FPS COST Action FP1202

Strengthening conservation:
a key issue for adaptation of marginal/peripheral populations
of forest trees to climate change in Europe (MaP-FGR)

SHORT TERM SCIENTIFIC MISSION REPORT

Assessment of genetic diversity of the Portuguese Pinus nigra Arn. Populations

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STSM Start at 01/09/2015 and End at 30/09/2015

Abstract

European Black Pine (*Pinus nigra* Arnold) belongs to the Family Pinaceae and to the Mediterranean pines group. This study constitutes the molecular characterization of six allochthonous Portuguese *P. nigra* populations using microsatellites (SSR) markers. These populations were installed 50 to 90 years ago and their provenances are unknown. The pool of the SSR data revealed 95% of genetic similarity among the *P. nigra* individuals that were structured into two differentiated populations with higher polymorphism within rather than among populations. According to the estimated genetic relationships, it seems to belong to two regional varieties of the same subspecies, supported by previous studies in the same populations.

List of contents

Pinus nigra is one of the three oldest pine species widely spread through the Iberian landscapes (García Antón et al. 1995; Carrión and Geel 1999; Rubiales et al. 2010). This species occupies about 1.4 million hectares in the Central and Southern Iberian Mountains and presents specimens with over 650 years old (Génova and Fernández 1998; Martín-Benito et al. 2008; Rubiales et al. 2010). *P. nigra* has an essential role in ecology,

preservation of ecosystems, soil protection, as well as high economic importance for wood production (Espelta et al. 2003; Martín-Benito et al. 2008, Lucas-Borja et al. 2012).

European *P. nigra* populations experienced different glacial and interglacial episodes of climatic changes and geological events (Thompson 2005) that resulted on a fragmented distribution (Isajev*et al*. 2004). Due to these features, these populations exhibit high variation at morphological, physiological, biochemical, genetic and ecological levels (Scaltsoyianne*set al.* 1994; Raffi *et al.* 1996; Bogunić *et al.* 2003; Afzal-Raffi and Dodd 2007; del Cerro Barja *et al.* 2009; Rubio-Moraga *et al*. 2012). Such variation among allopatric populations hampered their identification and resulted on taxonomic classification issues. Despite being considered a collective species (Villar 1947; Svoboda 1953; Fukarek 1958; Gaussen 1960; Debazac 1964; Pajares and Escudero 1989; Blanco 1998; Richardson 1998; Arbez and Millier 1971), other authors have proposed the botanic division of *P. nigra* species into allopatric subspecies and varieties (Delevoy 1949; Mirov 1967; Vidaković 1974; Barbéro et al. 1998; Greuter et al. 1984; Gaussen et al. 1964; 1993; Christensen 1997).

During the 20th century, several provenance trials were established independently in Europe, U.S.A. and New Zealand. The Corsican and Calabrian Black Pine provenances were found to be the best adapted genotypes. Nonetheless, most of the European plantations were installed with material from unknown origin or probably resulted on a mix of local and imported gene pools (Isajev et al. 2004).

Genetic diversity is fundamental for the maintenance and long term stability of forest ecosystems, since it determines the adaptive potential of the forestry species to the environmental conditions and climatic changes (Muller-stark et al. 1992; Çengel et al. 2012; Rubio-Moraga et al. 2012). The assessment of genetic variability among forestry populations

could contribute for the definition of strategies for *in situ* conservation, exploitation and restoration of genetic resources, and forest management (Lucić et al. 2010).

2. Objective of the STSM

Since the Portuguese *P. nigra* populations were never studied at molecular level and the origin of the reproductive material used in their installations is unknown, the goals of the Short Term Scientific Mission were: i) to evaluate their genetic diversity at the intra- and inter-population levels; ii) to infer about their relationships and structure; iii) to screen for the existence of different subspecies of *P. nigra* in Portugal by comparison of the SSR molecular patterns achieved with foreign samples; iv) and to study a putative correlation between molecular SSR patterns and wood density traits.

3. Links with Cost Action FP1202 MaP FGR

The *Pinus nigra* forest present in Portugal is allochthonous of unknown origin, constituted by small patches limited to the north and centre of the country. This species has an important economical and ecological value and due to different climatic pressures and geological modifications, it presents high morphological, physiological and genetic variability. Its adaptability to high altitude ecosystems and resistance to bad weather conditions offers advantages comparatively to other pines such as Maritime Pine and Scots Pine. However the lack of information concerning the ecologic and genetic background is a concern and in order to create management processes for this species and develop gene pool conservation it is necessary to investigate its adaptation and potential growth as a mountain species in Portugal to ascertain the continuity of this marginal pine forests.

The main objectives of this study are directly linked with Cost Action FP1202 MaP FGR that supports investigation of forest populations that are in the edge of species range. As in the case of *Pinus nigra* forests in Portugal that are isolated and spread in small forest

patches, the research of its genetic background allows the development of conservation strategies to create conditions for its survival.

4. Materials and methods

4.1 Plant Material

A total of 127 individuals were sampled for needle tissue in six allochthonous populations of *P. nigra* located at North and Centre of Portugal (Table 1). These populations present a regular and clean spacing among trees, typical of planted stands and good forestry management for avoidance of fires.

Table 1. Number and average dendrometric measurements of the *P. nigra* individuals sampled per population. In all populations the individuals were casually selected for sampling. Notes: Age – average age per population; h – average height of the individuals per population; DBH – Average diameter at breast height per population.

Local of the sampled population	Coordinates	Number of sampled individuals	Age (years)	Height (m)	DBH (cm)
Manteigas	40º22’47.00’’N; 7º33’18.00’’W	20	93.33	24.35	34.07
Vale do Zêzere	40º19’19.00’’N; 7º34’26.00’’W	20	58.79	14.95	24.8
Paredes de Coura	41º52’0.00’’N; 8º36’21.00’’W	20	53.47	14.76	21.09
Caminha	41º50’15.00’’N; 8º43’57.00’’W	25	52.73	26.3	32.56
Campeã	41º19’9.12’’N; 7º53’28.35’’W	20	51.75	23.12	37.11
Vila Pouca de Aguiar	41º 31' 03'' N; 08º 36' 03''O	22	75.60	26.82	40.08

Additionally, 90 individuals were sampled for differentiating xylem in the six main populations to try the establishment of a correlation between the SSR molecular patterns and their wood quality traits. Wood samples from these individuals were previously submitted to X-ray densitometry procedures to study the following parameters: average ring

density (RD), minimum density (MND), maximum density (MXD), earlywood density (EWD), latewood density (LWD), earlywood width (EWW), latewood width (LWW), ring width (RW), latewood percentage (LWP) and heterogeneity index (HI).

4.2 – Genomic DNA Extraction

The frozen needles and differentiating xylem of the *P. nigra* samples were previously used for genomic DNA extraction using the CTAB-based protocol of Doyle and Doyle (1987) with some modifications (Lemos et al. 2015a, unpublished data). The integrity of the genomic DNA was evaluated after electrophoresis on 0.8% agarose gels stained with ethidium bromide and quantified in the Nanodrop ND-1000 (Thermo Scientific) spectrophotometer. The DNA samples were diluted to a concentration of 30ng/µL using ultra-pure distilled water (Gibco).

4.3– Amplification of microsatellites (SSRs)

Microsatellites or simple sequence repeats (SSRs) are commonly used in plant genetic research due to its characteristics such as codominant nature, high abundance in eukaryotic genomes, robustness, high information content and ability to detect high levels of polymorphism (Bandelj et al., 2004).

Initially 2 multiplex (Table 2), previously tested were elaborated with 8 primers each for DNA amplification by PCR.

Table 2. Properties of the *P. nigra* SSR primers tested

Multiplex	SSR *locus*	Repeat Motif	Product Size	Labelling
1	pn6360	$(TC)_{20}$	324	6-FAM
	pn7754	$(TA)_{12}$	114	6-FAM
	pn2153	$(TG)_{12}$	311	VIC
	SPAG_7.14	$(TG)_{17}$ $(AG)_{21}$	209	VIC
	pn4379	$(AT)_{15}$	450	VIC
	pn2246	$(TA)_{13}$	359	PET
	pn6175	$(AT)_{12}$	201	NED
	pn1403	$(GA)_{13}$	307	NED
2	PHA_6062	trinucleotide	367	6-FAM
	pn350	$(AT)_{12}$	415	VIC
	PHA_4783	trinucleotide	472	VIC
	PtTX4001	$(GT)_{15}$	224	VIC
	pn8747	$(AT)_{12}$	278	NED
	pn4386	$(AT)_{11}$	407	NED
	PtTX3107	$(CAT)_{14}$	182	PET
	pn6266	$(GT)_{14}$	317	PET

4.4 – Software Analysis

4.4.1 – Genotyping

Genotyping was performed using the "Gene Marker" software. This program is able to analyze genotype data from amplified fragment length polymorphism (AFLP), terminal-restriction fragment length polymorphism (T-RFLP) and microsatellite analysis among others.

4.4.2 – Statistical Analysis

The statistical analysis was conducted based on the pool of the SSR data using the software GenAlEx (Peakall and Smouse 2006), STRUCTURE Ver.2.3.4 (Falush et al. 2007; Pritchard et al. 2010), STRUCTURE Harvester (Earl and vonHoldt, 2012), POPGENE 1.32 (Yeh *et al.* 1999), and TreeView (Page, 1996). With the GeneAlEx software it was possible to determine the genetic frequency, the AMOVA, PCoA, and the Mantel test. The STRUCTURE and the STRUCTURE Harvester software were used to study the genetic structure and to determine the optimal number of clusters among the six studied populations (Pritchard et al.

2000; Falush et al. 2003; 2007). The POPGENE 1.32 software allowed the analysis of the genetic variation among and within populations and the construction of a phylogram with the software TreeView.

5. Results and discussion

The 6 populations representative of *P. nigra* distribution in Portugal were analyzed for the characterization of the genetic diversity, relationships and structure. In the SSRs amplification, two multiplex in a total of 16 primers tested were used based on previous results related with their amplification potential, discriminative pattern and/or reproducibility. Each pair of SSR primers revealed 100% of polymorphism among the studied *P. nigra* individuals. All primers tested, proved to be highly discriminative and allowed the detection of SSR polymorphism at the intra- and inter-population levels. AMOVA is recognized as an effective tool to define population structure and degree of genetic differentiation (Peakall and Smouse 2006, 2012). This analysis allowed us to verify a higher molecular variation within populations (95%) rather than among (5%). The present results are in concordance with other studies conducted with dominant molecular markers that indicated *P. nigra* as a highly polymorphic species (Lucić *et al.* 2010; Šarac *et al.* 2014), generating problems with the definition of taxonomy in terms of species, subspecies and varieties (Afzal-Raffi and Dodd 2007).

For the extrapolation of the genetic structure of the Portuguese *P. nigra* populations based on codominant markers, the 'admixture' parameter was chosen and both STRUCTURE and STRUCTURE HARVESTER software were used. After several runs of *K* (number of clusters or populations) based on the pool of the SSR data, the STRUCTURE software resolved $K = 2$ highly differentiated genetic clusters (Fig. 1 and 2; Table 3). This value was further confirmed

with the software STRUCTURE HARVESTER which defined *K*=2 as the optimal number of clusters.

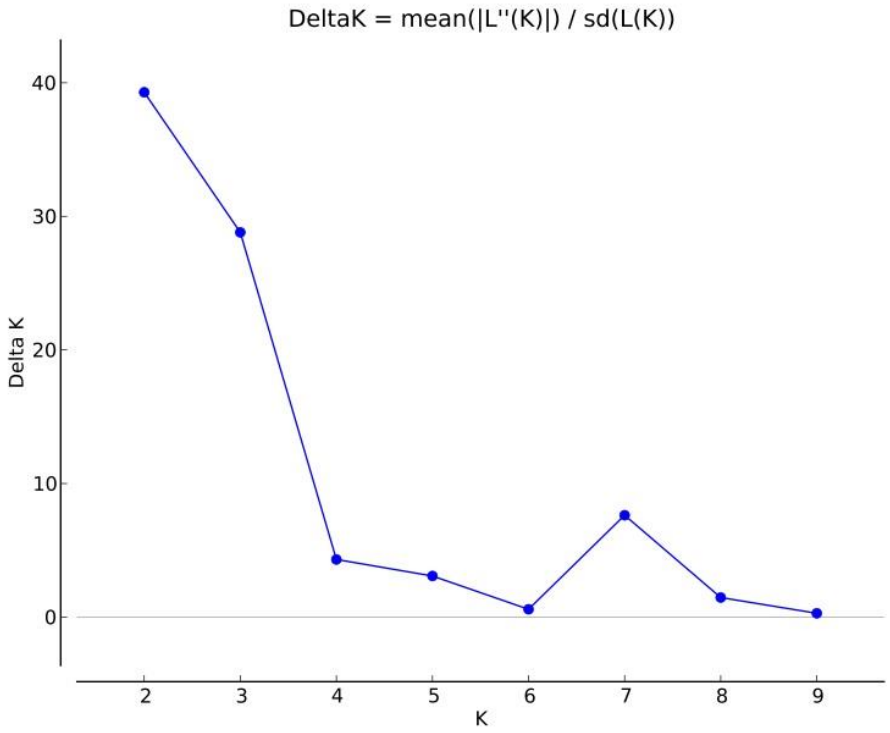

Figure 1. Diagram provided by STRUCTURE HARVESTER software for the estimation of optimal clusters number (Delta K), based on the pool of the SSR data.

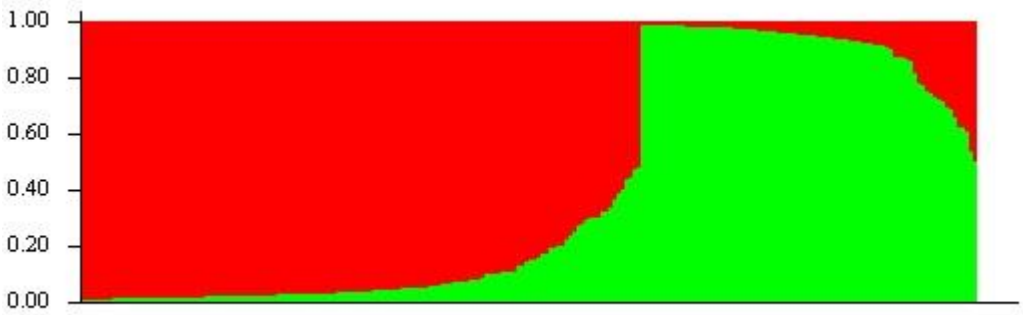

Figure 2. Bar plot diagram provided by the STRUCTURE software estimating the genetic structure of the Portuguese populations of *P. nigra* into two clusters (*K*=2) based on the pool of the SSR data.

The mean F_{ST} values attributed to each group were approximately of 0.035, indicating reduced genetic differentiation among the Portuguese *P. nigra* populations (Table 3).

Table 3. Mean F_{ST} values per group achieved with $K = 2$ using the STRUCTURE software based on the pool of SSR data.

Group	Mean F_{ST} value for $K = 2$
I	$F_{ST}\_1 = 0.0350$
II	$F_{ST}\_2 = 0.0345$

These results corroborated our previous studies performed with dominant markers on the same populations, once it was verified the existence of a single *P. nigra* subspecies in Portugal with the occurrence of 2 regional varieties, namely, *P. nigra* subsp. *laricio* var. *corsicana* and *P. nigra* subsp. *laricio* var. *calabrica* (Lemos et al. 2015b, unpublished data). The SSR data supports the assumption of existence of a single subspecies in Portugal and the occurrence of two varieties since a reduced value of molecular variation (5%) was determined among populations after AMOVA.

The PCoA based on the pool of the SSR data explained a cumulative percentage of total variation of 98.06% (Fig. 3). The populations with higher genetic identity were 'Caminha', 'Campeã' and 'Manteigas', while 'Vila Pouca de Aguiar' showed the lowest genetic identity with the remaining populations, explaining the occurrence of two clusters in the genetic structure.

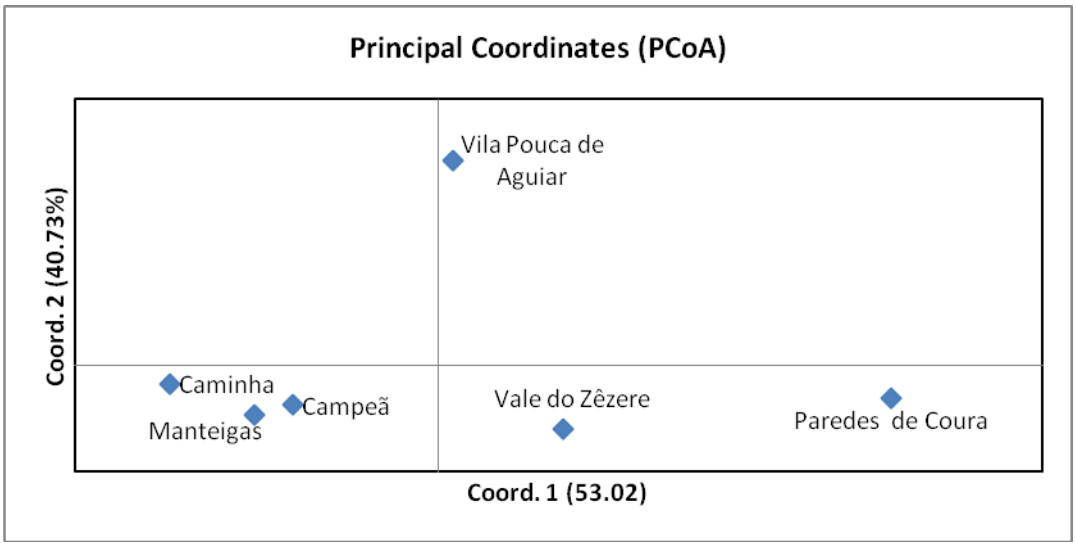

Figure 3. PCoA based on the Nei's genetic distance matrix among the 6 Portuguese populations of *P. nigra* based on the pool of the SSR data.

The SSR data was also analysed with the software POPGENE 1.32, which enabled the calculation of the following parameters: i) Shannon's information index (*I*; Lewontin, 1972), which measures gene diversity (Shannon and Weaver 1949); ii) estimation of the unbiased genetic identity (Nei 1973) and genetic distance (Nei 1978); iii) observed number of alleles (na); and iv) effective number of alleles (ne; Kimura and Crow, 1964) and Shannon's information index (I; Lewontin, 1972) (Table 5).

Table 5. Summary of genetic variation and gene diversity statistical analyses achieved per population, based on the pool of the SSR data.

Population	Mean ± standard deviation		
	na	ne	*I*
Manteigas	12.63 ± 6.21	5.62 ± 2.97	1.89 ± 0.60
Vale do Zêzere	12.50 ± 6.54	6.58 ± 4.37	1.93 ± 0.71
Campeã	12.75 ± 6.71	6.48 ± 4.52	1.90 ± 0.75
Paredes de Coura	12.63 ± 6.50	6.74 ± 4.41	1.93 ± 0.76
Vila Pouca de Aguiar	9.13 ± 4.94	4.71 ± 2.66	1.61 ± 0.65
Caminha	11.06 ± 5.77	5.56 ± 3.74	1.75 ± 0.71
TOTAL	20.31 ± 11.06	7.29 ± 5.06	2.08 ± 0.79

Population of 'Vale do Zêzere' and 'Paredes de Coura' present the highest values of Shannon's Information index (*I*) (Table 5). The results of the observed and expected homozygosity and heterozygosity (computed using Levene, 1949) and Nei's (1973) expected heterozygosity are presented in Table 6.

Table 6. Summary of heterozygosity statistical analyses achieved per population based on the pool of the SSR data.

Population	Observed homozygosity	Observed heterozygosity	Expected homozygosity	Expected heterozygosity	Nei	Average heterozygosity
Manteigas	0.51 ± 0.16	0.49 ± 0.16	0.23 ± 0.15	0.77 ± 0.15	0.76 ± 0.15	0.74 ± 0.18
Vale do Zêzere	0.42 ± 0.22	0.58 ± 0.22	0.23 ± 0.19	0.77 ± 0.19	0.76 ± 0.19	0.74 ± 0.18
Campeã	0.43 ± 0.20	0.57 ± 0.20	0.25 ± 0.19	0.75 ± 0.19	0.74 ± 0.19	0.74 ± 0.18
Paredes de Coura	0.40 ± 0.23	0.59 ± 0.23	0.24 ± 0.20	0.76 ± 0.20	0.75 ± 0.19	0.74 ± 0.18
Vila Pouca de Aguiar	0.41 ± 0.22	0.59 ± 0.22	0.29 ± 0.19	0.70 ± 0.19	0.69 ± 0.19	0.74 ± 0.18
Caminha	0.43 ± 0.22	0.57 ± 0.22	0.27 ± 0.19	0.73 ± 0.19	0.72 ± 0.19	0.74 ± 0.18
TOTAL	0.43 ± 0.19	0.57 ± 0.19	0.23 ± 0.18	0.77 ± 0.18	0.77 ± 0.18	0.74 ± 0.18

The highest values of Nei's expected heterozygosity are in 'Manteigas' and 'Vale do Zêzere', justifying the high polymorphism and molecular variation detected within populations reported above.

Tables 7 present the results of Nei's original measures and unbiased measures of genetic identity among the Portuguese samples revealed by the SSR markers.

Table 7. Nei's unbiased measures of genetic identity (top) and genetic distance (bottom) among the Portuguese populations of *P. nigra*, based on the pool of the SSR data.

Population	Manteigas	Vale do Zêzere	Campeã	Paredes de Coura	Vila Pouca de Aguiar	Caminha
Manteigas	****	0.9118	0.9470	0.7982	0.8389	0.9540
Vale do Zêzere	0.0924	****	0.9391	0.9189	0.8416	0.9072
Campeã	0.0545	0.0628	****	0.8251	0.8596	0.9622
Paredes de Coura	0.2254	0.0846	0.1922	****	0.8090	0.7829
Vila Pouca de Aguiar	0.1756	0.1725	0.1512	0.2119	****	0.8639
Caminha	0.0471	0.0973	0.0385	0.2447	0.1463	****

According to Nei's unbiased measures of genetic identity, the populations with higher values were found among 'Caminha', 'Manteigas' and 'Campeã', corroborating the PCoA results (Fig. 3).

The TreeView software allowed the construction of a phylogram based on the pool of the SSR data which allowed the determination of the genetic relationships among the studied populations (Fig. 4).

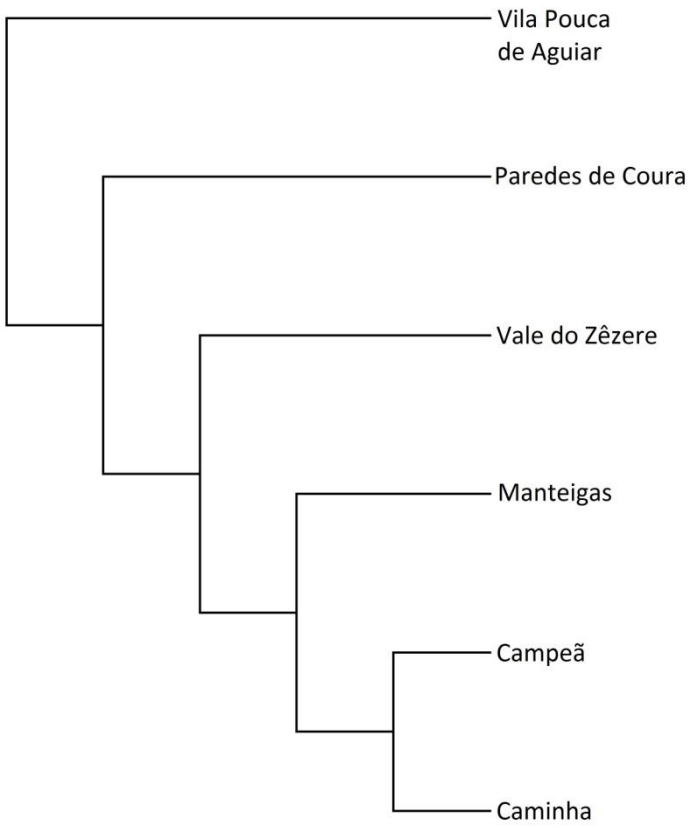

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Figure 4. Phylogram of the six Portuguese *P. nigra* populations based on the pool of the SSR data and constructed with the TreeView software.

The phylogram also corroborated the previous genetic analyses since it revealed a closest genetic relationships among the populations of 'Caminha', 'Campeã' and 'Manteigas' and a higher genetic distance among 'Vila Pouca de Aguiar' and the remaining populations (Fig. 4).

Based on our previous studies performed with dominant markers and the present results we could suggest that the Portuguese *P. nigra* populations belong to a single subspecies, namely, *laricio*, as determined by comparison of molecular patterns among Portuguese and foreign samples of this *taxon* (Dias *et al*. 2015 unpublished data). Furthermore, the SSR data achieved during the STSM contributed to our supposition of the existence of two regional varieties from subspecies *laricio* in Portugal. Additionally,

concerning the higher genetic distance of 'Vila Pouca de Aguiar' relatively to the remaining populations, such population might be installed with forest reproductive material belonging to a different infraspecific *taxon*. Additional genetic studies will be performed to confirm this hypothesis.

6. Conclusions

This study constituted the first molecular characterization of the six *P. nigra* populations that are representative of the distribution of this pine species in Portugal and that showed high adaptive potential to our country, as previously determined by dendrometric and ecological evaluations done during sampling (Dias *et al.* 2015).

Based on the molecular data provided by SSRs it was verified that these allochthonous populations have high genetic diversity, mostly at the intra-population level; a reduced genetic differentiation among them and, according to the estimated genetic relationships, it seem to belong to two regional varieties of the same subspecies, corroborating previous studies performed in the same populations. The understanding of the genetic diversity, structure and relationships of these *P. nigra* populations will be highly important under the scope of forestry management, genetic improvement and/or for the definition of afforestation and conservation strategies.

This report presents the main results achieved during the STSM. Nevertheless, additional data is currently being analyzed to infer about which subspecies of *P. nigra* is in fact present in Portugal, by comparison with SSR molecular patterns produced in foreign samples belonging to different infraspecific *taxa*. The genetic relatedness among molecular patterns and wood characteristics was partially accomplished by the analysis of this data in terms of the influence of demography. In order to further investigate this correlation, further collaboration with the host institution will be necessary to perform the analysis of single

nucleotide polymorphisms (SNPs). The detection of variations in the DNA sequence will possibly allow its correlation with wood characteristics.

The work carried in this STSM will result in a publication in an international scientific journal belonging to Scientific Citation Index.

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