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Studying the Genetic and the Epigenetic Diversity of the Endangered Species *Juniperus drupacea* Labill. towards Safeguarding Its Conservation in Greece

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Abstract: *Juniperus drupacea* Labill is a unique representative tree which, nowadays, has limited geographical range. In Greece, it exists only in the southeastern part of the Peloponnese, and it is labeled as endangered according to the IUCN in Europe. In the light of climatic changes, a conservation plan which will secure its adaptation and resilience is important. Knowledge of the genetic and the epigenetic diversity of *J. drupacea* in Greece can establish a pledge for sustainability. In this study, genetic diversity with amplified fragment length polymorphism (AFLP) markers and epigenetic diversity assessed with methylation-sensitive amplification polymorphism (MSAP) were used for eleven subpopulations of the species. Simultaneously, first assessment between midday water potential (Ψmd) and epigenetic diversity was calculated in order to determine drought response of the species. Results showed that genetic diversity was higher than epigenetic diversity and no subpopulation differentiation was observed. No significant correlations were found between geographic, epigenetic, and genetic diversity, indicating that the genetic diversity is uncoupled from epigenetic diversity. A significant negative correlation between epigenetic Shannon index and Ψmd was found. The holistic research of genetic and epigenetic diversity paves the way for an effective conservation plan for the species.

Keywords: genetic and epigenetic diversity; *Juniperus drupacea*; adaptation; midday water potential

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1. Introduction

The Syrian Juniper (*Juniperus drupacea* Labill) is the only representative tree of the section *Caryocedrus* Endl. of the genus *Juniperus* L. [1]. Nowadays its expansion is restricted, and in Greece exists only in one area in the southeastern part of the Peloponnese peninsula; it is labelled as endangered according to IUCN in Europe. According to NATURA 2000 sites in 1992, it was included in Annex I of Directive 92/43/EEC as a priority habitat type and at the Mt Parnon summit and Malevi Monastery as Special Protected Areas (code: GR 2520006).

J. drupacea is a special plant taxa as it is one of the few woody species that occurs in Europe and Asia, as Sobierajska, et al. [2] pointed out. According to the same authors, they studied the effect of the Aegean sea as a barrier between populations from Europe and Asia. Sobierajska, et al. [2] found six genetically and geographically diverse groups of populations, which probably reflects long-lasting genetic isolation during the Pleistocene. In addition, the location of the evolution and the divergence of the species remains unknown because fossil data is difficult to discover. Towards this direction and in order to investigate its evolution, extinction, and distribution, Boratyński et al. [3] studied genetic differentiation

between 41 populations of *Juniperus oxycedrus* spp. *oxycedrus*, *deltoides*, *macrocarpa*, etc., from the eastern and western Mediterranean habitats of the species. They found a significant increase in diversity from east to west in the Mediterranean Basin and a lack of significant differentiation between European and African populations by using three simple sequence repeat markers discovered from [4]. Furthermore, a significant study about past, present, and future geographic range of the species was implemented by [5] and pinpointed the fact that the species could become endangered in the future, and that conservation strategies should be adopted to allow for preservation of its genetic and morphological diversity.

In the face of the ongoing climatic changes and, due to the fact that forest trees are long-lived organisms with complex life cycles that lack mobility, adaptation is a crucial issue in terms of the sustainability and resilience of forest ecosystems. Forest managers and ecologists face a real challenge nowadays towards predicting the best situation which will favor migration or adaptation for tree species. Forest management and conservation relies on the relationships between genetic material and environmental changes [6]. Apart from the genetic diversity, which is the primary force of evolution, epigenetic forces are proved to significantly affect adaptability, phenotypic variation, expression of genes, drought resistance, etc., in various wood tree studies [7–9]. Epigenetics are defined as the changes that occur and are not obvious in the DNA sequence. DNA methylation is the most studied mechanism because it occurs in all living organisms [7,8]. Furthermore, DNA methylation is the most well studied epigenetic mechanism in forest trees in order to study heritability [10], drought resistance [11], climatic adaptation, which is affected by temperature [12], morphological differentiation [13] anthocyanin biosynthesis [14], floral development [15], and the relation between genetic and epigenetic variation [10,16].

In addition, plant water potential is used to assess plant water status [17]. Leaf water potential becomes more negative whenever the soil becomes drier or more water has to be transported though a plant's conduits to maintain transpiration. Thus, if the water potential in leaves of a similar age exposed to identical microclimatic conditions is compared, it can be used as a water stress indicator [18].

Understanding the connection between epigenetic and genetic diversity is a critical factor in determining the future resilience and survival of populations [17]. As noted by Richards [18], this link determines the extent to which phenotypic variation can be attributed solely to epigenetic effects. To gain insight into the complete adaptation profile and develop conservation and protection strategies, it is essential to examine both genetic and epigenetic profiles of plant populations.

Towards this goal, the primary study's objective is the estimation of genetic and epigenetic diversity for the threatened species of *Juniperus drupacea* in Greece in order to propose management actions for conservation of the species. Furthermore, a research question was also to figure out if this whole distribution of *J. drupacea* is one population subdivided by subpopulations and originated from one genetic pool. Lastly, the first results for water potential and relation to epigenetic and climatic profile will be presented and will be combined with the conservation and management plan in order to highlight potential adaptations of the population towards climatic changes.

2. Materials and Methods

Eleven natural subpopulations were sampled in order to cover all distribution of *J. drupacea* on the Parnonas mountain (Table 1, Figure 1). The study was focused on an area of 740 ha that has been declared as a "Monument of Nature to be preserved" according to the F.E.K. 121D/1980 national law. A total of 105 different trees were sampled, with 5 to 10 trees per subpopulation. We collected needles from trees spaced at least 50 m apart. We covered the whole distribution of the species in the protected area (Figure 1). Total genomic DNA was isolated using the extraction plant kit (Macherey Nagel, Duren, Germany). The DNA was quantified by a UV spectrophotometer (Eppendorf Biophotometer, Hamburg, Germany). After collection, during the same developmental stage and from the same crown aspect, samples were diluted to a working concentration of 10 ng/μL. To prevent any

possible alteration in DNA methylation status, the sampled leaves were promptly frozen, and all samples were subjected to identical treatment for DNA extraction, following the same protocol used for AFLP/MSAP markers. In the current study, we will refer to the sampled populations as subpopulations due to our hypothesis that they derive from a single population.

Table 1. Site code nomination of subpopulations, latitude, longitude, mean altitude, and number of trees studied.

Site Code	Latitude	Longitude	Altitude (m a.s.l.)	Number of Trees
A1	37.32904	22.58457	940	10
A2	37.32679	22.58515	1046	10
A3	37.33298	22.59284	949	10
A4	37.33182	22.59195	964	10
A5	37.32829	22.59287	1048	10
A6	37.32106	22.57229	1062	10
A7	37.31946	22.57489	1093	10
A8	37.31065	22.60086	1270	10
A9	37.31177	22.59781	1273	10
A10	37.32793	22.58405	950	5
A11	37.31380	22.60322	985	10

According to the data of the locally installed meteorological station, annual precipitation for the year 2022 was 774.4 mm, and mean, min, and max temperatures were 13.2 °C, 5.6 °C and 27.5 °C, respectively (Korakaki personal communication).

2.1. AFLP Procedure

Total genomic DNA (200 ng) was digested with 4 U of EcoRI and MseI for 3 h at 37 °C. Digested DNA fragments and EcoRI and MseI adapters were ligated with T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) for 3 h at 26 °C. The resulting DNA was used as primary template DNA in the AFLP analysis. A primer pair based on the sequences of the EcoRI and MseI adapters with one additional selective nucleotide at the 3′ end (EcoRI+A and MseI+C) was used for the first PCR step (pre-amplification, Table 2).

Pre-amplification PCR was performed in a total volume of 20 µL containing 1X Kapa Taq Buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 30 ng of each primer EcoRI+A, MseI+C, 1U Taq DNA polymerase (Kapa Biosystems; Wilmington, MA, USA), and 5 µL of diluted fragments (from the digestion and ligation reaction). Cycling was carried out in a BioRad (Hercules, CA, USA) thermocycler with a 95 °C hold for 30 s followed by 32 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min, followed by a final hold at 72 °C for 10 min. A 5 µL aliquot of the reaction was electrophoresed on agarose to verify amplification; the remaining 15 µL was diluted 5-fold with TE.

Selective amplifications were carried out in 10 µL total volumes consisting of 3 µL of diluted pre-selective template and using the same reaction conditions as for pre-selective amplification but using 30 ng of a MseI primer and 5 ng of an EcoRI primer per reaction. Selective amplification cycling was performed in a BioRad (Hercules, CA, USA) thermocycler with the following program: an initial cycle of 95 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min; then 12 cycles of 95 °C for 30 s with an annealing temp starting at 65 °C for 30 s but decreasing by 0.75 °C each cycle, 72 °C for 1 min; finally, 23 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, with a final hold at 72 °C for 30 min.

The f-AFLP product mixtures were denatured in formamide at 94 °C for 2 min and then subjected to electrophoretic separation using an ABI Prism 3730xl Genetic Analyzer (Applied Biosystems, Woburn, MA, USA). A total of ten selective primer combinations (Table 2) were used to screen for AFLP, with individual hybrids being assessed for the presence or absence of specific fragments. The size of the fragments detected was determined using the Genemapper v4.0 program and an internal standard (GS 500 LIZ, Applied Biosystems, Woburn, MA, USA). Only fragments with a size range

of 150 to 500 bases were counted and analyzed further, in order to minimize the impact of potential size homoplasy [19].

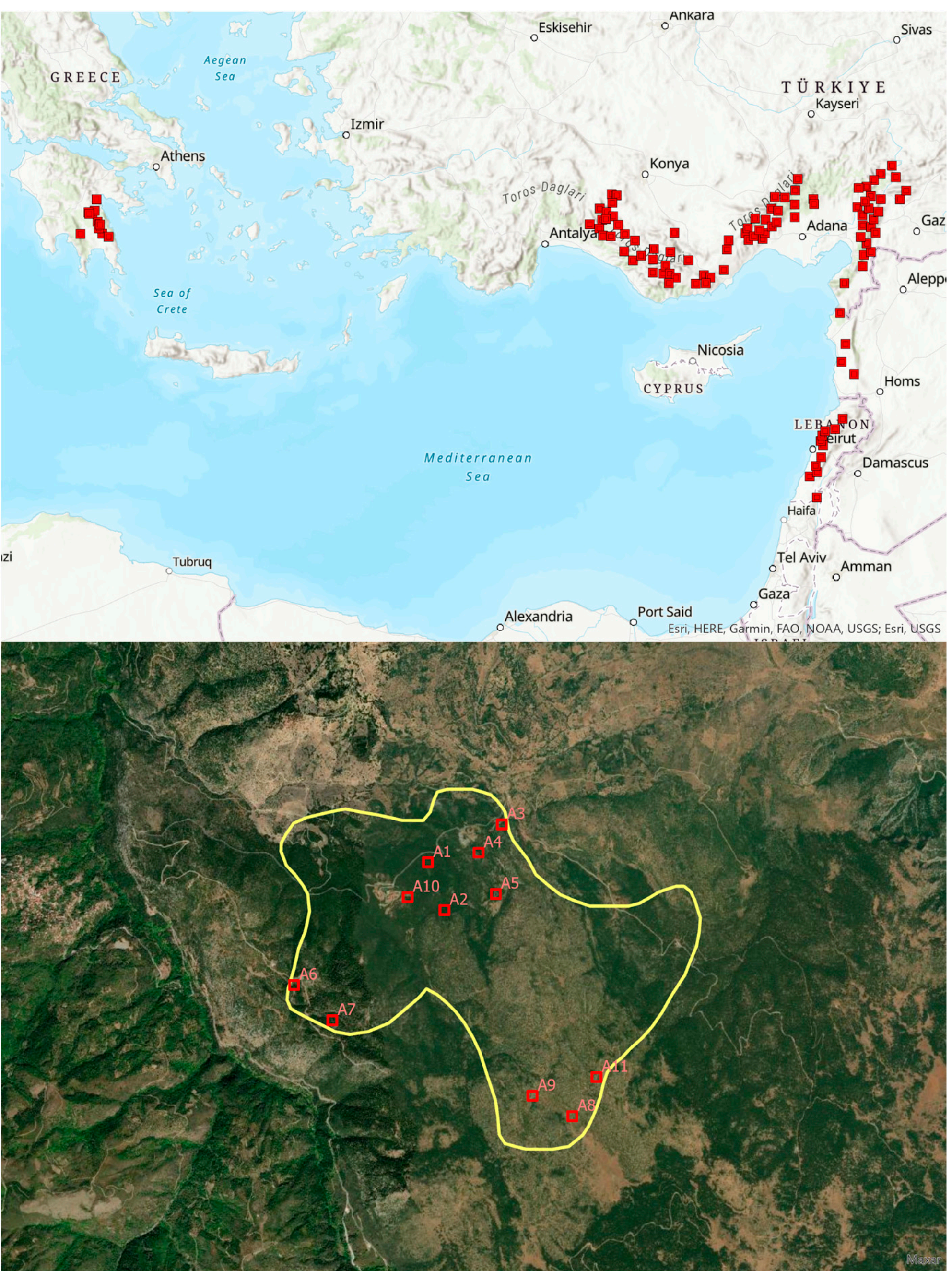

Figure 1. Geographical distribution of *J. drupacea* (**up**) and absolute protection zone of *J. drupacea*, with positions of the 11 subpopulations sampled (**down**).

2.2. MSAP Procedure

To perform the MSAP assay, double digests were carried out using either EcoRI/HpaII or EcoRI/MspI restriction enzymes (Table 2). Genomic DNA aliquots (200 ng) were digested in a 20 µL reaction volume containing 1X "One-for-All" buffer, 4 U EcoRI (New England, Biolabs, Ipswich, MA, USA), and 4 U of either HpaII or MspI enzyme (New England, Biolabs, Ipswich, MA, USA) at 37 °C for 3 h. Following digestion, two different adaptors, one for EcoRI sticky ends and one for HpaII/MspI sticky ends, were ligated to the DNA by adding a mix containing 5 pmol of EcoRI adapter, 50 pmol of HpaII/MspI adapter, 1 mM ATP, 1X "One-for-All" buffer, and 1 U of T4 DNA ligase (Invitrogen) to

each final digestion, and then the solution was incubated at 25 °C for 3 h. The resulting digested and ligated DNA fragments were diluted 5-fold and used as templates for the pre-selective amplification reaction. Pre-amplification reactions were performed using MspI/HpaII-primers in a total volume of 20 µL containing 1X Kapa Taq Buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 30 ng of each primer EcoRI+A, MspI/HpaII+A, 1U Taq DNA polymerase (Kapa Biosystems, Wilmington, MA, USA), and 5 µL of the diluted fragments. PCR pre-amplifications were carried out using two different sets of primers. The pre-amplified fragments were then diluted 10-fold and used as templates for the selective amplifications. Only the EcoRI primers were labeled for selective amplification, using the primer combinations shown in Table 2. Selective PCR was performed in a 10 µL reaction volume containing 1X Kapa Taq Buffer, 2.5 mM MgCl2, 0.08 mM of each dNTP, 5 ng of labeled EcoRI primer, 30 ng of HpaII/MspI primer, 1 U of Taq DNA polymerase (Kapa Biosystems, Wilmington, MA, USA), and 3 µL of diluted pre-amplified DNA. Four primer combinations were used during the selective amplification stage. The entire experiment was repeated twice to ensure fully reproducible MSAP bands for further processing.

Table 2. EcoRI/MseI and HpaII/MspI adapters, and pre-selective and selective primers used for the AFLP and MSAP analysis.

Primer Name	5′ to 3′ Sequence
EcoRI adapter	CTCGTAGACTGCGTACC AATTGGTACGCAGTC
MseI adapter	GACGATGAGTCCTGAG TACTCAGGACTCAT
HpaII/MspI adapter	GACGATGAGTCTCGAT CGATCGAGACTCAT
Pre-selective EcoRI primer	GACTGCGTACCAATTC-A
Pre-selective MseI primer	GATGAGTCCTGAGTAA-C
Pre-selective HpaII/MspI primer	ATGAGTCTCGATCGG-A
Selective EcoRI primers	GACTGCGTACCAATTC+ATG (FAM) GACTGCGTACCAATTC+ACT (HEX) GACTGCGTACCAATTC+AAC (ROX) GACTGCGTACCAATTC+AAG (TAMRA)
Selective MseI primer	GATGAGTCCTGAGTAA-CAA GATGAGTCCTGAGTAA-CAC GATGAGTCCTGAGTAA-CGT GATGAGTCCTGAGTAA-CTC
Selective HpaII/MspI primer	ATGAGTCTCGATCGGATC ATGAGTCTCGATCGGACT ATGAGTCTCGATCGGAAT
EcoRI adapter	CTCGTAGACTGCGTACC AATTGGTACGCAGTC

2.3. Needle Water Potential

Midday water potentials (Ψ_{md}, water potential under maximum daily water demand) were measured concurrently at six out of the eleven natural subpopulations on plants grown under field conditions. Plants were the same as those used for the collection of samples for genetic and epigenetic analyses. Two samples (twigs with needles) were obtained from five *J. drupacea* individuals per natural population, totaling sixty samples. WPmd measurements were conducted between 12:00 to 14:00 using a portable pressure chamber (model PMS 1003, PMS Instruments, Corvallis, OR, USA) [20].

2.4. Data Collection and Statistical Analysis

To convert allele size data from GeneMapper4.0 (Applied Biosystems, USA) into binary form, an AFLP Excel Macro was utilized, which indicated the presence of fragments as "1" and their absence as "0". In order to decrease the effect of potential size homoplasy,

only reproducible fragments within the range of 150 to 500 bases were counted and further analyzed [19]. In addition, GenAlex v6.0 [21] was used in order to calculate parameters, such as the percentage of polymorphic bands (P), Shannon's information index (I), and genetic diversity (He). GenAlex v6.0 was also used to conduct an analysis of molecular variance (AMOVA) and to calculate genetic distances and principal coordinate analysis (PCoA). To further evaluate the population's differentiation, the software AFLP-Surv 1.0 [19] was used to calculate the Fst. The omputation was made after 10,000 permutations and 1000 bootstraps. Moreover, Nei's genetic distance was also calculated with the same program.

For MSAP analyses, comparison of the banding patterns of EcoRI/HpaII and EcoRI/MspI reactions results in four conditions of a particular fragment, as follows: I—fragments present in both profiles (1/1), indicating an unmethylated state (u alleles); II—fragments present only in EcoRI/MspI profiles (0/1), indicating hemi- or fully methylated CG sites (m alleles); III—fragments present only in EcoRI/HpaII profiles (1/0), indicating hemimethylated CHG sites (h alleles); IV—absence of fragments in both profiles (0/0), representing an uninformative state caused either by different types of methylation, or due to restriction site polymorphism [22]. To separate unmethylated and methylated fragments and to test for the particular impact of the methylated conditions II and III, we used the "Mixed-Scoring 2" approach [22].

Epigenetic diversity within subpopulations was quantified using the R script MSAP_calc.r [22] as follows: (i) number of total and private bands (polymorphic subepiloci), (ii) percentage of polymorphic subepiloci (Pepi), and (iii) mean Shannon's information index (Iepi). GenAlEx 6 [21] was employed to compute haploid gene diversity (h) within subpopulations. GenAlEx was also used to conduct an analysis of molecular variance (AMOVA)—separately for each subepilocus class—in order to study the variation of CCGG methylation states (epiloci) among the eleven subpopulations. Separate principal coordinate analyses (PCoAs) were employed to determine the correlation between matrices derived from different marker systems, namely MSAP and AFLP, and the standardized Mantel coefficient [23] was employed. This test was also utilized to assess the similarity between geographic and genetic distances, as well as between geographic and epigenetic distances [23].

For needle water potential, regression analysis was used to compare mean values of Iepi and Ψmd of all six natural subpopulations, using Sigmaplot (v.14.0, Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Genetic Diversity

A total of 10 AFLP selective primer combinations were used and produced 1883 fragments for the 11 subpopulations studied. Mean percentage of polymorphism was 50.11% and ranged from 40.15% for the A10 to 60.28% for the A5 subpopulation, respectively (Table 3). The mean expected heterozygosity (He) ranged from 0.076 to 0.125, with a mean 0.104. The Shannon diversity index (I) offers an alternative method of quantifying biological diversity across multiple scales (genes to landscapes, GenAlex 6.5b) and ranged from 0.127 (A4 population) to 0.202 (A9 population). The number of effective alleles ranged from 0.810 (A10 population) to 1.082 (A8 population) presented a mean of 1.138. The mean expected heterozygosity for genetic diversity was 0.104 and ranged from 0.072 to 0.118.

Analysis of molecular variance portioned 99% of the genetic variation within subpopulations and 1% only among subpopulations (Table 4), whereas principal coordinate analysis explained only 13% of the variance. Parameters for genetic diversity are presented in Table 3 along with the epigenetic results.

Furthermore, Wright's fixation index (Fst) among subpopulations was −0.0037, with low 0.9868 and high 0.0133 p values, respectively, indicating no significant genetic differentiation among subpopulations. Nei's genetic distance also showed the small differentiation between subpopulations and is presented in Table 5.

Table 3. Collection sites of *J. drupacea* subpopulations, total epigenetic diversity, and comparison with genetic diversity indexes at the same individual plants (**P_{epi}**: percentage of polymorphic subepiloci, **I_{epi}**: Shannon's information index based on epiloci, **He and Hepi**: haploid genetic and epigenetic diversity, **P:** percentage of polymorphic bands, **I:** Shannon's information index based on genetic loci, **N.B.:** number of bands, **N.P.B.:** number of private bands.

		AFLP				MSAP		
Population	**P**	**I**	**He**	**Pepi**	**Iepi**	**Hepi**	**N.B.**	**N.P.B.**
A1	47.16	0.15	0.093	30.26	0.20	0.068	486	9
A2	44.77	0.14	0.088	48.91	0.31	0.098	794	125
A3	53.00	0.17	0.109	37.43	0.26	0.088	621	59
A4	41.53	0.12	0.076	32.38	0.21	0.071	533	8
A5	60.28	0.20	0.124	37.37	0.24	0.080	606	48
A6	53.00	0.17	0.106	32	0.21	0.078	533	32
A7	48.43	0.15	0.095	37.37	0.26	0.088	625	33
A8	53.90	0.17	0.107	42.36	0.27	0.089	683	74
A9	59.85	0.20	0.125	40.17	0.26	0.086	658	48
A10	40.15	0.17	0.123	28.51	0.23	0.077	480	22
A11	49.12	0.15	0.096	37.55	0.25	0.084	620	40
Mean	50.11	0.16	0.104	36.75	0.25	0.083	603.5	4.40

Table 4. Hierarchical AMOVA for AFLP and MSAP data (all subepiloci, as well as different subepiloci classes separately) performed by grouping subpopulations according to regions of origin.

Loci/Groups	**Source of Variation**	**d.f.**	**Variance Component**	**Total Variance (%)**	**Φ-Statistics (Φ_{ST})**	***p* Value**
AFLP loci	Among populations	10	1.88	1		
	Within populations	94	152.88	99	0.012	<0.001
	Total	104	154.77	100		
MSAP all subepiloci	Among populations	10	6.80	6		
	Within populations	94	97.28	94	0.061	>0.001
	Total	104	104.09	100		
MSAP *m*-subepiloci	Among populations	10	2.527	6		
	Within populations	94	38.673	94	0.038	>0.001
	Total	104	41.200	100		
MSAP *h*-subepiloci	Among populations	10	1.968	5		
	Within populations	94	34.796	95	0.054	>0.001
	Total	104	36.764	100		
MSAP *n*-subepiloci	Among populations	10	2.311	9		
	Within populations	94	23.819	91	0.088	>0.001
	Total	104	26.130	100		

3.2. Epigenetic Diversity

A total of 8 MSAP selective primer combinations were used and produced 1603 fragments for the 11 subpopulations studied herein. The number of markers per population ranged from 480 (A10 subpopulation) to 794 (A2 subpopulation). The mean percentage of polymorphism was 36.75% and ranged from 32% for A6 to 48.91% for the A2 population, respectively. The mean epigenetic expected heterozygosity Hepi ranged from 0.068 to 0.098

for A1 and A2, respectively. The epigenetic Shannon diversity index Iepi had a mean of 0.25 and ranged from 0.20 to 0.31 for the A2 and A1 subpopulations, respectively.

Table 5. Nei's genetic distance between populations.

Population	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
A1	0.000	0.000	0.000	0.0005	0.0001	0.000	0.000	0.000	0.0008	0.000	0.000
A2	0.000	0.000	0.000	0.000	0.0005	0.000	0.000	0.000	0.000	0.0007	0.000
A3	0.000	0.000	0.000	0.0002	0.000	0.000	0.000	0.000	0.000	0.000	0.000
A4	0.0005	0.0005	0.0002	0.000	0.0007	0.0006	0.000	0.0009	0.0006	0.0002	0.0003
A5	0.0001	0.000	0.000	0.0007	0.000	0.000	0.000	0.000	0.000	0.000	0.000
A6	0.000	0.0001	0.000	0.0006	0.000	0.000	0.000	0.000	0.000	0.000	0.002
A7	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.0002	0.000	0.000
A8	0.000	0.000	0.000	0.0009	0.000	0.000	0.000	0.000	0.000	0.000	0.000
A9	0.0008	0.0007	0.000	0.0006	0.000	0.000	0.0002	0.000	0.000	0.000	0.0007
A10	0.000	0.000	0.000	0.0002	0.000	0.000	0.000	0.000	0.000	0.000	0.000
A11	0.000	0.000	0.000	0.0003	0.000	0.0002	0.000	0.000	0.0007	0.000	0.000

Analysis of molecular variance portioned 94% of the genetic variation within subpopulations and 6% only among subpopulations (Table 4), whereas principal coordinate analysis explained only 17.03% of the variance (Figure 2). Parameters for genetic diversity are presented in Table 2 along with the epigenetic results.

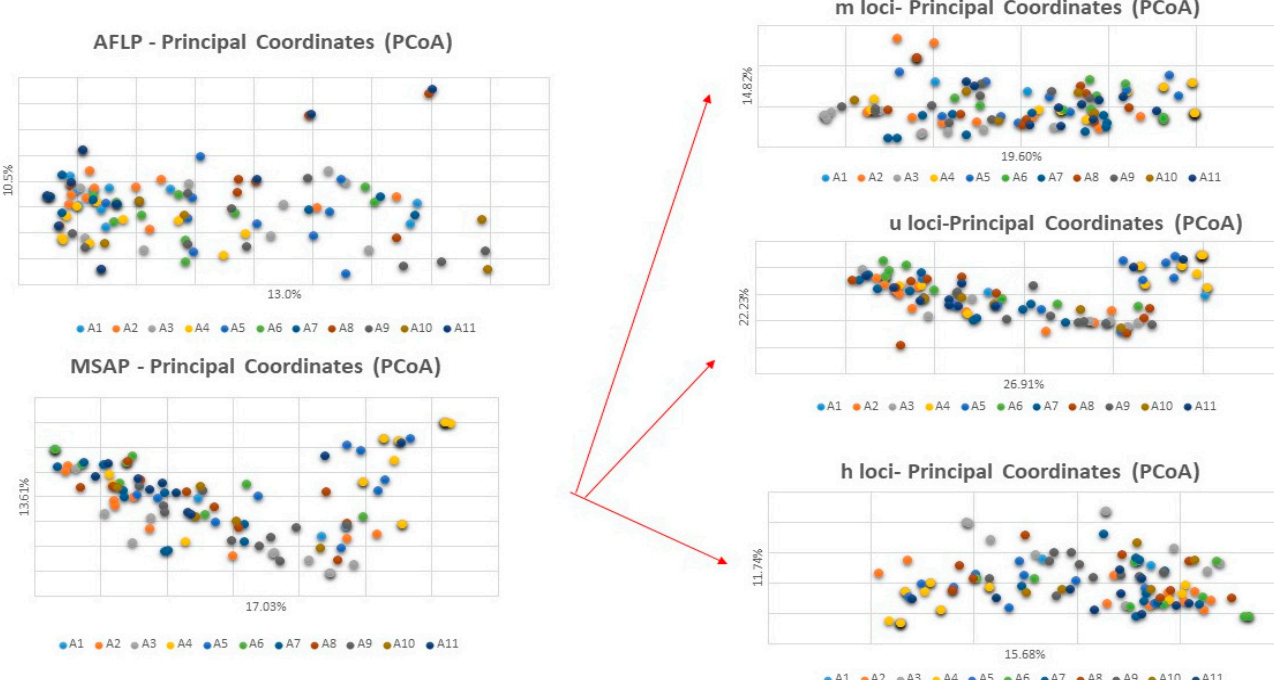

Figure 2. Principal coordinate analysis (PCoA) of genetics (AFLP), epigenetics (MSAP), and partition into three distinct methylation types, namely m-loci, u-loci, and h-loci.

According to the "Mixed-Scoring 2" approach [22] different parameters for each of the methylation profile was calculated for u, m, and h alleles. Each different approach is presented in Table 6.

Table 6. Different methylation profiles for u, m, and h alleles; Ne: number of effective epigenetic alleles; I_{epi}: Shannon's information index based on epiloci, Hepi: haploid epigenetic diversity.

	u Alleles			m Alleles			h Alleles		
Subpopulation	**Ne**	**I**	**He**	**Ne**	**I**	**He**	**Ne**	**I**	**He**
A1	1.081	0.093	0.055	1.128	0.122	0.078	1.081	0.102	0.058
A2	1.136	0.137	0.086	1.158	0.185	0.108	1.114	0.148	0.084
A3	1.122	0.134	0.081	1.126	0.138	0.084	1.124	0.141	0.085
A4	1.100	0.113	0.068	1.126	0.130	0.081	1.075	0.095	0.054
A5	1.132	0.145	0.088	1.141	0.155	0.094	1.072	0.093	0.053
A6	1.133	0.126	0.080	1.126	0.133	0.082	1.094	0.104	0.063
A7	1.143	0.141	0.089	1.145	0.158	0.096	1.098	0.117	0.068
A8	1.196	0.214	0.129	1.130	0.144	0.086	1.078	0.100	0.057
A9	1.140	0.151	0.092	1.127	0.147	0.087	1.101	0.121	0.070
A10	1.134	0.136	0.086	1.126	0.132	0.083	1.100	0.110	0.068
A11	1.162	0.162	0.101	1.130	0.144	0.087	1.083	0.103	0.059
Mean	1.134	0.141	0.087	1.133	0.145	0.088	1.093	0.112	0.065

Separate principal coordinate analysis was performed for AFLP, MSAP, and different types of loci, and the m, u, and h loci and presented in Figure 2. For the genetic analysis (AFLP data), the first two axes explained 13.0% of the total variation and absence of population differentiation. For epigenetic analysis, the first two axes explained 17.0% of the total variation, and, furthermore, 19.6%, 26.91%, and 15.68% for m, u, and h loci, respectively.

3.3. Comparison of Genetic and Epigenetic Indexes

The mean epigenetic diversity (H = 0.083), based on MSAP markers, was slightly lower than the genetic diversity (He = 0.104), but the difference was not significant (t = 4.203, $p = 0.054$, as shown in Table 2). The percentage of polymorphic loci in the epigenetic AFLP structure (36.75%) was slightly lower than in the genetic AFLP structure (50.11%) of the same individuals (Table 2), but the difference was not statistically significant (t = −4.99, $p = 0.518$). Furthermore, the Shannon genetic index (I) did not differ significantly from the Shannon epigenetic index (Iepi, t = 6.840, $p = 0.39$).

3.4. Correlation between Geographic, Genetic, and Epigenetic Variability

Correlation between the genetic and epigenetic variability was explored using a Mantel test. There was a non-significant positive correlation (R = 0.072, $p = 0.087$) between the pairwise genetic (AFLP) and geographic distances. Similarly, there was a non-significant positive correlation (R = 0.076, $p = 0.057$) between epigenetic (MSAP) and geographic distances (Figure 3). Lastly, we also detected a negative non-significant correlation between both genetic distance matrices assessed from the AFLPs and MSAP (R = −0.050, $p = 0.257$).

3.5. Correlation between Epigenetic Variability and Tree Water Status

The correlation between Iepi and Ψmd was tested using regression analysis to compare mean values of all six natural populations. A significant negative correlation was identified (Figure 4, $R^2 = 0.673$, $p < 0.05$).

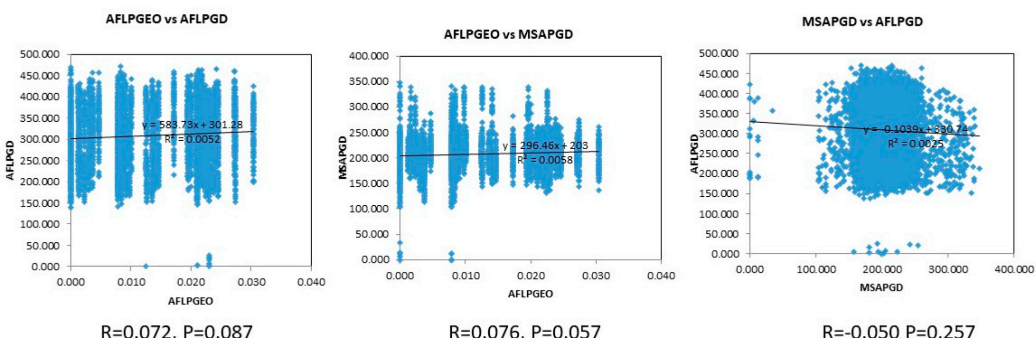

Figure 3. Separate Mantel tests for genetic and geographic, geographic and epigenetic, and epigenetic and genetic distances (from left to right).

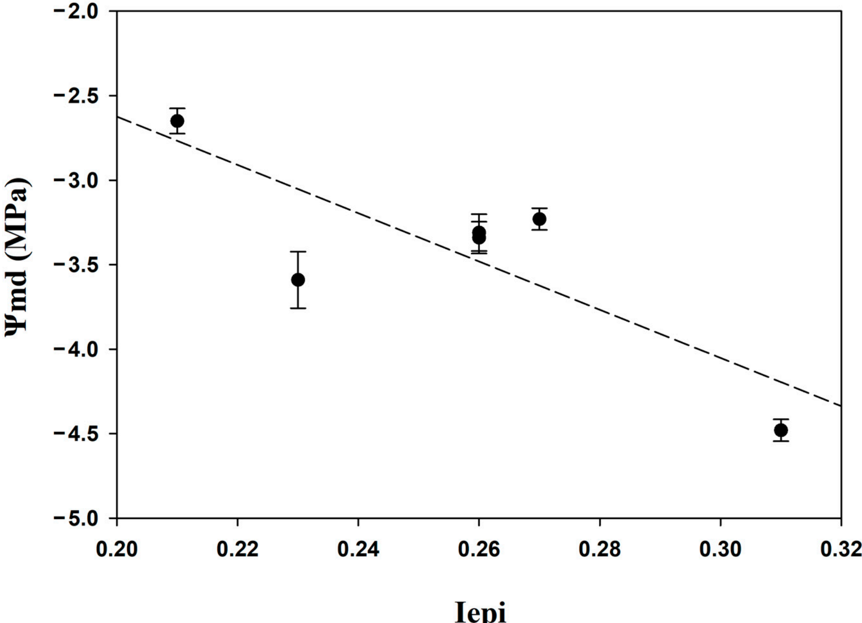

Figure 4. Correlation between Iepi and Ψmd.

When Ψmd was plotted against genetic diversity indexes, non-significant relations were identified, indicating that species genetic diversity was not affected by variations in tree water status.

4. Discussion

Genetic diversity has been traditionally considered as the primary source for biodiversity, evolution, and adaptive capacity. However, in recent years, numerous studies have shown the significant role that epigenetic plays in adaptation ([7,13,24–28] and in phenotypic plasticity [7,29]. Variation in DNA methylation may or may not depend on underlying genetic variation in DNA sequences, which further complicates efforts to resolve the role of DNA methylation [9]. According to Richards [25], there are two main extreme relations between genetic and epigenetic variation that have a profound effect on adaptation. In the light of climatic changes, the in-depth analysis of genetic and epigenetic diversity and their relation is considered crucial for the resilience and adaptation of forest ecosystems [7,28,30].

In this study, we studied genetic and epigenetic diversity of the endangered Syrian juniper (*J. drupacea*) and we attempted to present a first insight towards needle water potential and epigenetic correlation. We found that epigenetic diversity was lower than genetic diversity in both parameters of h and I but not statistically different. The same

results were obtained from other studies [13,31] when they compared Shannon's index diversity. In detail, Rico et al. (2014) analyzed natural *Querqus ilex* populations in relation to the genetic and epigenetic structure. They also found higher genetic than epigenetic diversity (but not statistically significant) and higher percentage of genetic polymorphic loci than from the epigenetic loci, in agreement with our results. Furthermore, in a previous study for natural populations of *Prunus avium* L. we have also found higher genetic than epigenetic diversity [10].

Concerning the correlations between genetic and epigenetic distances, we found a non-significant negative correlation, contrary to [28], where *Fagus sylvatica* populations were studied, and to [32], who studied *Viola elatior* natural populations, as both found significant positive correlations. Furthermore, mean percentage of polymorphic genetic and epigenetic variation was found to be 50.11% and 36.75%, respectively, indicating comparable lower results with previous studies for *Prunus avium* [10], *Pinus nigra* [16], *Fagus sylvatica* [28], and *Quercus ilex* [31]. AMOVA results partitioned 94% and 99% of epigenetic and epigenetic variation, respectively, within subpopulations, and results were in agreement with previous published studies for *F. sylvatica* [28], *P. avium* [10], *P. nigra* [30], etc.

Furthermore, in relation to the principal coordinate analysis (PcoA) performed for genetic and epigenetic diversity, no significant group was formulated, indicating no population differentiation. This is also an indication that the whole area comes from a single pool and that the 11 subpopulations studied are, in fact, subpopulations. Moreover, epigenetic analysis explained a higher percentage of variation (17%) in relation to genetic variation (13%). Furthermore, analysis of PcoA for the separate different methylated loci (m, u, and h loci) indicated that the higher percentage was formulated for the u loci that contained both unmethylated loci and methylated loci in external and internal cytosine. We have to highlight in this part that the study should also be enriched by employing codominant markers, which can help us estimate in-depth more genetic parameters, such as gene flow, number of private alleles, genetic structure, inbreeding coefficient, etc.

Water potential is the driving force for the movement of liquid water through the plant [33]. It is, therefore, a measure of the tree's energy water status. Negative values represent the negative pressure water needs to move downwards from the leaf to the petiole. The more negative the water potential, the more water-stressed the plant is. Furthermore, stomatal activity has been shown to be highly correlated with Ψmd [34]. Our findings are in agreement with the outcomes of the analysis of [35] in 49 studies, who found frequent variations in plant methylation patterns exposed to different environmental stress conditions. In addition, [36] found that natural intraspecific variation in several leaf traits of *Helleborus foetidus* individuals, such as stomatal size and density, which are key factors in water economy, is more frequently related to epigenetic than to genetic markers. On the contrary, in *Quercus ilex*, [31] found that after a natural population in Spain was exposed to a 12-year water stress, the percentage of the hypermethylated loci increased, whereas of the fully methylated loci decreased. However, they found epigenetic but no genetic differentiation. We present this important finding here as an indication for future resilience of the plant, as it is also correlated with epigenetic indexes, which are the immediate response of the plant to biotic and abiotic pressures and can serve as a reference point for management plans.

In-depth analysis of *Juniperus drupacea* subpopulations according to our results showed that whereas mean genetic was higher than epigenetic diversity, the relation between subpopulation's indexes of diversity was not in agreement. For example, the A5 subpopulation 's genetic diversity was 0.124, whereas the epigenetic diversity was 0.080. All results showed that genetic variation for *J. drupacea* was uncoupled from epigenetic diversity, possibly highlighting the long-term existence of the species in the area and the isolation by distance of the subpopulations. A similar result of the unique genetic pool was also found in the study from [2], where the Peloponnese population was separated from the others. Moreover, a recent study pointed out that the Syrian juniper has proven to be really difficult for in vitro culture according to [37]. Due to the abovementioned reasons,

and according to Walas et al. [5], the importance of in situ and ex situ protection of the species is highlighted for ensuring future survival adaptation and resilience in the face of ongoing climatic changes. Therefore, our proposal is to firstly conserve the species in situ and to prioritize the protection of the A9, A5, and A10 subpopulations for the genetic diversity and, afterwards, to conserve the A2, A8, and A3 subpopulations, which show higher epigenetic diversity indexes, indicating higher capability for adaptation. For ex situ protection, cones of the species must be preserved in the national gene bank of Greece in order to maintain the unique genetic pool of the species. Lastly, experiments about drought tolerance in relation to genetic and epigenetic diversity should be further implemented in order to qualify the adaptation ability of *J. drupacea*.

5. Conclusions

Juniperus drupacea is a valuable species, which, nowadays, has restricted geographical range. To enable its sustainability, conservation measures should be taken in the light of climatic changes [5]. In this study, an evaluation of its unique genetic and epigenetic diversity was assessed covering the species range in the area of the Peloponnese. Future research should also incorporate codominant markers in order to further access the genetic parameters of this important population of *J. drupacea*. Furthermore, we correlated water potential with epigenetic and genetic diversity indexes, and we discovered that epigenetic diversity plays a significant role in the ability of the tree to cope with future drought periods. The study proposes first planning conservation actions, both in situ and ex situ, in order to pave the way for the sustainability of the species in relation to the epigenetic and genetic pool of the subpopulations. Beyond this study, we should also focus on understanding the water stress and epigenetic correlation in order to assess the capability of the species to retain and pass the epigenetic marks through generations.

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