ONLINE RESOURCES

Evaluation of genetic diversity of Portuguese *Pinus sylvestris* L. populations based on molecular data and inferences about the future use of this germplasm

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Introduction

Pinus sylvestris (Scots pine) is one of the most abundant tree species on the globe (Matías and Jump 2012). Palaeontological data show that the largest refuges of Scots pine are located in the Balkans, the Alps and the Iberian peninsula (Bennett *et al.* 1991). Labra *et al.* (2006) pointed out that the postglacial expansion occurred from remaining local populations or, as also stated by Soranzo *et al.* (2000), through the northward expansion of southern refuge populations, following withdrawal of the ice sheets. The actual distribution of *P. sylvestris* extends from western Scotland to the Okhotsk Sea in eastern Siberia, and from beyond the subarctic forests of northern Scandinavia to arid, mountainous southern areas of Spain (Labra *et al.* 2006; Pyhäjärvi *et al.* 2008; Scalfi *et al.* 2009), with Portugal being the westernmost limit of its distribution. The Holocene postglacial history of *P. sylvestris* resulted in many possible refuges and colonization routes throughout Europe, which strongly influenced its genetic diversity and local adaptation (Matías and Jump 2012). Over recent decades, increased reproduction and growth have been detected at the northern limit of *P. sylvestris* as a response to increased temperature, whereas at its southern limit increased drought stress has resulted in decreased growth and, in some cases, massive mortality. According to Garzón *et al.* (2011), the contribution of local adaptation and plasticity of populations could be crucial for the persistence of species under the global warming scenario. Direct climatic effects on the species are acting together with indirect effects due to altered biotic interactions, including outbreaks of insects, pathogens and parasites, and increase of herbivore populations linked to declining ecosystem productivity. Predictive studies indicate a gradual decline of *P. sylvestris* at the southern range limit and expansion to higher latitudes (Matías and Jump 2012).

Genetic diversity constitutes the fundamental basis for evolution, adaptation, and ability to adapt to adverse environmental conditions (Laurentin 2009). Studies of genetic diversity, therefore, could also provide information about breeding strategies and use of forest germplasm, as well as definition of genetic relationships (Laurentin 2009). Under the scenario of global climatic changes that are negatively affecting the distribution of Scots pine, there is urgency of evaluation of the genetic diversity, structure and genetic relationships of populations with high adaptability potential, such as the Portuguese ones. Portugal has five representative areas of distribution that have never been characterized at the molecular-genetic level. Additionally, there is no information about their provenances. Thus, with this study, we intend to evaluate the genetic diversity, structure and relationships of 149 Portuguese Scots pine individuals from five representative plantation areas and 40 foreign Scots pine individuals from Germany, north and south of Spain, and Sweden, with intersimple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) markers. We also aim to infer probable provenances of the Portuguese populations, and future distribution of Scots pine in Portugal and worldwide in light of global climatic changes.

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Materials and methods

Plant material and genomic DNA extraction

Young needles were collected from 149 Scots pine individuals in five Portuguese representative areas of distribution (see figure 1), and 40 *P. sylvestris* individuals from Germany, north and south Spain (Puebla de Lillo and Montes Universales, respectively), and Sweden (table 1), and used for genomic DNA extraction following a CTAB-based protocol (Doyle and Doyle 1987).

Table 1. Details of Scots pine individuals used in the study.

Sampled area	Area code	Individuals
Tras-os-Montes (north of Portugal)	TM	40
Peneda (north of Portugal)	P	30
Pedra Bela (north of Portugal)	PB	27
Vinhais (north of Portugal)	V	25
Serra da Estrela (centre of Portugal)	SE	27
Puebla de Lillo (north of Spain)	PL	10
Montes Universales (south of Spain)	MU	10
Sweden	SW	10
Germany	GM	10
Total		189

ISSR and RAPD amplifications

We used 25 ng/μL of genomic DNA for ISSR and RAPD amplifications. Twenty SSR primers from the set 9/100 (University of British Columbia, Vancouver, Canada), previously used by our research group in other *Pinus* sp., were tested in this study for usefulness in revealing ISSR amplifications. The ISSR reaction mixture, amplification and electrophoresis conditions were performed as described by Carvalho *et al.* (2005).

A total of 43 oligonucleotides from the sets A (OPA), H (OPH) and C (OPC) from Operon Technologies (Alameda, USA) were tested here. The RAPD amplification mixture, conditions and electrophoresis followed the Lima-Brito *et al.* (2006) protocol.

For both dominant marker systems, each amplification product (band) was considered an RAPD or ISSR marker. Reactions were repeated twice and only reproducible bands were considered for the presence (1) / absence (0) analysis and for the construction of binary matrices. ISSR or RAPD bands with the same molecular weight were considered as the same marker.

Figure 1. Portugal has five representative plantation areas of *Pinus sylvestris* and we collected plant material in each one (indicated by the tree symbol).

Statistical analysis

We combined the ISSR and RAPD binary matrices for construction of the genetic similarity UPGMA (unweighted pair group method with arithmetic means) dendrogram and used the NTSYS pc ver. 2.02 software (Rohlf 1998) and the simple matching (SM) coefficient. The dendrogram groups were defined based on a cutoff line value. The goodness of fit of the UPGMA clustering to the data matrix was calculated by using COPH and MXCOMP programs after running the sequential agglomerative hierarchical nested (SAHN) cluster analysis module from the NTSYS software.

To evaluate the genetic structure of the plant material studied here and also to confirm the UPGMA clustering performed by NTSYS, we used STRUCTURE 2.3 software (Falush *et al.* 2007) with the 'no admixture' parameter (suitable for dominant markers), using 50,000 generations of burn-in period followed by 100,000 Markov chain Monte Carlo (MCMC) iterations, and different values of K (number of populations). A principal coordinates analysis (PCA) was also performed using the software GenALEX 6 (Peakall and Smouse 2006).

The ISSR and RAPD data were also analysed with the software POPGENE 1.32 (Yeh *et al.* 1999) which enabled us to calculate the following parameters: Shannon's information index (I), which measures gene diversity (Shannon and Weaver 1949); Nei's gene diversity index ($h = 1 - \sum p_i^2$, where p_i is the frequency of the ith allele at the locus; Nei 1973); total genetic diversity (H_T); genetic diversity within populations (H_S); relative magnitude of differentiation among populations (G_{ST}) (Nei 1987); and the interpopulation genetic diversity ($D_{ST} = H_T - H_S$).

Table 2. Primers and respective sequences.

Primer	Sequence	T	P	%*P*
ISSRs				
817	$(CA)_8A$	36	36	100
827	$(AC)_8G$	34	34	100
834	$(AG)_8YT$*	43	43	100
835	$(AG)_8YC$*	34	34	100
836	$(AG)_8YA$*	36	36	100
841	$(GA)_8YC$*	38	38	100
850	$(GT)_8YC$*	32	32	100
Total		253	253	100
RAPDs				
OPA-9	GGGTAACGCC	48	48	100
OPA-10	GTGATCGCAG	44	44	100
OPA-15	TTCCGAACCC	37	37	100
OPA-17	GACCGCTTGT	41	40	97.56
OPA-19	CAAACGTCGG	68	68	100
OPH-4	GGAAGTCGCC	63	63	100
OPH-7	CTGCATCGTG	63	63	100
OPH-19	CTGACCAGCC	51	51	100
Total		415	414	99.76

T, total bands; P, polymorphic bands; %*P*, percentage of polymorphism.* Y = C or T.

Results and discussion

Percentage of ISSR and RAPD polymorphism

Among the 20 primers tested, only seven produced amplification of polymorphic ISSR patterns (table 2). In the case of RAPD, eight presented amplification and/or polymorphism out of 43 primers tested (table 2).

All ISSR and RAPD primers except OPA-17 showed 100% polymorphism among the 189 Scots pine individuals studied (table 2). Primer OPA-17 revealed a monomorphic RAPD marker of 1000 bp (present in all samples from all locations), which could be considered specific for this species. Some of the SSR primers used in this study were previously used for ISSR amplifications in Scots pine by Li *et al.* (2005) and Labra *et al.* (2006). Nonetheless, we achieved higher number of amplified ISSR bands, higher number of amplified bands per primer, and higher total mean percentage of ISSR polymorphism, in particular, with primers UBC827, UBC834, UBC835 and UBC836 compared to the results reported by Li *et al.* (2005) and Labra

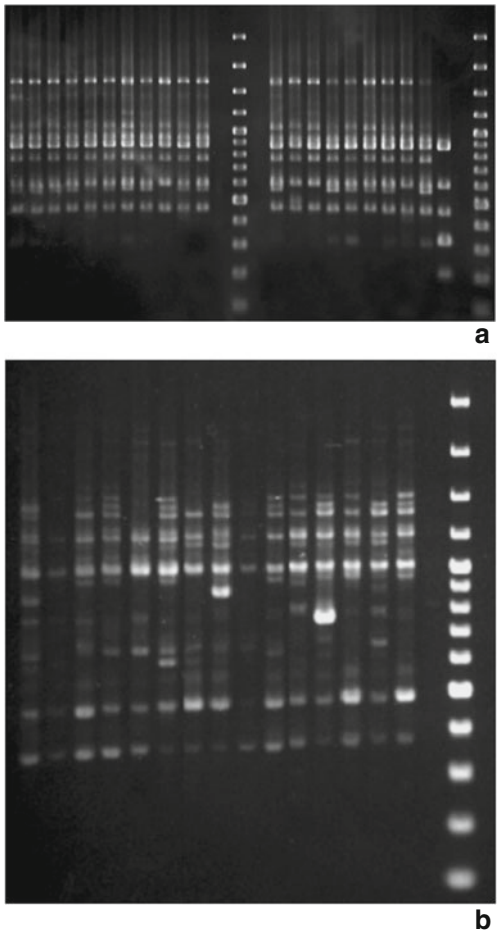

Figure 2. Intrapopulation polymorphism revealed by (a) ISSR markers produced with primer 841 in 21 individuals from Tras-os-Montes population, and (b) RAPD markers produced with primer OPA-10 in 15 individuals from Tras-os-Montes population.

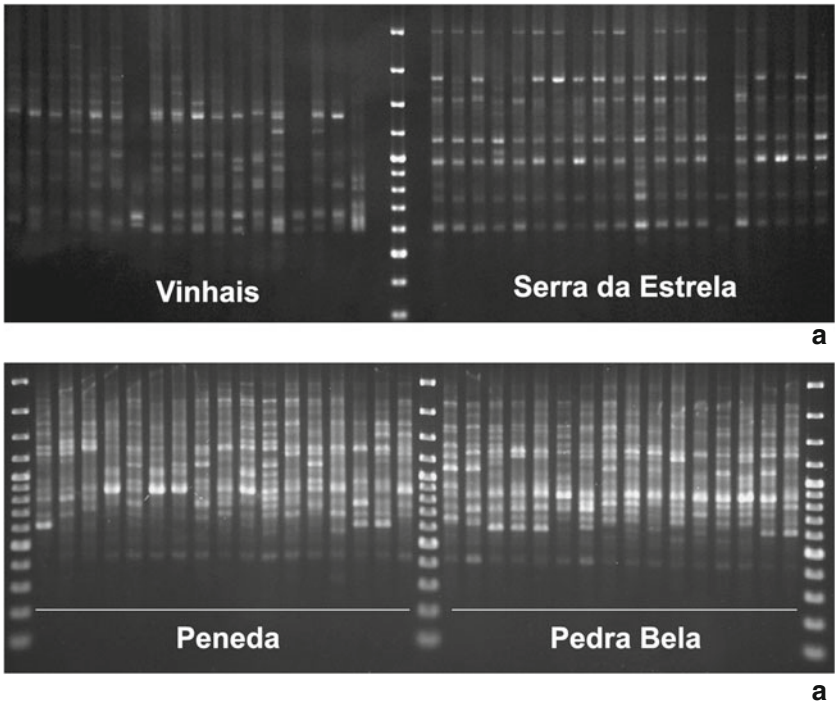

Figure 3. Interpopulation polymorphism revealed by (a) ISSR markers produced with primer 827 in individuals from Vinhais and Serra da Estrela populations, and (b) RAPD markers produced with primer OPA-10 in individuals from Peneda and Pedra Bela populations.

et al. (2006). These differences could arise from the fact that we are studying different genotypes from other locations but could also be due to a higher degree of genetic diversity of the samples studied here. The seven and eight ISSR and RAPD primers, respectively, showed polymorphism among individuals from the same population (intrapopulation polymorphism) (figure 2).

We also detected polymorphism among different populations/locations with the same primer for both marker systems (figure 3).

Genetic variation and diversity analyses

To statistically determine the degree of genetic variation and gene diversity within and among the nine populations of Scots pine under study, we analysed the molecular data using POPGENE 3.2 software. Unfortunately, due to the high number of amplified loci and limitation of the software, it was not possible to analyse the combination of ISSR and RAPD data. We therefore performed a separate analysis for each marker system. The summaries of the analyses performed for ISSR and RAPD loci are presented in table 3.

The genetic diversity of each population was highlighted by the estimates of of Shannon index (I) and Nei index (h) based on the ISSR markers, which were higher than those based on RAPDs (table 3). Further, the Shannon and Nei indexes estimated in this study are higher than those reported by Li *et al.* (2005) for other populations of *P. sylvestris*.

Table 4 presents the summaries of Nei's analysis of gene diversity of the nine populations under study based on the pool of the ISSR and RAPD data.

Despite the higher values of total (H_T) and intrapopulation (H_S) genetic diversity estimated using ISSRs, the RAPD markers allowed estimation of a significant value, namely 48.90%, of differentiation among populations (G_{ST}) which measures the proportion of gene diversity that is distributed among populations (table 4). RAPDs also evidenced a D_{ST} value of 10.55% which represents the degree of interpopulation genetic diversity (table 4). Although both types of markers showed high levels of polymorphism (table 2), the results of the genetic diversity analysis suggest that RAPDs may be more suitable for genetic diversity studies and estimation of differentiation among populations in this pine species, given that RAPD produced a higher number of bands than ISSRs. On the other hand, for studies of intrapopulation genetic variation in Scots pine, the ISSR markers are recommended. Tikhonova (2009) reported that the study of intrapopulation polymorphism provides much valuable information in terms of understanding mechanisms of population adaptation and species temporal and spatial self-maintenance. Use of multivariate analysis in intrapopulation polymorphism investigations allows consideration of different aspects of Scots pine, such as tree form diversity and forest stand condition. According to Tikhonova (2009) the strategy to conserve diversity of Scots pine from natural stands and plantations

Table 3. Summaries of genetic variation statistic analyses performed for ISSR and RAPD loci amplified in the nine Scots pine populations, being:

Population	Mean ± standard deviation	
	I	*h*
ISSRs		
Tras-os-Montes	0.681±0.018	0.488±0.018
Peneda	0.676±0.021	0.483±0.021
Pedra Bela	0.676±0.025	0.483±0.024
Vinhais	0.672±0.034	0.480±0.033
Serra da Estrela	0.674±0.026	0.481±0.025
Germany	0.636±0.087	0.447±0.075
Puebla de Lillo	0.642±0.074	0.452±0.067
Montes Universales	0.640±0.073	0.450±0.065
Sweden	0.637±0.079	0.447±0.068
Mean (189 individuals)	0.6907±0.004	0.4975±0.004
RAPDs		
Tras-os-Montes	0.228±0.271	0.150±0.187
Peneda	0.150±0.252	0.100±0.174
Pedra Bela	0.155±0.259	0.105±0.180
Vinhais	0.153±0.251	0.102±0.172
Serra da Estrela	0.147±0.249	0.098±0.171
Germany	0.172±0.267	0.116±0.186
Puebla de Lillo	0.178±0.268	0.120±0.185
Montes Universales	0.151±0.253	0.101±0.175
Sweden	0.150±0.259	0.102±0.180
Mean (189 individuals)	0.351±0.198	0.216±0.148

I, Shannon's information index; *h*, Nei's gene diversity index (Nei 1973).

should focus on big populations or groups proceeding from northwest to southeast (i.e. along the vector of changes of hydrothermal conditions), and special emphasis should be directed to highly diverse populations that constitute ecological niches, such as those found in refuges (Tikhonova 2009).

The high level of intrapopulation and interpopulation genetic diversity of the nine Scots pine populations under study are clearly reflected in the UPGMA clustering analysis (figure 4).

Genetic relationships and clustering analysis

To estimate the genetic relationships among the Portuguese populations, and Portuguese and foreign Scots pine individuals, we combined the binary matrices of polymorphic bands for both ISSRs and RAPDs and constructed an UPGMA dendrogram of genetic similarity using the simple matching coefficient and SAHN algorithm (figure 4).

The simple matching coefficient ranged from 0.69 to 0.94, corresponding to 25% genetic similarity among the 189 *P. sylvestris* individuals (figure 4). This low value of genetic similarity could be explained by the fact that we are studying Scots pine from different plantations with high intrapopulation and interpopulation genetic diversity, as previously demonstrated (tables 3 and 4). In the UPGMA dendrogram, we can consider two main groups. The first main group encloses four subgroups, I to IV, corresponding to Portuguese Scots pine individuals from Peneda, Vinhais, Serra da Estrela and Pedra Bela populations. The second main group includes the remaining five subgroups, V to IX, namely the Portuguese population of Tras-os-Montes and the four foreign populations (figure 4). Nonetheless, if we consider the middle cut-off value of 0.82 in the dendrogram, we find nine subgroups that correspond exactly to the number of populations/locations under study (figure 4). In addition, the high cophenetic correlation coefficient ($r = 0.9480$) calculated for this UPGMA tree indicates that it is a good representation of our molecular data. This dendrogram allows us to suggest that both marker systems have enough specificity to discriminate Scots pine individuals per population despite their dominant and arbitrary nature. A previous study had already demonstrated the reliability of these markers in assessment of genetic diversity and relationships among *Pinus* sp. (Gad and Mohamed 2012).

As mentioned, other goal of this work was to find the probable provenances of the Portuguese populations of Scots pine based on the ISSR and RAPD data. This goal was partially accomplished by the evidence of a close relationship among the German samples and those from the Portuguese population of Tras-os-Montes (see figures 4 and 5). Nonetheless, studies involving Scots pine individuals from other countries should be performed to find probable provenances of the populations in the remaining Portuguese plantation areas. PCA also revealed high cumulative percentage of total genetic variation (83.5%) explained only by the first three axes.

Genetic structure

Wright's *F* statistic analyses the structure of subdivided populations. One of the *F* statistic indexes is F_{ST} or

Table 4. Summaries of Nei's analysis of gene diversity performed for ISSR and RAPD loci amplified in the nine Scots pine populations.

ISSRs	H_T	H_S	G_{ST}	D_{ST} ($H_T - H_S$)
	0.4965±0.00001	0.4678±0.0003	0.0578	0.0287
RAPDs	H_T	H_S	G_{ST}	D_{ST} ($H_T - H_S$)
	0.2158±0.0238	0.1103±0.0090	0.4890	0.1055

H_T, total genetic diversity; H_S, intrapopulation genetic diversity; G_{ST}, relative magnitude of differentiation among populations; D_{ST}, interpopulation genetic diversity.

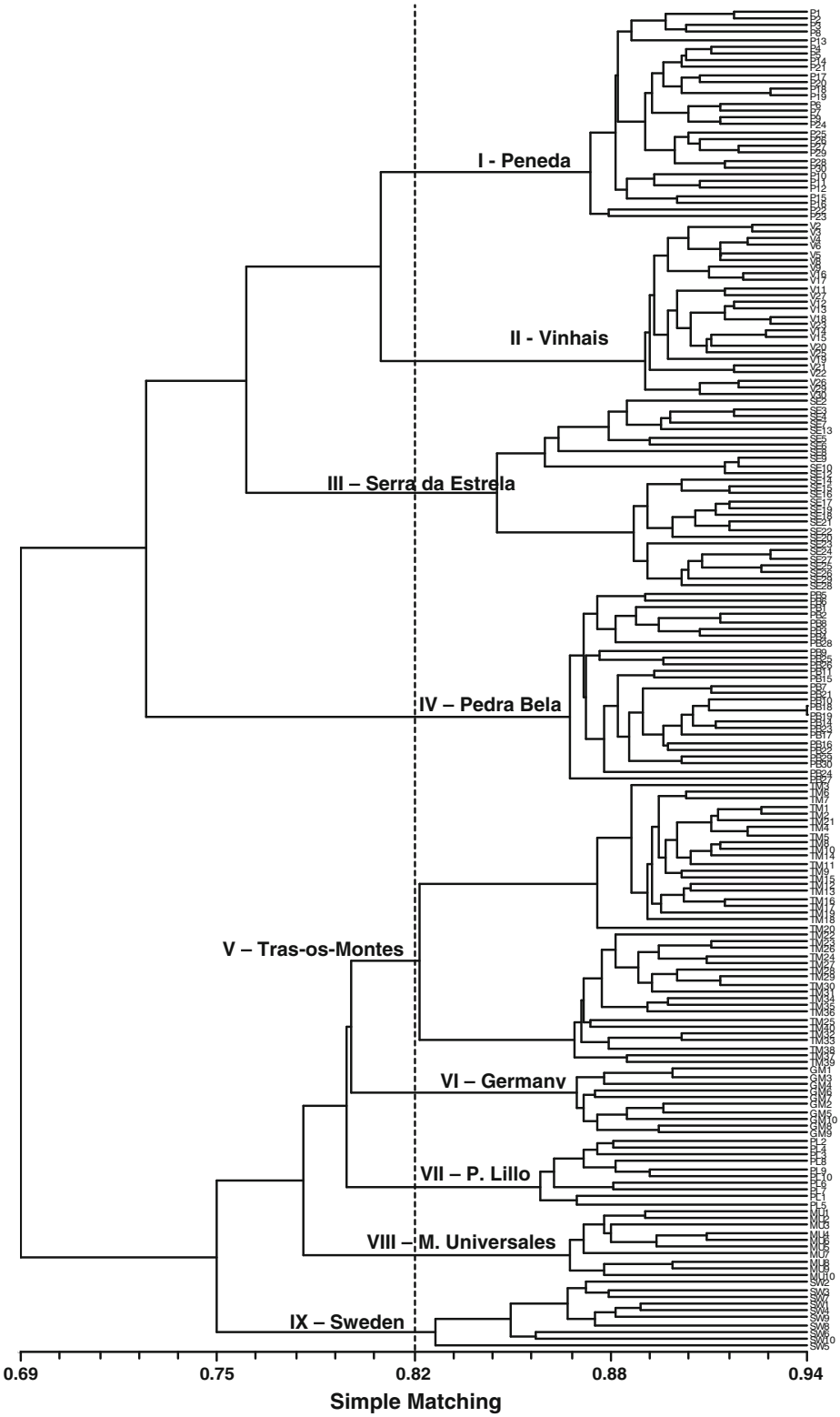

Figure 4. UPGMA dendrogram of genetic similarity among 189 *P. sylvestris* individuals, belonging to nine populations, based on the pool of the ISSR and RAPD data. The cophenetic correlation coefficient (*r*) for this matrix was 0.9480.

fixation index, which estimates the difference between observed and expected heterozygosity under conditions of Hardy–Weinberg equilibrium (HWE). Values for F_{ST} range from 0 (non-differentiation or no genetic divergence) to 1 (complete differentiation between the original group and their subgroups or fixation for alternative alleles in different

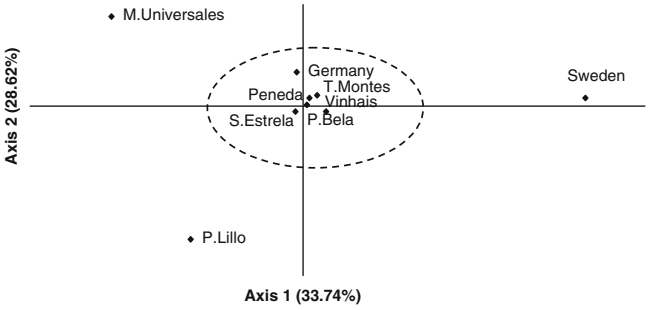

Figure 5. Principal coordinate (or component) analysis where the first three axes explained a cumulative 83.5% of total variation. The PCA grouping revealed the closest relationship of the German samples with the Portuguese ones, in particular those from Tras-os-Montes, and three separate groups composed by the three foreign-origin Scots pine samples.

subpopulations) (Laurentin 2009). In other words, it estimates the degree of gene differentiation among populations in terms of allele frequencies, and was determined as: $F_{ST} = 1 - (H_S/H_T)$ (Laurentin 2009). Thus, for the pool of the molecular data and for several runs of K (number of populations) ranging from 1 to 9, the structure analysis performed using STRUCTURE 2.3 software resolved $K = 5$ highly differentiated genetic clusters (table 5).

All the mean F_{ST} values attributed to each group were higher than 0.25, indicating significant genetic differentiation among populations (strong genetic structure). This feature could be explained by local adaptation (Savolainen *et al.* 2007). Therefore, five highly differentiated groups among the 189 Scots pine individuals were deduced. At first glance, we could consider that these groups correspond to the four foreign populations and one group enclosing all the Portuguese ones. Nonetheless, the five genetic clusters suggested by STRUCTURE are those that could be differentiated if we consider a cut-off value of 0.75 in the UPGMA dendrogram (figure 4), namely one group constituted by the Tras-os-Montes population and four foreign populations and the remaining four groups of Portuguese populations. STRUCTURE analysis provided an estimation of five subgroups being highly discriminative about the genetic structure of the germplasm under study. Since it was not possible to use POPGENE to analyse the combination of ISSR and RAPD data, we used STRUCTURE to deduce the genetic structure based on the pool of molecular data. Thus, considering the STRUCTURE analysis, which is more reliable because it is based on a higher number of markers (ISSRs and RAPDs), we might say that the population of Tras-os-Montes should be considered apart from the remaining Portuguese ones, and these results should be taken into account in future strategies of genetic resources conservation and use of germplasm for afforestation. On the other hand, all the Portuguese populations of Scots pine showed high interpopulation and intrapopulation genetic diversity, which reinforces the importance of developing efforts for their conservation.

Based on our research, we are confident that the use of this highly genetically diverse germplasm, with high adaptation potential and plasticity, can be used for afforestation in other countries, particularly in localities with environmental factors that constrain Scots pine survival.

Table 5. Mean F_{ST} values per group achieved with $K = 5$ using STRUCTURE 2.3 software based on the pool of ISSR and RAPD data.

Group	Mean F_{ST} for $K = 5$
I	$F_{ST}\_1 = 0.6585$
II	$F_{ST}\_2 = 0.5467$
III	$F_{ST}\_3 = 0.6357$
IV	$F_{ST}\_4 = 0.6101$
V	$F_{ST}\_5 = 0.4424$

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