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The World's oldest living grapevine specimen and its genetic relationships

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Summary

The Old Vine from Lent (Maribor, Slovenia) which belongs to the 'Modra Kavčina' group (*i.e.* 'Blauer Kölner' in the Vitis International Variety Catalogue and 'Žametovka' in the official varietal list of Slovenia) is considered to be the oldest living specimen of cultivated grapevine (at least 400 years old). The aim of our study was to determine the genetic relationships among different accessions of the 'Žametovka' group, the position the Old Vine within this group, and the relationship between the Old Vine and other red varieties grown in Slovenia and neighbouring countries. The molecular genetic analysis was based on microsatellite data. The study shows that the 'Žametovka' group is genetically completely different from other red varieties studied. Among these genetically distant varieties, in our study, 'Chasselas red' appears to be the closest. The 'Žametovka' group is genetically highly homogenous, and half of the studied accessions probably belong to the same clone. The 'Old vine' cannot be considered as a significantly different genotype. The minor differences detected by microsatellite markers are probably due to mutations accumulated over a long period of time and possibly to epigenetic changes.

Key words: *Vitis vinifera*, 'Žametovka', 'Blauer Kölner', microsatellites, Old Vine.

Introduction

The 'Žametovka' grapevine from Lent in Maribor, called the 'Old Vine' is considered to be the oldest living and fruiting specimen of cultivated grapevine on our planet. Its age was determined in 1972 by Prof. R. Erker, a dendrologist from the Department of Forestry and Renewable Forest Resources at the University of Ljubljana, Biotechnical Faculty, using drilling and microscopy. He found that the vine showed an age of 375 years, and might be even older: 400 or more (Zafošnik 2010). This grapevine can also be seen in paintings of Maribor dating from the years 1657 and 1681, which are kept in the Styrian Provincial Museum in Graz (Austria). In these paintings, one can clearly see the frontage of the same house, built in the 16th century, which was already vigorously overgrown with the same grapevine called 'the Old Vine'. The name 'Žametovka' in the International Vitis Variety Catalogue corresponds to 'Blauer Kölner'.

Today, 'Žametovka' is cultivated mainly in the Dolenjska wine region (SE Slovenia), as the main variety for the traditional cuvée wine called Cviček (a recognized traditional denomination). In the late 19th century, this wine was produced from four varieties: 'Žametovka', 'Plavec', 'Sylvaner' and 'Chasselas' (Kaserer *et al*. 1923).

The first ampelographic description of the 'Žametovka' can be found in the "Systematische Classification und Beschreibung der im Herzogthume Steiermark vorkomenden Rebsorten" (Trummer 1841). Trummer described three different types of the 'Kölner' variety, depending on the colour of berry skin (blue, red or white). Trummer listed several different synonyms that were used in different wine-growing areas (Tab. 1).

In 1905, Zweifler recommended three red varieties for cultivation in Styria: 'Blaufränkisch', 'Blauer Kölner' and 'Blauer Wildbacher' called 'Vranek' in Styrian Slovenia (Vršič 2001). Some authors considered 'Blauer Kölner' to be similar to the 'Scheibkörner' variety in Lower Austria (Babo and Mach 1881, Kaserer *et al*. 1923). In the grapevine collection of M. Oberlin (in France), it was found to be identical with the 'Enforiné du Jura' variety ('Gouais noir') (Viala and Vermorel 1909).

The main objective of our study was to determine genetic relationships of the Old Vine with other 'Žametovka' vines and other red varieties grown Slovenia and neighbouring countries.

Material and Methods

Plant material: A total of 31 grapevine (*Vitis vinifera* L.) genotypes were included in the study. The analyzed samples involved the germplasm of 'Žametovka', which was collected from different wine growing regions of Slovenia (Dolenjska 10 samples, Slovenian Styria 6 samples; including Old vine), and other varieties from the gene bank of the University Centre of Viticulture and Enology Meranovo, Faculty of Agriculture and Life Sciences (Fig. 1). As reference varieties, six commercial cultivars of grapevine were included: 'Merlot', 'Pinot Noir', 'Cabernet Sauvignon', 'Sultanine', 'Touriga nacional' and 'Barbera'.

DNA isolation: DNA was extracted from fresh, young leaves using the CTAB protocol. To approximately 2-3 cm^2 of fresh leaf tissue, one mL of preheated (68 °C) CTAB extraction buffer (Doyle and Doyle 1987) was added and well homogenized with a mortar and pestle, and transferred to a 1.5 mL tube. Samples were incubated for

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Table 1

Main synonyms and homonyms of 'Žametovka'

Synonyms or homonyms	Wine-cultivation region (or district or author)
Bleu de Cologne[4]	France
Kölner Kek[4], Korai Kek[4,7],	Hungary
Blauer Kölner[1], Blauer Luttenberger[1], Großschwarze[1], Rothe Ungarische[1], Scheibkörner[4,5,6], Schwarze[1], Schwarzer Muscateler[1],	Lower Austria
Modra kavčina[3,4], Žametasta črnina[3],	Croatia
Blauer Kölner[1, 9], Černila[1], Černina[1], Černa Laška[1,7], Černa Spania[1], Černi Spanier[1], Černi Zeleniak[1], Kapčina[1], Kapšina[8], Kolonjka[4], Kosavina[4], Modra Hlapčovina[1], Modra Kavčina[2], Urnik[1], Velka Černa[1], Velka Modrina[1], Velka Plava[1], Velka Sipa, Vranik[1,7], Zeleniak[1], Žametna Črnina[2], Žametovka[2], Windische Kavka[1],	Styria (Slovenia)
Blauer Hainer[1], Blauer Kölner[1], Blauer Cölner[8] , Blauer Milcher[1], Großblaue[1], Großkölner[1], Großmilcher[1,8], Kölner[1], Kölinger[1,8], Ordinare Schvarze[1]	Styria(Austria)
Kavčna[1], Kavčina[1], Modra Kavčina[2], Žametna Črnina[2], Žametovka[2]	Bizeljsko (Slovenia)
Frankenthaler[1,7]	Burger s.56
Große Wälsche[1]	Rath s. 55
Columella parientalis[1]	Von Vest s.48

[1]TRUMMER, [2]HRČEK and KOROŠEC-KORUZA, [3]TURKOVIĆ, [4]VIALA and VERMOREL, [5]BABO and MACH, [6]KASERER *et al.*, [7]ALEWELDT and DETTWEILER-MÜNCH, [8]HOHENBRUCK, [9]GOETHE.

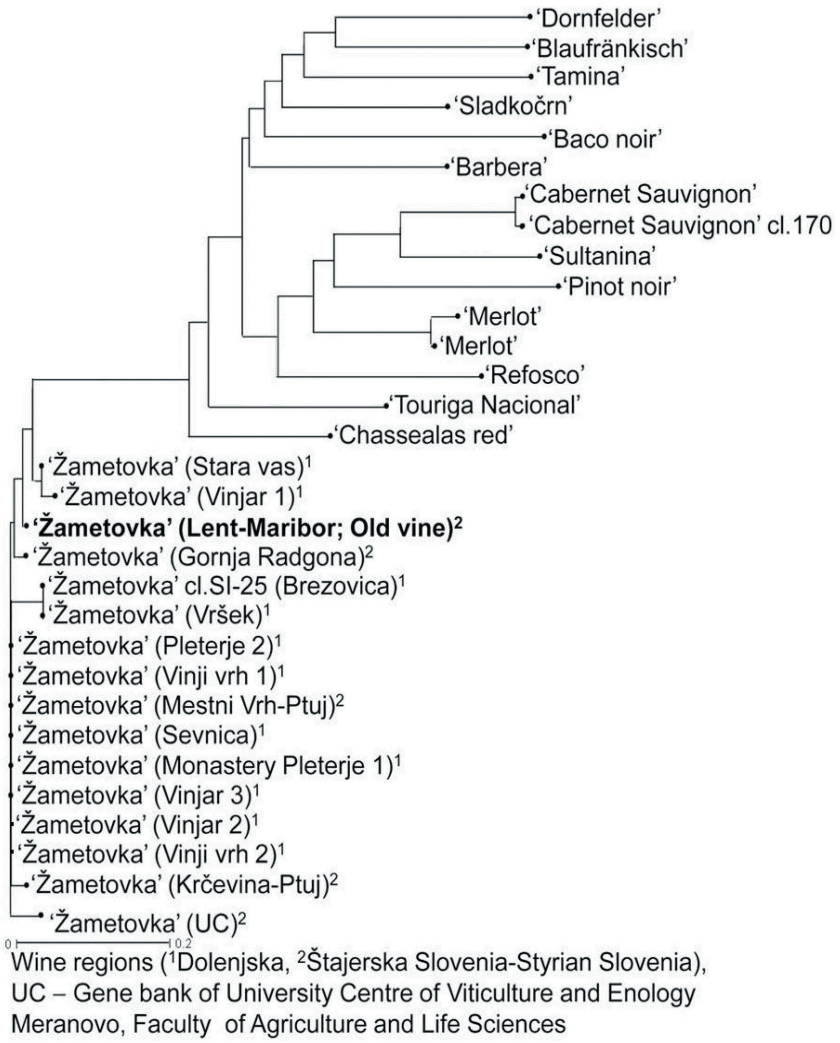

Fig. 1: Neighbor-joining tree based on microsatellite data involving 31 cultivars. The 'Žametovka' group involves 15 accessions from different locations as well as the Old vine from Lent (Maribor).

1.5 h at 68 °C in a water bath. After incubation, 600 µL of chloroform:isoamyl alcohol in a 24:1 proportion was added, and the samples were thoroughly stirred. The mixtures were centrifuged at 14.200 g_n for 10-15 min. After centrifugation, the supernatant was transferred to a fresh tube, and the DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate and 1 volume of ice cold isopropanol and kept at -20 °C for 20-30 min. Samples were again centrifuged at 14.200 g_n for 10-15 min. The pellet was washed in 70 % ethanol, air dried and rehydrated in 100 µL of TE buffer (Šiško *et al*. 2009). The DNA concentration was estimated using a DNA fluorometer DQ 300 (Hoefer, Inc., Holliston, Massachusetts). Two separate extractions per plant were performed.

Microsatellite analysis: Twelve microsatellite loci were used: 8 of the VVMD series (VVMD5, VVMD6, VVMD7 (Bowers *et al*. 1996), VVMD24 VVMD25 VVMD27 VVMD28, VVMD36 (Bowers *et al*. 1999), 3 of the ssrVrZAG series (ssrVrZAG62, ssrVrZAG79, ssrVrZAG112 (Sefc *et al*. 1999) and VVS2 (Thomas and Scott 1993).

PCR: Ten µl of PCR mixture contained 20 ng DNA, 0.25 U Taq DNA polymerase (Fermentas), 1 x PCR buffer (Fermentas), 2 mM $MgCl_2$ (Fermentas), 0.5 µL of each primer and 0.2 mM of each dNTP's (Sigma). The PCR condition consisted of a hot start for 5 min at 95 °C; 26-40 cycles of denaturation at 94 °C for 30-45 s, annealing at 50-58 °C for 30-45 s and an extension step at 72 °C for 90 s. Reactions were completed by incubation at 72 °C for 8 min (Štajner *et al*. 2008). The polymerase chain reaction (PCR) was performed using a Whatman Biometra T-Gradient thermocycler (Goettingen, Germany). Capillary electrophoresis of PCR products was performed on the Beckman Coulter CEQ8000 according to the manufacturer's instructions. Fragment size analysis was done with the built-in software. A fluorescent-labeled size marker (Beckman Coulter DNA Size Standard Kit 400 bp) was used as a molecular weight reference.

Data analysis: All unambiguous fragments were scored for the presence (1) or absence (0) of each band. Only clear and reproducible fragments were taken for data analysis. The binary data matrix was used to calculate Dice's similarity coefficients (Dice 1945). Values for Dice's coefficients fall between 0 (there is no common band) and 1 (two genotypes have identical markers, so they are identical). Dice similarity coefficients were calculated using the DARWIN computer package (Perrier and Jacquemond-Collet 2005). For each microsatellite locus, the number of alleles per locus (*n*), allele frequencies, observed heterozygosity (H_O), expected heterozygosity (H_E) and probability of identity (PI) were calculated using the 'IDENTITY 1.0' computer program (Wagner and Sefc 1999). The average distance between pairs of accessions was obtained by taking into account microsatellite data, and a neighbor-joining tree was constructed using the DARWIN computer package (Perrier and Jacquemond-Collet 2005). A matrix of Dice similarity coefficients was used for assessing relationships among 31 genotypes, using the neighbor-joining algorithm developed by Saitou and Nei (1987).

Results and Discussion

Molecular analysis: SSR analysis revealed 96 polymorphic alleles at 12 microsatellite loci (Tab. 2). The number of alleles detected per locus ranged from 4 (VVMD 25) to 12 (VrZag 79), with an average of 8 alleles per locus. The observed heterozygosity ranged between 0.226 (locus VVMD36) and 0.968 (loci VVMD 27 and VrZag 112), with an average of 0.806. The expected heterozygosity ranged between 0.607 (locus VVMD 24) and 0.814 (locus VVMD28), with an average of 0.728. The differences between the observed and expected heterozygosity were examined for all investigated loci. The largest difference was observed on locus VVMD 24 (0.328) and the lowest on locus VrZag 7 (0.012). The average of observed (0.806) and expected (0.728) heterozygosity was quite similar. At 10 out of 12 loci, the observed heterozygosity (H_0) was higher than expected (H_E), but at two loci (VVMD25 and VVMD36) H_0 was lower than H_E.

The most informative locus for this set of genotypes was VVMD28, with a probability of identity (PI) of 0.100, and the least informative locus was VVMD36, with a PI of 0.398. The cumulative probability of obtaining identical genotypes using all 12 loci was low (3.29×10^{-9}). The number of primers sufficient for reliable variety identification depends on the nature and discriminating power of each primer (Tessier *et al*. 1999); normally, six primer pairs are sufficient for differentiating between genotypes (Zulini *et al*. 2002). Closely related cultivars require larger numbers of pairs (Meredith *et al*. 1999).

Genetic relationships: The dendrogram based on microsatellite data (Fig. 1) indicates that the 'Žametovka' group is genetically completely different from the rest of this group of red varieties (which includes all the important red varieties from the official varietal list of Slovenia). Among the genetically distant varieties in this group, 'Chasselas red' appears to be the closest. In future investigations, it may be useful to include red varieties from other wine growing regions, especially from the Balkans, which is considered one of the centres of origin for the *Proles pontica* eco-geographical group. The Old Vine belongs to the 'Žametovka' group, which is genetically highly homogenous. Half of the accessions probably belong to the same clone. Small differences among the rest of the accessions (of the 'Žametovka' group) could be explained by mutations accumulated over a long period of time (possibly several centuries), and probably by epigenetic changes.

Ampelographic traits of the 'Žametovka' group and their phenotypic uniformity: According to the descriptors, the 'Žametovka' group is characterised by the following traits: the apex of a shoot is widened, the leaf margin is carmine, and the blade is five-sectioned, with teeth in the lateral sinuses (Fig. 2). Teeth, however, are not present in each lateral sinus. The blade surface is green, shiny and wavy, and the abaxial side is covered with fine woolly hair. The infructescence is big, medium compact and winged; berries are large, round, dark blue and waxy; the skin of berries is thick. Growth is medium vigorous, the grapes are late-ripening, the yield is high, and the plants are highly sensitive to low tem-

Table 2

Allele sizes (bp) of 16 accessions of 'Žametovka' from different locations in Slovenia and 6 reference varieties at 12 SSR loci

Sample	VVS2		VVMD24		VrZag112		VVMD27		VVMD5		VVMD7		VVMD6		VVMD36		VVMD25		VRZAG62		VVMD28		VRZAG79	
Žametovka UC Meranovo	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	239	245
Žametovka Vinjar 1	132	152	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	270	245	259
Žametovka Vinjar 2	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	245	259
Žametovka Vinjar 3	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	245	259
Žametovka Vršek	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	245	259
Žametovka cl. SI-25 Brezovica	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	245	259
Žametovka(Old Vine) Lent	132	132	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	245	259
Žametovka Gornja Radgona	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	270	245	259
Žametovka Krčevina-Ptuj	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	259	259
Žametovka Mestni vrh-Ptuj	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	245	259
Žametovka Vinji vrh 1	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	245	259
Žametovka Vinji vrh 2	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	245	259
Žametovka Sevnica	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	245	259
Žametovka Pleterje 1	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	245	259
Žametovka Pleterje 2	132	154	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	245	259
Žametovka Stara vas	132	152	207	211	230	242	181	189	234	236	248	254	190	208	261	261	245	245	200	204	270	280	245	259
Merlot	140	152	207	211	230	242	187	191	224	234	238	246	200	208	251	251	243	253	194	194	230	236	259	259
Pinot noir	136	152	213	215	240	242	185	189	226	236	238	242	200	200	251	251	243	253	188	194	220	238	239	245
Cabernet Sauvignon	138	152	207	217	230	234	175	189	230	238	238	238	208	208	251	261	243	253	188	194	236	238	247	247
Sultanine	144	152	207	217	230	234	181	195	232	232	238	252	208	211	247	251	243	253	188	194	220	236	247	247
Touriga Nacional	142	152	207	211	230	236	181	189	224	234	238	252	208	208	261	273	253	259	188	194	220	270	245	245
Barbera	132	134	207	217	230	230	185	189	224	224	248	252	190	208	263	263	243	259	192	200	236	262	243	259

Fig. 2: A typical leaf blade of the 'Žametovka' variety with a tooth in the lateral sinus (indicated by arrow).

peratures. Our observations of numerous phenotypic traits showed that the 'Žametovka' group is not very uniform. Typical ripe infructescences of the original (not improved genotypes, such as 'Žametovka' - Vinjar 2 from the Dolenjska wine growing district, SE Slovenia) contain numerous hard green berries. With systematic selection, breeders gradually changed this undesirable characteristic and developed improved genotypes – e.g. 'SI-25', the clone, which is characterised by much more homogenous berry colour. Another trait that has to be improved is the infructescence size. The selection should take into consideration genotypes (mutants) with smaller fruit clusters. Owing to a lower yield load, canes will be able to reach full maturity and therefore become more tolerant of winter frost.

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