View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by JKI Open Journal Systems (Julius Kühn-Institut)

Vitis **48** (3), 115–121 (2009)

# Genetic diversity in native Bulgarian grapevine germplasm (*Vitis vinifera* L.) based on nuclear and chloroplast microsatellite polymorphisms

T. Dzhambazova[1)], I. Tsvetkov[1)], I. Atanassov[1)], K. Rusanov[1)], J. M. Martínez Zapater[2)], A. Atanassov[1)] and T. Hvarleva[1)]

[1)] AgroBioInstitute, Sofia, Bulgaria
[2)] Instituto de Ciencias de la Vid y del Vino, (CSIC, Universidad de La Rioja, Gobierno de La Rioja), Logroño, La Rioja, Spain

## Summary

**Fifty one wild specimens collected in different areas in Bulgaria and nineteen native Bulgarian grapevine cultivars were genotyped with 7 nuclear and 5 chloroplast SSR markers. Based on the microsatellite allelic profile six wild samples, collected from the Danube Riverbank, were considered non *vinifera* genotypes. The genetic diversity for nuclear loci observed in the cultivated grapevines was comparable to that found in other cultivated collections. However, lower genetic diversity was observed in the set of wild samples. The dendrogram based on nuclear SSRs separated most of the cultivated grapevines from the wild samples. Four chlorotypes corresponding to previously determined chlorotypes A, B, C and D, were identified in the analyzed samples that occurred with different frequencies in groups of wild and cultivated plants. The most frequent chlorotype among wild samples was A, while it was C in the cultivated samples. The differentiation of Bulgarian grape chlorotypes in the context of differentiation of chlorotypes in Eurasian grape flora is discussed.**

K e y w o r d s : wild grapevines, native grapevines, microsatellite markers, chlorotypes, genetic diversity.

## Introduction

The Eurasian grapevine (*Vitis vinifera* L.) is one of ancient crops tightly linked to the human history. The fruits of the plant have been harvested for thousands of years and currently grapevine is the most widely cultivated fruit crop in the word. It is believed that the modern Eurasian grape cultivars (*V. vinifera* ssp. *sativa*) originated from the domestication of wild progenitors (*V. vinifera* ssp. *silvestris*) (Levadoux 1956). The place and period of primo-domestication events still remain uncertain. There are two main hypotheses that differ according to geographic locations and number of domestication events. The first hypothesis suggests the existence of a major domestication event occurred in the Neolithic time from a limited wild stock in the Near East (Olmo 1976). According to different archeological evidence, the roots of viticulture stretch back to about 5000-6000 BC on the territory of the Caucasus Mountains where native Neolithic populations discovered winemaking (McGovern 2003, McGovern *et al*. 1997). From this region the selected grapevines were spread to Egypt (ca. 3,000 BC) and later on to the Italian and Iberian peninsulas (ca 800 BC) (McGovern 2003). The second hypothesis proposes that domestication events occurred from multiple wild stocks and along the entire region of distribution of *V. vinifera* (Mullins *et al.* 1992). This hypothesis is supported by recent studies indicating the contribution of local wild grapevines in the development of current grapevine cultivars in Western and Central Europe (Arroyo-Garcia *et al*. 2002, Aradhya *et al.* 2003, 2006, Grassi *et al*. 2003).

Nuclear microsatellite markers have been extensively exploited for evaluation of genetic diversity and investigation of the genetic structure and relationships among grapevine cultivars in different collections, countries and populations (Thomas *et al*. 1993, Thomas and Scott 1993, Sefc *et al*. 1998, 2000, 2003, Lefort and Roubelakis-Angelakis 2001, Moncada *et al*. 2006, Almadanim *et al.* 2007, Stainer *et al.* 2008). However, the high level of polymorphism revealed by nuclear microsatellite markers hampers the determination of relationships between the DNA profile and the geographical distribution of grapevines. Chloroplast microsatellites are more suitable for this purpose due to the low rate of mutations and recombination in the chloroplast genome (Provan *et al*. 1999, 2001) as well as because of their maternal inheritance in grapevine (Arroyo-Garcia *et al*. 2002). The genotyping of grapevines with chloroplast SSR markers resulted in the identification of a few chlorotypes allowing to trace the frequency and geographical distribution of the studied genotypes (Arroyo-Garcia *et al.* 2002, 2006, Grassi *et al.* 2003, 2006, Imazio *et al.* 2005)

In the 19th and 20th century wild grapes (*Vitis vinifera* ssp. *sylvestris)* became close to disappearing because of a number of diseases and anthropogenic factors. Nowadays, wild grapevines exist in small populations in diverse natural ecosystems throughout Europe, the Mediterranean region of Northern Africa, the Middle East, and Western Asia (Ocete *et al*. 2002). In addition to the *sylvestris* populations, the existence of populations formed by naturalized rootstocks that are reproduced sexually giving rise to hybrids with higher genetic diversity than *Vitis vinifera* ssp *sylvestris,* have been recently reported (Arrigo and Arnold 2007).

Bulgaria is located near Transcaucasia, at the crossroads between Asia and Europe, within the area where primitive cultivars were disseminated from east to west. Bulgarian populations of wild *Vitis vinifera* ssp. *sylvestris*

Correspondence to: Dr. T. Hvarleva, AgroBioInstitute, 8 Dragan Tzankov blvd., 1164 Sofia, Bulgaria. Fax: +359-2-963-5408. E-mail: hvarleva@abi.bg

grapevines, and their genetic relationship with native Bulgarian cultivars, have not yet been investigated and their analyses could help understanding the process of grapevine domestication. Within this context our study aimed to: 1) evaluate the genetic diversity in native Bulgarian *V. vinifera* (*sylvestris* and *sativa*) grapes as well as the presence of non *V. vinifera* wild grapevines in the country and 2) estimate the genetic relationships between native *sylvestris* and *sativa* grapes.

## Material and Methods

Plant material: 47 specimens of Eurasian wild grape (putative *Vitis vinifera* ssp. *sylvestris*) were selected from six geographic regions on the territory of Bulgaria. The wild samples were collected in their natural habitats (river banks, forests) on the base of morphological characteristic of their flowers (female or male only) and leaves according to the phenotypic description of wild grapes in Katerov *et al*. (1990). The studied wild specimens were grouped in five populations according to their locations: the Danube River (D), Strandzha Mountain (SM), the Rhopothamo River (RR), Tatul (T) and Semchinovo (Sem). The last two populations are respectively located in the Central and Northern part of Rhodopa Mountains. In addition, two plants were collected from Cherven castle (Ch) and four wild specimens were obtained from the ampelographic collection of the Institute of Viticulture and Enology at Pleven. (Fig. 1). Nineteen native Bulgarian grape cultivars (*Vitis vinifera ssp. sativa*) were also analyzed. Seven of them, maintained in the collection of AgroBioInstitute, Sofia, were considered as ancient cultivars (Babrikov *et al*. 2000). They were previously characterized at 12 microsatellite loci (Hvarleva *et al.* 2004, 2005). The remaining twelve grapevine accessions were obtained from the collection of the Institute of Viticulture and Enology, Pleven (Pl). They are rare cultivars collected in the last century from different vineyards in Bulgaria. Only for four of them there is available information (Nedelchev 1951, Nedelchev and Kondarev 1962, Radulov and Babrikov 1986). The list of analyzed genotypes, their geographical locations, chlorotypes and nuclear microsatellite genotypes are given in Tab. 1.


Fig. 1: Location of wild *Vitis* populations in the physical map of Bulgaria. The circles indicate the location of the wild populations analyzed.

DNA extraction and microsatellite analysis: Genomic DNAs were extracted from 100 mg of frozen leaf tissue following the procedure described by Murray and Thompson (1980). Twelve microsatellite markers corresponding to 7 nuclear and 5 chloroplast loci were used. The following nuclear microsatellite loci were chosen for genotyping of wild grapes: VVS2, (Thomas and Scott, 1993), VVMD5, VVMD7, VVMD27 (Bowers *et al*. 1996, Bowers and Meredith 1999), ssrVrZAG21, ssrVrZAG62, ssrVrZAG79 (Sefc *et al*. 1999). The analyzed chloroplast loci were ccmpSSR3, cpSSR5, cpSSR10 (Weising and Gardner 1999), ccSSR5 and ccSSR9 (Chung *et al.* 2003). Amplification was performed in a volume of 20 µl containing PCR buffer (Fermentas), 50 ng DNA, 1 µM of each primer, 100 µM of each dNTP and 1U Pfu DNA polymerase (Fermentas) in a GeneAmp® PCR System 2700 (Applied Biosystem). PCR amplification was performed with the following thermal cycles: 4 min at 94 ºC; 10 cycles of denaturation (15 s at 94 ºC), annealing (15 s at 52 ºC, 48 ºC for loci NTCP8 and cpSSR10) and extension (15 s at 72 ºC), followed by 23 cycles of denaturation (15 s at 89 ºC), annealing (15 s at 52 ºC), and extension (15 s at 72 ºC) with a final step for 7 min at 72 ºC. PCR products were analysed on ALF Express II sequencer (GE Healthcare). Fragment lengths were estimated with the help of internal standards, produced by amplification of pUC19 fragments with sizes 50, 100, 150, 200, 250, 300 and 350 bp. The lengths of the alleles were automatically sized with software AlleleLocator 1.03 (GE Healthcare).

Genetic diversity analyses: For the calculation of allele frequencies, expected (*He*) and observed (*Ho*) heterozygosity, probability of identity (*PI*) and probability of null alleles, the software GENALEX (Peakall and Smouse 2006) was used. Genetic distances between grapevine genotypes were calculated as [-ln (proportion shared alleles)] using Microsat (Minch *et al.* 1997). The obtained data was used for the construction of a dendrogram using the programs KITSCH from the PHYLIP package software (Felsenstein 1989) and Treeview (Page 1996).

## Results and Discussion

Genetic diversity in wild and cultivated grapes based on nuclear polymorphisms: Nuclear microsatellite genotypes are presented in Tab. 1. The comparison of the obtained microsatellite profiles revealed the presence in all samples of the Danube population of VVMD5 (262bp, 264bp) and VVMD27 (207bp, 211bp, 215bp, 219bp) alleles with sizes that were outside of the range determined for V. *vinifera* (Bowers *et al*. 1996, Bowers and Meredith 1999, This *et al*. 2004). Given the possibility that these samples could correspond to other *Vitis* species they were excluded from the group of putative *Vitis vinifera ssp. silvestris* accessions and respectively from the analyses of their genetic diversity. The total number of alleles estimated for the remaining 45 wild samples was 66 for all 7 nuclear loci, with mean number of

Table 1

List of 70 analyzed wild and cultivated genotypes, their microsatellite profiles at 7 nuclear loci, chlorotype determined at 5 chloroplast loci and geographical location of wild samples

| Sample | Geographical location* | Chlorotype | VVMD5 | | VVMD7 | | VVMD27 | | VVS2 | | ZAG21 | | ZAG62 | | ZAG79 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strandzha Mountain (SM), Veleka River "Kachul" (VK), Veleka River "Sredok" (VS), Veleka River "Kovach" (VKO) | | | | | | | | | | | | | | | | |
| S 1 | SM-VK | A | 222 | 230 | 258 | 258 | 187 | 193 | 132 | 140 | 190 | 190 | 193 | 193 | 242 | 248 |
| S 2 | SM-VK | A | 222 | 230 | 258 | 258 | 187 | 195 | 132 | 140 | 190 | 190 | 193 | 193 | 244 | 244 |
| S 3 | SM-VK | A | 230 | 230 | 256 | 256 | 181 | 189 | 142 | 154 | 190 | 194 | 193 | 193 | 240 | 250 |
| S 4 | SM-VK | A | 222 | 230 | 246 | 256 | 189 | 189 | 154 | 154 | 190 | 190 | 193 | 193 | 240 | 250 |
| S 5 | SM-VK | A | 230 | 230 | 236 | 246 | 189 | 189 | 140 | 154 | 182 | 190 | 193 | 195 | 240 | 250 |
| S 6 | SM-VS | A | 230 | 230 | 246 | 260 | 189 | 189 | 132 | 154 | 190 | 190 | 193 | 193 | 246 | 252 |
| S 7 | SM-VKO | D | 226 | 232 | 244 | 260 | 189 | 189 | 132 | 140 | 190 | 206 | 193 | 203 | 240 | 248 |
| S 8 | SM-VKO | D | 226 | 232 | 244 | 260 | 189 | 189 | 132 | 140 | 190 | 206 | 193 | 203 | 242 | 250 |
| Samples from Rhopothamo River (RR), reserve "Water Lilies" (RWL), reserve "Arkutino" (RA) | | | | | | | | | | | | | | | | |
| RR1 | RR-RA | A | 224 | 224 | 250 | 260 | 189 | 189 | 142 | 154 | 190 | 202 | 191 | 199 | 236 | 246 |
| RR2 | RR -RA | A | 234 | 238 | 258 | 258 | 189 | 189 | 140 | 154 | 190 | 194 | 191 | 193 | 248 | 248 |
| RR3 | RR -RA | A | 224 | 230 | 260 | 260 | 189 | 189 | 140 | 146 | 182 | 190 | 193 | 193 | 248 | 248 |
| RR4 | RR -RA | A | 226 | 230 | 236 | 260 | 183 | 189 | 132 | 154 | 190 | 190 | 193 | 195 | 244 | 244 |
| RR5 | RR -RWL | A | 234 | 238 | 258 | 258 | 189 | 189 | 140 | 154 | 190 | 194 | 191 | 193 | 246 | 250 |
| RR6 | RR -RWL | A | 224 | 238 | 246 | 260 | 189 | 189 | 140 | 140 | 190 | 190 | 193 | 193 | 246 | 250 |
| RR7 | RR -RWL | A | 224 | 224 | 236 | 260 | 189 | 189 | 132 | 142 | 190 | 190 | 187 | 191 | 246 | 258 |
| RR8 | RR -RWL | A | 226 | 234 | 260 | 260 | 189 | 189 | 140 | 142 | 190 | 190 | 193 | 193 | 246 | 250 |
| RR9 | RR -RWL | A | 226 | 234 | 260 | 260 | 189 | 189 | 140 | 140 | 190 | 190 | 193 | 193 | 246 | 246 |
| RR10 | RR -RA | A | 224 | 224 | 254 | 254 | 189 | 189 | 154 | 156 | 190 | 190 | 193 | 193 | 240 | 250 |
| RR11 | RR -RA | D | 230 | 230 | 256 | 260 | 189 | 189 | 132 | 146 | 190 | 194 | 193 | 195 | 242 | 258 |
| RR12 | RR -RA | D | 228 | 230 | 256 | 260 | 189 | 189 | 132 | 154 | 190 | 190 | 193 | 193 | 242 | 246 |
| Samples from Danube River (D), Oriahovo (Dr-O) | | | | | | | | | | | | | | | | |
| D 1 | Dr-O | B | 264 | 264 | 240 | 248 | 197 | 215 | 132 | 144 | 204 | 214 | 189 | 189 | 254 | 254 |
| D 2 | Dr-O | B | 264 | 264 | 250 | 262 | 199 | 219 | 136 | 140 | 204 | 208 | 191 | 199 | 256 | 256 |
| D 3 | Dr-O | B | 264 | 264 | 236 | 248 | 197 | 207 | 124 | 140 | 204 | 210 | 189 | 189 | 254 | 254 |
| D 4 | Dr-O | B | 264 | 264 | 236 | 250 | 207 | 207 | 136 | 142 | 206 | 212 | 189 | 189 | 244 | 254 |
| D 5 | Dr-O | B | 262 | 262 | 240 | 250 | 199 | 207 | 142 | 142 | 212 | 212 | 191 | 199 | 254 | 258 |
| D 6 | Dr-O | B | 264 | 264 | 240 | 248 | 207 | 211 | 136 | 136 | 204 | 210 | 189 | 189 | 254 | 258 |
| Samples from Cherven castle (Ch), Rousse region | | | | | | | | | | | | | | | | |
| Ch 1 | Ch | C | 224 | 226 | 236 | 244 | 193 | 193 | 132 | 154 | 190 | 206 | 195 | 203 | 256 | 256 |
| Ch 2 | Ch | A | 226 | 226 | 252 | 260 | 181 | 189 | 132 | 146 | 190 | 190 | 195 | 203 | 250 | 254 |
| Samples from northern Rhodopa Mountains,Semchinovo (SEM) | | | | | | | | | | | | | | | | |
| Sem 1 | Sem | C | 226 | 230 | 244 | 254 | 189 | 189 | 140 | 142 | 188 | 188 | 193 | 199 | 254 | 258 |
| Sem 2 | Sem | C | 224 | 230 | 236 | 250 | 189 | 189 | 142 | 154 | 194 | 194 | 187 | 193 | 246 | 254 |
| Sem 3 | Sem | C | 224 | 230 | 254 | 260 | 181 | 189 | 132 | 140 | 190 | 206 | 193 | 195 | 254 | 254 |
| Sem 4 | Sem | C | 230 | 234 | 254 | 264 | 189 | 189 | 150 | 154 | 194 | 200 | 193 | 193 | 246 | 254 |
| Sem 5 | Sem | C | 224 | 230 | 254 | 258 | 189 | 189 | 150 | 150 | 190 | 202 | 193 | 195 | 246 | 246 |
| Sem 6 | Sem | C | 230 | 234 | 236 | 250 | 189 | 189 | 150 | 154 | 190 | 190 | 187 | 193 | 246 | 254 |
| Samples from central Rhodopa Mountains,Tatul (T), Tatul, sanctuary of Orfeus (T-KOrf), Kardzhali region (T-K) | | | | | | | | | | | | | | | | |
| T 1 | T-KOrf | D | 230 | 230 | 244 | 246 | 189 | 189 | 140 | 140 | 194 | 194 | 193 | 201 | 242 | 254 |
| T 2 | T-KOrf | D | 238 | 244 | 246 | 250 | 181 | 181 | 134 | 142 | 190 | 206 | 193 | 193 | 242 | 254 |
| T 3 | T-KOrf | D | 230 | 236 | 236 | 260 | 183 | 189 | 132 | 132 | 194 | 204 | 195 | 195 | 240 | 240 |
| T 4 | T-KOrf | A | 230 | 230 | 246 | 258 | 183 | 189 | 132 | 150 | 194 | 202 | 193 | 193 | 244 | 254 |
| T 5 | T-KOrf | A | 226 | 238 | 236 | 250 | 189 | 189 | 132 | 150 | 190 | 206 | 187 | 203 | 244 | 244 |
| T 6 | T-KOrf | D | 226 | 236 | 246 | 258 | 189 | 191 | 140 | 146 | 190 | 190 | 193 | 195 | 246 | 246 |
| T 7 | T-KOrf | D | 226 | 236 | 246 | 258 | 189 | 191 | 140 | 146 | 190 | 190 | 193 | 195 | 244 | 244 |
| T 8 | T-K | D | 224 | 238 | 236 | 260 | 179 | 189 | 142 | 146 | 190 | 206 | 187 | 195 | 240 | 250 |
| T 9 | T-K | D | 226 | 230 | 258 | 258 | 189 | 195 | 132 | 138 | 190 | 194 | 193 | 195 | 242 | 248 |
| T 10 | T-K | A | 224 | 232 | 236 | 236 | 183 | 189 | 132 | 142 | 190 | 194 | 187 | 193 | 240 | 250 |
| T 11 | T-K | D | 226 | 230 | 258 | 258 | 189 | 189 | 132 | 140 | 190 | 190 | 193 | 203 | 240 | 250 |
| T 12 | T-K | A | 226 | 230 | 254 | 264 | 181 | 189 | 132 | 150 | 190 | 190 | 195 | 195 | 250 | 258 |
| T 13 | T-K | D | 234 | 234 | 230 | 244 | 181 | 195 | 140 | 154 | 200 | 200 | 195 | 203 | 236 | 258 |
| Samples from Pleven (Pl), gene bank of the "Institute of Viticulture and Biology" | | | | | | | | | | | | | | | | |
| Pl 1 | Pl | C | 222 | 226 | 256 | 256 | 189 | 189 | 154 | 154 | 190 | 190 | 193 | 193 | 242 | 250 |
| Pl 2 | Pl | B | 224 | 232 | 236 | 248 | 189 | 189 | 154 | 154 | 190 | 206 | 177 | 193 | 244 | 248 |
| Pl 3 | Pl | C | 222 | 226 | 256 | 260 | 189 | 189 | 154 | 154 | 190 | 190 | 193 | 193 | 242 | 250 |
| Pl 4 | Pl | C | 230 | 230 | 256 | 260 | 195 | 195 | 154 | 154 | 190 | 190 | 193 | 193 | 242 | 242 |
| Ancient Bulgarian cultivars | | | | | | | | | | | | | | | | |
| Bolgar | | A | 224 | 230 | 236 | 246 | 185 | 185 | 132 | 134 | 190 | 214 | 185 | 187 | 242 | 250 |
| Gamza | | D | 224 | 224 | 244 | 252 | 185 | 185 | 132 | 134 | 206 | 206 | 187 | 203 | 248 | 248 |
| Dimyat | | C | 238 | 244 | 236 | 246 | 179 | 181 | 140 | 142 | 200 | 202 | 187 | 203 | 236 | 258 |
| Mavrud | | C | 230 | 238 | 236 | 246 | 179 | 181 | 132 | 144 | 206 | 206 | 187 | 193 | 236 | 242 |
| Misket cherven | | C | 234 | 244 | 236 | 246 | 179 | 179 | 134 | 142 | 202 | 206 | 187 | 193 | 250 | 258 |
| Pamid | | C | 224 | 244 | 236 | 236 | 183 | 189 | 134 | 142 | 200 | 206 | 187 | 187 | 242 | 250 |
| Tamyanka | | D | 226 | 234 | 230 | 246 | 179 | 195 | 132 | 132 | 206 | 206 | 185 | 195 | 250 | 254 |
| Rare Bulgarian cultivars (accessions from Pleven-Pl) | | | | | | | | | | | | | | | | |
| Chaush | | C | 226 | 226 | 244 | 244 | 179 | 183 | 134 | 134 | 206 | 206 | 187 | 203 | 248 | 248 |
| Orlovi nokti beli | | C | 234 | 244 | 236 | 244 | 181 | 181 | 142 | 148 | 202 | 206 | 187 | 195 | 250 | 258 |
| Bodliv prast | | C | 236 | 236 | 236 | 236 | 179 | 179 | 132 | 142 | 200 | 200 | 187 | 187 | 250 | 258 |
| Lisicha opashka byala | | B | 234 | 266 | 230 | 262 | 203 | 211 | 144 | 144 | 196 | 210 | 199 | 213 | 244 | 244 |
| Ribi mehur | | C | 224 | 234 | 246 | 250 | 181 | 181 | 132 | 142 | 202 | 202 | 187 | 203 | 242 | 258 |
| Lisicha opashka chervena | | C | 230 | 244 | 230 | 244 | 179 | 195 | 144 | 148 | 202 | 202 | 203 | 203 | 238 | 242 |
| Orlovi nokti cherni | | C | 234 | 244 | 246 | 246 | 179 | 179 | 142 | 148 | 200 | 206 | 193 | 203 | 250 | 258 |
| Cherno izreslivo | | D | 226 | 232 | 236 | 240 | 185 | 195 | 134 | 134 | 200 | 206 | 185 | 185 | 250 | 256 |
| Chaush rozov | | C | 236 | 244 | 246 | 246 | 179 | 179 | 134 | 142 | 200 | 206 | 187 | 195 | 242 | 248 |
| Kadarka byala | | C | 238 | 238 | 236 | 236 | 179 | 185 | 134 | 134 | 204 | 204 | 195 | 195 | 250 | 250 |
| Garvan | | C | 226 | 232 | 246 | 246 | 179 | 185 | 142 | 142 | 200 | 206 | 193 | 203 | 236 | 250 |
| Mavrud varnenski | | C | 226 | 232 | 246 | 246 | 179 | 185 | 132 | 144 | 200 | 206 | 193 | 203 | 236 | 250 |

alleles per locus (*Na*) of 9, 43±1.62. (Tab. 2). These values were higher than those obtained for the 19 native cultivars investigated in this study, 55 alleles in total and an average of 7.86 ± 1.06 alleles per locus (Tab. 2). This difference can be due to the higher number of wild samples analyzed compared to the number of grapevine cultivars as evidence by the similar values of effective alleles (*Ne*) per locus.

The estimated average value of genetic diversity (expected heterozygosity, *He*) for cultivated grapevine accessions was 0.78 ± 0.05. The value of *He* for the individual microsatellite loci is similar to those obtained by Sefc *et al*. 2000 for cultivars grown in different European regions. The resulting average value of *He* for wild grapes, 0.71 ± 0.17, was lower than that observed in cultivated samples (Tab. 2). On the other hand the lower *Ho* values observed in both wild and cultivated samples suggest the existence of inbreeding in both types of samples. The cumulative value of probability of identity for cultivated samples ($1.4 \times 10^{-8}$) was lower than that obtained for wild grapevines ($5.0 \times 10^{-8}$) in agreement with their higher *He*.

To characterize the genetic structure of wild and cultivated Bulgarian grape germplasm, a dendrogram based on the proportion of shared alleles was constructed (Fig. 2). The analyzed grapevine samples, with the exception of cultivar Lisicha opashka byala, formed two main groups, one (I) consisting of cultivated grapes and wild samples, and the other (II) containing only Danube wild samples.

Both, the unusual allele sizes obtained in individuals from Danube population and their genetic distance from the other analyzed *V. vinifera* samples suggest that the samples of Danube population could belong to a different *Vitis* species. Their microsatellite profiles did not show identity with available microsatellite profiles of rootstocks, *i.e.* Lin and Walker (1998), Sefc *et al.* (1998), De Andres *et al*. 2007, Djambazova *et al*. 2007, GMC database: www.iasma.it/areabioav/gmc.html. Thus we suggest that they could be interspecies hybrids. The cultivar 'Lisicha opashka byala, remained outside of both clusters. It also contained alleles that fall outside of the range of allele sizes, characteristic for *V. vinifera* at loci VVMD5 (266bp), VVMD27 (211 bp) and ssrVrZAG62(213bp), suggesting that this cultivar may have a interspecific hybrid origin.

The *V. vinifera* genotypes formed two separate groups within cluster I, the first one included almost all cultivars and two wild samples, T2 and T13, while the second group contained all remained wild grapes. (Fig. 2). The events of outcrossing of wild accessions with cultivated ones could be a possible reason for grouping of wild samples T2 and T13 with cultivated grapes. A clear separation between *sylvestris* and *sativa* Italian grapes, based on nuclear

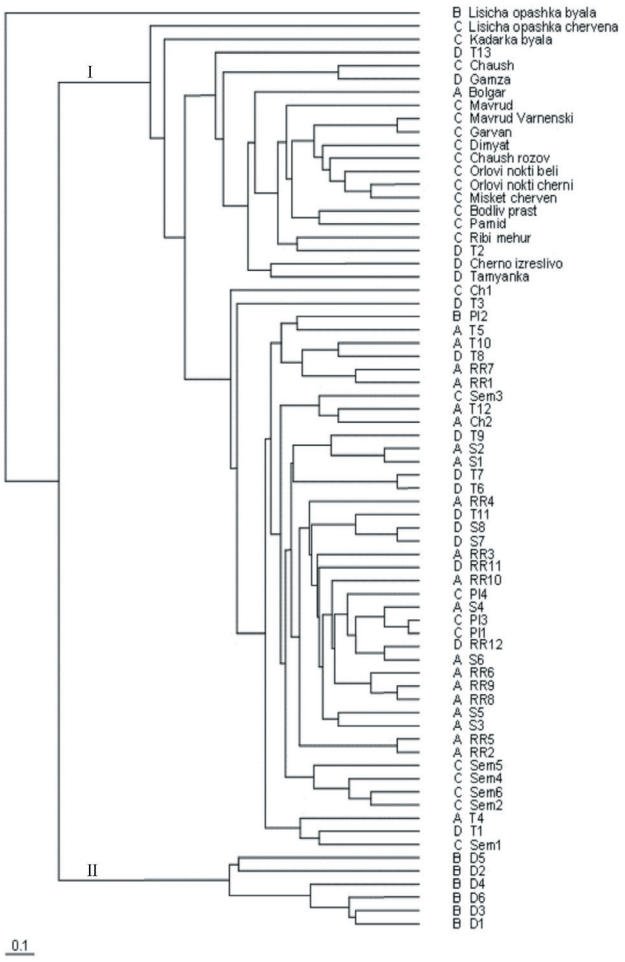


Fig. 2: Genetic relationships among 70 wild and cultivated accessions, based on data of seven nuclear microsatellite loci. The letters **A**, **B**, **C** and **D** in front of the name/abbreviation of each grape sample denotes the chlorotype of the sample.

SSR analysis, was also shown by Grassi *et al.* (2003). Two cultivars, 'Lisicha opashka chervena' and 'Kadarka byala' remained outside of the clusters of *sativa* and *sylvestris* grapes. These two cultivars do not have alleles that are unusual for *V. vinifera*, like those shown in the allelic profile of Danube samples and cultivar 'Lisicha opashka chervena'. Their position outside the cluster of cultivated grapes remain unclear.

Genetic diversity in wild and cultivated grapes based on chloroplast polymorphism: To further analyze the genetic structure and differentiation of wild grapevines as well as the genetic relationship between wild and cultivated grapevines native to Bulgaria, we investigated the variation in the chloroplast genome among the studied accessions. The chlorotype of

Table 2

Genetic diversity in wild and cultivated accessions. Na = number of alleles; Ne = number of effective alleles; Ho = observed heterozygosity; He = expected heterozygosity; Pi = probability of idendity

| | Na cumulative | Na average | Ne cumulative | Ne average | Ho average | He average | PI cumulative | PI average |
|---|---|---|---|---|---|---|---|---|
| wild | 66 | 9.43 ± 1.62 | 32 | 4.60 ± 2.49 | 0.62 ± 0.16 | 0.71 ± 0.17 | $5.0 \times 10^{-8}$ | 0.13 ± 0.11 |
| cultivated | 55 | 7.86 ± 1.07 | 33 | 4.78 ± 1.39 | 0.68 ± 0.10 | 0.78 ± 0.05 | $1.4 \times 10^{-8}$ | 0.08 ± 0.03 |

each specimen was determined based on the polymorphism at 5 chloroplast SSR loci, originally characterized for the chloroplast genome of tobacco (*Nicotiana tabacum* L) and more recently used for analysis of genetic relationship between wild and cultivated grape accessions (Arroyo-Garcia *et al*. 2002, 2006, Imazio *et al.* 2006). Polymorphisms at those five chloroplast microsatellite loci allowed the differentiation of all described chlorotypes. cpSSR loci were amplified in the investigated set of wild and cultivated grapevines, which confirms the observed earlier conservation of the chloroplast SSR loci sequences between the genus *Nicotiana* and *Vitis* (Arroyo-Garcia *et al*. 2002). Allele number per locus ranged from 2, for loci cpSSR3 (106, 107), cpSSR5 (104, 105), ccSSR5 (254, 255), and ccSSR9 (165, 166) to 3 for locus cpSSR10 (114, 115, 116).

The obtained allele variants at the analyzed chloroplast loci were identical to those reported in previous studies (Arroyo-Garcia *et al*. 2002, 2006). They combined in chlorotypes A, B, C, and D in the studied grape samples, according to Arroyo-Garcia *et al*. (2002, 2006; Tab. 3).

Most samples from each particular population were found to share the same chlorotype (Tab. 1). Most of the wild plants from Strandja mountain (SM population, 6 out of 8) and Ropothamo riverbank (RR population,10 out of 12) located in the south-east part of the country belonged to chlorotype A, while all representatives collected along the Danube river (D population) had chlorotype B. The two populations found in two locations in Rhodopa Mountains had different chlorotypes. Chlorotype C was found in all samples collected in the northern part of the mountain (Semchinovo, Sem population), while chlorotype D was obtained in 9 out of 13 samples collected from central part (Tatul, T population). Interestingly, chlorotype B was also found in *Vitis* species used as rootstocks (Arroyo-García *et al*. 2002) what supports the possibility that the Danube wild population derives from such an origin.

When Danube wild samples are excluded, the most frequent chlorotype in the Bulgarian wild grapevines was chlorotype A (47 %), followed by chlorotypes D (29 %), C (22 %) and B (2 %) (Tab. 3). These data were consistent with the results of Arroyo-Garcia *et al*. (2006) regarding the coexistence of chlorotypes A, C, and D in the Balkan Peninsula. In the same study chlorotype B was suggested as being the ancestral one since it didn't show a marked geographical distribution and was represented evenly and at a low frequency in the Eurasian region. This chlorotype was not previously found in wild grapes on the Balkan Peninsula as well as on the Italian Peninsula and the Middle East. In the present study chlorotype B was detected at a low frequency of 2 % (one out of 46 samples) among the Bulgarian wild samples.

Chlorotypes G, H, and E detected only in Near and Middle East regions were not found in the Bulgarian wild grapes. According to Arroyo-Garcia *et al.* (2006) chlorotypes C and D were not observed in Western and Central European wild grapevine populations, while chlorotype A was absent in the Near East. The presence of chlorotype A, C, and D in Bulgarian wild grapes suggests that the genetic diversity of Bulgarian wild populations occupies an intermediate place between the Near East region and Central and Western Europe, in agreement with its geographic location.

The same chlorotypes (A, B, C and D) were found in the set of 19 native Bulgarian cultivars, but with different frequencies than in the wild samples. The most abundant chlorotypes were C (74 %) and D (16 %), while A and B were bore only by one cultivar each (equivalent to 5 % each; Tab. 1). In fact, the cultivar carrying chlorotype B could be considered a hybrid cultivar given its location in the dendrogram and based on the presence of specific alleles at given nuclear loci. The lack of correlation between the frequency of different chlorotypes in native cultivated and wild grapes in Bulgaria suggest a reduced exchange of materials between the cultivated and the wild compartments in this region likely as a result of a higher exchange of cultivated grapevine genotypes between different vine growing regions in Bulgaria and Eastern regions.

The chlorotype frequencies determined in the set of Bulgarian native cultivars were quite different from those obtained for other countries in the Balkan area. According to Arroyo-Garcia *et al*. (2006) the prevalent chlorotype in a set of cultivars from the Balkan Peninsula (Greece, Bulgaria, Albania and Croatia) is D, followed by chlorotype C. In Arroyo-Garcia *et al.* (2002) studies chlorotype D was the predominant chlorotype (74 %) in a set of 39 Greek cultivars, while chlorotype C found in 18 % of these cultivars. Furthermore, studying a set of cultivars from the Balkan region (Romania and Serbia), Imazio *et al.* (2006) observed the lack of chlorotype V (ccmp3-106 bp, ccmp10-116 bp) that corresponds to chlorotype C in the study of Arroyo-Garcia *et al.* (2002, 2006). The small number of Balkan grapevine samples considered in those studies can result in these differences of chlorotype frequencies.

In conclusion, the characterization of the genetic diversity among wild and cultivated grapevines allowed the identification of native Bulgarian cultivars and natural

Table 3

Chlorotypes and allele sizes (bp) at 5 polymorphic chloroplast markers observed in this study and the frequency (%) of chlorotypes in wild and cultivated grapevine samples

| Chlorotype | cpSSR3 | cpSSR5 | cpSSR10 | ccSSR5 | ccSSR9 | Frequency *sylvestris* | Frequency *sativa* | Frequency total |
|---|---|---|---|---|---|---|---|---|
| A | 106 | 105 | 114 | 255 | 166 | 47 | 5 | 34 |
| B | 106 | 105 | 115 | 255 | 165 | 2 | 5 | 3 |
| C | 106 | 105 | 116 | 255 | 165 | 22 | 74 | 38 |
| D | 107 | 104 | 115 | 254 | 165 | 29 | 16 | 25 |

populations of wild grapevines as well as the evaluation of their genetic relationships. The results obtained on chlorotypes identification and distribution among Bulgarian wild grapes demonstrate a complex structure of the *sylvestris* gene pool consisting of a number of small groups of plants possessing predominantly the same chlorotype, likely as a consequence of inbreeding. At the same time the subgroup of *sylvestris* accessions formed after nuclear SSRs analysis do not match their geographical location, with the exception of four samples from Sem population (Fig. 2). The possible reason for this could be the rare events of out crossing between wild and cultivated grapes. Introduction of new genotypes in the population by the spread of seeds by birds and animals, could not be excluded as a factor that leads to genetic diversity enrichment in wild populations. Further analyses including wild and native cultivated grapevines from different regions of the Balkan Peninsula are necessary to determine the pattern of distribution of chlorotypes in this region and to complete the picture of grapevine chlorotype distribution in Europe

## Acknowledgements

The authors are grateful to Assoc. Prof. V. Dimitrova, Research Associate Z. Nakov and Research Associate M. Ivanov for kindly providing 12 genotypes from genebank of Institute of Viticulture and Enology, Pleven, Bulgaria. This research was supported by the Ministry of Education and Science, National Scientific Program "Genomics", project №: G-5-01/2003.

## References

Almadanim, M.; Couto, M.; Pereira, H.; Fevereiro, M.; 2007: Genetic diversity of the grapevine (*Vitis vinifera* L.) cultivars most utilized for wine production in Portugal. Vitis **46**, 116-119

Arrigo, N.; Arnold, C.; 2007: Naturalised *Vitis* rootstocks in Europe and consequences to native wild grapevines. Adv. Biochem. Eng. Biotechnol. **107**, 173-205.

Aradhya, M.; Dangl, G.; Prins, B.; Boursiquot, J.; Walker, M..; 2003: Genetic structure and differentiation in cultivated grape *Vitis vinifera* L. Genet. Res. **81,** 179-192.

Arroyo-Garcia, R.; Lefort, F.; de Andres, M.; Ibañez, J.; Borrego, J.; Jouve, J.; Cabello, F.; Martinez-Zapater, J. M.; 2002: Chloroplast microsatellites polymorphisms in *Vitis* species. Genome **45**, 1142-1149.

Arroyo-Garcia, R.; Ruiz-Garcia, L.; Bolling, L.; Ocete, R.; Lopez, M.; Arnold, C.; Ergul, A.; Söylemezoğlu, G.; Uzun, H.; Cabello, F.; Ibañez, J.; Aradhya, K.; Atanassov, A.; Atanassov, I.; Balint, S.; Cenis, J.; Constantini, L.; Gorislavets, S.; Grando, M.; Klein, B.; McGovern, P.; Merdinoglu, D.; Pejic, I.; Pelsy, F.; Primikirios, N.; Risovannaya, V.; Roubelakis-Angelakis, K.; Snoussi, H.; Sotiri, P.; Tamhankar, S.; This, P.; Troshin, L.; Malpica, J. M.; Lefort, F.; Martinez-Zapater, J. M.; 2006: Multiple origins of cultivated grapevine (*Vitis vinifera* L. ssp. *sativa*) based on chloroplast DNA polymorphisms. Mol. Ecol. **15**, 3707-3714.

Babrikov, D.; Barikov, D.; Pandeliev, C; 2000: Viticulture with Ampelography (in Bulgarian),Videnov, Sofia, Bulgaria.

Bowers, J.; Dangl, G.; Meredith, C.; 1999: Development and characterization of additional microsatellite DNA markers for grape. Am. J. Enol. Vitic. **50**, 243-246.

Bowers, J.; Dangl, G.; Vignani, R.; Meredith, C.; 1996: Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). Genome **39**, 628-633.

Chung, S.; Staub, J.; 2003: The development and evaluation of consensus chloroplast primer pairs that possess highly variable sequence regions in a diverse array of plant taxa Theor. Appl. Genet. **107,** 757-767.

de Andrés, M.; Cabezas, J.; Cervera, M.; Borrego, J.; Martínez-Zapater, J. M.; Jouve, N.; 2007: Molecular characterization of grapevine rootstocks maintained in germplasm collections. Am. J. Enol. Vitic. **58,** 75-86

Dzhambazova, T.; Hvarleva, T.; Hadjinicolis, A.; Tsvetkov, I.; Atanassov A.; Atanassov I.; 2007: Characterization of grapevine rootstocks using microsatellite markers. Biotechnol. Biotechnolog. Equipment, **21,** 58-62.

Felsenstein, J.; 1989: Phylogeny inference package. Cladistics **5**, 164-166.

Grassi, F.; Labra, M.; Imazio, S.; Ocete Rubio, R.; Failla, O.; Scienza, A.; Sala, F.; 2006: Phylogeographical structure and conservation genetics of wild grapevines. Conservat. Genet. **7**, 837-845.

Grassi, F.; Labra, M.; Imazio, S.; Spada, A.; Sgorbati, S.; Scienza, A.; Sala, F.; 2003: Evidence of secondary grapevine domestication centre detected by SSR analysis. Theor. Appl. Genet. **107**, 1315-1320.

Hvarleva, T.; Rusanov, K.; Lefort, F.; Tsvetkov, I.; Atanassov, A.; Atanassov, I.; 2004: Genotyping of Bulgarian Vitis vinifera L. cultivars by microsatellite analysis. Vitis **43,** 27-34.

Hvarleva, T.; Hadjinicoli, A.; Atanassov, I.; Atanassov, A.; Ioannou N.; 2005: Genotyping of Cyprus *Vitis vinifera* L. cultivars by microsatellite analysis.Vitis, **44**, 93-97.

Imazio, S.; Labra, M.; Grassi, F.; Scienza, A.; Failla, O.; 2005: Chloroplast microsatellites to investigate the origin of grapevine. Genet. Resour. Crop Evol. **00**, 1-9 (online publication).

Katerov, K.; Donchev, A.; Kondarev, M.; Getov, G.; Nachev, N.; Hershkovich, E.; Vulchev, V.; Markowa, M.; Braikov, D.; Todorov, H.; Mamarov, P.; Ivanov, I.; Zankov, Z.; Tsankov, B.; Raduilov, L.; Ivanov, M.; Djekova, M.; 1990: Bulgarian Ampelography volume 1 (in Bulgarian). Bulgarian Acad. Sci., Sofia, Bulgaria.

Lefort, F.; Roubelakis-Angelakis, K.; 2001: Genetic comparison of Greek cultivars of *Vitis vinifera* L. by nuclear microsatellite profiling. Am. J. Enol. Vitic. **52**, 101-108.

Levadox, L.; 1956: Les populations sauvages de *Vitis vinifera* L. Ann. Amelior. Plantes **6**, 59-118.

Lin, H.; Walker, A.; 1998: Identifying grape rootstocks with simple sequence repeat (SSR) DNA markers. Am. J. Enol. Vitic. **49**, 403-407.

McGovern, P.; 2003: The search for the origins of viniculture. In: Princeton Univ. Press (Eds.). Ancient Wine, 96-122.

McGovern, P.; Hartung, U.; Balder, V.; Glusker, D.; Exner, L.; 1997: The beginnings of winemaking and viniculture in the ancient near East and Egypt. Expedition **9**, 3-21.

Minch, E.; Ruiz-Linares, A.; Goldstein, D.; Feldman, M.; Cavalli-Sforza, M.; 1997: Microsat 1.5d: A computer program for calculating various statistics on microsatellite allele date. http://hpgl.stanford.edu/projects/microsat/).

Moncada, X.; Pelsy, F; Merdinoglu, D; Hinrichsen, P.; 2006: Genetic diversity and geographical dispersal in grapevine clones revealed by microsatellite markers. Genome **49**, 1459-1472.

Mullins M.; Bouquet A.; Williams L.; 1992: Biology of the Grapevine. Cambridge Univ. Press, Cambridge.

Murray, M.; Thompson, W. F.; 1980: Rapid Isolation of high molecular weight plant DNA. Nucl. Acids Res. **8**, 4321-4325.

Nedelchev, N.; 1951: Ampelography (in Bulgarian). Nauka i Izkustwo, Sofia, Bulgaria

Nedelchev, N.; Kondarev, M.; 1962: Viticulture (in Bulgarian). Hristo Danov, Plovdiv, Bulgaria.

Ocete, R.; Cantos, M.; Lopez, M.; Gomez, I.; Troncoso, A.; 2002: Wild grapevine populations in the Ossa-Morena mountain range (Portugal, Spain): Location, characterization and sanitary state. Vitis **41**, 51-56.

Olmo H. P.; 1976: Grapes, 294-298. In: N. W. Simmonds (Ed.): Evolution of crop plants. Longman, London.

Page, R.; 1996: Treeview: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12**, 357-358.

Peakall, R.; Smouse, P.; 2006: GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes **6**, 288-295.

Provan, J.; Powell, W.; Hollingsworth, P.; 2001: Chloroplast microsatellites: New tools for studies in plants ecology and evolution. Trends Ecol. Evol. **16**, 142-147.

Provan, J.; Soranzo, N.; Wilson N.; Goldstein D.; Powell, W.; 1999: A low mutational rate for chloroplast microsatellites. Genetics **153**, 943-947.

Radulov, L.; Babrikov, D.; 1986: Ampelography and Viticulture In: S. Ivanov and G. Tsankov (Ed.): Zemzdat (in Bulgarian).

Sefc, K.; Lopes, M.; Lefort, F.; Botta, R.; Roubelakis-Angelakis, K.; Ibanez, J.; Pejic, I.; Wagner, H.; Glössl, J.; Steinkellner, H.; 2000: Microsatellite variability in grapevine cultivars from different European regions and evaluation of assignment testing to assess the geographic origin of cultivars. Theor. Appl. Genet. **100**, 498-505.

Sefc, K.; Regner, F.; Glössl, J.; Steinkellner, H.; 1998: Genotyping of grapevine and rootstock cultivars using microsatellite markers. Vitis **37**, 15-20.

Sefc, K.; Regner, F.; Glössl, J.; Steinkellner, H.; 1999: Identification of microsatellite sequences in *Vitis riparia* and their applicability for genotyping of different *Vitis* species. Genome **42**, 367-373.

Sefc, K.; Steinkellner, H.; Lefort, F.; Botta, R.; da Camara Machado, A.; Borrego Palanco, L.; Maletic, E.; Glössl, J.; 2003: Substantial genetic contribution of local wild vines to European grapevine cultivars. Am. J. Enol. Vitic. **54**, 15-21.

Stajner, N.; Korosec-Koruza, Z.; Rusjan, D.; Javornik, B.; 2008: Microsatellite genotyping of old Slovenian grapevine varieties (V*itis vinifera* L.) of the Primorje (coastal) winegrowing region. Vitis, **47**, 201-204.

This, P.; Jung, A.; Boccacci, P.; Borrego, J.; Botta, R.; Costantini, L.; Crespan, M.; Dangl, G. S.; Eisenheld, C.; Ferreira-Monteiro, F.; Grando, S.;Ibáñez, J.; Lacombe, T.; Laucou, V.; Magalhães, R.; Meredith, C. P.; Milani, N.;Peterlunger, E.; Regner, F.; Zulini, L.; Maul, E.; 2004: Development of a standard set of microsatellite reference alleles for identification of grape cultivars. Theor. Appl. Genet. **109,** 1448-1458.

Thomas, M.; Matsumoto, S.; Cain, P.; Scott, N.; 1993: Repetitive DNA of grapevine: Classes present and sequences suitable for cultivar identification. Theor. Appl. Genet. **86**, 173-180.

Thomas, M.; Scott, N.; 1993: Microsatellite repeats in grapevine reveal DNA polymorphisms when analyzed as sequence-tagged sites (STS). Theor. Appl. Genet. **86**, 985-990.

Weising, K.; Gardner, R.; 1999: A set of concerved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. Genome **42**, 9-19.

*Received November 5, 2008*