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Morphological, Genetic and Biochemical Evaluation of *Dasypyrum villosum* (L.) P. Candargy in the Gene Bank Collection

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Abstract: The *Dasypyrum villosum* gene bank collection, comprising 32 accessions, was characterized morphologically and genetically for resistance to leaf diseases and for quality parameters of seeds with specific accent to protein polymorphism and protein and starch composition. The collected material represented nearly the whole distribution area in the Mediterranean. For SSR analysis, a set of 40 SSR markers for wheat was selected. A matrix of distances between genotypes was calculated using Simple Matching dissimilarity coefficient in the DARwin software. The collection was scored for resistance to powdery mildew, brown, stripe and stem rusts. A modified SDS-PAGE method with clear interpretation of high and low molecular glutenin subunits (HMW, LMW) was used for characterization of accessions. Morphological phenotyping revealed considerable diversity allowing the distinguishing of clusters tracing the geographical origin of accessions. Genetic diversity showed three groups but without significant bootstrap support. All tested accessions were resistant to the applied races of powdery mildew and leaf rust. Three accessions were moderately susceptible to currently available races of yellow rust. Biochemical analyses of seeds in selected populations showed a high content of crude proteins with a significant proportion of prolamins and Σglutelins. The SDS-PAGE of HMW and LMW glutelins confirmed both the high population polymorphism and the intra-population differences. Apart from the recent research in CWR breeding, *Dasypyrum villosum* is still an underrepresented species in germplasm collections and an underutilized species in breeding.

Keywords: *Dasypyrum villosum*; genetic resources; habitats; phenotyping; genotyping; seed composition; storage grain protein; SDS Page; HMW-LMW-Glu

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1. Introduction

Crop Wild Relative species (CWR) are unique genetic resources for breeding of related cultivated species. Having avoided the genetic bottleneck associated with cultivated crops CWR contain at least some of the necessary genetic diversity for crop improvement [1]. The reduction in genetic diversity during domestication is exacerbated by the demand for high crop productivity and crop uniformity in the field and the marketplace. The unintended consequence of recurrent selection is that potentially valuable genetic variants and associated phenotypes have been filtered out of crop gene pools [2]. CWR face threats such as intensive agriculture, urban development, pollution and biological invasions [3,4] and thus need conservation attention, but not many gene banks keep them *ex situ*. Their frequent occurrence in unfavorable conditions and their frequent resistance to common cereal diseases predestinate them as potential donors of important genes for cereal breeding.

CWR possess many desirable traits, especially for resistance to pests and diseases or tolerance to abiotic stresses like drought, heat, and flooding [1]. Such traits can be (and have been) bred into crops to modify yields, nutritional quality, and husbandry requirements to meet changing environmental or market demands [5].

Among CWR, the genus *Dasypyrum* (Cosson and Durieu) T. Durand (family *Poaceae*, tribe *Triticeae*, subtribe *Triticineae*) was given insufficient attention by breeders. It comprises two allogamous species: *Dasypyrum villosum* (L.) P. Candargy (syn. *Haynaldia villosa* Schur) and *Dasypyrum breviaristatum* (H.Lindb.) Fred. (syn. *Dasypyrum hordeaceum* Cosson and Durieu) Candargy. *Dasypyrum villosum* is an annual diploid allogamous grass, 2n = 2x = 14, with its genome designed by Sears [6] as VV. It is native to the north-eastern part of the Mediterranean region: South Europe from southern France to the Caspian Sea and the Caucasus areas [7,8].

Dasypyrum villosum possesses high levels of resistance to several important wheat diseases, such as rusts and wheat spindle streak mosaic virus (WSSMV) [9] and thus it is considered as a suitable donor of resistance genes [10]. *Dasypyrum* introgressions in wheat possess effective resistance genes *Pm21* to powdery mildew [10]. Adult-plant resistance gene against powdery mildew *Pm62* was transferred from *D. villosum* into common wheat in the form of Robertsonian translocation T2BS.2VL#5 [11]. The stripe rust resistance gene *Yr26* was introduced to wheat by Yildirim and Jones [12] and the *Pch3* gene for resistance to eyespot disease caused by *Pseudocercosporella herpotrichoides* was introduced by Murray et al. [13]. Liu [14] summarized up to date designated genes originated from wheat alien species: 17 stripe rust resistance genes, 35 leaf rust resistance genes, 30 stem rust resistance genes, 41 powdery mildew resistance genes, 3 Fusarium head blight-resistance genes, one wheat blast resistance gene, one *Septoria tritici* blotch resistance gene, one *Septoria nodorum* blotch resistance gene, 4 tan spot resistance genes, 2 eyespot resistance genes, one wheat spindle streak mosaic virus resistance gene, 2 wheat streak mosaic virus resistance genes and 2 cereal yellow dwarf resistance genes. As far as powdery mildew is concerned, to date, 58 resistant genes have been formally designated (*Pm1- Pm58*). Among them, some *Pm* genes were identified from the species in the tertiary gene pool, including *Pm7, Pm8, Pm17*, and *Pm20* from *Secale cereale*, *Pm40* and *Pm43* from *Thinopyrum intermedium*, and *Pm21* and *Pm55* from *Dasypyrum villosum* [15,16]. Abiotic stress tolerance was reported by [17]. Single kernel weight was affected less by heat stress in *D. villosum* than in common wheat.

Simultaneously, *Dasypyrum* possess genes that can increase the amount of seed storage of protein, lysine content and gluten strength [18]. Each of the *Dasypyrum villosum* chromosomes 1V, 4V and 6V contains gene(s) encoding resistance to at least one wheat disease and genes encoding seed storage proteins. On chromosome 1V, there are genes at complex loci coding for high molecular weight prolamins (Glu-V1) and low molecular weight prolamin proteins (Glu-V3) [19,20]. Kozub et al. [21] identified eight alleles at the Glu-V1 locus and four Gli-V1 alleles encoding ω-gliadin in the two Crimean populations of *D. villosum*. The α-gliadins, as the major initiators of celiac disease, were used to study phylogenetic affinities within the *Triticeae* tribe [22] α-gliadin gene sequences of *Dasypyrum, Australopyrum, Lophopyrum, Eremopyrum* and *Pseudoroengeria* species have amplified several times. The α-gliadins of wild *Secale, Australopyrum* and *Agropyron* genomes lack all four typical toxic epitopes for celiac disease, while other *Triticeae* species have accumulated these epitopes, suggesting that the evolution of these toxic epitope sequences occurred during the course of speciation, domestication or polyploidization of *Triticeae.*

Until recently, it was believed that inter-specific hybridization is not possible because 'V' genome chromosomes do not pair well with wheat chromosomes [12]. However, Chen et al. [10] have successfully recovered genotypes containing spontaneous translocations between wheat and *D. villosum* chromosome 6V with resistance to powdery mildew. Pollen fertility appears to be the main problem in crosses performed between *D. villosum* and wheat, especially in F1 generation [23].

The other related species, tetraploid *D. breviaristatum* is considered an allotetraploid based on the sequences comparison of nr5S DNA multigene family. Baum et al. [24] suggested that the genome constitution of the tetraploid should be considered VVVbVb. Given the widespread success of this introgression from *D. villosum*, research has been conducted with a similar aim to transfer useful genes from *D. breviaristatum* into wheat. Subsequently, wheat, *D. breviaristatum* introgression lines with multiple disease resistances have developed [22,25]. A large divergence between *D. breviaristatum* and *D. villosum* with respect to the organization of different repetitive sequences has been found [26].

Thus, these facts mentioned above make *Dasypyrum* an important genetic resource for wheat breeding and also an important item of gene bank collections [27]. Its distribution in large areas of the Mediterranean and long-term evolution in specific localities assume a high variability in many parameters. Nevertheless, numbers of *Dasypyrum* accessions in gene banks are still very low in comparison with cultivated cereal species and their direct ancestors e.g., *Aegilops*.

The *Dasypyrum villosum* collection comprising 32 accessions is a part of the *Triticeae* CWR collection maintained in the Gene Bank, Prague, Czech Republic. The aim of our study was to characterize the collection morphologically, genetically, from the point of resistance to leaf diseases and to obtain detailed information about selected quality parameters of seeds with specific accent to protein polymorphism and protein and starch composition.

2. Materials and Methods

2.1. Plant Material and Cultivation

The Gene Bank *Dasypyrum villosum* (L.) P. Candargy collection was used for this study (Table S1). Passport data for *Dasypyrum villosum* accessible accessions is available in the documentation system GRIN Czech (GRIN Czech Release 1.10.3 (vurv.cz)). The material was collected in the Mediterranean area between years 1988 and 2019 (Figure 1). In total 29 accessions were used for morphological evaluation and phenotyping and 21 accessions for genotyping. Selected 18 accessions were used for protein seed storage polymorphism study. The accessions used represent most of the diversity of the *Dasypyrum villosum* species in the Czech Gene Bank. The evaluation was done in field regeneration trials in small row plots separated by other wild *Triticeae* species and in the background of wheat in the Crop Research Institute Prague-Ruzyně, repeatedly for 3 or more years in the period 2008 to 2020. Each plot had about 20 to 40 plants. The crop treatment was carried out according to standard and certified protocols of the Gene Bank for cereal genetic resources and their CWR. For morphological evaluation, the descriptor list for *Aegilops* [28] and the descriptor list for wheat [29] were used. Voucher specimens for spike collection are collected from each regeneration. Morphological data were evaluated by Principal Component Analysis VD.

2.2. Genetic Evaluation

DNA was extracted using CTAB according to the optimized protocol based on Saghai-Maroof et al. [30]. For SSR analysis, a set of 40 SSR markers developed for wheat and published in Rőder et al. [31] was selected. PCR reactions were carried out according to Roussel et al. [32]. Products of PCR reactions were separated using capillary electrophoresis in ABI PRISM 3130 (Applied Biosystems, San Francisco, CA, USA) in quadruplex configuration with internal standard LIZ500 (Life Technologies, Carlsbad, CA, USA). Electrophoretograms were evaluated using GeneMapper software (Life Technologies, Carlsbad, CA, USA). A matrix of distances between genotypes was calculated using Simple Matching dissimilarity coefficient in the DARwin software [33]. For clustering, an unweighted neighbor-joining method was used. The support for the tree branches was obtained using 2000 bootstrap re-samplings. The diversity indices were calculated using POPGENE software, version 1.32 [34].

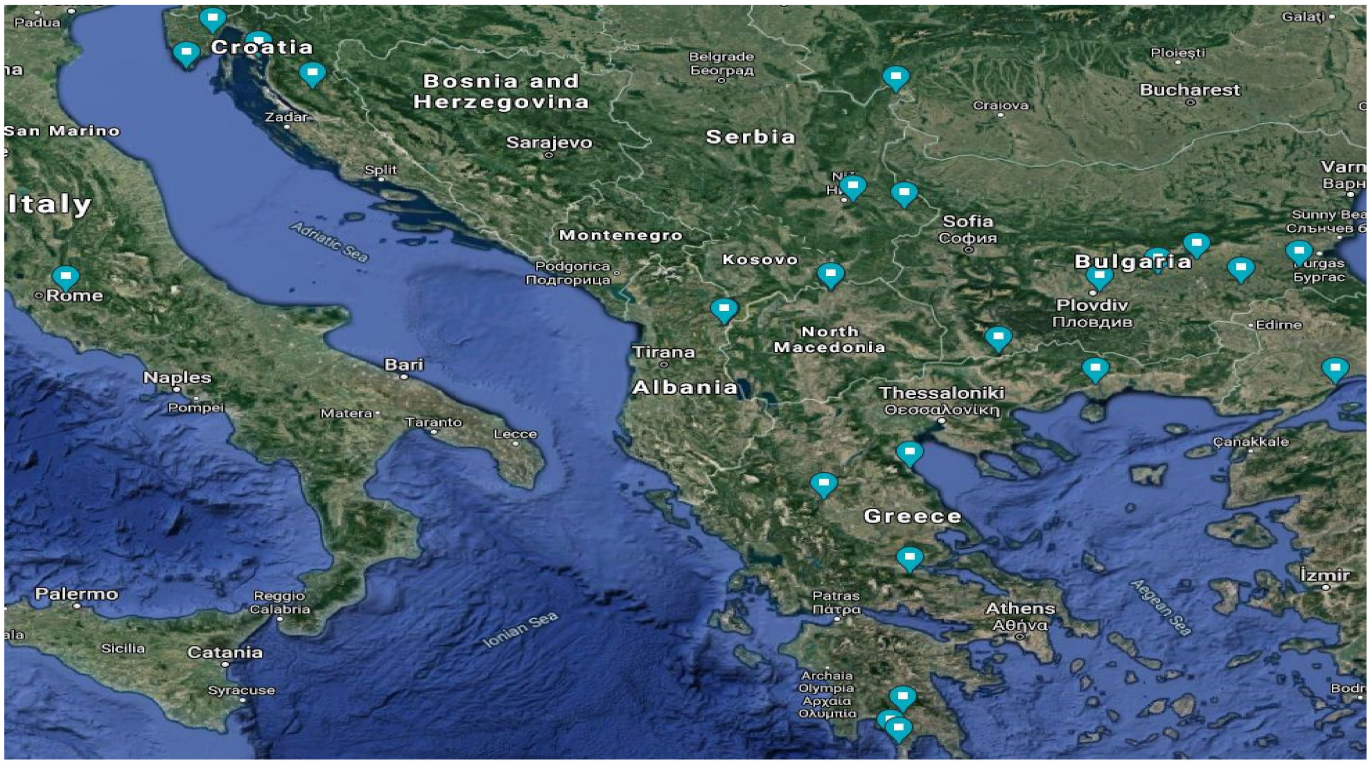

Figure 1. Map of collected accessions of *Dasypyrum villosum*.

2.3. Disease Resistance Evaluation

The collection was scored for field resistance to leaf diseases and the results were summarized over the testing years. The scoring was done on adult plants in trials neighboring the infection field with good access of infection. The plants in the infection field were inoculated by a mixture of the most virulent races currently available, or single races collected in the Czech Republic of leaf rust (*P. recondita* Rob. ex Desn.) and yellow rust (*P. striiformis* Westend). The stem rust (*Puccinia graminis* f. sp. *tritici* Erikss. & Henn) and powdery mildew (*Blumeria graminis* (DC.) Speer f. sp. *tritici* (DC.) Marchal) were from natural infections. The testing plants were exposed to natural infection from inoculated susceptible spreaders. Plants with various hypersensitivity reactions were considered as resistant. The scoring was done according to the descriptor list *Aegilops*. Additional evaluation was done according to Stakman et al. [35] where infection types were evaluated and % leaf area affected was recorded.

2.4. Biochemical Evaluation

The study of seed storage protein polymorphism was carried out in 8 original seed samples. A modified SDS-PAGE method according to Singh et al. [36] with clear interpretation of high molecular glutenin subunits (HMW-GS) and low molecular glutenin subunits (LMW-GS) routinely applied in wheat cultivars was also used for characterization of above mentioned 18 *Dasypyrum* populations (Table S1). The indication of protein bands (alleles) for HMW-Glu (glutelins) and LMW-Glu (glutelins) in *D. villosum* partially corresponded with the description of Zhong and Qualset [37]. Regarding the absence of any official allelic Glu catalogue for *Dasypyrum*, detected HMW- and LMW-Glu proteins were numerically marked (e.g., HMW-Glu-V1: 1) and characterized by their relative mobility and molecular weight in polyacrylamide gel.

After harvest, grain samples were analyzed for crude protein content according to the Kjeldahl method (ČSN EN ISO 20483), starch content according to the Ewers polarimetric method (ČSN EN ISO10520), and Micro-SDS sedimentation test (according to Axford [38]).

A slightly simplified Osborne method according to Dvořáček et al. [39] was used for content analyses of 3 individual protein fractions: albumins and globulins (Alb + Glo), prolamins (Pro) and Sum of glutelins (ΣGlu). Content of ΣGlu was calculated as a difference between the content of crude protein and sum of albumins + globulins and gliadins. The content of amylose was detected by enzymatic method (Amylose/Amylopectin assay kit) according to the company protocol [40].

Statistica 7.0 CZ statistical software (StatSoft, Inc., Tulsa, OK, USA) was used for basic statistical parameters (Mean, Standard Deviation–St. Dev. and Relative Standard Deviation–RSD). The software was also used for multiple comparison test (Tukey HSD test) as part of a multifactor analysis of variance ANOVA, further for calculations of linear regressions Pearson correlations (r) and for construction of PCA score plot.

The calculated dendrogram based on positions of HMW- and LMW glutelins was constructed from a binary data using the Jaccard's dissimilarity index. The generated matrix was used to form genetic relationships based on neighbor-joining algorithm using the unweighted pair group mean arithmetic (UPGMA). The DARwin software [33]. was used for the dendrogram construction.

3. Results

3.1. Characterization of Original Sites

The accessions of *Dasypyrum* were collected in the large geographical area of North Eastern Mediterranean between latitude 36°40′ and 45°14′ and longitude 12°50′ and 27°30′. This area covers most of the known distribution area [7,41]. The collected sites consist of natural habitats with original or near original vegetation (primary P) as secondary (S) and disturbed habitats with ruderal and segetal vegetation (Table S1). The primary sites comprise steppes on shallow soil on limestone, karst terraces with shallow soil pockets, limy gravel coastline and transition to the Mediterranean hard leaf shrubland like garrigue and maquis. Secondary habitats were roadsides, pastures, field margins and urban disturbed habitats. Both primary and secondary habitats were covered equally and represent typical sites of annual *Triticeae* grasses. The most northern collecting sites were in karst grassland and especially in littoral evergreen forests and maquis with *Quercus ilex* in Premantura, Istria, in fact in the most northern distribution of this vegetation (Table S1).

3.2. Morphological Phenotyping of the Dasypyrum Collection

Phenotyping of 20 morphological characters revealed a considerable diversity among accessions (Table 1). Plant height varied in the wild sites and similarly it varied also in standard conditions in cultivation. The mean value of plant height varied between 70 and 130 cm. The lowest height was found in populations collected in primary habitats from Korab Mts., Albania and S Peloponnese, Greece. The largest plant height was noted in populations formerly collected on secondary sites with deep sand and rocky soil. Leaves were scattered to densely pilose adaxially and glabrous abaxially, with ± ciliate margin and auricles. The spike length without awns was between 3.4 and 8.3 cm. The length of awns on top of the spikes also varied considerably, between 1.2 and 4.2 cm. While the spike length varied among years the awn length was uniform. Short spikes usually had longer awns. All awns were densely scabrid. Palea was rather membranaceous, 9–14 mm long. The color of spikes and awns at flowering time in the wild and in cultivation was uniformly green or brown or mixed with transitions. The dark colors were intensified with maturity being dark brown to black, with awns usually darker. Selecting dark spikes from white resulted in a combination of both in the next progeny. Structure of spikes was also variable showing remarkable visible variation in the spike texture. Spikelet length varied from 13 to 23 mm and there was similar variation in lower, upper glume and lemma. The upper glume was usually equal or longer than the lower glume. The length of awns on the lower glume was 10 to 36 mm, while on the upper glume 12 to 49 mm. There is no correlation between size of awns among accessions.

Table 1. Phenotyping of *Dasypyrum villosum* collection.

Accession Number	Pl height (cm)	Spike Length (cm)	Awn Length, Apical Spikelets (cm)	Spike Color in Flower	Awn Color in Flower	Spike Color maturity	Awn Color Maturity	Spikelet Length (mm)	Glume1_Length (mm)	Awn glume2 Length (mm)	Glume2 Length (mm)	Awn Glume2 Length (mm)	Lemma Length (mm)	Awn Lemma Length (mm)	Caryopsis Length (mm)	Caryopsis Width (mm)	Caryopsis Color	Anther Length (mm)	Anthocynin in Anthers	Glume Indumentun
01C2300004	107	8.0	2.0	9	9	8	8	16	6	18	6	16	12	12	5.5	1.4	8	6	0	1
01C2300005	88	6.4	2.4	8	8	3	3	14	5	17	6	26	11	18	4.9	1.3	8	7	1	2
01C2300006	88	6.4	2.3	9	9	8	8	17	6	18	7	30	11	25	5.8	1.6	4	7	0	1
01C2300007	90	8.0	3.0	9	9	8	8	18	7	30	7	35	13	30	5.8	1.6	8	6	0	2
01C2300008	100	7.0	2.0	3	9	9	9	14	6	22	6	17	11	19	6.0	1.4	4	6	0	1
01C2300009	130	7.0	3.0	3	9	9	9	18	7	18	7	18	14	32	5.5	2.0	9	6	0	1
01C2300010	100	8.0	2.5	3	3	1	1	13	5	18	6	25	12	24	5.3	1.7	9	6	0	1
01C2300011	83	8.0	2.0	9	9	8	8	16	7	18	7	30	15	35	5.1	1.2	9	7	0	2
01C2300013	85	8.0	3.0	3	8	1	3	15	5	23	5	24	12	9	6.2	1.5	9	6	1	2
01C2300014	93	7.4	1.9	8	8	3	3	19	7	18	8	30	12	13	4.4	1.3	4	5	0	2
01C2300015	99	6.2	2.8	9	9	8	8	17	6	28	7	33	11	30	5.5	1.6	8	6	1	1
01C2300016	73	6.0	2.5	9	9	8	8	15	5	22	6	22	10	13	6.2	1.6	4	7	0	1
01C2300017	77	4.7	1.2	3	9	9	9	18	6	15	7	17	11	10	5.5	1.8	4	7	0	1
01C2300018	86	6.4	2.2	9	9	8	8	15	4	12	5	20	10	11	5.1	1.4	8	6	0	1
01C2300019	85	7.0	2.1	9	9	8	8	16	5	15	5	16	10	22	5.0	1.4	4	6	0	1
01C2300020	90	4.5	3.6	3	3	1	1	17	6	17	7	32	13	45	6.8	1.9	9	6	1	2
01C2300021	90	3.4	3.2	3	3	1	1	13	6	10	6	12	13	16	5.5	1.6	9	6	1	1
01C2300022	105	4.6	2.0	3	3	1	1	14	8	31	8	29	11	8	5.5	1.8	9	6	0	2
01C2300023	105	4.1	3.1	8	8	3	3	14	8	31	8	29	11	8	5.2	1.5	4	6	0	2
01C2300026	80	5.8	1.6	3	3	1	1	16	6	13	7	15	12	8	4.3	1.5	4	6	0	1
01C2300027	80	7.3	2.2	8	8	3	3	15	7	12	7	15	11	8	5.1	1.4	4	5	0	2
01C2300028	104	8.3	3.5	9	9	4	9	20	9	31	8	41	12	10	5.5	1.9	4	7	0	1
01C2300029	80	6.8	2.3	9	3	4	1	16	7	26	9	22	11	11	6.0	1.5	4	7	0	1
01C2300030	70	3.9	3.1	3	9	1	8	19	8	26	11	43	11	19	5.3	1.8	4	6	0	2
01C2300031	84	3.6	4.2	9	9	8	8	23	9	18	13	34	12	16	5.6	1.9	8	6	0	2
01C2300032	84	5.0	3.1	3	8	1	3	18	7	36	9	49	11	12	5.5	1.8	4	6	0	2

Legend to Table 1. Description of characters (Descriptor list genus *Triticum* and *Aegilops*)

18. spike color after heading	
3	green (spike after heading)
8	violet-brown (spike after heading)
9	mixed
35. glume and awn color in ripeness	
1	white (glumes and awns in ripeness)
3	black (glumes and awns in ripeness)
4	brown
8	mixed white-black
9	mixed white-brown
40. caryopsis color in ripeness	
4	light brown (caryopsis in ripeness)
5	dark brown (caryopsis in ripeness)
9	mixed light brown-dark brown
glume indumentum:	
1	coarse, bristle-like hairs, rather remote
2	soft, softer hairs, usually connected
anthers: anthocyanin presence	
1	anthocyanin present
0	anthocyanin absent

Variation among the mean caryopsis size is rather medium, the length varies between 4.3 and 6.8 mm and width between 1.2 and 2.0 mm. Caryopsis color was defined in three categories dark brown, light brown, and mixed. There is no correlation between spike and caryopsis color. Anthocyanin in anthers was present in 5 accessions. Glume indumentum is very prominent in the *Dasypyrum* genus, it is expressed as branched bristles coming out of the glume keel. It was possible to divide it into two types: 1. coarse, bristle-like hairs, rather remote and 2. soft, softer hairs, usually interconnected to each other. The indumentum was coarse for 14 accessions and soft for 12 accessions.

Principal component analysis based on morphological characters revealed a possibility to separate four clusters corresponding to geography (Figure 2). The large cluster represents accessions from central Balkan area from Croatia over Albania to Bulgaria. Accessions from North Croatia can be separated. It is possible to distinguish another cluster in southern Balkan Greece to European Turkey. A separate isolated cluster corresponds to Peloponnese.

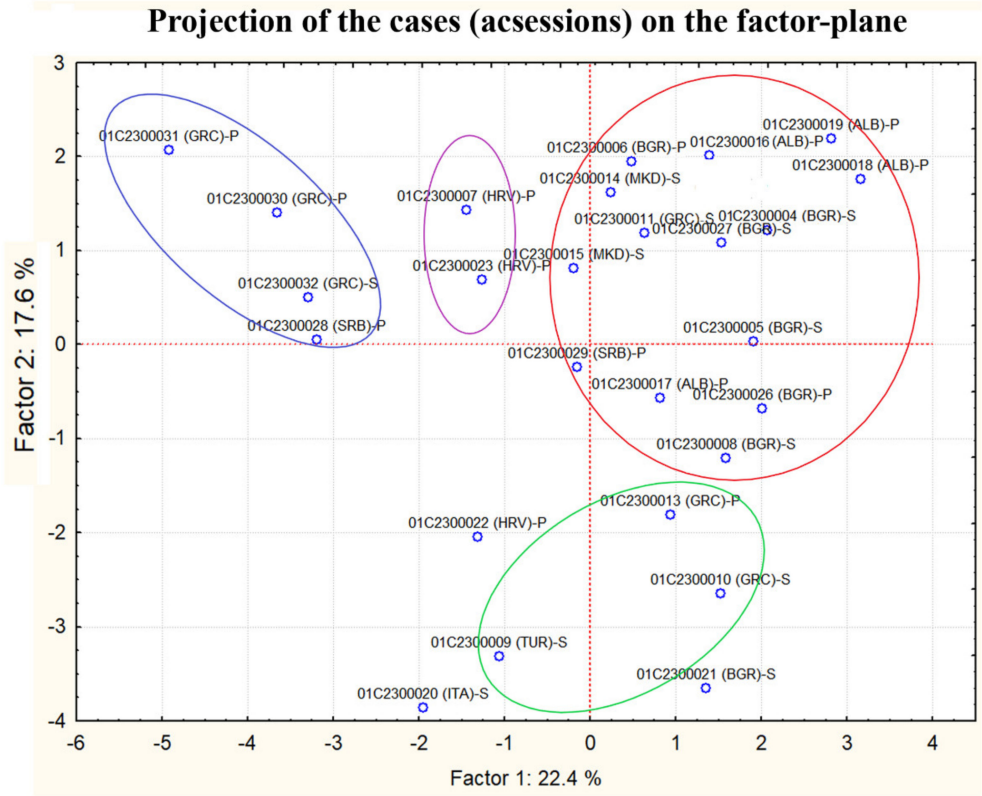

Figure 2. Principal component scores for collection of different *Dasypyrum* genotypes based on their morphological description (cyan-North Balkan, beige-Central Balkan, green-Southern Balkan, blue-Peloponnese).

3.3. Genotyping of the Dasypyrum Collection

In total, 21 genotypes were analyzed using microsatellite loci. As the used set of 40 SSR markers was developed for wheat, only 28 loci (70%) were polymorphic. On average, it was identified 5.3 alleles per locus. Effective number of alleles per locus was 2.8. Gene diversity [42] was 0.457 and Shannon information content [43] value 0.980. Cluster analysis showed to be three groups but without significant bootstrap support. This means that there is shallow genetic structure in data and studied *Dasypyrum* genotypes form a likely panmictic population. However, more samples from each collection locality should be analyzed to prove this hypothesis.

3.4. Resistance of Dasypyrum villosum to Leaf Diseases

The field resistance scoring was done repeatedly up to 9 years (Table 2). All tested accessions were resistant to powdery mildew and brown rust. Three accessions were susceptible to yellow rust. The infection type was marked 2 (moderately susceptible), lesions on leaves appeared as narrow stripes surrounded by a mild hypersensitivity reaction. Percentage of affected leaf area was 10–60%. Stem rust was detected only in two accessions. Infection type was 2 (moderately susceptible). Only a few small lesions were found and therefore the percentage of affected leaf surface was below 1%.

Table 2. Field resistance of *Dasypyrum villosum* accessions to powdery mildew, yellow, leaf and stem rusts. The worst score during the evaluation years.

National Acc. No.	No of Eval. YY	Powdery Mildew *	Yellow Rust *	Leaf Rust *	Stem Rust *
01C2300004	7	9	9	9	9
01C2300005	9	9	9	9	9
01C2300006	9	9	3 (2/50) **	9	9
01C2300007	2	9	9	9	9
01C2300008	1	-	-	9	9
01C2300009	1	-	-	9	9
01C2300010	5	9	2 (2/60) **	9	9
01C2300011	6	9	9	9	9
01C2300013	1	9	9	9	9
01C2300014	7	9	9	9	9
01C2300015	7	9	9	9	9
01C2300016	3	9	9	9	9
01C2300017	2	9	9	9	9
01C2300018	7	9	9	9	9
01C2300019	3	9	9	9	9
01C2300020	7	9	6 (2/10) **	9	9
01C2300021	5	9	9	9	9
01C2300026	4	9	9	9	9
01C2300027	4	9	9	9	8 (2/1) **
01C2300028	4	9	9	9	9
01C2300029	5	9	9	9	7 (2/1) **
01C2300030	1	9	9	9	9
01C2300031	1	9	9	9	9

* scale 9–1, 9—resistant, according to the descriptor list (Holubec and Matejovič, 2020); ** scale 0–4, % of leaf surface affected (Stakman et al. 1962).

3.5. Biochemical Evaluation of the Dasypyrum Collection

The examples of intra- and inter-accession variability based on HMW- (approximately 70–116 kDa) and LMW- (29–66 kDa) glutelin peptides are visualized in Figure 3. The schematic interpretation of all detected glutelin peptides in 18 tested *Dasypyrum* accessions is further described in Figure 4. It is evident that all *Dasypyrum* seeds showed a significantly lower number of HMW glutelins and higher number of LMW glutelin peptides (area 45–66 kDa) compared to standard wheat cultivar. Simultaneously, we also detected a number of intra population variabilities in glutelin peptides. In total, 52 glutelin peptides were identified in 18 different *Dasypyrum* accessions. Only 6 glutelin peptide combinations were detected belonging to the category of wheat HMW-glutenins with the molecular weight from 80 to 93 kDa. In contrast, in total 46 LMW-glutelin peptides were identified with the molecular weight from 24 to 63 kDa. In spite of the fact that almost every second grain of detected populations of *Dasypyrum* showed different glutelin spectrum, we also found *Dasypyrum* accession from Albania (01C2300017) which was characterized by uniform glutelin spectrum in all detected 8 original seeds (HMW-Glu-V1: '1' and LMW-Glu-V1: '15', '16', '20', '25' '27'). A higher spectral homogeneity of glutelins was also found in another of the Albanian accessions (01C2300016 and 01C2300019), where about 60% of those analyzed were identical.

National Access. No.	Genotype designation
01C2300005/1	BGR Grudovo E 354
01C2300005/2	BGR Grudovo E 354
01C2300005/3	BGR Grudovo E 354
01C2300005/4	BGR Grudovo E 354
Chinese Spring	Standard wheat cultivar
Weight marker	SigmaMarker wide range
01C2300004/1	BGR Rakitnica E 340
01C2300004/2	BGR Rakitnica E 340
01C2300004/3	BGR Rakitnica E 340
01C2300004/4	BGR Rakitnica E 340
01C2300017/1	ALB Korab 1640 m E 1177
01C2300017/2	ALB Korab 1640 m E 1177

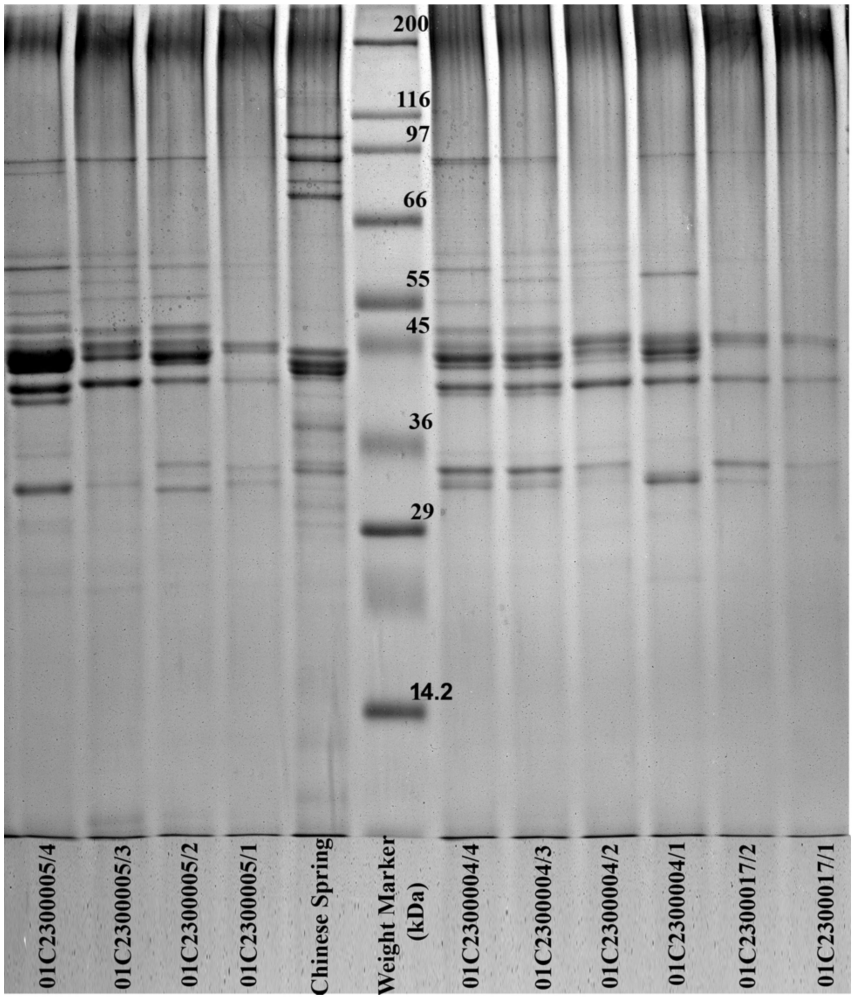

Figure 3. The examples of intra- and inter-population variability of HMW- (70–116 kDa) and LMW- (29–66 kDa) glutelins in 3 collected populations of *Dasypyrum villosum* including comparison with model common wheat cultivars (SDS-PAGE).

A cluster analysis involving the individual variability of the 18 harvested populations of *Dasypyrum vilosum* according to their glutelins composition is shown in Figure 5. In comparison with the PCA morphological analysis (Figure 2), it interpreted a narrower set representing in Figure 2 only two different zones: Z1: central Balkan area from Croatia over Albania to Bulgaria and Z2: southern Balkan Greece to European Turkey.

The analysis separated collected accessions into 2 main clusters (top and down cluster) which were further divided into the next 3 distinctive clusters (I.–III.) for the main upper- and (IV.–VI.) for the main down cluster.

In most cases, intra-accession diversity was less than inter-accession variability. The Greek and Albanian materials (01C2300010, 01C2300013 and 01C2300016) showed a specific separate position within cluster I. The accessions 01C2300013 and 01C2300016 were also identified as primary sources. Cluster III also included accessions from Albanian and further Bulgarian populations. It is interesting to note the close similarity of Bulgarian and Albanian accessions 01C2300026 and 01C2300018, which were indicated as primary sources. Due to the greater geographical distance, the location of Italian 01C2300020 and Turkish accessions 01C230009 into the common cluster VI is also interesting.

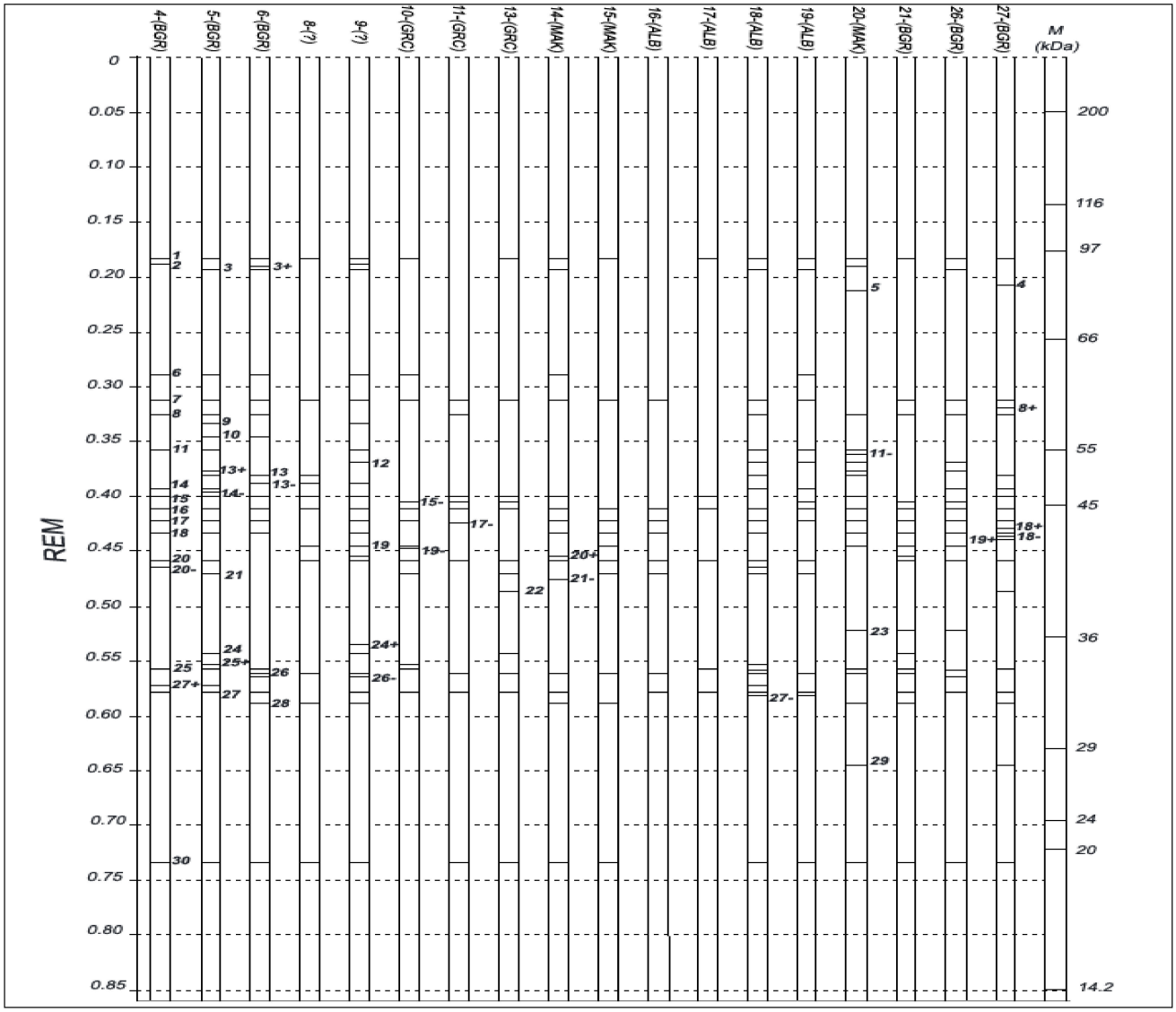

Figure 4. Schematic electrophoreogram (SDS-PAGE) of all detected HMW- and LMW-Glu combinations in individual populations of *Dasypyrum villosum*.

The most frequent Bulgarian accessions were incorporated into all clusters and were thus the bearers of the widest variability in the composition of glutelins. Extreme glutelin variability was detected in Serbian accessions 01C2300027 whose 3 identified Glu-lines included 3 different clusters III, IV and VI. It is also appropriate to mention two Macedonian accessions 01C2300014 and 01C2300015, which were very similar in morphological properties (see Figure 2), but were very different in the composition of glutelins.

A comprehensive three-year evaluation of grain composition was obtained for a total of seven accessions, which represented the Bulgarian, Albanian and Macedonian populations. With the exception of cluster I, these accessions were represented in the other 5 clusters (Tables 3 and 4).

Table 3 indicates on the one hand a higher content of proteins, two storage protein fractions (prolamins and Σglutelins) including Micro-SDS test compared to standard spring wheat cultivar Granny (*T. aestivum* L.). In contrast, the content of starch and amylose was lower compared to the wheat control. The most variable parameter in the tested accessions was the content of albumins and globulins (RSD = 20.8%,) followed by the content of Σglutelins (RSD = 17.6%) and prolamins (RSD = 15.9%).

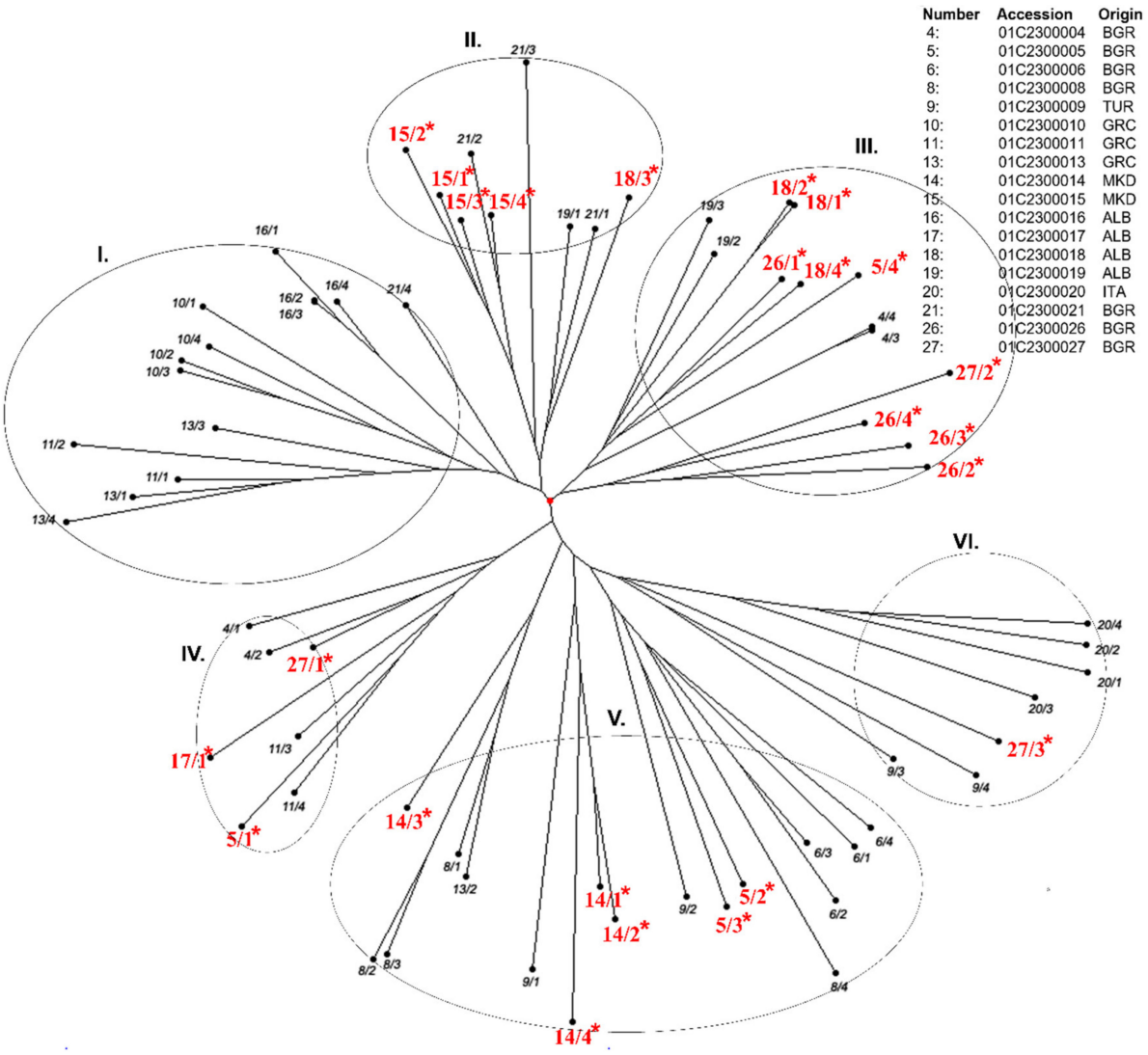

Figure 5. Cluster Analysis depicting genetic relationships among individual *Dasypyrum* accessions on basis of SDS-PAGE determining positions of seed HMW- and LMW glutelins. * Those in red were further used for evaluation of seed composition. Individual Roman numerals (I-VI) define more mutually similar groups of tested accessions.

Although the tested set of accessions showed wide variability, the detected 3-year mean values were not always significantly different among *Dasypyrum* accessions. This is especially evident for the amylose content. Moreover, the achieved average protein content or Micro-SDS test were significantly different only for accessions 27 and 17 respectively. On the other hand, the contents of protein fractions in the tested accessions were significantly different and simultaneously, their variability was significantly affected by the year too.

The calculations of the regression models and the correlation coefficients between the protein content and the protein fractions, including the Micro-SDS test, are characterized in the Figure 6.

The observed correlations between protein content and content protein fractions were only moderately strong (r = 0.4–0.5). The correlation between protein content and Micro-SDS test was even very low (r = −0.08). The slope of the regression equations characterizing the degree of change between the total protein and the protein fraction contents increased in order from albumins and globulins through prolamines to Σglutelins.

Table 3. Variability of seed composition in 7 selected populations of *Dasypyrum villosum* and spring wheat cultivar Granny (*T. aestivum* L) in tested years: 2008, 2009 and 2011.

Parameter	Mean	Min.	Max.	St. Dev.	RSD (%)	Granny (*T. aestivum*) Mean ± St. Dev.
Crude Protein (%)	23.7	21.2	29.7	1.9	8.0	13.6 ± 0.4
Starch (%)	51.3	41.2	55.9	3.6	7.0	63.8 ± 1.4
Alb + Glo (%)	3.7	2.4	5.5	0.8	20.8	3.6 ± 0.1
Pro (%)	8.4	6.1	10.8	1.3	15.9	4.6 ± 0.2
ΣGlu (%)	11.6	8.4	16.9	2.0	17.6	5.4 ± 0.3
Amylosa (%)	26.3	20.1	34.9	3.4	12.9	27.7 ± 2.3
Micro-SDS test(ml)	8.2	5.8	9.5	1.1	13.7	6.7 ± 0.3

Alb + Glo: albumins and globulins; Pro: prolamins; ΣGlu: Sum of glutelins; RSD: Relative standard deviation.

Table 4. Comparison of seed compositions among individual populations of *Dasypyrum villosum* in tested years: 2008, 2009 and 2011.

Genotype	Origin	CP (%)	Starch (%)	Alb + Glo (%)	Pro (%)	ΣGlu (%)	Amylose (%)	M-SDS Test (ml)
01C2300005	BGR	22.2 ^{a}	53.7 ^{a}	3.2 ^{a}	8.5 ^{ab}	10.5 ^{a}	29.5 ^{a}	8.6 ^{a}
01C2300014	MAK	23.7 ^{a}	50.1 ^{ab}	4.0 ^{b}	9.8 ^{b}	9.9 ^{a}	25.3 ^{a}	7.8 ^{a}
01C2300015	MAK	23.1 ^{a}	53. 7 ^{a}	3.5 ^{ab}	8.8 ^{ab}	10.7 ^{ab}	24.9 ^{a}	7.6 ^{a}
01C2300017	ALB	23.5 ^{a}	53.3 ^{a}	3.6 ^{ab}	7.5 ^{a}	12.4 ^{c}	26.0 ^{a}	9.3 ^{b}
01C2300018	ALB	22.8 ^{a}	50.9 ^{ab}	3.6 ^{ab}	7.3 ^{a}	11.9 ^{bc}	25.7 ^{a}	8.6 ^{a}
01C2300026	BGR	22.7 ^{a}	52.3 ^{a}	3.7 ^{ab}	7.7 ^{a}	11.4 ^{abc}	27.2 ^{a}	7.9 ^{a}
01C2300027	BGR	26.5 ^{b}	47.0 ^{b}	3.6 ^{ab}	8.0 ^{ab}	14.9 ^{d}	25.3 ^{a}	7.8 ^{a}
Year—2008		23.5 ^{a}	51.7 ^{a}	3.8 ^{b}	8.5 ^{b}	11.2 ^{b}	23.9 ^{b}	8.8 ^{a}
Year—2009		22.9 ^{a}	51.1 ^{a}	4.3 ^{c}	9.0 ^{b}	9.6 ^{a}	26.6 ^{a}	8.3 ^{a}
Year—2011		23.6 ^{a}	52.5 ^{a}	2.9 ^{a}	7.3 ^{a}	13.4 ^{c}	28.5 ^{a}	7.6 ^{b}

Values with no common letter indexes are statistically significant at $p \leq 0.05$; CP: Crude protein; Alb + Glo: albumins and globulins; Pro: prolamins; ΣGlu: Sum of glutelins; M-SDS test: Micro- sedimentation test.

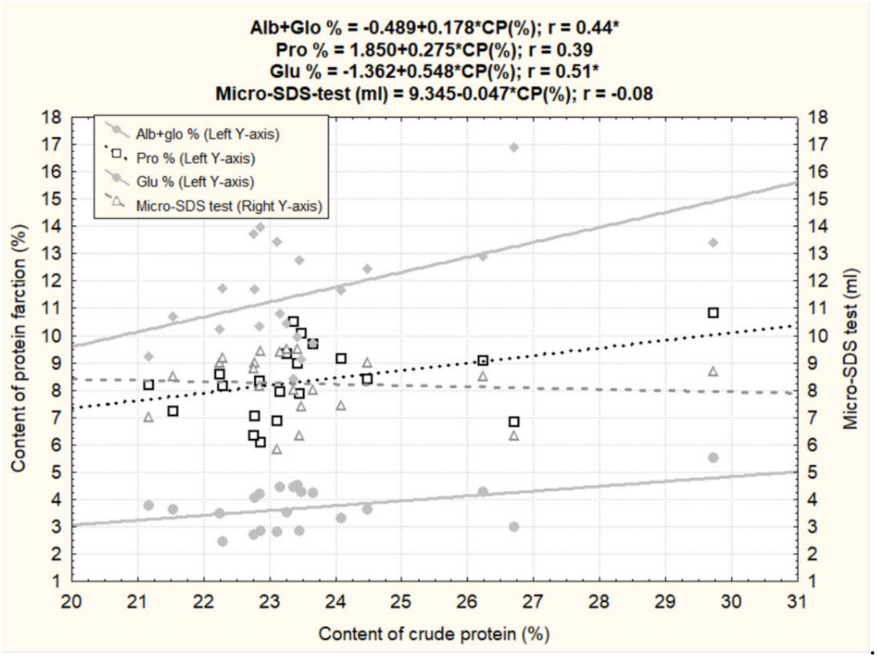

Figure 6. Regression and correlation analyses among crude protein content and content of protein fractions in the tested accessions of *Dasypyrum villosum.* * correlation values significant at $p \leq 0.05$; CP: Crude protein; Alb + Glo: albumins and globulins; Pro: prolamins; Glu: Sum of glutelins.

4. Discussion

The presented Czech Gene Bank collection of *Dasypyrum villosum* covers the area of Central and East Mediterranean, counting about 80% of the known distribution area. It does not include accessions from France, North Africa and Caucasus. In Italy and Western Greece *Dasypyrum villosum* dominates in at least five associations: *Aveno-Brometum* Biondi & Baldoni 1991, *Vulpio-Dasypyretum* Fanelli 1998, *Rapistro-Dasypyretum* ad. int., *Knautio-Galactitetum* Bolos et al. 1996, *Laguro-Dasypyretum* Fanelli 1998 [44]. The most northern collecting sites were in karst grassland in Dugo Polje and especially in littoral evergreen forests and maquis with *Quercus ilex* in Premantura, Istria (Table S1), which is the most northern distribution of this hard leaf Mediterranean vegetation. The occurrence continues inland to pseudo-steppe with grasses and annuals of the *Thero-Brachypodietea* class [45].

The postulated primary habitats of this species are fore dunes, from where it colonized secondary habitats, in particular fallows, where it is presently most abundant [44]. We marked as primary habitats those on the sandy/rocky coast and adjacent limestone cliffs and also limestone outcrops at higher altitudes and surrounding undisturbed steppe habitats (Korab Mts.). Here we assume *Dasypyrum villosum* can be primary or at least archaeophytic. Among secondary habitats *D. villosum* rapidly colonizes abandoned fields. Thereafter, it maintains a high cover for many years. Species coexisting with *D. villosum* are also quite stable, although it is possible to observe an increase in species that have relatively high requirements for nutrients, such as *Avena sterilis* [44]. In rocky primary habitats *Dasypyrum villosum* is accompanied by annual grasses (e.g., *Aegilops* spp. in Premantura or Peloponnese) that complete their life cycle before the drought period in summer. Drought conditions on the coast are often combined with salinity when within reach of sea water (accompanied by *Pholiurus incurva*) or salt spray from the sea (*Hordeum* spp.). The salt and drought tolerance of *Dasypyrum villosum* was repeatedly reported [46,47].

Morphological measurement of characters was done repeatedly in revolving regeneration trials, where low number of testing years refers to a low hardiness of the material and therefore cultivation problems. Diversity in characters in our study revealed larger intervals than shown by Cabi et al. [48] for west Turkey, most likely because we covered a geographically larger area—nearly the whole area of distribution including a wider ecological amplitude of habitats. Palynological analysis [48] also revealed some variation, but does not allow distinguishing among different *Dasypyrum* populations, only distinguishing among related genera being closest to *Secale*.

Passport data on *Dasypyrum villosum* are available in 7 European country collections, altogether for 124 accessions (EURISCO: European Search Catalogue for Plant Genetic Resources (ipk-gatersleben.de)). Among them Germany lists 48 accessions and UK 23 accessions. The Czech documentation system (GRIN Czech Release 1.10.3 (vurv.cz)) lists 22 currently available accessions. Phenotyping data including morphological, phenological and biochemical evaluation of the Czech *Dasypyrum* collection was uploaded to the documentation system GRIN Czech as raw experimental data and will be available after completing the *Dasypyrum* descriptor list. Currently phenotyping data are not available in gene banks holding *Dasypyrum* collections (e.g., GBIS/I (ipk-gatersleben.de).

Phenotyping data are less supported by genotyping because of low significance of bootstrap support. The second cluster in the dendrogram (Figure 7) comprise accessions of central Balkan and is comparable to the main cluster of PCA (Figure 2). Among phenotyping data the most important are data on accession resistance. A high resistance of the tested *Dasypyrum villosum* accessions to leaf diseases is in accordance with the findings of many authors; genes encoding resistance to at least one wheat disease are located at each of the *D. villosum* chromosomes 1V, 4V and 6V (summarized by [7]. The powdery mildew resistance gene Pm21, originating from wheat wild relative *Dasypyrum villosum*, confers immunity to all known races of *Blumeria graminis* f. sp. *tritici* [49]. They found only 4 accessions susceptible from the screening of more than 100 lines. [50] Li et al. (2016)

reported increased resistance to yellow rust (*Puccinia striiformis* f. sp. *tritici)* at adult plant stage in *Dasypyrum*- wheat hybrids.

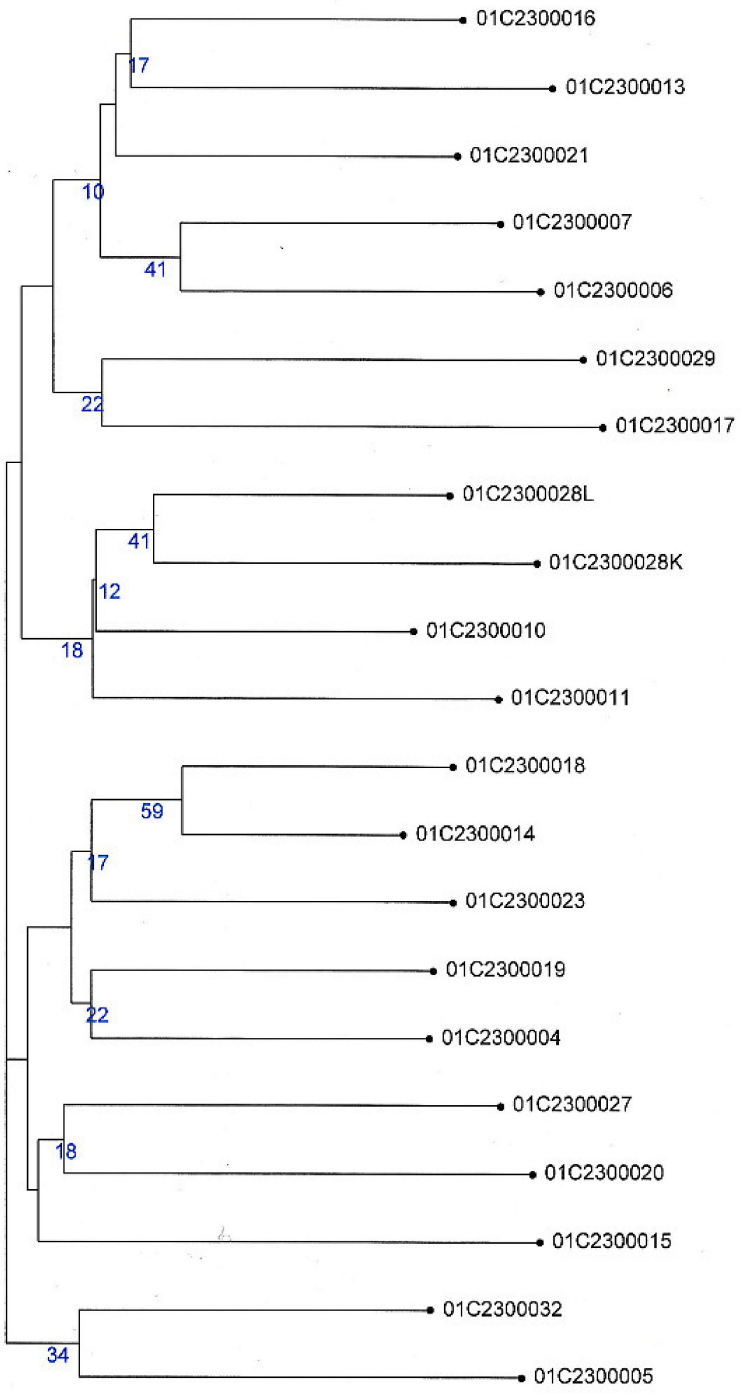

Figure 7. Dendrogram constructed by UNJ clustering method from SM dissimilarity matrix computed from SSR data.

Compared to morphological data, the evaluation of seed storage protein polymorphism is more genotypically bound, therefore storage peptides are a suitable marker for the study of the genetic diversity of the *D. villosum* populations [7]. On the other hand, the share of the studied genome responsible for protein polymorphism is significantly lower in comparison with the number of genes influencing the observed morphological parameters.

Compared to Zhong and Qualset [37], who detected a total of 14 alleles for HMW Glu-V1, our numbers of 6 combinations of HMW glutelins were lower. Consistent with these authors, combinations only null, single and two HMW-glutelin peptides (subunits) were observed in the region between 66–119 kDa. This is a significantly smaller number compared to HMW-GS (glutenin subunits) for bread wheat [51]. On the other hand, the detected polymorphism of LMW-glutelins in the studied populations was high and indicated a large variability (plasticity) of this species. The observed intra-accession variability of glutelins is thus probably the interplay of several factors related to the development of the population at a certain locality and possible genetic drift in case of accidental contact with another population of the same species.

The observed uniform Albanian accession 01C2300017 may also indicate low initial genetic variability at the beginning of its formation and its subsequent isolation from neighboring populations. However, for this hypothesis, hundreds of plants (seeds) would have to be evaluated. To the contrary, the high polymorphism of the Bulgarian accessions of *Dasypyrum* could be related to the frequent contacts of different populations in this area, e.g., through human activity. However, to confirm this hypothesis, it would also be necessary to form more extensive collections and perform significantly higher numbers of electrophoretic analyses.

D. villosum is not only considered a significant donor of resistance for wheat breeding, but is also considered a promising source for improving the quality of wheat grain. Nevertheless, we do not notice any detailed studies aimed at seed composition of *D. villosum*. In this respect, we consider the three-year screening analysis of grain composition (despite only a small collection of 7 *D. villosum* accessions) to be unique.

The high protein values found and the Micro-SDS test further confirm the results of some wheat breeders, where the incorporation of gene segments at the Glu V1 locus from *D. villosum* significantly improved the protein content and baking quality of wheat grain [52].

Analysis of the content of protein fractions confirmed a high proportion (over 85%) of so-called gluten proteins (prolamins and Σglutelins) in crude protein and a lower proportion of albumin and globulin fraction. The values of correlations between protein fractions and crude protein were not as close as the correlations found in the case of durum wheat mentioned by Mefleh et al. [53]. However, in accordance with durum wheat, our regression models confirmed that the increase in crude proteins is mainly related to a proportionally higher increase in both storage protein fractions–prolamins and Σglutelins. To compare polymorphism of varieties based on molecular markers and HMW/LMW-glutelins is complicated due to a genetically narrower region of genes encoding glutelins compared to SSR markers, which cover polymorphism of a significantly larger number of genes. Mutual evaluation is also complicated by the identified intraspecific polymorphism of glutelin peptides. Nevertheless, similarly clustered accessions can be found for both methods, e.g., 01C2300013 (GRC), 01C2300016 (GRC) and 01C2300021 (BGR), or accessions 01C2300010 (GRC) and 01C2300011 (GRC) or also 01C2300004 (BGR) and 01C2300019 (ALB).

On the other hand, regarding clearly similar genotypes 01C2300005 (BGR) and 01C2300006 (BGR) according to the composition of glutelins, the evaluation of the polymorphism of SSR markers classified them completely differently in the dendrogram. There is also a similar discrepancy in accessions 01C2300015 (MKD) and 01C2300020 (ITA) with a closer relationship according to SSR markers and significant dissimilarity according to glutelins. The application of both methods can thus be understood rather independently, as complementary approaches suitable for the detailed definition of genetic variability of the studied populations.

The genotypes tested were generally highly resistant. The exception was mainly 3 accessions (01C2300006 (BGR), 01C2300010 (GRC) and 01C2300020 (ITA)) with a significantly higher susceptibility to yellow rust. Due to the use of non-specific molecular markers or storage proteins without a declared link to the observed resistance, they can hardly be

expected to segregate into a common cluster. This was also confirmed by the obtained results, when in both dendrograms (based on SSR and Glutelins) their occurrence was in significantly different clusters.

Apart from the recent research in CWR breeding, *Dasypyrum villosum* is still an underrepresented species in germplasm collections and an underutilized species in breeding.

5. Conclusions

The *Dasypyrum villosum* accessions have been subsequently collected all over the European territory and evaluated at the Czech Gene Bank. Information on sites and habitats were summarized in order to document representativeness of the collection over Europe, comprising diverse ecological conditions of the source material. Morphological phenotyping revealed considerable diversity, while allowing the distinguishing of clusters tracing the geographical origin of accessions. Genetic diversity showed three groups but without significant bootstrap support. All tested accessions of *Dasypyrum villosum* were resistant to the applied races of powdery mildew and leaf rust. Three of 21 accessions were moderately susceptible to currently available races of yellow rust. Two from 23 accessions were slightly affected by stem rust from natural infection.

The SDS-PAGE of HMW- and LMW glutelins confirmed both the high accession polymorphism and also the intra-accession differences. Biochemical analyses of seeds in selected accessions of *Dasypyrum villosum* showed a high content of crude proteins with a significant proportion of their storage components (prolamins and Σglutelins) and their statistically significant differences among populations. At the same time, the declared favorable technological (baking) quality of these storage proteins was also confirmed in our accessions, through high values of the Micro-SDS test.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11071316/s1, Table S1. *Dasypyrum villosum* collection, Gene Bank, CRI, Prague, CZE.

Author Contributions: Conceptualization V.H. and V.D.; methodology V.H., V.D. and L.S.L.; software, V.D. and L.S.L.; validation, V.H.; formal analysis, L.S.L.; investigation, V.H.., V.D., L.S.L. and S.E.; resources, V.H.; data curation V.D. and L.S.L.; writing—original draft preparation, V.H., V.D. and L.S.L.; writing—review and editing, V.H., V.D., L.S.L. and S.E.; visualization, V.D.; supervision V.H.; project administration V.H.; funding acquisition V.H. and V.D. All authors have read and agreed to the published version of the manuscript.

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