Origin of Slovenian wild grown grapevines and their genetic relationships

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The spread of phylloxera at the end of the 19th century caused a significant genetic erosion and reduction of Slovenian areas under grapevine. One of the consequences was the introduction of resistant rootstocks from North America. Twelve SSRs markers well distributed through the Vitis genome were screened on 70 grapevine genotypes with focus on Slovenian grapevines growing in the wild, which could hypothetically include descendants of the North American germplasm introduced more than a century ago, along with Slovenian wild indigenous genotypes. The results suggest that the Slovenian wild genotypes can be grouped into five clusters: (1) species or hybrids involving *Vitis labrusca* L**.**, *Vitis riparia* Michx*., Vitis rupestris* Scheele and *Vitis longii* W.R. Prince & Prince; (2*) Vitis vinifera* subsp*. vinifera* L. germplasm and its hybrids with the North American germplasm; (3) V. *vinifera* subsp*. sylvestris* (C.C. Gmel.) Hegi and descendants of its natural crosses; (4) descendants of crosses involving *Vitis berlandieri* Planch. and (5) North American germplasm and its hybrid descendants collected in the Southwest of the country. Allelic diversity of the genetically 'pure' *V*. *vinifera* subsp*. sylvestris* has been partly preserved through natural intercrosses with cultivated *V*. *vinifera* or with the much more vigorous and resistant American genotypes. For the survival of the North American germplasm, vegetative propagation has been crucial; however, such high levels of genetic variation cannot be explained without the presence of natural hybridization involving genetically very diverse genotypes. Most Slovenian wild grapevines are well adapted to the local environmental conditions, and some can be considered as potential rootstock material or as a source of allelic diversity for genetic breeding.
Keywords: grapevine, rootstocks, microsatellites, SSR, *Vitis* spp., genetic diversity

Die Herkunft slowenischer wildwachsender Weinreben und deren genetisches Verhältnis. Die Ausbreitung der Reblaus Ende des 19. Jahrhunderts verursachte eine signifikante genetische Erosion bei Weinreben und eine Verringerung der Weinanbauflächen. Eine der Folgen war die Einführung resistenter Wurzelstöcke aus Nordamerika. Zwölf SSRs-Marker, die gut über das Vitis-Genom verteilt sind, wurden an 70 Rebgenotypen untersucht mit dem Fokus auf slowenische wildwachsende Weinreben, die rein hypothetisch gesehen Nachkommen des vor mehr als einem Jahrhundert eingeführten nordamerikanischen Keimplasmas beinhalten könnten, sowie auf slowenische wildwachsende indigenen Genotypen. Die Ergebnisse legen nahe, dass sie in fünf Cluster eingeteilt werden können: (1) Arten oder Hybriden, die *Vitis labrusca* L*., Vitis riparia* Michx*., Vitis rupestris* Scheele und *Vitis longii* W.R. Prince & Prince einbeziehen, (2) *Vitis vinifera* subsp*. vinifera* L. Keimplasma und seine Hybriden mit dem nordamerikanischen Keimplasma, (3) *V. vinifera subsp. sylvestris* (C.C. Gmel.) Hegi und Nachkommen seiner natürlichen Kreuzungen, (4) Nachkommen von Kreuzungen, die *Vitis berlandieri* Planch. einbeziehen und (5) nordamerikanisches Keimplasma und seine hybriden Nachkommen, die im Südwesten des Landes gesammelt wurden. Die allelische Vielfalt der genetisch "reinen" *V. vinifera* subsp*. sylvestris* wurde teilweise durch natürliche Kreuzungen mit kultivierten *V. vinifera* oder mit viel kräftigeren und resistenteren amerikanischen Genotypen erhalten. Für das Überleben des nordamerikanischen Keimplasmas war die vegetative Vermehrung von entscheidender Bedeutung. Allerdings konnte ein derart hohes Maß an genetischer Variation nicht ohne das Vorhandensein einer natürlichen Hybridisierung mit genetisch sehr unterschiedlichen Genotypen erklärt werden. Die meisten wildwachsenden slowenischen Weinreben sind gut an die örtlichen Umweltbedingungen angepasst, und einige von ihnen können als geeignetes potenzielles Unterlagenmaterial oder als Quelle allelischer Vielfalt für die genetische Züchtung angesehen werden.
Schlagwörter: Weinrebe, Unterlagen, Mikrosatelliten, SSR, *Vitis* spp*.,* genetische Vielfalt

The common grapevine (*Vitis vinifera* L.) is one of the oldest cultivated fruit species and is subdivided into two subspecies: subsp. *vinifera* L. (the cultivated subspecies), and subsp. *sylvestris* (C.C. Gmel.) Hegi, which is thought to be the wild progenitor of the cultivated grapevines (Levadoux, 1956; Zohary and Hopf, 2000) and used to be abundant from the western Himalayas to the European Atlantic coast (Lacombe et al., 2003; Bacilieri et al., 2013); however, the appearance of downy mildew (*Plasmopara viticola* (Berk. et Curtis ex de Bary) Berl. et de Toni), powdery mildew (*Erysiphe necator* Schwein.) and phylloxera (*Daktulosphaira vitifoliae* Fitch) significantly reduced natural populations of *V. vinifera* subsp. *sylvestris* and caused genetic erosion. At present, this wild relative of the common grapevine is restricted to small, isolated populations along riverbank forests (Arnold, 1998; Zohary and Hopf, 2000; Schneider et al., 2015). During the last century, pests and diseases, together with various economic factors, led to a significant decrease in the number of cultivated grapevine varieties. Among these factors, the spread of phylloxera at the end of the 19th century was probably the most important. The areas under grapevine were almost halved. Since chemical eradication of this pest was unsuccessful, it was decided to introduce resistant grapevine rootstocks from North America (Granett et al., 2001; This et al., 2006), a measure which was also important for managing drought stress (Pavlousek, 2011; Zhang et al., 2016; Fort et al., 2017).

In Slovenia, *Vitis* spp. plants can be found growing wild in many forests, especially forest edges, and abandoned vineyards. They may originate from various sources such as subsp. s*ylvestris*, various rootstock material and numerous natural hybrids. Slovenian vineyards are characterized by the presence of both indigenous (autochthonous) and introduced (allochthonous) varieties (Štajner et al., 2011; Maul et al., 2015). The first introductions of *V. vinifera* germplasm took place before the appearance of phylloxera. The first North American materials were introduced to Northeast Slovenia at the end of the 19th century (Skalicky, 1907a, b) and belonged to *Vitis* acerifolia Raf. (syn. *V. solonis* hort. Berol. ex Planch.) and *Vitis riparia* Michx. At approximately the same time, the Austro-Hungarian agricultural authorities began to introduce grapevines belonging to *Vitis labrusca* L. Many growers expected that some of the *V. labrusca* genotypes would gradually replace traditional *V. vinifera* germplasm. Later introductions involved interspecific hybrids (*Vitis berlandieri* Planch. *× V. riparia*) and other species such as *Vitis rupestris* Scheele and *V. riparia.* The interspecific hybrid *V. berlandieri × V. riparia* was already present in 1906 (Skalicky, 1907a, b). Owing to vegetative propagation, some of these early introduced materials most probably survived in the wild and have been occasionally used as rootstock.

V. vinifera has been present since prehistoric times across most European regions with a temperate climate. It has always been an important member of the existing plant communities. The discovery of America and the introduction of new plant species was associated with the transfer of pests and diseases. For *V. vinifera*, especially *V. vinifera* subsp. *sylvestris,* the consequences were drastic. Since the existing genotypes of this indigenous taxon were highly susceptible to new pests and diseases, their presence in plant communities began to decrease, and in many cases, they gradually died out. The cultivated taxon (i.e., *V. vinifera* subsp. *vinifera*) was in a better position because there were numerous cultivars and it was possible to select at least partly tolerant ones, or to use pesticides. Regarding phylloxera, the only reasonable solution was grafting on a resistant rootstock imported from North America. For general agricultural practice, this was a good solution, but it was a disaster for the indigenous wild *V. vinifera* subsp. *sylvestris*. Due to resistance to pests and diseases, many of the introduced North American *Vitis* species and their interspecific hybrids became invasive in the new environment and gradually changed the original structure of plant communities where *V. vinifera* subsp. *sylvestris* used to be a stable member. More than a century after the introduction of the American germplasm, there were significant changes in plant communities involving *Vitis* species. Natural interspecific crossings between feral North American *Vitis* species and the native grapevine *V. vinifera* have been documented, and these have led to the emergence of a genetic complex of wild forms, rootstocks, naturalized domesticated forms and hybrids derived from spontaneous hybridizations and introgressions among these taxa (Bodor et al., 2010; Ocete et al., 2011; Cunha et al., 2020; D'Onofrio, 2020; Arnold and Schnitzler, 2020). This process has contributed to the eradication

of the endemic wild grapevine *V. vinifera* subsp. *sylvestris*, already endangered by American pests and pathogens and large-scale habitat destruction. Recent studies have shown that wild grapevines survived as small populations in remote mountain sites, screes, floodplain forests of large rivers, their deltas, and their tributaries (Arnold et al., 2010; Regner et al., 2004; Tiefenbruner et al., 2015; Arnold et al., 2017).

The present study involves a molecular analysis of grapevine genotypes growing in the wild across Slovenia (hypothetically wild relatives of cultivated *V. vinifera*, various feral genotypes and their hybrids), locally grown grapevines and reference genotypes of cultivated and wild *V. vinifera*. Microsatellite markers (SSRs) have been found to be very useful for grapevine varietal identification and genetic characterization (Vršič et al., 2011; Maul et al., 2015). Our study had two main purposes: (1) to document the presence/absence of the indigenous *V. vinifera* subsp. *sylvestris* and (2) to find out what happened to early North American grapevine materials, especially in association with their genetic and taxonomic background. Because the Slovenian climate is highly favorable for many grapevine pests and diseases, we assumed that it would be very difficult, or impossible, to find a genuine *V. vinifera* subsp. *sylvestris*. We also wanted to elucidate some of the problems associated with the evolution of the North American germplasm that escaped from cultivated areas. We assumed that both vegetative and seed propagation took place. Given the differences in sexual expression of plants, hybridization was probably frequent and also involved the indigenous wild taxon of *V. vinifera*. The emphasis was placed on molecular analyses of plants growing in the wild. According to our hypothesis, some of the introduced materials were lost, some were preserved by vegetative multiplication and remained genetically more or less unchanged, while the rest were subjected to genetic recombination, involving self- and cross-fertilization (i.e., intra- and interspecific hybridization). We assumed that genetic recombination probably played a significant role, resulting in high genetic and morphological variation.

Materials and methods

Plant material

A total of 70 grapevine genotypes were included in the study (Table 1): (1) 13 cultivated genotypes involving 6 reference varieties ('Merlot', 'Pinot Noir', 'Cabernet Sauvignon', 'Sultanine', 'Touriga Nacional', 'Barbera') and 7 rootstock genotypes; (2) 3 accessions of *V. vinifera* subsp. *sylvestris*, two of which were obtained from the Botanical Garden of the University of Vienna and one from the Botanical Garden of the University of Graz (Austria); (3) 54 samples of wild-growing and feral genotypes collected randomly across Slovenia from abandoned vineyards and nearby areas such as forests or forest edges and river banks. For the ampelographic characterisation of young shoots and leaves, some descriptors from the list of descriptors developed by the International Organization of Vine and Wine (OIV, 2009) were used.

DNA isolation and microsatellite analysis

DNA was extracted from fresh, young leaves using the CTAB method (Doyle and Doyle, 1987), with some modifications as described by Šiško et al. (2009). Two separate extractions per plant were performed.

Twelve microsatellite loci were used. Eight SSR-markers (VVS 2, VVMD 5, VVMD 7, VVMD 25, VVMD 27, VVMD 28, VrZAG 62, VrZAG 79) recommended by the European project GrapeGen06 were applied (Maul et al., 2012). Additionally, we used four markers: VVMD 6 (Bowers et al., 1996), VVMD24, VVMD 36 (Bowers et al., 1999), VrZAG 112 (Sefc et al., 1999). Fifteen µl of PCR mixture contained 20 ng DNA, 0.45 U Taq DNA polymerase (Fermentas, Waltham, Massachusetts, USA), 1x reaction buffer (Fermentas, Waltham, Massachusetts, USA), 4 mM $MgCl_2$ (Fermentas, Waltham, Massachusetts, USA), 0.5 µM of each primer (Sigma, Darmstadt, Germany) and 0.2 mM of each dNTP's (Sigma, Darmstadt, Germany). The polymerase chain reaction (PCR) was performed using a Whatman Biometra T-Gradient thermocycler (Göttingen, Germany). Capillary electrophoresis of PCR products was performed on a Beckman Coulter CEQ8000 (Brea, California, USA) according to manufacturer's instructions. Fragment size

analysis was done with the built-in software. A fluorescently labelled size marker (Beckman Coulter DNA Size Standard Kit 400 bp (Brea, California, USA) was used as a molecular weight reference.

Table1: Plant materials used in the investigation

Label/Name	Typ[1]	Origin[2]	Location	Label/Name	Typ[1]	Origin[2]	Location
220 *V. riparia*	R	SPGB	46°32'16.6"N 15°33'24.2"E	191 Nunska graba 1	W	ab. vineyard	46°29'22.4"N 16°14'08.5"E
207 'Boerner'	R	SPGB	46°32'16.6"N 15°33'24.2"E	192 Nunska graba 2	W	ab. vineyard	46°29'25.1"N 16°14'08.4"E
215 *V. rupestris*	R	SPGB	46°32'16.6"N 15°33'24.2"E	194 Nunska graba 3	W	ab. vineyard	46°29'28.5"N 16°14'09.9"E
217 'M *IV*'	R	SPGB	46°32'16.6"N 15°33'24.2"E	193 Ivanjkovci	W	river bank	46°27'38.6"N 16°09'28.8"E
218 'SO4'	R	SPGB	46°32'16.6"N 15°33'24.2"E	197 Lahonci 1	F	forest edge	46°28'49.3"N 16°07'33.9"E
R1 'Merlot'	cv.	UL BF	46°02'58.2"N 14°28'28.4"E	195 Lahonci 2	F	forest edge	46°28'48.4"N 16°07'50.5"E
R2 'Pinot noir'	cv.	UL BF	46°02'58.2"N 14°28'28.4"E	196 Lahonci 3	F	forest edge	46°28'28.5"N 16°08'01.2"E
R3 'Cabernet sauvignon'	cv.	UL BF	46°02'58.2"N 14°28'28.4"E	198 Trnovci 1	W	forest edge	46°30'04.9"N 16°02'17.2"E
R4 'Sultanine'	cv.	UL BF	46°02'58.2"N 14°28'28.4"E	199 Trnovci 2	F	forest edge	46°29'59.7"N 16°02'22.0"E
R5 'Touriga nacional'	cv.	UL BF	46°02'58.2"N 14°28'28.4"E	201 Trnovci 3	F	forest edge	46°29'54.0"N 16°02'31.8"E
R6 'Barbera'	cv.	UL BF	46°02'58.2"N 14°28'28.4"E	200 Vitomarci	W	ab. vineyard	46°30'53.8"N 15°56'27.2"E
R7 *V. rupestris*	R	UL BF	46°02'58.2"N 14°28'28.4"E	202 Sencak	F	forest edge	46°30'32.6"N 16°00'33.3"E
249 *V. v.* subsp. *sylvestris*	sp.	BG Wien	48°11'31.9"N 16°23'00.6"E	295 Hrastje	W	forest edge	46.618226°N 16.086525°E
246 *V. v.* subsp. *sylvestris*	sp.	BG Wien	48°11'31.9"N 16°23'00.6"E	271 Vinje 1	W	forest	46°09'15.8"N 14°43'40.9"E
250 *V. v.* subsp. *sylvestris*	sp.	BG Graz	47°04'54.5"N 15°27'25.6"E	272 Vinje 2	W	forest	46°09'14.0"N 14°43'01.1"E
180 *V. riparia* Graz	R	BG Graz	47°04'54.5"N 15°27'25.6"E	292 Kostel	W	river bank	45°30'34.7"N 14°54'48.2"E
175 Kalvaria 1	W	ab. vineyard	46°34'08.9"N 15°38'20.5"E	274 Puce	W	ab. vineyard	45°29'11.6"N 13°43'56.1"E
171 Kalvaria 2	W	ab. vineyard	46°34'11.3"N 15°38'15.7"E	275 Bric 1	W	forest	45°27'59.4"N 13°44'09.1"E
172 Kalvaria 3	W	ab. vineyard	46°34'21.7"N 15°37'58.6"E	287 Bric 2	W	forest	45°27'50.9"N 13°44'05.7"E
179 Kalvaria 4	W	ab. vineyard	46°34'20.9"N 15°37'50.6"E	288 Bric 3	W	forest	45°27'53.1"N 13°44'10.4"E
173 Kalvaria 5	W	ab. vineyard	46°34'11.8"N 15°38'14.0"E	289 Bric 4	W	forest	45°27'55.6"N 13°44'14.9"E
236 Vurberk 1	W	forest edge	46°29'06.8"N 15°47'43.8"E	290 Bric 5	W	forest	45°28'00.3"N 13°44'21.9"E
237 Vurberk 2	W	forest edge	46°29'14.6"N 15°47'32.4"E	291 Bric 6	W	forest	45°28'03.9"N 13°44'30.9"E
238 Korena	W	forest edge	46°31'10.2"N 15°47'03.3"E	273 Nova vas	W	forest	45°29'00.6"N 13°42'20.9"E
239 Vodole	F	forest edge	46°33'50.9"N 15°41'22.8"E	276 Dragonja river	W	ab. vineyard	45°28'10.7"N 13°45'31.0"E
240 Malečnik 1	W	ab. vineyard	46°33'05.2"N 15°42'04.0"E	277 Dragonja 1	W	forest edge	45°27'32.9"N 13°42'47.7"E
241 Malečnik 2	F	forest edge	46°33'22.9"N 15°42'07.1"E	278Dragonja 2	W	forest edge	45°27'30.4"N 13°42'44.6"E
242 Zavrh	W	forest edge	46°32'12.1"N 15°50'02.2"E	279 Dragonja 3	W	forest edge	45°27'29.8"N 13°42'23.9"E
245 Selce	W	forest edge	46°30'53.6"N 15°49'31.6"E	280 Dragonja 4	W	forest edge	45°27'26.4"N 13°42'12.3"E
244 Rospoh	W	forest edge	46°35'53.2"N 15°37'50.2"E	281 Dragonja 5	W	forest edge	45°27'23.9"N 13°42'06.7"E
243 Ciglence	W	forest edge	46°30'35.2"N 15°46'53.1"E	282 Secovlje 1	W	ab. vineyard	45°28'12.2"N 13°37'14.3"E
186 Moravci	W	ab. vineyard	46°30'47.9"N 16°01'01.8"E	283 Secovlje 2	W	ab. vineyard	45°28'14.4"N 13°37'14.2"E
187 Kamenscak	W	forest edge	46°30'51.2"N 16°08'01.2"E	284 Secovlje 3	W	ab. vineyard	45°28'20.4"N 13°37'12.0"E
188 Vidanovci	W	forest edge	46°30'44.6"N 16°07'41.4"E	285 Secovlje 4	W	ab. vineyard	45°28'21.2"N 13°37'11.3"E
189 Runcetov breg	W	ab. vineyard	46°30'14.9"N 16°13'14.7"E	286 Sv. Peter	W	forest	45°27'40.8"N 13°40'18.6"E

[1]Type of plant material: R - rootstock, cv.-cultivar, sp. - species, W - wild, F - feral genotype not considered as cultivar, [2]Origin: SPGB - Slovenian plant gene bank of the University Center for Viticulture and Enology of the Faculty of Agriculture and Life Sciences, UL BF - University of Ljubljana Biotechnical Faculty, BG Wien - Botanischer Garten der Universität Wien (Austria), BG Graz-Botanischer Garten der Karl-Franzens-Universität Graz (Austria) ab. vineyard-abandoned vineyard

Data analysis

All unambiguous fragments were scored for the presence (1) or absence (0) of each band. The binary data matrix was used to calculate Dice's similarity coefficients (Dice, 1945), and a neighbor-joining tree was constructed using the DARWIN computer package (Perrier and Jacquemond-Collet, 2005). For each microsatellite locus, the number of alleles per locus (n), allele frequencies, observed (H_0) and expected heterozygosity (H_E) and polymorphic information content (PIC) were calculated using the Cervus 3.0.7 computer program (Marshall et al., 1998, 2014 version).

Results

A total of 188 alleles were detected at 12 microsatellite loci, while the number of alleles detected per locus ranged from 9 (VVMD 24) to 25 (VVMD 28), with an average of 16.33 alleles per locus (Table 2). The observed heterozygosity ranged between 0.583 (locus VVMD 36) and 0.931 (loci VVS 2, VVMD 7 and VVMD 28), with an average of 0.812. The expected heterozygosity ranged between 0.809 (locus VVMD 24) and 0.930 (loci VVMD 27 and VVMD 28), with an average of 0.877. The differences between the observed and expected heterozygosity were observed on all studied loci. The largest difference was observed on the locus VVMD 36 (0.223) and the lowest on the locus VVMD 28 (0.001). The averages of observed (0.812) and expected (0.877) heterozygosity were quite similar. The highest PIC value (polymorphic information content, PIC, is a measure of the quality of informativeness of molecular markers) was obtained on locus VVMD 28 (0.919) and the lowest on locus VVMD 24 (0.784). The obtained allele sizes and their frequencies are presented in Table 3, specific allele sizes for each cluster are listed in Table 4.

The dendrogram based on microsatellite data arranged the analyzed samples into five main clusters (Fig. 1), each consisting of several groups. In the first main cluster, there are the descendants of genetic recombination among American species, most probably involving *V. berlandieri*. 'SO4' and 'M *VI*' originate from crosses between *V. berlandieri* and *V. riparia*. The accessions Kalvaria 3, Kalvaria 2 and Kalvaria 4 (taken in a forest covering a surface of ca. 3 ha) represent one genotype, most probably the rootstock originating from the nearby abandoned vineyard, which 'escaped' to the forest by long vines. They could also be descendants of a single plant developed from a seed carried by a bird. All wild-grown genotypes of the fourth group of this cluster were collected in Northeast Slovenia and share several common morphological traits similar to the rootstock 'SO4' and 'M *VI*'.

The second cluster includes the three *V. vinifera* subsp. *sylvestris* reference genotypes and most probably the descendants of natural crosses involving (1) *V. vinifera* subsp. *sylvestris* and *V. vinifera* subsp. *vinifera*, and/or (2) *V. vinifera* subsp. *sylvestris* and the North American germplasm. The first possibility appears to be most probable and can be supported by the findings of Salayeva et al. (2010), which showed that the wild populations of *Vitis* from regions near the Caspian Sea in Azerbaijan were molecularly similar to the gene pool of *V. vinifera* subsp. *vinifera* cultivated in that area. Based on molecular evidence, indications of natural hybridization between *V. vinifera* subsp. *sylvestris* and *V. vinifera* subsp. *vinifera* were also noted by Jahnke et al. (2016) in Hungary.

The third cluster includes accessions having the American germplasm which were collected in the Southwest part of the country, in Istria and neighboring regions, close to the Northeast Adriatic Coast. From the results of the molecular analysis, it is possible to assume that they are genetically diverse: some may represent original American rootstocks brought from Italy and some could be descendants of various artificial or natural crosses among the American genotypes. Considering their young shoots and leaves, it can be assumed that they combine traits of *V. berlandieri*, *V. riparia* and *V. rupestris.*

Table 2: SSR loci analyzed and parameters of genetic variability calculated for different microsatellite loci of the 70 Vitis genotypes: number of alleles (n), observed (H0) and expected (HE) heterozygosity, and polymor-phic information content (PIC).

Locus	n	H_0	H_E	PIC
VVS 2	20	0.931	0.929	0.917
VVMD 5	14	0.722	0.900	0.884
VVMD 6	10	0.764	0.849	0.823
VVMD 7	17	0.931	0.908	0.894
VVMD 24	9	0.833	0.809	0.784
VVMD 25	13	0.792	0.862	0.840
VVMD 27	22	0.917	0.930	0.918
VVMD 28	25	0.931	0.930	0.919
VVMD 36	16	0.583	0.816	0.791
VrZag 62	15	0.694	0.887	0.870
VrZag 79	15	0.806	0.870	0.851
VrZag 112	12	0.847	0.833	0.806
Average	16.33	0.812	0.877	0.858

Table 3: Allele size (bp) and allele frequency (in parenthesis) of the 70 genotypes, at twelve microsatellite loci

VVS 2	VVMD 5	VVMD 6	VVMD 7	VVMD 24	VVMD 25	VVMD 27	VVMD 28	VVMD 36	VRZag 62	VrZag 79	VRZag 112
122 (0.0139)	205 (0.0069)	190 (0.0278)	230 (0.0347)	201 (0.0694)	239 (0.0347)	175 (0.0069)	200 (0.0069)	237 (0.0139)	174 (0.0069)	237 (0.0069)	230 (0.2569)
124 (0.0417)	224 (0.0833)	194 (0.0069)	232 (0.0694)	203 (0.1042)	241 (0.1736)	177 (0.0069)	202 (0.0069)	239 (0.3403)	182 (0.0208)	239 (0.0278)	234 (0.0208)
126 (0.0208)	226 (0.1111)	198 (0.0069)	234 (0.0556)	205 (0.3681)	243 (0.2361)	179 (0.0139)	218 (0.1042)	241 (0.0139)	188 (0.0903)	241 (0.0208)	236 (0.0556)
128 (0.0278)	230 (0.0625)	200 (0.0347)	238 (0.1042)	207 (0.0903)	245 (0.0556)	181 (0.0417)	220 (0.0417)	243 (0.0139)	190 (0.1042)	243 (0.0208)	238 (0.0139)
132 (0.0694)	232 (0.1181)	202 (0.1319)	242 (0.0417)	209 (0.0833)	247 (0.0139)	183 (0.0139)	222 (0.0347)	247 (0.0278)	192 (0.1181)	245 (0.0764)	240 (0.0694)
134 (0.0903)	234 (0.1458)	206 (0.2222)	244 (0.0417)	211 (0.1458)	251 (0.0764)	185 (0.0764)	228 (0.0069)	249 (0.0625)	194 (0.1458)	247 (0.0694)	242 (0.1875)
136 (0.0694)	236 (0.0486)	208 (0.1597)	246 (0.0278)	213 (0.0069)	253 (0.1597)	187 (0.0625)	230 (0.0417)	251 (0.2292)	196 (0.2222)	249 (0.0417)	244 (0.2361)
138 (0.0417)	238 (0.0139)	210 (0.1806)	248 (0.0139)	215 (0.0833)	255 (0.0417)	189 (0.1319)	234 (0.0069)	253 (0.0972)	198 (0.0139)	251 (0.1875)	248 (0.0486)
140 (0.0278)	240 (0.0139)	212 (0.0694)	250 (0.1806)	217 (0.0486)	259 (0.1389)	191 (0.1319)	236 (0.0486)	259 (0.0278)	200 (0.0486)	255 (0.1389)	250 (0.0347)
142 (0.1250)	248 (0.0278)	216 (0.1597)	252 (0.0625)	/	261 (0.0069)	193 (0.0486)	238 (0.1319)	261 (0.0417)	202 (0.0625)	257 (0.0764)	254 (0.0069)
144 (0.0278)	250 (0.0417)	/	254 (0.0208)	/	271 (0.0347)	195 (0.0417)	240 (0.0208)	263 (0.0278)	204 (0.0069)	259 (0.2431)	256 (0.0625)
146 (0.0903)	260 (0.0417)	/	256 (0.0278)	/	273 (0.0139)	197 (0.0069)	242 (0.0208)	265 (0.0208)	206 (0.0486)	261 (0.0278)	262 (0.0069)
148 (0.0208)	262 (0.1458)	/	258 (0.0347)	/	275 (0.0139)	201 (0.0139)	244 (0.0556)	273 (0.0069)	210 (0.0139)	263 (0.0278)	/
150 (0.1111)	264 (0.1389)	/	260 (0.0347)	/	/	203 (0.0069)	246 (0.1389)	277 (0.0069)	214 (0.0278)	267 (0.0139)	/
152 (0.1181)	/	/	262 (0.0625)	/	/	205 (0.0278)	248 (0.0694)	292 (0.0347)	216 (0.0694)	273 (0.0208)	/
154 (0.0208)	/	/	264 (0.1736)	/	/	207 (0.0556)	250 (0.0417)	294 (0.0347)	/	/	/
156 (0.0486)	/	/	274 (0.0139)	/	/	209 (0.0972)	252 (0.0069)	/	/	/	/
158 (0.0069)	/	/	/	/	/	211 (0.0625)	254 (0.0833)	/	/	/	/
160 (0.0208)	/	/	/	/	/	213 (0.0764)	256 (0.0417)	/	/	/	/
162 (0.0069)	/	/	/	/	/	217 (0.0417)	262 (0.0347)	/	/	/	/
/	/	/	/	/	/	219 (0.0208)	268 (0.0278)	/	/	/	/
/	/	/	/	/	/	221 (0.0139)	270 (0.0069)	/	/	/	/
/	/	/	/	/	/	/	274 (0.0069)	/	/	/	/
/	/	/	/	/	/	/	280 (0.0069)	/	/	/	/
/	/	/	/	/	/	/	286 (0.0069)	/	/	/	/

Table 4: Specific allele sizes (bp) and their frequencies for each cluster

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
VVS 2	150 (0.4231)	152 (0.351)	136 (0.1818)	124, 136, 142, 146 (0.1250)	134, 152 (0.2083)
VVMD 5	234 (0.4615)	226 (0.3214)	264 (0.2273)	262 (0.2500)	232 (0.2917)
VVMD 6	210 (0.5000)	202 (0.4643)	206 (0.4091)	216 (0.3750)	208 (0.3750)
VVMD 7	264 (0.3462)	264 (0.3214)	250 (0.2273)	250 (0.3250)	238 (0.5000)
VVMD 24	205 (0.3077)	211 (0.6071)	205 (0.6364)	205 (0.6000)	207 (0.2917)
VVMD 25	243, 251 (0.2692)	259 (0.5714)	243 (0.3636)	241 (0.3750)	243 (0.3750)
VVMD 27	191 (0.2692)	189 (0.4643)	193, 213 (0.1818)	209 (0.2000)	189 (0.2500)
VVMD 28	254 (0.3462)	238 (0.3571)	248 (0.1818)	246 (0.3750)	220, 238 (0.2083)
VVMD 36	239, 251 (0.3077)	251 (0.2143)	239 (0.3636)	239 (0.6250)	251 (0.4167)
VrZag 62	216 (0.2308)	196 (0.5000)	192 (0.3182)	190 (0.2500)	188,194 (0.3333)
VrZag 79	251 (0.2692)	251 (0.4643)	255 (0.3182)	259 (0.3000)	259 (0.2500)
VrZag 112	230, 242 (0.3846)	230 (0.5357)	244 (0.4091)	244 (0.3000)	230, 242 (0.2500)

The fourth cluster includes four separate groups and a distinct genotype 274. In group 1, there are two *V. riparia* genotypes and their hybrids. This species also involves the rootstock 'Boerner'. The second group most probably includes three genotypes of *V. rupestris*; two are named as such and the third is Kalvaria 1, while the accession 191 (Nunska graba 1) could be a backcross hybrid (*V. riparia* × *V. rupestris*) × *V. rupestris*. In the third group, there are four accessions which probably represent direct hybrids or backcrosses of *V. labrusca* with *V. riparia* or *V. rupestris*, or hybrids involving three species (i.e., *V. labrusca, V. riparia, V. rupestris*). In the fourth group, there are only three accessions, and according to the morphological characteristics of the leaves and young shoots, these accessions (186, 188 and 242) could be natural hybrids involving *V. riparia* and *V. longii.* Accession 274 appears to be different. It possibly combines the gene pool of *V. longii* with *V. rupestris* and *V. riparia*. The fifth cluster consists of *V. vinifera* subsp. *vinifera* germplasm, including reference cultivars.

Discussion

The majority of wild-grown accessions included in the study can be considered as descendants of the North American germplasm. Most of them originate from four American species (*V. berlandieri*, *V. labrusca*, *V. riparia*, *V. rupestris*) and their hybrids brought to the country as rootstock material by the agricultural institutions of the Austro-Hungarian Empire, and later by the Kingdom of Yugoslavia and the Kingdom of Italy. The first North American genotypes were introduced in the mid-19th century, during the period of the Austro-Hungarian Empire which included a great part of Northeast Italy. The plant materials were introduced (1) from the west or south-west of the country (most probably from Italy) (e.g., cluster III) and (2) from the east or south-east (most probably from Hungary and the Balkans) (e.g., cluster I).

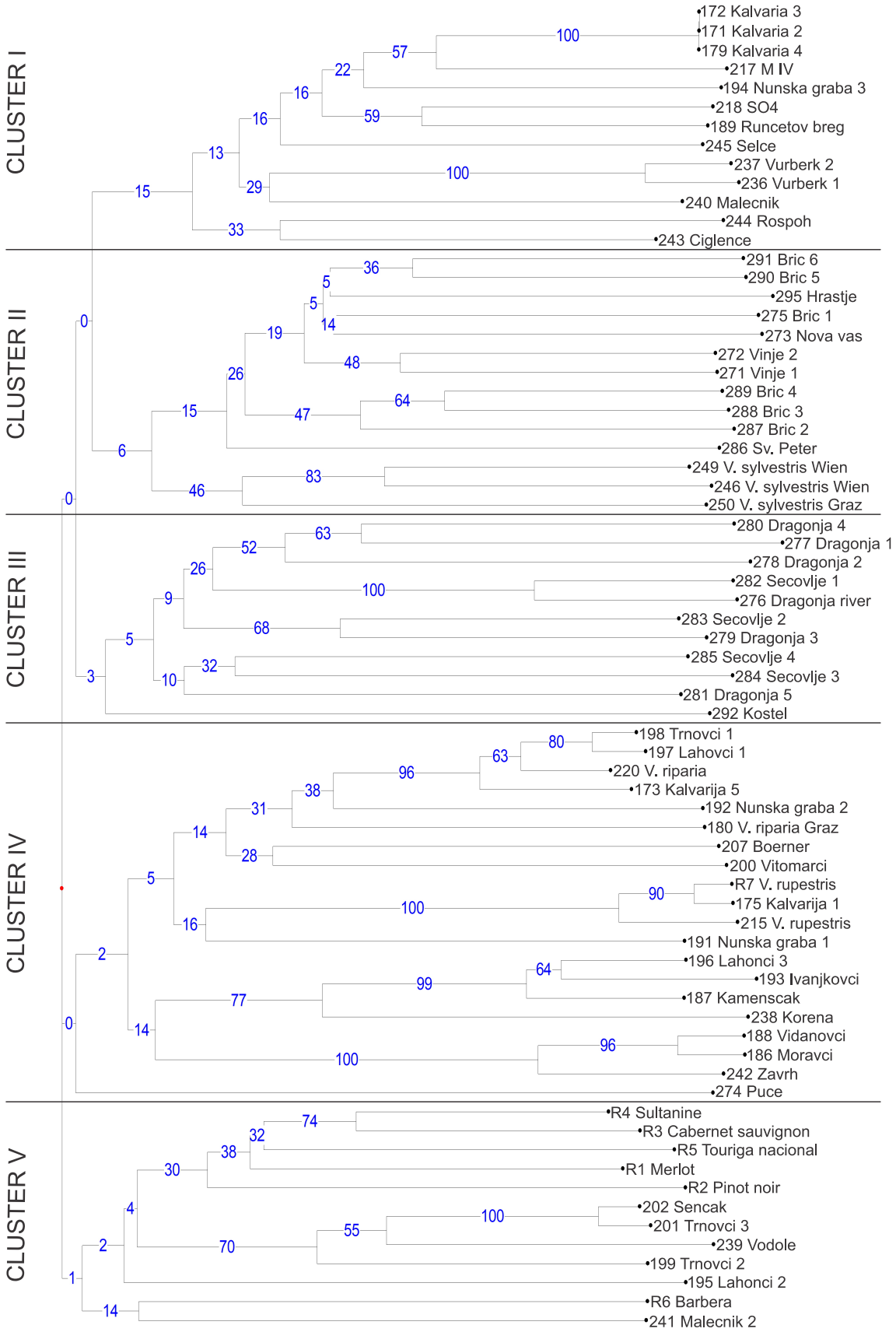

Fig. 1: Neighbor-joining tree based on microsatellite data involving 70 genotypes

V. berlandieri × V. riparia, *V. riparia* and *V. rupestris* dominated in and around abandoned vineyards, away from settlements, while *V. labrusca* appeared to be more common close to settlements. The majority of introduced *V. labrusca* genotypes were planted as replacements for the traditional, susceptible table and wine cultivars. With the development of new production technologies (i.e., the use of phylloxera-resistant or tolerant rootstocks and fungicides), *V. labrusca* and its hybrids began to lose importance. For successful traditional growers, they became undesirable and were consequently reduced or exterminated. However, it was very difficult to remove them completely because the plants were vigorous and resistant to most diseases. One consequence was the 'migration' of *V. labrusca* and its hybrids away from vineyards. Other rootstock materials (*V. berlandieri × V. riparia*, *V. riparia* and *V. rupestris*) survived in more or less the same way.

The wild relative, genetically 'pure' *V. vinifera* subsp. *sylvestris* was not found among the collected and analyzed wild-grown plants. If it still exists, it is probably very rare. Before the appearance of phylloxera, it was probably very common. There were probably three main reasons for the drastic reduction of its presence in natural habitats: (1) susceptibility to phylloxera, (2) intensive agriculture and (3) natural and artificial reforestation. Farmers and foresters treated these plants as weeds and tended to remove them because they could cause significant damage, especially to young trees. This is practiced even today. According to Bodor et al. (2010), the wild grape (*V. vinifera* subsp. *sylvestris*) has become a highly threatened species in Europe mainly because of habitat loss, competition with alien grape species and intensive forest exploitation. Regarding neighboring countries, only small populations could be found in Austria in the riparian woods and floodplaines of Danube and March east of Vienna (Regner et al., 2004; Tiefenbrunner et al., 2015; Arnold et al., 2017), in the eastern Adriatic coast in Croatia and Bosnia and Herzegovina (Zdunic et al., 2017, 2020) in ten Italian regions (Biagini et al., 2014) and in Hungary (Jahnke et al., 2016; Bodor et al., 2010). However, allelic diversity of wild grape could be efficiently preserved in descendants of its crosses with cultivated *V. vinifera* subsp. *vinifera* or with much more resistant American genotypes. In our study, these hypothetical genotypes are listed in cluster 3. Similar conclusions are mentioned by Bodor et al. (2010), who identified interspecific hybrids of *V. sylvestris* and *V. riparia* on the territory of Szentendre Island, Hungary.

Vegetative propagation was probably crucial for the survival of the North American germplasm in this part of the world. As a vineyard was abandoned or cleared for replanting or another purpose by pushing the old grapevine plants to the edges, many of the rootstock plants began to regrow and spread with long vines to the nearby areas, which were often bushy. They easily climbed the trees and began to dominate. However, our analysis suggests that sexual propagation was probably also present. Vegetative propagation alone cannot explain such a high level of genetic and phenotypic variation, even if we assume that there were several introductions, which could have involved genetically diverse materials. In abandoned vineyards, pest- and disease-resistant rootstocks sprouted, flowered (often being dioecious plants) and produced fruit, which were eaten by various animals, particularly birds, and people. In this way, the seeds were spread around.

Wild-growing grapevine plants of North American origin can also be found far from their original vineyards, for example in remote forests. The forest environment is probably much more favorable for seeds to germinate than open grassland. In late autumn, the seeds are covered by fallen leaves, which maintain the moisture and temperature levels necessary for wintering and germination in early spring.

Our evidence suggests that sexual propagation is probably most frequent among *V. labrusca* genotypes. Numerous genotypes belonging to this species are grown for consumption or production of juice or low-quality wine. However, this is not the case with other species like *V. riparia*, *V. rupestris*, or *V. berlandieri*. During processing of *V. labrusca*, the seeds and residue of processed fruit are not destroyed but usually deposited in various places around settlements, forest edges or fields, and some of these seeds germinate and develop into mature plants. The wild-growing grapevines in this study belonged to a range of

species and interspecific hybrids. Natural hybridization probably involved intra- and interspecific combinations, although we did not study the share of each. Considering the dioecy, we can assume that interspecific hybridization was probably frequently present. As indications, some of the specimens included in the study and classified as *V. riparia* or *V. rupestris* exhibited some, although limited, morphological differences and greater variation, and they appeared to be much more vigorous than the plants considered as standards of each species being studied. This could be explained if specimens belonging to the *V. riparia* cluster were descendants of more complex crosses (e.g. a backcross (*V. riparia* × *V. rupestris*) × *V. riparia*).

Very complex hybrids probably do not exist. There are several reasons for that: (1) the time period since the first introduction of North American germplasm is relatively short (ca. 130 years); (2) individual plants may live and be fruitful for several centuries; (3) in the less favorable environmental conditions of Southwest and Northeast Slovenia, vegetative propagation is much more efficient than propagation by seed, and (4) in natural conditions, crosses involving different species are generally less frequent. It is also possible that some of the wild-grown genotypes may belong to the original clones that were introduced more than a century ago.

The existing wild-growing grapevines are the result of sophisticated genetic processes that involve natural intra- and interspecific crosses, genetic segregation associated with natural and artificial selection, and epigenetic processes. The genetic changes, however, have been limited because of predominant clonal reproduction and the lengthy life cycle of plants. Since the plants have been able to survive in tough natural conditions for so long, they can be a very useful source of allelic diversity for genetic breeding or can be used directly as rootstock material.

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