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Genetic Variation of a Lentil (*Lens culinaris*) Landrace during Three Generations of Breeding

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Abstract: Genetic differentiation between 40 lentil genotypes was tested using molecular markers. The genotypes were produced from a Greek landrace of commercial interest via the honeycomb breeding methodology, i.e., single-plant selection in the absence of competition, across three successive pedigree generations. The selected genotypes from each generation were examined for genetic relationships using 15 SSR molecular markers with HRM analysis. As expected, low variation among consecutive generations at the level of 2.5–7.7% was detected. Analysis of molecular variance (AMOVA) revealed that partitioning of this variation was at higher percentage within each generation's population than between them. Population structure analysis indicated that ongoing selection could effectively shift the allelic composition in each generation. The applied honeycomb breeding methodology that effectively improved progeny yield and seed quality increased the percentage of favorable alleles altering allelic composition but not eliminating genetic variation of the breeding population.

Keywords: plant breeding; molecular markers; SSR; HRM

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1. Introduction

Lentil is one of the most important cultivated pulses worldwide, being high in protein, a good source of vitamins A and B, fiber, potassium, and iron, ideal for vegetarians, and an inexpensive food for low-income people [1]. Even though lentil has been cultivated for almost 10,000 years, systematic breeding research has been carried out only recently [2]. Due to the shortage of improved varieties, farmers prefer to cultivate landraces because they adapt readily to local environments [3].

Lentil cultivation suffers frequent production failures due to biotic and abiotic constraints. A wide range of pathogens can infect lentils, with fungal diseases being the most important [4]. Although focus has been concentrated on biotic stresses because of the obvious and serious reduction in yield and quality, abiotic stresses are also a major factor that cause a great loss in lentil production. Climate and soil variation and their interaction in different regions, affect lentils' productivity by influencing foliar and soil-borne diseases, pests, and associated rhizobia [5]. Characterization of available germplasm is significant for breeders aiming to breed improved high yielding varieties with excellent quality and value [6].

Breeding strategies could exploit the existing variation within landraces to broaden the genetic base of commercial crops [7]. Landraces are the primary material used by breeders due to their contribution as a gene pool from which breeders can derive elite varieties [1]. Lentil is a self-pollinated species due to cleistogamy and dehiscence of anthers before opening of the flower. Thus, naturally evolving lentil landraces are highly heterogeneous populations, which consist of a mixture of several fairly homozygous genotypes [2]. Nevertheless, field studies indicate that the degree of outcrossing in lentil

can range from 0.06 to 5.12% between cultivars and can be, depending on environmental conditions and the genotype, as high as 22% within the same cultivar [8], which may further expand genetic diversity within a lentil landrace. Therefore, lentil landraces offer the option to apply single-plant selection as a short-time tool to breed high-yielding and stable pure-line varieties [3].

Plant breeding collects, induces, and rearranges genetic diversity by selecting superior genotypes conforming to specific criteria. A balance between the positive and negative effects of breeding on allelic richness determines the increase or loss of diversity [9]. Applying a breeding methodology, breeders should know how selection influences genetic diversity, how it affects the number of alleles in the breeding population, and what are the effects on the genetic background of the improved gene pool [10].

The honeycomb pedigree breeding program was carried out within a lentil landrace at an ultra-low density to prevent plant-to-plant interference for any input; absence of competition was assumed to facilitate the identification of genotypes of both high yield capacity and the ability to escape seed-borne and aphid-transmitted viruses [2]. Single-plant selection for three successive generations led to promising sister lines on grain yield and virus tolerance [2,11,12]. The breeding scheme implied that the selected single-plant progeny sister lines should have similar genetic identity, which could be followed in next generations. This property would enable a study of genetic relationships of the selected genotypes and answer to specific questions: (a) what is the extent of genetic diversity of the mother population; (b) what are the genetic relationships of selected plants in the first cycle; (c) how consistent is the selection procedure regarding the genetic makeup of each selection generation, and (d) what is the allelic composition of the improved selected plants compared to the original population. Addressing these questions is important for application of modern selection and breeding technology tools to shape landraces into useful cultivars preserving their agricultural significance [13]. Thus, we set up a study to estimate the genetic diversity of 40 1st, 2nd, and 3rd generation sister lines, using Simple Sequence Repeats (SSR) molecular markers to answer these questions.

2. Materials and Methods

2.1. Plant Material

A total of 40 lentil single-plant sister lines were evaluated for genetic relationships (Table 1). These genotypes were obtained via the honeycomb breeding model at the ultra-low density of 1.2 plants/m^2 (i.e., absence of competition) within the 'Evros' landrace for three successive generations [2]. The mother population (MP) was a well-adapted and commercially cultivated landrace in northeast Greece. In each generation, virus-free (asymptomatic) and highest-yielding plants were selected to form the respective single-plant progeny lines, i.e., 1st (1GSLs), 2nd (2GSLs), and 3rd (3GSLs) generation sister lines. Details of the breeding methodology are described by [2]. For the sake of clarity, henceforth, the lines with a common genealogy constitute a family, i.e., the 1GSL coded 8 and its progenies (8-. and 8-.-) constitute the family 8.

Ten seeds from every line were planted into pots with three replications; leaf samples from each line were collected at plants' full growth state and kept at −20 °C until further processing.

2.2. DNA Extraction

Samples (300 mg of frozen leaves) from each line were ground to a fine powder in liquid nitrogen and DNA was extracted using the DNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality of isolated DNA was assessed and visualized in 1% agarose gel electrophoresis in 1× TAE buffer using λHindIII ladder as size marker and then quantified using Gel Analyzer 19.1 software (www.gelanalyzer.com (accessed on 3 January 2022)).

Table 1. The genealogy of 40 lentil lines examined in the present study, obtained through single-plant selection for three successive generations within a landrace. Numerical code indicates the line's origin, i.e., the 3GSL coded 8-2-2 was originated from a single plant of the 2GSL coded 8-2, stemmed from a single plant of the 1GSL coded 8, ancestor of which was a single plant of the initial landrace.

1GSLs		2GSLs		3GSLs	
Sample Code	**Line Code**	**Sample Code**	**Line Code**	**Sample Code**	**Line Code**
1	1	15	20-13	30	8-2-2
2	2	16	2-4	31	8-11-7
3	3	17	2-10	32	15-5-10
4	4	18	18-15	33	2-4-6
5	5	19	15-5	34	8-2-4
6	6	20	1-14	35	8-2-3
7	7	21	8-1	36	8-1-9
8	8	22	8-3	37	15-5-11
9	9	23	2-9	38	8-3-8
10	10	24	15-6	39	8-2-1
11	11	25	8-2	40	15-7-5
12	12	26	15-7		
13	13	27	8-11		
14	15	28	2-8		
		29	15-12		

2.3. PCR Amplification and HRM Analysis

Fifteen Simple Sequence Repeats (SSR) primer sets designed by [14] were used for genetic differentiation analysis of 40 lentil samples. The PCR amplification and HRM analysis on a Light Cycler 96 System (Roche, Mannheim, Germany) for real-time PCR and HRM analysis were performed in a total volume of 11 µL, containing 20 ng genomic DNA, 1× KAPA HRM FAST master mix, 2.5 mM $MgCl_2$, 0.2 µM forward and reverse primer. The PCR protocol included the following steps: preincubation of 95 °C for 180 s, followed by 45 cycles of 95 °C for 5 s (denaturation), 51–58 °C (depending on the primer pair) for 20 s (annealing) and 72 °C for 45 s (extension). The optimal annealing temperature for each primer pair (Table 2) was defined testing them in gradient Real-time PCR with a specific range of temperatures (data not shown). The PCR was followed by HRM analysis using SYBR Green I dye with the following steps: 95 °C for 60 s, 40 °C for 60 s, and then 65 °C to 97 °C allowing 25 data acquisitions per 1 °C. PCR products were visualized in 2% agarose gel electrophoresis in 1× TBE buffer using 1kb ladder as a size marker.

Table 2. The annealing temperatures (°C) of the SSR primer sets used in the research; further information on primer's sequence, band size and inheritance are given in [14].

Primer	Tm (°C)
SSR19	58
SSR33	58
SSR59-2	58
SSR80	58
SSR107	54
SSR113	54
SSR154	57
SSR156	57
SSR167	56
SSR184	56
SSR199	58
SSR204	56
SSR302	56
SSR317-2	56
SSR323	58

2.4. Statistical Analysis

Discrimination of different alleles was performed with analysis of HRM profiles of the samples using the Roche Light Cycler 96 software. The samples were assigned to groups, according to the amplicons melting temperature (Tm), the shape of the normalized melting curves, and the difference plots. Each distinct group was assumed as a different SSR allele and was numerically coded for further analysis. GenAlex 6.5 software [15,16] was used to calculate allele frequencies and parameters of genetic diversity including the number of alleles (Na), the effective number of alleles (Ne), the information index (I), diversity (h), and unbiased diversity (uh) for each locus, the mean overall loci, and the grand mean. A matrix of pairwise genetic distance based on allele dissimilarities was calculated and Analysis of Molecular Variance (AMOVA) was performed to estimate the partitioning of the variance within and between GSLs and PCoA to find and plot the major patterns within allele loci and different GSLs samples. The genetic distance matrix was used to construct a dendrogram with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using MEGAX (http://www.megasoftware.net (accessed on 3 January 2022)) [17]. The PIC (Polymorphic Information Content) was calculated using Equation (1) as described by [18]:

$$PIC = 1 - \sum_{i=1}^{n} f_i^2 \quad (1)$$

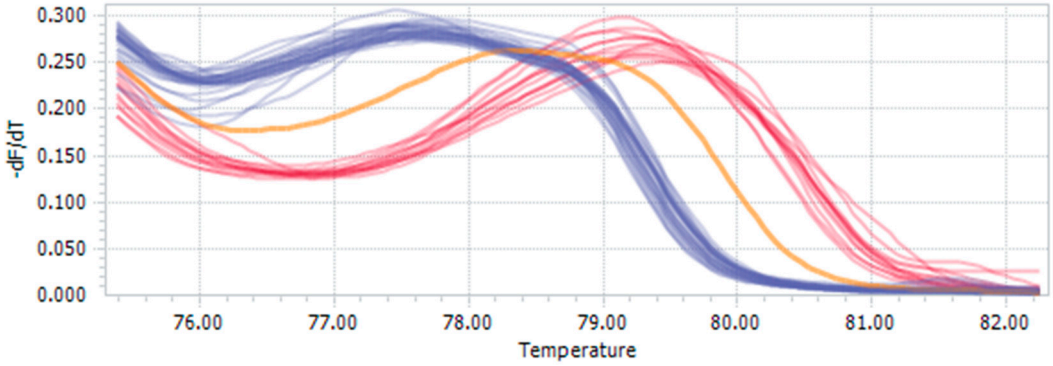
where f_i^2 the frequency of the ith allele.

The STRUCTURE 2.3.4 software [19] based on the Bayesian model ran three iterations from K = 2 to K = 10 with a 5000 burnin period and 50,000 Markov Chain Monte Carlo (MCMC) repeats after burn-in using the admixture model. Then the results were analyzed with STRUCTURE HARVESTER [19,20] to infer population structure and assign individuals to groups, based on the Δk method [21].

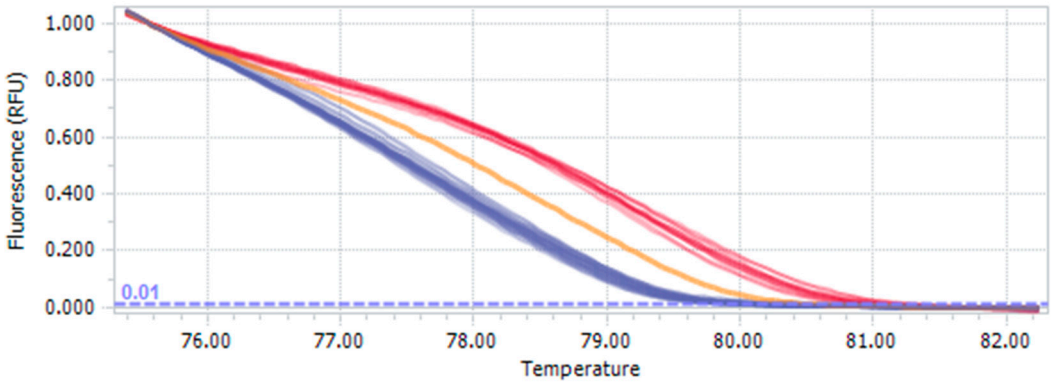
3. Results

Data were analyzed with the Roche Light Cycler 96 software with the appropriate adjustments. The pre- and post- melt range of melting curves were selected for optimum discrimination considering the pedigree of the lines, with settings for normalization to proportional and sensitivity for Delta Tm and curve shape discrimination adjusted to 50%. Grouping of the samples was automatic depending on normalized melting curves (Figure 1a) and normalized melting peaks (Figure 1b), while the presence of a group was also examined comparing the banding patterns on the agarose gel. In several SSRs it was possible to confirm the presence of different alleles from the gel band patterns (Supplementary Figure S1). Each group was depicted with a different color. A numeric matrix was created assuming that each group represented a different allele of the respective SSR locus.

A total number of 48 alleles were detected for the 15 SSR primer sets in the 14 1GSLs. Nine alleles were lost in the second cycle of selection (18.75%), and from the remaining 39 alleles, five were lost (12.82%) in the third cycle. Fourteen alleles were lost in total representing 29.1% of the allelic richness of the 1st GSLs. Two to five alleles per locus were detected (Figure 2a). Analysis of allelic patterns across breeding generations indicated a decrease of mean numbers of alleles (Na), alleles with a frequency higher than 5% (Na Freq. $\geq$ 5%), and the number of private alleles (No. Pr. Al.) that was zero at the 3GSLs, while effective alleles (Ne), information index (I), and haploid diversity increased from 1GSL to 2GSL and then declined from 2GSL to 3GSL (Figure 2b).

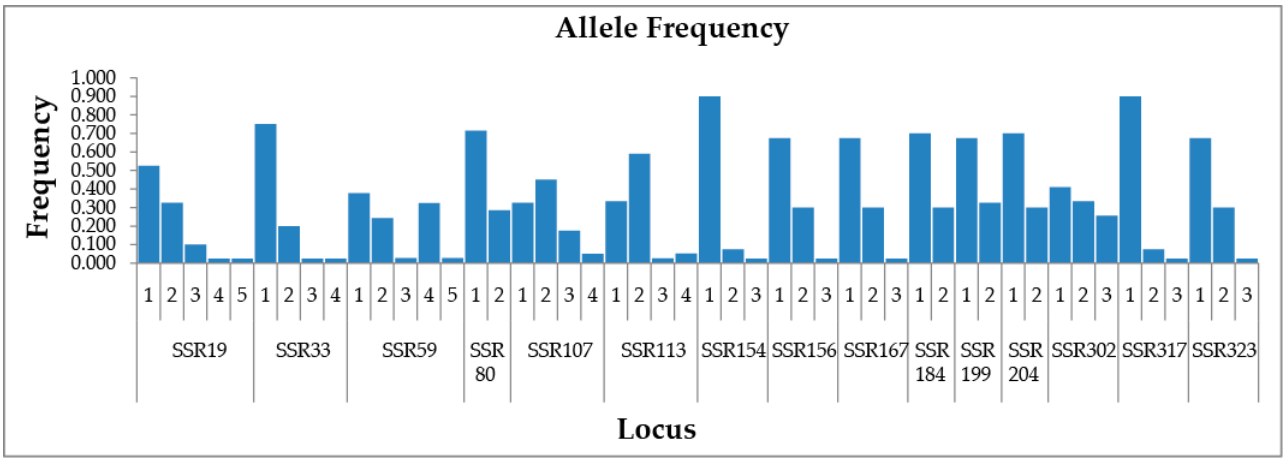


(**a**)



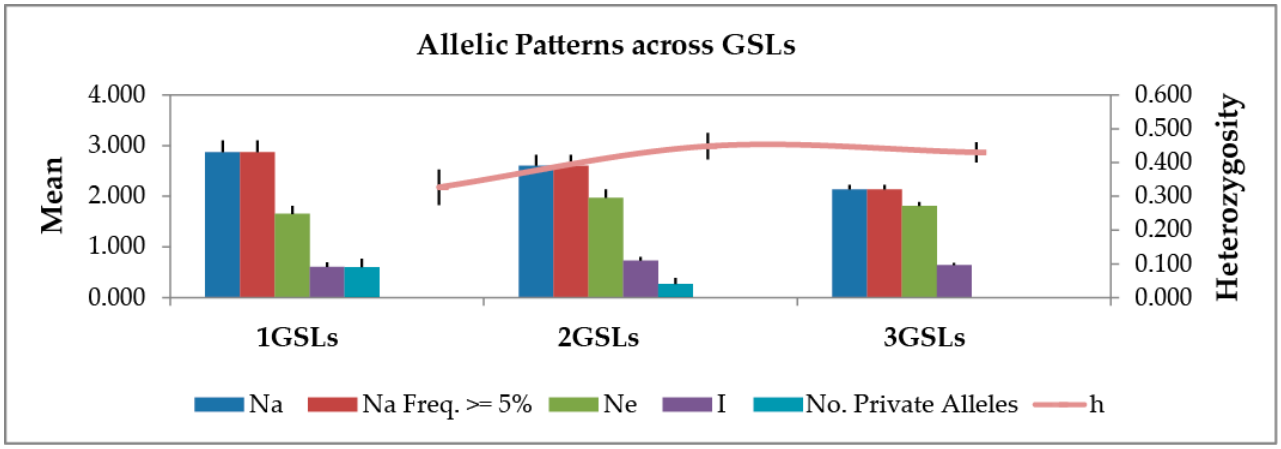
(**b**)

Figure 1. Graphs of (**a**) Normalized melting peaks and (**b**) normalized melting curves generated by analysis of SSR156 with Roche Light Cycler 96 software from data of the 40 lentil lines. Lines of family 8 are in red, lines of family 5 in orange, and all the rest in blue.



(**a**)

Figure 2. *Cont.*



(**b**)

Figure 2. (**a**) Allele frequencies: Histogram depicting the allele frequency per locus varying from 0.025 for unique alleles to as high as 0.90 for the major alleles of SSR154 and SSR317; (**b**) Allelic patterns across GSLs: histogram depicting the mean and standard errors across loci by breeding generation for the following statistics: Mean numbers of alleles (Na), alleles with a frequency higher than 5% (Na Freq. ≥ 5%) effective alleles (Ne), information index (I), number of private alleles (No. Pr. Al.), and haploid diversity (h).

Genetic diversity parameters for the 15 SSRs were calculated to assess their overall informativeness (Table 3) finding that the number of different alleles (Na) varied from two to five, the number of effective alleles (Ne) from 1.225 to 3.236, information index (I) between 0.381 and 1.272, PIC ranged from 0.664 to 0.925, diversity (h) from 0.184 to 0.691 and unbiased diversity (uh) from 0.188 to 0.710. In general, all markers were polymorphic and had high discrimination capacity. The most informative was SSR59 with 5 alleles and the highest values for all indices while less informative was SSR154 with the lowest values.

Table 3. SSRs' parameters obtained after statistical analysis of data from 15 SSR loci of 40 lines: Na (number of different alleles), Ne (Number of effective alleles), I (Shannon's information index), PIC (Polymorphic Information Content), h (diversity), and uh (unbiased diversity).

SSR	Na	Ne	I	PIC	h	uh
SSR19	5	2.548	1.118	0.906	0.608	0.623
SSR33	4	1.656	0.722	0.848	0.396	0.406
SSR59	5	3.236	1.272	0.925	0.691	0.710
SSR80	2	1.690	0.598	0.677	0.408	0.420
SSR107	4	2.930	1.179	0.889	0.659	0.676
SSR113	4	2.164	0.924	0.877	0.538	0.552
SSR154	3	1.225	0.381	0.723	0.184	0.188
SSR156	3	1.831	0.719	0.790	0.454	0.465
SSR167	3	1.831	0.719	0.790	0.454	0.465
SSR184	2	1.724	0.611	0.664	0.420	0.431
SSR199	2	1.782	0.631	0.683	0.439	0.450
SSR204	2	1.724	0.611	0.664	0.420	0.431
SSR302	3	2.897	1.081	0.859	0.655	0.672
SSR317	3	1.225	0.381	0.727	0.184	0.188
SSR323	3	1.831	0.719	0.790	0.454	0.465
Average	3.2	2.2	0.778	0.787	0.454	0.465
SE	0.262	0.158	0.071	0.024	0.039	0.040

Genetic diversity parameters at population level indicated that the number of different alleles (Na) varied from 2.13 to 2.87, the number of effective alleles (Ne) varied from 1.65 to

1.97 while Shannon information index (I) got values between 0.60 (1GSLs) and 0.73 (2GSLs). Diversity (h) and unbiased diversity (uh) indicators took values from 0.33 (1GSLs) to 0.45 (2GSLs, 3GSLs) and from 0.35 (1GSLs) to 0.49 (2GSLs), respectively (Table 4). AMOVA (Table 5) showed that diversity within populations (88%) was much greater than between them (12%). The highest percentage of polymorphic loci was observed in 2GSLs and 3GSLs (100%) followed by 1GSLs with 93.33%. The observed private alleles were 0.6 in 1GSLs, 0.2 in 2GSLs, and 0.067 in 3GSLs. A distance matrix based on φPT between GSLs showed that the genetic distance between 1GSLs and 2GSLs was lower (2.5%) than the observed between 2GSLs and 3GSLs (7.7%) while the highest difference (26.7%) was recorded between the 1GSLs and 3GSLs (Table 6).

Table 4. Genetic diversity parameters at population level (1GSLs, 2GSLs, 3GSLs) for the 15 SSR loci: Na (number of different alleles), Ne (number of effective alleles), I (Shannon information index), h (diversity), uh (unbiased diversity).

Populations	Na	Ne	I	h	uh
1GSLs	2.87 ± 0.24	1.65 ± 0.16	0.60 ± 0.09	0.33 ± 0.05	0.35 ± 0.06
2GSLs	2.60 ± 0.21	1.97 ± 0.17	0.73 ± 0.07	0.45 ± 0.04	0.49 ± 0.04
3GSLs	2.13 ± 0.09	1.81 ± 0.08	0.64 ± 0.04	0.45 ± 0.03	0.48 ± 0.03
Mean	2.53 ± 0.12	1.81 ± 0.08	0.66 ± 0.04	0.41 ± 0.03	0.44 ± 0.03

Table 5. Analysis of Molecular Variance (AMOVA) within and between GSLs.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	Estimate of Variance	Percentage of Total Variation (%)
Among Pops	2	18.973	9.486	0.463	12%
Within Pops	37	124.277	3.359	3.359	88%
Total	39	143.250		3.822	100%

Table 6. Pairwise Population φPT Genetic Distance between GSLs.

	1GSLs	2GSLs
2GSLs	0.025	
3GSLs	0.267	0.077

The UPGMA dendrogram of 40 lines based on the dissimilarity distance matrix is depicted in Figure 3. Lines of family 8 form a separate branch in a bifurcated tree, indicating their close genetic relationships. The second branch of the tree is divided into a clade solely comprising the family 5 while the rest of the families are mixed in a separate clade. Principal Coordinates Analysis (PCoA) was employed to find and plot the major patterns between the families based on their distance matrix (Figure 4). The first two PCoA axes explained the 69.81% of total variation within lentil families. Samples were separated into three major groups along the first axis with family 8 and its progeny to form a cluster, family 5 alone, and the rest of the samples to form a second group. No specific grouping was detected for samples derived from family 2 or family 15.

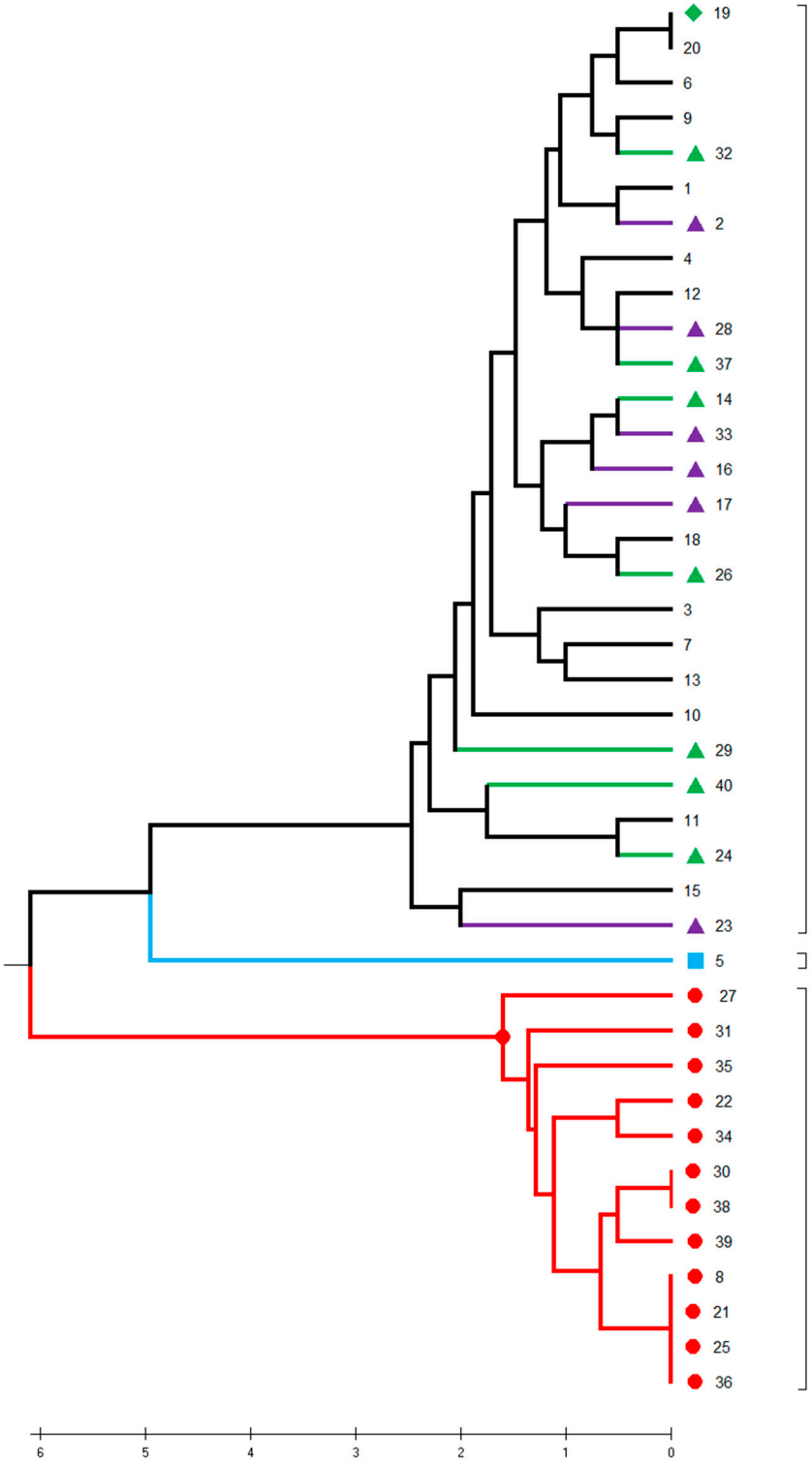


Figure 3. UPGMA dendrogram of genetic distances based on a dissimilarity matrix between the 40 lentil lines of the three GSLs. Progeny derived from family 8 is in red, from 2 in mauve, and from 15 in green. Family 5 branch is in turquoise and the rest lines in black. Scale represents the percentage of dissimilarity.

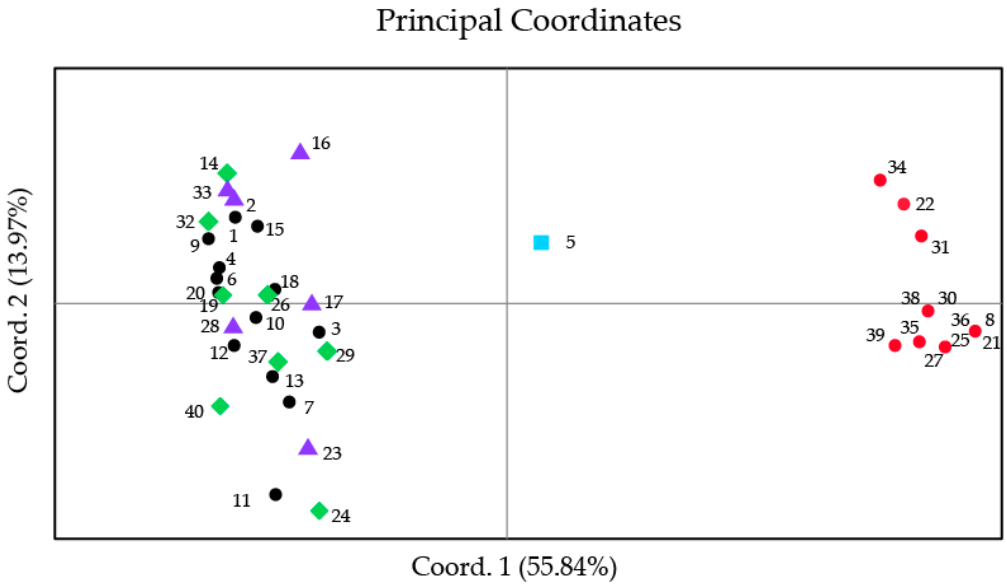


Figure 4. Principal Coordinate Analysis (PCoA) for 40 lentil lines using a dissimilarity distance matrix. Different families are indicated by red circle (family 8), mauve triangle (family 2), green rhombus (family 15), turquoise square (family 5) and black circles (rest of the families).

Structure analysis and Evanno's method revealed that the optimum ΔK was at K = 3 (Figure 5), while Figure 6 presents the composition of generations.

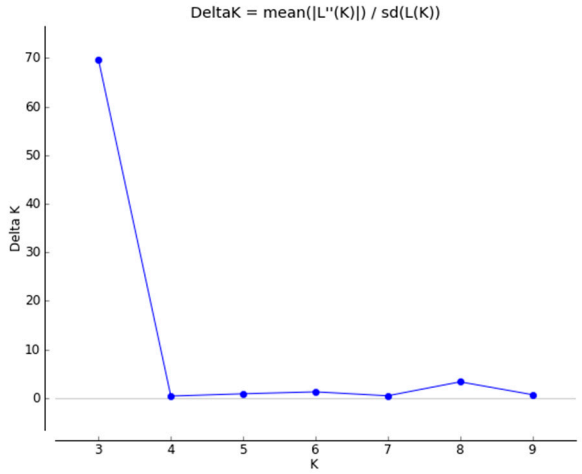


Figure 5. ΔK graph for every K value calculated with Evanno's method [21] and the STRUCTURE HARVESTER software.

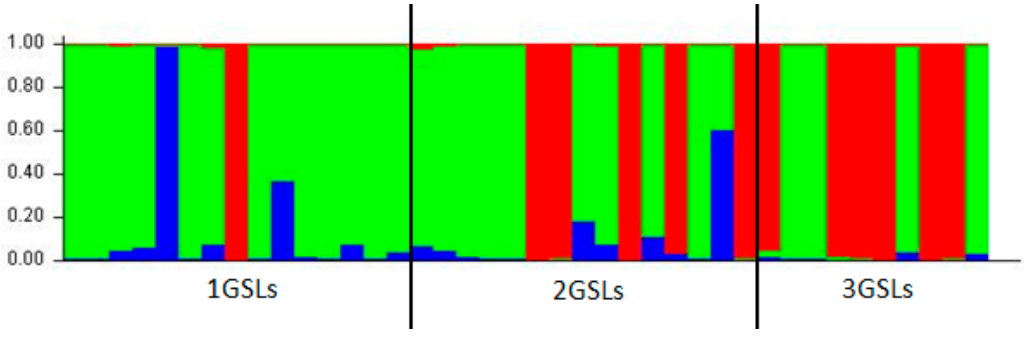


Figure 6. The population structure barplot generated with STRUCTURE software at K = 3 for the 40 lentil lines.

4. Discussion

The objective of this study was to examine genetic variation within and between pedigree sister lines derived from the commercially cultivated lentil landrace "Evros", using SSR molecular markers and HRM analysis. The 15 SSR molecular markers applied with HRM analysis based on melting curves and difference plot of the markers efficiently discriminated the 40 lentil lines according their genealogy.

Researchers have used molecular markers to study genetic diversity in lentil and other legumes [22–26], but genetic differentiation among successive generations of breeding populations has been considered granted and has not been thoroughly investigated. Generally, it is considered that genetic diversity decreases through the breeding of landraces and their transformation to inbred varieties after the first cycle of breeding. The transient fluctuations in the variability led to the bottleneck effect, possibly due to industry and consumers' preferences of seed quality characteristics [27]. All plant species went through two major bottlenecks, the first during domestication and the second through breeding [28]. However, some breeding programs reversed the bottleneck effect by increasing the level of genetic variation. According to studies, genetic diversity increased through breeding over the years or at certain breeding cycles [29–33]. In addition, there are studies where genetic diversity through breeding is preserved, such as in wheat, barley, maize, and flax [34–44] while others indicated that genetic diversity decreases, such as in wheat and maize [45–49].

Several studies examining lentil polymorphism using agromorphological traits, molecular markers and imaging analysis indicate that genetic variation between lentil species, varieties, and within or between landraces is high [50–55]. Even if genetic variation during breeding decreases, there is evidence that this reduction in variation is minor while the allelic reduction at individual chromosomal segments is significant [10]. In this study, the level of diversity of the 14 lentil families was high enough as determined by the presence of 48 alleles, indicating adequate variation in the "Evros" landrace to encourage the breeding effort. Although the progenies were selected from a limited number of mother plant-families in successive breeding generations, allelic variation was not diminished. Lost alleles in the second cycle were at the level of 18.75%, while at the end of the third cycle 29.1% of allelic richness (14 alleles) of 1GSLs was lost. Considering that the final selection (3GSLs) discarded 11 out of the 14 initially selected plants (1GSLs), allelic richness was not substantially affected. This could be due to similarities of the discarded families with the selected families 2 and 15, as revealed in the UPGMA dendrogram and PCoA analysis. As evident in Figures 3 and 4, the unique genotypic category that is not represented in the 3GSLs, is the family 5. Diversification of this family is characteristic and could point to the utility of molecular markers in better management of genetic diversity during selection. This family could be included in the successive generations to avoid further loss of genetic diversity in the breeding population. Family 8 and its progeny are grouped in a separate clade, and the rest of 1GSLs are grouped with families 2 and 15, indicating a common pool of alleles in these materials.

Crossing genetically different plants leads to significant genetic diversity, which could be further material for selection. In such a way, breeders can develop uniform new varieties, which replace polymorphic landraces [9]. Selection within a landrace may similarly result in uniform new varieties. Our study found that genetic diversity through breeding a landrace decreased but not diminished. The selection of plants with desirable traits narrows the genetic diversity in marker loci and contributes to selecting co-inherited beneficial characteristics. Moreover, the objective of plant breeding is to combine as many favorable traits as possible in only one genotype or even increase the presence of such traits in a population [9].

Interestingly, examining allele frequencies by generation we observed that unique alleles appeared for markers SSR19, 33, 59, and 154 in 2GSLs and then lost in 3 GSLs. This finding merits an explanation and could be due to the way heterozygous loci are detected and recorded with HRM-PCR. This analysis assumes each different melting curve as a single different allele. However, heterozygous loci are also recorded as a single allele,

and allelic separation in the next generation will give rise to three different allelic HRM patterns: one for each homozygous and one for the heterozygous genotype. In such a way and due to Mendelian segregation, successive generations detect heterozygous loci from the declining genetic variability. As an example, this is evident in the analysis of HRM curves for SSR107. In 1GSLs, electrophoresis of the PCR products indicates the presence of two alleles in homozygous and heterozygous patterns (Supplement Figure S2). Samples with the one allele (1–4, 6–7, 11–14) display a slow band, while samples 5 and 8 with the other allele display a fast band. Luckily enough, one sample (10) is heterozygous for SSR107 and displays both bands. However, HRM analysis indicates the presence of three curve patterns, which is interpreted as the presence of three alleles. In the consecutive breeding generations, no samples are deriving from family 10. Thus, a biallelic pattern is observed in HRM and the analysis records a loss of one allele (the heterozygous curve). However, as heterozygosity in lentil is extremely low, this should not significantly affect estimation of genetic variation. Furthermore, the high selection pressure in the breeding population resulted in a small number of lines in succeeding generations with similar genetic background. Selection and fixation of the favorable alleles would decrease the genetic variation. Accordingly, a low genetic distance between GSLs is expected by the way each GSL was formed. The above hypothesis is in accordance with AMOVA, which showed that this low diversity is partitioned rather within (88%), than between (12%) GSLs, which is expected for self-pollinated species.

PCoA and analysis with the STRUCTURE software revealed the structure within GSLs. Estimation of hypothetical subpopulations using K-values indicated the presence of three subpopulations that in 1GSLs prevailed two subpopulations (blue and green colors) while at the end of the breeding (3GSLs) prevailed the third sub-population (red color) (Figure 4). These results indicate a shift in allelic composition among the populations and the effect of breeding through GSLs. Furthermore, the highest genetic distance between 1GSLs and 3GSLs (0.267) could provide support for the efficiency of the breeding methodology. The observed breeding populations' composition throughout breeding cycles indicates the prevalence of selected genotypes with the desirable characteristics.

In conclusion, this study provided supportive evidence for the genetic diversity of the breeding population, as was indicated by allele richness of the 1GSLs. Selection at ultra-low density in consecutive cycles decreased genetic diversity and maintained the genetic relationships of selected plants, revealing proper management of the breeding material. The allelic composition of the improved selected plants compared to the original population was shifted, indicating the prevalence of improved genotypes in the final population. Molecular markers could provide an effective tool for proper management of allelic composition in landrace breeding to avoid extreme loss of diversity and potentially useful alleles for future variety development.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app12010450/s1, Figure S1: Banding patterns of the 40 lentil lines after electrophoresis of the PCR products for SSR167. All lines derived from family 8 are highlighted in yellow color and show a distinct band pattern from the rest of the families. Vertical lines distinguish breeding generations; Figure S2: A. Top: Banding patterns of the14 1GSLs after electrophoresis of the PCR products for SSR107. Samples with the one allele (1–4, 6–7, 11–14) display a slow band while samples 5 and 8 with the other allele display a fast band. One sample (10) is heterozygous for SSR107 and displays both bands. Middle: difference plot and bottom: normalized melting curves of the HRM profiles of the PCR products for SSR107. Separation of curves for lines 5–8 and 10 from the rest (blue curves) is indicated. B. Difference plot (up) and normalized melting curves (down) of the HRM profiles of the PCR products for SSR107 in samples of 2GSLs and 3 GSLs. The profiles of samples derived from family 8 are in blue while the rest are in red.

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References

1. Polidoros, A.N.; Avdikos, I.D.; Gleridou, A.; Kostoula, S.D.; Koura, E.; Sakellariou, M.A.; Stavridou, E.; Gerasopoulos, D.; Lagopodi, A.; Mavromatis, A.; et al. Lentil Gene Pool for Breeding. In *Cash Crops*; Priyadarshan, P., Jain, S.M., Eds.; Springer: Cham, Switzerland, 2022. [CrossRef]
2. Kargiotidou, A.; Chatzivassiliou, E.; Tzantarmas, C.; Sinapidou, E.; Papageorgiou, A.; Skaracis, G.N.; Tokatlidis, I.S. Selection at ultra-low density identifies plants escaping virus infection and leads towards high-performing lentil (*Lens culinaris* L.) varieties. *J. Agric. Sci.* **2014**, *152*, 749–758. [CrossRef]
3. Vlachostergios, D.N.; Tzantarmas, C.; Kargiotidou, A.; Ninou, E.; Pankou, C.; Gaintatzi, C.; Mylonas, I.; Papadopoulos, I.; Foti, C.; Chatzivassiliou, E.K.; et al. Single-plant selection within lentil landraces at ultra-low density: A short-time tool to breed high yielding and stable varieties across divergent environments. *Euphytica* **2018**, *214*, 1–15. [CrossRef]
4. Taylor, P.; Lindbeck, K.; Chen, W.; Ford, R.; Al, T. El Lentil Diseases. In *Lentil: An Ancient Crop for Modern Times*; Yadav, S.S., McNeil, D.L., Stevenson, P.C., Eds.; Springer: Dordrecht, The Netherlands, 2007; pp. 291–313.
5. Materne, M.; Neil, D.M.C.; Hobson, K.; Ford, R. Abiotic Stresses. In *Lentil: An Ancient Crop for Modern Times*; Yadav, S.S., McNeil, D.L., Stevenson, P.C., Eds.; Springer: Dordrecht, The Netherlands, 2007; pp. 315–329.
6. Nasim, N.; Sandeep, I.S.; Sahoo, A.; Das, S.; Panda, M.K.; Acharya, L.; RamaRao, V.V.; Nayak, S.; Mohanty, S. Population genetic structure and diversity analysis in economically important Pandanus odorifer (Forssk.) Kuntze accessions employing ISSR and SSR markers. *Ind. Crops Prod.* **2020**, *143*, 1–12. [CrossRef]
7. Tokatlidis, I.; Vlachostergios, D. Sustainable stewardship of the landrace diversity. *Diversity* **2016**, *8*, 29. [CrossRef]
8. Horneburg, B. Outcrossing in lentil (*Lens culinaris*) depends on cultivar, location and year, andvaries within cultivars. *Plant Breed.* **2006**, *125*, 638–640. [CrossRef]
9. Louwaars, N.P. Plant breeding and diversity: A troubled relationship? *Euphytica* **2018**, *214*, 1–9. [CrossRef] [PubMed]
10. Fu, Y.B. Impact of plant breeding on genetic diversity of agricultural crops: Searching for molecular evidence. *Plant Genet. Resour.* **2006**, *4*, 71–78. [CrossRef]
11. Kargiotidou, A.; Chatzivassiliou, E.K.; Tzantarmas, C.; Tokatlidis, I.S. Seed propagation at low density facilitates the selection of healthy plants to produce seeds with a reduced virus load in a lentil landrace. *Seed Sci. Technol.* **2015**, *43*, 31–39. [CrossRef]
12. Kargiotidou, A.; Vlachostergios, D.N.; Tzantarmas, C.; Mylonas, I.; Foti, C.; Menexes, G.; Polidoros, A.; Tokatlidis, I.S. Addressing huge spatial heterogeneity induced by virus infections in lentil breeding trials. *J. Biol. Res.* **2016**, *23*, 1–8. [CrossRef]
13. Casañas, F.; Simó, J.; Casals, J.; Prohens, J. Toward an evolved concept of landrace. *Front. Plant Sci.* **2017**, *8*, 145. [CrossRef]
14. Hamwieh, A.; Udupa, S.M.; Choumane, W.; Sarker, A.; Dreyer, F.; Jung, C.; Baum, M. A genetic linkage map of Lens sp. based on microsatellite and AFLP markers and the localization of fusarium vascular wilt resistance. *Theor. Appl. Genet.* **2005**, *110*, 669–677. [CrossRef]
15. Peakall, R.; Smouse, P.E. GenALEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—An update. *Bioinformatics* **2012**, *28*, 2537–2539. [CrossRef]
16. Peakall, R.; Smouse, P.E. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* **2006**, *6*, 288–295. [CrossRef]
17. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef]
18. Smith, J.S.C.; Chin, E.C.L.; Shu, H.; Smith, O.S.; Wall, S.J.; Senior, M.L.; Mitchell, S.E.; Kresovich, S.; Ziegle, J. An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): Comparisons with data from RFLPS and pedigree. *Theor. Appl. Genet.* **1997**, *95*, 163–173. [CrossRef]
19. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **2000**, *155*, 945–959. [CrossRef] [PubMed]
20. Earl, D.A.; vonHoldt, B.M. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* **2012**, *4*, 359–361. [CrossRef]
21. Evanno, G.; Regnaut, S.; Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* **2005**, *14*, 2611–2620. [CrossRef] [PubMed]

22. Dissanayake, R.; Braich, S.; Cogan, N.O.I.; Smith, K.; Kaur, S. Characterization of Genetic and Allelic Diversity Amongst Cultivated and Wild Lentil Accessions for Germplasm Enhancement. *Front. Genet.* **2020**, *11*, 1–14. [CrossRef]
23. Mbasani-Mansi, J.; Ennami, M.; Briache, F.Z.; Gaboun, F.; Benbrahim, N.; Triqui, Z.E.A.; Mentag, R. Characterization of genetic diversity and population structure of Moroccan lentil cultivars and landraces using molecular markers. *Physiol. Mol. Biol. Plants* **2019**, *25*, 965–974. [CrossRef]
24. Aci, M.M.; Lupini, A.; Badagliacca, G.; Mauceri, A.; Lo Presti, E.; Preiti, G. Genetic diversity among lathyrus ssp. Based on agronomic traits and molecular markers. *Agronomy* **2020**, *10*, 1182. [CrossRef]
25. Mohamed, A.; García-Martínez, S.; Loumerem, M.; Carbonell, P.; Ruiz, J.J.; Boubaker, M. Assessment of genetic diversity among local pea (*Pisum sativum* L.) accessions cultivated in the arid regions of Southern Tunisia using agro-morphological and SSR molecular markers. *Genet. Resour. Crop Evol.* **2019**, *66*, 1189–1203. [CrossRef]
26. Pereira, H.S.; Mota, A.P.S.; Rodrigues, L.A.; de Souza, T.L.P.O.; Melo, L.C. Genetic diversity among common bean cultivars based on agronomic traits and molecular markers and application to recommendation of parent lines. *Euphytica* **2019**, *215*, 1–16. [CrossRef]
27. Smith, S.; Bubeck, D.; Nelson, B.; Stanek, J.; Gerke, J. Genetic Diversity and Modern Plant Breeding. In *Genetic Diversity and Erosion in Plants*; Ahuja, M.R., Mohan Jain, S., Eds.; Springer: Cham, Switzerland, 2015; ISBN 9783319256375.
28. Sthapit, S.R.; Marlowe, K.; Covarrubias, D.C.; Ruff, T.M.; Eagle, J.D.; McGinty, E.M.; Hooker, M.A.; Duong, N.B.; Skinner, D.Z.; See, D.R. Genetic diversity in historical and modern wheat varieties of the U.S. Pacific Northwest. *Crop Sci.* **2020**, *60*, 3175–3190. [CrossRef]
29. Parker, G.D.; Fox, P.N.; Langridge, P.; Chalmers, K.; Whan, B.; Ganter, P.F. Genetic diversity within Australian wheat breeding programs based on molecular and pedigree data. *Euphytica* **2002**, *124*, 293–306. [CrossRef]
30. Roussel, V.; Leisova, L.; Exbrayat, F.; Stehno, Z.; Balfourier, F. SSR allelic diversity changes in 480 European bread wheat varieties released from 1840 to 2000. *Theor. Appl. Genet.* **2005**, *111*, 162–170. [CrossRef]
31. Maccaferri, M.; Sanguineti, M.C.; Donini, P.; Tuberosa, R. Microsatellite analysis reveals a progressive widening of the genetic basis in the elite durum wheat germplasm. *Theor. Appl. Genet.* **2003**, *107*, 783–797. [CrossRef] [PubMed]
32. Smale, M.; Reynolds, M.P.; Warburton, M.; Skovmand, B.; Trethowan, R.; Singh, R.P.; Ortiz-Monasterio, I.; Crossa, J. Dimensions of diversity in modern spring bread wheat in developing countries from 1965. *Crop Sci.* **2002**, *42*, 1766–1779. [CrossRef]
33. Christiansen, M.J.; Andersen, S.B.; Ortiz, R. Optimal portfolio choice under a liability constraint. *Ann. Oper. Res.* **2000**, *97*, 131–141. [CrossRef]
34. Donini, P.; Law, J.R.; Koebner, R.M.D.; Reeves, J.C.; Cooke, R.J. Temporal trends in the diversity of UK wheat. *Theor. Appl. Genet.* **2000**, *100*, 912–917. [CrossRef]
35. Fu, Y.-B.; Peterson, G.W.; Richards, K.W.; Somers, D.; DePauw, R.M.; Clarke, J.M. Allelic reduction and genetic shift in the Canadian hard red spring wheat germplasm released from 1845 to 2004. *Theor. Appl. Genet.* **2005**, *110*, 1505–1516. [CrossRef]
36. Orabi, J.; Jahoor, A.; Backes, G. Changes in allelic frequency over time in European bread wheat (*Triticum aestivum* L.) varieties revealed using DArT and SSR markers. *Euphytica* **2014**, *197*, 447–462. [CrossRef]
37. Fu, Y.B.; Kibite, S.; Richards, K.W. Amplified fragment length polymorphism analysis of 96 Canadian oat cultivars released between 1886 and 2001. *Can. J. Plant Sci.* **2004**, *84*, 23–30. [CrossRef]
38. Fu, Y.B.; Rowland, G.G.; Duguid, S.D.; Richards, K.W. RAPD analysis of 54 North American flax cultivars. *Crop Sci.* **2003**, *43*, 1510–1515. [CrossRef]
39. Fu, Y.B.; Peterson, G.W.; Scoles, G.; Rossnagel, B.; Schoen, D.J.; Richards, K.W. Allelic Diversity Changes in 96 Canadian Oat Cultivars Released from 1886 to 2001. *Crop Sci.* **2003**, *43*, 1989–1995. [CrossRef]
40. Khan, I.A.; Awan, F.S.; Ahmad, A.; Fu, Y.B.; Iqbal, A. Genetic diversity of Pakistan wheat germplasm as revealed by RAPD markers. *Genet. Resour. Crop Evol.* **2005**, *52*, 239–244. [CrossRef]
41. Khlestkina, E.K.; Röder, M.S.; Efremova, T.T.; Börner, A.; Shumny, V.K. The genetic diversity of old and modern Siberian varieties of common spring wheat as determined by microsatellite markers. *Plant Breed.* **2004**, *123*, 122–127. [CrossRef]
42. Koebner, R.M.D.; Donini, P.; Reeves, J.C.; Cooke, R.J.; Law, J.R. Temporal flux in the morphological and molecular diversity of UK barley. *Theor. Appl. Genet.* **2003**, *106*, 550–558. [CrossRef] [PubMed]
43. Lu, H.; Bernardo, R. Molecular marker diversity among current and historical maize inbreds. *Theor. Appl. Genet.* **2001**, *103*, 613–617. [CrossRef]
44. Manifesto, M.M.; Schlatter, A.R.; Hopp, H.E.; Suárez, E.Y.; Dubcovsky, J. Quantitative evaluation of genetic diversity in wheat germplasm using molecular markers. *Crop Sci.* **2001**, *41*, 682–690. [CrossRef]
45. Fu, Y.B.; Peterson, G.W.; Yu, J.K.; Gao, L.; Jia, J.; Richards, K.W. Impact of plant breeding on genetic diversity of the Canadian hard red spring wheat germplasm as revealed by EST-derived SSR markers. *Theor. Appl. Genet.* **2006**, *112*, 1239–1247. [CrossRef] [PubMed]
46. Hazen, S.P.; Zhu, L.; Kim, H.-S.; Tang, G.; Ward, R.W. Genetic diversity of winter wheat in Shaanxi province, China, and other common wheat germplasm pools. *Genet. Resour. Crop Evol.* **2002**, *49*, 437–445. [CrossRef]
47. Le Clerc, V.; Bazante, F.; Baril, C.; Guiard, J.; Zhang, D. Assessing temporal changes in genetic diversity of maize varieties using microsatellite markers. *Theor. Appl. Genet.* **2005**, *110*, 294–302. [CrossRef]
48. Reif, J.C.; Hamrit, S.; Heckenberger, M.; Schipprack, W.; Maurer, H.P.; Bohn, M.; Melchinger, A.E. Trends in genetic diversity among European maize cultivars and their parental components during the past 50 years. *Theor. Appl. Genet.* **2005**, *111*, 838–845. [CrossRef]

49. Tian, Q.Z.; Zhou, R.H.; Jia, J.Z. Genetic diversity trend of common wheat (*Triticum aestivum* L.) in China revealed with AFLP markers. *Genet. Resour. Crop Evol.* **2005**, *52*, 325–331. [CrossRef]
50. Toklu, F.; Karaköy, T.; Hakli, E.; Bicer, T.; Brandolini, A.; Kilian, B.; Özkan, H. Genetic variation among lentil (*Lens culinaris* Medik) landraces from Southeast Turkey. *Plant Breed.* **2009**, *128*, 178–186. [CrossRef]
51. El-Nahas, A.I.; El-Shazly, H.H.; Ahmed, S.M.; Omran, A.A.A. Molecular and biochemical markers in some lentil (*Lens culinaris* Medik.) genotypes. *Ann. Agric. Sci.* **2011**, *56*, 105–112. [CrossRef]
52. Fikiru, E.; Tesfaye, K.; Bekele, E. Morphological and molecular variation in Ethiopian lentil (*Lens culinaris* Medikus) varieties. *Int. J. Genet. Mol. Biol.* **2011**, *3*, 60–67.
53. Torricelli, R.; Silveri, D.D.; Ferradini, N.; Venora, G.; Veronesi, F.; Russi, L. Characterization of the lentil landrace Santo Stefano di Sessanio from Abruzzo, Italy. *Genet. Resour. Crop Evol.* **2012**, *59*, 261–276. [CrossRef]
54. Alghamdi, S.S.; Khan, A.M.; Ammar, M.H.; El-Harty, E.H.; Migdadi, H.M.; El-Khalik, S.M.A.; Al-Shameri, A.M.; Javed, M.M.; Al-Faifi, S.A. Phenological, nutritional and molecular diversity assessment among 35 introduced lentil (*Lens culinaris* medik.) genotypes grown in saudi arabia. *Int. J. Mol. Sci.* **2013**, *15*, 277–295. [CrossRef]
55. Tsanakas, G.F.; Mylona, P.V.; Koura, K.; Gleridou, A.; Polidoros, A.N. Genetic diversity analysis of the Greek lentil (*Lens culinaris*) landrace "Eglouvis" using morphological and molecular markers. *Plant Genet. Resour. Characterisation Util.* **2018**, *16*, 469–477. [CrossRef]