Final report

Quest for the *Vitis sylvestris* (GMEL.) Populations of the Szigetköz and Fertő-Hanság National Park, Ex Situ Conservation, Propagation and Comparative Analysis by Molecular Markers

INTRODUCTION

The evolution of cultivated plants played important role in the ascent of humanity. Research of their origin and evolution started at the beginning of the 20th century, but till nowadays a lot of questions remain open.

A large number of theories exist about the evolution of the European grapevine (*Vitis vinifera* L.). According to De Candolle (1894), the grapes originate from the Trans-Caucasus. Based on the geographical principle in evolution by Darwin (1883), the regions of the primary origin of cultivated plants was created by Vavilov (1932) based on the diversity of the wild relatives of the given species. In this system, the European grapevine (*Vitis vinifera* L.) was classified (together with pistachio and almond) to the Central Asiatic Center.

In the after-glacial Eurasia, the existed woodland grape (*Vitis sylvestris* GMEL.) spread in whole Europe, and existed even in the southern part of Scandinavia. Man liked its fruits in its natural territory, collected and consumed them. The first *Vitis vinifera* type seeds (long seeds with well-developed "beak") were found between the excavation finds originating from the 2nd millennium BC. Keeping to west and south, the *Vitis vinifera*-like seed findings turned up gradually from later and later ages. This proves that once the *Vitis sylvestris* was taken into cultivation from the Trans-Caucasus by the people of the ancient Asia. Later the already *Vitis vinifera* was received by the people of the antique West-Asia and the people, who lived in the islands of the Aegean-see, spread it on the northern and southern bank of the Mediterranean-see (Kozma, 1991).

According to Terpó (1986), the *Vitis vinifera* is not uniform, but the progeny of more original grape species, the main fundaments between the *Vitis sylvestris* GMEL. could be the hermaphrodite flowered *V. hissarica* and the *V. nuristanica*. In 1988 he developed a new intraspecific system of *Vitis sylvestris* GMEL. The substance of his taxonomy was that he sorted the woodland grapes into subspecies based on the hairs of the leaves, and into varietas based on the shape of the leaves. He deduced the eco-geographical groups (convarietas) of *Vitis vinifera* directly or indirectly from these varieties (Jahnke et al., 2014).

The ancient cultivars of *Vitis vinifera* were classified in three con-varieties: pontica, orientalis and *occidentalis* by Negrul (1969). In his work he pointed, that the crosses between cultivars of different con-varieties lead to valuable types: those between pontica and orientalis give some promising wine types; and those of *orientalis* x *occidentalis* give early-ripening types; those of *occidentalis* x *pontica* segregate and give some high-quality wine types.

Accordingly this geographical cultivar groups (convarieties) of *Vitis vinifera* were not likely to have simultaneously developed, but they formed from the different woodland grape types side by side, or crossing one another respectively, as follows: First the pontican cultivar group (convar. pontica) developed in West- and East-Georgia and in Asia Minor. Its initial form could be the local woodland grape, the *Vitis sylvestris* GMEL. var. balcanica, *Vitis sylvestris* GMEL. var. *typica* (var. *sylvestris*). Inside the eastern cultivar group (convar. orientalis), the subconvar. caspica was born in the grape-growing countries of the antique Asia Minor, from the woodland grapes near the places bordering on the Caspian-lake (*Vitis sylvestris* GMEL. var. abberans). The origin of the convar. orientalis subconvar antasiatica dates from the later period . The hybrids of the pontican cultivars and the local woodland grapes (*Vitis sylvestris* GMEL.) were the starter forms of the western (convar. *occidentalis*) cultivars (Kozma, 1991).

The *Vitis sylvestris* GMEL. in Hungary is a protected species (Farkas, 1999). The quest and reservation of its populations are significant in terms of nature conservation and reserve of biodiversity as well. As pointed before, it is supposed, that this species itself, or crossing with other species could be the progenitor of the European grapevine (*Vitis vinifera* ssp. sativa). The ex situ conservation of the quested individuals has a great importance in the practical point of view as well, as they can serve as a resistance source in the future breeding programs.

The fundamental questions of this research were: on the desired area where can be found woodland grape (*Vitis sylvestris* GMEL.) individuals or populations; these individuals, into which type (varietas) owe, can be considered as the „clear" individuals of the race, how much they are genetically different, and how much they differ from the cultivated European grapevine (*Vitis vinifera* L.); whether the quested individuals are classifiable into the types (varietas) of Terpó (1988), and what is the connection between these types and the geographical-ecological groups (convarietas) of the European grapevine (*Vitis vinifera* L.).

MATERIALS AND METHODS

Plant material

The phenology and ampelography of 32 *Vitis sylvestris* GMEL. genotypes were recorded. (The origins of the accessions are described below.) For isozyme and SSR analyses 18 *Vitis vinifera* L. cultivars and 20 rootstock accessions were added for comparison.

Phenology and ampeloraphy

Phenology of the different genotypes were recorded based on the BBCH scale (Lorenz et al., 1995) in 2014-2016. Morphology of the *Vitis sylvestris* GMEL. accessions were characterised by 30 OIV descriptors (OIV, 2015).

Isozyme analyses

Dormant canes were collected in January, 2016; subsequently stored in plastic bags at 4 ^{o}C until processing at the longest for 2 days. Active enzymes were extracted from the dormant canes as described by Arulsekar and Parfitt (1986). Vertical polyacrylamide gel electrophoreses were carried out and gels were stained for AcP, CO, PER nad GOT as described by Royo et al. (1997). Results were evaluated visually. Isozyme bands were digitally scored (1-present, 0-absent).

SSR analyses

DNA extraction and SSR analyse procedures for 8 loci (VMC6F1, VVMD27, VVMD5, VMC6E1, VMC6G1, VVMD7, VMCNG4b9, VVMD28) are described in Jahnke et al. (2016).

RESULTS

Quest and conservation of the individuals

The woodland grape stocks were labelled with plastic stripes in-situ in the Szigetköz, in 2013. All of the stocks were marked on map, were located and the GPS coordinates were saved. Photos were taken in spring and autumn of 2013 and 2014 about all of the individuals. In June 2013, young shoots from the individuals were collected and grafted to rootstocks in Badacsony, Hungary.

Seeds were collected from 5 (genetically identical) female flowered stocks in the Szigetköz, in autumn 2013. The seedlings were planted outdoor in early spring, 2014, and young shoots of 23 seedlings were successfully grafted.

Phenology

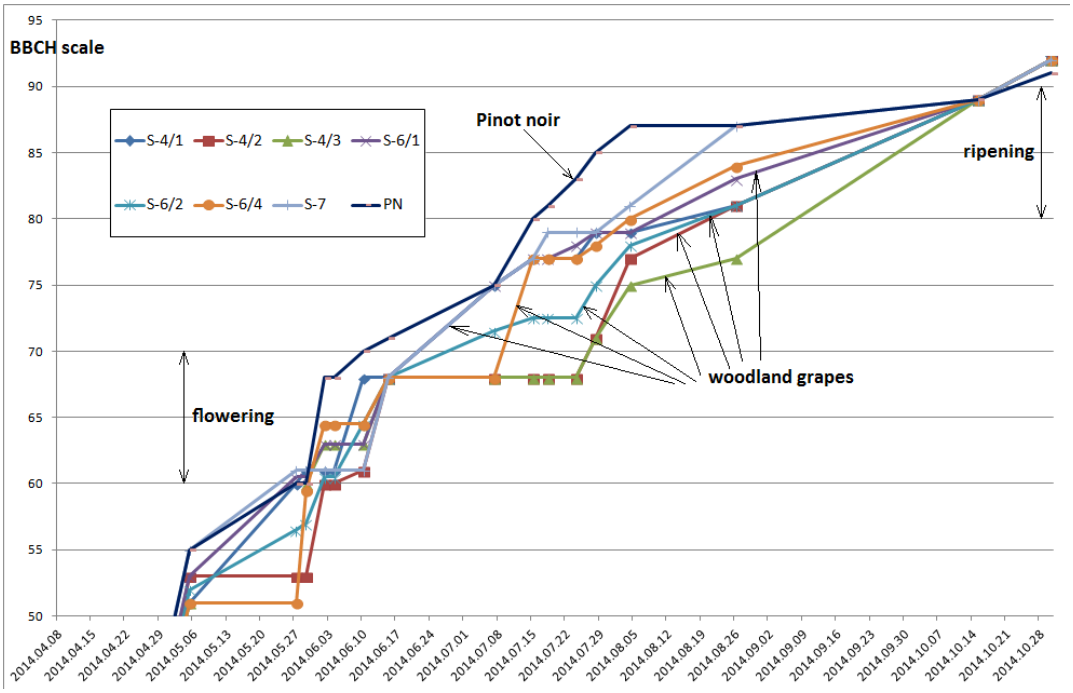

Fig. 1. Phenology of female flowered woodland grape (*Vitis sylvestris* GMEL.) genotypes and *Vitis vinifera* L. cv. 'Pinot noir' based on the BBCH scale (Badacsony, 2014.)

Based on our results it can be established, that there are differences between the time of flowering and fruit set between the different woodland grape (*Vitis sylvestris* GMEL.) genotypes, and an average *Vitis sylvestris* stock flowers and ripe later comparing to 'Pinot noir' (*Vitis vinifera* L.) (see Fig. 1.), but the difference depends on the weather as well. No correlation was found between the flowering dynamics and sex of woodland grape genotypes.

Ampelography

Differences were found between *Vitis sylvestris* GMEL functional female flowered, functional male flowered accessions and *Vitis vinifera* L. cv. 'Pinot noir'.
Based on the OIV descriptors the following conclusions can be drown:

- All of the woodland grapes were either functional female or functional male flowered, none of them were hermaphrodite (OIV 151: 2 or 4).
- The petiole sinus of the mature leaves characteristically is widely opened for the woodland grape accessions (OIV 079: 1-2).
- The prostrate hairs between main veins on lower side of blade of the mature leaves absent for female flowered woodland grapes and sparse for male flowered ones. (OIV 084: 1 or 3)
- The density of erect hairs on main veins on lower side of blade is low for female flowered, and medium for male flowered woodland grape individuals (OIV087: 3 or 5).
- The bunches of the female flowered woodland grapes are very short (OIV 202:1), very loose (OIV 204:1), the berries are very short (OIV 220: 1), globose shaped (OIV 223: 2) with blue black coloured skin (OIV 225: 6).
- Based on the results it can be established, that all of the woodland grape accessions belongs to *Vitis sylvestris* GMEL. var. *typica*

Isozyme analyses

Results of isozyme analyses of the 32 *V sylvestris* accessions and 18 *V. vinifera* cultivars are presented in table 1. Based on these results UPGMA dendogram was constructed (Figure 2). The European grapevine cultivars and the woodland grapes separate well in the dendogramm, supporting the different genetic background. Only the ancient cultivar 'Szürkebarát' (Pinot gris) of *Vitis vinifera* L. convar. *occidentalis* falls to the "*sylvestris*" cluster, supporting the morphological data, that these woodland grapes belong to the *typica* varietas.

Accession ID	Origin	AcP								CO						GOT				PER					
		1	2	3	4	5	6	7	8	1	2	3	4	5	6	1	2	3	4	1	2	3	4	5	6
Sziren	*Vitis vinifera* L.	1	1	1	1	1	1	1	0	0	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1
Trilla		1	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1
Gesztus		1	0	1	1	1	1	1	1	0	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1
Heuréka		1	0	1	1	1	1	1	0	0	1	1	0	1	1	1	1	0	0	0	0	1	1	1	1
Generosa		1	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	0	1	0	1	1	1	1	1
Kecskemét 7		1	0	1	0	1	1	1	1	1	0	1	0	1	0	1	1	0	1	0	1	1	1	1	1
Cserszegi fűszeres		1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	1	1	1	1	1	1
Irsai_oliver		1	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	1	1	0	1	1	1	1	1
Kovidinka		0	1	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1
Szurkebarat		1	1	0	1	1	1	1	0	1	1	0	0	1	1	1	1	1	0	0	0	0	1	1	1
Ezerjo		1	1	1	1	1	1	1	0	1	1	0	0	1	0	1	1	1	1	0	1	1	1	1	1
Pozsonyi fehér		1	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	0	0	0	1	1	0	1	1
Kadarka		0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	0	1	1	0	1	1
Muscat Lunel		1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	0	0	1	1	1	1
Muscat Ottonel		1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	1	1
Piros Tramini		1	0	1	1	1	1	1	1	1	0	1	0	1	1	1	1	0	0	0	1	1	1	1	1
Cabernet sauvignon		1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	1
Chardonnay		1	1	1	0	1	1	1	0	0	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1
B1	*Vitis sylvestris* GMEL.	1	0	1	1	1	1	1	0	1	0	0	1	0	0	0	0	1	1	0	1	1	1	1	1
B2		1	0	1	1	1	1	1	0	1	1	0	0	0	0	1	1	1	1	0	1	1	1	1	1
B5		1	1	1	1	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	1	1	0	1	1
B10		1	1	1	0	0	0	0	0	1	0	1	0	0	1	1	1	0	0	0	0	1	0	1	1
B12		0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	1	1	0	1	0	0	1	1
B13		0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	1	1	0	1	1	1	1	1
B16		0	1	1	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	1	1	1	1	1
B19		0	1	1	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0	1	0	0	1	1
B21		1	0	1	1	1	1	1	0	0	1	1	0	1	0	0	1	1	0	0	1	1	0	1	1
B24		0	1	0	1	1	1	1	0	0	1	0	0	1	0	1	1	0	0	0	1	1	0	1	1
B26		0	1	1	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	1	0	1	0	1	1
B27		1	1	1	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	1	1	0	1	1	1
B30		1	0	1	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	1	1	1	1
B31		1	0	1	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	1	0	1	1
B33		1	0	1	0	1	1	1	0	0	1	1	1	0	0	1	1	1	1	0	0	1	0	1	1
B34		0	1	1	0	0	0	0	0	0	1	1	1	0	0	1	1	1	1	1	1	1	0	1	1
B36		1	1	1	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0	0	0	0	1	1
B37		1	1	1	0	1	1	1	0	0	1	0	0	1	0	1	1	0	0	0	0	1	0	1	0
B41		1	1	1	0	1	1	1	0	0	1	0	0	1	0	1	1	1	1	0	0	0	0	1	0
B47		1	1	1	1	1	1	1	0	0	1	0	0	1	0	1	1	1	0	0	1	0	0	1	1
B48		1	1	1	1	1	1	1	0	0	1	0	0	1	0	1	1	0	0	0	1	0	0	1	0
B49		1	1	1	0	1	1	1	0	0	1	0	0	1	0	1	1	1	0	0	0	0	0	1	1
B50		1	1	0	1	1	1	1	0	0	1	0	0	1	0	1	1	1	1	0	0	0	0	1	1
B51		1	1	1	0	1	1	1	0	0	1	0	0	1	0	1	1	1	1	0	0	0	0	1	1
S 4/1		1	1	1	0	1	1	1	0	1	0	0	1	0	0	1	1	0	0	1	1	0	0	1	1
S 4/2		1	1	1	0	1	1	1	0	1	0	0	1	0	0	1	1	0	0	1	1	1	0	1	1
S 4/3		1	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1	0	0	1	1	1	0	1	1
S 6/1		1	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1	0	0	1	1	0	0	1	1
S 6/2		1	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1	0	0	1	1	0	0	1	1
S 6/4		1	1	0	0	1	1	1	0	1	0	0	1	0	0	1	1	0	0	1	1	0	0	1	1
S7		1	1	1	1	1	1	1	0	1	0	0	1	1	0	1	1	0	0	1	0	1	1	1	1
S1		1	1	1	0	1	1	1	0	1	0	0	1	0	0	0	0	1	1	0	1	1	0	1	1

Table 1. I isozyme analyses results for AcP, CO, GOT and PER for V. *sylvestris* accessions anf V. *vinifera* cultivars.

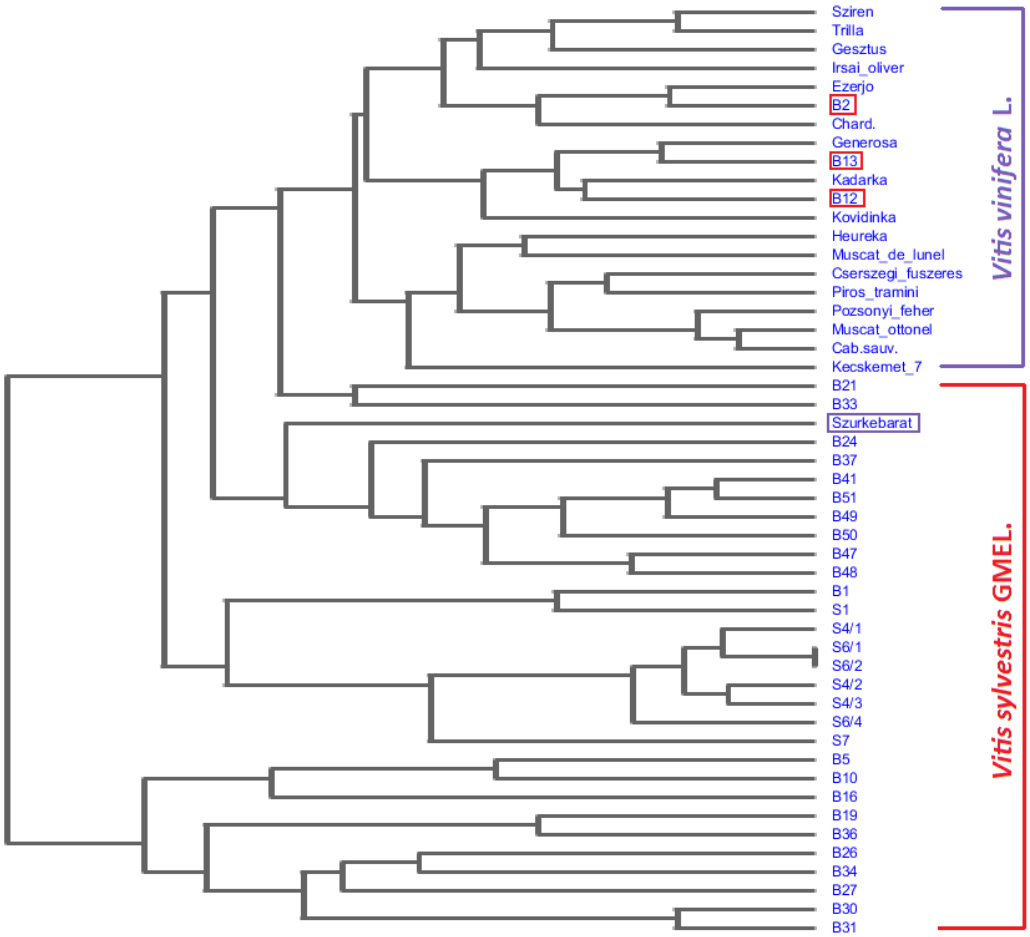

Fig. 2. UPGMA dendogram of the accessions based on isozyme results.

Acid phosphatase isoenzyme patterns consist of 2 zones. The presence of a maximum of 4 bands in the faster migrating region represents a distinct locus. This region consists of 3 or 4 bands in the case of *Vitis vinifera* ssp. sativa cultivars, and 3 bands for all analysed rootstocks and for the majority of the woodland grapes, but is totally absent in some *Vitis vinifera* ssp. *sylvestris* accessions. This phenomenon can be used as a marker for true-to-typeness of *Vitis sylvestris* accessions in future studies (Jahnke et al, 2017).

SSR analyses

Based on the SSR results in 8 loci (Jahnke et al. 2016) dendogram was constructed (Fig. 3.). The main groups (V. *sylvestris*, V. *vinifera* and rootstocks) mainly form 4 distinct groups. *Vitis sylvestris* GMEL. accessions form two distinct groups in the dendogram. The rootstocks Aramon Ganzin N1 and N2 (V. *vinifera* x V. *rupestris*) shows similarity to *Vitis vinifera* ssp. sativa variety 'Cserszegi fűszeres', which is not surprising, taking into accent the hybrid origin of this accessions. Most of the V. *vinifera* varieties form a large group with most of the V. *sylvestris* genotypes, but the two species form distinct sub-groups. Most of the rootstock accessions form a distinct group. The *Vitis sylvestris* genotypes form distinct groups and show similarity with *Vitis vinifera* genotypes, which clearly shows their true-to-typeness.
Only 3 *Vitis vinifera* L. cultivars, the ancient cultivar 'Traminer' (*Vitis vinifera* L. convar. *occidentalis*) and 2 offsprings (Generosa=Ezerjó x Traminer; Heuréka= Pozsonyi fehér x Traminer) fall to the *Vitis sylvestris* GMEL. cluster, which similarly to the isozyme results supports the morphological data.

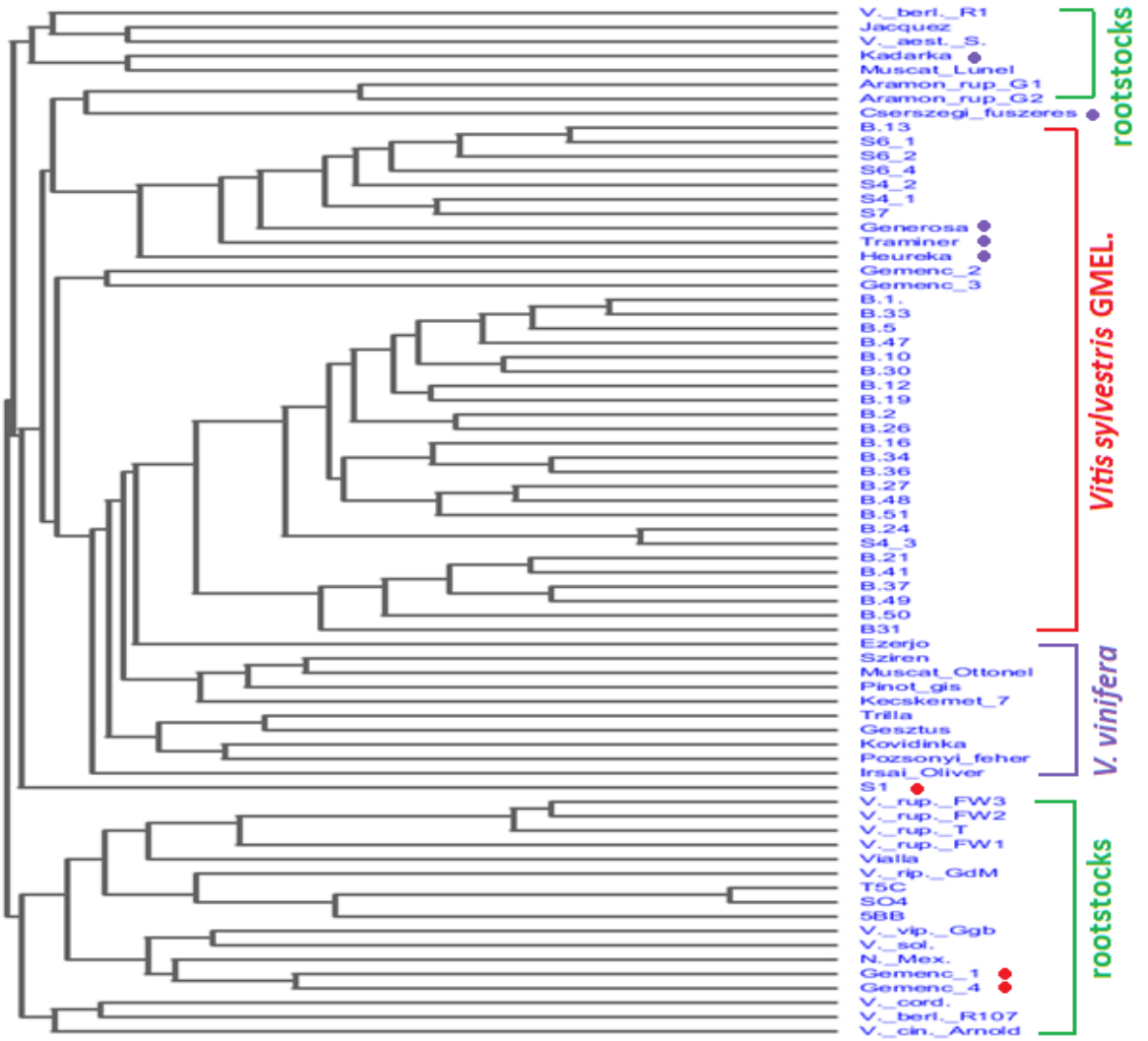

Fig. 3. UPGMA dendogram of the accessions based on SSR results.

SUMMARY

In the years of 2012-2015, 32 woodland grape genotypes were collected in the Szigetköz and Fertő-Hanság National Park, Hungary and ex-situ preserved in the genebank National Agricultural Research and Innovation Centre, Research Institute for Viticulture and Enology, in Badacsonytomaj, Hungary. Phenological development stages based on the BBCH scale and ampelographical data by 30 OIV descriptors were recorded. Isozyme and SSR analyses were carried out in the preserved genotypes, 18 Vitis vinirera and 20 rootstocks were also analysed for comparison. Summarising the results it can be established, that the ex-situ preserved genotypes are true-to-type woodland grapes, belonging to Vitis sylvestris GMEL. var typica.
The results support – as suggested by Bodor et al. (2010) - the further quest, ex-situ and in-situ preservation and analyses of the *Vitis sylvestris* GMEL. germplasm in Hungary.

ADDITIONAL INFORMATION

SNP polymorphism analyses were carried out in 10 loci, but resulted no differences between accessions. SSR analyses in 11 additional loci were done, repetition for the confirmation of the results are in progress.
Berries collected from *Vitis sylvetris* GMEL. accessions in 2014 - 2015 were analyses for chemical composition (nutrient elements and polyphenols). Wine was fermented from the must of the berries collected in 2015, and analysed for nutrients, polyphenols and biogen amines.

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