agriculture

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Monitoring and Genetic Characterization of Historical Grapevine Varieties (*V. vinifera* ssp.) from Styria in Slovenia

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Abstract: The aim of this research work was to find historical varieties that existed in this area before phylloxera and to identify them on the basis of historical written sources and genetic analyses. At the beginning of the 19th century, around 300 grape varieties were cultivated in Styria. Between 2020 and 2022, old vineyards were monitored at 115 locations in Styria (between the Mura and Sava rivers) in Slovenia. The directly collected samples (340 grapevine accessions) were determined by molecular analysis with 24 SSR markers. A total of 66 different genotypes were detected. After comparison with the available databases, 29 historical varieties and 37 unknown historical genotypes were identified. Several parameters were calculated to evaluate the usefulness of the selected loci in this work, and a dendrogram representing the genetic similarities between the origins was created using the neighbor-joining method to investigate possible ancestry relationships in the sample set. The most common historical varieties were 'Belina' ('Heunisch weiss'), 'Vrbovec' ('Tantovina Eihenblaetrige'), 'Ranfol' ('Ranfol beli'), and 'Pelesovna' ('Vulpea'). Varieties from the current variety list were also frequently found, such as 'Frankinja' ('Blaufraenkisch') and 'Žametovka' ('Kavčina črna'). In a few locations, one of the most important red varieties from the beginning of the 20th century was also found in this area (alongside 'Frankinja' and 'Žametovka'), i.e., 'Vranek' ('Zimmettraube balu'). At that time, this variety was planted in multi-variety vineyards and was preserved, but its importance in single-variety vineyards quickly declined due to female flower. In addition, genetic analyses have shown that 37 unknown historical genotypes have been found in this area. These genotypes need to be described ampelographically and technologically evaluated in the future. Most of the vegetative offsprings of these genotypes have already been transferred to the Meranova gene bank, where they can be accurately described ampelographically under the same pedoclimatic conditions.

Keywords: *V. vinifera* L.; historical varieties; genetic analyses; Styria; Slovenia

Citation: Vršič, S.; Trapp, O.; Maul, E.; Röckel, F.; Perko, A. Monitoring and Genetic Characterization of Historical Grapevine Varieties (*V. vinifera* ssp.) from Styria in Slovenia. *Agriculture* **2024**, *14*, 640. https://doi.org/10.3390/agriculture14040640

Academic Editor: Domenico Pignone

Received: 12 March 2024
Revised: 4 April 2024
Accepted: 18 April 2024
Published: 22 April 2024

1. Introduction

Today, the *Vitis* international variety catalogue (VIVC) contains 23,000 cultivars, breeding lines, and *Vitis* species and slightly more than 12,000 prime names of *V. vinifera* L. varieties [1]. There are many synonyms and homonyms of varieties, so that there are about 6000 to 10,000 individual varieties [2]. In the last 100 years, however, several factors have led to a sharp decline in this number. The spread of phylloxera (*Daktulosphaira vitifoliae* Fitch) and serious fungal diseases from America in the second half of the 19th century, such as powdery mildew (*Erysiphe necator* Schwein.) and downy mildew (*Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & De Toni) [3–5], led to many native varieties being replaced by European–American hybrids. Moreover, in areas with traditional viticulture, most of the autochthonous varieties have gradually been replaced by a small group of varieties due to the transition from mixed plantations to monovarietal vineyards. This phenomenon is

known as the "genetic erosion of the grapevine" and has led to many indigenous varieties being threatened with extinction. A greater diversity of varieties has been preserved mainly at a local level, especially where intensive viticulture is not practiced [6,7]. Historical varieties are an important genetic resource for grapevine breeding. It is assumed that their genotypes have oenologically and agronomically interesting characteristics and are well adapted to specific environments. Most cultivars have not yet been evaluated but could contribute to the flavor characteristics of wines in the future and be a source of disease resistance or adaptation to stress conditions. However, for successful breeding, the conservation of the vine's genetic resources is of utmost importance, as this enables the appropriate selection of starting material for the breeding process.

Styria is the most extended wine-growing region in Slovenia, with a strong viticulture and winemaking tradition dating back to Roman times [8]. In 1822, an internationally important grapevine research station was established on the estate in Vrhov dol near Maribor, today the University Centre of Viticulture and Enology Meranovo (VEM), Slovenia, whose main aim was to introduce new grapevine varieties (mainly from the Rhineland) into practice. This was the beginning of the first erosion of grapevine varieties in this area, half a century before the appearance of phylloxera. The initiator of these activities was Archduke Johann, the great-grandson of Maria Theresa. During this period, many experts also described the grape varieties [9–11] in this wine-growing region [12–15]. The exact and systematic description of the grapevine varieties in Styria was made by Franz Trummer (1841) [16,17], who described 282 varieties, of which more than 100 already had Slovenian synonyms. The synonyms led to increasing confusion in the nomenclature of grapevine varieties, which still exists today. In 2000, a new grape variety collection was established at the VEM, which is now the largest gene bank for grapevine varieties in Slovenia, with around 450 accessions of *Vitis* species, *vinifera*, and *sylvestris* grapevines, rootstocks, and interspecific crosses. Thus, the loss of one of the largest gene banks for grape varieties in Central Europe (more than 900 accessions) in Maribor in the last century was partially compensated [18].

In this wine-growing region, the varieties introduced at that time, such as 'Riesling', 'Traminer', the Pinot group, 'Welschriesling', etc., are cultivated today [18]. In particular, extensive cultivation in small vineyards (200–500 plants) to produce wine for personal consumption has influenced the preservation of several different varieties in this area. Although this area is still suitable for the conservation of several grape varieties (on-farm conservation), this is already being negatively affected by demographic factors.

The aim of this research was to find historical varieties that existed in old vineyards in Styria in Slovenia and that were described in the Trummer ampelography in 1841.

2. Materials and Methods

2.1. Plant Material and Study Site

Some historical grapevine varieties were transferred to the collection of the University Centre of Viticulture and Enology Meranovo (VEM) before 2010, and in 2020, the planned monitoring of 115 old vineyards began. This was conducted in the area between the Mura and Sava rivers (Figure 1). Styria in Slovenia includes large parts of the Podravje wine-growing region (with the exception of the area between the Mura river and the Hungarian border) and a smaller part of the Posavje wine-growing region up to the Sava river. In general, these vineyards are between 70 and more than 100 years old and are grown on old, low "single vines trellis" next to the stakes. Most of them were restored after the phylloxera, when the vines in this area were mainly grafted onto Rupestris. This was confirmed by the outgrowth of the rootstock. Later, grafting was performed on (berlandieri × riparia) rootstocks, e.g., on Teleki from No. 4 to 9, which were tested at the beginning of the 20th century [19]. In 1912, the cane of Teleki 4 was sent to Oppenheim, from which the rootstock SO4 was selected. The approximate age of some of them was also confirmed by the owners.

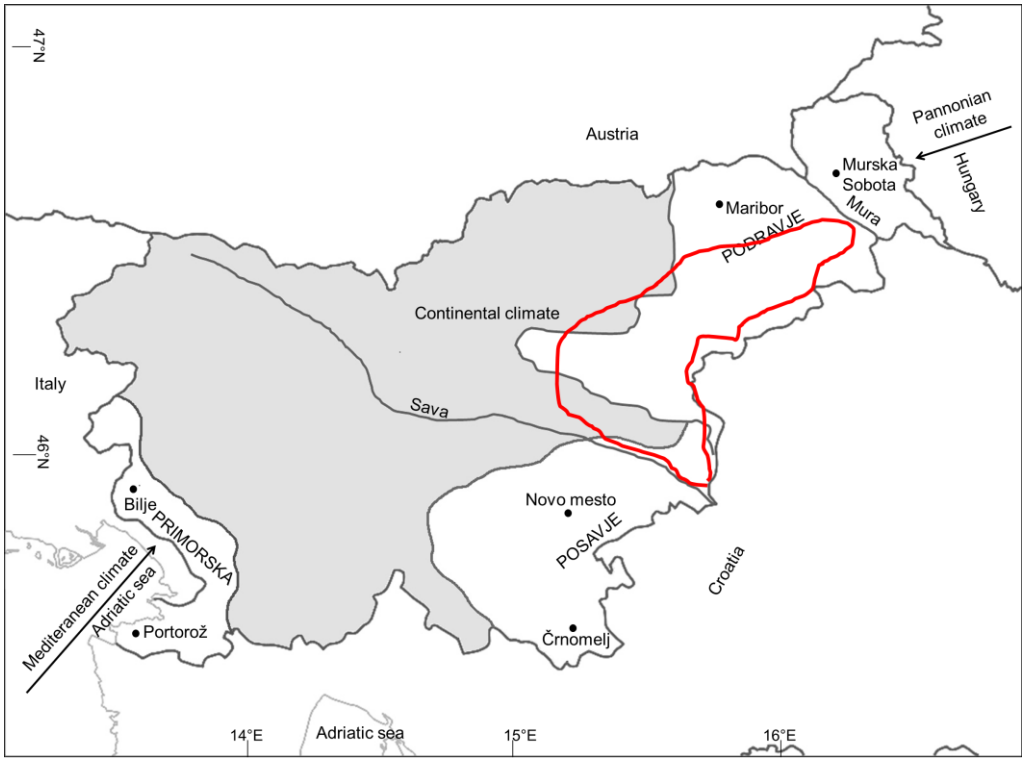

Figure 1. Areas of monitoring of old vineyards and identification of historical varieties in Styria between the Mura and Sava rivers (marked with a red line) in 2020, 2021, and 2022.

Some of the varieties were identified based on the grape varieties described by Trummer (1841) and ampelographic characteristics directly in the vineyards according to the OIV descriptors [20]. All varieties were then genetically identified and confirmed at the Julius Kühn Institute, Institute for Grapevine Breeding Geilweilerhof, Siebeldingen, Germany, for genetic analyses. Samples of young leaves from 340 individual accessions were taken in June, and the ampelographic characteristics of the shoot tip, young leaves, and adult leaves were described according to the primary and secondary OIV descriptors used in Genres-Projects (Genres081/GrapeGen06) [21,22]. The characteristics of the canes and bunches were described when fruits were fully ripened. The ampelographic characterization has not yet been completed, as the vegetative growth potential in old vineyards was very different. This will be conducted over the next few years at the grapevine gene bank Meranovo, where the complete copy of the accessions was made as part of this research work. During the winter (December–January), individual canes were stored in a cold room at 2 °C until grafting in spring. Recently grafted plants were grown in the greenhouse before planting in the UC Meranovo gene bank.

2.2. DNA Extraction and Microsatellite (nSSR) Analysis

The varieties were determined by microsatellites or simple sequence repeat (SSR) markers, which have been greatly proven to be a reliable tool for the identification and genetic characterization of grapevine varieties and have been used in recent years either for germplasm identification or for parentage studies [23,24]. Total genomic DNA was extracted from young leaf tissue, stored at −80 °C until use, and grinding with the MM 300 Mixer Mill System (Retsch, Haan, Germany) occurred before use. Grapevine DNA was extracted using the NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany). The extracted DNA was quantified by spectrometry and diluted to a concentration of 1 ng/µL. The microsatellite fingerprinting of genotypes was performed at 24 microsatellite loci: VVS2, VVMD5, VVMD7, VVMD21, VVMD24, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62, VrZAG67, VrZAG79, VrZAG83, VMC4f3.1, VMC1b11, VVIb01, VVIn16, VVIh54,

VVIn73, VVIp31, VVIp60, VVIv37, VVIv67, and VVIq52 [25–31]. All forward primers were 5′ end-labeled with fluorescent dyes (FAM, HEX, TAMRA, ROX, and PET).

The fragment length was determined by capillary electrophoresis using the ABI 3130xlGenetic Analyzer (Applied Biosystems, Life Technologies, Waltham, MA, USA). The combinations of microsatellite loci (multiplexes) were optimized in the laboratory of the Julius Kühn Institute in Germany. The use of different markers and different fragment lengths allowed for the multiplexing of polymerase chain reactions (PCRs) with up to five SSR markers characterized by similar annealing temperatures. In total, 1 ng of DNA was mixed with the 2x KAPA2G Fast PCR Kit (Duren, Germany) to prepare 5 µL reaction mixes containing a master mix and 100 pmol of each primer. Amplification was performed in ABI 9700 thermal cyclers (Applied Biosystems, Foster City, CA, USA), starting with an initial denaturation of 3 min at 95 °C, followed by 30 cycles of denaturation at 95 °C (15 s), annealing at 60 °C (30 s), and extension at 72 °C (30 s). A final extension was performed at 72 °C for 7 min. In total, 1 µL of PCR product was used to determine the fragment length, and the results were processed using GeneMapper 5.0 software (Applied Biosystems, Foster City, CA, USA) based on a fluorescently labeled size marker ranging from 35 to 500 bp [32]. To correct for amplification shifts between different multiplexes, SSR profiles (Table S1) were adjusted by adding DNA from standard cultivars from the Julius Kühn Institute laboratory, 'Muscat a petit grains' and 'Cabernet franc', to each PCR amplification run.

2.3. Genetic Diversity Analysis

Various measures of genetic variability were calculated among 70 *V. v.* subsp. *vinifera* genotypes (66 historical and 4 reference varieties), 12 *V. v.* subsp. *sylvestris*, and 13 rootstocks (Supplementary Material Table S2) collected in old vineyards or abandoned in nature. The number of distinct alleles per locus (Na), number of effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), Shannon's information index (I), and fixation index (F) were calculated for each locus for historical grapevine varieties and other profiles used for comparison (rootstocks, reference varieties). GenAlEx software, version 6.5, was used to calculate the genetic diversity statistics for each locus [33,34]. Genetic relationships among accessions were assessed by distance-based cluster analysis using the neighbor-joining method (NJ) [35], as implemented in MEGA 11.0 software [36].

2.4. Population Structure

Cluster analysis based on the Bayesian model was performed using the STRUCTURE V2.3.4 software package [37] to determine the optimum genetically supported groupings based on the SSR markers data. The STRUCTURE configuration was set to use an admixture model and independent allele frequencies in the population. The allele frequency parameter (lambda) was set according to the STRUCTURE manual. Different numbers of putative populations (K) were tested, ranging from 1 to 10. The burnin period and number of Markov Chain Monte Carlo (MCMC) repetitions after burnin were set to 100,000 and 100,000, respectively, in each independent run with 10 iterations. The choice of the most likely number of clusters (best K) was assessed using the ad hoc delta K statistic, as described according to Evanno [38] with the Clumpak (Clustering Markov Packager Across K) program [39].

Principal coordinate analysis (PCA) was used to indicate genetic divergence between samples in a multidimensional space over a distance matrix with data standardization, using GenAlEx software, version 6.5 [33,34].

3. Results and Discussion

3.1. Genetic Diversity of the Historical Grapevine Varieties from Styria, Slovenia

Statistical indices were calculated on the basis of the allele profiles, and the genetic diversity of the identified historical varieties, unknown historical genotypes, the *sylvestris*, and the rootstocks was determined (Tables 1 and 2). Table 1 shows the descriptive statistics and genetic diversity of 95 samples, including historical varieties, unknown historical

genotypes, *sylvestris*, and rootstocks. The number of alleles per SSR locus (Na) ranged from 5 (VVIQ52) to 28 (VMC4f3.1), and the number of effective alleles (Ne) ranged from 2099 (VVIN73) to 10,000 (VVIP31), with an overall average of 5.477. The highest Shannon' information index was observed at the VVIP31 locus (2.595) and the lowest at the VVIQ52 locus (1.111), while the mean value of the SSR loci was 1.958. Observed heterozygosity (Ho) was highest at VVIN73 and lowest at VVMD31, ranging from 0.415 to 0.905, with an overall mean of 0.713. The expected heterozygosity (He) values ranged from 0.524 (VVIN73) to 0.900 (VVIP31) with a mean of 0.783. The mean F value for the dataset was 0.096, with the lowest F value of −0.079 for VMC1B11 and the highest of 0.236 for VVIQ52.

Table 1. Descriptive statistics and genetic diversity at 24 microsatellite loci from 95 genotypes of *Vitis vinifera* subsp. *sylvestris* Slovenian population (*sylvestris*), *Vitis vinifera* subsp. *vinifera* (cultivars), hybrids, and rootstocks.

Locus	Ra (bp)	N	Na	Ne	I	Ho	He	F
VVS2	123–163	95	16	5.364	2.066	0.747	0.814	0.081
VVMD7	231–265	95	16	5.275	2.133	0.716	0.810	0.117
VVMD5	198–270	94	15	7.457	2.219	0.819	0.866	0.054
VRZAG62	186–214	95	11	6.104	1.987	0.800	0.836	0.043
VRZAG79	237–265	94	13	7.472	2.210	0.787	0.866	0.091
VVMD28	216–278	93	23	8.741	2.475	0.882	0.886	0.004
VVMD32	237–272	89	13	5.268	1.927	0.742	0.810	0.085
VVMD25	235–269	95	13	4.579	1.833	0.642	0.782	0.178
VVIV67	329–398	95	21	7.056	2.336	0.695	0.858	0.191
VRZAG83	156–201	95	15	4.476	1.825	0.705	0.777	0.092
VVIN16	141–171	95	8	2.141	1.113	0.432	0.533	0.190
VVIN73	256–270	94	7	2.099	1.138	0.415	0.524	0.208
VVIP60	306–346	95	15	7.440	2.268	0.884	0.866	0.022
VMC1B11	167–197	94	13	5.505	1.960	0.883	0.818	0.079
VVIB01	269–313	95	12	3.746	1.650	0.663	0.733	0.095
VVIH54	139–187	95	17	6.668	2.193	0.747	0.850	0.121
VVMD24	200–215	95	8	3.637	1.582	0.632	0.725	0.129
VVMD27	176–218	95	20	6.449	2.178	0.863	0.845	0.022
VVIQ52	70–82	95	5	2.632	1.111	0.474	0.620	0.236
VRZAG67	118–171	95	23	7.648	2.465	0.800	0.869	0.080
VVIP31	169–209	95	21	10.000	2.595	0.905	0.900	0.006
VVMD21	219–267	95	15	2.924	1.668	0.505	0.658	0.232
VVIV37	144–174	95	12	3.825	1.747	0.716	0.739	0.031
VMC4f3.1	158–232	94	28	4.934	2.319	0.660	0.797	0.173
Total			360					
Min			5	2.099	1.111	0.415	0.524	−0.079
Max			28	10.000	2.595	0.905	0.900	0.236
Mean			15.000	5.477	1.958	0.713	0.783	0.096

Na—number of different alleles; Ne—effective alleles; I—Shannon's information index; Ho—observed heterozygosity; He—expected heterozygosity; F—fixation index.

The number of alleles per locus (Na) was 7.625 for the historical varieties, 8.208 for the unknown historical genotypes, 4.292 for the *sylvestris*, and 8.542 for the rootstock samples. The *sylvestris* samples had the lowest Ne value (2.519) and the highest Na value in the rootstocks (5.408). The observed heterozygosity (Ho) was highest in the samples of the historical varieties and lowest in the *sylvestris* samples. The expected heterozygosity was highest in the rootstock (0.766) and lowest in the *sylvestris* population (0.540). The fixation index (F) was negative in historical varieties (−0.074) and unknown historical genotypes (−0.024), while it was positive in *sylvestris* (0.037) and rootstocks (0.126) (Table 2).

Table 2. Genetic diversity estimates for each analyzed population of historical varieties and unknown historical genotypes, *sylvestris*, and rootstocks.

Population		N	Na	Ne	Ho	He	F
Historical varieties	Mean	33.000	7.625	4.133	0.774	0.719	−0.074
	SE	0.000	0.528	0.329	0.032	0.023	0.022
Unknown historical genotypes	Mean	36.792	8.208	4.003	0.722	0.704	−0.024
	SE	0.085	0.640	0.346	0.032	0.026	0.028
Sylvestris	Mean	12.000	4.292	2.519	0.528	0.540	0.037
	SE	0.000	0.332	0.225	0.051	0.033	0.065
Rootstocks	Mean	12.667	8.542	5.408	0.694	0.766	0.126
	SE	0.253	0.637	0.441	0.057	0.031	0.064
Total	Mean	33.000	7.625	4.133	0.774	0.719	−0.074
	SE	0.000	0.528	0.329	0.032	0.023	0.022

Na—number of different alleles; Ne—effective alleles; Ho—observed heterozygosity; He—expected heterozygosity; F—fixation index.

The pairwise Nei's genetic distance and Fst values for historical varieties, unknown historical genotypes, *sylvestris*, and rootstock samples are shown in Table 3. The Nei's distance ranged from 1.410 (*sylvestris*–rootstock) to 0.053 (historical varieties–historical unknown genotypes). The Fst values confirmed the pattern with the highest value of 0.173 (rootstock–*sylvestris*) and the lowest value of 0.011 (historical varieties–historical unknown genotypes). This shows, on the one hand, that the rootstocks and *sylvestris* form a separate group, and on the other hand, it shows that the historical varieties and the unknown historical genotypes belong to the same group. The Nei´s distance and the Fst values for the included accessions show that the historical varieties and the unknown historical genotypes are closest to each other and both belong to vinifera.

Table 3. Estimates of pairwise population Fst values (below the diagonal) and pairwise population matrix Nei´s genetic distance (above the diagonal).

	Historical Varieties	Unknown Historical Genotypes	Sylvestris	Rootstocks
Historical varieties		0.053	0.692	1.194
Unknown historical genotypes	0.011		0.721	1.181
Sylvestris	**0.132**	**0.135**		1.410
Rootstocks	**0.110**	**0.113**	**0.173**	

In bold significant Fst values with $p \leq 0.01$ calculated over 999 permutations.

Based on the available ampelographic descriptions according to the OIV descriptors, the literature sources, genetic analysis, and comparison with the VIVC database [1], we identified a total of 66 different grapevine genotypes in the wine-growing region of Styria by the end of 2022. Most of the identified varieties are not on the national variety list of this wine region, except 'Frankinja', 'Kraljevina', 'Portugalka', 'Ranfol', 'Rumeni plavec', and 'Žametovka' (Table 4). Out of 66 genotypes, 29 genotypes were confirmed as historical varieties (Table 1). These varieties were cultivated in this area more than 200 years ago, which is also confirmed by written sources from this period [12–17,40,41]. However, this is only about 10% of the varieties that Trummer described in this area in 1841. In the old vineyards (115 locations), the most frequently represented varieties were 'Belina' ('Heunisch weiss') and 'Vrbovec' ('Tantovina Eihenblaetrige') in more than half of the monitored locations, followed by 'Ranfol' ('Ranfol beli'), 'Pelesovna' ('Vulpea'), 'Frankinja' ('Blaufraenkisch'), 'Žametovka' ('Kavčina črna'), 'Peček' ('Elbling weiss'), 'Kraljevina' ('Kraljevina'), and unknown variety 'Zelenec' ('Zelenec') (Figure 2). In at least 5 locations out of 115, the following varieties were also found: 'Lipna' ('Prsljivka'), 'Velika črnina'

('Bettlertraube'), 'Beli kozjak' ('Coarna alba'), 'Svetla belina' ('Svjetlak'), 'Portugalka' ('Portugieser blau'), 'Rumeni plavec' ('Plavec žuti'), 'Trda belina' 'Hartheunisch', and 'Topolina' ('Lisztes feher') (Table 4). The other 37 genotypes were confirmed as unknown historical genotypes (Table 5) when comparing the data to the VIVC database. According to the existing ampelographic descriptions and existing written sources, these genotypes are written with local names in Table 5. All these genotypes will ampelographically be described in detail in the following years. In the 19th century, with the introduction of new varieties, the arrangement of vineyards also changed. Due to the transition from mixed plantations to monovarietal vineyards, varieties with female flowers began to lose their importance. Thus, the 'Vranek' ('Zimmettraube') variety (Table 4), which was the main red variety in mixed vineyards alongside 'Žametovka' and 'Frankinja' at the beginning of the 19th century [42], was lost very quickly.

Table 4. Historical vine varieties identified by genetic analysis in Styria (Slovenia) between 2020 and 2022 with the Slovenian names and prime name in VIVC.

Name of Variety SLO	Prime Name VIVC	Name of Variety SLO	Prime Name VIVC
'Bela kavka'	'Knipperle'	'Portugalka' *	'Portugieser blau'
'Beli javor'	'Javor Weiss'	'Ranfol' *	'Ranfol beli'
'Beli kozjak'	'Coarna alba'	'Svetla belina'	'Svjetlak'
'Belina'	'Heunisch weiss'	'Rumeni plavec' *	'Plavec žuti'
'Črni šipon'	'Kadarka kek'	'Topolina'	'Lisztes feher'
'Frankinja' *	'Blaufraenkisch'	'Trda belina'	'Hartheunisch'
'Gosjenog'	'Gänsfüsser blau'	'Velika črnina'	'Bettlertraube'
'Kraljevina' *	'Kraljevina'	'Volovnik'	'Vela pergola'
'Lepa zastavica'	'Zierfandler rot'	'Vranek' **	'Zimmettraube blau'
'Lipna'	'Prsljivka'	'Vrbovec'	'Tantovina Eihenblaetrige '
'Ložinka'	'Pescsi Szagos'	'Zagajec'	'Blaue Batttraube'
'Modri naprstnik'	'Augster blau'	'Žametovka' *	'Kavčina črna'
'Peček'	'Elbling Weiss'	'Rdeča Šopatna'	'Roter Veltliner'
'Pelesovna'	'Vulpea'	'Babov grozd'	'Frühroter veltliner'
'Nojburgar'	'Neuburger'		

VIVC—Vitis International Variety Catalogue, SLO—Slovenia, * variety is on the list of grapevine varieties of wine-growing region Styria in, Slovenia, ** female plants.

Table 5. Unknown historical genotypes identified by genetic analysis in Styria (Slovenia) between 2020 and 2022 with identification of plants gender.

Name of Variety	Gender	Name of Variety	Gender	Name of Variety	Gender
'Antonija'	HH	'Ivek'	HH	'Pohorka'	HH
'Banovina'	F	'Jelovec'	F	'Poklek'	F
'Birna'	HH	'Jurjevina'	HH	'Rohlin'	HH
'Blanca'	HH	'Ledina'	HH	'Skok'	HH
'Bučanka'	HH	'Martinka'	HH	'Sojek'	HH
'Cirjan'	HH	'Okič'	F	'Strmec'	HH
'Dolka'	HH	'Paradiž'	HH	'Tična'	HH
'Furman'	F	'Pečica'	HH	'Topolka'	HH
'Gajka'	HH	'Pečovka'	HH	'Varnica'	HH
'Gruška'	HH	'Pikica'	HH	'Viderman'	HH
'Gruškovec'	HH	'Planina'	HH	'Zabukovka'	HH
'Habjana'	HH	'Podboč'	F	'Zelenec'	HH
'Haložanka'	HH				

HH—hermaphrodite, F—female.

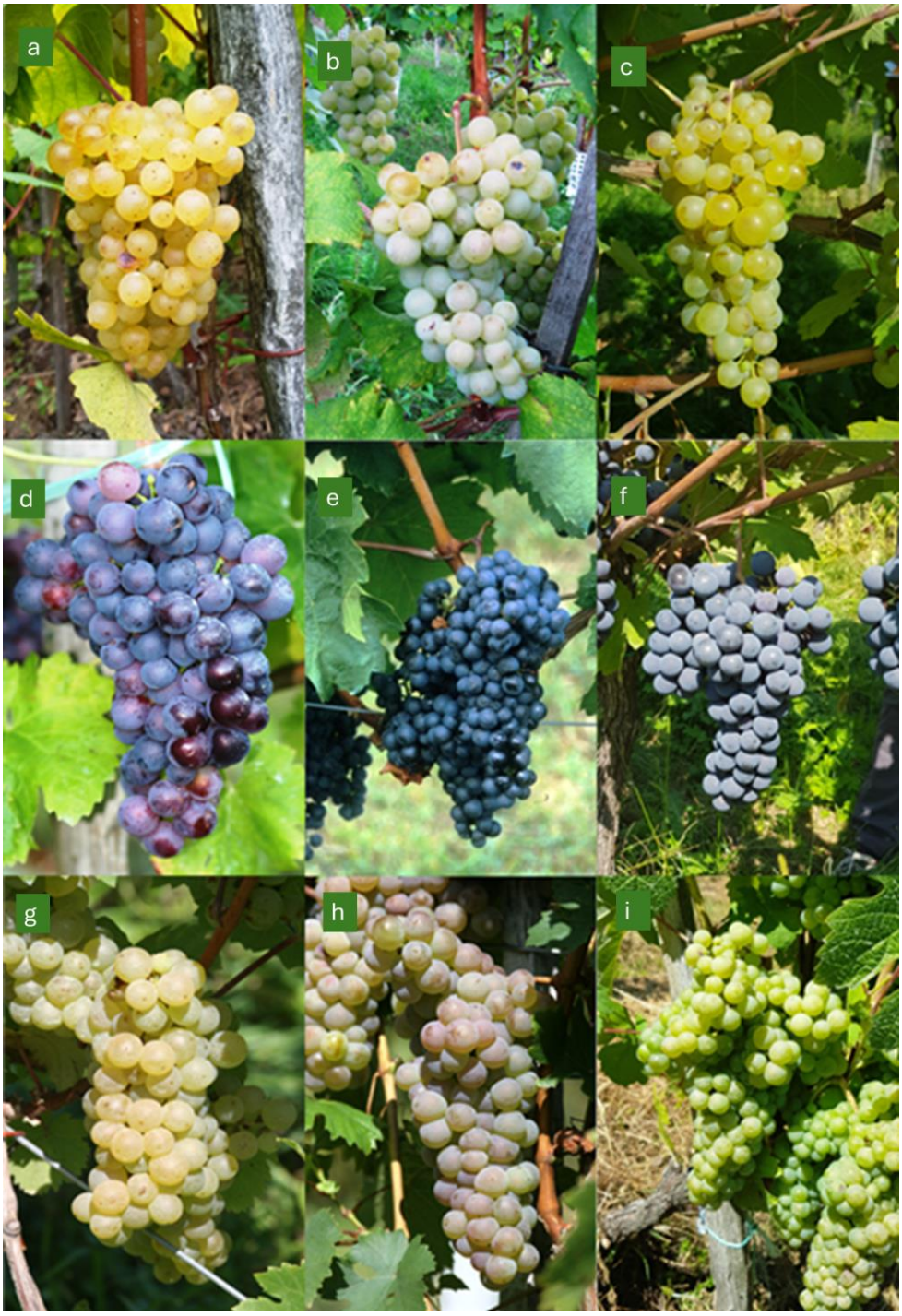

Figure 2. The most common varieties in old vineyards in Styria in Slovenia (from left to right and from top to bottom; in parentheses is the prime name from the VIVC grapevine database): (**a**)—'Belina' (Heunisch weiss), (**b**)—'Vrbovec' ('Tantovina Eihenblaetrige'), (**c**)—'Ranfol' ('Ranfol beli'), (**d**)—'Pelesovna' ('Vulpea'), (**e**)—'Frankinja' ('Blaufraenkisch'), (**f**)—'Žametovka' ('Kavčina črna'), (**g**)—'Peček' ('Elbling weiss'), (**h**)—'Kraljevina' ('Kraljevina'), and (**i**)—'Zelenec' ('Zelenec') (photo: S. Vršič. 2019–2022).

3.2. Genetic Characterization

The neighbor-joining method was used to construct a phylogenetic tree (dendrogram) based on the frequency of alleles at 24 loci for 66 genotypes found in old vineyards in Styria, composed of 29 historical varieties and 37 unknown historical genotypes (according to the

VIVC database and historical writings). In addition, 4 traditional (reference) grapevine varieties, 12 Slovenian *sylvestris*, and 13 rootstocks (Teleki 8 B and 9B from the UC Meranovo gene bank, the rest as escaped in the wild) are included in the dendrogram. In the dendrogram (Figure 3), the included genotypes are genetically very close to each other and form a group (cluster), whereas in the rootstocks, they form a separate group and are clearly separated from the vinifera group. Nevertheless, the group can be divided into several subgroups with varieties that are closer to each other in terms of genetic profile or related to the parent candidate and its offspring.

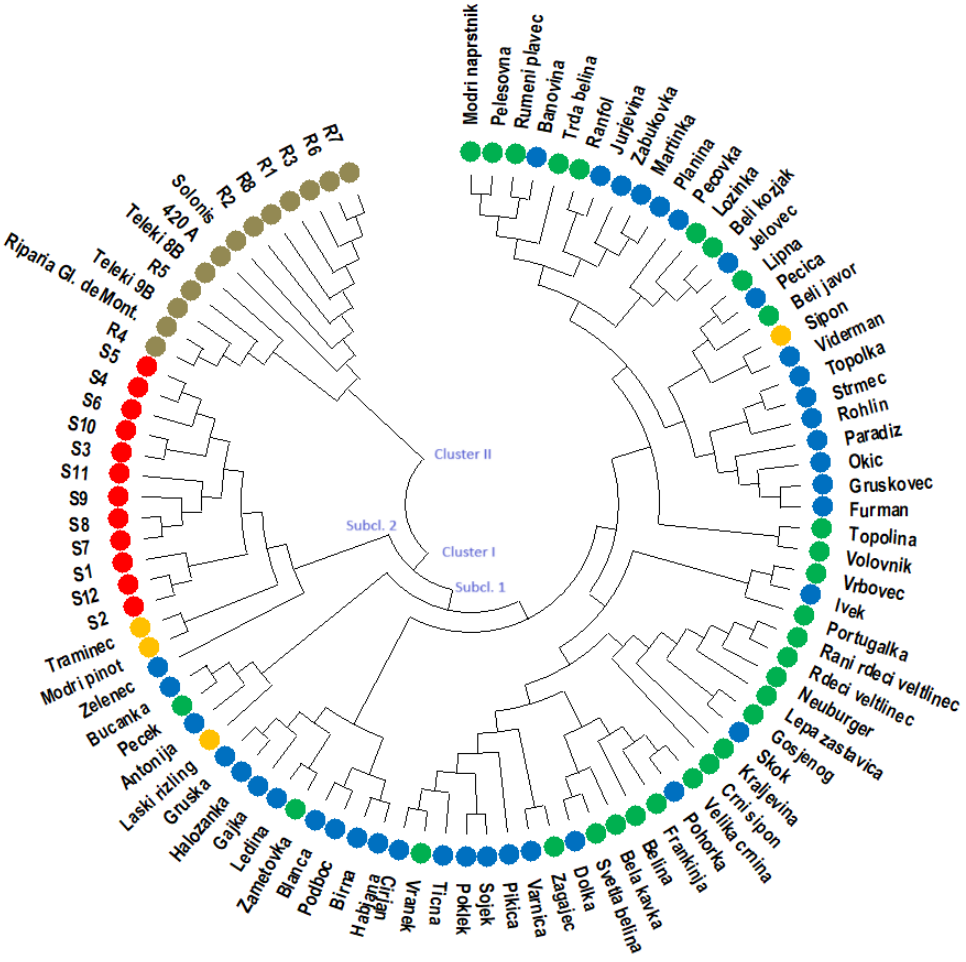

Figure 3. Phylogenetic tree (dendrogram) using the neighbor-joining (NJ) method of genetic links between reference varieties (orange). Historical grapevine varieties (blue) and unknown historical genotypes (green) sampled in old vineyards in Styria in Slovenia and Slovenian *sylvestris* (red) and rootstocks (gold).

Based on the included samples, the vinifera group is divided into two subgroups. The first includes the varieties close to Slovenian *sylvestris*, namely reference varieties 'Traminec' ('Traminer') and 'Modri pinot' ('Pinot noir') and unknown historical variety 'Zelenec'. The second subgroup has five major groups into which the rest of the vinifera genotypes are classified. The first of the five is the group close to 'Laški rizling' ('Welschriesling') and 'Peček'. In the second are those classified around 'Žametovka'. In a larger third group, genotypes are clustered close to varieties 'Vranek', 'Belina', 'Gosjenog', and 'Vrbovec'. The smallest fourth group has only two varieties; one of them is 'Volovnik'. In the fifth largest group are genotypes close to 'Šipon' ('Furmnt') and 'Ranfol'.

The genotypes in the dendrogram are closely related to each other within the subgroups, and there is often a relationship between the parent candidate and the offspring.

Based on genetic analyses of the parentage, it was found that the variety 'Belina' ('Heunisch weiss') is one of the parents in most cases, e.g., 'Belina' is one of the parents of the variety 'Peček' [1]. This also confirms the widespread nature of the variety 'Belina' in the past. The variety 'Pelesovna' is indicated as one of the potential parental candidates of the varieties 'Modri naprstnik', 'Topolina', and 'Trda belina', but more precise analyses are still needed. In the old vineyards, all the parental varieties of 'Frankinja' and 'Portugalka' were found. According to the findings of Maul et al. 2016 [43], these are 'Vranek' ('Zimmettraube blau') in both varieties and 'Belina' ('Weiser Heunisch') and 'Zeleni silvanec' ('Silvaner grün'). 'Belina' is the most common variety in the old vineyards even today, and 'Zeleni silvanec' is still on the list of wine varieties for this wine-growing region. The 'Vranek' variety was the main red variety at the beginning of the 20th century [42]. Also, one of the grandparents of the 'Frankinja' and 'Gosjenog' varieties was found [1].

3.3. Population Structure

The non-hierarchical horizontal clustering method applied using the Structure software effectively classified the 95 genotypes into three distinct clusters. These clusters corresponded to *V. vinifera* L. subsp. *vinifera* (DC.) Hegi., which included historical varieties and unknown historical genotypes; *V. vinifera* L. subsp. *sylvestris* (C. C. Gmelin) Hegi (*sylvestris*); and rootstocks. The determination of the optimal number of clusters (K) was based on the methodology described by [38], seen in Supplementary Figure S1, with the calculation of K facilitated by the Clumpak program. The separation of these clusters was evident, although some varieties and unknown historical genotypes showed partial admixture, suggesting a *vinifera*/*sylvestris* ancestry (Figure 4).

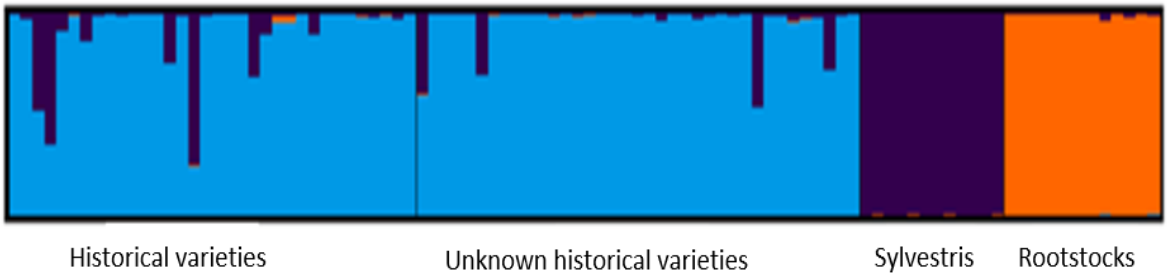

Figure 4. Bar plot displaying the admixture proportions of 95 genotypes (historical varieties, unknown historical genotypes, *sylvestris*, and rootstock) as estimated by Structure analysis at K = 3. Each accession is represented by a single vertical bar divided into K colors.

The historical varieties and certain unknown historical genotypes showed varying degrees of admixture. In particular, the reference varieties 'Traminec' ('Traminer') and 'Modri pinot' ('Pinot noir') showed the highest percentage of admixed genomes among the historical varieties. Other historical cultivars such as 'Pecek', 'Bela kavka', and certain unknown historical genotypes, such as 'Zelenec', 'Bučanka', 'Podboč', and 'Cirjan', also showed mixed *sylvestris*/*vinifera* ancestry, as evidenced by the Supplemental Table S3. The clustering patterns observed in Structure were consistent with those determined by the neighbor-joining (NJ) analysis.

4. Conclusions

After 2010, the monitoring of old vineyards at 115 sites and the genetic identification of historical varieties were carried out with the aim of preserving the genetic resources of the vines still present in situ. Genetic analyses and ampelographic descriptions confirmed 29 historical varieties. Among them, the most common varieties were 'Belina' (Heunisch weiss), 'Vrbovec' ('Tantovina Eihenblaetrige'), 'Ranfol' ('Ranfol beli'), and 'Pelesovna' ('Vulpea'). In some locations, one of the most important red varieties from the beginning of the 20th century was also found in this area, i.e., 'Vranek' ('Zimmettraube blau'), which has disappeared in single-variety vineyards due to female flower. In addition, 37 unknown

historical genotypes were found during genetic analyses. These genotypes have to be described ampelographically and evaluated technologically in the future. Most of the vegetative offsprings of these genotypes have already been transferred to the Meranovo gene bank, where they can be accurately described ampelographically under the same pedoclimatic conditions. Without conservation measures, the historical varieties from old vineyards with all their diverse genetic material will be lost forever. Some of them were lost during the research work due to disease (Grapevine flavescence dorée) and also due to demographic influences. The results of this research work were a first step towards preserving diversity, reducing genetic erosion, and the possibility of reviving historical varieties in this wine-growing region.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14040640/s1. Figure S1: Results of STRUCTURE analyses. (a) Calculating Best K by Evanno. (b) Using median values of Ln Prob of Data to calculate Prob (K = k). Three different genetic groups (populations) were suggested (ΔK = 3). The test was run from K = 1 to 10 using STRUCTURE software. Table S1: SSR profiles of analyzed samples. Table S2: List of samples used in this study. Table S3: Q values of 95 samples obtained from Structure software analysis

Author Contributions: Conceptualization, S.V.; formal analysis, A.P., F.R., E.M. and O.T.; funding acquisition, S.V.; investigation, S.V. and A.P.; methodology, S.V. and A.P.; visualization, S.V.; writing—original draft, S.V. and A.P.; writing—review and editing, S.V., A.P., O.T. and F.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Slovenian national research agency and the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (grant no. CRP V4-0475) via the project "Determining the optimal grape production technology in terms of predicted climate changes and the future of Slovenian viticulture".

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Data are available from the authors.

Acknowledgments: This work was supported by University Centre of Viticulture and Enology Meranovo, Faculty of Agriculture and Life Sciences, University of Maribor.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

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