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SRAP markers for characterization of the genetic diversity and differentiation of *Pinus nigra* populations in protected forested areas in Bulgaria, Greece, and Cyprus

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ABSTRACT
The present study aimed to evaluate the genetic diversity and differentiation of *Pinus nigra* Arnold, the European black pine, populations located in protected forested areas in three countries from the Balkan and Mediterranean region: Bulgaria, Greece, and Cyprus. Totally, 175 DNA samples from *P. nigra* plants collected from eight populations in these countries were analyzed using three SRAP primer pairs. The applied SRAP markers demonstrated high-resolution power, resulting in the identification of an average of 215 ± 67 polymorphic loci per SRAP primer pair. The analysis of molecular variance (AMOVA) showed that 82% of the observed variation was due to intra-population variation, while 18% was due to inter-population variation. The overall analysis of the population structure suggested low (Fst ≤ 0.01) intra-country differentiation for the populations from Bulgaria and Cyprus and moderate (0.15 ≤ Fst ≤ 0.17) inter-country differentiation. On the contrary, high differentiation between the populations (0.06 ≤ Fst ≤ 0.20) and complex genetic structure were characteristic of the three populations from Greece, relatively closely located in the area of the Pindos National Park. Analysis of the population structure also revealed that one of the Pindos populations belonged to the cluster of Bulgarian populations (Fst ≤ 0.01), showing moderate (Fst ≤ 0.11) to high (Fst ≤ 0.20) genetic differentiation from the other analyzed Pindos populations. The further use of SRAP markers for mapping the genetic diversity among *P. nigra* populations and identification of local populations diverted from the overall phylogeography-driven pattern in the studied region are discussed.

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Introduction

The genus *Pinus*, or pines, consists of approximately 100 species of economically important coniferous trees in the Pinaceae family [1]. Conifers, with *Pinus* on top, dominate the world's timber production, mostly through plantation forestry, and are preferred over angiosperms because of their faster growth [1]. The European black pine, *Pinus nigra* Arnold, is a Mediterranean representative of the genus, widely but fragmentedly distributed across Southern Europe, Northwest Africa, Turkey, Corsica, and Cyprus [2,3]. It is highly valued because of the high quality of the wood [4] and also broad tolerance to different soil types and climatic conditions [2,3,5,6].

Several subspecies have been recognized across its natural range [3], predetermined by the fragmented and isolated populations. The fragmentation of black pine populations has occurred mainly due to anthropogenic impact and deforestation, as well as forest fires, etc. [7,8]. Black pine is also among the most preferred species for reforestation purposes [9–12]. Reforestation activities in recent years have increasingly used clonal orchards, taking into account the

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Table 1. Geographic coordinates of *Pinus nigra* Arn populations used in this study. BG, Bulgaria; GR, Greece; CY, Cyprus.

P. nigra Arn. population	Population designation	Geographic coordinates	Natura 2000 protected area	Number of samples
Lepenitsa (BG)	LE	41.96675 N, 23.97714 E	Rodopi-Zapadni	22
Chepino (BG)	CH	41.99581 N, 23.9856 E	Rodopi-Zapadni	22
Draginovo (BG)	DR	42.07095 N, 24.02333 E	Yadenitsa	22
Konitsa (GR)	KO	40.0591 N, 20.77605 E	n/a	21
Samarina (GR)	SA	40.09765 N, 21.04548 E	n/a	20
Aoos lake (GR)	AO	39.84352 N, 21.07539 E	Valia Kalnta kai Techniti Limni Aoou	20
Troodos mountains-1 (CY)	TR1	34.93566 N, 32.90276 E	Ethniko Dasiko Parko Troodous	25
Troodos mountains-2 (CY)	TR2	34.94624 N, 32.85111 E	Ethniko Dasiko Parko Troodous	25

existing genetic diversity in reforested areas. Therefore, the application of affordable and efficient methods for the characterization of the black pine inter- and intra-population genetic diversity and structure will be very useful for forest management and conservation practices [7,13,14].

Sequence-related amplified polymorphism (SRAP) molecular markers, developed by Li and Quiros [15], have proved successful in a wide range of genetic studies in model and crop plant species [15–17]. SRAP markers have also been employed in many genetic studies on cultivated tree species, including Korean pine (*Pinus koraiensis* Sieb. et Zucc. [18]), rubber tree (*Hevea brasiliensis* Müll.Arg. [19], *Acer* spp. [20], etc., but still have been rarely used for the characterization of natural forest species populations. We recently demonstrated that SRAP markers could represent a valuable tool for the characterization of the genetic diversity of tree species in forest areas [21]. Unlike other commonly employed molecular markers, SRAP markers are highly reproducible, independent of particular genetic loci and relatively inexpensive [15].

Black pine has been the subject of many population studies employing inter-simple sequence repeat (ISSR), simple sequence repeat (SSR), and single nucleotide polymorphism (SNP) markers [3, 13,22–26]. DNA marker studies suggested that the present black pine populations in Europe originate from three previously differentiated genetic groups corresponding to three geographical areas: Western Mediterranean, Balkan Peninsula, and Asia Minor [27,28]. The studies further demonstrated a high level of fragmentation and the occurrence of bottlenecks for some of the studied populations [3,22,25,26,29]. To the best of our knowledge, no study has so far reported the application of SRAP markers for the genetic characterization of black pine populations, although these markers have been effectively applied in population studies in other plant species.

In this study, SRAP markers were applied to characterize the genetic structure and differentiation of *P. nigra* populations located in protected forested areas in Bulgaria, Greece, and Cyprus.

Materials and methods

Plant material and genomic DNA purification

Pinus nigra Arn. plant material from a total of 175 trees was collected from 3 populations in Bulgaria (municipality of Velingrad, southwest Bulgaria), 3 populations in Greece (the Pindos National Park, northwest Greece), and 2 populations in Cyprus (the region of the Troodos mountains), as shown in Table 1 and Figure 1. Only big-sized trees with an estimated age of over 80–100 years were sampled. Each sample consisted of 50–100 fully developed pine needles, which were collected from the lower pine tree branches, packed in plastic bags, stored in a cooling transportation box until transferred to the laboratory facilities and stored at −80 °C until DNA purification. The frozen pine needles were milled to a fine powder using a TissueLyser II laboratory mill (Qiagen). Genomic DNA was extracted from 100 mg of ground material using a modified CTAB protocol [30]. The concentration of the resulting genomic DNA was determined using NanoDrop 2000 (Thermo Scientific) and was adjusted to a working concentration of 25 ng μL^{-1} with ultra-pure water.

Analysis of SRAP markers

Polymerase chain reaction (PCR) reactions were performed with 50 ng of genomic DNA, 10 pmol from each forward and reverse primer, 2× DreamTaq PCR Master Mix (Thermo) in a total volume of 25 µL. Thirty-five different SRAP primer combinations, according to Li and Quiros [15], were tested. Three primer pairs were selected for analysis of the genetic structure and population differentiation among the analyzed *P. nigra* populations based on the efficiency of the PCR reaction, number of bands and clarity of the obtained electropherograms. The PCR primers were purchased from Macrogen Europe BV. The selected combinations of primer pairs, as well as their sequences, are shown in Table 2. Each EM primer was 5′ labelled with fluoresceine amidite (FAM). The PCR conditions were as follows: initial denaturation for 4 min at 94 °C, 3 cycles of 94 °C for 1 min, 35 °C for 1 min and 72 °C for

Figure 1. Locations of the analyzed *P. nigra* Arn. Populations in Bulgaria, Greece, and Cyprus. The designations and geographic coordinates of the populations are presented in Table 1.

Table 2. Nucleotide sequences of SRAP primers used in the study.

	Forward primer		Reverse primer
EM2	5′-TGAGTCCAAACCGGAGC-3′	ME3	5′-GACTGCGTACGAATTATT-3′
EM7	5′-TGAGTCCAAACCGGTCC-3′	ME1	5′-GACTGCGTACGAATTAAA-3′
EM1	5′-TGAGTCCAAACCGGAAA-3′	ME8	5′-GACTGCGTACGAATTCAC-3′

1 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and final elongation at 72 °C for 1 min. DNA fragments were analyzed on an Applied Biosystems 3130 Genetic Analyzer (Thermo) using 36-cm long capillaries (Thermo), POP 7 polymer (Thermo), and LIZ500 (cat. number 4322682, Thermo) as a size standard. Fragment sizing was performed using the GeneMapper 4.0 software (Thermo).

Data analysis and graphical presentation

GeneAlex 6.5 [31,32] was used for the analysis of molecular variance (AMOVA) and Principal Coordinate Analysis (PCoA). AFLP-surv [33] was used for the calculation of the pairwise fixation index Fst. The genetic structure was analyzed *via* Structure v. 2.3.4, with admixture as the ancestry model, a burn-in of 100,000 iterations and 200,000 MCMC repeats after burn-in. The number of assumed genetic clusters (K) was set from 1 to 10, with 10 iterations for each value of K. The most probable number of clusters (K) was inferred by the Evanno method using Structure harvester v. 0.6.94 (http://taylor0.biology.ucla.edu/structureHarvester/) [34,35]. Clumpak (http://clumpak.tau.ac.il/) was used to calculate an average result over the 10 simulation runs at different K values. ArcMap 10.6.1 (Esri, Redlands, CA, USA) was used to build geographic maps of populations.

Results

Black pine is one of the abundant and economically important native conifers in Bulgaria and Greece and is also abundant in the Troodos mountains in Cyprus. One hundred and seventy-five samples from eight *Pinus nigra* Arn. populations located in protected forested areas in Bulgaria, Greece, and Cyprus were analyzed using three SRAP primer pairs, namely EM2 × ME3, EM7 × ME1, and EM1 × ME8. The primer pairs were selected to generate electropherograms with distinct SRAP peaks and have been efficiently applied for genotyping of other plant species [21,36]. The SRAP analysis with these primer pairs resulted in the identification of 138, 252, and 257 polymorphic bands, respectively (Table 3). Overall, the SRAP genotyping of the entire set of samples with the three primer pairs resulted in the detection of 1804 amplified SRAP fragments. Of these, 647 ones appeared to be

polymorphic, accounting for an average of 215 ± 67 polymorphic loci scored per analysis with a single SRAP primer pair.

The results from the SRAP analysis were used to characterize the genetic structure of the studied populations and cluster the samples using the Principal Coordinate Analysis (PCoA; Figure 2). The calculated Delta K showed a maximum at $K = 2$, indicating that the studied populations formed two major genetic clusters (Figure 2(a)). The constructed barplot, which represents the genetic structure of the analyzed populations at $K = 2$ (Figure 2(b)), demonstrated the formation of one cluster by the three Bulgarian (LE, CH, DR) and one Greek (KO) populations and a second cluster by the two Cyprus (TR1, TR2) and the remaining two Greek (SA, AO) populations. The results from the PCoA analysis (Figure 2(d)) corresponded well to this clustering, as the samples from the two population clusters were well separated on both sides along Coord. 1. Only the samples from the Greek population SA were

Table 3. Summary of SRAP genotyping data obtained following SRAP analysis of 175 studied black pine samples.

Primer pair	Total number of SRAP loci	Number of polymorphic loci	Percent of polymorphic loci
EM7 × ME1	609	252	41.4%
EM3 × ME3	526	138	26.2%
EM1 × ME8	669	256	38.3%
Total	1804	646	38.3%
Average per primer pair	601 ± 72	215 ± 67	

located centrally along Coord. 1, which is related to the admixed genetic structure of this population (Figure 2(b)). However, the genetic structure and PCoA analyses also indicated that further genetic substructure existed among the set of analyzed populations.

The obtained SRAP data was subjected to a second round of analysis assuming the affiliation of the populations to four genetic clusters ($K = 4$). The results showed that the first major cluster from the $K = 2$ analysis remained, including the Bulgarian (LE, CH, DR) and one Greek (KO) population (Figure 2(c)). At the same time, the populations affiliated to the second major $K = 2$ clusters were separated into three distinct clusters, one of which involved the two Cyprus (TR1, TR2) populations, whereas the other two clusters involved samples from the SA and AO Greek populations (Figure 2(c)). The results from the PCoA analysis support the described $K = 4$ clustering pattern, as the two populations from Cyprus (TR1 and TR2) were separated along Coord. 2 from populations SA and AO from Greece, and most of the samples from the AO population were separated along the Coord 3 from the samples of the SA population (Figure 2(e)). The pairwise analysis of the genetic differentiation between the populations using the pairwise Fst values provided further support to the described genetic clustering (Table 4). The lowest Fst value of Fst = 0 was observed between the two Cyprus populations TR1 and TR2, indicating a lack of differentiation

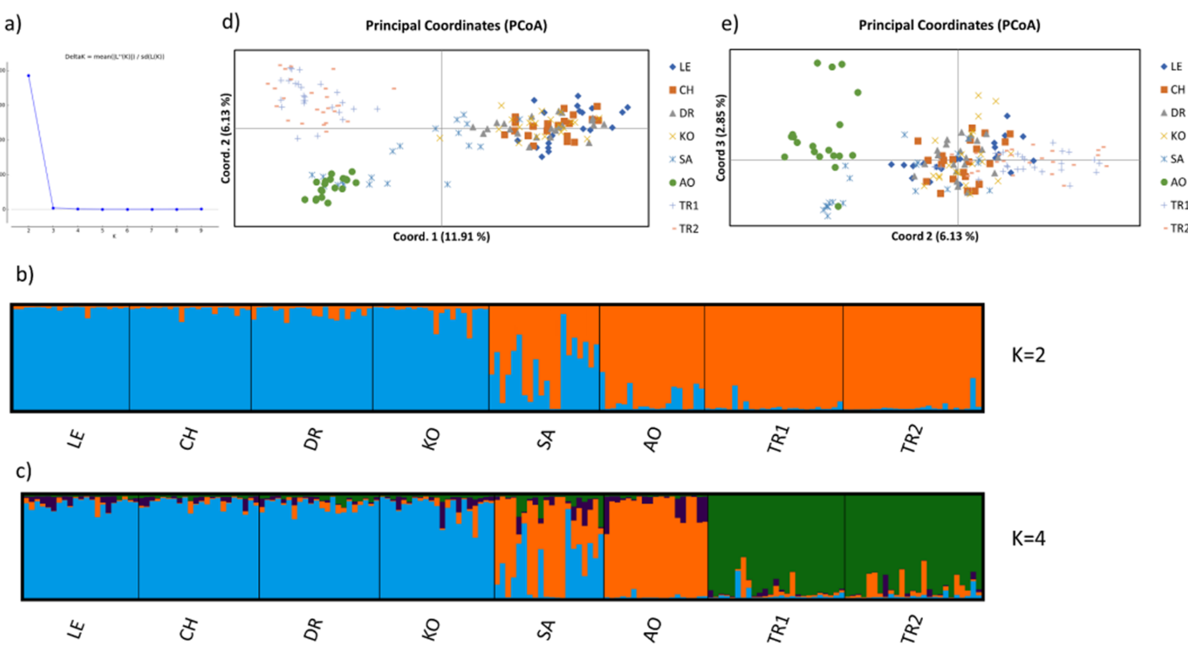

Figure 2. Genetic structure and Principal Coordinate Analysis (PCoA) based on SRAP marker data: (a) estimation of the most probable number of genetic clusters (K), (b) bar plot representing the genetic structure at $K = 2$, (c) bar plot representing the genetic structure at $K = 4$, (d) PCoA (Coord. 1 vs. Coord. 2), (e) PCoA (Coord. 2 vs. Coord. 3). LE, CH, DR, KO, SA, AO, TR1, and TR2—designations of the studied populations according to Table 1.

Table 4. Pairwise comparison of Fst values between populations as a measure of their genetic differentiation. LE, CH, DR, KO, SA, AO, TR1, and TR2—designations of the studied populations according to Table 1.

Country and population		Bulgaria			Greece			Cyprus	
		LE	CH	DR	KO	SA	AO	TR1	TR2
Bulgaria	LE		0.01	0.01	0.01	0.11	0.21	0.17	0.17
	CH	0.01		0.00	0.01	0.11	0.20	0.15	0.16
	DR	0.01	0.00		0.00	0.10	0.20	0.15	0.15
Greece	KO	0.01	0.01	0.00		0.11	0.20	0.14	0.15
	SA	0.11	0.11	0.10	0.11		0.06	0.12	0.11
	AO	0.21	0.20	0.20	0.20	0.06		0.18	0.18
Cyprus	TR1	0.17	0.15	0.15	0.14	0.12	0.18		0.00
	TR2	0.17	0.16	0.15	0.15	0.11	0.18	0.00	

between these two populations. Low genetic differentiation with Fst ≤ 0.1 was also observed between the three populations from Bulgaria (LE, CH, and DR) and population KO from Greece. The highest genetic differentiation, Fst value in the range between 0.11 and 0.21, was found between the populations affiliating to the two major $K=2$ clusters. As expected, an intermediate genetic differentiation was observed between the populations within the second cluster, with Fst = 0.06 for the SA and AO populations and Fst in the range between 0.1 and 0.18 for the SA, AO and TR1, and TR2 populations. The AMOVA results showed that 82% of the observed variation was within the populations, while 18% was among the populations.

Discussion

In this study, the application of SRAP markers on a set of 175 samples from 8 *Pinus nigra* populations resulted in the scoring of an average of 215±67 polymorphic loci per analysis with a single SRAP primer pair, which once again demonstrated the efficiency of this type of molecular markers for characterization of the genetic diversity and differentiation of sets of populations from various plant species reported in other studies [16,17,36–38]. It is difficult to use SRAP genotyping data to set up a molecular clock and carry out simulation analyses on the evolution and speciation of plant species and to evaluate some genetic parameters like heterozygosity, inbreeding coefficient, and haplotype diversity, for example, the application of various SNP and SSR markers in several *P. nigra* studies [3,24,27,39–41]. On the other hand, the high polymorphic information content of SRAP markers makes them very useful for geographic and environmental mapping of the level of genetic diversity and differentiation of local populations of plant species. The results from such applications of SRAP markers could be further used for the identification of local populations, which divert from the overall phylogeographic and environmentally driven pattern of genetic diversity and differentiation of the populations in various plant species.

The phylogeographic structure and evolution of *P. nigra* populations at the European level have been the subject of several studies using chloroplast, mitochondrial, and nuclear SNP and SSR markers [3,24,27,28,39–41]. The studies suggest a complex pattern of differentiation of the European *P. nigra* populations. Their present state is a result of initial fragmentation of an ancestral population into three to six distinct genetic lineages and further complex differentiation of the local populations driven by geographic isolation, environmental conditions, climate changes, or other factors [3,27,39,41]. The results of these studies also indicate that *P. nigra* remains a rather genetically homogeneous species, affected relatively weakly from the isolation by distance and a subject of rather strong gene flow [39,41]. Part of the results from this study agree well with the pattern of genetic diversity and differentiation of *P. nigra* populations reported in the previous studies using various SNP and SSR markers [24,39,41]. Correspondingly, the SRAP analysis showed a lack of or very low intra-country differentiation between the geographically closely located *P. nigra* populations in Cyprus and Bulgaria and a relatively high level of inter-country differentiation between the populations from these geographically distant countries. Both groups of populations affiliate to two separate country-associated clusters in the genetic structure bar plots, constructed at $K=2$ and $K=4$. The genetic differentiation of the three populations (KO, SA, and AO) from Greece was more complex. All three populations are rather closely geographically located in the area of the Pindos National Park of Greece. Surprisingly, one of the tested *P. nigra* populations from the Konitsa (KO) region appeared to be genetically very close to the three distantly located populations from the Rhodope mountains in Bulgaria. At the same time, the KO population showed a high level of differentiation from the geographically much closer AO population from the area of Aoos Lake and moderate differentiation from the other tested population (SA) from the region of Samarina in the Pindos National Park. Considering that the three populations from the Pindos National Park are at geographically close locations, allowing effective gene flow among them [42,43] and that they are less affected by human activities, the most likely cause for the observed inter-population genetic diversity is the environmentally driven differentiation of these populations and/or possible impact of reproductive isolation. The SRAP markers employed in this study could provide a valuable tool for a better understanding of the differentiation process through fine mapping of the *P.*

nigra genetic diversity in the Pindos National Park and subsequent application of parental and by-parental SNP and SSR markers to the selected set of samples from the region. The described differentiation pattern of *P. nigra* populations from the Pindos National Park in Greece corresponds well to the proposed existence of a patchy *P. nigra* differentiation pattern related to the action of complex factors. Overall, the results of this study demonstrate that SRAP markers can be efficiently employed for screening and mapping of the pattern of black pine intra- and inter-population genetic diversity and differentiation, which can later be taken into consideration in forest management and conservation practices or as a basis for further detailed population studies.

Conclusions

This SRAP marker study of the genetic diversity and differentiation of *P. nigra* populations from protected forested areas in three countries demonstrated that SRAP markers are highly efficient for characterization of the population structure, suggesting that they can be successfully applied for higher resolution mapping of the genetic diversity and differentiation of local *P. nigra* populations located in the investigated geographic area. Thus, SRAP markers could be used as an affordable and efficient tool for identifying local population(s) that divert from the observed overall genetic diversity and differentiation pattern of the studied region. SRAP genotyping results can also be effectively used in the design of forest conservation and management practices. The present SRAP study further revealed that the three studied populations from the Pindos National Park, Greece, divert from the established differentiation pattern, as one of the populations showed low genetic differentiation from the *P. nigra* populations in Bulgaria and much higher differentiation from other closely located populations in the Pindos National Park.

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Author contributions

Conceptualization, pm, V.F. and I.A.; Sampling, K.R., pm, V.F., A.S., F.U. and I.A. Methodology, T.Z., K.R., pm, V.F. and I.A.; Software, K.R. and T.Z.; Investigation, T.Z., E.B., A.S. and M.R.; Funding Acquisition, pm, V.F., and I.A.; Writing—original draft, review and editing, T.Z., I.A. and K.R. All authors have read and approved the final version of the paper.

Disclosure statement

The authors declare no conflict of interest

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Data availability statement

All data included in this study are available from the corresponding author [I.A.] upon request.

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