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Brief Report

Conservation Genetics of the Only Honeysuckle Azalea (*Rhododendron luteum*) Population Present in Greece

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Abstract: Honeysuckle azalea (*Rhododendron luteum*) has only a single population occurrence in Greece, on Lesvos Island of the north-eastern Aegean Sea. The genetic diversity of this population was studied in a population of $n = 37$ individuals randomly sampled in a transect spanning between the highest and lowest natural altitudinal distribution limits in Mt. Ordymnos, SW Lesvos. A modified DNA extraction and isolation protocol was used to overcome problems of DNA quality due to secondary metabolite activity. Genetic variation was investigated based on molecular Inter Simple Sequence Repeat (ISSR) markers. Results showed the presence of a sufficient amount of genetic diversity for the maintenance of adaptive potential. Genetic diversity was lower but comparable to that of other *Rhododendron* species sampled from the centre of their natural distribution, despite the relatively small population size, negative anthropogenic pressure and population isolation due to the island environment. Some structuring of genetic diversity was indicated based on a PCoA analysis and the genetic distance dendrogram, while spatial autocorrelation was highly significant. Results point towards the need to assign a protection status to the whole area of the species' natural distribution on Lesvos Island. Moreover, it is proposed that an in situ Gene Conservation Unit (GCU) be established in the core of this population in Lesvos as part of the Network of the European Forest Genetic Resources Programme, while the establishment of ex situ conservation is also advised.

Keywords: *Rhododendron luteum*; azalea; conservation genetics; ISSR; GCU; Lesvos Island

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1. Introduction

Honeysuckle azalea, or yellow azalea (*Rhododendron luteum* (L.) Sweet; Ericaceae), is a deciduous, insect-pollinated shrub with a mature height of about 2–4 m. The *R. luteum* genome size is rather large for a plant genome (approximately 6.2 pg per nucleus; [1]). Honeysuckle azalea is an ecologically valuable species due to its soil stabilisation properties. It has an economic value as well due to its importance in landscape architecture. The most attractive feature of *R. luteum* is its fragrant funnel-shaped yellow flowers, which typically appear in late spring to early summer [2]. Their prominent stamens are borne in clusters, creating a striking display, and this stunning inflorescence (usually in April and May) classifies the species as an important ornamental plant. Through breeding, more than 1000 varieties and cultivars [3] with a variety of flower colour and growth habits have been developed to capitalise on the aesthetic value of the species.

Moreover, *R. luteum* stems, leaves and flowers contain ample amounts of highly bioactive phenols and flavonoids [4] but, most importantly, grayanotoxins [5]. These are also known as mad honey toxins and can be lethal for animals and poisonous to humans when consumed in sufficient quantities, affecting the cardiovascular and nervous systems [4,6]. When consumed in the form of honey, they present psychoactive and hallucinogenic effects [4]. Grayanotoxins present an economic opportunity for pharmaceuticals due to their medicinal potential; they have proven analgesic, antiviral and antifungal properties and are considered effective for sexual dysfunction, hypertension, heart disease, diabetes and

gastrointestinal disorders, while *R. luteum* leaf extracts are cytotoxic for human cancer cell lines [7–10]. *R. luteum* leaf extracts exhibit selective cytotoxicity, especially against colon and liver cancer cells, compared to normal fibroblast cells [10]. Grayanotoxins also have many uses in traditional medicine in China, Nepal and Turkey [8]. An *R. luteum* leaf tea is used to that effect as an antifungal, anti-inflammatory and analgesic remedy [8,9,11].

R. luteum is native with sporadic appearances in southern Poland, Belarus, Ukraine, Austria, Slovenia, Croatia, northern Asia Minor and the Caucasus Mountains. It is also found in Greece, but it is rare, being present in only one location, on the island of Lesvos. Lesvos is a large island (1636 km^2), the third-largest island in Greece and the eighth-largest in the Mediterranean, located in the north-eastern Aegean Sea, not far from the Asia Minor coast (Figure S1). Lesvos was part of Aegiis, the large land massif that connected the Hellenic mainland with Asia Minor in pre-historic times [12], and its current climate depicts a transition from the Mediterranean winter rain climate to the continental steppe climate of Asia Minor [13]. Lesvos has a unique, extremely heterogeneous geomorphology, which is strongly linked to its high floristic diversity [14,15]. Limestones, marbles and schists dominate the south, southeast, east and north-west of the island, while volcanic tuffs, volcanic rocks and basalts dominate the central, west and south-west parts [12,16]. The diverse flora of the island consists of 1516 species that belong to 597 genera και 119 families [13,17]. The *R. luteum* population is considered a relic of the Pontic flora [14,15,18,19] and is found in the understory of *Pinus nigra* and *Pinus brutia* forests, either as the dominant understory species or in mixture mostly with *Arbutus unedo* [14,15,20]. It is present at an elevation of 60–760 m in a locally restricted natural distribution of about 40 km^2 [18,21]. It has been traditionally suppressed by herders and farmers due to its toxic effects on grazing animals [4,6]. The species is classified under the IUCN category VU (vulnerable) [20] due to its small area of natural distribution and the small size of the local (sub)-populations.

This unique and isolated island population is the focus of this study, which aims to discern the extent and structure of its genetic diversity. The specific objectives of this study pertain to the investigation of (a) the levels of genetic diversity of this island population compared with core/mainland populations of *R. luteum* and other *Rhododendron* species; (b) the presence of genetic structure, i.e., whether genetic diversity is purely distributed at random in a spatial sense or not; (c) the evaluation of contemporary standing genetic diversity; and (d) if the levels of genetic diversity signal a need for the species genetic conservation and—in conjunction to the above—if the population genetic parameters associated to genetic diversity and structure are within the minimum critical values needed for conservation measures to be meaningful.

This study explores the importance of rare, remote and isolated populations that nevertheless represent flag species in their restricted environment and investigates their value in plant conservation genetics and, more broadly, in the biodiversity preservation of highly diverse island ecosystems.

2. Materials and Methods

2.1. Plant Material

In total, $n = 37$ sampled individuals of *R. luteum* were collected from Mt. Ordymnos, Lesvos Island, Greece (39°12′27″ N 26°06′01″ E), at the end of April 2019. This population is found mostly in the understory of *Pinus brutia* and *Pinus nigra* forests. Sampling followed an altitudinal transect progressing from high to low elevation (from the vicinity of Profetes Elias peak towards Zoodochos Pigi, Parakoila, Greece), starting from the highest altitudinal occurrence of the species and keeping a minimum of 30 m between successive samples to reduce the possibility of sampling filial structures. It has been shown that genetic variation in plant species depends on altitude [22] since altitudinal gradients are associated with a number of environmental variables that may potentially affect population genetic variation [23]. Besides environmental variables, human pressure may also be a factor in the *Rhododendron* population studied, as negative anthropogenic pressure from herders decreases with altitude.

2.2. DNA Extraction

Obtaining high-quality and -quantity DNA from leaf samples proved to be a challenge. We used the following protocols: (1) CTAB [24], (2) NucleoSpin® Plant II Mini kit for Genomic DNA from plants (Macherey—Nagel; (https://www.mn-net.com/nucleospin-plant-ii-mini-kit-for-dna-from-plants-740770.50 (accessed on 1 December 2023)), (3) NucleoSpin® Tissue Mini kit for Genomic DNA from cells and tissue (Macherey—Nagel (https://www.mn-net.com/nucleospin-tissue-mini-kit-for-dna-from-cells-and-tissue-740952.50 (accessed on 1 December 2023)), (4) Qiagen DNeasy Plant Pro Kit (Qiagen; https://www.thermofisher.com/order/catalog/product/4452222 (accessed on 1 December 2023)) with the provided PS solution for removing products of the plant's secondary metabolism and (5) Kobayashi et al. [25] with modifications [26]. The purity and quality of the extracted DNA were determined by electrophoresis on 1% (w/v) agarose gel and using a Quawell Q5000 spectrophotometer (Quawell Technology, Inc., Sunnyvale, CA, USA).

2.3. PCR Amplification Using Inter-Simple Sequence Repeat (ISSR)

Six ISSR markers (University of British Columbia, UBC), which were unambiguously scorable, were selected to investigate genetic diversity statistics. These were UBC807, UBC811, UBC827, UBC834, UBC841 and UBC860). ISSR markers that provide repeatable results are a sound choice for organisms when genetic information is lacking. The primers used have been successfully applied before in plant population genetics research and in *Rhododendron* studies in particular [27–30]. PCR reactions were performed in a final volume of 10 µL and contained ~25 ng of DNA, 1× Kapa Taq Polymerase Buffer (Kapa Biosystems, Wilmington, NC, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.65 mM primer and 0.3 U Taq polymerase. The PCR program used in an Eppendorf Mastercycler ep thermocycler (Eppendorf, Hamburg, Germany) was 95 °C for 15 min, followed by 35 cycles with the following steps: 45 s at 95 °C, 1 min at 50 °C and 2 min at 72 °C and a final elongation time of 8 min at 72 °C.

2.4. Gel Electrophoresis

PCR products were stored at −20 °C until they were visualised on 1.5% (w/v) agarose gels after staining with ethidium bromide along with an MWD100 DNA ladder (NIPPON Genetics EUROPE, Düren, Germany). The stability of the results was checked using twice- and thrice-repeated PCRs.

2.5. Data Analysis

Genetic diversity statistics were calculated using GenAlEx 6.5.03 [31,32]. Genetic distances from binary genotypic data were calculated according to Huff et al. [33], which essentially provides a Nei and Li non-Euclidian distance [33]. This distance was chosen as it could be employed to investigate genetic differentiation between individual samples and then be utilised in the spatial autocorrelation analysis. The genetic distance matrix was imported to MEGA 10.2.6 [34] to construct a UPGMA dendrogram. A principal coordinate analysis (PCoA) was also performed in GenAlEx. Sample spatial autocorrelation was investigated according to the methodology of Smouse et al. [35,36]. The analysis was performed in multivariate space, taking into account all loci, and results were summarised in a correlogram. The overall statistical significance was assessed based on the nonparametric heterogeneity test suggested by Smouse et al. [36] and implemented in GenAlEx. The COLONY software 2.0.6.7 (London, UK) [37] was employed to estimate effective population size by utilising the sibship assignment method [38]. This method is suitable for estimating effective population sizes from single-timepoint data of a population. Furthermore, it is one of the few methods available for binary haploid genetic data, making it ideal for the purposes of the present study.

3. Results

3.1. DNA Extraction and Genetic Diversity

Out of the five DNA extraction and isolation protocols tested, the highest quantity and best quality of extracted and isolated DNA resulted from two: the Qiagen DNeasy Plant Pro Kit and the Kobayashi et al. [25] modified protocol [26]. All six ISSR primers used were applied successfully, and a total of 51 ISSR loci were detected (8.50 loci per primer). Polymorphism was high (P = 90.2%). The following genetic diversity estimates were revealed (standard errors in parentheses): number of different alleles Na = 1.902 (0.042), number of effective alleles Ne = 1.446 (0.050), unbiased expected heterozygosity (Nei's gene diversity) He = 0.275 (0.025) and Shannon's information index I = 0.410 (0.032) (Table 1). Na presents the number of alleles detected in the population on a per locus basis, while Ne is the number of equally frequent alleles that it would take to achieve the same expected heterozygosity as in the studied population. The expected heterozygosity (He) indicates the proportion of heterozygous genotypes in the population expected under Hardy–Weinberg equilibrium, whereas the Shannon information index (I) refers to the weighted arithmetic mean of the proportional abundances of different alleles in the population [31,32]. The above results were compared to those in the available literature on genetic diversity revealed by dominant markers (ISSR, AFLP and RAPD) in rhododendrons (Table 1).

Table 1. Genetic diversity results in honeysuckle azalea (*Rhododendron luteum*) from Lesvos Island, Greece, and results obtained from other *Rhododendron* species using dominant molecular genetic markers (P: percent polymorphic loci; Ne: number of effective alleles; He: expected heterozygosity (Nei's gene diversity); I: Shannon information index).

Species	Genetic Entry	Marker	P (%)	Ne	He	I	Reference
R. luteum	Natural population	ISSR	90.20	1.446	0.275	0.410	This study
R. triflorum	Natural populations	ISSR	98.30	1.575	0.338	0.508	[27]
R. aureum	Natural populations	ISSR	87.43	-	-	0.459	[28]
R. moulmainense	Natural populations	ISSR	57.58	-	0.163	0.256	[39]
Rhododendron sp.	Accessions	ISSR	91.90	1.690	0.390	0.570	[29]
Rhododendron sp.	Cultivars	ISSR	96.99	-	-	-	[30]
R. triflorum	Natural populations	AFLP	95.86	1.959	0.306	0.464	[27]
R. protistum var. *giganteum*	Natural populations	AFLP	66.67	-	0.240	0.358	[40]
Rhododendron, 5 species	Natural populations	AFLP	92.84	-	-	0.556	[41]
R. aureum	Natural populations	RAPD	95.16	-	-	0.479	[28]
Rhododendron, 29 species	Accessions	RAPD	98.03	-	-	-	[42]

We have made an attempt to provide a draft estimate of effective population size (Ne) and pertinent confidence intervals (CIs) using COLONY. The software produced results assuming random mating (Ne = 26, 95% CIs = 15–46) and rather more conservative estimates without this assumption (Ne = 20, 95% CIs = 12–38).

3.2. Genetic Differentiation

The PCoA results resolved, in two-dimensional multivariate space, about 25% of the total variation and portrayed the presence of a single but loosely formed cluster (Figure 1), as expected by the occurrence of single random-mating populations. However, some structure with the cluster could be discerned, as many of the individuals of the higher altitude end of the sampling area were present in opposite quadrats compared to the individuals sampled from the lower end of the transect (Figure 1).

The UPGMA dendrogram of the sampled individuals depicted rather more clearly the loose substructure seen in PCoA (Figure 2). One large cluster included most of the sampled individuals, while a small second cluster contained primarily the individuals at the end of the sampling transect.

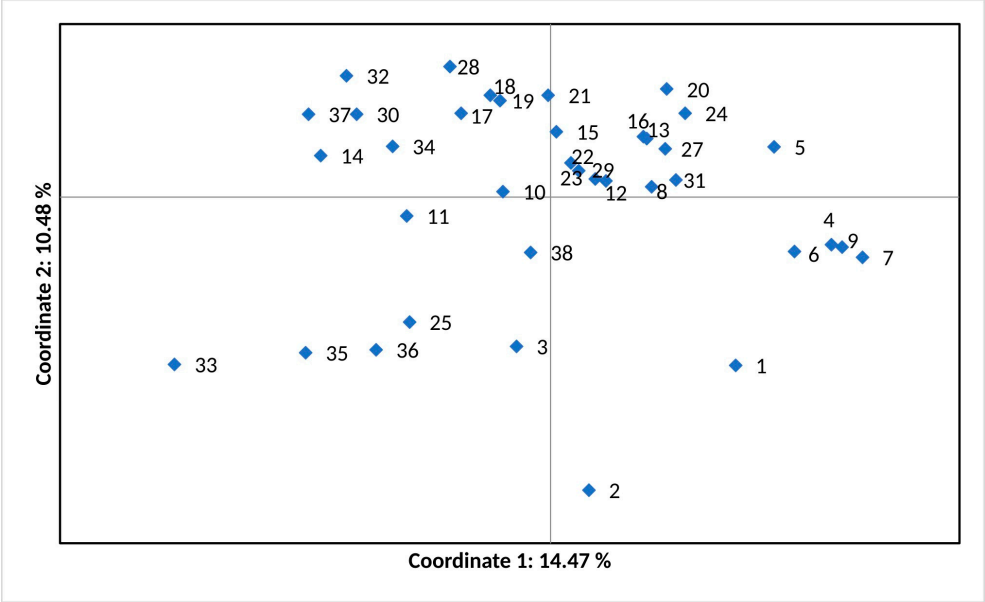

Figure 1. PCoA biplot of the honeysuckle azalea (*Rhododendron luteum*) individuals from Lesvos Island, Greece, used in this study.

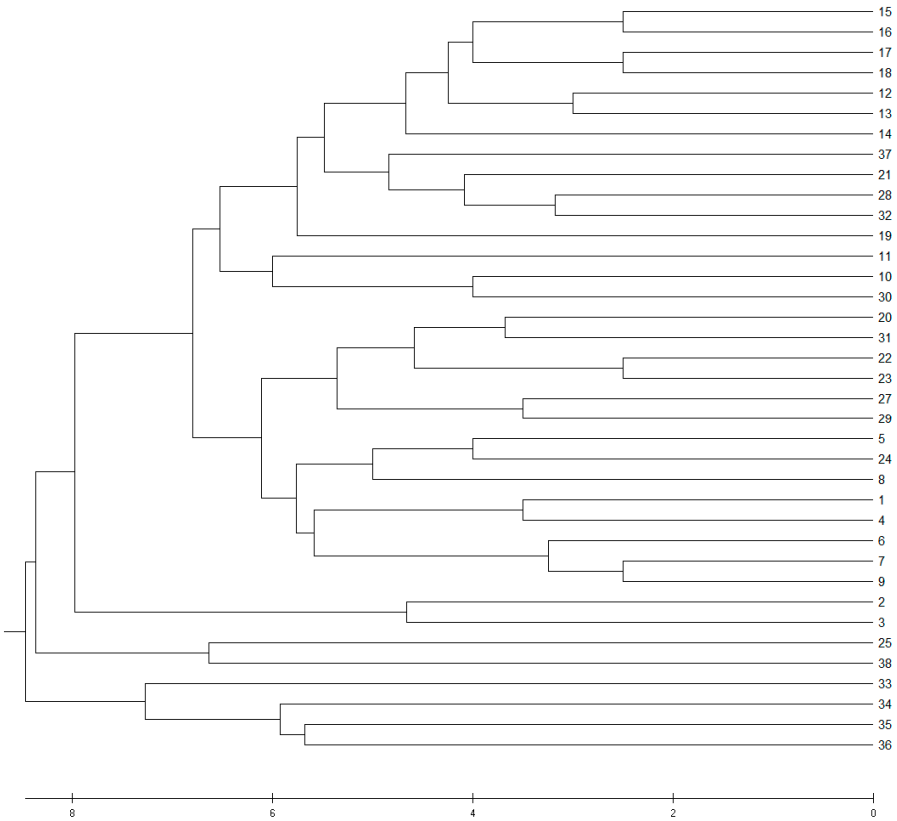

Figure 2. UPGMA dendrogram based on pairwise genetic distances [33] of the Honeysuckle azalea (*Rhododendron luteum*) individuals from Lesvos island, Greece, used in this study.

3.3. Spatial Autocorrelation Analysis

In order to investigate the above observations further, we performed a spatial autocorrelation analysis [35,36]. A non-random distribution of genotypes in space was indicated. Results (Figure S1) showed that there is indeed a highly significant correlation between the genetic distance and the geographic location of the individuals in space (Ω = 60.017,

$p = 0.001$), in this case, with particular regard to their altitudinal position. Individuals in different altitudinal classes showed a small-scale genetic structure that correlated with their geographic location.

4. Discussion

The percentage of polymorphic loci was within the range reported for other *Rhododendron* species using ISSR markers (P = 57.58–98.30; Table 1) and also within the range reported when other dominant molecular markers were employed. On the other hand, the number of effective alleles (Ne) was lower compared to a study in *R. triflorum* using ISSR and AFLP markers in investigating natural populations [27] (Table 1) and in an ISSR analysis of *Rhododendron* sp. accessions [29] (Table 1). The expected heterozygosity (Nei's gene diversity) was also lower compared to all studies in other *Rhododendron* species (Table 1), except one study in natural populations of *R. moulmainense* [39] that also used ISSR markers (Table 1); however, this study reported the use of populations declining in size [39].

Shannon's diversity index (I) [43] is perhaps a parameter that is more optimal to be used for comparisons across diverse study types and different dominant molecular markers. This is because Shannon's diversity index (I) is simply related to the weighted arithmetic mean of the proportional abundances of different alleles and can offer some very good statistical properties for measuring biological information across multiple scales, from individual loci to natural populations [31]. The Shannon diversity index (I) found in this study was lower than the values reported in the majority (75%) of other studies in *Rhododendron* using dominant molecular markers and different genetic entries (Table 1). Moreover, the only studies that presented lower Shannon index values were the ISSR study on the declining *R. moulmainense* natural populations [39] discussed above and the AFLP study of the endangered *R. protistum* var. *giganteum* that used only two known endemic populations present in a restricted geographical area [40]. The levels of genetic diversity found are considered to be representative of those of the underlying population, as our sample size ($n = 37$) is sufficient for this type of study, where $n = 20$–30 is considered adequate [44,45]. The other ISSR studies in rhododendrons that considered natural populations, accessions and cultivars had an average sample size of $n = 20$ (range $n = 3$ to $n = 46$) (Table 1), while, when only natural populations were considered, the average sample size used was $n = 18$ (range $n = 10$ to $n = 28$) (Table 1). The only study with a sample size similar to ours can be considered as the study of Wu et al. [40] ($n = 28$), which employed AFLP markers (Table 1) where genetic diversity was lower (I = 0.358, whereas in our study I = 0.410). When considering natural population studies using ISSRs, the most similar to our sample size was the study of Trung et al. [39], which used a population sample size almost half that of ours ($n = 20$). In this study, genetic diversity was also found to be lower (I = 0.256; Table 1) than in the present study.

The two estimates of effective population size derived under the assumption of random mating (Ne = 26) and under the assumption of non-random mating (Ne = 20) are both very small. The results above indicate the presence of a small-scale genetic structure, which implies some non-random mating; hence, the actual Ne may be closer to the lower end of this range than the upper end. In any case, these estimates are half, or less than half, the critical population size where genetic drift becomes much more important than selection [46] and 20–25 times lower than the current acceptable level of effective population size for maintaining genetic diversity and adaptive capacity ($Ne \geq 500$; see [35] and references therein).

The results from the UPGMA dendrogram, the PCoA biplot and the spatial autocorrelation analysis indicated the presence of a fine-scale genetic structure, as the groups of individuals from the high and low limits of the sampling transect clustered separately. It appears that the environmental variables associated with the altitudinal gradient and potentially a differential anthropogenic pressure (it can be safely postulated that herders may suppress the rhododendrons more at the lower than at the higher end of the gradient) may have contributed to such a fine-scale structure. This finding comes somehow in agreement

with the floristic report of Bazos and Yannitsaros [18] who consider the extremes of the sampled transect as different localities in the occurrence of *R. luteum* in Lesvos. Adaptation to different altitudes has been implied in rhododendrons, for instance, in *R. ovatum* (Wang et al. 2021), while the association of genetic diversity and differentiation with altitude has also been suggested in *R. aureum* [28]; however, these studies did not investigate small-scale differences in genetic diversity and structure as this study did. Overall, in spite of its small census (and effective) population size, the population under study portrays some sub-structuring associated with distance and altitude.

The *R. luteum* population in Lesvos presents a lower genetic diversity and low effective population size when compared to populations of other *Rhododendron* species from continental areas and from the core of their natural distribution. However, the genetic diversity parameters obtained were considerably higher than those of *Rhododendron* species populations that are either of declining size or endangered. Island populations often contain lower levels of gene diversity [47] and, in fact, lower genetic diversity and low effective population size have been reported in *Rhododendron* species as well, for instance, in *R. tsusiophyllum* in Izu island, Japan [48]. Lower island genetic diversity can be the result of a founder effect, absence of gene flow from the source population and stochastic processes, such as genetic drift owing to restricted habitats as well as limited census and effective population size [49]. Nevertheless, strong selection, especially due to environmental differences between the island and continental populations, may also come into effect as a significant determinant of genetic diversity in islands [50], as it was also found in *R. tsusiophyllum* in Izu island [48].

5. Conclusions

In the *R. luteum* population of Lesvos island, genetic diversity remains at considerable levels despite genetic isolation, a restricted habitat, small census and effective population size, and adverse human impacts in the past. As the species is predominantly outcrossing and substantial gene flow has been observed in other *Rhododendrons* [51], the maintenance of a strong metapopulation structure in the *R. luteum* (sub)-populations in Lesvos Island coupled with a reduction in grazing and human impact in recent years may have contributed to the maintenance of notable genetic diversity. In this respect, future research should focus on completing the analysis of sub-populations in the entire natural distribution and the use of codominant markers such as SSRs and SNPs.

In conclusion, the following measures, regarded as applications of this research, are advised:

1. Expansion of the GR4110003 NATURA2000 protected area in order to include all of the *R. luteum* natural distribution in Lesvos Island.
2. Establishment of an in situ gene conservation unit (GCU) within the European Forest Genetic Resources Programme (EUFORGEN; https://www.euforgen.org/) network; as all the respective minimum requirements [52] are met, the species is characterised in Greece as vulnerable (VU; IUCN) while this population is the only natural occurrence of the species in the country. This unit can also take the form of a conglomeration of small conservation micro-reserves [53], depending on the distribution of genetic variability within and among sub-populations, and given the association between altitude and genotype already found.
3. Establishment of genetic monitoring [54–56] to ensure long-term maintenance of genetic diversity.
4. Ex situ conservation, which can be enacted initially by the establishment of (a) a seed collection according to genetic conservation principles and (b) a plantation by seed outside the natural range.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f15010005/s1, Table S1: Molecular genetic data from 6 ISSR primers and 51 loci used in the study of in honeysuckle azalea (*Rhododendron luteum*) from Lesvos Island, Greece. Figure S1: Spatial autocorrelation analysis [35,36] of the Honeysuckle azalea *Rhododendron luteum*

individuals used in this study. Upper (U) and lower (L) confidence limits bound the 95% confidence interval about the null hypothesis of no spatial structure as determined by 999 permutations. Error bars bound the 95% confidence interval about r as determined by 999 bootstrap resamplings.

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Data Availability Statement: The data presented in this study are available as Supplementary Materials in Table S1.

Conflicts of Interest: The authors declare no conflict of interest.

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