GENOTYPING GRAPEVINE ACCESSIONS WITH CHLOROPLAST MARKERS

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Using chloroplast markers ccmp 2, 3, 4 and 10 as well as ccSSR 4, 5, 6, 9, 12, 13, 15, 17 and 19, 210 accessions of grapevine were analysed. Due to the occurrence of seventeen different chloroplast genotypes we could characterize several grapevine cultivars and accessions. According to their maternal inheritance chloroplast markers are useful to prove part of the maternal parentage. We could also verify the kinship of cultivars like 'Zweigelt' ('Rotburger'), 'Blauburger' and 'Roesler' derived from newer crossings. In contrast to the previously determined parentage in the case of 'Sylvaner' chloroplast markers did not coincide. Finally, the 'Sylvaner' origin was confirmed by more than 40 nuclear SSR markers. Additionally weak polymorphism was observed between some European cultivars and genetically far distant rootstocks based on American Vitis species. For instance 'Pinot noir' and 'Blauburger' displayed the same profile with chloroplast markers than 'Kober 5BB' despite numerous different chloroplast genotypes within *Vitis vinifera*. On the other hand the analysis of several accessions of different old cultivars by chloroplast markers revealed unexpected variability within 'Traminer', 'Sylvaner' and 'Pinot gris' accessions. In the case of 'Traminer' we verified the differences by cloning and sequencing of the fragments. It seems that despite generally high stability of the chloroplast genome, mutations can never be excluded. However, it can be concluded that cultivars with more than one chloroplast genotype were frequently propagated in the past and were distributed wider than others. Hence, differences of chloroplast genotypes within grapevine cultivars are rather the result of intensive vegetative propagation than that of natural evolution.
Keywords: *Vitis vinifera*, chloroplast genotype, genome, genetic profile, polymorphism, microsatellite

Charakterisierung von Rebakzessionen mittels Chloroplasten-Markern. Die Chloroplasten-Marker ccpm 2, 3, 4 und 10 sowie ccSSR 4, 5, 6, 9, 12, 13, 15, 17 und 19 wurden verwendet, um 210 Rebsorten und Typen zu analysieren. Es traten 17 verschiedene Chlorotypen auf, die zur weiteren Charakterisierung von Rebsorten und ihren Typen verwendet wurden. Durch die maternale Vererbung der Chloroplasten können diese Marker zur Abstammungsüberprüfung verwendet werden. Die Elternschaft der relativ jungen Rebsorten 'Zweigelt' ('Rotburger'), 'Blauburger' und 'Roesler' konnte bestätigt werden. Andererseits konnte für die Sorte 'Sylvaner' die bereits definierte Abstammung auf Basis von 40 SSR-Markern mit den Chloroplasten-Markern nicht bestätigt werden. Außerdem ergaben sich gemeinsame Profile von Sorten mit großer genetischer Unterschiedlichkeit, wie den europäischen Sorten und den Unterlagsreben. 'Pinot noir' und 'Blauburger' zeigten mit den Chloroplasten-Markern dasselbe Profil wie 'Kober 5BB' trotz guter Variabilität innerhalb der Vitis vinifera-Sorten. Zusätzlich entdeckten wir auch unerwartete Variabilität innerhalb der Sorten 'Traminer', 'Sylvaner' und 'Ruländer'. Für die Sorte 'Traminer' wurden die Unterschiede durch Klonieren der Fragmente und Sequenzierung der DNA bestätigt. Auch wenn die Chloroplasten-DNA als sehr stabil gilt, kann eine Mutation nie ausgeschlossen werden. Andererseits lässt eine höhere Variabilität innerhalb der Sorte auf eine stärkere Vermehrung oder größere Verbreitung schließen. Unterschiede der Chlorotypen innerhalb einer Sorte dürften eher der Vermehrung als der natürlichen Evolution zu zuordnen sein.
Schlagwörter: *Vitis vinifera*, Chloroplasten-Genotyp, Genom, genetisches Profil, Polymorphismus, Mikrosatelliten

The chloroplast genome in higher plants is more conserved than other DNA regions. It is often difficult to discriminate chloroplast profiles within one species (Grassi et al., 2002). Therefore chloroplast markers show low polymorphism and are not appropriate for identification systems within one species (Chung et al., 2006). Nevertheless mononucleotide repeats could be found within the chloroplast genome of higher plants and rarely these allow to find polymorphism. The flanking sequences are conservative enough that the primers can be used for numerous dicotyledonous plants. (Weising and Gardner, 1999).

These rare polymorphic loci within the chloroplast DNA are maternally inherited. Simple sequence repeats in the chloroplast genome are useful to investigate the spread of plant material by characterizing chloroplast genotypes. These biotypes were used to define several gene pools for grapevine in Europe (Imazio et al., 2006). Domestication centers for grapevine could be defined far away from Transcaucasia (Arroyo Garcia et al., 2006). The genetic characterization of grapevine is done nowadays by genotyping with six or nine nuclear SSR markers (This et al, 2004). Considering the genetic profile information can be gained about true-to-typeness and relationship of genetically close varieties.

In order to detect single nucleotide polymorphisms in the chloroplast genome with markers is more complicated than detection of other polymorphisms. Mutations happen not all the time at defined regions and can only be used if they are located between the primer regions. If the primer sequence is concerned the loss of the annealing side will result in loss of the alleles (Thomas et al., 1993). On the other hand chloroplast SSR markers are also available where the diversity mainly is based on polymorphism within dinucleotide repeats (Chung and Staub, 2003). The idea of the current study was to get additional information about grapevines and their relationship by analyzing the chloroplast genome of about 200 accessions, which were already characterized by numerous nuclear SSR markers. With maternally inherited markers the direction of a cross can be defined easily. The hypothesis that accessions of the same chloroplast genotype represent members of the same grapevine family is not consistent with gained results.

MATERIAL AND METHODS

The DNA of plant material was mainly gained from genotypes of the collection of the HBLA für Wein- und Obstbau Klosterneuburg. Some rare cultivars were used from collections of Freiburg and Neustadt in Germany and were involved in this study. 'Traminer' samples were received from the vine monument in Rhodt in Germany and 'Savagnin' samples from Colmar in France. 'Sylvaner' samples were introduced from non-clonal material of autochthonous samples, from 'Sylvaner blau' ('Szilvani kek') from Pécs in Hungary and from the only blue 'Sylvaner' clone ST 25 from Germany.

DNA was extracted from young leaves by following the protocol described by Thomas et al. (1993) and modified by Regner et al. (1998). The varieties involved in this study were characterized at least with 22 SSR and 4 chloroplast markers. The true-to-typeness was verified for all involved accessions.

The VVS and the VVMD markers were developed by Thomas and Scott (1993) and by Bowers et al. (1996 and 1999), respectively. The VRZAG markers were created by Sefc et al., (1999). Chloroplast markers ccmp 2, 3, 4, 10 and ccSSR 4, 5, 6, 9, 12, 13, 15, 17 and 19 were published by Weising and Gardner (1999) and by Chung and Staub (2003).

Amplification was performed in 20 µl volume containing 16 mM $(NH_4)2SO_4$, 67 mM Tris-HCl pH = 8.8, 1.5 mM $MgCl_2$, 0.01 % Tween 20, 0.1 mM each dNTP (GenXpress, Maria Wörth, Austria), 0.2 µM primer, 1 Unit SAVADY Taq DNA polymerase (Peqlab, Erlangen, Germany), and 50 ng genomic DNA of grapevine.

A Mastercycler thermocycler (Eppendorf, Hamburg, Germany) was used for SSR analysis in 40 cycles. The amplification of the SNP and SSR loci was performed by following our general protocol, but by applying specific annealing conditions. The general PCR protocol applied for these studies was 2 min. denaturation at 94 °C and 40 cycles with annealing phase for 30 seconds (temperature between 50 °C and 55 °C) and denaturation for 15 sec. at 92 °C. The annealing temperature for each locus was set to a temperature 10 °C below the estimated T_m. In order to avoid irregular shorter fragments a final extension of the fragments was performed at 72 °C for 5 min. Due to the different size range of the involved loci multiplex

PCR was feasible. At least the alleles of three loci were separated on one sequencing gel.

Yield of DNA fragments was estimated by running an aliquot of the sample on a 2 % agarose gel stained with Midori Green. The samples were denaturated by heating up with formamide and loaded together with a size standard (50 bp DNA ladder; Roth, Karlsruhe, Germany) to a 6 % polyacrylamid gel. Detection of the SSR fragments labelled with the fluorescent dyes (IRDye-700, IRDye-800; Metabion, Martinsried Germany) was carried out by an automated sequencer (NEN Model 4300 DNA Analyzer; Licor, Lincoln, USA). Labelling with these different fluorescent colouring agents facilitated the application of multiplex PCR. In some cases of labelling annealing of primers is of decreased efficiency. Therefore chloroplast markers were not labelled and fragments were detected by silver staining (Grassi et al., 2002).

RESULTS

Two hundred and ten accessions were genotyped with chloroplast markers ccmp 2, 3, 4 and 10 (Weising and Gardner, 1999) as well as ccSSR 4, 5, 6, 9,12,13,15, 17 and 19 (Chung and Staub, 2003). Polymorphism in chloroplast DNA is rare, nevertheless the markers ccmp 3 and 10 as well as ccSSR 5 and 9 provided single or dinucleotide differences (Fig. 1). We could detect 17 different combinations out of 36 possible ones (Table 1 and 1a). We did not use the null alleles for further creation of diversity in chloroplast genotypes though the lack of the allele at a locus was confirmed by the fact that other loci of the same genotype worked well. In 11 samples showed, at least one chloroplast locus failed in priming and amplification of the allele. It seems very clear that in contrast to other authors (Arroyo-Garcia et al., 2006) the differences observed by aid of this limited amount of markers will not allow calculating a relevant genetic distance between these accessions. We defined an identical profile within the most rootstock cultivars and it was not discernible from the profile of 'Pinot noir' (Table 2). The same chloroplast profile as 'Kober 5BB' was obtained with other rootstocks like 'Couderc 3309', 'Ruggeri 140', 'Teleki 5C', as well as the grapevine cultivars 'Blauburger' and 'Zierfandler'.

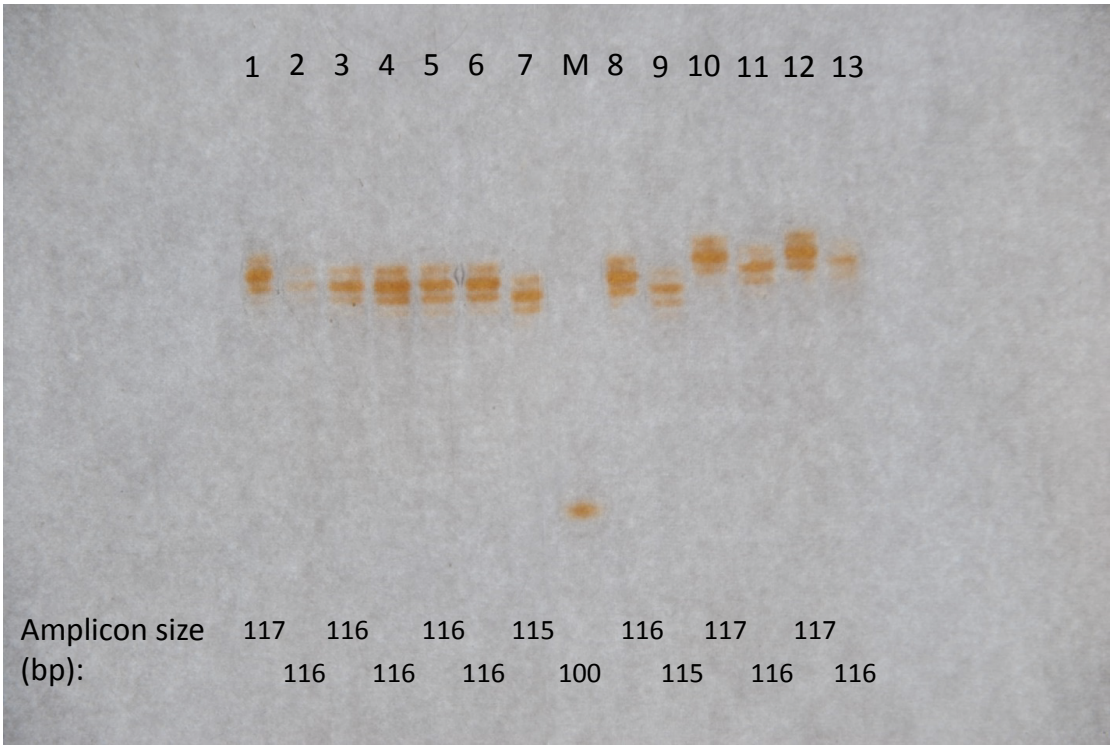

Fig. 1: PAGE gel for separation of chloroplast alleles ccmp10 stained with silver and 50bp length standard (lane M fragment 100bp); varieties: lane 1: 'Bl. Portugieser'; lane 2: 'Blauburger'; lane 3: 'Veltliner Red'; lane 4: 'Sylvaner'; lane 5: 'Pinot blanc'; lane 6: 'Sauvignon blanc'; lane 7: 'St. Laurent'; lane M: length standard; lane 8: 'Kober 5BB'; lane 9: 'Pinot gris'; lane 10: 'Österr. Weiß'; lane 11: 'Pinot noir'; lane 12: 'Traminer'; lane 13: 'Traminer' main.

Table 1: Variability of chloroplast genotypes within genotyped accessions

ccmp 10	ccmp 3	ccSSR 5	ccSSR 9	frequency
115	106	238	172	6
115	106	240	172	35
115	106	240	170	4
115	107	238	170	2
115	107	240	170	4
116	106	238	172	2
116	106	238	170	5
116	106	240	172	3
116	106	240	170	18
116	107	236	170	3
116	107	238	170	56
116	107	240	170	7
117	106	238	170	6
117	106	240	170	33
117	106	240	172	2
117	107	238	170	4
117	107	240	170	5

Table 1a: Occurring alleles of the 4 polymorphic chloroplast loci

ccmp 10	ccmp 3	ccSSR 5	ccSSR 9
115	106	236	170
116	107	238	172
117		240	

For some already defined crosses such as 'Zweigelt' ('Rotburger') and 'Roesler' the maternal inheritance from 'St. Laurent' was confirmed. (Table 3). We also verified a close relationship and possible maternal relationship for 'Veltliner Red' and 'Neuburger' as well as for 'Lagrein' and 'Teroldego'. In contrast at least in the parentage of 'Sylvaner' as a cross of 'Traminer' with 'Österreichisch Weiß' chloroplast markers did not prove the already satisfyingly confirmed heritage (Table 4). Therefore, which of the parents was the female vine, remains still unclear. An example for a newer cross with deviation of chloroplast genotype is 'Blauburger' which does not maintain only the parental alleles but shows a new size. At the locus ccmp10 'Blauburger' shows an allele with 116bp while the parents 'Portugieser' and 'Blaufränkisch' reach 117bp.

Among some experts chloroplast markers are regarded as very conservative and stable. Nevertheless in the current study we could detect variability of chloroplast markers within several varieties. We found allelic differences within the 'Traminer' accessions, within 'Sylvaner' and within 'Pinot' samples (Table 5). These differences were amazing and it was necessary to fortify the data by repetitions and independent reference samples. For instance the specific profile of 'Sylvaner' with red/blue berries was confirmed by one 'Szilvány kek' sample from Hungary and the only registered clone of this accession ST 25 from nursery Steinmann in Germany (Table 5). Finally we decided to sequence at least the polymorphism found within 'Traminer' types (Fig. 2) at the chloroplast marker ccmp10. In addition our study revealed a lack of coincidence of chloroplast alleles within the 'Pinot' family is not given despite a clear common SSR profile (Regner et al., 2006). Especially 'Pinot gris' differs from the profile of 'Pinot' (noir and blanc). Pinot gris rendered furthermore two independent profiles, one is identical with the profile of 'Schwarzriesling' ('Pinot Meunier') and the second one is an individual Pinot gris profil.

Table 2: Variability of chloroplast genotypes of a small sample of cultivars (same profile for 'Kober 5BB', 'Blauburger' and 'Pinot noir')

cultivar	ccmp 10	ccmp 3	ccSSR 5	ccSSR 9
Portugieser (N)	117	106	240	170
Blauburger	116	106	240	170
Merlot	117	106	240	170
Sankt Laurent	115	106	240	172
Zweigelt	115	106	240	172
Veltliner (R)	116	107	238	170
Kober 5BB	116	106	240	170
Pinot noir	116	106	240	170
Härtling	116	107	236	170

Table 3: Cultivars with genetic relationship by maternal inheritance show the same chlorotype

cultivar	ccmp 10	ccmp 3	ccSSR 5	ccSSR 9
Sankt Laurent	115	106	240	172
Zweigelt	115	106	240	172
Roesler	115	106	240	172
Veltliner (R)	117	106	238	170
Neuburger	117	106	238	170
Lagrein	117	106	238	170
Teroldego	117	106	238	170

Table 4: Parentage of well-known crosses with deviation (bold) of the chlorotype

cultivar	ccmp 10	ccmp 3	ccSSR 5	ccSSR 9
Traminer	117	107	238	170
Sylvaner	**115**	107	240	170
Österr. Weiß	117	106	240	170
Portugieser	117	106	240	170
Blauburger	**116**	106	240	170
Blaufränkisch	117	106	240	170

Table 5: Variability of chloroplast genotypes within traditional cultivars (deviation in bold)

cultivar/accession	ccmp 10	ccmp 3	ccSSR 5	ccSSR 9
Traminer	**117**	107	238	170
Traminer /main	**116**	107	238	170
Traminer Kl 42	**115**	107	238	170
Sylvaner main	116	107	**240**	170
Sylvaner (R/N) St 25	116	107	**238**	170
Pinot blanc	**116**	106	240	**170**
Pinot gris	**115**	106	240	**170**
Pinot gris /85	**115**	106	240	**172**
Schwarzriesling	**115**	106	240	**172**
Pinot noir	**116**	106	240	**170**

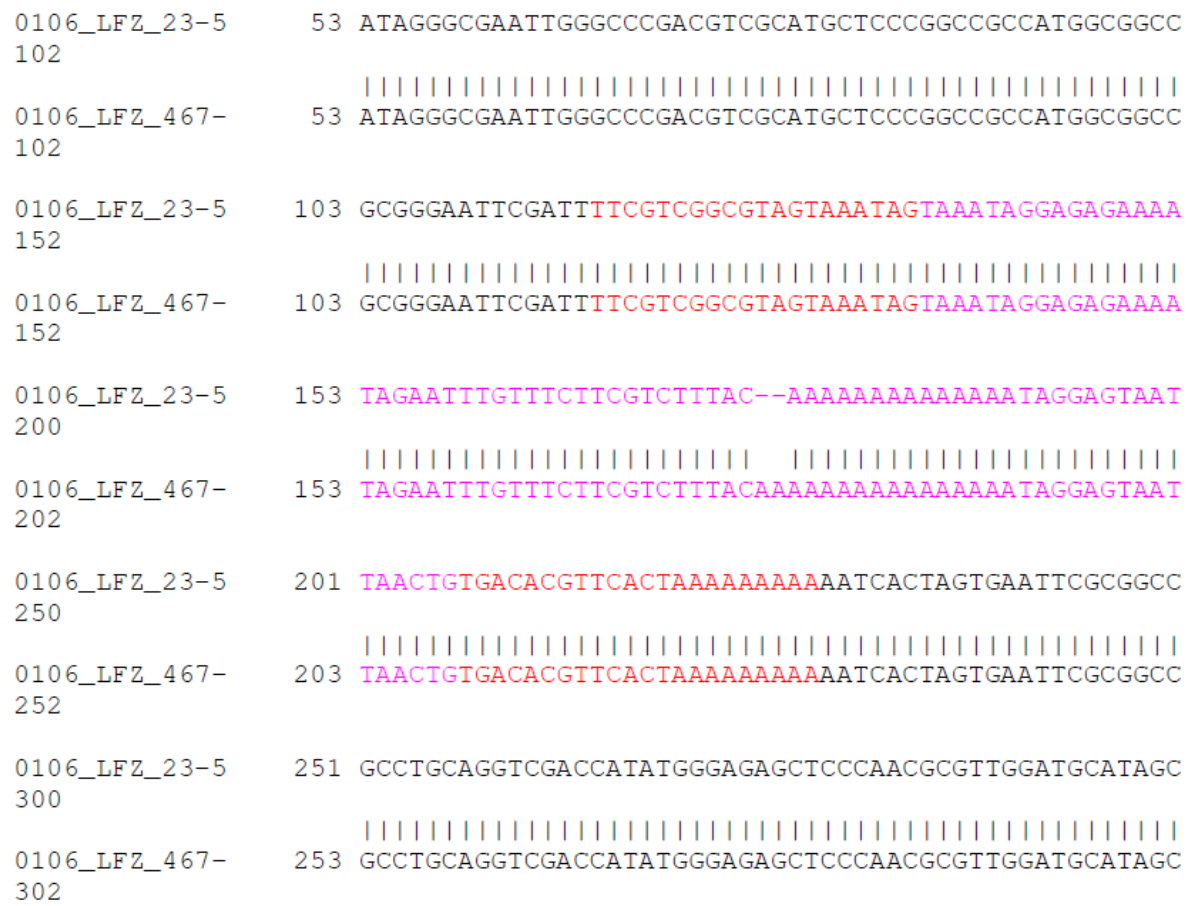

Fig. 2: Mutation at the position 177/178 of ccmp10 in 'Traminer' genotypes

DISCUSSION

Despite the observed stability of chloroplast markers in some other reports (Arroyo- Garcia et al., 2002; Imazio et al., 2006) within the genepool of grapevine we could not confirm this behaviour. We found a different situation when we analysed several types and accessions of one cultivar. It seems that especially old traditional varieties dispose of more variability than estimated by ampelographic deviations or fingerprinting with SSR markers (Pervaiz et al., 2016). Nevertheless, chloroplast markers can be used to follow the maternal inheritance with the restriction that mutations never can be excluded and occur more frequently in genotypes with high rate of propagation cycles (Regner et al., 2001). Deviations in chloroplast markers between the parents and the off-spring as observed for the allele ccmp 10 of 'Blauburger' could be caused by the hybridization process itself. The cultivar is still young, not widely distributed and the extent of propagation is limited. On the other hand we observed larger deviation in the inheritance of 'Sylvaner'. As the cultivar has been existing for centuries and intensively used in former viticulture the deviations from both parental profiles can be explained easily. Variation in chloroplast genotypes became even clearer when we also found variability within different samples of the cultivar 'Sylvaner' itself. The profile for the red/blue-berried 'Sylvaner' types would confirm the maternal parentage of 'Traminer' as already shown before, but such a conclusion neglects the gender direction (Regner and Kaserer, 2002). The variability within the cultivar 'Traminer' was only observed at one locus but is broader with two additional alleles. Cultivars with such flexible profile could easily be aligned with others on basis of few chloroplast markers only. Therefore numerous nuclear SSR markers allowed to reconstruct the genetic network of 'Traminer' (Myles et al., 2011).

Although the 'Pinot' colour types (noir, gris and blanc) share the same common genetic nuclear SSR profile, some smaller deviations (Jahnke et al., 2011; Hocquingny et al., 2004) could already be demonstrated previously. Considering variability 'Pinot gris' seems to be the oldest or most distinct of the 'Pinot' types (Hocquingny et al., 2004). The results of the presented study will confirm this hypothesis as we observed two chloroplast loci with clear deviations. The linkage of 'Schwarzriesling' to the main 'Pinot' profile could be explained by a development via 'Pinot gris' (Hocquingny et al., 2004) or a cross with Traminer (Myles et al., 2011). Our results allow a clear differentiation between the main 'Pinot' chloroplast genotype ('Pinot noir' and 'Pinot blanc') from that of 'Pinot gris'. Surprising was the fact that 'Schwarzriesling' and 'Pinot gris' shared the same chloroplast profile. In our study we could demonstrate two different profiles for 'Pinot gris'. That could mean a higher mutation rate due to intensive propagation and one type closer to the other 'Pinots' could indicate the former development. Therefore 'Schwarzriesling' could be favoured as the maternal parent of 'Pinot gris' while the paternal genome derived from 'Traminer'. Myles et al. (2011) confirmed the importance of 'Traminer' for the development of numerous European varieties with potential of high quality. They also considered the parentage of 'Traminer' for 'Pinots' (Imazio et al., 2002). Currently several hypotheses on the origin of the 'Pinots' exist, maybe our results can contribute to clarification of the confusion. If 'Traminer' crossed with 'Schwarzriesling' would cover all alleles of 'Pinot', why should such a combination not have occurred. If mutations changed 'Schwarzriesling' to 'Pinot gris' and finally to 'Pinot noir' and 'Pinot blanc' with more time and high frequency of mutations different developments could be possible. Some individual alleles of 'Schwarzriesling' (Table 6) are neither common ones in *Vitis vinifera* nor common for the 'Pinots'. It can be assumed that this discussion could not be solved as too many genotypes are available at a genetic small base and a lot of former ancient types are lost. Today genotypes do not reflect the variability that might have been reached for a given cultivar in earlier times. At least, loss of variability is the consequence of using clones instead of mass selection. Additional data would be helpful and therefore this work will only be a further puzzle detail to create a realistic picture of the 'Pinots'. Sometimes a single locus with a rare allele or high variability sometimes could be more convincing than large amounts of data with low potential.

Hence, genotyping with chloroplast markers is a useful tool for studying genetic evolution by maternal inheri-

tance but it never can be excluded that deviations derived by mutations will appear and get the chance to be propagated. Considering that chloroplast markers also show variability can reinforce genetic analysis of grapevine resources.

Table 6: Relationship of 'Pinot' with 'Traminer' and 'Schwarzriesling' (rare alleles are bold)

Locus	Traminer	Pinot	Schwarzriesling
VVS 2	151	137 : 151	**129** : 137
VVMD 5	232 : 238	228 : 238	228 : 238
VVMD 7	243 : 257	239 : 243	239 : 243
VVMD 36	254 : 264	254	**240** : 254
VrZag 62	189 : 195	189 : 195	189 : 195
VrZag 79	246 : 252	240 : 246	240 : 246
VMC 8g9	177	177 : 187	187 : **222**

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