PhD THESIS

Rise of the effectiveness of the grape breeding with the investigation of the genetic background of the species

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Budapest, 2006

PhD School

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(1.Life Sciences, /1.5. Biological Sciences/; 4. Agricultural Sciences, /4.1. Plant Production and Horticultural Sciences/)

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1. Introduction, goals

The grape is one of the most ancient cultivated plants of the mankind. Extraordinary rich assortment came into existence through the time between the changing environmental conditions to the satisfaction of economical-commercial intentions. The success of table- and wine-grape growing is basically determined by the use of varieties.

Today, it is more important than ever to identify varieties correctly. Not only is the accurate identification important to nurseries, growers and winemakers, but modern international trade regulations and wine labelling laws require that varietally labelled wines be correctly identified (MEREDITH, 2001).

The developments of molecular markers, within that first of all the application of DNA technologies provide several new opportunity to the augmentation of the genetic knowledge of the grape. The DNA markers make the research of the origin of present grape varieties easier, and they help to breed new varieties.

In the case of grape the analysis of more molecular markers became widespread. From these, in other countries, first the analysis of isoenzymes, in the last years followed by SSR (Simple Sequence Repeats) analysis was done. In my dissertation I intend to analyse 48 *Vitis vinifera* L. varieties by these methods.

In the frame of isoenzyme analysis our aims were to characterise the investigated varieties by the most polymorph enzymes reported in the literature (catechol-oxidase, acid phosphatase, glutamate-oxalacetate transaminase, peroxidase, esterase, glucose-phosphate isomerase and phosphoglucomutase), and to develop the most appropriate gel-system for this.

The microsatellite markers belong to the most efficient types of DNA markers, providing individual profile of every variety, allowing unambiguous identification, which is not

influenced by the environment, diseases, or the growing technology.

For the microsatellite analyses we wish to characterise *Vitis vinifera* L. varieties and intraspecific hybrids by seven primer pairs, reported to be efficient for variety identification (VVS2, VVS16, VVMD7, VMC4G6, VMC4H6, VMC4A1 and VrZag79). As primary conditions, we aimed to optimize the PCR reactions and the constitution of the reaction solution.

In the evaluation of our results our aim was to investigate the suitability our results for taxonomical research, to look for the connection between the isoenzyme and microsatellite profile and the geographical-ecological group (*convarietas*) of one variety.

The name of the grapevine cultivar 'Kéknyelű' has become inseparable from the name of the Badacsony vine region, it's fame is well known beyond our frontier as well. In the Internet, the Vitis International Variety Catalogue (http://www.genres.de/idb/vitis/) we find the 'Kéknyelű', as the synonym of the Italian grapevine cultivar 'Picolit'. The morphological similarity of these varieties was reported further as well. As the name of 'Kéknyelű' itself has a serious marketing value, I kept it extremely important to confirm the difference of these varieties, by molecular markers.

2. Results, evolutions and conclusions

2.1. Results of the isoenzyme analysis, evolution of the results and conclusions

The isoenzyme patterns of 8 enzymes were analysed by vertical polyacrilamide gel electrophoresis. The same enzyme extract of one variety was used for the analysis of all the 8 enzymes. Samples were collected seven times (in 2003 and in 2004 3 times, after defoliation, in January and in March, in 2005 ones in January). The analyses were made in 3 repetitions per

samples.

Based on the enzyme patterns of cathecol-oxidase, glutamate-oxalacetate transaminase, acid phosphatase and peroxidase, the varieties were characterised and sorted. The patterns by enzymes are shown in **figure 1**.

There was no differences between the varieties in the case of leucine aminopeptidase (LAP), the same pattern was observed for all the samples. It failed to get rateable patterns in the case of glucose-phosphate isomerase (GPI) and phosphoglucomutase (PGM) even with the modification of the gel system. In the case of esterase (EST), the pattern was so comlex, that without the help of computer program it is almost impossible to evaluate it. The isoenzyme patterns were not or just partly reproducible. Because of the hard evoluation and the lack of reproducibility the results of esterase were not used in the establishment of the genetic distances of the varieties.

Summarizing the results it can be established, that according to literature data (ROYO et al., 1997), the isoenzyme banding pattern of the varieties for cathecol-oxidase (CO), acid phosphatase (AcP), glutamate-oxalacetate transaminase (GOT), peroxidase (PER), when the sampling is in the resting period, not depend of the place and time of the sampling.

From the characterised varieties Cabernet sauvingnon and Chardonnay were analysed with poliacrilamide gel electrophoresis in similar gel-system by ROYO et al. (1997). Comparing the results reported in this article with our results for these two varieties it can be established, that for all of the 4 enzymes (AcP, CO, GOT, PER) the same number of bands were detected. When you use one of these varieties for standard, the pattern of the other variety is the same, so these results supposedly are the same.

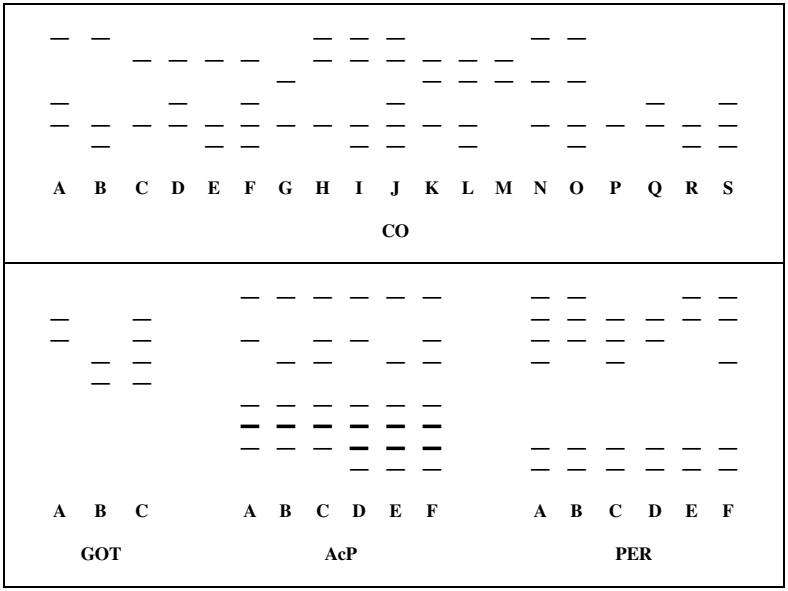

Figure 1: Isoenzyme banding pattern types for CO, GOT, AcP and PER

Out of the isoenzyme patterns of native gel electrophoresis the cathecol-oxidase showed the highest polymorphism. The acid phosphatase system showed quite good diversity, while in the peroxidase and glutamate-oxalacetate transaminase systems, the polymorphism of the patters was lower.

I tried to separate the varieties based on the isoenzyme banding patterns. According to the used marks (**Figure 1**), the varieties were ranked (**Table 1**). Most of the warieties can be identified in this way. The non-identifiable varieties were grouped based on their isoenzym patterns.

Table 1: Separation of the analysed varieties based on their isoenzyme patterns

Variety	CO	GOT	ACP	PER	Type no.	Variety	CO	GOT	ACP	PER	Type no.
Leányka	A	A	B	D	1	**Ezerjó**	H	C	B	C	22
Királyleányka	A	C	A	D	2	**Sauvignon**	H	C	C	A	23
Pozsonyi fehér	B	A	F	D	3	**Semillon**	H	C	C	A	23
Zefir	C	A	A	B	4	**Csomorika**	H	C	D	B	24
Tramini	D	A	A	C	5	**Vulcanus**	I	A	A	F	25
Kékoportó	D	A	B	D	6	**Cabernet franc**	I	A	C	D	26
Fehér góhér	D	A	D	D	7	**Cabernet sauvignon**	I	A	C	D	26
Arany sárfehér	D	C	F	D	8	**Pintes**	I	A	D	D	27
Rajnai rizling	E	A	C	E	9	**Bakator (tüdőszínű)**	I	B	D	A	28
Zeus	E	A	C	D	10	**Zengő**	J	C	C	D	29
Chardonnay	E	C	A	C	11	**Ottonel muskotály**	K	A	A	D	30
Zenit	E	C	A	D	12	**Kövérszőlő**	L	C	F	D	31
Bouvier	F	A	A	D	13	**Kövidinka**	M	C	D	C	32
Zöld szilváni	F	A	C	B	14	**Sárga muskotály**	N	C	C	D	33
Cirfandli	F	A	F	B	15	**Kékfrankos**	N	C	C	A	34
Juhfark	F	C	A	D	16	**Picolit**	O	A	E	E	35
Pinot blanc	F	C	A	D	16	**Hárslevelű**	O	C	D	D	36
Pinot noir	F	C	A	D	16	**Kadarka**	O	C	F	D	37
Szürkebarát	F	C	A	D	16	**Olasz rizling**	Q	A	A	B	38
Zöld veltelíni	F	C	A	C	17	**Rózsakő**	Q	A	A	F	39
Chasselas (fehér)	F	C	C	C	18	**Badacsony-43**	Q	C	A	B	40
Budai	G	A	C	B	19	**Badacsony-15**	Q	C	B	B	41
Furmint	G	A	E	D	20	**Kékmedoc**	R	B	C	D	42
Kéknyelű	G	C	A	D	21	**Zéta**	S	A	E	D	43

Varieties with the same patterns for 4 enzymes were got into one group. They were the followings: group no. 16: Pinot blanc, Szürkebarát, Pinot noir, Juhfark; group no. 23: Sauvignon blanc, Semillon; group no. 26: Cabernet franc, Cabernet sauvignon.

It is apparent from the results, that most of the varieties (40 from 48) can be distinguished by their isoenzyme patterns for this 4 enzymes.

It is characteristic for most of the non-distinguishable varieties, that they are morphologically very similar, and some

of them (the Pinots from the group no. 16) – based on the literature -are not or hardly distinguishable even with DNA markers as well (CIPRIANI et al., 1994; HALÁSZ et al., 2005).

For the establishment, if there is a connection between the phenotypic features and the patterns get from the isoenzyme analyses, the connection between that one variety belongs to one convarietas, and the isoenzyme features of this variety, and the determination the convarietas of one variety from isozyme data were investigated. For the findings of this, cluster analysis was performed by SPSS 14.0 statistic analysis program. The varieties were recorded by the origin system into 3 groups (according to NÉMETH, 1967, or TÓTH and PERNESZ, 2000). The preset of one isoenzyme band of one variety was recorded to the program as independent variable. The separation of the grape varieties by two discrimination function is presented (**Figure 2**).

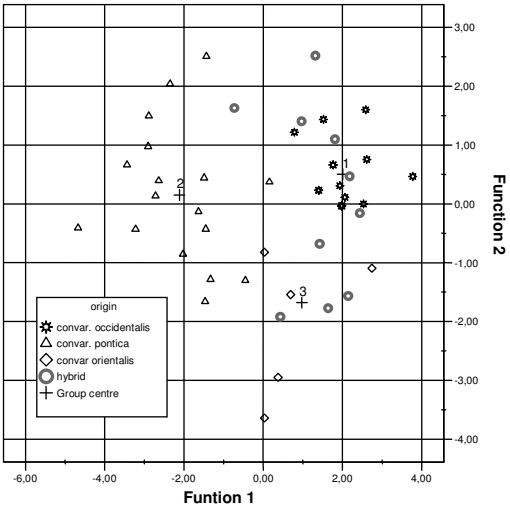

Figure 2: Separation of the origin groups of *Vitis vinifera* L. varieties by isoenzyme patterns.

The highest coefficient in function 1 in absolute value was detected in AcP7: 1,008. If you have a look at , in what kind of varieties the acid phosphatase band no. 7 appears, than you can find, that almost solely appears in the range of pontican varieties, in more than 2/3 part of them have this band in their pattern, in the other two groups it appears only in the 'Cirfandli' variety. To find out, if the connection between the appearance of this isoenzyme band and that one variety belongs to the pontican group, χ^2 test was made. The value of χ^2 was: 15,28. The critical value of χ^2 in this case is: 3,84, as this value is much lower, than the calculated value, the connection is significant in 95% significance level. The index, which indicates the closeness of the connection is the contingence coefficient, of which value in this case: r=0,63.

You can draw the inference from this, that the indicated isoenzyme form is characteristic for the pontican varieties.

2.2. Results, discussion of the microsatellite analysis and conclusions

In table 1 indicated 48 grapevine varieties were analyses by microsatellite (SSR) analyses. The quantity of DNA in the samples were determined by a photometric method. According to the measure, the quantity of DNA is connected with the time of sampling. The quantity and quality of DNA was better in the resting period from the phloem extracted samples, than in the extractions made from the young (before bloom sampled) leves.

The PCR reaction was optimized according to literature data (HAJÓSNÉ NOVÁK, 1999). In the optimizing reactions the DNA extract of 5 varieties were used. The reaction products were verified in TAE- agarose gel, the exact length of the fragments were determined by automatic sequencing apparatus. The results are summarized in **table 2**.

Table 2: Microsatellite (SSR) fragment lengths

Name of the variety	VMC4A1		VVS2		VrZag79		VMC4G6		VVMD7		VVS16	
Arany sárfehér	259	261	140	150	232	238	122	126	237	251	291	291
Badacsony-15	265	275	140	148	234	246	122	124	235	241	285	285
Badacsony-43	269	271	134	150	232	232	122	122	237	247	285	285
Bakator (tüdőszínű)	259	261	134	140	244	246	122	130	237	251	285	285
Bouvier	265	267	130	148	234	246	122	122	241	241	285	285
Budai	271	275	140	150	246	246	136	138	247	247	285	291
Cabernet franc	269	271	136	144	242	254	128	132	237	261	283	285
Cabernet sauvignon	267	271	136	148	242	242	122	132	237	237	285	285
Chardonnay	271	275	134	140	238	240	122	126	237	241	285	291
Chasselas	273	275	130	140	246	254	132	138	237	245	285	285
Cirfandli	265	267	130	130	240	246	122	122	241	251	263	285
Csomorika	261	275	130	130	232	254	120	124	237	247	289	291
Ezerjó	265	267	130	140	232	244	120	126	237	237	285	285
Fehér góhér	261	261	130	130	244	254	122	126	237	247	285	289
Furmint	269	271	130	150	232	244	128	138	237	247	285	291
Hárslevelű	267	271	130	142	232	246	122	128	237	245	285	285
Juhfark	263	263	132	142	230	242	120	128	237	245	285	291
Kadarka	259	261	130	140	246	252	130	140	237	247	263	291
Kékfrankos	277	279	140	142	232	246	122	128	237	247	263	289
Kékmedoc	275	275	142	150	244	248	122	132	241	245	285	285
Kéknyelű	273	275	130	148	246	246	120	128	237	241	285	285
Kékoportó	265	267	142	150	242	252	122	128	241	253	263	285
Királyleányka	269	271	128	130	242	244	126	126	245	247	285	291
Kövérszőlő	267	267	130	142	232	246	130	140	237	253	285	291
Kövidinka	259	261	130	132	242	244	128	128	245	253	285	285
Leányka	269	271	130	130	232	246	122	124	247	251	263	285
Olasz rizling	271	279	132	148	244	246	122	122	245	255	285	285
Ottonel muskotály	273	275	130	140	248	252	122	132	237	241	285	285
Picolit	265	267	132	136	234	254	120	120	243	243	285	285
Pinot blanc	267	275	134	148	234	240	122	122	237	241	285	285
Pinot noir	267	275	134	148	234	240	122	122	237	241	285	285
Pintes	265	267	136	150	238	240	122	122	237	255	285	285
Pozsonyi fehér	259	261	132	152	244	246	124	128	247	253	285	289
Rajnai rizling	269	271	140	150	238	240	122	126	247	255	285	291
Rózsakő (B-36)	275	275	148	150	246	246	128	128	237	247	285	285
Sárga muskotály	267	279	130	130	246	250	122	138	231	247	285	285
Sauvignon	267	267	130	148	240	242	122	124	237	255	285	285
Semillon	265	267	130	130	242	246	122	138	237	255	285	285
Szürkebarát	267, 265	275, 273	134	148	234	240	122	122	237	241	285	285
Tramini	265	267	148	150	238	244	122	122	241	255	285	285
Vulcanus (B-38)	269	271	134	150	232	246	122	138	237	247	285	291
Zefir	267	267	146	148	234	246	122	122	231	241	285	285
Zengő	265	265	130	140	232	246	120	122	237	241	285	285
Zenit	265	267	130	148	232	246	122	126	237	241	285	285
Zéta	267	271	130	130	234	244	122	138	241	247	285	291
Zeus	265	267	130	140	232	246	120	122	237	241	285	285
Zöld szilváni	265	267	150	150	242	244	122	128	241	245	285	291
Zöld veltelíni	269	271	130	150	240	244	122	128	245	255	285	285

The analyses of some varieties of these were made by other researchers as well, so we had the opportunity partly compare the results. If the Pinot varieties are used as standards in the evaluation of results, it can be established, that

the fragment lengths in our results are the same in almost every case, that the other researchers get, but there are small differences in some case. There can be more reasons of the deviations. Possible reason can be the different plant material, it is just possible, that in this cases the reason can be the variability in one variety. Where there is only 1-2 bp. difference between the fragment lengths, there it can originated from measure mistake.

There was no opportunity for the comparison of the results in the case of the VMC4A1, VVS16 and VMC4G6 microsatellite primers, as we find no data in the literature. Based on literature data it can be established, that our fragment lengths are in the expected range.

Based on the results, the loci were statistically evaluated. Based on this results it can be established, that the number of genotypes were the highest in the case of the VVS2 and VVMD7 primers, so most of the varieties were identificable by these SSR markers.

The SSR markers proposed by the Vitis Microsatellite Consortium showed high variability. Few of varieties were analysed by the VMC4A1 and VMC4G6 primers hitherto, but it can be established, that these show quite high variability, so their use in the future is suggestible. Based on our results, the VVS16 primer showed low variability, only 4 genotype were detected in the range of the analysed varieties.

On the dendrongram based on the results, you can't see clear groups according to the geographical-ecological variety groups, but the varieties in the same group are generally closer to each other, than to the others. It can be established, that the Italian variety 'Picolit' isolate from the others, so from the 'Kéknyelű' as well (**Figure 3**).

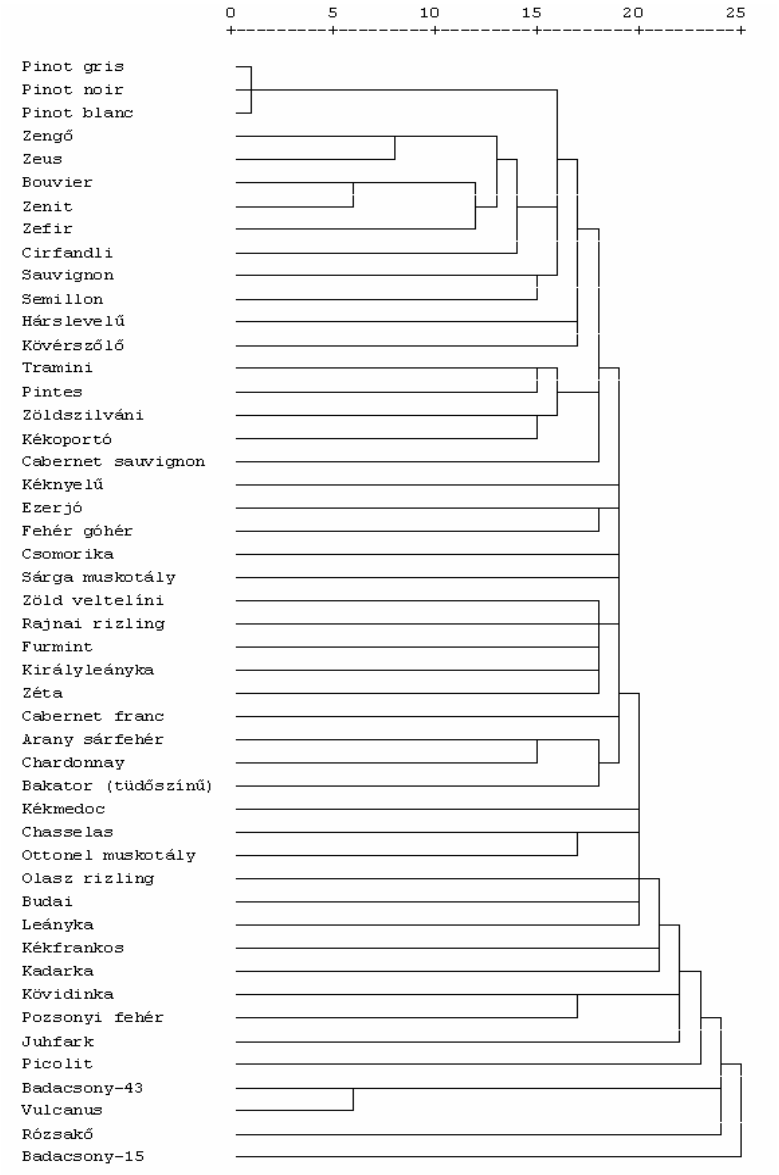

Figure 3: The dendrogramm of the analysed varieties based on the microsatellite result**s**

For the establishment, if there is a connection between the phenotypic features and the results get from the SSR analyses, the connection between that one variety belongs to one convarietas, and the microsatellite alleles of this variety, and the determination the convarietas of one variety from microsatellite data were investigated.

For the findings of this, cluster analysis was performed, with the same method as in the isoenzyme analyses by SPSS 14.0 statistic analysis program.

The program separated the varieties by two functions. For the analyses of the connection between the microsatellite alleles and the convarietas group of one variety χ^2 tests were made in the case of coefficients of high value, but we find no connection in 95% significance level.

By the study of the results it struck, that the 'Szürkebarát' variety gave 4 fragments instead of 2, in every repetitions of the VMC4A1 primer, it gave the same fragments as its close relative the 'Pinot blanc' (267, 275), and 2 more alleles, where the fragments lengths were 2 bp. Shorter (265, 273).

Although in the analyses the same alleles with the 'Pinot blanc' were taken into consideration, the additional two alleles were formed in every case during the PCR reaction, in the comparison of the two varieties it must taken into account in any case. On the basis of all these it can be stated that, most of the analysed varieties can be separated from each other by the 6 used primers.

2.3. Evaluation of the isoenzyme and microsatellite results

The data get from the alternative molecular markers show more precisely the similarity or diversity of the varieties. Considering that, the results were evaluated together as well.

Table 3: The classification of the varieties based on the isoenzyme or microsatellite results

Variety	isoenzyme		microsatellite		Variety	isoenzyme		microsatellite	
	original	calculated	original	calculated		original	calculated	original	calculated
Cabernet franc	1	1	1	1	Budai	3	1	3	3
Cabernet sauvignon	1	1	1	1	Csomorika	3	3	3	3
Cirfandli	1	1	1	1	Ezerjó	3	2	3	3
Olasz rizling	1	1	1	1	Fehér góhér	3	3	3	3
Pinot blanc	1	1	1	1	Furmint	3	3	3	3
Pinot gris	1	1	1	1	Hárslevelű	3	3	3	3
Pinot noir	1	1	1	1	Kadarka	3	3	3	3
Sauvignon	1	1	1	1	Kéknyelű	3	3	3	3
Semillon	1	1	1	1	Kövidinka	3	3	3	3
Tramini	1	1	1	1	Királyleánynka	4	2	4	2
Zöld veltelíni	1	1	1	1	Kövérszőlő	3	3	3	3
Zöld szilváni	1	1	1	1	Kékfrankos	2	2	2	2
Bouvier	1	1	1	1	Picolit	3	3	3	3
Zéta	3	3	3	3	Pozsonyi fehér	3	3	3	3
Chasselas	2	1	2	2	Rajnai rizling	1	1	1	1
Juhfark	2	1	2	2	Chardonnay	1	1	1	1
Pintes	3	3	3	3	Zenit	4	1	4	1
Leányka	2	2	2	2	Zengő	4	2	4	2
Kékmedoc	2	2	2	2	Zefir	4	1	4	3
Kékoportó	2	2	2	2	Zeus	4	1	4	1
Sárgamuskotály	3	3	3	3	Badacsony-15	4	2	4	3
Ottonel muskotály	4	3	4	3	Badacsony-43	4	2	4	2
Arany sárfehér	3	3	3	3	Rózsakő	4	1	4	1
Bakator	3	3	3	3	Vulcanus	4	1	4	3

The discriminant analysis was made by the results of the alternative genetic markers together, but because the variability of microsatellite markers was much higher, the program took only these into consideration. During the discriminant analysis with the two different markers the program classified

the hybrid varieties as well; the results are shown in **table 3**.

In some varieties the original and the calculated group based on isoenzyme data are different (Chasselas, Juhfark, Budai, Ezerjó), they are underlined in the table. In this varieties it possible happened because they are originally of hybrid origin, only the parents were not identified yet.

The classification of the hybrid varieties were made by the results of both of the markers. The alternative markers gave the same results in most of the cases (seven from ten), when the parents of the hybrid variety are known and the two classification are different, there generally the first marker sorted the hybrid to one parent, the second to the other one, instead of the case of the Badacsony-15 candidate.

The similarity (Jaccard) indexes were calculated by the two results together, and dendrogramm was drown (**Figure 4**). Based on these results it is more conspicuous, tht the 'Picolit' variety how far differs from the other nalysed variety. It may be concluded that, the geographical diversity of the varieties can be related to their genetic background.

This supposition could be particularly true for the autochthon varieties, as in the Badacsony Wine District the 'Kéknyelű' or in the Friuli-Venesia Wine District the 'Picolit'. This feature can be more strong in the autochton varieties, because they adapted to a given growing area, this is the reason because they are grown only in a defined area.

It can be clearly established based on the results of the discriminant and cluster analysis that, there is a connection between the origin (convarietas) of the varieties and their profile with genetic markers, which confirm the hypothesis, that the forming of convarietases could have a strong genetic basis.

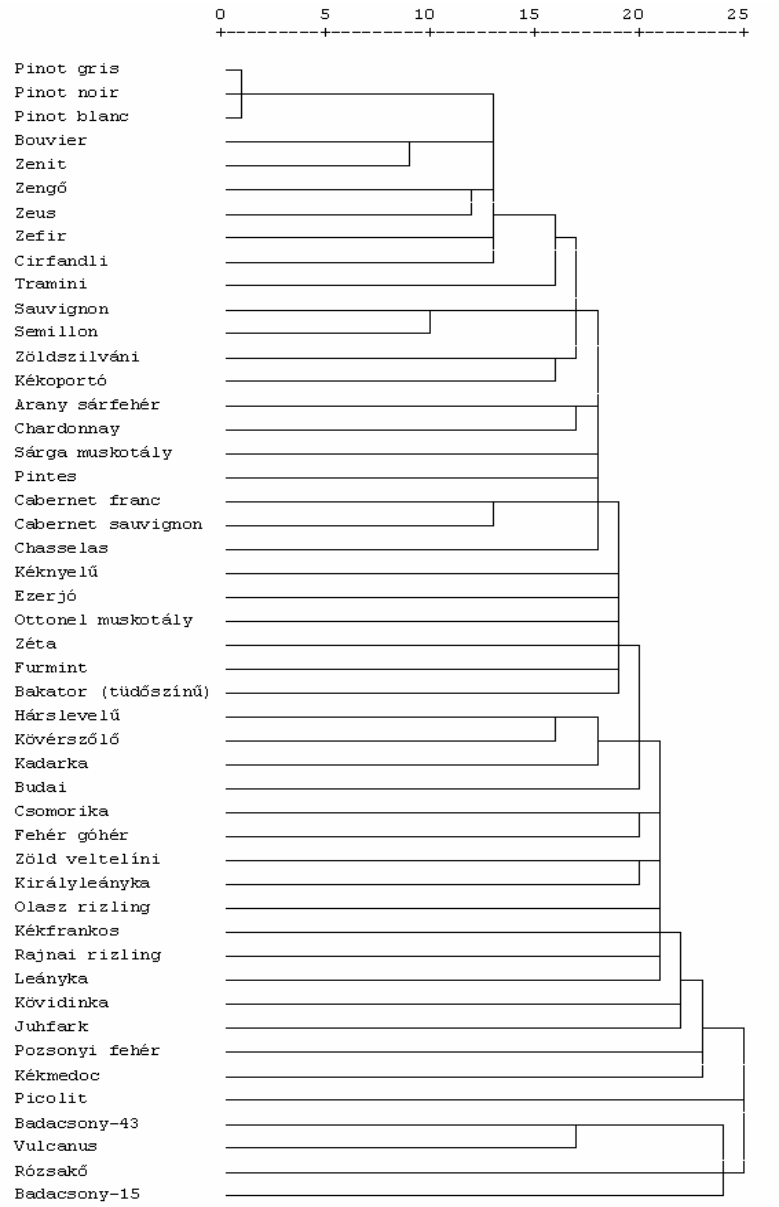

Figure 4: Dendrogramm of the varieties based on isoenzyme and microsatellite results

2.4. New scientific results

The following new scientific results could drown:

— An acid phosphatase pattern was found by isoenzyme analyses, which is characteristic for the pontican cultivars, the connection was verified by statistic analyses
— Connection was found between the origin group of the varieties and the isoenzyme pattern and microsatellite profile of the varieties
— The difference between the varieties ‘Kéknyelű’ and ‘Picolit’ was verified by isoenzyme and microsatellite analyses.
— The separation between the Pinot conculta of the ‘Pinot blanc’ and ‘Szürkebarát’ varieties by microsatellite marker was successful.
— In the grape growing of Hungary important varieties Were characterised by isoenzyme analyses.
— The analysed varieties were characterised by the VMC4A1 and VMC4G6 microsatellite markers for the first time.
— By the developed microsatellite and isoenzyme systems most of the varieties can be identified (46 from the 48), so this system can be used in the practice for the verification of variety identity.

3. Summary

The success of plant breeding is basically determined by the genetic polymorphism of the stock material, in the case of all species. During the cross breeding of plants, the highest the genetic variability of the offspring population is, the highest the genetic distance between the crossed parents is.

My aims were to investigate the isoenzyme patterns in 8 enzyme systems (CO, GOT, AcP, PER, EST, LAP, GPI, PGM), and to determine the microsatellite profile in 7 loci of 48 grapevine varieties.

In the case of the CO, GOT, AcP and PER enzymes the results were reproducible and the patterns of the woody stems were independent from the time of sampling in the resting period of the grape. Based on the isoenzyme patterns of these 4 enzymes the most of the investigated 48 varieties (40 varieties) were identifiable.

I find correlation between the isoenzyme patterns and the pertain to convarietas of the varieties. It was established, that while the varieties of the convarietas pontica differentiate from the varieties of the convarietas orientalis and occidentalis, the last two groups don't differentiate strongly from each other. I identified a special acid phosphatase isoenzyme banding pattern, which is characteristic for the pontican cultivars, but it seldom appears in other two groups. It was possible to evidence the otherness of the grapevine cultivars 'Kéknyelű' and 'Picolit' with both isoenzyme and microsatellite markers.

Based on my microsatellite analyses I was able to identify 46 varieties from the 48 investigated ones. Based on the results of the misrosatellite analyses with the VMC4A1 primers it was possible to differenciate 2 cultivars of the Pinot conculta, the 'Pinot blanc' and the 'Szürkebarát'.

Main publications of the author in the topic of the thesis

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