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## Article

# Exploring Genetic Diversity in Romanian *Lycium* Species: A Comparative Study of Wild and Cultivated Genotypes

Roxana Ciceoi <sup>1</sup>, Adrian Asănică <sup>2</sup>, Vasilica Luchian <sup>2</sup> and Mihaela Iordachescu <sup>1,\*</sup>

<sup>1</sup> Research Center for Studies of Food Quality and Agricultural Products, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59, Mărăști Bd., 011464 Bucharest, Romania; (R.C.) roxana.ciceoi@qlab.usamv.ro

<sup>2</sup> Faculty of Horticulture, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59, Mărăști Bd., 011464 Bucharest, Romania; (A.A.) adrian.asanica@horticultura-bucuresti.ro; (V.L.) vasi\_botanica@yahoo.com

\* Correspondence: mihaela.iordachescu@qlab.usamv.ro (M.I.)

**Abstract:** Goji berries, long valued in Chinese medicine and cuisine for their wide range of medicinal benefits, are considered a 'superfruit' and functional food. Out of the nearly 100 *Lycium* species known for their genetic diversity, *L. barbarum* and *L. chinense* currently dominate the market. Due to increasing market demand and concerns about food safety and sustainability, Europe and the Americas are expanding the local goji berry production, using as starting material plants originated from China. European breeding programs are focusing on *Lycium* to develop varieties adapted to local conditions, especially in response to climate change. By 2023, Romania registered seven goji berry varieties, both from *L. barbarum* and *L. chinense* species, without incorporating local germplasm. Advanced genomic studies, including NGS sequencing of five cultivated and three wild goji berry plant genomes, have been conducted to analyse their genetic variation, with a particular focus on the *BODYGUARD* 3 and 4 genes. This research uncovered significant differences between cultivated and wild genotypes, both in the entire genome and specifically in the *BODYGUARD* genes, providing crucial insights for goji berry breeders to support the development of goji berry cultivation in Romania.

**Keywords:** goji berry; breeding; plant resistance; Whole Genome Sequencing; *BODYGUARD* genes

## 1. Introduction

Goji berry plants have been used in Chinese medicine and cuisine for thousands of years [1] and presently, the berries are acknowledged as one of the most recognized 'superfruits' of the 21st century [2–4], being considered a functional food [1,5]. The goji berry has attracted significant attention in Western countries due to its nutritional profile, especially for its abundant vitamins and antioxidants. Its ORAC (Oxygen Radical Absorbance Capacity) values, which lie between 25,000 and 30,000, surpass those of other nutritionally beneficial fruits like pomegranates and blueberries, indicating its superior antioxidant capacity [6]. Its medicinal uses go from improving visual acuity [5,7,8], infertility [5], abdominal pain [5], dry cough, fatigue, and headache [5], immune system support, cancer prevention [7,8], antidiabetic activity [7,8] to increased longevity [8–10] and enhanced fertility [10–13].

In China, out of the existing nine *Lycium* taxa [14], only four are traditionally utilized, with *L. barbarum* and *L. chinense* being the main species traded worldwide [14,15]. For over two millennia, various pharmacological substances have been collectively referred to as "gou qi" [4,5,16], although it is likely that a diverse range of genetic materials is hidden behind this generalized term. The genus *Lycium*, distributed in temperate and subtropical regions worldwide [17–19], actually comprises 436 species names [20]. Out of these, only 92 are acknowledged as accepted species. 241 of these names are considered synonyms, while the remaining 103 names are categorized as unplaced [20]. Yao and al. (2018), cites 97 *Lycium* species, out of which 35 species and 2 varieties with recorded uses of food and/or medicine worldwide [14]. The Plants of the World Online records 101 officially recognized

*Lycium* species, indicating their indigenous presence in 71 countries, across 130 regions [21]. The database also indicate a list of 91 countries and 73 regions where *Lycium* species have been newly introduced, including Romania as one of these recent additions [21].

The flora of Romania recognizes *Lycium halimifolium* L. as a native species [22], however, a manuscript from 1867, referenced by Marian (2008), documents the traditional usage of *Lycium vulgare* Dun. in Romania, also mentioning its identification as *L. barbarum* in the Transylvania region [23]. Although it is scientifically acknowledged that *L. halimifolium* is typically considered a synonym of *L. barbarum* [24–26], widespread public belief, likely shaped by online media and local traditions, treats *L. halimifolium* and *L. barbarum* as separate species, attributing different culinary and toxicological properties to them [27,28]. In fact, both *L. halimifolium* and *L. vulgare* are today synonym names of *L. barbarum* [25,29]. Evidence of the long-standing presence of wild goji berry species in Romanian regions is found in the variety of local names used historically. These include names like 'licie', 'lician', 'lițian', 'răchișoară', 'răchiță', and 'zaharică' in Bucovina, 'licină' in Transylvania, and 'cătină' and 'cătină de garduri' in Moldova region [23]. The plant is characterized as a shrub with red berries, which not only serves as a natural fence in the countryside, but it is also recognized for its medicinal properties. Traditionally, it has been used to treat conditions related to fear and anxiety, demonstrating psychological effects, and for epilepsy and spasms, indicating neurological benefits [30]. In few Romanian regions it is considered an invasive plant, as in Oltenia, Danube riverbanks and Dobrogea [31,32].

Due to goji berry increased fame, the market demand grew exponentially in the last two decades [33,34]. China dominates goji berry production, particularly in the Northwest regions, like Ningxia and Xinjiang, two main exporting regions [9,35,36]. In contrast, production in North America and Europe is limited due to a lack of traditional use and the challenge of adapting Chinese varieties to these regions [37–40]. Ideal U.S. cultivation spots include Northern California and parts of the Northwest, while in Europe, Italy and Romania are leading in production [41,42]. Italy boasted the largest European goji farm in 2013, with 35 hectares and 60,000 plants [6]. Romania has emerged as a significant producer of goji berries [41], focusing also on plant material for cultivation [43], with a market that is showing a rising trend [42]. Especially in the difficult context of climate change constraints, goji berry planting material adapted to local conditions is required by European farmers. Therefore, initiatives on identifying new cultivars and crop production processes were launched, together with breeding programs that considered *Lycium* as an important target [44]. In Romania, by 2023, seven varieties of *Lycium* spp. were registered on the Official catalogue of cultivated plant varieties: 'Erma', 'Transylvania', 'Kirubi', 'Kronstadt', 'Bucur', 'Sara' and 'Anto', belonging to both *L. barbarum* and *L. chinense* [45].

Genetic diversity is the cornerstone of biodiversity, offering significant value in numerous areas, both scientific and practical [46]. It plays a crucial role in comprehending the origins and evolutionary processes of species, conserving genetic resources, investigating valuable germplasm resources and use the genetic materials displaying valuable traits [47,48]. Having a deeper understanding of native goji berry genetic resources is important both for preserving local biodiversity and for the breeding sector [36,47,49]. Since the rise of goji berry market demand, extensive molecular studies have been undertaken, to determine valuable genes in both cultivated and native or spontaneous plants, as a resource for valuable traits in the future breeds [1,18,36,49–51].

The process of crop plant breeding focuses on identifying and cultivating new plant varieties that exhibit beneficial characteristics like higher yields, better disease resistance, and enhanced nutritional value [52]. The high-throughput technologies, including genomics, transcriptomics, and metabolomics, have opened a new phase in crop breeding, enhancing the efficiency and precision of this process [52,53]. The last two decades have seen a significant growth in both the volume and quality of publicly available plant genomes [53]. This progress is largely due to advancements in sequencing technologies such as PacBio Sequel, Illumina HiSeq, Oxford Nanopore PromethION, and Illumina NovaSeq, alongside bioinformatic tools like the chromosome conformation capture technique (Hi-C) and BioNano optical maps. These developments have greatly enhanced the efficiency of genome sequencing, assembly, and annotation.[53–55].

In the Solanaceae family, which includes around 90 genera and 3,000 species, 170 full genomes of 46 species have been reported [54]. These genomic studies primarily targeted horticultural crops and their wild relatives, such as the cultivated tomato (*Solanum lycopersicum*) and its wild counterpart (*Solanum pimpinellifolium*), potato (*Solanum tuberosum*), hot pepper (*Capsicum annuum*), and eggplant (*Solanum melongena*). They also extend to tobacco (*Nicotiana tabacum*), decorative flowers like *Petunia inflata* and *Petunia axillaris*, and herbs with medicinal properties, including *Datura stramonium* and *Lycium barbarum* [52,56], or its invasive relative, *L. ferocissimum* [57]. *L. barbarum* genome contains 12 chromosomes [34] ( $2n = 2x = 24$ ) and is 1.8 Gb in size, with a level of heterozygosity of approximately 1% [56]. The sequenced and annotated genome ASM1917538v2 [58] was obtained by sequencing a haploid plant developed from pollen culture, using PacBio Sequel technology [56]. The annotation allowed identification of 47,740 genes and 34,339 protein-coding sequences. The availability of another annotated genome, of *L. ferocissimum*, of 1.2 Gb size, 40,291 genes and 30,549 protein-coding genes [57] will ease even more the characterization of the future goji berry sequenced genomes, allowing identification of new genes of interest.

Exploring the genetic diversity in Romanian wild and cultivated genotypes of *Lycium* species could bring the breeding sector one step closer to rapidly developing new varieties, adapted for specific ecopedological conditions and market requirement, indicating potential genetic markers for required traits. Generally, the goji berry in Romania has three major biotic threats, the powdery mildew, the goji berry gall mite and the stink bugs [59,60], being much more suitable for organic production than in China [40,61]. The escalating threat of extreme weather events caused by climate change is set to pose an increasingly serious challenge to goji berry production, major threats being extreme drought and insolation [62,63]. The Romanian homologated varieties were developed based on Chinese varieties germplasm, due to their high fruit quality traits [64,65], without using the local germplasm.

The current study marks the initial phase of a broader project focused on genes related to resistance to abiotic and biotic stress, focusing here on cuticle thickness. This aspect is critically important due to cuticle significant influence on resistance to drought, insolation, and pests [66–68], as the cuticle is essential for plant survival in periods of severe drought [69] and pests and disease attack [70]. *BODYGUARD* genes in plants typically encode for proteins that are directly involved in defending the plant against various stresses, including the biosynthesis of cutin in *Arabidopsis* [69,71,72], mainly the  $\alpha/\beta$  hydrolase protein [69,71,73]. Analysing the genetic diversity of cultivated and wild goji plants genes has the final aim of providing information required by the goji berry breeders supporting the development of goji berry production in Romania.

## 2. Results

### 2.1. NGS Data Analysis

#### 2.1.1. Sequencing Data Quality Control

The genomes of eight Romanian *L. barbarum* varieties, out of which five were cultivated varieties, part of a population obtained from Chinese seeds [74] and three were spontaneous plants growing in the wild in three different Romanian counties [31,75] were sequenced using NGS technology. The quality distribution of sequencing was analysed across the entire length of all sequences to identify any locations with abnormally low sequencing quality, that could indicate the inclusion of incorrect bases at higher-than-normal rates. The proportion of clean data relative to raw data, referred to as the effective rate, was higher than 99.52% for all reads. For 1,669,720,889 bp reference genome, the mapping rate of each sample ranged from 96.66% to 99.36%. Referring to the reference genome (without Ns), the average depths were between 10.01 X and 9.29 X, and 1 X coverages ranged from 77.43% to 97.41%, the results falling within the acceptable normal range and could be utilized in variation detection and genetic analysis.

#### 2.1.2. SNP Detection, Distribution, and Mutation Frequency

SNP (Single Nucleotide Polymorphisms) variations were observed in all eight genotypes, but the quantity and genomic distribution of these variations differed across the genotypes. A total of 108,290,958 SNPs were identified within the eight genotypes, with an average ranging from 14,079,300.6 SNPs/genome for the cultivated specimens and 12,631,485 SNPs/genome for the wild specimens. The Lb2 genome exhibited the largest quantity of SNPs, totalling 15,983,773.

In the eight genotypes, the transitions – point mutations that change one purine nucleotide to another or one pyrimidine to another – were more frequent, with an average count of 8,559,837.5. This was higher than the number of transversions, which are mutations that switch a purine for a pyrimidine or vice versa, averaging at 4,976,532.25. This resulted in an average ts/tv ratio (transitions to transversions) of 1.72, which was relatively consistent across the two categories of genotypes, cultivated and wild. However, there was a difference between cultivated and wild-grown plants: the average ts/tv ratio was higher in cultivated genotypes at 1.736, compared to 1.688 in wild-grown plants.

The genotype Lb7w exhibited the highest heterozygosity rate, measured as 5.037‰ (per thousand), whereas the lowest rate was found in genotype Lb4, at 3.245‰. On average, wild plants showed a higher heterozygosity rate, averaging at 4.851‰, compared to cultivated plants, which had an average heterozygosity rate of 3.607‰.

Figure 1 illustrates the distribution of the six types of SNP mutations. Genotypes Lb1 and Lb2 showed the highest number of SNPs across all six SNP types, while genotype Lb8w had the lowest count. Among these six types of SNP mutations, for every genotype, the most common was the C:G > T:A mutation, followed by T:A > C:G. Conversely, the least frequent type of SNP was the C:G > G:C mutation.

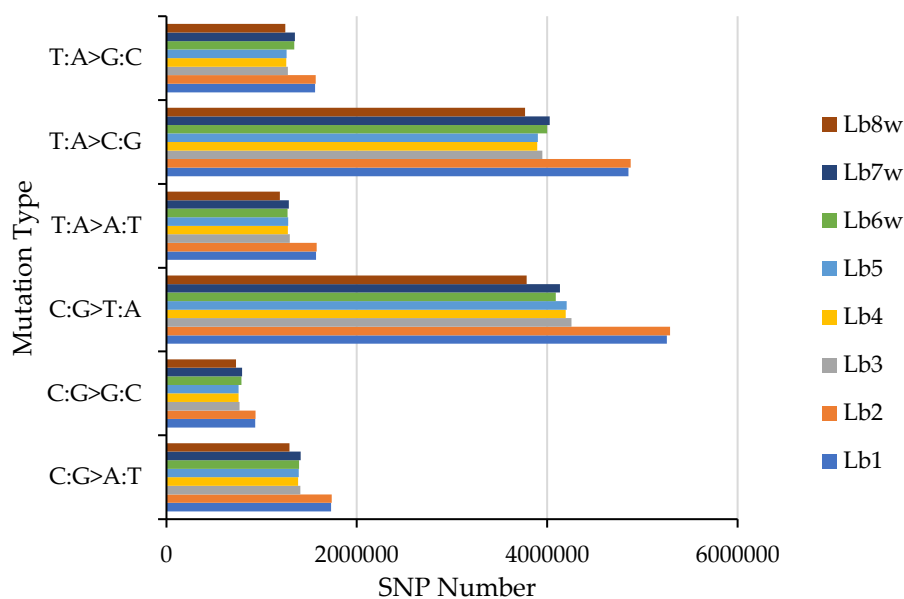


Figure 1. SNP mutation type distribution.

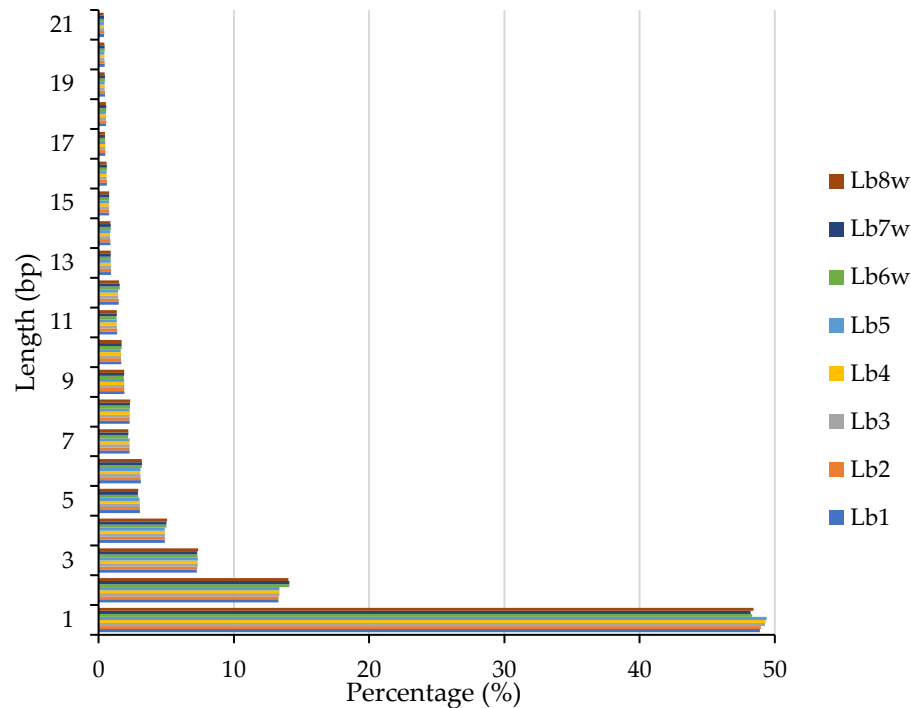
### 2.1.3. Insertion/Deletion Detection and Distribution

InDel (Insertions and Deletions) variations were detected in all the studied genotypes, amounting to a total of 11,225,960 InDels, averaging 1,403,245 InDels per genome. The genotype Lb2 had the highest number of InDels at 1,773,325, whereas Lb8w had the fewest, totaling 1,197,605. For cultivated specimens, the average InDels per genome was 1,495,506.4, compared to 1,249,476 for wild specimens. When examined individually, the overall count of insertions, which is 5,166,205, is less than the total total number of deletions, totaling 6,043,098. However, the mean count of insertions in cultivated specimens, at 684,984.8, exceeds the average insertion count in wild specimens, which is 580,427. In the case of deletions, the average number of deletions in cultivated specimens, amounting to 808,410.6, is greater than the average deletion count in wild specimens, recorded at 667,015.



The InDel heterozygosity rate, expressed in per mille (‰) and calculated as the ratio of InDels to the total genomic bases, is 0.381‰. This value is lower on average for cultivated specimens, at 0.335‰, compared to the higher average rate for wild specimens, which is 0.458‰.

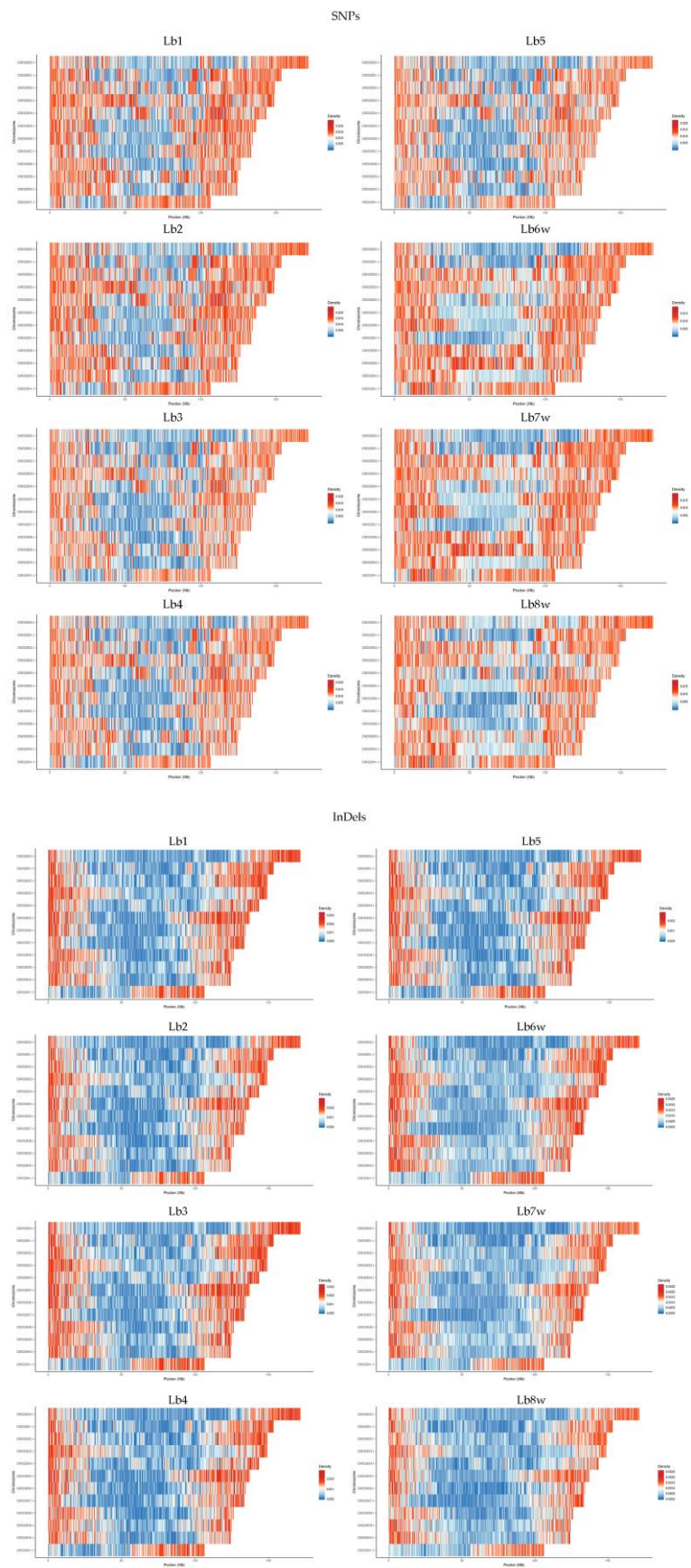
InDels distribution within the genome (Figure 2) was the highest for 1 bp insertion/deletion and decreased with the increase in sequence length.



**Figure 2.** Length distribution of InDels in the eight Romanian goji berry genomes.

In the analysis of the eight genotypes, the densities of SNPs and InDels across each chromosome appeared to be relatively similar. However, a visible reduction in InDel density was observed (Figure 3). Additionally, distinct differences were evident between the genomes of cultivated and wild plants. Generally, the ratio of SNPs to InDels was around 10. This ratio was slightly lower in cultivated plants, ranging from 9.29 to 9.52, and somewhat higher in wild plants, with values ranging from 10.03 to 10.16.

For both SNPs and InDels, it is visible that the density of variation is higher at the end of the all 12 chromosomes. In addition: (1) all genomes of cultivated specimens have a high variation density, for both SNPs and InDels, almost in the middle



**Figure 3.** SNPs (Top) and InDels (Bottom) densities per chromosome, per genotype of the 5<sup>th</sup> chromosome, the same area being observed also in the genomes of wild plants, but with a lower density; (2) all genomes have the first half of the 12th chromosome with a very low density of both

SNPs and InDels, with the exception of wild plants, where SNPs are present in a very high density; (3) the first chromosome, although is the longest one, has the longest area with low density of SNPs and InDel variations, except the Lb8w; (4) there is a clear distinction between cultivated and wild plant genomes, easier to be observed at the level of SNPs.

2.1.4. Sequence analyses of BODYGUARD genes in Romanian goji berry genomes

In both *Lycium* species available genomes, three BODYGUARD (BDG) genes have been identified (Table 1). However, these genes are situated on different chromosomes in each species, on chromosomes 4, 8, and 9 in *Lycium barbarum*, and on chromosomes 1, 3, and 9 on *Lycium ferocissimum*.

**Table 1.** Name, description and location of the BODYGUARD gene in the *L. barbarum* and *L. ferocissimum* genomes.

Name/Gene ID	Description	Location
LOC132634709 ID: 132634709	probable lysophospholipase BODYGUARD 3 [ <i>Lycium barbarum</i> (goji berry)]	Chromosome 4, NC_083340.1
LOC132607278 ID: 132607278	probable lysophospholipase BODYGUARD 4 [ <i>Lycium barbarum</i> (goji berry)]	Chromosome 8, NC_083344.1
LOC132609965 ID: 132609965	probable lysophospholipase BODYGUARD 3 [ <i>Lycium barbarum</i> (goji berry)]	Chromosome 9, NC_083345.1
LOC132060388 ID: 132060388	probable lysophospholipase BODYGUARD 3 [ <i>Lycium ferocissimum</i> ]	Chromosome 1, NC_081342.1
LOC132049371 ID: 132049371	probable lysophospholipase BODYGUARD 4 [ <i>Lycium ferocissimum</i> ]	Chromosome 3, NC_081344.1
LOC132030714 ID: 132030714	probable lysophospholipase BODYGUARD 3 [ <i>Lycium ferocissimum</i> ]	Chromosome 9, NC_081350.1

Analyzing the eight studied genomes using Genome Workbench, a distinct divergence was noted between the genomes of cultivated and wild plants.

Regarding the BDG gene situated at LOC132634709 (Table 2) on Chromosome 4, 22 SNPs were identified within its coding region. Out of these, 14 are synonymous mutations, meaning they do not change the amino acid sequence. With the exception of the SNP at the position 1312, situated within a codon that encodes for lysine/arginine (basic amino acids), the rest of the SNPs are situated within codons that encode for either nonpolar (10) or polar amino acids (11), and none of these SNPs changed the polarity or charge of the encoded amino acid. A key finding was the apparent distinction in most SNPs between cultivated and wild varieties of the plant, differences being observed as homozygous versus heterozygous SNPs.

In the BDG gene at LOC132607278 (Table 3) on Chromosome 8, analysis revealed 28 SNPs within its coding sequence, including 4 synonymous mutations. Notably, SNPs at positions 287-288 and 392-294 each impact a single codon, changing AAA to CGA (Lysine to Arginine) and AAA to GAC (Lysine to Aspartic Acid), respectively. A distinct pattern was observed between cultivated and wild genotypes in 17 specific SNP locations: 174, 209, 288, 392-394, 617, 648, 688, 694, 799, 891, 1030, 1049, 1076, 1150, 1151, 1266, and 1304, predominantly presenting as homozygous versus heterozygous SNPs. Apart from the SNPs at positions 146 and 1274, which alter the charge of the encoded amino acids (Lysine to Glutamic Acid and Glutamic Acid to Lysine, respectively) from basic to acidic and vice versa, the other SNPs do not cause any changes in either the polarity or charge of the encoded amino acids.

**Table 2.** Sequence analysis of goji berry BDG gene, LOC132634709, on Chromosome 4.



SNP	DNA sequence	Amino acid	Reference genome	Cultivated					Wild		
			ASM1917538v2	Lb1	Lb2	Lb3	Lb4	Lb5	Lb6w	Lb7w	Lb8w
517	AAT/AGT	Asn/Ser	Asn	h69	h59	h67	h50	h75	Asn	Asn	Asn
529	AGC/ATC	Ser/Ile	Ser	h69	h57	h40	h50	h75	Ser	Ser	Ser
537	TTA/CTA	Leu/Leu (silent)	Leu(TTA)	Leu(CTA)	Leu(CTA)	Leu(CTA)	Leu(CTA)	Leu(CTA)	h33	h20	h30
590	CTT/CTC	Leu/Leu (silent)	Leu(CTT)	Leu(CTC)	Leu(CTC)	Leu(CTC)	Leu(CTC)	Leu(CTC)	h37	Leu(CTT)	h40
746	TGC/TGT	Cys/Cys (silent)	Cys(TGC)	Cys(TGT)	Cys(TGT)	Cys(TGT)	Cys(TGT)	Cys(TGT)	h50	h17	h30
749	CTG/CTC	Leu/Leu (silent)	Leu(CTG)	h35	h40	h50	h33	h20	h43	h29	h36
767	CTA/CTG	Leu/Leu (silent)	Leu(CTA)	Leu(CTG)	Leu(CTG)	Leu(CTG)	Leu(CTG)	Leu(CTG)	h50	h37	h30
804	CCA/ACA	Pro/Thr	Pro	h78	h45	h25	h80	h60	Pro	Pro	Pro
873	GCT/TCT	Ala/Ser	Ala	h67	h56	h18	h60	h80	Ala	Ala	Ala
956	ATG/ATT	Met/Ile	Met	h42	h35	h37	h40	h80	Met	Met	Met
1190	TCA/TCT	Ser/Ser (silent)	Ser (TCA)	h46	h92	h50	h45	h60	Ser (TCA)	Ser (TCA)	Ser (TCA)
1208	GCA/GCC	Ala/Ala (silent)	Ala (GCA)	h50	h7	h50	h60	h40	h75	h40	h33
1247	AGT/AGC	Ser/Ser (silent)	Ser (AGT)	h50	h20	h57	h70	h25	h80	h45	h40
1312	AAA/AGA	Lys/Arg	Lys	Arg	Arg	Arg	Arg	Arg	h80	h40	h50
1352	TGC/TGT	Cys/Cys (silent)	Cys(TGC)	h42	h39	h53	h57	h44	Cys(TGC)	Cys(TGC)	h14
1439	AAA/AAG	Lys/Lys (silent)	Gly	h59	h65	h60	h18	h60	h67	h57	h57
1457	CAG/CAA	Gln/Gln (silent)	Gln (CAG)	Gln (CAG)	Gln (CAG)	Gln (CAG)	Gln (CAG)	Gln (CAG)	h67	h57	h57
1520	ATG/ATT	Met/Ile	Met	h43	h54	h67	h83	h45	Met	Met	Met
1631	TAC/TAT	Tyr/Tyr (silent)	Tyr (TAC)	Tyr (TAT)	Tyr (TAT)	Tyr (TAT)	Tyr (TAT)	Tyr (TAT)	h75	h25	h73
1715	ATA/ATT	Ile/Ile (silent)	Ile	h61	h47	h50	h50	h50	h67	h33	h60
1761	ACG/TCG	Thr/Ser	Tyr	h67	h71	h50	h37	h33	Tyr	Tyr	Tyr
1886	GGC/GGG	Gly/Gly (silent)	Gly (GGC)	Gly (GGC)	h36	h54	h86	h30	h83	h43	h75

nonpolar

polar

basic

acidic

Table 3. Sequence analysis of goji berry BDG gene, LOC132607278, on Chromosome 8.

SNP	DNA sequence	Amino acid	Reference genome	Cultivated					Wild		
			ASM1917538v2	Lb1	Lb2	Lb3	Lb4	Lb5	Lb6w	Lb7w	Lb8w
129	TGG/TTG	Trp/Leu	Trp	h50	h67	h67	h57	h50	h33	h75	h75
146	AAA/GAA	Lys/Glu	Lys	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu
174	GTA/GCA	Val/Ala	Val	h45	h58	h17	h33	h71	Val	Val	Val
202	GAG/GAC	Glu/Asp	Glu	Asp	h93	Asp	Asp	Asp	h41	h62	Asp
209	TTT/CTT	Phe/Leu	Phe	Phe	Phe	Phe	Phe	Phe	h46	h62	h67
287-288	AAA/CGA	Lys/Arg	Lys	h50	h47	h20	h50	h82	Lys	h14/Lys	Lys
392-394	AAA/GAC	Lys/Asp	Lys	h35	h53	h33	h33	h67	Lys	Lys	Lys
617	GAA/AAA	Glu/Lys	Glu	Glu	Glu	Glu	Glu	Glu	h57	h75	h75
648	GCA/GGA	Ala/Gly	Ala	Ala	Ala	Ala	Ala	Ala	h57	h12	h14
688	AAC/AAT	Asn/Asn (silent)	Asn	h41	h36	h57	h50	h25	Asn	Asn	Asn
694	TGC/TGT	Cys/Cys (silent)	Cys	h41	h36	h57	h43	h14	Cys	Cys	Cys
799	GTA/GTG	Val/Val (silent)	Val(GTA)	Val(GTA)	Val(GTA)	Val(GTA)	Val(GTA)	Val(GTA)	h50	h40	Val(GTG)
824	TCT/CCT	Ser/Pro	Pro	Pro	Pro	Pro	Pro	Pro	h43	h45	Pro
864	TAC/TTC	Tyr/Phe	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	h50	h33	Tyr
891	AGT/ATT	Ser/Ile	Ser	h53	h58	h56	h78	h67	Ser	Ser	Ser
1023	TGG/TTG	Trp/Leu	Trp	h6	Trp	Trp	Trp	Trp	h50	h71	h43
1030	GGA/GGT	Gly/Gly (silent)	Gly (GGA)	h58	h22	h43	h56	h33	Gly (GGA)	Gly (GGA)	Gly (GGA)
1049	TGG/GGG	Trp/Gly	Trp	h38	h78	h43	h44	h71	Trp	Trp	Trp
1076	ATT/GTT	Ile/Val	Ile	h38	Ile	Ile	Ile	Ile	h40	h50	h67
1150	TTT/TTA	Phe/Leu	Phe	Phe	Phe	Phe	Phe	Phe	h29	h50	h50
1151	ATG/GTG	Met/Val	Met	Met	Met	Met	Met	Met	h29	h50	h50
1266	CCT/CTT	Pro/Leu	Pro	h64	h75	h60	h64	h50	Pro	Pro	Pro
1270	GAA/GAT	Glu/Asp	Glu	Asp	Asp	Asp	Asp	Asp	h60	h62	Asp
1274	GAG/AAG	Glu/Lys	Glu	Glu	Glu	Glu	Glu	Glu	h60	h57	Lys
1304	ACT/TCT	Thr/Ser	Thr	h64	h73	h71	h86	h40	Thr	Thr	Thr
1338	TGT/TCT	Cys/Ser	Cys	h36	h23	h37	Ser	h71	Cys	Cys	Cys

nonpolar

polar

basic

acidic

The BDG gene at LOC132609965 (Table 4) on chromosome 9 features 29 SNPs within its coding region, with 18 of these being silent mutations. Two SNPs at positions 555-557 lead to the formation of four different codons: TCT, GCT, TCA, and TCG, which correspond to the amino acids Serine, Alanine, Serine, and Serine, respectively. In a similar manner, the two SNPs at positions 1671-1672 result in the codons GGA, AGA, and GAA, which encode for the amino acids Glycine, Arginine, and Glutamic Acid, respectively. The SNPs at positions 555-557 are unique in that they alter the polarity of the encoded amino acid from Serine (as found in the reference genome) to Alanine (as observed in the cultivated genotypes). The other SNPs, however, do not cause any changes in the polarity or charge of the amino acids they encode. Approximately half of these SNPs show sequence-level differences between the cultivated and wild type genotypes.

Table 4. Sequence analysis of goji berry BDG gene, LOC132609965, on Chromosome 9.

SNP	DNA sequence	Amino acid	Reference genome	Cultivated						Wild		
			ASM1917538v2	Lb1	Lb2	Lb3	Lb4	Lb5	Lb6w	Lb7w	Lb8w	Lb9w
473	CCT/CCC	Pro/Pro (silent)	Pro	Pro	Pro	Pro	Pro	Pro	h75	h56	h33	Pro
479	TAC/TAT	Tyr/Tyr (silent)	Tyr (TAC)	Tyr(TAT)	Tyr(TAT)	Tyr(TAT)	Tyr(TAT)	Tyr(TAT)	h50	h44	h33	h33
498	GCC/ACC	Ala/Thr	Ala	h61	h41	h67	h67	h17	h40	h50	h33	Ala
548	TGT/TGC	Cys/Cys (silent)	Cys (TGT)	Cys (TGT)	Cys (TGT)	Cys (TGT)	Cys (TGT)	Cys (TGT)	h50	h54	Cys (TGT)	Cys (TGT)
555-557	TCT/GCT/TCA/TCG	Ser/Ala/Ser/Ser	Ser	Ala	Ala	Ala	Ala	Ala	h50	h46	h33	h33
563	TCT/TCC	Ser/Ser (silent)	Ser (TCT)	Ser(TCC)	Ser(TCC)	Ser(TCC)	Ser(TCC)	Ser(TCC)	h67	h46	Ser(TCT)	Ser(TCT)
625	GCG/GTG	Ala/Val	Ala	Val	Val	Val	Val	Val	h75	h70	h40	h40
627	GCT/TCT	Ala/Ser	Ala	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	h40
643	TTC/TCC	Phe/Ser	Phe	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
668	CTT/CTC	Leu/Leu (silent)	Leu (CTT)	Leu(CTC)	Leu(CTC)	Leu(CTC)	Leu(CTC)	Leu(CTC)	h33	h60	h50	h50
713	TCG/TCC	Ser/Ser (silent)	Ser (TCG)	Ser(TCC)	Ser(TCC)	Ser(TCC)	Ser(TCC)	Ser(TCC)	h25	h33	h75	h75
977	TCG/TCA	Ser/Ser (silent)	Ser (TCG)	Ser (TCA)	Ser (TCA)	Ser (TCA)	Ser (TCA)	Ser (TCA)	h33	h20	h60	h60
982	TAT/TGT	Tyr/Ser	Tyr	h50	h67	h50	h50	h50	Tyr	Tyr	Tyr	Tyr
986	CGG/CGA	Arg/Arg (silent)	Arg (CGG)	Arg (CGA)	Arg (CGA)	Arg (CGA)	Arg (CGA)	Arg (CGA)	h33	h20	h63	h63
1076	GAG/GAA	Glu/Glu (silent)	Glu(GAG)	Glu (GAA)	Glu (GAA)	Glu (GAA)	Glu (GAA)	Glu (GAA)	h33	h20	h70	h70
1079	AAA/AAG	Lys/Lys (silent)	Lys (AAA)	h58	h47	h40	h43	h67	h25	h25	h70	h70
1223	CCA/CCC	Pro/Pro (silent)	Pro (CCA)	h50	h48	h22	h60	h57	Pro (CCA)	Pro (CCA)	Pro (CCA)	Pro (CCA)
1256	AGG/AGA	Arg/Arg (silent)	Arg (AGG)	h41	h56	h71	h56	h50	h37	h67	Arg(AGA)	Arg(AGA)
1285	GTG/GCG	Val/Ala	Val	h42	h44	h67	h60	h62	h37	h775	Ala	Ala
1304	TCG/CTC	Ser/Ser (silent)	Ser(TCG)	h65	h48	h17	h50	h33	Ser (TCG)	Ser (TCG)	Ser (TCG)	Ser (TCG)
1394	CTG/CTT	Leu/Leu (silent)	Leu(CTG)	Leu(CTC)	Leu(CTC)	Leu(CTC)	Leu(CTC)	Leu(CTC)	h50	h50	Leu(CTC)	Leu(CTC)
1421	TTA/TTG	Leu/Leu (silent)	Leu (TTA)	h67	h62	h60	Leu (TTG)	Leu (TTA)	Leu (TTA)	Leu (TTA)	Leu (TTA)	Leu (TTA)
1466	ACT/ACA	Thr/Thr (silent)	Thr(ACT)	Thr (ACA)	Thr (ACA)	Thr (ACA)	Thr (ACA)	Thr (ACA)	h50	h40	h57	h57
1550	ACA/ACG	Thr/Thr (silent)	Thr(ACA)	Thr (ACG)	Thr (ACG)	Thr (ACG)	Thr (ACG)	Thr (ACG)	h33	h50	h57	h57
1580	ATC/ATA	Ile/Ile (silent)	Ile (ATC)	Ile (ATA)	Ile (ATA)	Ile (ATA)	Ile (ATA)	Ile (ATA)	h33	h57	h71	h71
1671-1672	GGA/AGA/GAA	Gly/Arg/Glu	Gly	h16	h9	h16	h11	h6	Gly	Gly	Gly	Gly
1689	GCT/TCT	Ala/Ser	Ala	h23	h15	h21	h20	h24	Ala	Ala	Ala	Ala
1726	ACA/AAA	Thr/Lys	Thr	h10	h17	h33	h18	h23	h57	h17	h67	h67

nonpolar

polar

basic

acidic

3. Discussion

The sequencing and annotation of the goji berry genome in 2023 [56] represents a crucial resource for future resequencing projects. The advancements in Next Generation Sequencing/Whole Genome Sequencing (NGS/WGS) [55] are poised to generate a wealth of data, which will be instrumental in developing new goji berry varieties. The examination of variations in density at the genomic level, particularly for SNPs and InDels, highlights specific genome areas that warrant further investigation, to identify potentially beneficial genes from wild genotypes that could be integrated into new varieties. The observed increased polymorphism near the ends of chromosomes can be attributed to the higher frequency of recombination in these areas [76].

In Romania, seven new goji berry varieties have already been created and registered, originating from Chinese genomic material, without genetic elements from native plants. Present research is dedicated to enriching the diverse gene pool found in wild germplasm, potentially enhancing the unique characteristics of Romanian goji berries. By examining the morphological and phenological traits of wild goji berries and correlating them with genetic data, characteristics like early or late flowering, high drought tolerance, strong resistance to low temperatures (Figure 4), as well as features like thicker cuticles and leaves, could become valuable assets in breeding programs.

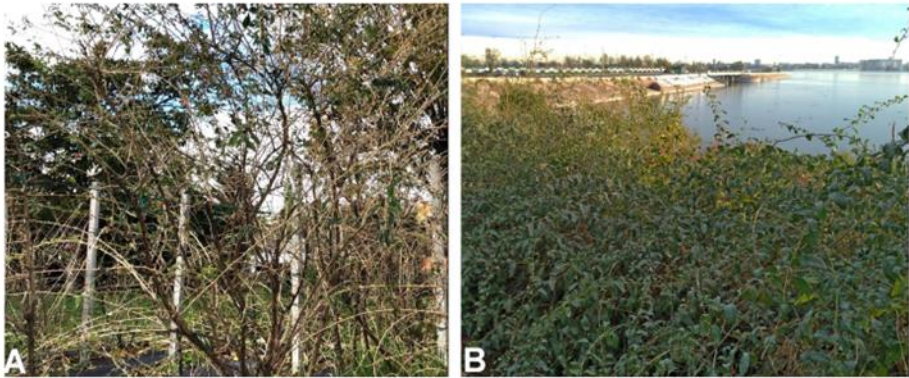
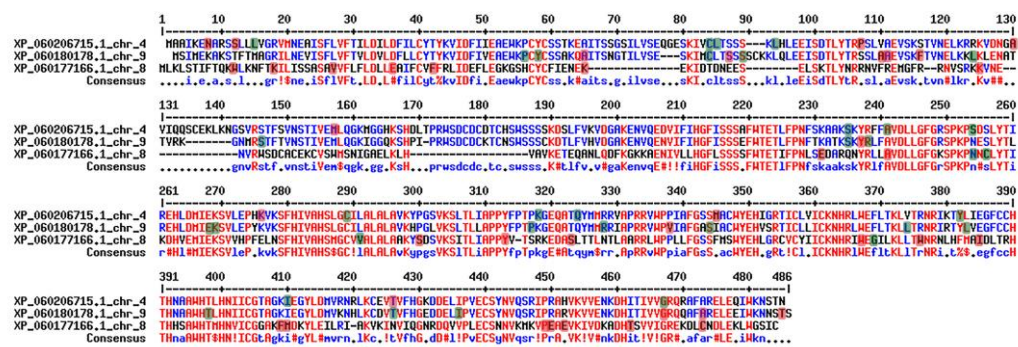


Figure 4. Cultivated (A) and wild (B) *L. barbarum* plants on 13 November 2023.

In earlier research, the morpho-anatomical features of leaves and flowers of both wild and cultivated goji berries in the Bucharest region were analysed. The study aimed to identify key traits of interest to both goji berry breeders and taxonomists [31]. An other study also involved mapping

the spontaneous genetic resources found across Romania [75]. Notable morphological distinctions were observed in leaf shape, orientation, and width of Romanian *L. barbarum*, results that are similar with findings reported in Republic of Moldova, in a similar study between cultivated and wild goji berry [77]. The leaf anatomical characteristics are particularly significant in relation to biotic and abiotic stress factors, the wild plants having the leaves covered with a thick cuticle, prominently developed vascular bundles, and sheaths surrounding the vascular bundles within the mesophyll. Additionally, the palisade cells in these plants were observed to be considerably larger than in cultivated plants [31]. These findings motivate further investigation into genes putatively linked to these phenotypic differences. The formation of the plant cuticle involves several proteins that play crucial roles in the biosynthesis and regulation of cutin and waxes, as BDG, CER, KCS, VLCFAs, GPAT, LACS, ABC, SHN/WIN, LTPs, CD1 [69,71,78,79].

The sequence analyses of the *BDG* genes in Romanian goji berry genomes revealed several differences among the three genes. The genes located on chromosomes 4 and 9 are encoding probable lysophospholipase BODYGUARD 3 proteins, whereas the gene located on chromosome 8 encodes a probable lysophospholipase BODYGUARD 4 protein [56]. The *BDG 4* gene from chromosome 8 is shorter than the *BDG 3* genes, from chromosomes 4 and 9. Although located on different chromosomes, two of the genes presented SNPs affecting the same amino acid, as in 12, 65, 84, 235, 254, 410, 426, 467, and 473 positions. Even if some SNPs are located within conserved regions, many of them are silent (Figure 5).



**Figure 5.** Alignment of the three BDG proteins. Highlighted with red are the non-synonymous SNPs, with green are the synonymous SNPs.

## 4. Materials and Methods

### 4.1. Plant Material

In this study, five selected genotypes from Romanian goji berry cultivated and three spontaneous-growing genotypes were examined. The five genotypes are integral to an extensive breeding program for goji berries that commenced in 2014 at the Experimental Field of the Faculty of Horticulture, at University of Agronomic Sciences and Veterinary Medicine in Bucharest [64,65,74]. The initial biological samples were derived from the seeds of *Lycium barbarum* L., including five distinct biotypes: Lb1-Lb5 [74]. The native plant samples were chosen from robust and well-established populations in the counties of Bucharest (Lb6w), Ilfov (Lb7w), and Călărași (Lb8w). Specifically in Bucharest, specimens were gathered from the shores of Morii Lake (44.453424, 26.013337), a natural area on the periphery of the western segment of the Romanian capital. This location was also selected for a comparative morpho-anatomical study of the leaves and flowers of both wild and cultivated goji berry plants [31]. The plants encountered in Ilfov county are believed to have originated from cultivated specimens within a military base, subsequently becoming naturalized in the area. (44.447382, 26.019239). The specimens from Călărași were found proliferating along a roadside on Lehliu city (44.434389, 26.858775).

### 4.2. DNA Extraction

Genomic DNA from fresh goji berry leaves was isolated using the InnuPure C16 automated system (Analytik Jena GmbH, Jena, Germany), which employs magnetic particle separation technology for the fully automated extraction and purification of DNA. This process took place at the Research Center for Studies of Food Quality and Agricultural Products at the University of Agronomic Sciences and Veterinary Medicine in Bucharest, Romania. For genomic DNA extraction was used the InnuPREP Plant DNA I Kit-IPC16 (Analytik Jena GmbH, Jena, Germany), adhering to the protocols provided by the manufacturer. Initial processing involved breaking down the plant material externally, with the sample being mashed into a fine powder under liquid nitrogen and then homogenized with a SLS lysis solution (with CTAB as detergent), proteinase K, and an RNase A solution. Following this external lysis step, the automatic DNA extraction continued in the InnuPure C16 automated system, as per the manufacturer's guidelines. DNA quantification was performed with the NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) [80].

#### 4.3. Sequencing

Whole-genome sequencing (WGS) was conducted using the next-generation sequencing (NGS) technology of an Illumina platform by Novogene Co., Ltd. (Cambridge, UK). The original image data from Illumina's high-throughput sequencing were converted into sequenced reads (raw data) through the CASAVA base recognition process (Base Calling) at Novogene Co., Ltd. These raw data were saved in FASTQ (.fq) format files [81], which included the sequencing reads along with their respective base quality scores.

#### 4.4. Computational Data Processing, and Sequencing Analysis

The BWA software was utilized to align the effective sequencing data with the reference sequence, using the parameters: `mem -t 4 -k 32 -M` [82]. The alignment outcomes were used to calculate the mapping rate and coverage. To ensure the quality of subsequent analyses, reads of low quality or those containing adaptors were filtered out to achieve clean reads. The Phred quality score (Qphred), which is indicative of the base quality, was determined by the formula  $Q_{phred} = -10\log_{10}(e)$ , with 'e' being the error rate of the sequencing.

The reads were aligned with the reference genome of the goji berry, GCF\_019175385.1, downloaded from the NCBI database [56]. This process produced sequence alignment format files, which were subsequently transformed into binary sequence alignment format (\*.bam) files. These were then processed to generate a variant file containing SNP (Single Nucleotide Polymorphism) data. The mapping rates for the samples indicate the degree of resemblance between each sample and the reference genome. Additionally, depth and coverage serve as metrics for the consistency and extent of correspondence to the reference genome, as conducted by Novogene Co., Ltd.

#### 4.5. SNP Detection and Annotation

SNP detection was conducted using SAMtools with the specified parameters '`mpileup -m 2 -F 0.002 -d 1000`' [82], facilitated by Novogene Co., Ltd. To minimize the likelihood of errors in SNP identification, the data underwent a two-step filtration process: a SNP was only considered if it was supported by over four reads, and its mapping quality had to exhibit a root mean square value exceeding 20, based on the supporting reads' mapping qualities. The overall heterozygosity rate of SNPs across the genome (het rate, denoted in permille ‰) was determined by the number of heterozygous SNPs over the total count of genomic bases. SNPs were assorted into six mutation classifications: T:A > C:G, T:A > G:C, C:G > T:A, C:G > A:T, T:A > A:T, and C:G > G:C. Take, for instance, mutations from T:A to C:G, which entail alterations from T to C and A to G. A T-to-C mutation on one strand of the DNA double helix will correspond to an A-to-G mutation at the identical position on the opposite strand. As a result, mutations of T > C and A > G were grouped together in one category.

#### 4.6. Insertion/Deletion (InDel) Detection and Annotation



An InDel was identified as either an insertion or a deletion of a DNA sequence that is 50 base pairs (bp) in length or shorter. Detection of InDels was carried out using SAMTOOLS with the parameters set to 'mpileup -m 2 -F 0.002 -d 1000' [82], annotated with ANNOVAR software [83], by Novogene Co. The criteria for filtering InDels to enhance detection accuracy were consistent with those applied during SNP detection. The length distribution of InDels was examined as a proportion of the entire genome.

#### 4.7. Sequence Analysis of the BDG genes

Next-generation sequencing BAM files containing the nucleotide sequence data for the eight goji berry genotypes were uploaded onto the NCBI Workbench software and aligned to the reference genome [84]. For each variety, the differences in nucleotide sequence were noted. Amino acid sequences of the three probable BDG proteins were aligned using the MultAlin software [85].

### 5. Conclusions

The present study re-sequenced the whole genome for eight *L. barbarum* genotypes, cultivated and wild-type, and analysed the variability of three *BDG* genes, involved in the cuticle biosynthesis, at the coding sequence level. NGS sequencing revealed clear differences between cultivated and wild type genotypes, not only in the whole genome, but also among the *BDG* genes. Future studies will be done to confirm the role of the *BDG* genes in cuticle biosynthesis, and furthermore, their implication in the resistance to biotic/abiotic stress. In addition, the data generated by the whole genome resequencing of these genotypes will allow for the analysis of additional useful genes that could be introgressed from the wild type into future varieties in the goji berry breeding programs in Romania.

**Supplementary Materials:** Not applicable.

**Author Contributions:** Conceptualization, R.C. and M.I.; methodology, R.C., M.I.; software, R.C., M.I.; investigation, R.C., M.I., A.A., V.L.; writing—original draft preparation, R.C., M.I.; writing—review and editing, R.C., M.I., A.A., V.L.; project administration, R.C.; funding acquisition, R.C. All authors have read and agreed to the published version of the manuscript.

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