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Comparison between phenetic characterisation using RAPD and ISSR markers and phenotypic data of cultivated chestnut (*Castanea sativa* Mill.)

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Abstract

Patterns of phenotypic and phenetic variability in six Portuguese cultivars of chestnut (*Castanea sativa* Mill.) are evaluated. Morphological characterisation was based on the quantification of seventeen traits. Variance analysis showed significant differences among cultivars, and cultivar × year for all the traits studied, and trees within cultivars showed also some significant differences for some of the morphological variables. A significant correlation was obtained between length of the leaf blade and the percentage of unisexual and androgynic inflorescence with the effective thermal index, accumulated rainfall from April to October and from July to October, or the accumulated temperature below seven during the dormant period. Principal Component and cluster analysis were performed to group the cultivars, according to their similarity coefficients. For molecular characterisation, 125 RAPD and 157 ISSR polymorphic markers were amplified using 28 and 7 primers respectively. High level of congruence among the two marker systems (r = 90.5%) was obtained from comparison of phenetic similarities based on the percentage of shared fragments. ISSR markers revealed important advantages over RAPDs, due to a high effective multiplex ratio (12.5 for ISSR compared with 2.2 for RAPD analysis) and reproducibility. Although morphological and molecular results are comparable, slight differences are showed in cluster analysis UPGMA dendrograms. Molecular analysis explained homonym situations among 'Martainha' and 'Longal' cultivars in Portugal.

Introduction

In Europe there are two main areas of particular biological value for sweet chestnut (*Castanea sativa* Mill.) genetic resources, Turkey and Iberian Peninsula, each of them showing peculiar characteristics (Villani et al. 1999). These two areas, together with Italy, are the leading European chestnut producers and collectively account for 55.3% of the 240000 tons world's annual production. In Portugal, chestnut has a relevant place at the socioeconomic level, reaching an annual fruit production of 20000 tons (INE 1997).

The genotype identification is traditionally based on the observation of morphological characters whose expression is largely influenced by development, environmental and cultivation factors. Furthermore it is necessary to find morphological descriptors that are able to distinguish among different cultivars, and make observations in different years and regions in order to reduce the environmental interactions (for chestnut, see Pereira-Lorenzo et al. (1996a), Oraguzie et al. (1998)). Isozymes (Pereira-Lorenzo et al. 1996b; Pereira et al. 1999) and molecular markers as RAPDs (Fineschi et al. 1993; Galderisi et al. 1998; Santana et al. 1999) or SSRs (Botta et al. 1999) have been used to classify cultivars of chestnut. The choice of appropriate markers for different aspects of germplasm evaluation is of the most interest, and different

results (phenetic dendrograms) of each approach have been reported for several species, as the basis for detecting each of these markers is different (Rafalski et al. 1996).

In this work, we aimed to compare RAPDs, ISSRs and morphological data, in order to evaluate the suitability and congruence of these different markers for estimating overall phenetic similarity between a group of important Portuguese chestnut cultivars. RAPD (Welsh and McClelland 1990; Williams et al. 1990) relies on the amplification of the genome, using a single randomly chosen 10-mer primer, under low annealing temperatures. ISSR (Inter simple sequence repeats) is based on the amplification of inter repeat regions using microsatellite-anchored primers (Zietkiewicz et al. 1994). Both RAPD and ISSR are dominant markers, present low start up costs, especially if the ISSR amplification fragments are detected by silver staining, and automation is possible. Whereas RAPD analysis can present reproducibility problems, ISSR combines two important features, high reproducibility due to the use of longer primers, and high multiplex ratio, since microsatellites are ubiquitous and abundant in eukaryotic genomes.

In fruit crops, several reports have compared different molecular markers, as in *Citrus* (Fang et al. 1997), *Vitis*(Bowers and Meredith 1998), *Ribes*(Lanham and Brennan 1999) and *Pyrus*(Monte-Corvo et al. 2000). Few studies have, however, related morphological data and molecular markers for estimating diversity between genotypes and, although in chestnut, studies that aimed to quantify the level of correlation between genetic, morphometric or physiological data have been published (Villani et al. 1992; Pereira-Lorenzo et al. 1996a; 1996b), they were based on isozyme markers. Comparison between different molecular markers and morphological characters were not accessed.

Molecular technique comparisons are important because, depending on the question being addressed, one technique is more appropriate than another. Furthermore, different techniques are informative at different taxonomic levels. With the increasing development and generalised use of a large number of different molecular markers during the last years, the debate about adequacy of morphological as opposed to molecular data for the reconstruction of phylogenies arouse (Petersen and Seberg 1998). It is important to point out that in this work we aim to estimate indicators of overall phenetic similarity and this approach is inappropriate for phylogenetic analysis. Accurate identification of the cultivars and the assessment of intra and intercultivar variability are important in clonal selection programs. Since Portuguese cultivars are not well characterised and are classified according to their origin, clarifying synonymy and homonymy situations, is important for management of germplasm collections.

Materials and methods

Plant materials

The six accessions studied belong to a collection of ENFVN (Estação Nacional de Fruticultura Vieira Natividade), Alcobaça, Portugal (Table 1). 'Martainha1' and 'Martainha2' as well as 'Longal5' and 'Longal6' were purposely selected since they are suspected to be homonyms. For morphological characterisation, four trees of each cultivar were analysed during a five years (1993–1997) period, for the morphological characters listed in Table 2. For RAPD and ISSR analysis one tree per cultivar was selected for DNA extraction, since in a previous assay, no differences between RAPD and ISSR patterns of different trees of each cultivar were detected (unpublished data).

Genomic DNA extraction

DNA was extracted from fresh newly expanded leaves, using a hexadecyltrimethylammonium bromide (CTAB) protocol adapted from Doyle and Doyle (1990) as described in Santana et al. (1999).

RAPD assays

The reaction mixtures had a total volume of 25 μL. The mixture contained 1.0 unit of *Taq* DNA polymerase (Promega), 0.4 μM primer, 0.16 mM of each dNTP (Promega), 2.5 mM $MgCl_2$, 1 × reaction buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.1 mM

Table 1. List of *Castanea sativa* Mill. Portuguese cultivars and corresponding provenience, analysed for phenetic and morphological variation.

Accessions	Source	Accessions	Source
Amarelal	Calvelo	Martainha1	Tamanhos
Longal5	Carvalho	Martainha2	Soito
Longal6	Espinhoso	Verdeal	Casteição

Table 2. Morphological traits observed for the analysis of the cultivars of *Castanea sativa* Mill.

Trait symbol	Trait description
LLB	Length of the leaf blade (cm)
WLB	Width of the leaf blade (cm)
LPE	Length of the petiole (cm)
NVE	Number of veins
LIN	Length of inflorescence (cm)
PUI	Percentage of unisexual inflorescences
PAI	Percentage of androgynic inflorescences
NFI	Number of feminine inflorescences
PBI	Percentage of brachystaminate inflorescences
PMI	Percentage of mesostaminate inflorescences
PLI	Percentage of longistaminate inflorescences
NNK	Number of nuts per kilogram
PN30	Percentage of nuts with diameter <30 mm
PN30–35	Percentage of nuts with diameter 30 to 35 mm
PN35–40	Percentage of nuts with diameter 35 to 40 mm
PN40	Percentage of nuts with diameter >40 mm
PS	Percentage of sinus

EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween™ 20, 0.5% Nonidet™ P40), and 50 ng of template DNA. Reactions were performed in a UNO-Thermoblock thermal cycler (Biometra version 2.72, Göttingen, Germany), programmed as follows: 5 min at 94 °C for initial denaturation, 45 cycles of 5 s at 94 °C (denaturation), 30 s at 36 °C (annealing), and 1 min at 72 °C (extension). A final extension step at 72 °C for 5 min followed. A total of twenty-eight 10-mer primers of arbitrary sequence (Operon Technologies, Alameda Calif.) were selected for PCR amplification (OPA01, OPA02, OPA03, OPA04, OPA07, OPA09, OPA10, OPA13, OPA15, OPA18, OPC05, OPC06, OPC08, OPC12, OPE01, OPE03, OPE04, OPE06, OPE07, OPE08, OPE11, OPE12, OPE14, OPE15, OPE16, OPE18, OPE19, OPE20). The amplification products were visualised on 1.5% agarose gels stained with ethidium bromide, using standard methods (Sambrook et al. 1989). Three replications of each reaction for each selected primer were carried out and only reproducible bands were scored.

ISSR assays

Amplification reactions were carried out in volumes of 20 μL containing 30 ng template DNA, 1.0 unit *Taq* DNA polymerase (Pharmacia, Biotech), 0.25 mM each dNTP (Gibco BRL) and 1 μM primer (Gibco BRL), in 1 × reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris HCl pH 9.0). PCR reactions were performed under the following conditions: 4 min at 94 °C for initial denaturation, 27 cycles of 30 s at 94 °C (denaturation), 45 s at 52 °C (annealing) and 120 s at 72 °C (extension), followed by 7 min at 72 °C for final extension of the single strands. Thirteen primers were screened using a bulked DNA sample and seven primers that produced a complex band pattern in agarose gels were selected for final amplifications (Table 3). ISSR amplified fragments were mixed with equal volume of formamide dye (98% formamide, 10 mM EDTA, 0.05% xylene cyanol), loaded onto pre-warmed denaturing 6% polyacrylamide gels 7.5 M urea, and electrophoresed in 1XTBE buffer, at 50 W constant power until the dye front reached the end of the gels. The gels were silver stained as described in Bassam et al. (1991).

Data analysis

Morphological traits. Data analysis followed three steps: analysis of variance, multivariate analysis and regression analysis between morphological and climate variables.

The effect of interaction cultivar × year and tree within cultivar were determined according to the following model:

$$X_{i(m)j} = \mu + C_m + T_{i(m)} + Y_j + (CY)_{mj} + (TY/C)i(m)_j \quad (1)$$

were $X_{i(m)j}$ is the observation of the tree i (i = 1 to 4) within the cultivar m (m = 1 to 6) in the year j (j = 1 to 5); μ is the mean value of all observations; C_m, $T_{i(m)} + Y_j$, $(CY)_{mj}$ and $(TY/C)_{i(m)j}$ are the effects of the cultivar m, the tree i within cultivar m, the year j, interaction cultivar × year, and the error associated to the interaction tree within cultivar × year.

Cluster and Principal Component Analysis (PCA) were performed using the NTsys-pc ver.1.8 (Rohlf 1993) software. For both analyses, a correlation matrix was computed from the standardised data to determine the similarity among cultivars. For cluster

Table 3. List of selected ISSR primers used to characterise *Castanea sativa* Mill. cultivars.

ISSR Primer sequence (5′ − 3′)	
HVH (CA)7	(CA)8 R
HVH (TG)7	(GA)8 YG
VHV (GT)7	(AG)8 YT
DBD (AC)7	

B = C or G or T; D = A or G or T; H = A or C or T; R = A or G; V = A or C or G; Y = C or T.

Table 4. Climatic variables calculated for morphological characterisation, during the five-year period. RAO accumulated rainfall from April to November, RJO accumulated rainfall from July to October, IET accumulated temperature (°C) above seven degrees between budbreak and harvesting time, COA accumulated temperature (°C) below seven degrees during the dormant period.

Variable	1993	1994	1995	1996	1997
RAO	504.1	315.7	169.6	240.6	355.7
RJO	286.4	81.3	82.5	98.7	160.5
IET	68.8	71.6	81.2	63.2	82.1
COA	809.1	918.2	569.4	501.8	525.9

analysis, the UPGMA (unweighted pair-group method with arithmetic averages) was used and dendrograms were constructed. In PCA, Eigenvectors were calculated to determine the contribution of each variable for the separation of the cultivars. A Minimum Spanning Tree (MST) was also included.

Environmental variables were computed from the meteorological station nearby (7 km) for the period 1992–1997, in order to perform regression analysis between the morphological data and climate variables. These variables were the same as reported by Pereira-Lorenzo et al. (1996a), plus the accumulated temperature (°C) below seven degrees during the dormant period, between October of the previous year and April of the current year (COA). The effective thermal index (ETI) is defined as the accumulated temperature degrees (°C) above seven between budbreak and harvesting time. The accumulated rainfall from April to October (RAO) and from July to October (RJO) were the other variables computed (Table 4).

Molecular markers. The information provided by each marker system was determined according to Powell et al. (1996) indices: Effective Multiplex Ratio (number of polymorphic products from a single amplification reaction), Expected Heterozygosity ($H = 1 - \Sigma p_i^2$, where p_i is the allele frequency for the ith allele) and Marker Index (the product of Effective Multiplex Ratio and Expected Heterozygosity). Similarity values were estimated based on the fraction of bands common to each pair of cultivars, according to Nei and Li (1979) coefficient. Cluster analyses were performed to construct dendrograms, using the unweighted pair-group method with arithmetic averages (UPGMA) from the similarity data matrices. The Numerical Taxonomy and Multivariate Analysis System program package for personal computer (NTSYS-pc version 1.8; Rohlf (1993)) was used in statistical calculations.

Results

Morphological traits

The F values resulting from the conducted analysis of variance are displayed in Table 5. The results of interaction cultivar × year revealed significant differences for all the morphological characters studied except for percentage of nuts with diameter 30–35 mm (PN30-35). Significant differences among trees within cultivars were found only for length of the leaf blade (LLB), width of the leaf blade (WLB), length of the petiole (LPE), number of veins (NVE) and length of inflorescence (LIN).

Dendrograms based on the correlation dissimilarity coefficients from cluster analysis of the six cultivars, calculated for each of the five years separately, re-

Table 5. F values from the analysis of variance of the morphological traits studied. See table 2 for trait abbreviations.

Traits	Source of variation Cultivar × Year	Tree/cultivar
LLB	24.17^{***}	5.44^{***}
WLB	7.76^{***}	21.91^{***}
LPE	5.68^{***}	6.32^{***}
NVE	13.52^{***}	7.70^{***}
LIN	4.65^{***}	2.46^{**}
PUI	157.38^{***}	1.34^{ns}
PAI	8.45^{***}	1.44^{ns}
NFI	[a]	[a]
PBI	6.13^{***}	1.06^{ns}
PMI	10.10^{***}	1.17^{ns}
PLI	13.56^{***}	0.99^{ns}
NNK	7.48^{***}	1.20^{ns}
PN30	6.81^{***}	1.43^{ns}
PN30–35	1.45^{ns}	0.46^{ns}
PN35–40	6.86^{***}	0.27^{ns}
PN40	5.03^{***}	0.44^{ns}
PS	11.62^{***}	1.63^{ns}

n.s., *, **, *** Nonsignificant or significant at $P \leq 0.05$, 0.01 or 0.001 respectively. [a] Not calculated, due to inexistent variability among trees within cultivars.

vealed different phenotypic classifications (Figure 1). This aspect was particularly true for the years 1993 and 1994, reinforcing the importance of collecting morphological data during several years or in different regions.

Principal Component Analysis associated with the Minimum Spanning Tree, based on the five-year averages, provided complementary information to cluster analysis, as it allows a graphical presentation of the distribution of the cultivars in a three-dimensional plot (Figure 2). In this representation 'Martainha1', 'Martainha2' and 'Verdeal' are clearly separated from 'Longal5', 'Longal6' and 'Amarelal'. 'Longal5' and 'Amarelal' are the most similar cultivars.

The Eigenvalues indicate that three components provide a very good description of the data, as account for 80.7% of the standardised variance (Table

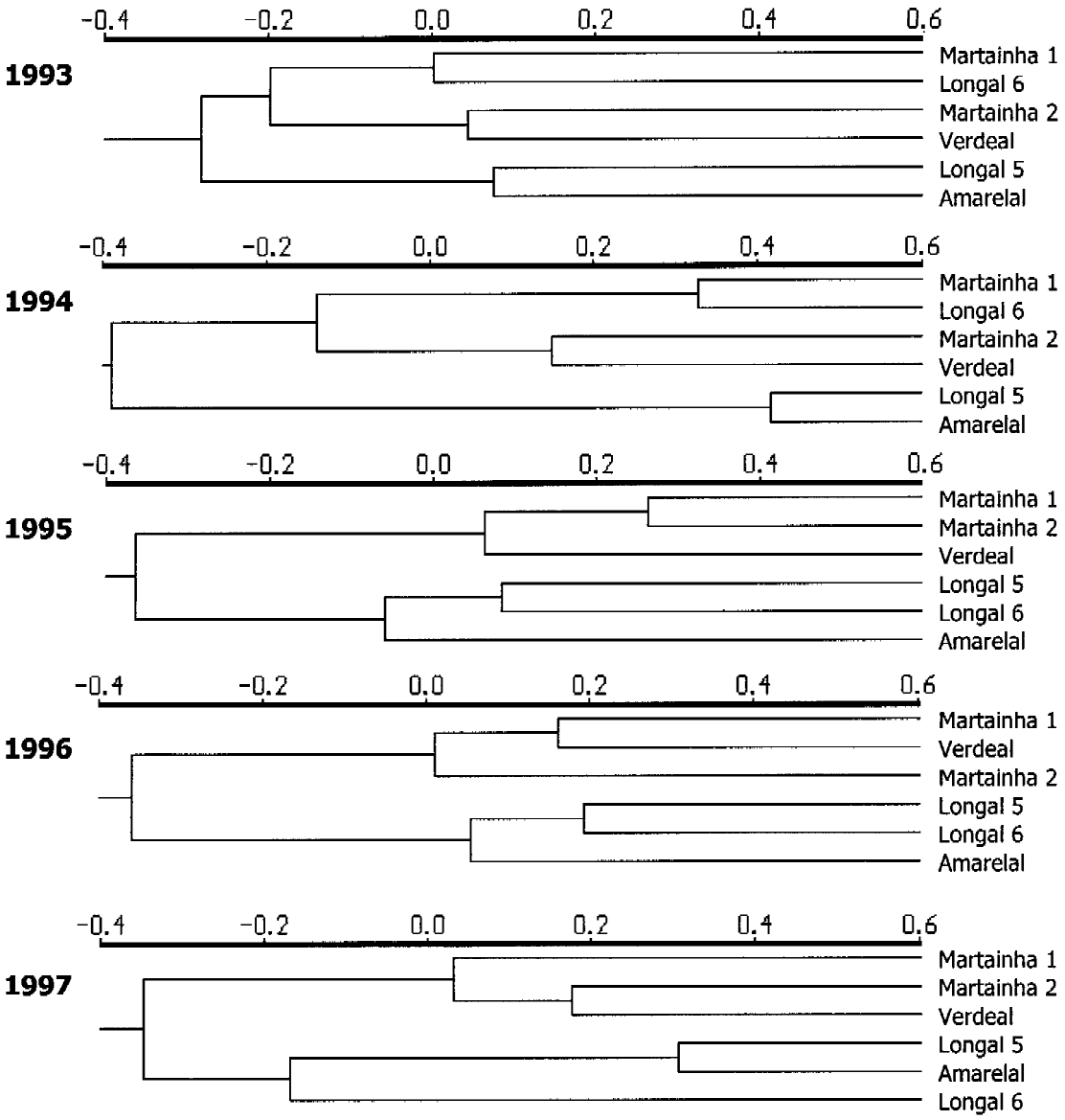

Figure 1. UPGMA dendrograms obtained by Cluster Analysis based on the morphological data collected by individual year.

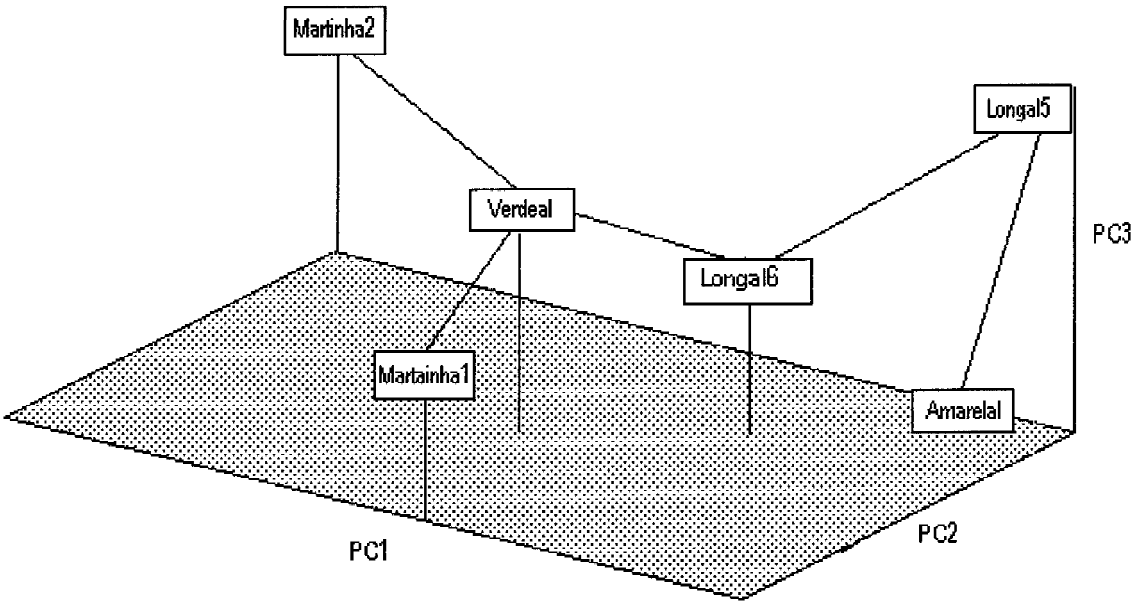

Figure 2. Projection of the cultivars and Minimum Spanning Trees on the first three principal components based on the five-year mean values of the morphological observations.

6). The analysis of Eigenvectors provides information about the traits responsible for the separations along the first three Principal Components. PC1 had 45.6% of the total variation. Percentage of nuts with diameter <30 cm (PN30) contributed positively to PC1. In contrast, number of veins in 10 cm (NVE), percentage of nuts with diameter 35 to 40 cm (PN35-40) contributed negatively. This Principal Component is responsible for the separation of 'Martainha2' from 'Longal5' and 'Amarelal'. PC2 exhibited 20.8% of the total morphological variability mainly caused by differences in the length of the leaf blade (LLB) and length of the petiole (LPE), and is responsible for the individualisation of 'Martainha1' apart from the other cultivars. PC3 had 14.2% of the total variation and was positively associated with the number of feminine inflorescences (NFI), whereas percentage of brachystaminate inflorescences (PBI) was negatively associated. PC3 is responsible for the separation of 'Longal5' from 'Amarelal'.

No significant correlations were found between morphological data and climatic variables except for length of the leaf blade, and percentage of unisexual and androgynic inflorescence (Table 7). Length of the leaf blade (LLB) was correlated ($p \leq 0.05$) with the two independent variables RAO/RJO, percentage of unisexual inflorescences (PUI) was highly correlated ($p \leq 0.001$) with IET and correlated ($p \leq 0.01$) with any combination of IET and other variables, while percentage of androgynic inflorescences (PAI) was correlated ($p \leq 0.01$) with COA and its combination with any of the other three variables.

Molecular markers

The twenty-eight selected primers yielded a total of 224 reproducible, well defined RAPDs, of which 125 (56%) were polymorphic. An average of 8.2 bands, ranging from 7 to 12, was observed for each primer.

A total of thirteen ISSR primers were tested. Primers (AT)8YC, (AG)8YC, (GA)8YC, (GT)8YC, (AGC)4YR and (TCC)5RY failed to amplify a sufficient number of fragments visible in agarose gels and were not used in the analysis. The seven primers used (Table 3) detected 157 polymorphic ISSR fragments, in a total of 291 bands resolved, accounting for 54% of polymorphisms. The average number of scored bands per primer was 41.6, ranging from 31 to 68, about 5-fold superior to RAPD analysis.

Both RAPD and ISSR fragments ranged from about 250 to 1750 bp in the scored region. Although a small percentage of RAPD markers were not reproducible among the three replication reactions, ISSR analysis revealed perfect reproducibility, even if performed by different operators, in different days and in different PCR reactions and electrophoresis.

Table 6. Correlation between original variables and the first three Principal Components used in the ordination of the *Castanea sativa* Mill. cultivars.

Variable	Principal Component 1	2	3
Length of the leaf blade (cm)	0.23877	0.85580	0.23036
Width of the leaf blade (cm)	−0.75034	0.43846	0.30965
Length of the petiole (cm)	0.48975	0.76980	−0.37485
Number of veins	−0.87611	−0.38563	−0.14602
Length of inflorescence	−0.79124	−0.38290	0.26104
Percentage of unisexual inflorescences	0.73067	−0.60951	0.15498
Percentage of androgynic inflorescences	−0.73067	0.60951	−0.15498
Number of feminine inflorescences	−0.08271	−0.15010	0.93017
Percentage of brachystaminate inflorescences	−0.40063	−0.34769	−0.79137
Percentage of mesostaminate inflorescences	0.79956	0.42575	0.06431
Percentage of longistaminate inflorescences	−0.50794	−0.16425	0.55926
Number of nuts per kilogram	0.68541	−0.55386	−0.18372
Percentage of nuts with diameter <30 mm	0.89815	−0.24186	0.13877
Percentage of nuts with diameter 30 to 35 mm	0.63864	0.34472	0.00571
Percentage of nuts with diameter 35 to 40 mm	−0.87599	−0.18866	−0.03376
Percentage of nuts with diameter >40 mm	−0.28913	0.06254	−0.48518
Percentage of sinus	0.78176	−0.55313	−0.10304
Eigenvalue	8.22	3.75	2.55
Percent variation	45.64	20.82	14.19
Cumulative percent variation	45.64	66.46	80.65

A summary of the effectiveness of the different markers is given in Table 8. ISSR markers proved to be more useful than RAPD markers, since it present a marker index of 8.12, while this indice is 1.54 for RAPDs. This aspect is due to the superior multiplex and effective multiplex ratios provided by ISSR analysis, since the calculated percentage of polymorphic bands and Expected heterozygosity were similar for both marker systems.

Similarity coefficients calculated according to Nei and Li (1979) coefficient revealed high mean similarities among cultivars of 82% and 83% for RAPD and ISSR analysis, respectively. The phenetic classification obtained using UPGMA as a clustering method is represented in the dendrograms of Figure 3. High congruence was obtained among the two markers. The similarity matrices calculated for RAPD and ISSR data were compared and a significant correlation (r = 90.5%) was obtained. In both dendrograms the cultivars are grouped in two clusters: The first cluster is composed of 'Amarelal' and 'Longal5', while in the second cluster, 'Longal6' and 'Martainha2' clustered together, as well as 'Verdeal' and 'Martainha1'. These two cultivars showed very high similarity in both analysis. Supposed homonym cultivars appeared clearly separated.

Table 8. Analysis of banding patterns generated by RAPD and ISSR assays for the six *Castanea sativa* Mill. cultivars.

	RAPD	ISSR
Number of scored bands	224	291
Number of polymorphic bands	125(56%)	157(54%)
Number of scored bands per primer	8.2 ± 1.9	41.6 ± 13.8
Multiplex ratio	5.8	31.6
Effective multiplex ratio	2.2	12.5
Expected heterozygosity	0.70	0.65
Marker index	1.54	8.12

Table 7. F-values for the morphological traits studied that showed correlation with climatic variables. RAO accumulated rainfall from April to November, RJO accumulated rainfall from July to October, IET accumulated temperature (°C) above seven degrees between budbreak and harvesting time, COA accumulated temperature (°C) below seven degrees during the dormant period. LLB length of the leaf blade (cm), PUI percentage of unisexual inflorescences, PAI percentage of androgynic inflorescences.

Trait	RAO	RJO	IET	COA	RAO/RJO	RJO/IET	RAO/IET	IET/ROA	RAO/ROA	RJO/ROA
LLB	0.07^{ns}	0.62^{ns}	0.33^{ns}	1.27^{ns}	3.04^{*}	0.54^{ns}	0.17^{ns}	0.90^{ns}	0.81^{ns}	1.07^{ns}
PUI	0.09^{ns}	0.04^{ns}	7.03^{***}	1.11^{ns}	0.06^{ns}	3.66^{**}	3.89^{**}	3.58^{**}	1.03^{ns}	0.66^{ns}
PAI	1.21^{ns}	1.05^{ns}	0.91^{ns}	4.16^{**}	0.60^{ns}	0.86^{ns}	0.88^{ns}	3.32^{**}	5.11^{***}	3.22^{**}

ns, *, **, *** Nonsignificant or significant at P ≤ 0.05, 0.01 or 0.001, respectively.

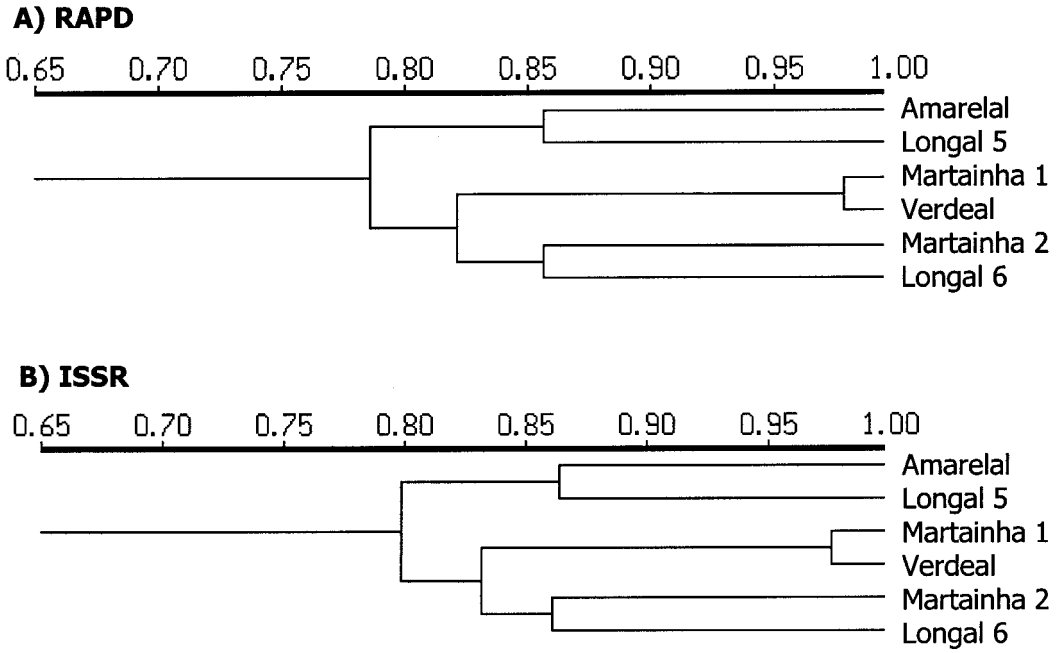

Figure 3. UPGMA dendrogram obtained by Cluster Analysis based on A) RAPD and B) ISSR amplified fragments, for the six cultivars studied.

Discussion

The results of this work point out the advantages of molecular markers over morphological data for diversity studies among European chestnut. Since RAPD and ISSR markers assess differences among the cultivars at the DNA level, no interaction with the environment is expected. On the contrary, morphological traits were affected by the particular conditions of each individual year. Considerable differences were obtained between cluster analysis performed with data from the years 1993 and 1994 and the remaining ones. This is not fully unexpected, since these years had particular climatic conditions, especially with respect to COA. The year 1993 showed also significantly higher rainfall, compared to the other years (Table 4). Correlation of individual morphological traits with climate variables showed significant correlation of variables related with temperatures (IET and COA) with the percentage of unisexual and androgynic inflorescences, respectively. The effect of temperature in flower physiology and sex expression is well known for many species (Sedgley and Griffin 1989). This correlation was also observed by Pereira-Lorenzo et al. (1996a). The length of the leaf blade was correlated with RAO/RJO. These correlations may have been responsible for the differences in the phenotypic classification observed, since when this three variables were removed from the analysis, a more congruent classification was obtained among the different years (data not shown). These aspect strengths the need to include only morphological characters strongly genetically determined in the descriptor lists for germplasm classification and evaluation.

The existence of significant differences among trees within cultivars was reported for some morphological traits and isozyme systems (Pereira-Lorenzo et al. 1996a; 1996b). Although morphological and isozyme characterisation are environmental-dependent, according to these authors, the genetic effects are expected to be more important than the environmental effects, so this suggests the existence of genetic variability within cultivars. In our study, however, although significant differences among trees were also observed for some traits, the intercultivar differences were higher than intracultivar variation, and the results of molecular characterisation provided indication that intracultivar variability should be minimal.

Both morphological and molecular data proved that the similarities observed among supposed homonym

cultivars, 'Martainha1' and 'Martainha2', as well as 'Longal5' and 'Longal6', are sufficiently low to be considered the same cultivar.

High congruence was obtained among phenetic classification using RAPD and ISSR data. This can be explain by the similar way that this two marker systems detect polymorphisms, since both are dominant markers, the primers anneal arbitrarily and the fragments detected present about the same size. ISSR markers proved, however, to be more useful, due to the high effective multiplex ratio and reproducibility. Even using polyacrylamide gels to resolve the ISSR amplification products, this technique can be less time and money consuming due to these two features. Yang et al. (1996) found lower relative costs for ISSR, compared to RAPD, since RAPD present extra costs due to the relatively low frequency of reproducible polymorphisms.

Despite the disadvantages of morphological classification, a significant correlation was obtained among similarity matrices of RAPD and ISSR with binarized morphological data, without the three variables correlated with climate variables (r = 71.8% and 71.3%, respectively). This aspect reveal that, for the cultivars employed in this study, morphological classification is comparable to molecular classification, if environmental-dependent variables were excluded from the descriptor lists. In many situations, the significance of the derived variables (principal components) may be useful in giving a general and biological description of the main traits of the cultivars with efficiency of discrimination greater than would be in the case if only non-mapped molecular markers were used.

In this study, the main difference among phenotypic and phenetic dendrograms, was about 'Longal6'. This cultivar was, with morphological characters, clustered together with 'Longal5' and 'Amarelal'. This is not fully unexpected, since both cultivars are named 'Longal', in different regions of Portugal, according to a morphological classification. This aspect points out other disadvantage of morphological data, since it only reveals information about a limited part of the genome.

In conclusion, characterisation based on molecular markers is faster, less expensive and more reliable that the one based on morphological characters. However, morphological classification can be useful in cases when it is necessary to obtain an agronomic description of the germplasm used. In these cases, only variables with a strong genetic control should be quantified. Mapped molecular markers closely associated to these characteristics (both single traits and Quantitative Trait Loci) can provide, however, the best solution for characterisation studies. Therefore, the development of mapping programs for all species, including chestnut, for which this information is still not available, is necessary.

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