

## Conservation value of the native Hungarian wild grape (*Vitis sylvestris* Gmel.) evaluated by microsatellite markers

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### Summary

**Wild grape (*Vitis sylvestris* GMEL.) has become a highly threatened species in Europe because of habitat loss, competition with alien grape species and intensive forest exploitation. Twenty-three *Vitis* spp. samples were analysed at 8 microsatellite loci to estimate the genetic diversity of the natural *Vitis sylvestris* specimens. In order to analyse the morphological traits and to perform molecular analysis, 11 native individuals were sampled from 6 remnant Hungarian habitats of the wild grape. To compare the genetic relationships among the wild specimens, samples from Turkish habitats, as well as cultivars of *Vitis vinifera*, *Vitis labrusca* and *Vitis riparia* were also included. Genetic diversity was higher within the Hungarian wild grape locations, with a mean of  $H_e = 0.74$ , which was higher than that of samples originating from a larger area of Turkey,  $H_e = 0.69$ . Most of the Hungarian samples formed a well-defined, separate branch on the NJ tree. Based on the morphological traits and molecular analysis on the territory of Szentendre Island, formerly considered to be one of the largest native locations of wild grape, interspecific hybrids of *Vitis sylvestris* and *Vitis riparia* were identified. It can be concluded from the results that most of the Hungarian habitats studied could be valuable for the conservation. This paper reports on Hungarian *Vitis sylvestris* habitats, providing the first genetic study on diversity and on the relationship of *Vitis sylvestris* to other *Vitis* specimens, wild or cultivated, in the central part of the Carpathian Basin.**

**Key words:** *Vitis sylvestris*, SSR, genetic diversity, NJ tree, conservation value.

### Introduction

Until the end of the 80s the morphological characterization of the wild grape (*Vitis sylvestris* GMEL.), the reviews of native Hungarian habitats were regularly published by TERPÓ (1962, 1988). It is presumed that wine grape (*Vitis vinifera* L.), one of the oldest cultivated plants in South-eastern Europe, was domesticated in Transcaucasia (Tur-

key, Iran and Armenia) from the wild species *Vitis sylvestris* GMELIN (RIAZ *et al.* 2007). These centres represent the source for modern grapevine cultivation and the starting point of its spread throughout the European continent (LEVADOUX 1957). In the postglacial period, *Vitis sylvestris* GMEL. escaped from its southern refugia, spread towards the north and became a common species in European forests, along riverbanks and on hillsides (ARNOLD *et al.* 1998, TERPÓ and BÁLINT 1987). However, in recent centuries the natural habitats have been subjected to strong human impacts and because of habitat loss the wild grape has only survived in small, scattered populations and has become a highly threatened species in Europe (SOÓ 1966; SIMON 2000, KIRÁLY 2007). Moreover, in the early 20<sup>th</sup> century the alien *Vitis* species that were introduced as rootstocks (*Vitis riparia* MICHX., *Vitis labrusca* L.) invaded the natural habitats of the wild grape in Europe. When cross-breeding of rootstock cultivars was begun in Hungary, the main aim was to produce cultivars that could adapt easily to calcareous soils and had good rooting capacity. Breeders mainly used the hybrids *V. Berlandieri* x *V. riparia* and *V. Berlandieri* x *V. rupestris* (CSEPREGI 1992). The cross-bred species quickly adapted to the new circumstances and had no difficulty in escaping from cultivated areas and deserted vineyards (ARRIGO and ARNOLD 2007). Due to dispersion by birds (from the mother-rootstock plantations to overland), the cultivated species were able to invade large territories and often hybridized with native *Vitis sylvestris* plants. Being strong competitors for the wild grape, rootstocks displaced autochthonous specimens from their natural habitats. The populations of wild grape thus decreased in size, leading to severe erosion of the genestock (CSEPREGI 1988). Naturalized alien locations of *Vitis riparia* MICHX., *Vitis rupestris* SCHEELE and *Vitis labrusca* L. and hybrid specimens of *V. sylvestris* x *V. riparia* hybrid origin were also mentioned by TERPÓ (1988).

A detailed inventory of European wild grape habitats, including Hungarian wild grape populations, was made by ARNOLD *et al.* (1998), who also summed up the most important factors threatening wild grape habitats: the decrease in the natural range due to alien invasion and human activities, such as forest exploitation; and the effect of rapid changes in ecological parameters (soil drying). OCETE *et al.* (2008) concluded that Spanish and French populations,

like those in other parts of Europe, had been impaired and the number of plants in each habitat had decreased. Optimal biota can be self-renewing in natural communities (FRANKEL 1974).

The genetic variation of natural wild grape in Danubian habitats in Austria was evaluated by REGNER *et al.* (2004). Based on SSR markers, the genetic profiles of five geographic areas were described, but no significant correlation was found between the genetic and geographical distances between the studied locations. Until now no other evaluation has been made of the genetic diversity of remnant wild grape locations in the Carpathian Basin.

In the present study DNA microsatellite markers were used to estimate the genetic variation of native *Vitis sylvestris* GMEL. specimens in Hungarian habitats. Besides the Hungarian plant material, wild grape samples from Turkey were included, together with other *Vitis* species; American species and *Vitis vinifera* L. cultivars. These grapevine cultivars represent the three main taxonomic groups or *convar.* identified by NÉMETH (1967), *convar.* occidentalis, *convar.* orientalis and *convar.* pontica, and reflect the different geographic origins of the grapevine cultivars. Microsatellite markers are ideal for estimating the genetic variability of wild grape (GRASSI *et al.* 2003; FORNECK *et al.* 2003). Former studies indicated the suitability of these SSR markers, because of being adequately variable for the evaluation of the genetic relationships among *Vitis* samples (ARRIGO and ARNOLD 2007).

The main objectives of this work were to evaluate (i) the genetic diversity of the native wild grape within the territory of Hungary and to compare diversity between locations in different habitat types, (ii) to compare Hungarian and Turkish wild grape samples, and (iii) to obtain an insight into the genetic divergence from the native species to widely cultivated *Vitis vinifera* cultivars and to alien American grape species.

### Materials and methods

**Plant material:** The samples (*Vitis sylvestris*, *Vitis vinifera*, *Vitis labrusca* and *Vitis riparia*) included in this study are listed in Tab. 1, together with the sampling regions and habitats. The Hungarian specimens were collected from native habitats following TERPÓ studies. To ensure the reproducibility of the investigations leaves and herbarium specimens were collected and in most of the cases GPS coordinates were registered.

**DNA extraction:** The extraction was performed on shoot tips or shoots (in the case of the specimens from Turkey). The samples were frozen in liquid nitrogen and squashed to a powder. Genomic DNA was extracted with the Qiagen Plant Mini Kit, according to the instructions of the manufacturer (Qiagen, Biomarker LTD. Gödöllő).

**Microsatellite analysis:** Eight pairs of nuclear microsatellites: VvS1, VvS2 (THOMAS and SCOTT 1993), VvMD5, VvMD7 (BOWERS *et al.* 1996), VvMD27 (BOWERS *et al.* 1999), VrZag21, VrZag62 and VrZag79 (SEFC *et al.* 1999) that had proved to be variable in former

studies were used. PCR amplification was performed with a Peltier Thermal Cycler (PTC, MJ Research) using the following program: 5 min denaturation at 94 °C; 35 cycles of 45 s denaturation at 94 °C, 30 s annealing at 52 °C and 1 min elongation at 72 °C, then a final elongation step for 7 min at 72 °C. The amplifications were checked on 1 % agarose gel. SSRs were analysed on an ABI 3100 analyser (Applied Biosystems).

**Data analysis:** Expected heterozygosity was calculated as  $H_e = 1 - \sum p_i^2$  with the software Identity 0.1 (WAGNER and SEFC 1999), where  $p_i$  is the frequency of the  $i^{\text{th}}$  allele in the sample set (NEI 1973). To evaluate the genetic divergence among the samples, the distances between the specimens were calculated based on the proportion of shared alleles using MSAT2 (MINCH *et al.* 1997), considering all possible bootstrap samplings. Neighbor joining (NJ) and consensus trees were calculated with the neighbor and consensus (extended majority rule) algorithms of the PHYLIP software package (FELSENSTEIN 1989). The dendrogram was edited manually and drawn using Mega4 (TAMURA *et al.* 2007).

### Results and Discussion

The fragment lengths of 23 plants based on 8 microsatellites were analyzed in this study to detect the rate of polymorphism and to describe the genetic relationships between the samples (Tab. 2). DNA was successfully extracted and amplified from one of the two herbarium specimens collected by TERPÓ (Mecseknádasd 1963). Recent field observations suggested that there were now fewer specimens in each habitat compared with the data published by TERPÓ (1988).

On the Neighbour-joining dendrogram the samples formed four main clusters, two consisting of Hungarian *Vitis sylvestris*, one of Turkish *Vitis sylvestris* and one of *Vitis vinifera* samples. Two branches connected the alien *Vitis* species (Figure) to the main clusters. The floodplain samples (Óbuda Island) showed a close relationship to each other, while samples from the Hungarian Central Mountains area (Pilis, Mád) clustered together. Among the three individuals collected from the Pilis Mt., two had no detectable differences at the 8 loci. The third sample (Pilisszentkereszt) differed at several loci thus, casting doubt on the clonal structure of the stand.

As expected, the genetic analysis confirmed expectations regarding morphology. The two wild grape samples from Szentendre Island showed conspicuous similarities with *Vitis riparia*. The leaves were “flame-shaped”, there were no or very few erect and prostrate hairs, and the leaf length was much greater than the width. These samples were hybrids, suggesting that the area had been colonised by a hybrid population of *Vitis sylvestris* x *Vitis riparia*. This habitat, previously studied by TERPÓ (1988), shows strong genetic impoverishment and the decay of the natural wild grape gene-stock. In consequence, genetic erosion strongly reduces the conservation value of this habitat. All the alien *Vitis* samples (including the above-mentioned

Table 1

Selected grapevine and wild grape samples with GPS coordinates of the habitats and ecological associations or germplasm collections

No.	Name	Habitat/Origin	GPS coordinates
1	Óbuda I.	Floodplain	47°33/19°03
2	Óbuda II.		
3	Óbuda III.		
4	Szentendre Island I.	Floodplain	47°43/19°06
5	Szentendre Island II.		
6	Pilis I.	Beech forest	
7	Pilis II:		
8	Pilisszentkereszt		
9	Visegrád	Gorge	47°45/18°59
10	Mecseknádasd	Beech forest	
11	Mád	original habitat: beech forest (sample preserved in the Soroksár Botanical Garden)	
12	Turkey I. (Dumanlı)		
13	Turkey II. (Yumaklar)		
14	Turkey III. (Macun)		
15	Turkey IV. (Dumanlı)		
16	Turkey V. (Yumaklar)		
17	Turkey VI. (Macun)		
18	Pinot noir	<i>Convar. Occidentalis</i>	Cultivar collection of Pécs (MARD)
19	Chasselas	<i>Convar. Orientalis</i>	Cultivar collection of Pécs (MARD)
20	White Goher	<i>Convar. Pontica</i>	Cultivar collection of Pécs (MARD)
21	Kadarka	<i>Convar. Pontica</i>	Cultivar collection of Pécs (MARD)
22	<i>Vitis riparia</i> MICHX		Cultivar collection of Pécs (MARD)
23	<i>Vitis labrusca</i> L.		Cultivar collection of Pécs (MARD)

Table 2

List of investigated samples and the genotypes at 8 SSR loci, fragment lengths are given by base-pairs (bp), where "n.d." means no data

No.	Name	VvS1	VvS2	VvMD5	VvMD7	VvMD27	VrZag21	VrZag62	VrZag79								
1	Óbuda I.	182	190	132	152	228	228	240	190	190	192	202	197	197	252	256	
2	Óbuda II.	n.d.	n.d.	132	152	228	228	240	248	190	190	192	196	197	205	248	256
3	Óbuda III.	162	190	132	146	228	228	248	264	180	190	192	196	199	205	252	256
4	Szentendre Island I.	198	198	134	142	266	266	250	252	206	216	212	214	195	195	254	256
5	Szentendre Island II:	194	194	138	142	264	266	252	252	206	214	214	214	195	195	256	256
6	Pilis I.	182	190	134	152	226	232	248	264	192	192	196	208	197	205	250	250
7	Pilis II.	182	190	134	152	226	232	248	264	192	192	196	208	197	205	250	250
8	Pilisszentkereszt	182	190	134	152	226	232	248	264	192	192	196	208	197	205	250	252
9	Visegrád	188	194	126	132	236	260	236	248	180	186	206	216	193	205	248	252
10	Mecseknádasd	182	184	132	154	228	238	244	240	204	208	182	194	191	199	252	262
11	Mád	190	190	132	152	228	232	240	266	170	192	190	190	197	199	252	252
12	Turkey I. (Dumanlı)	182	188	138	144	222	234	240	248	178	184	196	202	197	203	246	246
13	Turkey II. (Yumaklar)	182	188	144	150	232	232	234	234	182	182	192	204	203	203	248	260
14	Turkey III. (Macun)	182	188	136	136	222	234	240	248	178	184	196	202	197	203	232	246
15	Turkey IV. (Dumanlı)	182	182	142	142	222	232	248	260	176	178	192	208	197	197	240	254
16	Turkey V. (Yumaklar)	182	182	138	140	222	232	238	240	182	190	202	204	197	197	260	260
17	Turkey VI. (Macun)	182	182	138	146	222	228	240	250	186	190	192	204	197	211	260	260
18	Pinot noir	184	190	136	152	228	238	240	244	186	190	202	208	189	195	240	246
19	Chasselas	182	190	132	144	228	236	240	248	186	190	202	208	197	205	252	260
20	White Goher	182	182	132	154	238	240	240	250	182	182	204	208	191	205	250	260
21	Kadarka	182	184	132	132	226	226	248	256	186	194	206	208	189	205	250	250
22	<i>Vitis riparia</i>	184	194	140	144	266	266	252	266	206	212	206	210	195	201	256	260
23	<i>Vitis labrusca</i>	190	198	152	152	238	238	236	250	204	208	180	184	203	203	238	248

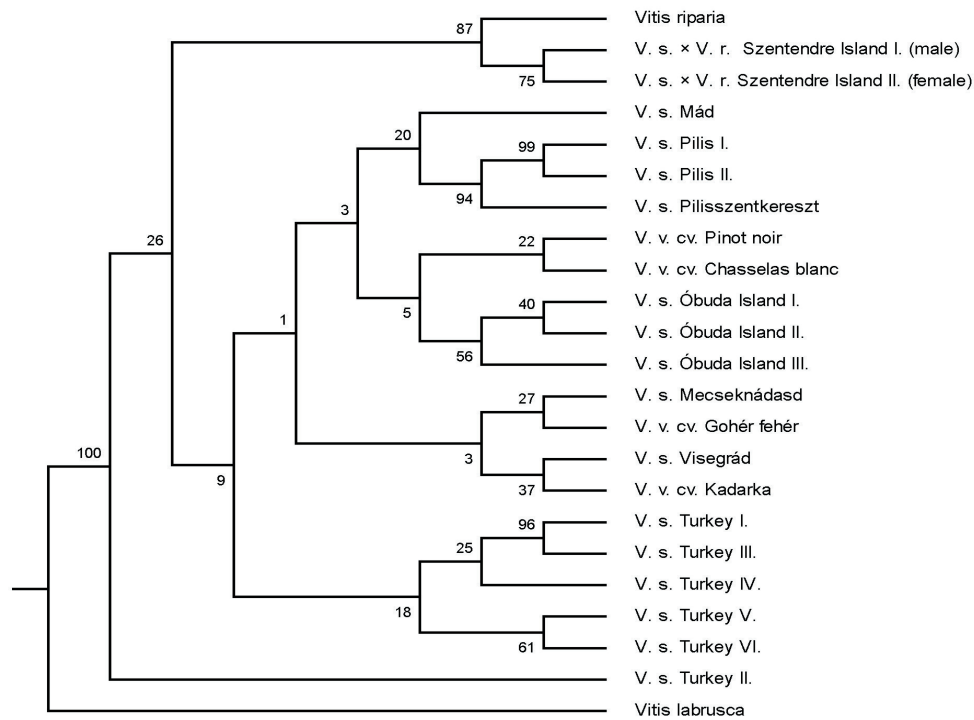


Figure: Consensus NJ tree representing relationships between the 24 *Vitis* samples based on microsatellite variability. Percentage bootstrap values are calculated from all possible (6,434) samplings of the 8 microsatellite loci. V. s.: *Vitis sylvestris*, V. v.: *Vitis vinifera*, V. s. x V. r.: hybrids of *Vitis sylvestris* and *Vitis riparia*.

hybrids) branched separately on the dendrogram, proving that these genetic markers are capable of distinguishing American species and their hybrids from autochthonous specimens.

As expected, the Hungarian and Turkish samples grouped in different clusters, thus suggesting that the genetic divergence between stands increased with the geographical distance. However, the Turkey II sample branched separately from the others, raising the need for further studies. The close relationship with the *Vitis labrusca* sample can be explained by the fact that this specimen is now the prevalent species in Turkey (CANGI *et al.* 2006).

A total of 94 loci were analysed. Following successful amplification, the expected size ranges were detected at all loci (Tab. 2). The expected heterozygosity values ranged from  $H_e = 0.7510$  (VvS1) to  $H_e = 0.8960$  (VvMD27), the average for the 8 loci being  $H_e = 0.8490$ . In the case of the Hungarian samples the lowest heterozygosity value was observed at locus VvS1 ( $H_e = 0.6953$ ) and the highest at VrZag21 ( $H_e = 0.8395$ ). Average genetic diversity within the Hungarian wild grape samples was  $H_e = 0.7404$ . Diversity within the Turkish samples was generally lower, with a range of  $H_e = 0.3750$  (VvS1) to  $H_e = 0.8333$  (VvS2). Genetic parameters such as the number of null alleles ( $n$ ), the expected ( $H_e$ ) and observed heterozygosity ( $H_o$ ) and the probability of identity (P.I.) are reported in Tab. 3.

A close relationship was detected between *Vitis vinifera* and *Vitis sylvestris* specimens in the case of Mecseknádasd and *V. vinifera* cv. 'Gohér white', and between wild grape samples from the Visegrád area and *V. vinifera* 'Kadarka', but this was only supported by low bootstrap values. According to NÉMETH (1967) the two above mentioned cultivars belong to convar. *pontica*, and are strongly

Table 3

Statistical results for samples from Hungary (HUN), Turkey (TUR) and all samples together (SUM), where:  $n$ : number of alleles,  $H_e$ : expected heterozygosity,  $H_o$ : observed heterozygosity,  $r$ : estimated frequency of null alleles

Locus	$n$	$H_e$	$H_o$	$r$	P.I.
VvS1 HUN	6	0.6953	0.8750	-0.1059	0.2326
VvS1 TUR	2	0.3750	0.5000	-0.9090	0.6015
VvS1 SUM	7	0.7510	0.6818	0.0395	0.1552
VvS2 HUN	6	0.7407	1.0000	-0.1489	0.1923
VvS2 TUR	7	0.8333	0.6666	0.0909	0.0921
VvS2 SUM	12	0.8733	0.8260	0.0252	0.0522
VvMD5 HUN	6	0.7160	0.6666	0.0287	0.1957
VvMD5 TUR	4	0.6805	0.8333	-0.0909	0.2782
VvMD5 SUM	11	0.8601	0.6521	0.1117	0.0640
VvMD7 HUN	6	0.7530	0.8888	0.0774	0.1815
VvMD7 TUR	6	0.7777	0.8333	-0.0312	0.1460
VvMD7 SUM	12	0.8431	0.8695	-0.0143	0.0742
VvMD27 HUN	7	0.7469	0.4444	0.1731	0.1691
VvMD27 TUR	6	0.8055	0.8333	-0.0153	0.1228
VvMD27 SUM	16	0.8960	0.6956	0.1056	0.0362
VrZag21 HUN	9	0.8395	0.8888	-0.0268	0.0799
VrZag21 TUR	5	0.7777	1.0000	-0.1250	0.1599
VrZag21 SUM	15	0.8913	0.9130	-0.0114	0.0408
VrZag62 HUN	5	0.7037	0.8888	-0.1086	0.2431
VrZag62 TUR	3	0.5416	0.5000	0.0270	0.4557
VrZag62 SUM	10	0.8119	0.6956	0.0641	0.1013
VrZag79 HUN	5	0.7286	0.6666	0.0357	0.2057
VrZag79 TUR	6	0.7361	0.5000	0.1360	0.1758
VrZag79 SUM	11	0.8657	0.6521	0.1144	0.0618
average HUN	6.25	0.7404	0.7898	-0.0094	0.1874
average TUR	4.875	0.6909	0.7083	-0.1146	0.2540
average SUM	11.75	0.8490	0.7482	0.0543	0.0732



associated with vineyards in the Eastern part of Europe. NÉMETH (1967) links the cultivar 'Kadarka' to Albania, but RAPAICS (1942) regards it as a Turkish cultivar, because the name of Üsküdar (a district of Istanbul) has a similar pronunciation. NÉMETH (1967) considers 'Gohér white' to be an old Hungarian cultivar, possibly suggesting that it was derived from *Vitis sylvestris*. On the other hand the two *Vitis vinifera* cultivars, 'Pinot noir' (convar. occidentalis) and 'Chasselas' (convar. orientalis), exhibited no relationship with any of the *Vitis sylvestris* samples.

The genetic divergence clearly detected by the 8 microsatellite loci, between both the geographically distant habitats and the different habitat types, suggests the existence of distinct gene-pools preserved along the species range. This might be attributed to historical events, *i.e.* to repeated cycles of range expansion and contraction of the species from the Pleistocene to the post-glacial period. This spatial distribution of intraspecific diversity along the populations, which usually exhibits distinct patterns in different species (AVISE 2000, HEWITT 2004), is the contemporary genetic imprint of the past and is accordingly one of the most important attributes for conserving species biodiversity in natural habitats.

In consequence, the results emphasize the high conservation value that should be attributed to the Hungarian habitats, most of which have preserved a distinct pattern of rich, uncontaminated genetic material. However, further studies that include other European specimens will allow us to form a general view on the genetic variation of the wild grape in south-eastern Europe.

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