MOLECULAR IDENTIFICATION OF FUNGI RESISTANT GRAPE GENOTYPES

PhD Thesis

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BACKGROUND AND OBJECTIVES

Production of grapevines is threatened by biotic (viruses, bacteria, fungi and insects) and abiotic stresses (i.e. drought, winter cold). From these stresses fungal infections reduce mostly the yield and damage fruit and wine quality, so viticulture requires substantial fungicide application. This has environmental risk, harmful for human health and it is very expensive (In Hungary the cost of chemical control of 1 hectare grapevine is 100 000 Ft, without the incidental expenses). Breeding resistant and high quality grape varieties can solve this problem, and it is very important both financially and environmentally.

Among fungal diseases, powdery mildew threatens the yield in the highest degree because it does not require specific weather conditions, i.e. adequate humidity and temperature conditions for infection, as in the case of downy mildew. Nowadays grapevine cultivation requires the use of chemical fungicides, like sulphur and sterol biosynthesis inhibitors. Most farmers must apply 6-10 fungicid sprays per season in order to avoid/control the powdery mildew infection, which can cause almost 90% yield loss. In France the cost of fungicides for powdery mildew is 75 million Euros per year, moreover appearance of resistant fungal strains can be expected against it.

Breeding new and fungi resistant grape (*Vitis vinifera* L.) varieties is time-consuming and resource-intensive, since grapes have a long generation cycle, and because the maintenance of hybrid progenies requires extensive area of land and rigorous cultivation. The use of DNA-based markers linked to genes of interest considerably reduces breeding costs. Molecular marker-assisted selection (MAS) facilitates the precise identification of seedlings that have inherited the desired gene shortly after germination, even before the expression of the trait is observable in the progeny. In this way, unwanted progeny can be eliminated and the size of the hybrid population can be reduced early during the breeding process. In recent years, considerable progress has been made in generating tools for MAS in grapes. Large number of DNA sequence-based markers have been developed which, in turn, made the construction of genetic linkage maps possible (Doligez et al., 2006, Di Gaspero et al., 2007). Many of the simple sequence repeats-based (SSR) markers are publicly available in Internet-accessible databases. The availability of linkage maps and molecular markers makes the mapping of agronomically favorable genes increasingly straightforward. The recent publication of the *V. vinifera* genome sequence (Jaillon et al., 2007, Velasco et al. 2007) accelerates the development of new SSR markers and allows them to be anchored to physical maps.

Breeding in viticulture aims at producing cultivars resistant to the most spread fungal pathogens: powdery (PM) and downy mildew (DM)(*Erysiphe necator* Schwein. Burr, *Plasmopara viticola* Berk. et Curtis).

Simple sequence repeats (SSR)-based markers are particularly useful in MAS, because they are co-dominant and, thus, allow the unambiguous identification of both the desired allele and its homologue. SSR markers enable breeders to simultaneously select for several genes in a progeny. This is particularly useful when multiple genes that encode the same phenotype are to be introgressed into a single genome. Combining multiple genes to confer the same phenotype is termed gene pyramiding. This approach is essential when breeders combine several qualitative resistance (*R*) loci against a disease into a hybrid plant. Different *R* genes are thought to detect the pathogen by different mechanisms, therefore, resistance conferred by a combination of various *R* genes is more difficult to overcome by the pathogen than resistance due to a single *R* gene (McDonald and Linde, 2002). Maximizing the durability of resistance is particularly important when fighting off rapidly evolving pathogens such as the grape powdery mildew.

In the Institute of Viticulture and Enology, Pécs different hybrid families were produced by Kozma et al. in order to combine PM and DM resistance genes. We analyzed four from these families: BC_4 (VRH 3082-1-42) x *V. vinifera* 'Kishmish vatkana', BC_4 x *V. vinifera* 'Kishmish moldavskij', *V. vinifera* 'Génuai zamatos' x *V. vinifera* 'Kishmish vatkana', (*V. vinifera* 'Dzhandzhal kara' x *Vitis* hibrid 'Laszta') x (*V. vinifera* 'Katta kurgán' x *V. vinifera* 'Perlette').

Since no *V. vinifera* L. cultivars carrying PM resistance genes were found till the mid '60-ies, wild *Vitis* species were applied as resistance gene sources. *Muscadinia rotundifolia* Michx. Small is an excellent gene source carrying the *Run1* dominant PM and the *Rpv1* major DM resistance genes. A (*M. rotundifolia* x *V. vinifera*) BC_4 hybrid of French origin (Bouquet,1986) has been applied in Hungary since 1996 in crosses with *V. vinifera* cultivars. However the *V. vinifera* cultivars is classified as susceptible, different cultivars show varying levels of susceptibility. Some Central Asian table grape varieties such as 'Dzhandzhal kara' (Korbuly, 1999) and 'Kishmish vatkana' (Kozma et al., 2006) show a marked resistance against powdery mildew. Dominant PM resistance gene of 'Kishmish vatkana' was named *Ren1*. For pyramiding the three mildew resistance genes 'Kishmish vatkana' (*Ren1*) was crossed with the *M. rotundifolia* x *V. vinifera* BC_4 (*Run1* and *Rpv1)* hybrid family, in the progeny it is possible to select genotypes carrying all the three resistance genes (*Run1/Rpv1/Ren1).*

Our aim was to select genotypes from the progenies carrying PM and DM resistance genes of different origin (*Muscadinia rotundifolia-Run1*, *Rpv1, V. vinifera-Ren1,* and 'Seyve-Villard' PM QTLs) with SSR, CB (designed on BAC libraries) and RAPD based SCAR markers; wild *Vitis* species and resistant varieties were characterized with these markers.

Objectives:

1. The purpose of our study was to use marker assisted selection (MAS) to identify the genotypes carrying pyramided resistance genes in the BC_4 x 'Kishmish vatkana' (BC_5) hybrid population (*Run1/Rpv1/Ren1* genotypes); it was also an objective to develop a multiplex PCR method for the improvement of MAS efficiency in order to be able to detect the different resistance genes in a single step, furthermore to select routinely the resistant genotypes from the sensitive ones in agarose gel;
2. Our aim was to develop a molecular marker based selection method, which can be applied in other populations as well;
3. To follow the resistance genes with molecular markers in the (*V. vinifera* 'Dzhandzhal kara' x *Vitis* hibrid 'Laszta') x (*V. vinifera* 'Katta kurgán' x *V. vinifera* 'Perlette') hybrid population; and to compare the PM resistance genes of 'Kishmish vatkana' and 'Dzhandzhal kara';
4. To characterize resistant varieties bred in Hungary with PM QTL linked markers;
5. To identify a molecular marker system which makes possible to distinguish *V. vinifera* varieties from wild *Vitis* species; and to prove that the PM resistant varieties ('Kishmish vatkana' and 'Dzhandzhal kara') belongs to *V. vinifera.*

MATERIALS AND METHODS

Plant materials

Different hybrid families were produced by Kozma et al. in the Institute of Viticulture and Enology in order to combine PM and DM resistance genes. We analyzed four from these families: BC_4 (VRH 3082-1-42) x *V. vinifera* 'Kishmish vatkana', (BC_4 x *V. vinifera* 'Kishmish moldavskij'), *V. vinifera* 'Génuai zamatos' x *V. vinifera* 'Kishmish vatkana', (*V. vinifera* 'Dzhandzhal kara' x *Vitis* hibrid 'Laszta') x (*V. vinifera* 'Katta kurgán' x *V. vinifera* 'Perlette').

Interspecific hybrids, resistant varieties bred in Hungary, cultivars from Asia, sensitive *V. vinifera* varieties (reference varieties), wild *Vitis* species and rootstocks were used for the PM QTL analysis, for determining the genetic distance of the PM resistant cultivars from Central-Asia, and to develop specific marker system in order to distinguish *V. vinifera* varieties from wild *Vitis* species.

DNA isolation

Genomic DNA was isolated from young leaves with DNeasy Plant Mini Kit according to the manufacturer's protocol (Qiagen). DNA quality and concentration was measured with a NanoDrop spectrophotometer.

PCR conditions and markers used in this study

PCR was performed in a reaction volume of 10 μL in BioRad iCycler thermocycler. The components of the reaction mixture were 20 ng of template DNA, 0.6 U of WTB-Taq polymerase (WestTeam Biotech, Pécs), 0.1 mM dNTP mix, 0.75 μM of each forward and reverse primer, and 1.25 mM $MgCl_2$ in 1x PCR buffer.

Simple sequence repeat and CB marker analysis

Markers linked to *Run1/Rpv1* resistance genes:

VMC8g9 and VMC4f3.1 were used to follow the inheritance of the *Run1* gene as described by Barker et al (2005), and VMC1g3.2 was used for *Rpv1* according to Wiedemann-Merdinoglu (2006). Following the *Rpv1* gene we tested SSR markers very close to VMC1g3.2: VVim11 and VVib32 (Doligez et al. 2006). CB markers, CB69.70 and CB137.138 and CB191.192 have been developed by Barker et al. (2005) using a bacterial artificial chromosome (BAC) library (Dry personal communication).

Markers linked to *Ren1* resistance genes:

Screening for the *Ren1* gene was undertaken using three linked SSR markers, UDV020a, VMC9h4.2 and VMCNg4e10.1, which were determined by Hoffmann et al. (2008) to be

located at a genetic distance of approximately 0.9 cM from the *Ren1* locus. This is the first time when SSR markers linked to *Ren1* have been used for MAS.

Markers linked to PM QTLs:

Three SSR markers, VMC4d9.2, UDV15b and VViV67 were used according to Eibach et al. (2007) and ScORA7-760 SCAR marker according to Akkurt et al. (2007).

SSR markers used constructing a dendrogram:

Microsatellite fingerprintings of the different grape varieties were made using 9 SSR markers on the proposal of the GrapeGen06 (http://www.montpellier.inra.fr/grapegen06) project:

VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VVS2, ssrVrZag62, ssrVrZag79 (Thomas and Scott 1993, Bowers et al. 1996, 1999, Sefc et al. 1999).

Development of a *Vitis* species specific marker system

In order to distinguish *V. vinifera* varieties from wild *Vitis* species we used the following markers: primer pairs designed on *rbcL* genes coded in plastids (Soltis et al. 2000), nuclear gibberellic acid gene sequences (*GAI1*) (Wen et al. 2007), *Vine-1* retrotransposon (Verriés et al. 2000) and the 20D18CB9 marker linked to *Vvmyb (which gene plays rule in the anthocyan biosynthesis)* (Walker et al. 2006). 20D18CB9 marker is developed by using BAC library (‘Cabernet Sauvignon’ Barker et al. 2005).

PCR conditions

Reaction conditions with CB primers were as follows: initiation at 94°C for 2 minutes; 40 cycles of denaturation at 94°C for 10 seconds, primer annealing at 57°C for 30 seconds; and DNA synthesis at 72°C for 1 minute; post-amplification at 72°C for 5 minutes.

For the amplification with the SSR markers, we performed touchdown PCR, which consisted of an initiation cycle at 94°C for two 2 min; 10 cycles of denaturation at 94°C for 30 seconds, primer annealing at 65°C for 30 seconds, and extension at 72°C for 1 minute, where the annealing temperature was decreased by 1°C at each cycle. This was followed by 24 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute. The reaction was completed with a post-amplification extension cycle at 72°C for 5 minutes.

Detection of the PCR products

To determine the exact size of PCR amplicons, they were fractionated in an 8% polyacrylamide gel (ReproGel™ High Resolution, GE Healthcare BioSciences, AP Hungary Kft, Budapest) in a vertical system (ALF-Express II). Fragments were detected by the Cy-5

fluorescent label attached to the forward primer. The precise size of the amplified SSR regions was determined relative to external and internal standards of known nucleotide length, using the ALFwin Fragment Analyser 1.0 software.

Products of the CB primers were detected in 1.2% agarose gel.

Products of the multiplex PCR were separated both in ALF-Express II. and in 4% Metaphor® (Cambrex Bioproducts, Biocenter Kft, Szeged) agarose gel.

Statistical evaluation and construction of a dendrogram

For the cluster analysis the UPGMA (’Unweighted Pair Group Method with Arithmetic mean’) method was used, which belongs to the hiearachical cluster methods. UPGMA method based on Jaccard’s similarity coefficients (Jaccard 1908).

Data gained from the microsatellite analysis were converted into binary codes then inserted into the table of the SPSS 11.0 for Windows software. A dendrogram was constructed to show the results, the genetic distances between the varieties.

RESULTS

Marker assisted selection (MAS) in different hybrid populations

Population No. 06-1: VRH 3082-1-42 BC_4 x 'Kishmish vatkana'

To generate multi-resistant grape genotypes that combine the *Ren1* and *Run1*/*Rpv1* genes, a cross was made by Kozma et al. between 'Kishmish vatkana' and VRH 3082-1-42 BC_4 (Bouquet, 1986), where the former was the male and the latter the female parent. The *Run1+*, *Ren1+* or *Run1+/Ren1+ g*enotypes showing same phenotype were identified by molecular markers. For MAS analysis, we randomly selected 440 plants from the segregated progeny, 410 plants from the PM-resistant and 30 from the PM-susceptible progeny. To find markers that can be used for routine genotyping in MAS, we evaluated SSR markers linked to *Ren1* and *Run1*.

For *Ren1*-linked markers, we assayed VMC9h4.2, UDV020a and VMCNg4e10.1, which were determined by Hoffmann and co-workers (2008) to be located at a genetic distance of approximately 0.9 cM from the *Ren1* locus. For all three of these markers, amplicon size differences allowed unambiguous distinction of *Ren1* and its homologous allele. Allele sizes for VMC9h4.2, VMCNg4e10.1, and UDV020a for the progeny under study are shown in Table 1. The three *Ren1*-linked alleles were always inherited together, confirming their tight linkage (Hoffmann et al., 2008) (**Table 1**). All plants that carrying the *Ren1*-linked markers were resistant to PM, and none of the 30 PM-susceptible plants inherited any of the marker alleles indicating PM resistance.

Table 1

Allele sizes of the SSR markers linked to the resistance genes detected in 06-1 population

	Ren1			*Run1*		*Rpv1*
	VMC 9h4.2	UDV20a	VMC Ng4e10.1	VMC 8g9	VMC 4f3.1	VMC 1g3.2
'Kishmish vatkana'	262:**<u>286</u>**	138:**<u>164</u>**	240:**<u>260</u>**	167:174	160:186	122:140
BC_4	282:298	148:148	260:260	**<u>160</u>**:167	184:**<u>186</u>**	**<u>122</u>**:140
'Cardinal'	289:307	140:160	265:286	179:179	162:162	135:140
BC_4 x 'Kishmish vatkana'						
Resistant genotypes	282:**<u>286</u>** **<u>286</u>**:298	148:**<u>164</u>**	260:**<u>260</u>**	**<u>160</u>**:167 **<u>160</u>**:174	160:**<u>186</u>** 186:**<u>186</u>**	**<u>122</u>**:140 **<u>122</u>**:122
Sensitive genotypes	262:282 262:298	138:148	240:260	167:167 167:174	160:184 184:186	122:140 140:140

Allele sizes previously associated with resistance markers are shown in bold and underlined.

For *Run1*-linked markers, we applied VMC8g9 and VMC4f3.1 SSR markers and 3 *Run1*-specific dominant markers (CB191.192, CB69.70, CB137.138), which had been designed on the basis of the BAC library clones, as described by Barker et al. (2005).

The allele sizes of VMC8g9 were 160 (*Run1*-linked), 167, and 174 bp, and were readily distinguishable from one-another, this marker is convenient/appropriate to select genotypes carrying the *Run1* gene. VMC4f3.1 marker was excluded because of detection difficulty (2-bp difference between the *Run1*-linked allele and its homologue)(**Table 1**). Data prove the tight correlation between VMC8g9 and CB markers. In those plants which do not contain the *Run1* gene and after all are PM symptomless the *Ren1* resistance gene from 'Kishmish vatkana' is present. Plants that are positive for both *Run1* and *Ren1* are valuable material for grape breeding since they have two dominant PM resistance genes from different sources and they are on different chromosomes (*Run1* is on *LG12* and *Ren1* is on LG13).

Screening downy mildew resistance in the progeny we applied VMC1g3.2 SSR marker linked to *Rpv1* gene according to Merdinoglu et al. (2003). The *Rpv1*-specific allele size of BC_4 of VMC1g3.2 is 122 bp. As the VMC1g3.2 primers also prime the synthesis of a 122-bp amplicon in 'Kishmish vatkana', only the individuals homozygous for the 122 bp allele could be identified as *Rpv1+* genotypes. We determined that individuals that are homozygous for this allele (122:122, *Rpv1+)* are also *Run1+*, which corroborates the findings by Merdinoglu et al. (2003) and Dry et al. (2010). The analysis of heterozygous individuals required the involvement of another markers. The analysis has been started with two new markers located in the vicinity of the VMC1g3.2 marker locus (VVIm11 and VVIb32) (Doligez et al. 2006). According to our results SSR marker VVim11 is appropriate to follow *Rpv1* DM resistance gene based on the analysis of BC_4 x 'Kishmish moldavskij' hybrid population. VVim11 marker has not been used earlier for MAS, it was the first time to apply to distinguish the sensitive and resistant genotypes.

To further streamline the selection process, we developed a multiplex PCR- and agarose gel electrophoresis-based method for the simultaneous detection of both *Run1* and *Ren1*. Multiplex PCR products were separated both on 8% polyacrylamide (ALF Express II.) and 4% Metaphor gel. In this way this method was suitable for separating the PM resistant and sensitive individuals through agarose-based electrophoresis. PCR products of CB markers – they have been developed on the basis of BAC-clones - could be separated and evaluated in 1.2% agarose gel. The results illustrate that MAS offers a rapid and accurate method to select hybrid genotypes that combine multiple loci of interest in grape.

None of the plants that supported powdery mildew growth on their leaves harboured either of the resistance genes. Among the 410 plants that were resistant to powdery mildew 36% contained both the *Run1* and *Ren1* resistance genes, while 28% were *Run1*-positive and 36% *Ren1*-positive. A great advantage of the multiplex PCR method is that it enables us to select the valuable genotypes in a single step, saving time, effort, and resources. Marker assisted selection is indispensable for selecting *Run1+/Ren1+* genotypes due to the same phenotypic effect of both resistance genes (Katula-Debreceni et al. 2010).

Population No. 06-3: V. vinifera 'Génuai zamatos' x *V. vinifera* 'Kishmish vatkana'

The results of our study demonstrate that SSR markers developed for the mapping of disease resistance loci in grape can be applied for MAS. Molecular markers tightly linked to *Ren1* loci are appropriate to select another hybrid population, where one of the parents is 'Kishmish vatkana'. The cross 'Génuai zamatos' x 'Kishmish vatkana' (78 symptomless and 68 sensitive progenies) was screened with VMC9h4.2 SSR marker, because the allele sizes of this marker made it possible to detect the results in agarose gel. The resistance allele could be detectable in the symptomless individuals. It is a rapid and efficient method to select the progeny, there is no need to evaluate of resistance to powdery mildew, which is laborious and costly and to maintain the huge segregating population.

Population No. 07-12: (V. vinifera 'Dzhandzhal kara' x *Vitis* hybrid 'Laszta'*) x* (*V. vinifera* 'Katta kurgán' x *V. vinifera* 'Perlette'*)*

We tested the (*V. vinifera* 'Dzhandzhal kara' x 'Laszta') x (*V. vinifera* 'Katta kurgán' x *V. vinifera* 'Perlette') hyibrid population (126 offspring) with markers linked to known resistance genes (*Ren1, Run1, Rpv1)* and PM QTLs (3 SSRs-VMC4d9.2, UDV15b, VViV67 and 1 SCAR-ScORA760) because of the 'Seyve Villard' origin of 'Laszta'. The population enabled us to compare the resistance genes of the two Central-Asian table grape cultivars, 'Kishmish vatkana' and 'Dzhandzhal kara'. The data showed that SSR markers linked to *Run1/Rpv1* resistance genes are not appropriate to select the resistant and sensitive genotypes, that means this hybrid population does not posses these genes. SSR profiles in *Ren1* linked loci on LG 12 showed that 'Kishmish vatkana' and 'Dzhandzhal kara' contain the same *Ren1* PM resistance gene, confirmed by the literature (Coleman et al. 2009) (**Table 2**).

Table 2
Allele sizes of SSR markers linked to *Ren1* and *Run1/Rpv1* resistance genes in (‘Dzhandzhal kara’ x ’Laszta’) x (’Katta kurgán’ x’Perlette’) progeny

	Ren1			*Run1/Rpv1*	
	VMC9h4.2	UDV20a	VMCNg4e10.1	VMC8g9	VMC1g3.2
‘Kishmish vatkana’	262:**286**	138:**164**	240:**260**	167:174	122:142
‘Dzhandzhal kara’	280:**286**	150:**164**	255:**260**	167:174	124:128
‘Laszta’	252:290	150:150	230:268	162:178	128:134
‘Dzhandzhal kara’ x ‘Laszta’	**286:**290	150:**164**	**260:**268	162:174	124:128
‘Katta kurgán’ x ‘Perlette’	262:286	138:150	238:260	178:178	122:128
Sensitive genotypes	262:290 286:290	138:150 150:150	238:268 260:268	162:178 174:178	124:128 122:128 122:124 128:128
Resistant genotypes	262:**286** 286:**286**	138:**164** 150:**164**	238:**260** 260:**260**	162:178 174.178	124:128 122:128 122:124 128:128

Allele sizes previously associated with resistance markers are shown in bold and underlined.

PM QTL analysis

Application of SSR markers linked to PM QTLs

‘Laszta’ is an interspecific resistant variety, PM and DM QTLs inherited from ‘Seyve Villard’ parents. SSR markers VMC4d9.2, UDV15b and VViV67 linked to PM QTL on LG 15 are applied according to Eibach et al. (2007) in 07-12 hybrid population. A multilocus marker UDV15b developed by Di Gaspero et al. (2005) generates multipeaks, which produces difficulties in analysis of results. Using the two other markers (VMC4d9.2 és VViV67) the results showed a variation between the resistant and sensitive springs. PM QTLs described in ‘Regent’ cultivar were not appropriate to analyze this population, because the resistant parent (‘Dzhandzhal kara’ x ‘Laszta’) is homozygous for the alleles of the linked SSR markers.

We suggest to generate a test population in order to identify new molecular markers linked to PM QTLs in ‘Laszta’.

Table 3
Allele sizes of SSR markers linked to PM QTLs on LG 15

	VMC4D9.2	VViV67
'Regent'	235:240	334:352:364
'S 7053'	230:235	334:352
'Laszta'	235:240	352:364
'SV 20365'	235:240	334:352:364
'SV 12375'	230:235	334:352:364
BC_4	244:244	352:364
V. labrusca	230:240	344:352:358
V. rupestris	235:240	358:358
V. berlandieri	235:235	330:352
V. lincecumii	235:235	330:338:352
Resistant parent: 'Dzhandzhal kara' x 'Laszta'	240:240	352:352
Sensitive parent: 'Katta kurgán' x 'Perlette'	226:240	352:364

Analysis of Hungarian bred resistant varieties by SCAR marker linked to PM QTL

We characterized wild *Vitis* species, PM resistant cultivars from Central-Asia, interspecific cultivars and Hungarian bred resistant cultivars with ScORA7-760 SCAR marker linked to PM QTL. Powdery mildew resistance can be followed by the SCAR marker in these varieties: 'Regent', 'Seibel 7053', 'SV 20365', 'Villard blanc', 'Seibel 4986', 'Viktória gyöngye', 'Nero', 'Zala gyöngye', 'Bianca', 'SV 12286'. Our data can be useful for a resistance breeding program, where the aim is disease resistance gene pyramiding with MAS. 'Viktória gyöngye', 'Nero' and 'Zala gyöngye' are not only resistant varieties, they are of high quality and early ripining table grapes, inherited from world wide known 'Csaba gyöngye' ('Pearl of Csaba'). In a breeding program crossing these varieties with 'Kishmish vatkana' or 'Dzhandzhal kara' durable resistance can be achieved by gene pyramiding. We can follow the dominant PM resistance gene (*Ren1)* and the PM QTLs of 'Seibel'/'Seyve Villard' origin by MAS. Furthermore applying genotypes *Run1+*/*Ren1+* from hybrid population No. 06-1 and PM resistant interspecific varieties as parents in a cross, new resistant varieties can be produced by MAS.

Characterization of different *V. vinifera* varieties with markers linked to resistance genes

Different *V. vinifera* varieties were characterized with molecular markers linked to *Run1/Rpv1* and *Ren1* resistance genes. Our aim was to test whether allele sizes of sensitive varieties correspond to allele sizes showing resistance (BC_4, 'Kishmish vatkana'), and to compare the resistance genes of different origin of resistant varieties to known genes (*Run1/Rpv1, Ren1*).

Neither of the sensitive varieties harboured alleles linked to resistance. PM resistant Asian varieties ('Kishmish vatkana', 'Dzhandzhal kara', 'Tagobi', 'Gordin', 'Alexandrouli', 'Tsitska', 'Bazaletouri tsolikouri', 'Kabarcik', 'Rezisztens magvatlan') and interspecific hybrids ('Regent', 'Laszta') do not have the *Run1/Rpv1* genes derived from *M. rotundifolia.* Varieties 'Kishmish vatkana' and 'Dzhandzhal kara' originating from the same place (Uzbekistan) share the same allele sizes linked to resistance (Coleman et al. 2009). We had no data about the origin of 'Rezisztens magvatlan' (Resistant seedless), according to our results this variety can derive from Central Asia, as the seedless and PM resistant 'Kishmish vatkana'. The other PM resistant Asian varieties do not possess the already known resistance genes (*Run1/Rpv1, Ren1*), so they can be new resistance sources for breeding. Our purposes to analyze and to map the resistance gene of 'Kabarcik' variety by creating a test cross. (Allele sizes of *V. vinifera* varieties are in **Appendix/Table 1, 2**.)

Microsatellite analysis of varieties of Asian origin

We have determined the SSR profile of Asian cultivars (PM resistant and sensitive varieties), 2 reference cultivars ('Chardonnay' and 'Pinot noir'), wild *Vitis* species and American rootstocks in 9 microsatellite locus (VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VVS2, ssrVrZag62, ssrVrZag79), recommended by GrapeGen06 project (allele sizes are in **Appendix/Table 3**). Our aim was to construct a dendrogram based on cluster analysis in order to show the genetic distance among these cultivars and species. All of the Central-Asian and reference *V. vinifera* varieties are included in Cluster 1, and varieties from Uzbekistan grouped together in a smaller group including the PM resistant 'Dzhandzhal kara' and 'Kishmish vatkana' as well. Wild *Vitis* species and American rootstocks are included different clusters (Cluster 2 and 3). The results confirm that

'Dzhandzhal kara' and 'Kishmish vatkana' derived from Central Asia belong to *V. vinifera*, and are not related to either wild *Vitis* species nor American rootstocks.

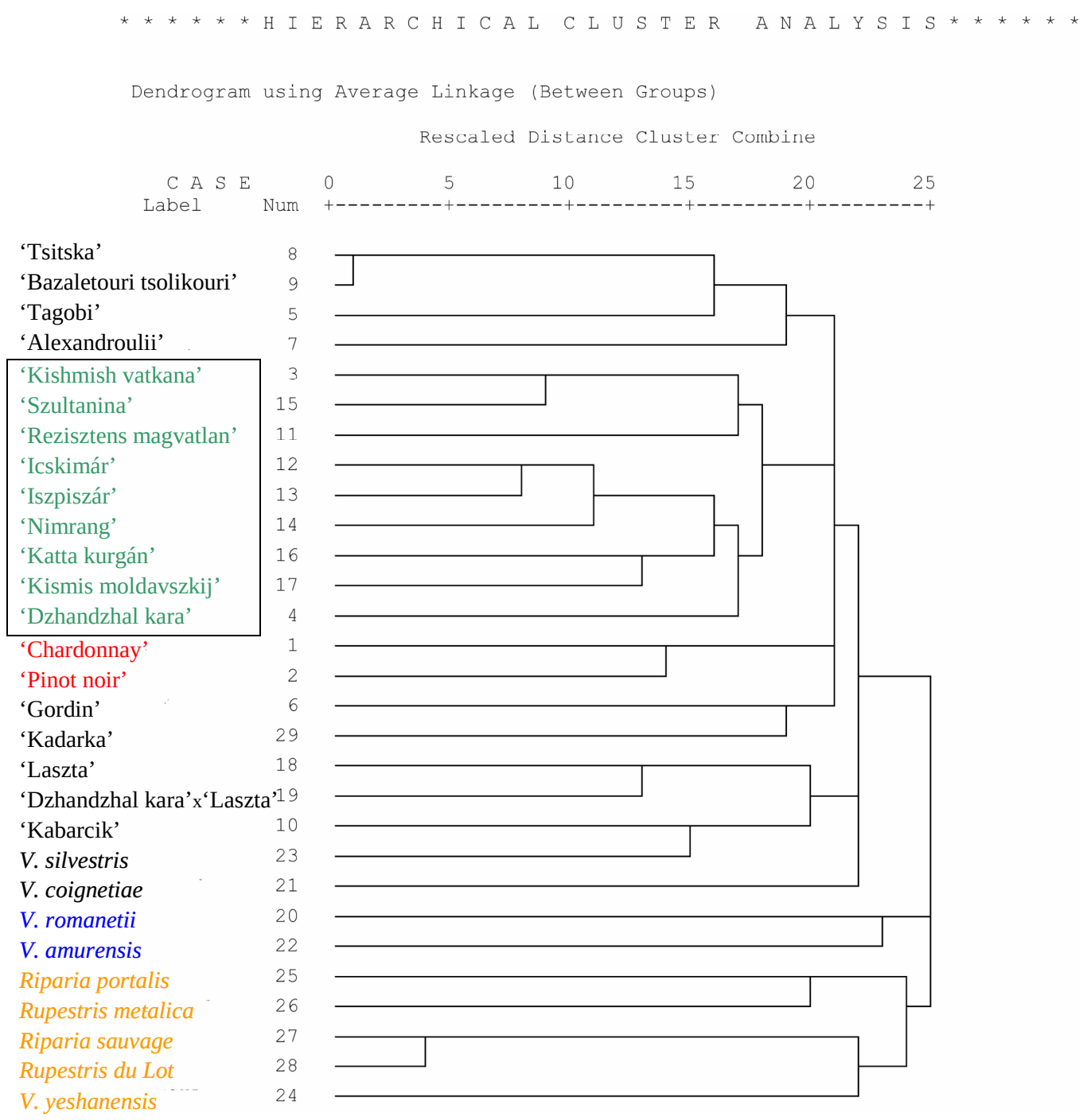

Figure 1: Cluster analysis results shown on dendrogram (using 9 SSR markers)

Identification of *V. vinifera* specific marker

Grape varieties belonging to *V. vinifera* var. *orientalis* convar. *antasiatica* (i.e. 'Nimrang', 'Icskimar', 'Iszpiszár', 'Katta kurgan', 'Sultanina', 'Kishmish vatkana', 'Dzhandzhal kara') have different morphological features than other *V. vinifera* varieties. One might assume that these varieties are not pure *V. vinifera*, perhaps recent interspecific hybridisation might have occurred with wild *Vitis* species or American rootstocks. We have processed a *V. vinifera* specific marker system to prove the pure *V. vinifera* origin of these PM resistant cultivars ('Dzhandzhal kara' and 'Kishmish vatkana'). We have identified a molecular marker which makes it possible to distinguish *V. vinifera* varieties from the wild ones after a Polymerase Chain Reaction (PCR) and polyacrilamid gel electrophoresis without sequencing.

Phylogenetic analysis of *Vitis* species mostly based on comparing coding and non-coding plastid sequences (Soltis et al. 2000), however these differences of sequences can be detected with difficulties. During the analysis 3 *V. vinifera* cultivars were used as references, 6 PM resistant Asian cultivars, 21 wild *Vitis* species, *M. rotundifolia*, *Parthenocissus quinquefolia*, and 3 rootstocks were examined by molecular markers.

The pattern of gel electrophoresis were near identical got by PCR primers designed on plastid genes (*rbcL, atpB*) (Soltis et al. 2000), or nuclear gibberellic acid gene sequences (*GAI1*) (Wen et al. 2007). Differences can be detectable only by sequencing.

A molecular marker (20D18CB9) (Walker et al. 2006) linked to *Vvmyb* gene, which plays a role in the anthocyan biosynthesis, showed slight polymorphism between *V. vinifera* cultivars and *Vitis* species. Determination of the size of the PCR product is not possible on agarose gel, so we detected the amplicons on ALF express II., making bigger internal and external standards used in SSR analysis (**Table 4**).

In *V. vinifera* cultivars (samples 1-3) used as references a 582 bp fragment was amplified by 20D18CB9 marker, same as the PM resistant Asian cultivars (samples 4-9). Among the wild *Vitis* species only *V. coignetiae* possessed this size DNA fragment. In the other wild species different size and/or different number of PCR fragments amplified. In all of the Asian wild *Vitis* species we got the 582 bp DNA fragment, while in North American wild *Vitis* species not. *V. silvestris* is native to Middle Asia and neighbourhood of Kaukazus, respectively the PM resistant *V. vinifera* cultivars also derive from Asia (Uzbekistan, Georgia).

Table 4
Allele sizes of different *V. vinifera* cultivars, wild species and rootstocks with 20D18CB9 marker

Cultivars/species	DNA fragment size (bp)	Species	DNA fragment size (bp)
'Barbera'	582	*Vitis cordifolia*	540
'Chardonnay'	582	*Vitis titanica*	540
'Pinot noir'	582	*Vitis arizonica*	540:575
'Kishmish vatkana'	582	*Vitis labrusca*	540:575
'Tagobi'	582	*Vitis lincecumii*	535:540
'Gordin'	582	*Vitis yeshanensis*	575
'Alexandrouli'	582	*Vitis solonis (syn. V. acerifolia)*	575
'Tsitska'	582	*Vitis vulpina*	535:540
'Bazaletouri tsolikouri'	582	*Vitis longii puncee*	540:575
'Dzhandzhal kara'	582	*Vitis pagnucci*	550
'Kabarcik'	582	*Vitis riparia*	535:540
Vitis romanetii	571:582	*Vitis slarini*	538
Vitis coignetiae	582	*Vitis dalniana*	540:575
Vitis amurensis	582:602	*Muscadinia rotundifolia*	570:575
Vitis silvestris	582:590	*Riparia portalis*	540
Vitis aestivalis	550	*Rupestris metallica*	570:575
Vitis candicans	550:560	*Riparia sauvage*	540:575
Vitis cinerea	550	*Parthenocissus quinquefolia*	440
Vitis monticola	550		

Vitis species of different origin are labelled with colours: blue: *Asian Vitis species*, purple: *North American Vitis species,* green: *Muscadinia rotundifolia and Parthenocissus quinquefolia*, black: *V. vinifera* cultivars and *V. silvestris*, orange: *rootstocks*.

The BAC libary based 20D18CB9 marker is suitable to distinguish *V. vinifera* cultivars from wild *Vitis* species without sequencing, making a PCR and a polyacrilamid gel electrophoresis. It has been proven that PM resistant cultivars of Middle Asian origin belong to *V. vinifera*.

The powdery mildew resistant 'Kishmish vatkana' and 'Dzhandzhal kara' varieties are valuable for grape breeders, because they open up the possibility of combining the resistance gene (*Ren1*) with high quality in *V. vinifera.*

New scientific results

1. We have developed a method to prove the presence of the pyramided resistance genes (*Run1, Rpv1, Ren1*) in the BC_4 x 'Kishmish vatkana' hybrid family, and we were able to select the genotypes carrying these resistance genes together or separately.
2. We were the first to prove that SSR markers used to map *Ren1* powdery mildew resistant gene is appropriate for MAS.
3. We have developed a method, based on multiplex PCR and agarose gel electrophoresis to select genotypes carrying *Run1/Rpv1/Ren1* resistance genes in a single step./ To further streamline the selection process, we developed a multiplex PCR-based method and agarose gel electrophoresis of the resulting amplicons.
4. We have proved that VVim11 SSR marker is appropriate to follow *Rpv1* downy mildew resistance gene, and can be used for MAS (results based on BC_4 x *V. vinifera* 'Kishmish moldavskij' population).
5. We have verified that SSR markers tightly linked to resistance genes can be applied to select another hybrid population, where one of the parents is the resistant donor.
6. With analysis of 07-12 hybrid population (*V. vinifera* 'Dzhandzhal kara' x *Vitis* hibrid 'Laszta') x *(V. vinifera* 'Katta kurgán' x *V. vinifera* 'Perlette') we have confirmed that PM resistant 'Kishmish vatkana' and 'Dzhandzhal kara' varieties have the same PM resistance gene (*Ren1*).
7. We have determined that molecular markers linked to PM QTLs in 'Regent' are not appropriate for marker assisted selection in 07-12 hybrid population. We proposed mapping QTLs in 'Laszta' interspecific hybrid in order to identify new molecular markers.
8. We were the first to determine that PM QTL on LG 15 of the Hungarian bred 'Viktória gyöngye', 'Nero', 'Zala gyöngye' and 'Bianca' can be follow by molecular markers, accordingly these varieties can be used in gene pyramiding breeding programs.
9. We have determined the microsatellite fingerprint of Asian cultivars using SSR markers recommended by GrapGen06 project, based on cluster analysis we have verified that PM resistant Central Asian varieties have smaller genetic distance to *V. vinifera* than to wild species or rootstocks.
10. We have proven by using molecular markers that PM resistant Asian varieties belong to *V. vinifera*.

DISCUSSION AND RECOMMENDATIONS

Our results have proven that genotypes showing the same phenotype but carrying different resistance genes can be selected by molecular markers tightly linked to these genes. Based on our method we are able to select the valuable, resistant offsprings from a segregating hybrid population in an early stage routinely, saving money and efforts. The results show that maintaining a huge segregating progeny, continuously screening the resistance status and reselecting the population can be avoided. We are able to determine after the process DNA isolation and PCR whether springs possess PM resistance gene or not, and which PM resistance gene they contain. Although the aim of the molecular analysis of the cross BC_4 x 'Kismis vatkana' was to pyramid PM resistance genes (*Ren1, Run1*) into one genotype, we propose to evaluate the resistance of downy mildew in order to confirm the applicability of VVim11 SSR marker to track DM resistance gene (*Rpv1*) in this population as well.

Screening the cross 'Génuai zamatos' x 'Kishmish vatkana' with the *Ren1* linked marker VMC9h4.2 enabled us to select the PM resistant genotypes easily in agarose gel. Our results show that the objective of combining resistance and high quality can be achieved by intraspecific crossing and via following the resistance gene by MAS.

The molecular analysis of (*V. vinifera* 'Dzhandzhal kara' x *Vitis* hibrid 'Laszta') x *(V. vinifera* 'Katta kurgán' x *V. vinifera* 'Perlette') hybrid population verified that the PM resistant 'Kishmish vatkana' and 'Dzhandzhal kara' originating from Central Asian harbour the same resistance gene, *Ren1*. The other resistant parent of this population is the interspecific variety 'Laszta'. The PM QTLs of 'Laszta' can not be followed with SSR and SCAR markers known so far linked to QTLs of 'Regent', therefore we propose to generate a test cross in order to map these QTLs and to identify new molecular markers.

According to our results we recommend to identify additional molecular markers linked to QTLs in varieties of 'Seibel' or 'Seyve Villard' origin bred in Hungary ('Duna gyöngye', 'Csillám', 'Palatina', 'Göcseji zamatos', 'Medina') that makes it possible to follow the PM and DM QTLs of these cultivars.

We have demonstrated the genetic distance of Asian cultivars (belonging to *V. vinifera* convar. *orientalis*) to other *V. vinifera* (convar. *occidentalis* and *pontica*) varieties, wild *Vitis* species and rootstocks on dendrogram based on SSR analysis (VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VVS2, VrZAG62, VrZAG79). We have involved reference varieties in the analysis to prove the correctness of our results. We have proven that

‘Kishmish vatkana’ and ‘Dzhandzhal kara’, which differ from other *V. vinifera* varieties morphologically and are PM resistant, belong to *V. vinifera*. We have identified a molecular marker enabled us to distinguish *V. vinifera* varieties from wild *Vitis* species without sequencing, applying only PCR and polyacrylamid gel electrophoresis.

We have characterized PM resistant Asian varieties with markers linked to known resistance genes (*Run1/Rpv1, Ren1*). Based on our results variety ‘Rezisztens magvatlan’ (Resistant seedless) has the same PM resistance gene than ‘Kishmish vatkana’, and they are closely related. In the case of the other varieties we have not found matching alleles, meaning that resistance genes of these varieties have not been identified yet. Crossing these varieties with sensitive ones, it is possible to map their resistance genes and it provides facilities to identify and involve new resistance sources into breeding programs. The Turkish variety ‘Kabarcik’ can be promising in this aspect.

According to our results gene pyramiding breeding program can be set up applying MAS, where resistance genes (*Ren1, Run1, Rpv1*, PM QTLs) can be followed reliably by molecular markers. For example applying genotypes from BC_4 x ‘Kismis vatkana’ population carrying resistance genes (*Ren1+/Run1+/Rpv1+* genotypes) in a cross with ‘Bianca’ (all PM QTLs-this Ph.D. thesis- and DM QTLs -*Rpv3* and *Rpv7*, Bellin et al. 2009- can be tracked by molecular markers), enabling us to select *Ren1+/Run1+/Ren3+/Rpv1+/Rpv3+/Rpv7+* genotypes from the progeny.

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APPENDIX

A/Table 1: SSR profile of sensitive varieties with markers linked to resistance

	Ren1		*Run1/Rpv1*	
Name of varieties	VMC9h4.2	UDV20a	VMC8g9	VMC1g3.2
BC_4	282:298	148:148	**160**:167	**122**:140
'Kishmish vatkana'	262:**286**	138:**164**	167:174	122:140
'Cardinal'	289:307	138:148:152:158	179:179	135:140
'Csaba gyöngye'	264:289	138:152:162	179:179	118:135
'Irsai Olivér'	289:312	138:152	179:202	118:140
'Madeleine angevine'	289:289	138:152	176.179	118:128
'Muscat Fleur d'Oranger'	264:312	138:162	179:205	128:135
'Kadarka'	289:307	135:148:158	179:179	140:140
'Pozsonyi'	282:312	138:148:162	167:202	128:140
'Kossuth szőlő'	289:289	138:152	176:179	118:128
'Duchess of Buccleugh'	264:282	138:148:162	164:205	128:128
'Izsáki'	262:262	128:152:162	167:174	118:128
'Kövérszőlő'	276:276	138:160	174:179	128:135
'Leányka'	282:282	128:138:152	167:172	128:128
'Királyleányka'	289:289	128:135:152:158	172:176	128:128

BC_4 and 'Kishmish vatkana' are references. Allele sizes previously associated with resistance markers are shown in bold and underlined.

A/Table 2: SSR profile of Asian varieties and interspecific hybrids ('Laszta' és 'Regent') with markers linked to resistance

	Ren1		*Run1/Rpv1*	
Fajta neve	VMC9h4.2	UDV20a	VMC8g9	VMC1g3.2
BC_4	282:298	148:148	**160**:167	**122**:140
'Kismis vatkana'	262:**286**	138:**164**	167:174	122:140
'Kabarcik'	262:276	138:148	167:176	135:140
'Dzsandzsal kara'	280:**286**	148:**164**	167:174	124-128
'Laszta'	252:290	148:148	162:178	128-135
'Regent'	262:282	142:148	174:174	128:140
'Tagobi'	266:298	148:162	176:176	124:128
'Gordin'	282:308	148:158	167:176	118:128
'Alexandrouli'	282:290	138:148:166	167:167	128:135
'Tsitska'	298:302	148:148	176:176	118:118
'Bazaletouri tsolikouri'	282:298	138:148	167:176	118:135
'Rezisztens magvatlan'	254:**286**	148:**164**	172:174	124:124
'Iszpiszár'	276:276	138:156:166	174:174	124:144
'Icskimár'	262:276	138:148:166	167:174	128:144

BC_4 and 'Kishmish vatkana' are references. Allele sizes previously associated with resistance markers are shown in bold and underlined.

APPENDIX

A/Table 3: Allele sizes of varieties, species and rootstocks applied to determine the genetic distance of Asian varieties in 9 SSR locus

	VVMD5	VVMD7	VVMD25	VVMD27	VVMD28	VVMD32	VVS2	Vrzag62	Vrzag79
'Chardonnay'	236:240	243:247	242:258	182:190	220:230	241:273	138:144	192:200	246:248
'Pinot noir'	230:240	243:247	242:252	186:190	220:238	241:273	138:152	192:198	242:248
'Kishmish vatkana'	236:242	243:253	242:242	180:196	220:236	251:273	138:146	192:206	250:262
'Dzandzsal kara'	236:242	247:253	244:248	180:196	236:260	251:273	126:156	192:200	250:250
'Tagobi'	230:236	249:257	244:258	180:186	246:246	259:259	126:144	192:200	250:254
'Gordin'	228:248	243:243	242:258	180:180	230:238	265:273	134:134	192:200	240:262
'Alexandroulii'	238:242	251:251	242:258	180:186	236:246	263:263	144:154	194:208	240:254
'Tsitska'	228:236	243:257	242:258	186:186	238:260	263:273	144:144	200:200	254:254
'Bazaletouri tsolikouri'	228:236	253:257	242:258	180:186	238:260	251:263	144:144	200:200	242:254
'Kabarcik'	238:242	251:251	242:252	186:186	238:250	251:273	138:138	192:192	254:260
'Rezisztens magvatlan'	236:240	239:257	252:252	186:196	220:228	251:251	126:152	192:206	254:270
'Icskimár'	236:242	247:257	252:260	186:196	236:246	251:257	142:152	192:200	252:260
'Iszpiszár'	226:242	247:257	250:260	186:196	246:246	251:257	142:156	192:192	254:260
'Nimrang'	230:236	247:251	252:260	186:196	238:246	251:273	144:152	192:200	254:260
'Szultanina'	236:236	243:257	242:252	182:196	220:246	251:251	146:152	192:192	250:262
'Katta kurgán'	236:242	251:257	242:250	182:196	236:246	257:273	134:156	192:192	250:260
'Kismis moldavszkij'	236:242	251:257	242:250	186:193	238:246	251:273	136:152	192:192	244:250
'Laszta'	240:240	253:255	242:252	180:190	238:238	257:257	134:150	190:198	258:264
'Dzhandhsal kara' x 'Laszta'	240:242	247:255	250:252	180:190	238:260	251:257	150:156	192:198	250:264
V. romanetii	248:248	247:249	254:258	186:186	222:240	249:249	130:130	220:220	250:250
V. coignetiae	236:242	243:255	243:246	184:188	236:236	239:239	134:140	192:198	242:242
V. amurensis	236:236	245:245	250:264	192:212	230:246	249:249	130:142	192:204	260:260
V. silvestris	238:238	243:251	242:252	184:190	238:242	251:273	134:134	194:198	254:254
V. yeshanensis	238:240	235:251	244:258	180:198	238:238	231:273	136:144	196:196	258:258
Riparia portalis	268:268	255:269	240:240	200:212	218:246	237:237	142:146	196:204	258:262
Rupestris metalica	254:254	255:265	240:246	186:206	218:248	241:241	142:142	202:208	262:264
Riparia sauvage	228:232	235:251	244:254	196:208	218:250	241:259	132:146	178:208	252:258
Rupestris du Lot	228:262	235:251	244:254	186:196:208	218:250	241:259	136:146	178:194:208	240:258
'Kadarka'	228:228	251:259	242:258	186:196	230:262	273:273	136:136	192:208	252:252

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