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Molecular Characterisation of Post-Fire Naturally Regenerated Populations of Maritime Pine (*Pinus pinaster* Ait.) in the North of Portugal

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Abstract: Wildfires act as a selection force threatening the sustainability and diversity of forest genetic resources. Few studies have investigated the genetic effects of forest wildfires. Species with perennial canopy seed banks in serotinous cones and soil or with long-distance seed and pollen dispersion can preserve genetic diversity and population differentiation under normal fire regimes. To test this hypothesis, we characterised molecularly *Pinus pinaster* Aiton (maritime pine) seedlings produced from seeds sampled in post-fire, naturally regenerated populations that had been subject to different fire regimes in the North of Portugal using inter-simple sequence repeats (ISSRs). The sampled populations burned once (A), twice (B), or three (D) times or had no prior fire history (C, control). Given the globally low seed germination ability, only 104 plantlets regenerated and were described. These plantlets were grouped according to their origin population. Intra-group ISSR polymorphism ranged from 72.73% (B) to 89.41% (D), revealing genetic differentiation among groups originating from populations that had experienced different fire recurrence. Overall, the unaffected genetic diversity of the regenerated plantlets allowed us to accept the hypothesis. Our findings enhance our understanding of the species ability to withstand fire-induced challenges and their responses to wildfires, guiding conservation endeavours and forest management strategies to bolster ecosystem resilience.

Keywords: Inter-Simple Sequence Repeat (ISSR) markers; genetic variability; differentiation and structure; maritime pine

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1. Introduction

Climate change is increasing the Mediterranean region's temperature, water deficit, fire recurrence and severity [1]. The increase in wildfires can threaten the sustainability of forest genetic resources and potentially impact genetic diversity and population structure [2]. The post-fire natural regeneration depends on the seed bank available in the canopy and in the ground that is released during the fire [2,3]. When the regenerated plants reach reproductive maturity, natural stands with high genetic diversity and random mating can occur [4,5]. Evaluating the genetic diversity in post-fire regenerated stands is paramount for their management [3]. Different authors have analysed the genetic diversity in post-fire regenerated populations of various forestry species with different molecular markers [2,3,6].

The maritime pine (*Pinus pinaster* Aiton) is an essential native conifer in the western Mediterranean basin. This species is abundant in Portugal, occupying approximately 22.1% of the forest area (713,300 ha) [7]. The sustainability of the species is commonly associated with the ability of stands to regenerate naturally in situations of final harvest (typical harvest age around 45–50 years) or post disturbance by fires. The incidence of forest fires in the Mediterranean region is high, leading to the explicit consideration of this threat in the silvicultural and forest management models, such as those proposed for the species by [8].

DNA markers such as the inter-simple sequence repeat (ISSR) have been used to assess the impact of fire recurrence, in the short-term, in the genetic variability of Portuguese populations of *P. pinaster* [6]. These authors analysed two Portuguese populations of *P. pinaster*, Vila Seca-2 and Seirós, from areas that burned once (in 2005) and twice (in 1990 and 2005), respectively, and compared them with a population that was not affected by fire (Vila Seca-1). The population that burned twice showed the highest percentage of ISSR polymorphism and genetic diversity indexes [6]. The authors concluded that, in the short term, the fire regime can affect genetic diversity without generating genetic erosion of *P. pinaster*. Time since fire occurrence has been highly discussed as a concern with respect to ecosystem trajectory and recovery [9]. Many studies have focused on the short-term tree regeneration patterns because the post-fire ecosystem maintains them in the long term [10,11]. However, given the projections of increased wildfire frequency and severity as conditions become warmer and drier [1], the future regeneration patterns may differ from the past ones [9]. For these reasons, evaluation of the long-term response to fire in regenerated populations, including tree regeneration ability [12] and genetic variability (this study), is required. The adaptive potential, distribution, and/or survival of any forestry species highly depend on its degree of genetic variability [2,6,13]. This information is crucial for defining forest management strategies to promote the natural regeneration of forest species [14,15]. Nonetheless, studies focusing on the genetic effects of forest wildfires are scarce.

For a predominantly outcrossing species with long-distance gene flow, such as *P. pinaster*, we hypothesised that, despite the recurrence of normal wildfires, the genetic variability, differentiation, and structure of the post-fire naturally regenerated populations would be preserved in the long term. To test this hypothesis, with this work, we aimed to analyse the genetic variability of seedlings regenerated from seeds isolated from fully closed pinecones that were collected in four post-fire regenerated *P. pinaster* populations (A-D), located in the North of Portugal, subject to different fire recurrence using the ISSR molecular markers. Population A burned once in 1987, population B burned twice in 1987 and 1998, population D burned three times (in 1989, 1990, and 1998), whereas population C had no prior fire history and was used as a control. Through this work, the analysed seedlings were considered as belonging to four groups of individuals identified by the same letter assigned to the populations from which they originated. Independently of the fire recurrence, the *P. pinaster* seedlings revealed high intra-group ISSR polymorphism, ensuring the genetic differentiation and structure and evidencing the preservation of genetic variability, in the long term, of post-fire naturally regenerated *P. pinaster* populations.

2. Materials and Methods

2.1. Sampling Area

In this work, we collected pinecones in four post-fire, naturally regenerated *P. pinaster* adult populations, located in the 'Vale do Tâmega' region, the largest continuous area covered with the species in the North of Portugal (Figure 1).

A complete dendrometric characterisation of these populations can be consulted in [16]. The areas of these populations range from 4.0 ha to 8.6 ha. The soil is predominantly classified as shale umbric cambisols. The climate is temperate, characterised by dry and mild summers with an average annual temperature of 9.8 °C and accumulated annual precipitation of 1489 mm. This site is classified as medium-high quality for the development of *P. pinaster*.

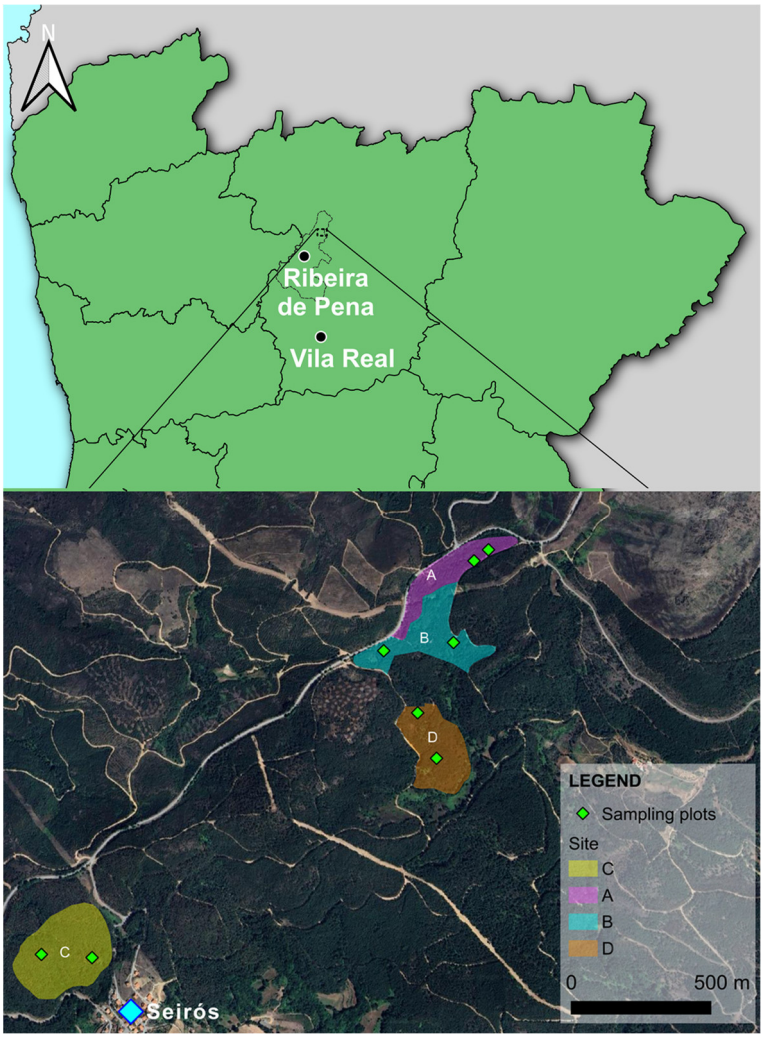

Figure 1. Geographic location of the study area and sampled populations.

The four populations will be named A, B, D, and C through the text according to their wildfire recurrence regimes, namely population A originated from a pine stand that was burned once (1987), population B originated from a pine stand which burned twice (1987 and 1998), and population D originated from a pine stand that was burned three times (1989, 1990, and 1998). Population C was never burnt, and it was used as a control.

2.2. Plant Material

Two sample plots representative of the stand conditions were identified for data and plant material collection in each stand. The plots were circular with an area of 200 m^2. We collected at least 40 fully closed pinecones at each site without visible insect attack or disease signs. The sample was collected on the ground near the canopy of the trees. The average size of the pinecones sampled in each population was similar. The collection of the pinecones was performed in February and April 2022.

The pinecones were incubated at 100 °C [17] for one hour to allow their opening. The seed's viability was assessed by manual compression [18]. The seeds were stored for a few days at room temperature [19].

One hundred seeds from different pinecones collected from each population were mixed and randomly selected to ensure the existence of genetic variability. The seeds were sown in trays (60 × 40 mm) containing soil from a pine population and peat in the 2:1 proportion. The trays were maintained at room temperature with 16 h of photoperiod

for 90 days to allow for seed germination and seedling emergence. The substrate was watered whenever required.

At the end of the seed germination test, 104 *P. pinaster* seedlings emerged: 32 from group A, 25 from group B, 32 from group D, and 15 from group C (control).

Seedlings with 10–12 cm height were collected and their aerial parts immediately frozen and maintained at −80 °C until genomic DNA extraction. This procedure was repeated periodically throughout the 90 days since seed germination varied within and among populations.

2.3. Genomic DNA Extraction

Each frozen seedling was grounded with liquid nitrogen, and the genomic DNA was isolated using the DNAeasy®Plant Mini Kit (Qiagen®, Valencia, CA, USA) following the manufacturer's instructions. After checking the genomic DNA sample integrity following electrophoresis on 0.8% agarose gel and purity with the Nanodrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), each genomic DNA sample was diluted to 20 ng μL^{-1}.

2.4. Amplification and Visualisation of ISSR Markers

Seven SSR primers from set #9–Microsatellite of the University of British Columbia (UBC; Table 1) were individually tested for the amplification of ISSR markers in the 104 genomic DNA samples.

Table 1. Primers individually tested for ISSR markers amplification. Note: (*)—R = (A,G) and Y = (C,T).

Primer Name	Sequence 5′→3′
UBC 825	ACA CAC ACA CAC ACA CT
UBC 826	ACA CAC ACA CAC ACA CC
UBC 846	CAC ACA CAC ACA CAC ART *
UBC 849	GTG TGT GTG TGT GTG TYA *
UBC 850	GTG TGT GTG TGT GTG TYC *
UBC 855	ACA CAC ACA CAC ACA CYT *
UBC 856	ACACACACACACACACYA *

The choice of these primers was based on the similarity of their motif repetition to the oligonucleotides used by [6] in *P. pinaster* and previous works conducted in other *Pinus* spp. [20,21].

For ISSR markers amplification, the reaction mixture (final volume of 20 µL) contained 20 ng of genomic DNA, 5 µM of primer (Table 1), and GRS 2x Mastermix (Grisp, Lda.—Research Solutions, Portugal). The ISSR amplification reactions were performed on a thermal cycler T-Professional (Biometra) under the conditions described in [22]: an initial denaturation step of 94 °C for 5 min, 45 cycles of 94 °C for 30 sec, 52 °C for 45 sec, and extension at 72 °C for 2 min, followed by a final extension of 5 min at 72 °C.

The ISSR amplified products were loaded on 1.5% agarose gels prepared with 1× TBE buffer and precast staining with 0.8% of Xpert Green DNA Gel Stain (Grisp, Lda.—Research Solutions, Portugal). The molecular weight marker GeneRuler DNA Ladder Mix (Thermo Fisher Scientific, Inc.) was loaded onto each agarose gel. During UV exposure, the gel images were captured with the Molecular Imager® Gel DocTM XR+ equipment using the Image Lab™ version 6.1 Software for Windows (BIO-RAD, Bio-Rad Laboratories, Inc.).

After the amplification and visualisation of the ISSR markers in the 104 DNA samples, the genetic variability within and among groups and their genetic relationships and structure were estimated.

2.5. Statistical Analyses

Each ISSR band was considered a marker or locus, and one band with the same molecular weight amplified in different individuals was considered the same ISSR marker or locus. Each amplification reaction was repeated twice, and only reproducible bands were considered for the binary matrix presence/absence analysis and construction.

The percentage of ISSR polymorphism (%P) generated per primer, group of individuals that regenerate from seeds collected in the populations A to D, and among individuals, was determined by %P = [(Number of polymorphic ISSR bands)/(total number of amplified ISSR bands)]$\times$]*ta*.

The binary matrix based on the pooled ISSR data was statistically analysed with different software to estimate the studied population's genetic variability, relationships, structure, and diversity parameters. The Numerical Taxonomy and Multivariate Analysis System–NTSYSpc version 2.2 [23] was used to construct a dendrogram of genetic similarity based on the simple matching coefficient, SIMQUAL format, SAHN (Sequential Agglomerative Hierarchical and Nested Clustering) module, and UPGMA (Unweighted Pair Group Method with Arithmetic Means) clustering method. For the estimation of the percentage of polymorphic loci (%P), Nei's gene diversity (*h*), Shannon's Information index (*I*), analysis of molecular variance (AMOVA), and Principal Coordinates Analysis (PCoA) based on the pairwise Nei's genetic distance [24] matrix, the software GenAlEx version 6.502 [25] was used. Assuming the Hardy–Weinberg equilibrium, the total genetic diversity (HT), genetic diversity within populations (HS), the relative magnitude of differentiation among populations (GST), genetic diversity among populations (DST = HT $-$ HS), and estimation of gene flow among populations (Nm) from GST [26] calculated by $Nm = [(0.5(1 - \mathrm{GST}))/\mathrm{GST}]$ were determined with the software POPGENE 1.32 [27], based on Nei's analysis of gene diversity in subdivided populations [28].

For the genetic structure inference, 10,000 'burn-in' settings by 20,000 Markov Chain Monte Carlo (MCMC) iterations were performed with the software STRUCTURE v.2.3.4 [29–32] using the 'admixture' and 'correlated allelic frequencies among populations' as parameters. Fifty runs were performed for each value of K (number of genetic clusters) ranging from 1 to 4. The mean values of gene differentiation among populations (FST), regarding allele frequencies, were determined as follows: $\mathrm{FST} = 1 - \mathrm{HS/HT}$ [32], using the STRUCTURE software.

To estimate the most likely number of genetic clusters (K), the output results of the STRUCTURE analysis were analysed with two online tools: (i) STRUCTURE HARVESTER [33] using the statistic deltaK, which represents the most significant rate of change between each subsequent K value [34], and (ii) CLUMPAK—Cluster Markov Packager Across K [35].

3. Results

All primers produced polymorphic ISSR patterns within and among groups of seedlings. Figure 2 presents this result, showing polymorphic ISSR patterns within and among groups, generated by the primer 850.

Table 2 presents the resulting data from the presence/absence analysis per primer in each group, per primer in the 104 individuals, as well as the genetic diversity parameters, h and I, per group.

Each primer detected high ISSR polymorphism at the intra-group level (Table 2). The primers 849, 855, and/or 856 produced the highest percentage values of ISSR polymorphism in the four groups, along with primers 825, 826, and 846 in group D (Table 2). The seven primers amplified a total of 89 markers that were polymorphic among the 104 individuals, resulting in 100% ISSR polymorphism (Table 2). Overall, the seven primers were highly informative for assessing the ISSR polymorphism at the intra- and inter-group levels.

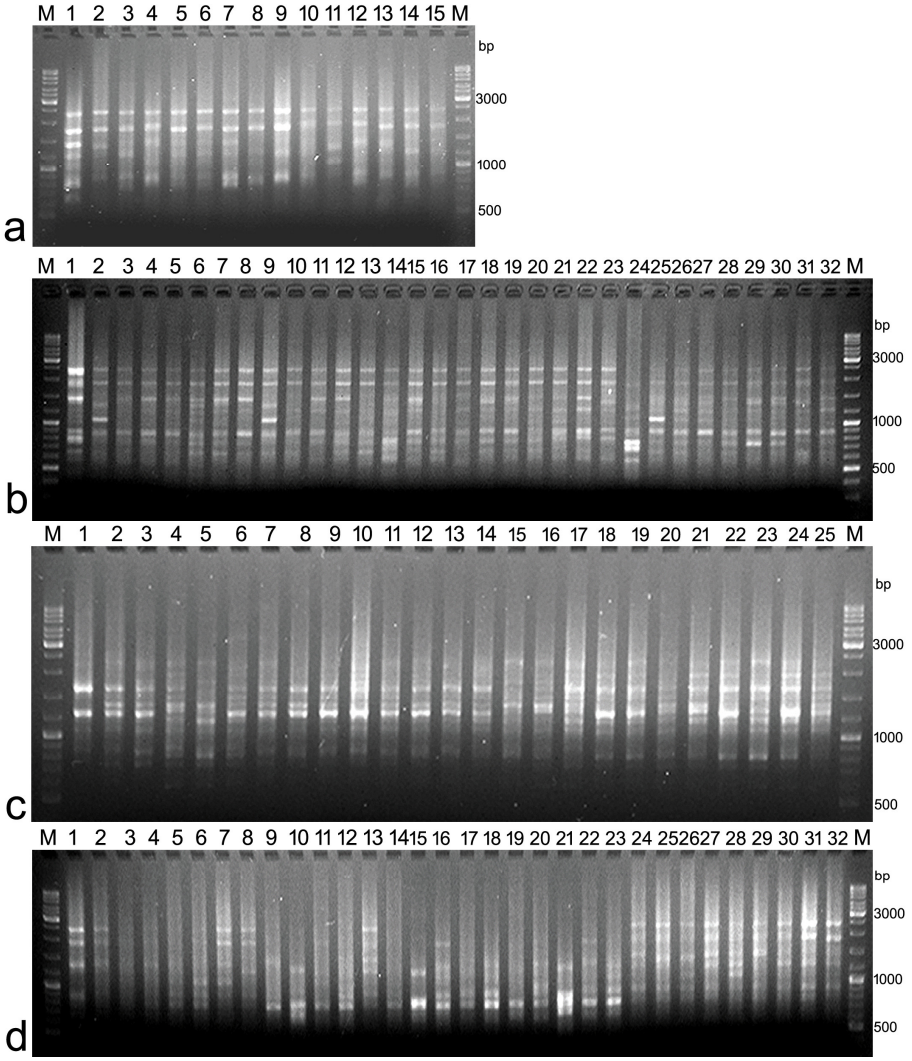

Figure 2. ISSR markers produced with primer 850 in the 104 maritime pine individuals (**a**–**d**) whose seeds were collected in the: (**a**) control (unburned) population, C; (**b**) burnt once (A) population; (**c**) burnt twice (B) population; and (**d**) burnt three times (D) population, visualised after electrophoresis on agarose gels stained with X-pert green. M—Molecular weight marker GeneRuler DNA Ladder Mix.

Table 2. Values of percentage of ISSR polymorphism (%P) per primer; *P. pinaster* individuals that originated from populations C, A, B, and D, and among individuals, as well as the Nei's gene diversity (*h*) and Shannon's Information index (*I*) parameters calculated per group of individuals. Notes: populations C—unburned (control); A—burnt once; B—burnt twice; and D—burnt three times.

Percentage of ISSR Polymorphism within the Group of Individuals Originating from:							
	Population C (control)				Population B		
Primer	T	P	% P	Primer	T	P	% P
825	7	6	85.71	825	6	4	66.67
826	4	1	25.00	826	7	4	57.14
846	9	5	55.56	846	8	5	62.50
849	8	8	100	849	8	7	87.50
850	9	6	66.67	850	11	6	54.55
855	6	6	100	855	7	7	100
856	8	8	100	856	8	7	87.50
Total	51	40	78.43	Total	55	40	72.73

Table 2. *Cont.*

Percentage of ISSR Polymorphism within the Group of Individuals Originating from:							
	Population A				Population D		
Primer	T	P	% P	Primer	T	P	% P
825	9	7	77.78	825	15	15	100
826	6	3	50.00	826	11	11	100
846	9	7	77.78	846	12	12	100
849	4	3	75.00	849	9	9	100
850	11	9	81.82	850	12	11	91.67
855	10	9	90.00	855	13	5	38.46
856	5	4	80.00	856	13	13	100
Total	54	42	77.78	Total	85	76	89.41

Percentage of ISSR Polymorphism among Individuals				Genetic Diversity Parameters (Mean ± Standard Error) Per Group of Individuals with Origin in:		
Primer	T	P	% P	Population	h	I
825	15	15	100	C	0.47 ± 0.004	0.66 ± 0.005
826	11	11	100	A	0.49 ± 0.002	0.68 ± 0.002
846	12	12	100	B	0.48 ± 0.002	0.67 ± 0.003
849	10	10	100	D	0.48 ± 0.002	0.68 ± 0.002
850	14	14	100	Mean	0.48 ± 0.002	0.67 ± 0.002
855	14	14	100			
856	13	13	100			
Total	89	89	100			

The ISSR polymorphism was higher in the seedlings that originated from the D population which burned three times (Table 2). On the other hand, the ISSR polymorphism was lower in the control seedlings that originated from the unburned population (Table 2). In addition, the ISSR molecular patterns presented by the control (C) individuals were more similar to those exhibited by the seedlings originating from population A (burned once), as verified by the UPGMA dendrogram (Figure 3). The ISSR patterns of individuals regenerated from seeds of the B and D populations were different and resulted in separate clustering, apart from those individuals regenerated from seeds collected in populations A and C (Figure 3).

The genetic diversity parameters, h and I, were quite similar among groups, but the lower mean values were shown by the control (unburned) one (Table 2). The total mean values (over all loci and groups) of the h and I parameters were 0.48 and 0.67, respectively (Table 2).

The high polymorphism among the individuals of each group was also confirmed by the analysis of molecular variance (AMOVA) performed with 9999 permutations. This test revealed only 0.09% of the molecular variation among groups, and 99.91% was attributed to differences among individuals within groups. The sources 'among groups' and 'within groups' contributed significantly ($p < 0.001$) to the genetic differentiation of the analysed individuals.

To estimate the genetic relationships among the analysed *P. pinaster* individuals and groups, the binary matrix based on the pooled ISSR data was used to construct a UPGMA dendrogram of genetic similarity (Figure 3).

Concerning the dendrogram topology, for a cut-off value of ~0.62, two main groups (I and II) could be considered (Figure 3). Group I was divided into two subgroups, I-a—composed of individuals regenerated from seeds collected in populations C and A (control and burnt once, respectively), and I-b—integrating exclusively individuals regenerated from seeds of population D (burnt three times) (Figure 3). Group II was constituted solely by individuals regenerated from seeds collected in population B (burnt twice) (Figure 2). The genetic similarity among the 104 *P. pinaster* individuals, estimated by the simple matching coefficient, was 39% (Figure 3). The individuals that originated from population D shared

65% of genetic similarity among them. In contrast, individuals from subgroup I-a and group II presented a genetic similarity higher than 70% among them (Figure 3).

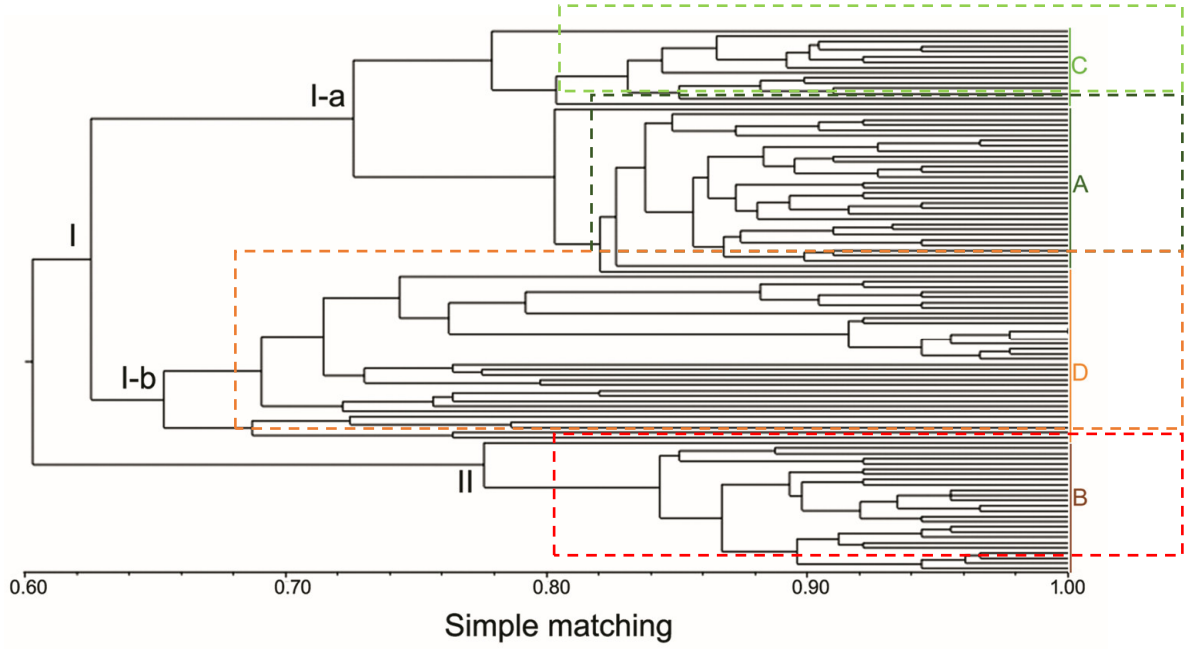

Figure 3. UPGMA dendrogram of genetic similarity, based on the pooled ISSR data and simple matching coefficient, among the 104 maritime pine individuals. The individuals were clustered per group according to their origin population, as indicated by the coloured boxes. Notes: populations C—unburned (control); A—burnt once; B—burnt twice; and D—burnt three times.

The UPGMA clustering (Figure 3) was corroborated by the values of Nei's genetic distance determined between the control (C) and D individuals (0.2890), between the control (C) and B individuals (0.2811), and between the control (C) and A individuals (0.1605). However, in the Principal Coordinates Analysis (PCoA) based on the pooled ISSR data and the pairwise Nei's genetic distance matrix, the control (C) individuals were projected separately from those originating from the burned populations (Figure 4).

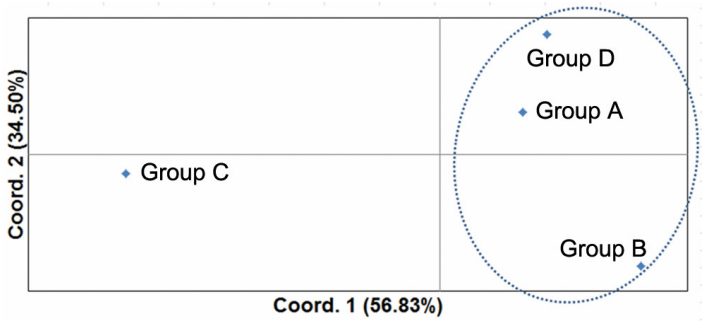

Figure 4. Principal Coordinates Analysis (PCoA) based on the pooled ISSR data and Nei's genetic distance matrix showing the projection of the groups of individuals (Group A to Group D) originating from the burned populations (A, B, and D) and those from the control group of individuals that originated from the unburned (C) population.

The PCoA is a multivariate technique that allows for the major patterns within multivariate data, such as multiple loci and samples, to be determined and plotted [25]. Despite the complex mathematics inherent to the PCoA, this process enables the detection of the major coordinates or axes of variation existent within a multidimensional dataset [25]. Each successive axis explains proportionately less of the total variation, such that, when there

are distinct groups, the first two or three axes will typically reveal most of the separation among groups, explaining the majority of total variation [25]. According to [36], the first three axes of a PCoA should explain a cumulative percentage of total variation higher than 70%. In this work, the first three coordinates demonstrated a cumulative rate of 100% total variation, evidencing statistical relevance.

The analysis of the Nei's gene diversity in the four groups of *P. pinaster* individuals indicated a mean (± standard deviation, s.d.) value of total genetic diversity (HT) of 0.32 ± 0.03 and a mean (± s.d.) value of gene diversity within groups (HS) of 0.17 ± 0.01. In addition, the relative magnitude of differentiation among groups (GST) was 0.4592. The genetic diversity among groups (DST), calculated as DST = HT − HS, was 0.15, and the estimation of gene flow among groups (Nm) was 0.5889.

The global analyses performed with STRUCTURE, STRUCTURE HARVESTER, and CLUMPAK estimated K = 3 as the most likely number of genetic clusters. The single line bar plot respecting the original order of the individuals in the binary matrix and the estimation of the optimal number of genetic clusters as K = 3 based on the statistic deltaK are presented in Figure 5.

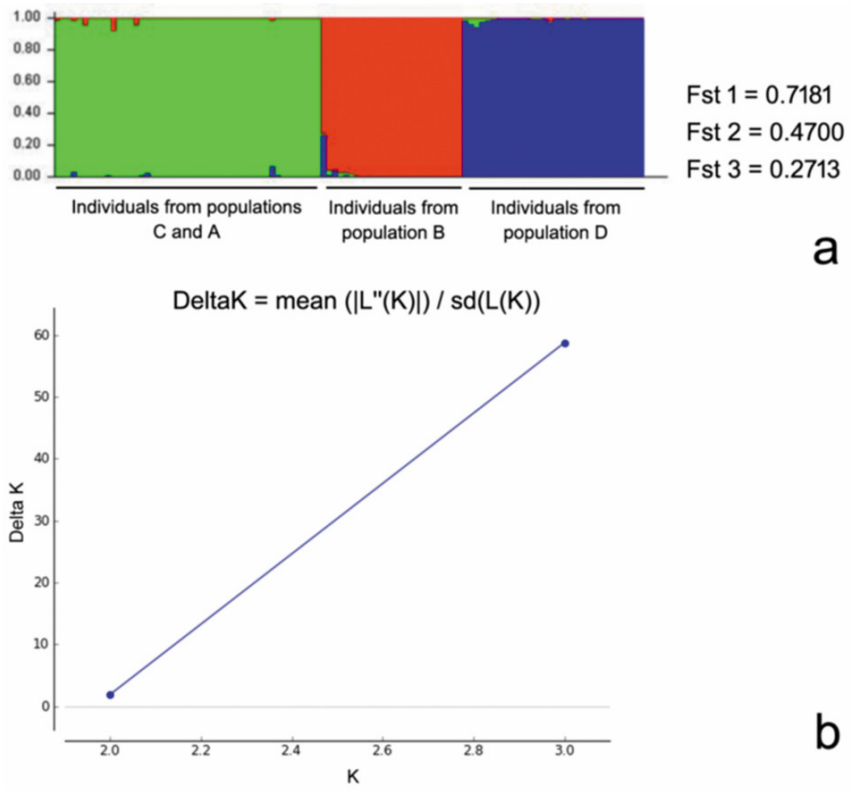

Figure 5. Genetic structure analyses based on the binary matrix constructed with the pooled ISSR data obtained from the 104 *P. pinaster* regenerated individuals that originated from seeds sampled in the post-fire, naturally regenerated populations C, A, B, and D, using the software (**a**) STRUCTURE and (**b**) STRUCTURE HARVESTER. (**a**) Single line bar plot representing an estimated genetic structure of three genetic clusters, for K = 3, and respective mean values of gene differentiation among populations (FST); and (**b**) the optimal number of genetic clusters (K) was retrieved as K = 3 by STRUCTURE HARVESTER after grouping the results provided by the STRUCTURE program and allowing the visualisation of likelihood of multiple K values after hundreds of iterations [33].

The single line bar plot displayed as the output result of the Bayesian analysis corresponds to the original order of the individuals in the binary matrix and evidenced that the first genetic cluster comprised individuals regenerated from seeds collected in the populations C and A (Figure 5a). Hence, this first genetic cluster matched with subgroup I-a of the UPGMA dendrogram (Figure 3). In addition, the second and third bar plots of

Figure 5a that correspond to individuals regenerated from seeds harvested from populations B and D match with group II and subgroup I-b, respectively, confirming the UPGMA clustering (Figure 3). In short, the UPGMA clustering was confirmed by the STRUCTURE analysis that retrieved K = 3 genetic clusters. The mean FST values provided per cluster, for K = 3, ranged from 0.27 to ~0.72 (Figure 5a). These FST values suggested high genetic differentiation among groups.

4. Discussion

Due to the reduced seed germination percentage, the molecular characterisation was conducted for DNA samples from only 104 *P. pinaster* seedlings. The low germination rate was not ascribed to fire recurrence, as it also occurred in the control seeds. A similar result was found in our previous work [16].

For this work, we hypothesised that a predominantly outcrossing species with long-distance gene flow, i.e., *P. pinaster*, would preserve the genetic variability, differentiation, and structure of the post-fire, naturally regenerated stands in the long-term, despite the normal wildfire recurrence. The molecular data obtained from the 104 *P. pinaster* individuals regenerated from seeds belonging to the analysed populations subject to different fire recurrence allowed us to accept the proposed hypothesis.

Concerning the molecular characterisation of the 104 *P. pinaster* regenerated seedlings with the ISSR markers, we verified high percentage of polymorphism within and among the groups analysed. The high intra-group genetic diversity is expected for an allogamous species, resulting in increased gene flow (Nm) and genetic differentiation of the sampled populations. In other pine species, ISSRs also revealed a higher genetic variability within rather than among populations [20,21]. AMOVA revealed a similar result in the present work. ISSRs have been widely used for the assessment of genetic diversity, relationships, and structure in different pine species ([20,21], among many others). In the particular case of *P. pinaster*, these markers have been used to assess the genetic variability in post-fire, naturally regenerated stands located in the vicinity of the ones analysed here [6]. The authors concluded that fire recurrence can affect the genetic variability of post-fire, naturally regenerated stands in the short term without causing genetic erosion. The present work complements such information by evidencing the preservation of genetic variability, differentiation, and structure of the naturally regenerated post-fire stands in the long term. Lucas-Borja and colleagues [6] detected higher values of the genetic diversity parameters, h and I, and the highest percentage of ISSR polymorphism in maritime pine individuals from the twice-burned population in relation to the population which only burned once. In this work, we found the highest genetic diversity parameters and percentage of ISSR polymorphism in the group of individuals originating from the D population (burned three times).

The reliability of the ISSR markers and the highly discriminative power of the used primers also enabled the projection of the groups of individuals regenerated from seeds collected in the burned populations (A, B and D) away from group C which originated from the control (unburned) population, as seen in the PCoA. The preliminary results achieved in this work evidence the suitability of the ISSR markers for analysing the genetic effects of fire recurrence. In future works involving ISSRs, a higher number of individuals and primers should be used, along with different DNA marker systems, to allow for an in-depth analysis of the genetic effects of fire recurrence in ecosystems prone to this natural phenomenon that will become more frequent due to climate change.

The impacts of wildfires are on the rise in the Mediterranean Basin due to increasingly extreme fire seasons marked by faster and more intense fires, often exceeding the capacity of fire suppression forces to respond effectively. With climate change, fire behaviour is anticipated to escalate further in severity [37]. Fire plays a pivotal role in driving plant evolution, particularly evident in pines. Evolutionary fire ecology emphasizes the necessity to select pine populations with traits conducive to the survival of expected fire regimes when restoring fire-prone ecosystems [38]. Among the species significantly affected by

forest fires in Portugal are the forests of *P. pinaster*, owing to their high spatial continuity and substantial fuel loads. Factors such as high flammability, high density of trees and shrubs in the stands, and expansive continuous areas further exacerbate fire propagation dynamics [39]. While *P. pinaster* is recognised as a fire-adapted species, this label primarily pertains to its adaptation to low-intensity fires. Survival in such fires is facilitated by specific traits such as height, self-pruning, and bark thickness [40]. *P. pinaster* and other conifer tree species develop physiological and reproductive strategies to protect the canopy seed bank and ensure survival [9]. *P. pinaster* reproductive strategies are accompanied by the preservation of high genetic diversity in the naturally regenerated post-fire stands, ensuring a broad adaptive potential of the species to fire-prone ecosystems. The high genetic variability of forest species is required for adaptation to and resilience in the face of abiotic and biotic stresses and, ultimately, for the species survival [6]. Nevertheless, the natural regeneration and seedling establishment depend on various ecological and silvicultural factors [10,15]. Addressing other fire regimes beyond the low-intensity ones, additional strategies, such as using sourcing seeds from populations with the appropriate adaptive characteristics, might be required. Incorporating seeds from diverse sources, including young post-fire populations [38], into restoration efforts can mitigate risks associated with reduced genetic diversity from reliance on a single post-fire population. This approach can serve as an adaptive management action in areas prone to recurrent forest fires, as is often the case in the Mediterranean region, particularly Portugal, where forest fires occur annually. By enhancing genetic diversity and resilience within restored ecosystems, this strategy contributes to a robust evolutionary potential, thereby fostering ecosystem sustainability in the face of ongoing fire challenges.

Despite the development of this work under controlled conditions, the results achieved afford valuable information for the future design of forest management strategies. These include, but are not restricted to, the convenience of identifying the history of fire disturbance in the catalogue record of the seeds that will be certified in the Region of Provenance (RP), as well as the use of assisted sowing in post-fire regenerated areas, with a mixture of seeds from distinct populations that facilitate the regeneration of new forests corresponding to populations with higher genetic variability and resilience.

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