanine ammonia-lyase (PAL)

The results obtained for the amplification of the regions encoding genomic regions for PAL show that only the PAL1/PAL2 primers have demonstrated polymorphism, and therefore, have been selected to analyse the genetic variability among the different populations. The statistical analysis of the obtained electrophoretic profiles provided the dendogram represented in Figure 1.

Dendrogram analysis demonstrates the large genetic variability of *Quercus suber* and shows that the distribution of trees is independent of their geographical origin.

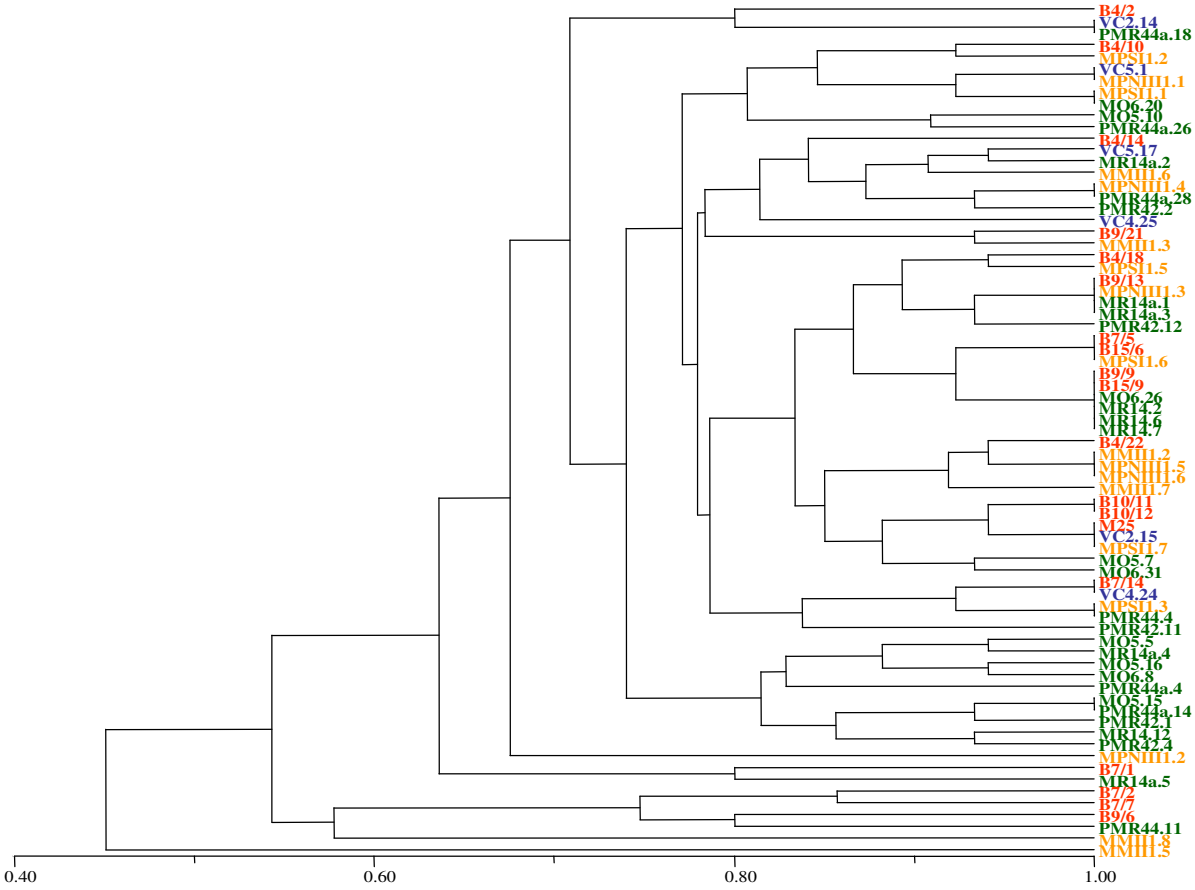

Figure 1 – Dendogram based on the UPGMA method applied to the similarity matrix (cophenetic correlation coefficient r = 0,799).

Dirigent proteins

Consistently the electrophoretic profiles showed a single PCR product with molecular size of approximately 300 bp.

Despite the fact that all PCR amplifications had the same or similar molecular size, sequencing results based on dirigent proteins showed small differences between nucleotide sequences. The alignment shows 97% similarity for the amplified region e also that there are 13 polymorphic locations. The respective peptidic sequences, presented in Figure 2, were analysed regarding their hydrophobicity indexes (Figure 3) by the algorithm developed by Kyte and Doolittle with a window size of nine amino acids (Ward, 2001).

Figure 2 – Alignment of the 19 peptidic sequences obtaining 98% similarity (Geneious v. 2.5.2).

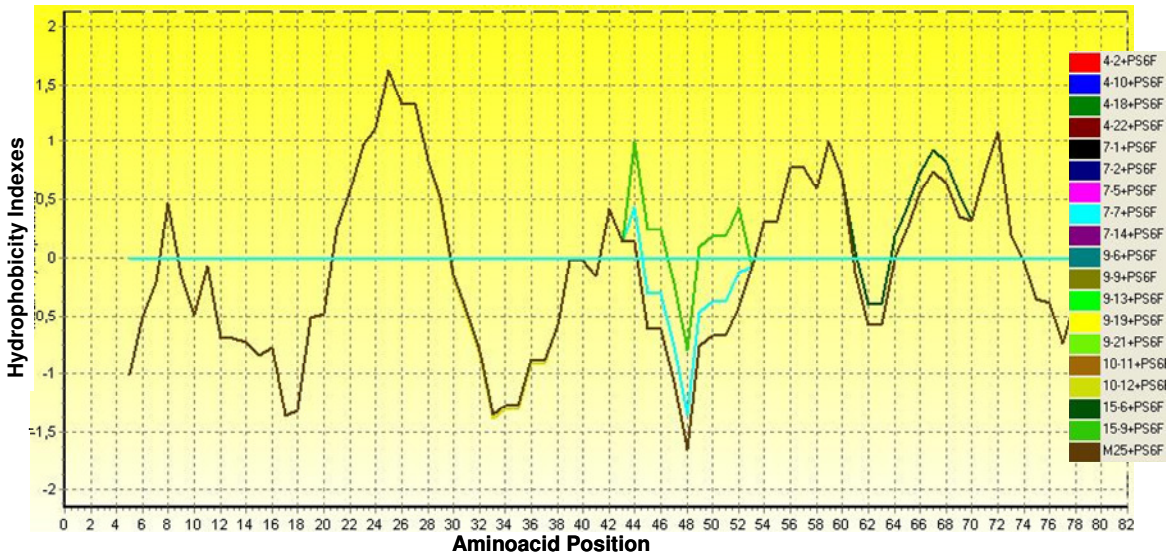

Figura 3 – Hydrophobicity graphic representing the 19 peptidic sequences obtained using the Kyte & Doolittle method (Bioedit v.7.0).

Based on the shapes of the hydrophobicity profiles, a comparison between the amino acid sequences suggest that a more efficient dirigent protein is synthesised for the producer of good cork quality than the producer of bad cork quality, which can justify the difference (at least, qualitatively) of these two kinds of cork quality.

Morphological analysis of cork

From the analysis of the seven assessed physical characteristics determined on the cork oak trees located in the 4 studied populations, the trees selected as good cork producers are presented in Table 3.

Table 3 – Selected cork oak trees as good or bad cork producers in the 4 populations.

Herdade	Cork oak trees
Monte Fava	B7/1, B7/5, B7/7, B7/14, B9/13, B10/11, B15/9, M25
Palma	VC2.14, VC2.15, VC5.1
Lezírias	MPSI.1.1, MPSI.1.3, MMII.1.7, MPNIII.1.1, MPNIII.1.2, MPNIII.1.3, MPNIII.1.4
Espirra	MO5.5, MO5.15, MO6.8, MO6.20, MO6.26, MO6.31, MR14.2, MR14.4, MR14.7, MR14a.1, MR14a.2, MR14a.4, PMR44.4

The phenogram represented in Figure 4 was obtained through the statistical analysis.

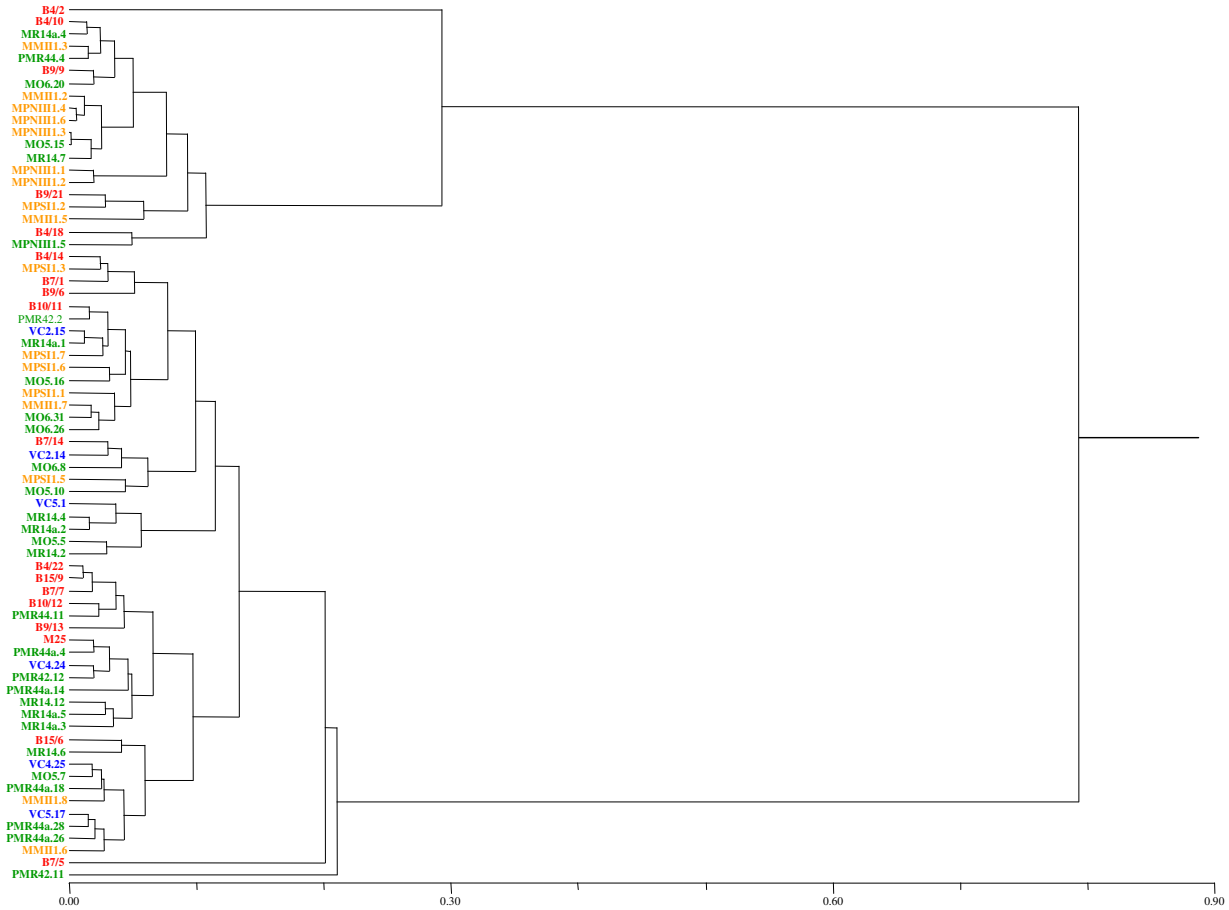

Figure 4 – Fenogram based on he UPGMA method applied to the distance matrix (cophenetic correlation coefficient r = 0,991).

These physical characteristics do not allow, distinctively, separating the trees. The analysis of their projections of their two main components shows the relative importance of each variable in the spatial settlement of the trees. In Figure 5, the projections of the physical variables of the cork were displayed regarding their first and second main components, and in Figure 6, the minimum spanning tree was represented overlapped with the projection of the trees on the plane defined by the first main component (58,49%) and by the second main component (30,25%) based on the morphological characteristics of the cork.

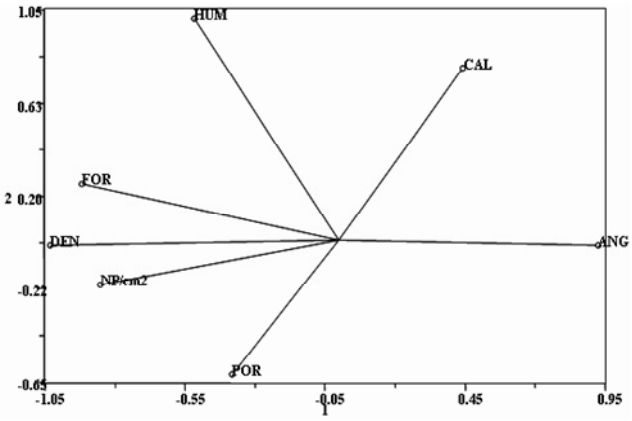

Figure 5 – Projections of the 7 physical characteristics of cork, on the plane of the first two main components.

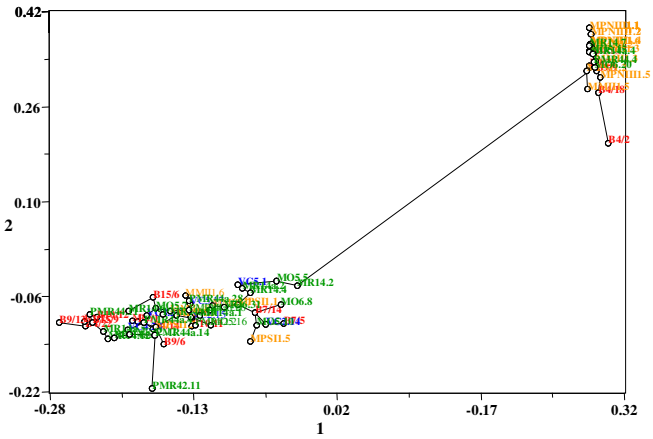

Figure 6 – Minimal connection tree overlapped with the projection of the trees on the plane of the first two main components.

Overlapping the projections of the cork's physical characteristics onto the projections of the trees basing on the cork's variables, it is shown that the first main component separates the trees by porosity, number of pores and density. These characteristics, which bear great influence on the cork quality, place to the left the trees that were classified as good quality cork producers. The second main component separates the trees based on their calibre, which is an equally important characteristic however with less influence on the cork quality.

Correlations between molecular analysis and morphological characteristics of cork

The comparison of the group including the good quality cork producers located on the left side of the first main component with the dendogram based on the molecular analysis shows that no there is correlations.

Conclusions

This study allowed some progress and clarification of some basic knowledge need of the genetic variability and its possible correlations with cork quality.

As a final conclusion, there is a large genetic diversity among the trees and populations, as it was demonstrated by the molecular and morphological analysis.

Eventually, due to the complexity of the involved factors, the detection of the differences that allowed differentiating cork oak trees and populations, as well as establishing correlations between the trees through their PAL electrophoretic profiles and nucleotide sequences of the encoding regions of the dirigent proteins with the cork quality was not possible. This means that the genomic regions analysed are not genetically correlated with the cork quality.

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