

Gene pool variation and phylogenetic relationships of an indigenous northeast Italian grapevine collection revealed by nuclear and chloroplast SSRs

Marzia Salmaso, Renzo Dalla Valle, and Margherita Lucchin

Abstract: A germplasm safeguard programme was set up with 19 grapevine varieties considered as indigenous to north-eastern Italy. To better estimate how genetic structure can be used to obtain a conservation perspective of local varieties, genetic variability was examined at 30 nuclear and 3 chloroplast polymorphic microsatellite loci in the native varieties plus 7 European cultivars taken as reference. The genetic profiles of all the cultivars were searched for possible parentage relationships and several suspected cases of the same variety having different names were investigated. The alleles shared at the loci suggest a parent–offspring relationship between Merlot and Cabernet Franc, ‘Gruaja’ and ‘Negrara Veronese’, and Marzemina Nera and Marzemina Bianca. Alleles at the 30 nuclear loci are consistent with Raboso Veronese being the progeny of Marzemina Bianca and Raboso Piave. Chloroplast-specific haplotypes were singled out for the first time in this indigenous germplasm and should be considered typical of the region. It is hypothesized that there are many specific haplotypes for the local varieties due to a past contribution of wild grapevine to the cultivated gene pool. The majority of investigated cultivars were demonstrated to constitute an independent source of genetic variation, and therefore a possible valuable resource of genetic traits for breeders.

Key words: *Vitis*, Italian grapevine varieties, genetic variation, nuclear microsatellites, chloroplast microsatellites.

Résumé : Un programme de sauvegarde des ressources génétiques a été mis sur pied avec les 19 cépages de vigne considérés indigènes du nord-est de l’Italie. Afin de mieux évaluer comment la structure génétique pouvait permettre d’obtenir une perspective sur la conservation des variétés locales, la variabilité génétique a été examinée sur 30 microsatellites nucléaires et 3 microsatellites chloroplastiques polymorphes chez les variétés indigènes ainsi que chez 7 cépages européens employés comme témoins. Les profils génétiques de chacun des cépages ont été examinés pour révéler de possibles relations de parenté et plusieurs cas d’homonymie suspectée ont été investigués. Les allèles partagés à ces locus suggèrent une relation parent–descendant entre le Merlot et le Cabernet Franc, ‘Gruaja’ et ‘Negrara Veronese’ et Marzemina Nera et Marzemina Bianca. Les allèles aux 30 locus nucléaires suggèrent également que Raboso Veronese serait un descendant du Marzemina Bianca et du Raboso Piave. Des haplotypes chloroplastiques spécifiques ont été documentés pour la première fois au sein de ces ressources génétiques indigènes et ils devraient être considérés comme typiques de la région. Les auteurs avancent l’hypothèse que la présence de plusieurs haplotypes spécifiques des variétés locales serait due à une contribution antérieure de la vigne sauvage au pool génique de la vigne cultivée. La plupart des cépages étudiés se sont avérés des sources indépendantes de variation génétique et constituent ainsi une source potentiellement utile de caractères génétiques pour les sélectionneurs.

Mots-clés : *Vitis*, cépages italiens, variation génétique, microsatellites nucléaires, microsatellites chloroplastiques.

Introduction

In Italy, as in other Mediterranean countries, grapevine (*Vitis vinifera* L.) used to be characterized by the diffuse

presence of indigenous cultivars originated through centuries of natural and human selection (Martin et al. 2003; Snoussi et al. 2004; Imazio et al. 2006; De Mattia et al. 2007; Santana et al. 2007). Local varieties thus evolved morphological structures and physiological mechanisms enabling them to cope with the local bioclimatic and edaphic conditions, and so have assumed the significance of invaluable genetic resources (This et al. 2006) whose conservation and valorization, because of their determinant role in characterizing the terroir, should be vigorously pursued. In northeast Italy, a grape-growing region with ancient traditions, there are still a large number of local varieties typical of different areas (Caló et al. 2004). Although some still form the basis of famous regional wines (Raboso Piave, Marzemina, Corbina), many risk extinction, since only isolated plants

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have been reported and no new vineyards are established with them. Furthermore, they not being registered in the Italian Catalogue of Cultivated Grapevine Varieties, the official document listing the varieties authorized for growing in Italy, no plant material is available for propagation in commercial nurseries.

According to Varanini (1988), at the beginning of the 15th century a Marzemino wine had already been recorded as a special wine able to fetch the best price in the Euganean area. Maffioli (1981) reported Beolco (1521) praising the characteristics of a sourish wine named “Rabosino” or “Rabosazza”, which was probably the ancestor of Raboso Piave. Ludovico Pastò, a Venetian doctor and writer who lived in the 18th century, extolled the fine aroma and pleasant flavour of Friularo, a wine produced from the cultivar ‘Friularo’ grown in the Euganean area and which still produces a DOC (Controlled Designation of Origin) wine (Pastò 1778). Unfortunately, historical information about the grapevine cultivars grown in northeast Italy is fragmented, but the specialized cultivation of some, such as ‘Corbinona’, ‘Corbinella’, Raboso Piave, Marzemina Bianca, and ‘Marzemina Nera Bastarda’, certainly dates back to the 17th century (Agostinetti 1679). A proper regional grapevine inventory was made only in the latter half of the 19th century. On the occasion of the first Primary Agricultural Products Show, held in Vicenza in 1855 (Clementi 1855), all the local varieties considered in our study or varieties with the same name were present, with only three exceptions: ‘Agostana Nera’, ‘Friularo’, and ‘Marzemina Cenerenta’. In the second half of the 19th century the spread of parasites from America dramatically changed the panorama of cultivar growing, causing a significant reduction in the cultivated grapevine germplasm in northeastern Italy and other European regions.

Diseases first and then negligence caused by two world wars led to a further loss of local germplasm, which has been substituted by foreign cultivars such as Merlot, Cabernet, Chardonnay, and Pinot. The current tendency to plant successful and famous cultivars throughout the vine-growing regions of the world is resulting in a globalization of wine taste, minimizing the contribution of the cultivar and environment of production, lessening regional specificity, and causing a rapid reduction in the number of local genotypes. This trend needs to be counterbalanced by preserving the typical varieties of the different regions to prevent genetic erosion (Sefc et al. 2000; Armanni et al. 2007). The only way to preserve this heritage is to identify, characterize, possibly exploit, and preserve it in germplasm collections. However, the conservation of ancient local varieties is frequently hindered by the difficulty of their unambiguous recognition; typical varieties often cannot be easily distinguished because they have been distributed throughout an area and have acquired new names, rendering accurate varietal labelling a legal requirement. Many cases of synonymy and homonymy burden these rare ancient varieties; different names for the same material (synonyms) do exist but, because of some peculiar features of the varieties or the regions where they were grown, a convergence of names for different cultivars (homonyms) also occurs (Fossati et al. 2001; Crespan and Milani 2001; This et al. 2004). In recent years results from molecular marker analyses have been helping to clarify some of the phylogeny, synonymy, and

homonymy of famous grapevine cultivars (Vouillamoz et al. 2003; Karatas et al. 2007; Vouillamoz et al. 2007). Simple sequence repeats (SSR) have been shown to be particularly useful for the characterization of germplasm collections because they are highly polymorphic, usually codominant, and reproducible (Thomas and Scott 1993; Cipriani et al. 1994; Bowers et al. 1999; Bowers and Meredith 1997; Di Gaspero et al. 2005; Goto-Yamamoto et al. 2006; Lopes et al. 2006).

This paper reports the results of a study of genetic diversity and relationships within an endangered grapevine germplasm collection performed using polymorphisms at nuclear and chloroplast microsatellite loci. Microsatellite analysis was conducted to determine genetic relationships and possible parent–offspring pairs in a set of 19 ancient local varieties from northeastern Italy, some of which are nearly extinct. To ensure that allele size of SSRs was properly scored, the SSR marker system relied on a *Vitis vinifera* microsatellite genotype database. For this purpose, the database of GENRES #081 (European Network for Grapevine Genetic Resources Conservation and Characterization; This et al. 2004) was utilized. Seven certified international *Vitis vinifera* varieties were also included for reference purposes. The aim of the research was to characterize the ancient local cultivars, identify the most similar groups from the matrix of similarity coefficients, and check whether these groupings are concordant with the known history of the cultivars in order to help with the efficient preservation of old local genotypes that could represent valuable genetic combinations. Furthermore, characterizing the genetic diversity of local varieties would allow a more efficient application of these materials in breeding and MAS (marker-assisted selection) programmes. Chloroplast-specific haplotypes have been found for the first time in this indigenous germplasm and should be considered typical of the region. We hypothesize that there are many specific haplotypes for the local varieties due to a past contribution of wild grapevine to the cultivated gene pool. This supports the hypothesis of secondary domestication events having occurred in the Mediterranean basin.

Materials and methods

Plant materials

Leaves were obtained from 19 local varieties held in two private collections near the Euganean Hills (Padua) and one near Breganze (Vicenza) in northeastern Italy. Five different accessions named ‘Friularo’, 3 named Raboso Piave, and 3 named Raboso Veronese were analysed. Seven international certified cultivars (Barbera VCR433, Chardonnay R8, Cabernet Franc VCR10, Cabernet Sauvignon R5, Merlot R3, Pinot Nero VCR20, and Traminer R1) were supplied by a certified commercial nursery (Table 1).

SSR analysis

Total DNA was extracted from leaves using the Nucleon PhytoPure Genomic DNA Extraction Kit (GE Healthcare Life Sciences). After RNase digestion (10 µg/µL RNase A), samples were resuspended in sterile water.

For nuclear SSR analysis, 30 marker loci were examined: UDV27, UDV109 (Di Gaspero et al. 2005); VMC2A3, VMC2C7, VMC5C5, VMC2G2 (developed by the Vitis Microsatellite Consortium, managed by Agrogen SA, Moissy-

Table 1. Grapevine cultivars investigated, listed in alphabetical order.

Cultivar	Source
'Agostana Nera' ^a	Euganean Hills
<i>Barbera</i> ^b	Certified nursery
'Cabernet Colli Euganei' ^a	Euganean Hills
<i>Cabernet Franc</i> ^b	Certified nursery
<i>Cabernet Sauvignon</i> ^b	Certified nursery
<i>Chardonnay</i> ^b	Certified nursery
'Corbinella' ^{a,b}	Euganean Hills
'Corbinona' ^{a,b}	Euganean Hills
'Friularo' (5)	Euganean Hills
'Gatta' ^{a,b}	Euganean Hills
'Gruaja' ^a	Breganze, Vicenza
Marzemina Bianca ^b	Euganean Hills
'Marzemina Cenerenta' ^a	Euganean Hills
Marzemina Nera ^b	Euganean Hills
'Marzemina Nera Bastarda' ^{a,b}	Euganean Hills
<i>Merlot</i> ^b	Certified nursery
'Negrara Veronese' ^{a,b}	Euganean Hills
'Pataresca' ^{a,b}	Euganean Hills
<i>Pignola</i> ^b	Euganean Hills
<i>Pinot Nero</i> ^b	Certified nursery
'Raboso Friularo' ^a	Euganean Hills
Raboso Piave ^b (3)	Euganean Hills
Raboso Veronese ^b (3)	Euganean Hills
'Schiavetta Doretta' ^a	Euganean Hills
'Tintoria' ^a	Euganean Hills
<i>Traminer</i> ^b	Certified nursery

Note: The international varieties used as reference are in italics. For three cultivars the number of different accessions assayed is given in parentheses.

^aVarieties not registered in the Italian Catalogue of Cultivated Varieties.

^bVarieties present in the Vitis International Variety Catalogue (<http://www.vivc.bafz.de/index.php>).

Cramayel, France); VrZAG21, VrZAG25, VrZAG62, VrZAG64, VrZAG79, VrZAG83, VrZAG93, VrZAG112 (Sefc et al. 1999); VVMD5, VVMD6, VVMD7, VVMD8, VVMD25, VVMD26, VVMD27, VVMD36 (Bowers et al. 1996, 1999); VVS2 (Thomas and Scott 1993); and VVIP10, VVIQ61, VVIP77, VVIS70, VVIU37, VVIV16, VVIV37 (Merdinoglu et al. 2005). Six of the investigated markers (VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, and VrZAG79) have recently been chosen as a core set for the screening of grapevine collections in Europe covered by the GENRES #081 research project (<http://www.genres.de/eccdb/vitis/>). For chloroplast SSR analysis, 3 universal microsatellite markers designed for dicotyledonous angiosperms were used: ccmp3, ccmp5, and ccmp10 (Weising and Gardner 1999). Nuclear microsatellites were amplified in a volume of 12.5 µL containing 10 ng of template, 0.25 µmol/L forward primer and 0.37 µmol/L reverse primer, 100 µmol/L dNTPs, 1.2 mmol/L MgCl₂, 0.4 U of *Taq* polymerase (Bioline), and 1× *Taq* polymerase buffer. Chloroplast microsatellites were amplified in a volume of 20 µL containing 12 ng of template, 0.4 µmol/L forward primer and 2.4 µmol/L reverse primer, 4 µmol/L dNTPs, 2.5 mmol/L MgCl₂, 0.5 U of *Taq* polymerase (Bioline), and 1× *Taq* polymerase buffer.

Amplifications were performed using the PCR conditions recommended by the GENRES #081 research project. A Biometra TGradient thermocycler was used with the following temperature conditions: 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at the annealing temperature, and 1 min at 72 °C (45 s for chloroplast microsatellite amplification), followed by a final step of 30 min at 72 °C (5 min for chloroplast microsatellite amplification). The annealing temperatures were as follows: 49 °C for VVIP10 and VVIQ61; 50 °C for VVIU37 and VVMD5; 51 °C for ccmp3; 52 °C for VrZAG112; 53 °C for UDV27, UDV109, VrZAG64, and VVS2; 54 °C for VVMD27 and VVMD7; 55 °C for ccmp10, ccmp5, VMC2C7, VVMD6, and VVMD8; 56 °C for VVIP77, VVIV16, VVIV37, and VVIVS70; 57 °C for VrZAG25, VrZAG83, VrZAG93, and VVMD36; 58 °C for VrZAG79, VVMD25, and VVMD26; and 59 °C for VMC2A3, VMC2G2, VMC5C5, VrZAG21, and VrZAG62. PCR amplification products were subjected to agarose gel electrophoresis. Once the melting temperature was optimized for each primer pair, the entire population was screened at that optimal temperature. The choice of markers previously used in other European studies, cultivars in common with those studies, and PCR conditions as described in GENRES #081 ensures that our results are comparable with those of other laboratories and will contribute to the ongoing international genotype identification effort. The forward primers were labelled with ABI fluorescent dyes at the 5' ends and PCR products were analysed by capillary electrophoresis with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, California). Chromatograms were analysed using the software GeneMapper 4.0 (Applied Biosystems, Monza, Italy). The automatic peak labelling of the ABI PRISM Genetic Analyzer required an additional visual check of individual peak positions so that values could be rounded to obtain integer allele numbers. Previously labelled reference peak profiles aided the decision making.

SSR data analysis

Chromatograms were scored as genotypes based on the allele composition. Samples in which only a single allele per locus was detected were considered homozygous instead of heterozygous with a null allele for computing genetic diversity parameters.

The pairwise genetic divergences between cultivars were estimated as $1 - P_s$, where P_s (the genetic similarity parameter, or proportion of shared alleles) is the number of shared alleles summed over loci divided by twice the number of loci compared (Bowcock et al. 1994). The expected heterozygosity (H_e ; Nei 1973), the effective number of alleles (n_e ; Hartl and Clark 2006), the probability of identity (I ; Paetkau et al. 1995), and the frequency of null alleles (r ; Brookfield 1996) were estimated as follows: $H_e = 1 - \sum_{i=1}^n p_i^2$, $n_e = 1/(1 - H_e)$, $I = \sum_i p_i^4 + \sum_i \sum_{j>i} (2p_i p_j)^2$, and $r = (H_e - H_o)/(1 + H_e)$, where p_i and p_j are the frequencies of the i th and j th alleles in a given population. The expected and observed (H_o) heterozygosity and the probability of identical genotypes (PI) were calculated using IDENTITY 1.0 software (Wagner and Sefc 1999). Likelihood statistics corresponding to the detected possible parent-offspring combinations

were calculated as described elsewhere (Bowers and Meredith 1997; Sefc et al. 1997). MICROSAT (Minch 1997) software was used to create a genetic distance matrix between all pairwise combinations and to summarize information on unique alleles and allele distribution. A UPGMA dendrogram (Sneath and Sokal 1973) and a neighbour-joining network (Saitou and Nei 1987) for nuclear microsatellite loci were constructed from the matrix of pairwise genetic divergences between cultivars using PHYLIP (Felsenstein 1989) for the estimation and Tree-View (Page 1996) for the visualization of the dendrograms. Chloroplast haplotypes (plastotypes) were identified based on the three loci. Phylogenetic relationships of the detected plastotypes were evaluated based on the number of different alleles among haplotypes.

Results

Investigation of genetic relationships among grapevine cultivars

The ability to unambiguously distinguish cultivars and clarify cases of synonymy and homonymy is important for solving problems concerning the management of grapevine germplasm (Lopes et al. 1999). Among the 26 varieties analyzed on the basis of 30 nuclear SSR loci, 23 different SSR profiles were detected. Except for VVIU37 and VVMD26, each locus identified at least 2 varieties in a specific way. The markers were found to have high discriminative power: except for 'Corbinella', virtually all cultivars were distinguished with a combination of 3 loci. At the 30 loci 'Friularo' was shown to be synonymous for Raboso Piave, and 'Raboso Friularo' was shown to be synonymous for Raboso Veronese. The variety 'Corbinona' was identical to 'Corbinella' except for one allele at the locus VrZAG25. For the gene pool analysis of the germplasm, the two varieties were considered as synonymous. Marzemina Nera and 'Marzemina Cenerenta' were identical at the 30 loci, but different from Marzemina Bianca and 'Marzemina Nera Bastarda'. 'Cabernet Colli Euganei' differed from Cabernet Franc at 18 alleles out of 60 (Table 2). The results at these loci also suggest interesting suppositions about the genetic relationships between some varieties. Marzemina Bianca and Marzemina Nera shared one allele per locus at all 30 loci, indicating a possible parent-offspring relationship. The same was true for Traminer and Pinot Nero according to the observation of Regner et al. (2000), who proposed Riesling Renano and Traminer as the progenitors of Pinot Nero. A new putative genetic relationship was also observed for the first time between Cabernet Franc and Merlot, as they share at least one allele per locus (Table 2). Alleles at 28 of the 30 microsatellite loci were consistent with a parent-offspring relationship between 'Gruaja' and 'Negrara Veronese'. The difference at the loci VrZAG64 and VMC2A3 could be due to clonal mutation, which is frequent in ancient cultivars. A somatic mutation was also found by Vouillamoz et al. (2003) in an analysis of parent-offspring relationships of ancient alpine grape cultivars.

A likely cross was also identified: alleles at all 30 loci are consistent with Raboso Veronese being the progeny of Raboso Piave and Marzemina Bianca. The detailed parentage indices for Raboso Veronese and its putative parents are shown in Table S1², where the likelihood-ratio values compare the probability of the parentage of the suggested cultivars with (i) the probability that two random cultivars are the parents, (ii) the probability that one of the suggested cultivars and a random cultivar are the parents, and (iii) the probability that one of the suggested cultivars and a close relative to the second suggested cultivar are the parents. The likelihood ratios show that the putative parents are 1×10^{17} times more probable than two random cultivars. If Marzemina Bianca is one of the parents, the likelihood of the observed Raboso Veronese alleles is 2.77×10^{11} times higher if Raboso Piave is the second parent rather than a random cultivar. Likewise, if Raboso Piave is one of the parents, the likelihood of the observed Raboso Veronese alleles is 3.28×10^{11} times higher if Marzemina Bianca is the second parent rather than a random cultivar. The likelihood of the Raboso Veronese alleles is 9390 times higher if Raboso Piave is a parent versus a close relative of Raboso Piave and 6370 times higher for Marzemina Bianca versus a close relative of Marzemina Bianca. The allele frequency computations utilized were based on the 22 different varieties. Because this is a relatively small number of individuals, to compensate for possible errors the likelihood ratios were also calculated from 95% upper confidence limits of the observed allele frequencies. Even in this case, the putative parents are more probable than two random cultivars. The Raboso Veronese alleles were in fact 4.27×10^6 times more likely if the parents were Raboso Piave and Marzemina Bianca rather than two random cultivars (Table S1).

Estimation of genetic diversity among the local varieties, based on nuclear SSR markers

Although these nuclear SSR markers have already been evaluated in grapevine, their information content in this indigenous northeastern Italian grapevine germplasm was assessed here for the first time. Evidence of three alleles was found in several varieties instead of the usual one or two expected for a diploid species (Table 2). Microsatellite somatic mutations were detected for 18 loci, representing 2.3% of total cases. To confirm the presence of more than two alleles at the same locus, the microsatellite PCR products were sequenced in 9 of the 18 observed cases. The sequencing results showed that the flanking regions of the microsatellite loci were conserved, confirming that the amplified fragments were allelic. An example of aligned partial sequences of 3 microsatellite alleles for one of the sequenced loci is shown in Fig. S1². At the 30 SSR loci the mutated alleles were found 7 times in 'Tintoria' and 11 times in 'Schiavetta Doretta'. Fourteen loci presented a tri-allelic profile in one variety (VMC2A3, VMC2G2, VMC5C5, VrZAG21, VrZAG25, VrZAG62, VVIP77, VVIV37, VVMD5, VVMD7, VVMD8, VVMD26, VVMD27, VVMD36), whereas VrZAG64 and VrZAG79 evidenced the highest

²Supplementary data for this article are available on the journal Web site (<http://genome.nrc.ca>) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 3808. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/cms/unpub_e.html.

Table 2. Molecular data of the 23 different genotypes identified at 30 nuclear SSR loci, listed in alphabetical order.

Cultivar	UDV109	UDV27	VMC2A3	VMC2C7	VMC2G2	VMC5C5	VrZAG21	VrZAG25	VrZAG62	VrZAG64
'Agostana Nera'	124, 124	154, 162	148, 148	144, 152	119, 123	116, 120	190, 206	227, 235	192, 202	133, 161
Barbera	124, 146	162, 184	162, 164	142, 152	119, 123	116, 120	190, 200	227, 227	190, 198	157, 161
'Cabernet Colli Euganei'	146, 160	140, 158	164, 182	138, 152	123, 125	120, 122	200, 200	227, 235	186, 202	133, 157
Cabernet Franc	124, 146	144, 158	164, 182	144, 152	121, 125	118, 122	190, 200	227, 227	192, 202	155, 157
Cabernet Sauvignon	124, 146	158, 158	174, 182	144, 152	121, 125	118, 122	200, 206	227, 239	186, 192	135, 157
Chardonnay	124, 124	162, 174	148, 158	142, 154	119, 119	116, 116	200, 206	227, 227	186, 194	157, 161
'Corbinella'	124, 124	140, 154	162, 164	136, 150	119, 121	116, 118	190, 206	239, 239	190, 194	135, —
'Corbinona'	124, 124	140, 154	162, 164	136, 150	119, 121	116, 118	190, 206	235, 239	190, 194	135, —
'Friularo'(5) / Raboso Piave (3)	124, 124	140, 162	148, 164	152, 152	123, 123	120, 120	206, 206	227, 227	194, 194	133, 161
'Gatta'	124, 124	140, 162	148, 182	138, 152	123, 123	120, 120	190, 206	235, 235	192, 194	133, —
'Gruaja'	124, 124	158, 162	148, 182	136, 138	119, 121	116, 118	190, 200	227, 227	192, 192	157, —
Marzemina Bianca	124, 146	158, 162	164, 182	138, 142	119, 125	116, 122	190, 202	235, 249	192, 198	135, 139
Marzemina Nera / 'Marzemina Cenerenta'	124, 124	158, 162	148, 182	138, 152	119, 123	116, 120	190, 190	227, 249	192, 192	139, 161
'Marzemina Nera Bastarda'	124, 146	158, 158	148, 148	136, 150	119, 119	116, 116	202, 206	227, 235	190, 202	135, —
Merlot	124, 124	154, 158	164, 182	142, 152	119, 121	116, 118	200, 200	227, 227	192, 192	133, 157
'Negrara Veronese'	124, 124	154, 162	162, 164	138, 150	119, 121	116, 118	200, 206	227, 235	192, 202	133, 161
'Pattaresca'	124, 124	140, 162	150, 164	144, 152	119, 121	116, 118	190, 206	227, 235	190, 194	135, 161
Pignola	124, 124	162, 162	148, 182	142, 152	123, 127	120, 124	200, 206	227, 235	190, 192	133, 157
Pinot Nero	124, 124	162, 174	148, 174	138, 142	119, 121	116, 118	200, 206	227, 239	186, 192	135, 161
'Raboso Friularo' / Raboso Veronese (3)	124, 124	162, 162	148, 164	138, 152	123, 125	120, 122	190, 206	227, 249	192, 194	139, 161
'Schiavetta Doretta'	124, 146	140, 158	150, 164	136, 144	119, 121, 125	116, 118, 122	190, 200, 204	227, 249	186, 190, 194	135, 139, 161
'Tintoria'	124, 140	150, 156	148, 152, 164	138, 152	117, 119	114, 116	190, 220	227, 239, 245	192, 192	135, 139, 157, 161
Traminer	124, 124	154, 174	158, 174	142, 152	119, 121	116, 118	200, 206	239, 249	186, 192	135, 161

Table 2. (continued).

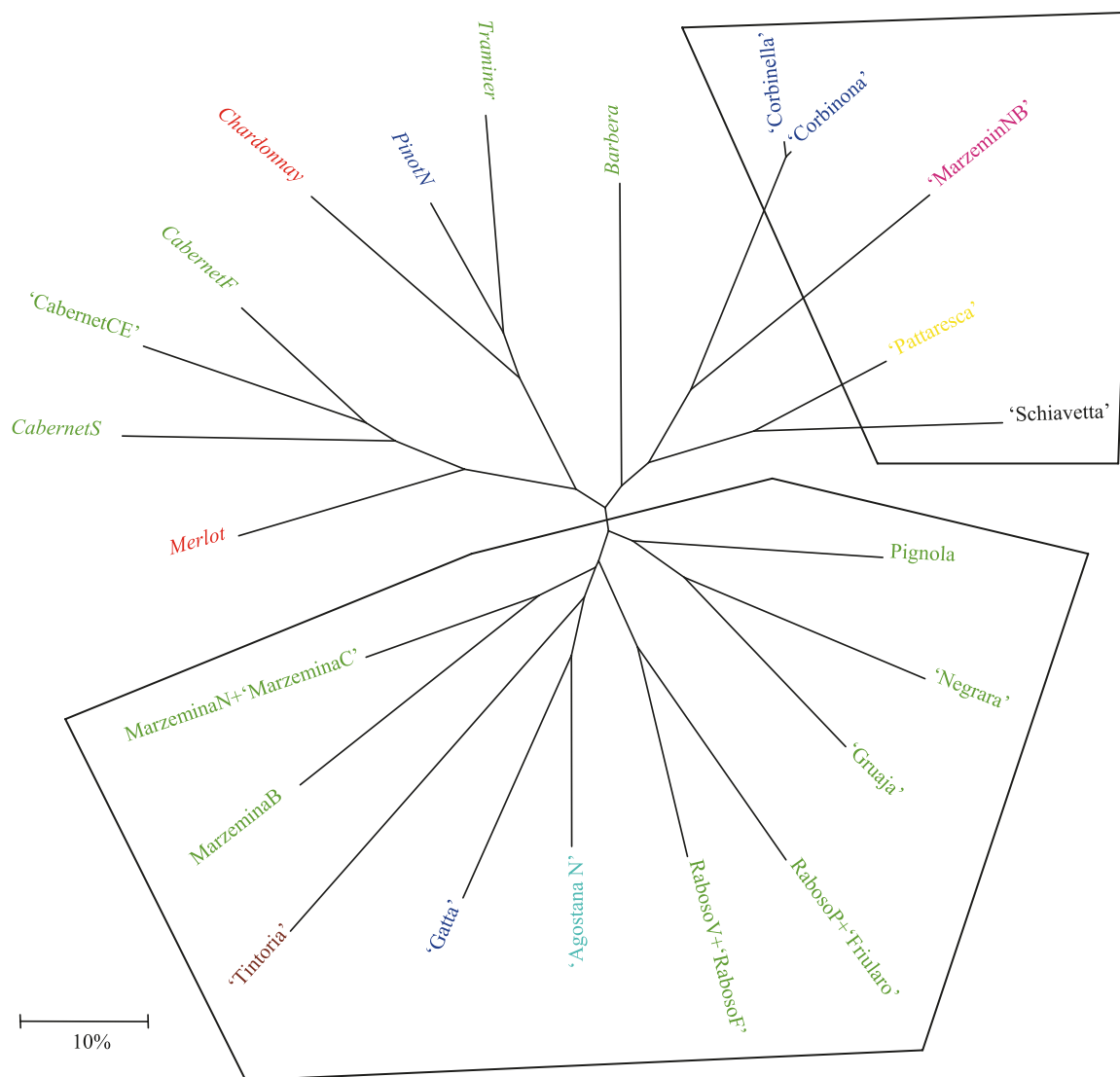
Cultivar	VrZAG79	VrZAG83	VrZAG93	VrZAG112	VVIP10	VVIP77	VVIQ61	VVIS70	VVIU37	VVIV16
'Agostana Nera'	252, 260	195, 195	187, 195	229, 235	280, —	174, 180	357, 361	187, 187	228, 234	103, 103
Barbera	244, 260	189, 195	187, 231	229, 229	280, 294	174, 192	357, 361	187, 187	228, 228	101, 103
'Cabernet Colli Euganei'	248, 248	201, 201	197, —	229, 243	276, 280	186, —	361, 363	187, 187	228, 234	101, 103
Cabernet Franc	248, 260	195, 201	197, —	229, 243	276, 280	186, 196	357, 363	187, 187	228, 234	101, 103
Cabernet Sauvignon	248, 248	201, 201	197, —	229, 235	276, 294	182, 186	357, 363	187, 187	234, 234	101, 101
Chardonnay	244, 246	189, 201	187, —	241, 241	294, —	182, 192	357, 361	187, 189	228, 234	89, 101
'Corbinella'	244, 252	195, 201	187, 231	229, 235	276, —	174, 210	357, 357	187, 187	228, 228	103, 103
'Corbinona'	244, 252	195, 201	187, 231	229, 235	276, —	174, 210	357, 357	187, 187	228, 228	103, 103
'Friularo'(5) / Raboso Piave (3)	244, 252	195, 221	187, 231	235, 241	280, —	180, —	357, 357	187, 189	228, 228	103, 103
'Gatta'	252, 260	195, 195	195, 231	233, 233	280, —	180, 186	357, 361	187, 189	228, 234	103, 103
'Gruaja'	252, 260	191, 195	187, —	235, 241	280, 294	186, —	357, 361	187, 189	228, 228	101, 103
Marzemina Bianca	252, 252	195, 195	187, 189	241, 243	276, —	174, 186	361, 361	187, 187	228, 234	103, 103
Marzemina Nera / 'Marzemina Cenerenta'	244, 252	195, 201	187, —	241, 243	276, 282	174, 184	357, 361	187, 187	228, 228	103, 103
'Marzemina Nera Bastarda'	250, 250	195, 201	187, —	235, 241	276, —	174, 210	357, 357	187, 187	228, 234	103, 103
Merlot	260, 260	195, 201	187, 197	229, 243	280, 284	186, —	357, 363	187, 187	234, 234	103, 103
'Negrara Veronese'	246, 260	195, 195	187, —	241, 243	276, 294	186, —	357, 361	187, 187	228, 228	101, 103
'Pattaresca'	244, 252	195, 201	231, —	235, 243	280, —	174, 210	357, 357	187, 189	228, 228	101, 103
Pignola	252, 260	195, 195	187, —	241, 243	274, 280	186, —	357, 361	187, 187	228, 228	101, 103
Pinot Nero	240, 246	189, 201	187, —	241, 243	280, 294	182, 192	357, 357	187, 187	228, 234	101, 103
'Raboso Friularo' / Raboso Veronese (3)	244, 252	195, 221	189, 231	235, 241	276, 280	174, 180	357, 361	187, 187	228, 228	103, 103
'Schiavetta Doretta'	244, 250, 260	189, 191	197, 231	235, 243	280, —	174, 190, 210	357, 357	189, 207	228, 234	101, 103
'Tintoria'	246, 252, 260	195, 195	187, 225	229, 251	280, —	182, —	357, 361	189, 189	228, 228	103, 105
Traminer	246, 252	189, 201	187, —	235, 241	294, —	182, 192	357, 357	187, 187	234, 234	101, 103

Table 2. (concluded).

Cultivar	VVIV37	VVMD5	VVMD6	VVMD7	VVMD8	VVMD25	VVMD26	VVMD27	VVMD36	VVS2
'Agostana Nera'	148, 148	232, 236	206, —	242, 254	137, 137	244, 258	252, 258	177, 189	254, 254	128, 138
Barbera	148, 160	226, 226	186, 204	250, 254	137, 137	242, 258	252, —	181, 185	264, 264	128, 130
'Cabernet Colli Euganei'	148, 160	226, 238	204, —	242, 264	139, 141	242, 258	252, —	171, 185	254, 270	134, 142
Cabernet Franc	148, 160	226, 240	196, 204	242, 264	141, 152	242, 252	252, —	177, 185	254, 254	134, 142
Cabernet Sauvignon	160, —	232, 240	204, —	242, —	137, 152	242, 252	252, 254	171, 185	254, 262	134, 148
Chardonnay	148, 160	234, 238	196, 206	242, 246	135, 141	242, 258	252, 258	177, 185	254, 274	132, 138
'Corbinella'	156, 160	232, 232	204, —	250, 258	137, 152	258, 258	252, —	181, 185	268, 268	130, 152
'Corbinona'	156, 160	232, 232	204, —	250, 258	137, 152	258, 258	252, —	181, 185	268, 268	130, 152
'Friularo'(5) / Raboso Piave (3)	160, 162	228, 232	196, 204	250, 260	135, 152	258, 258	252, 254	181, 185	254, 254	130, 138
'Gatta'	162, —	232, 238	206, —	250, —	135, 137	244, 258	252, —	177, 189	254, 254	138, 152
'Gruaja'	148, 162	226, 232	204, 206	250, —	135, 152	244, 252	252, 254	177, 181	262, 262	128, 148
Marzemina Bianca	148, 158	226, 238	196, —	242, 256	135, 137	244, 244	252, 264	181, 189	254, 254	128, 128
Marzemina Nera / 'Marzemina Cenerenta'	148, —	226, 232	196, 206	242, 264	135, 137	244, 258	252, 258	181, 185	254, 254	128, 128
'Marzemina Nera Bastarda'	160, 168	228, 236	204, 206	234, 250	137, 137	258, 262	252, —	181, 181	248, 248	130, 138
Merlot	160, 170	226, 236	196, 204	242, 250	137, 152	242, 252	252, 256	185, 187	254, 254	134, 148
'Negrara Veronese'	148, —	224, 232	186, 206	250, —	135, 135	244, 260	252, 258	177, 185	262, 262	130, 130
'Pattaresca'	148, 160	224, 238	186, 204	250, 256	135, 137	244, 258	252, —	181, 181	254, 264	130, 152
Pignola	148, 162	228, 232	196, 204	240, 248	135, 137	242, 258	252, 258	177, 185	254, 254	128, 130
Pinot Nero	148, 160	228, 238	196, —	242, 246	135, 137	242, 252	252, 258	181, 185	254, 254	132, 148
'Raboso Friularo' / Raboso Veronese (3)	158, 162	232, 238	196, —	242, 260	137, 152	244, 258	252, 264	181, 181	254, 254	128, 130
'Schiavetta Doretta'	160, —	228, 138, 148	186, 204	240, 248, 254	135, 137, 141	242, 244	252, 254	177, 181, 189	254, 264	130, 152
'Tintoria'	148, 158, 160	232, 252	198, —	242, 254	137, 137	240, 244	252, 134, 158	187, 197	238, 254, 264	128, 128
Traminer	160, —	232, 238	196, 204	246, 260	135, 137	252, 252	252, 254	185, 185	254, 264	148, 148

Note: — indicates that the cultivar is either homozygous or heterozygous with a null allele. Tri-allelic and tetra-allelic profiles are in bold.

Fig. 1. Neighbour-joining network of 26 grapevine varieties based on the phenetic distances estimated from polymorphism at 30 nuclear SSR loci. The scale indicates 10% divergence. International cultivars are in italics. Indigenous varieties are grouped in the two enclosed areas. The plastotypes are represented by eight colours as follows: I(D), green; II(A), blue; III(C), red; IV(E), light blue; V, magenta; VI, yellow; VII(F), black; VIII(B), brown. ‘AgostanaN’ = ‘Agostana Nera’, CabernetF = Cabernet Franc, ‘CabernetCE’ = ‘Cabernet Colli Euganei’, CabernetS = Cabernet Sauvignon, MarzeminaN = Marzemina Nera, ‘MarzeminaC’ = ‘Marzemina Cenerenta’, MarzeminaB = Marzemina Bianca, ‘MarzeminNB’ = ‘Marzemina Nera Bastarda’, ‘Negrara’ = ‘Negrara Veronese’, PinotN = Pinot Nero, RabosoP = Raboso Piave, ‘RabosoF’ = ‘Raboso Friularo’, RabosoV = Raboso Veronese, and ‘Schiavetta’ = ‘Schiavetta Doretta’.



mutation rate, showing a chimeric state in two varieties (one tri-allelic and one tetra-allelic profile for VrZAG64, and two tri-allelic profiles for VrZAG79). Three alleles at locus VVMD7 were also observed by Crespan (2004) in two accessions of Primitivo and by Hocquigny et al. (2004) in one accession of Pinot Grigio. It is sometimes difficult to evaluate a chimeric state at microsatellite loci if the differences in length or quantity of the amplified products are small and if the microsatellite locus also shows either extra base additions or stutters. Automatic sequencing seems less subject to these errors but nevertheless they arise (This et al. 2004). In our study the chimeric situation was clear in all the described cases.

Only the distinguishable local genotypes and the genotypes with a bi-allelic profile were included in the character-

ization of SSR polymorphisms. Moreover, ‘Cabernet Colli Euganei’ (not a native variety but introduced from France as Cabernet Franc) and ‘Tintoria’ were excluded from the gene pool analysis of the local grapevine germplasm. Indeed, after this analysis at the nuclear and chloroplast levels, ‘Tintoria’ was considered as an interspecific hybrid with non-*vinifera* *Vitis*. This variety shows both very different genetic characterization (Fig. 1) and phenotype (Tocchetti 1978) from any other *Vitis vinifera* cultivar, so it cannot be considered for a *Vitis vinifera* gene pool analysis. The genetic analyses were therefore carried out on 13 non-redundant genotypes.

In addition to a commercial molecular weight marker, PCR fragments of 7 previously analyzed international cultivars were used as internal size standards for fragment size

Table 3. Genetic diversity parameters from DNA profiles of 13 local northeastern Italian varieties at 30 nuclear SSR loci.

Locus	No. of alleles	H_o	H_e	n_e	PI	r	Allele size (bp)	Codes from This et al. (2004)	Allele frequency (%)
UDV027	4	0.769	0.683	3.155	0.251	-0.051	162		46.1 (9.8)
UDV109	2	0.231	0.204	1.256	0.696	-0.022	124		88.5 (6.3)
VMC2A3	5	0.846	0.731	3.717	0.204	-0.067	148		38.5 (9.5)
VMC2C7	6	0.923	0.796	4.902	0.128	-0.071	152		30.8 (9.0)
VMC2G2	5	0.750	0.712	3.472	0.233	-0.022	119		37.5 (9.9)
VMC5C5	5	0.750	0.712	3.472	0.233	-0.022	116		37.5 (9.9)
VrZAG21	4	0.833	0.663	2.967	0.303	-0.102	206		41.7 (10.1)
VrZAG25	4	0.769	0.642	2.793	0.331	-0.077	227		46.1 (9.8)
VrZAG62	5	0.750	0.719	3.559	0.211	-0.018	192 ^a	CF1	41.7 (10.1)
VrZAG64	5	0.667	0.781	4.566	0.155	0.064	133/135/161		25.0 (8.8)
VrZAG79	5	0.833	0.694	3.268	0.230	-0.082	252	TR2	45.8 (10.2)
VrZAG83	5	0.615	0.535	2.151	0.313	-0.052	195		65.4 (9.3)
VrZAG93	5	0.538	0.624	2.660	0.296	0.053	187		53.8 (9.8)
VrZAG112	5	0.923	0.745	3.922	0.196	-0.102	235/241		30.8 (9.0)
VVIP10	5	0.385	0.621	2.639	0.342	0.15	280		50.0 (9.8)
VVIP77	5	0.667	0.743	3.891	0.199	0.044	186		33.3 (9.6)
VVIQ61	2	0.538	0.453	1.828	0.607	-0.059	357		65.4 (9.3)
VVIS70	3	0.384	0.369	1.585	0.534	-0.011	187		76.9 (8.3)
VVIU37	2	0.385	0.311	1.451	0.620	-0.056	228		80.8 (7.7)
VVIV16	2	0.385	0.311	1.451	0.620	-0.056	103		80.8 (7.7)
VVIV37	6	0.692	0.734	3.759	0.198	0.024	148		38.5 (9.5)
VVMD5	6	0.917	0.753	4.049	0.149	-0.093	232	TR1	41.7 (10.1)
VVMD6	4	0.615	0.725	3.636	0.232	0.063	204/206		30.8 (9.0)
VVMD7	10	0.750	0.774	4.425	0.111	0.014	250 ^b	ME2	41.7 (10.1)
VVMD8	3	0.750	0.621	2.639	0.379	-0.079	137		45.8 (10.2)
VVMD25	6	0.769	0.663	2.967	0.293	-0.064	258		42.3 (9.7)
VVMD26	4	0.692	0.529	2.123	0.334	-0.106	252		65.4 (9.3)
VVMD27	4	0.750	0.687	3.195	0.246	-0.037	181 ^c	PI1	45.8 (10.2)
VVMD36	5	0.154	0.580	2.381	0.274	0.270	254		61.5 (9.5)
VVS2	5	0.769	0.737	3.802	0.204	-0.019	130 ^d	BA2	34.6 (9.3)
Average	4.6 (1.6)	0.660	0.628	—					
Multi-locus probability of identity					1.13×10^{-17}				

Note: H_o , observed heterozygosity; H_e , expected heterozygosity; n_e , effective number of alleles; PI, probability of identity; r , probability of null alleles. Size and frequency are shown for the most frequent allele, and size is also expressed in This et al. (2004) codes. Standard deviations are in parentheses. The synonyms Marzemina Nera and 'Marzemina Cenerenta', 'Raboso Friularo' and Raboso Veronese, and 'Friularo' and Raboso Piave are treated as one cultivar each. 'Corbinella', 'Cabernet Colli Euganei', and 'Tintoria' are not included in the analysis of local cultivars.

^aAll alleles are shifted by -2 nucleotides with respect to This et al. 2004.

^bAll alleles are shifted by 2 nucleotides with respect to This et al. 2004.

^cAll alleles are shifted by -4 nucleotides with respect to This et al. 2004.

^dAll alleles are shifted by -5 nucleotides with respect to This et al. 2004.

determination. For some loci, a shift of 2 to 5 nucleotides was found in the reference cultivars. Such small systematic differences in allele size scoring are to be expected when using different fragment separation systems (Sefc et al. 2001).

In the 13 indigenous northeastern Italian varieties the number of alleles per locus ranged from 2 at loci UDV109, VVIQ61, VVIU37, and VVIV16 to 10 at locus VVMD7 (Table 3), with an average of 4.6 alleles per locus. Grapevine has been demonstrated to have a high degree of heterozygosity in its genome (Aradhya et al. 2003; Lijavetzky et al. 2007; Salmaso et al. 2004). The expected heterozygosity over the 13 varieties ranged from 0.204 (UDV109) to 0.796 (VMC2C7) and the observed heterozygosity was higher than the expected heterozygosity at 22 of the 30 examined loci. As a consequence, the probability of occurrence of null alleles was positive at 8 loci. Attention should be paid in the

scoring of these loci, as heterozygosity could be underestimated. To characterize the usefulness of the 30 SSR markers, the information content of each locus was assessed as the probability of identical genotypes (PI). Cumulative probabilities to obtain identical genotypes from different cultivars at each of the 30 loci were in the order of 1×10^{-17} . The number of observed alleles and the PI values indicate VVMD7 as the locus with the highest information content (10 alleles, PI = 0.111), whereas UDV109 (2 alleles, PI = 0.696) is the least informative. In addition, the effective number of alleles (n_e), i.e., the number of equally frequent alleles that would be required to produce the same homozygosity, was highest in VMC2C7 (4.902) and lowest in UDV109 (1.256). For UDV109, the distribution of allele frequencies was clearly skewed, with the most common of two alleles (124 bp) accounting for a cumulative frequency

Table 4. Allele size, allele frequency, and genetic diversity values for the three polymorphic chloroplast SSR loci in 22 distinct grapevine varieties and in 13 native northeastern Italian varieties ('Corbinella', 'Tintoria', and 'Cabernet Colli Euganei' are not included).

Locus	Allele size (bp)	22 varieties		13 native varieties	
		Allele frequency (%)	H_e	Allele frequency (%)	H_e
ccmp3	101	4.54	0.492	7.69	0.461
	102	31.82		23.08	
	103	63.64		69.23	
ccmp5	100	63.64	0.463	69.23	0.426
	101	36.36		30.77	
ccmp10	107	18.18	0.483	23.08	0.461
	108	68.18		69.23	
	109	13.64		7.69	
Average	103.87 (3.56)	37.5 (24.99)	0.479 (0.015)	37.5 (27.41)	0.449 (0.020)

Note: Standard deviations are in parentheses; H_e , expected heterozygosity.

of 88.5%. Considering the 22 different grapevine varieties, 46 variety-specific alleles (alleles detected in only one variety) were produced from 23 of the 30 SSR markers (Table S2²). Markers UDV27, VVMD7, and VVMD36 showed the highest number of variety-specific alleles in the population (four). Moreover, the local germplasm showed a higher proportion of unique alleles than the international cultivars, which showed only 12 unique alleles. The varieties showing the highest numbers of accession-specific alleles were 'Marzemina Nera Bastarda', Pignola, and 'Tintoria', with 5, 5, and 13 alleles, respectively.

Phylogenetic relationships among the grapevine varieties estimated by nuclear SSR markers

The proportion of shared alleles was used to calculate genetic similarity between all pairwise combinations in the population. This parameter assumes that all alleles are equally related (Bowcock et al. 1994) and this appears to be appropriate, as there is no sequence information on the evolution (stepwise mutation or infinite allele) of these alleles. The main advantage of this genetic similarity measure is that it requires no assumptions about the structure of the population or the allele frequencies within the population (Dangl et al. 2001). This parameter is therefore appropriate for unnatural populations such as grapevine cultivars. The average pairwise genetic similarity at 30 SSR loci among the 13 distinct local varieties was $P_s = 46\% \pm 10\%$. Based on the matrix of pairwise divergence values derived from the matrix of similarity, a dendrogram was obtained according to the UPGMA procedure (data not shown) and a network was derived by the neighbour-joining method (Fig. 1). The results obtained with both methods were generally congruent except for the Raboso varieties, which were clustered together in the network but separately in the cladogram, and the indigenous varieties, which were clustered together in the UPGMA dendrogram and in two groups in the neighbour-joining network. Both grouping analyses showed a clear separation between the international and indigenous cultivars. 'Corbinona' and 'Corbinella', 'Marzemina Nera Bastarda', 'Pattaresca', and 'Schiavetta Doretta' stand apart from a second native group. The average similarity between these varieties and the other local varieties was 44%, 40%, 50%, and 40%, respectively. With the exclusion of the 50% of 'Pattaresca', these values, with the 42% of average simi-

larity of Marzemina Bianca, represent the greatest average distance among the local varieties. In contrast, the highest pairwise similarities were detected between 'Gatta' and 'Agostana Nera' (65%), Raboso Veronese and Marzemina Nera (65%), and 'Schiavetta Doretta' and 'Pattaresca' (68%). Raboso Veronese presents 58% similarity with its putative parent Marzemina Bianca and 63% with the other putative parent, Raboso Piave. In both methods 'Tintoria' resulted as an outgroup with respect to the other varieties.

Genetic diversity and phylogenetic relationships among varieties estimated by chloroplast SSR markers

The consensus primers designed by Weising and Gardner (1999), which have been demonstrated to amplify polymorphic products in *Vitis* spp. (Arroyo-Garcia et al. 2002), were tested on the 22 distinct varieties. All three primer pairs yielded polymorphic products in our samples. Amplified fragments at each locus were found to be in the range of previously reported sizes for angiosperm species (Weising and Gardner 1999). All alleles differed by 1 bp, which is consistent with variation in the number of residues within a mononucleotide repeat. As in Arroyo-Garcia et al. (2002), two size variants were detected for ccmp5 (100 and 103 bp) and three were detected for ccmp10 (107, 108, and 109 bp), but three and not two were detected for ccmp3 (101, 102, and 103 bp) (Table 4). At locus ccmp3 the most frequent fragment was the 103 bp fragment (63.6%), at locus ccmp5 it was the 100 bp fragment (63.6%), and at locus ccmp10 it was the 108 bp fragment (68.2%). 'Pattaresca' was the only variety showing the 101 bp allele at locus ccmp3.

To characterize the chloroplast genetic diversity of our materials, the allele variation at the three loci was evaluated in the 22 distinct grapevine varieties and 13 native ones. Expected heterozygosity was similar in both groups, varying from 0.463 to 0.492 for the 22 distinct varieties and from 0.426 to 0.461 for the local ones.

Allele variants of the three loci combined in a total of 8 plastotypes of the 18 expected by random combination (Table 5). Haplotype I, corresponding to haplotype D of Arroyo-Garcia et al. (2002), was the most frequent (frequency higher than 50%). The 7 international cultivars showed much less genetic diversity than the indigenous ones, with only 3 plastotypes (Fig. 2): 4 cultivars belonged

Table 5. Haplotypes defined on the basis of eight polymorphic alleles found at loci ccmp3, ccmp5, and ccmp10 (the letters correspond to the plastotype nomenclature of Arroyo-Garcia et al. 2006), their frequencies, and the corresponding varieties (the international cultivars are in italics).

Haplotype	Allele size (bp)			Frequency			7 international varieties (%)	Varieties
	ccmp3	ccmp5	ccmp10	ccmp10	22 varieties (%)	13 native varieties (%) ^a		
I (D)	103	100	108	54.55	53.85	57.14	57.14	Group of 15 varieties ^b
II (A)	102	101	107	13.64	15.38	14.29	14.29	'Corbinona'/'Corbinella', 'Gatta', <i>Pinot Nero</i>
III (C)	102	101	109	9.09	0	28.57	28.57	<i>Chardonnay</i> , <i>Merlot</i>
IV (E)	103	100	109	4.55	7.69	0	0	'Agostana Nera'
V	102	100	108	4.55	7.69	0	0	'Marzemina Nera Bastarda'
VI	101	101	107	4.55	7.69	0	0	'Pataresca'
VII (F)	103	101	108	4.55	7.69	0	0	'Schiavetta Doretta'
VIII (B)	102	101	108	4.55	0	0	0	'Tintoria'

^a'Corbinella', 'Tintoria', and 'Cabernet Colli Euganei' are not included.

^b*Barbera*, *Cabernet Franc*, *Cabernet Sauvignon*, 'Cabernet Colli Euganei', 'Gruaja', Marzemina Bianca, 'Marzemina Cenerenta' / Marzemina Nera, 'Negrara Veronese', Pignola, Raboso Piave / 'Friularo', Raboso Veronese / 'Raboso Friularo', *Traminer*.

to haplotype I (D), 1 belonged to haplotype II (A), and 2 belonged to haplotype III (C). Haplotypes I (D) and II (A) were still rather frequent among the local varieties, whereas haplotype III (C) was not present. Varieties 'Agostana Nera', 'Marzemina Nera Bastarda', 'Pataresca', 'Schiavetta Doretta', and 'Tintoria' showed a cultivar-specific haplotype. Haplotype VIII (B) of 'Tintoria' is present in many non-*vinifera* *Vitis* species (Arroyo-Garcia et al. 2002). Haplotype diversity was higher in the indigenous varieties (66.27%) than in the international ones (57.14%).

Discussion

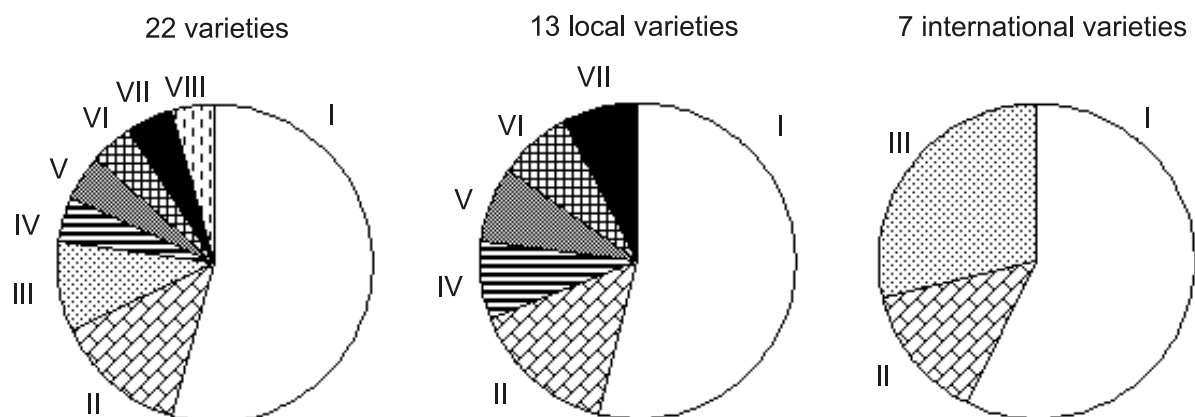
One of the major applications of microsatellite markers in viticulture is to identify and distinguish cultivars (Thomas et al. 1994; Bowers et al. 1996; Sefc et al. 1998). Microsatellite analysis is a powerful tool for characterizing a germ-plasm collection (Sefc et al. 2000; Lopes et al. 1999) and clarifying the origins of grapevine cultivars.

An analysis of genetic variation by nuclear and chloroplast SSR markers of 19 native grapevine varieties of northeastern Italy is reported in this paper. Samples from 7 international cultivars were also included as reference to the local materials. Except for 'Tintoria' and 'Cabernet Colli Euganei', the native varieties formed two tight clusters in both the dendrogram and the network on the basis of nuclear SSR markers, thus confirming their local origin (Fig. 1). The two clusters of indigenous varieties were also different at the chloroplast level (Table 5). The first included varieties that are very different from each other in the plastotype: 'Corbinona' and 'Corbinella', plastotype II(A); 'Marzemina Nera Bastarda', plastotype V; 'Pataresca', plastotype VI; and 'Schiavetta Doretta', plastotype VII(F). The second cluster instead shares plastotype I(D), except for 'Gatta', which has plastotype II(A), and 'Agostana Nera', which has plastotype IV(E).

The most economically important of the local varieties here analyzed is 'Friularo', which produces the homonymous DOC wine and has been cultivated mainly in the province of Padova since the 18th century (Caporali 1972; Pastò 1778). To investigate whether the name 'Friularo' is used for genotypes distinct from Raboso Piave, an old variety cultivated throughout northeastern Italy, 5 different accessions of 'Friularo' and 3 of Raboso Piave were analyzed at 30 SSR loci. Identical genotypes were detected in all 8 plants, confirming that the names 'Friularo' and Raboso Piave unambiguously refer to a single genotype.

In the past, the 'Marzemina' group was strongly widespread in northeastern Italy. Agostinetti mentioned 'Marzemina Bastarda' and Marzemina Bianca in 1679. The former has almost disappeared during the last 50 years as a cultivated grapevine because of the poor quality of its wine; the latter has lately been recovered and entered in the Italian Catalogue of Cultivated Varieties. In our study 'Marzemina Nera Bastarda' was genetically very different from the other 'Marzemina' varieties: a very low genetic similarity (38%) was observed between Marzemina Bianca and 'Marzemina Nera Bastarda'. Marzemina Nera, 'Marzemina Cenerenta', and Marzemina Bianca are phenotypically similar varieties that differ by berry colour: black in the first, grey in the second, and white in the third. Marzemina Nera and 'Marze-

Fig. 2. Frequency of eight plastotypes in the varieties analysed. The numbers correspond to the different haplotypes of Table 5.



mina Cenerenta' were found to be synonymous varieties at the chloroplast and nuclear SSR loci. Berry colour is regulated by a single locus in chromosome 2 (Doligez et al. 2002; Fischer et al. 2004; Salmasso et al. 2008) that comprises two very similar genes, *VvMYBA1* and *VvMYBA2*, located in a cluster (Walker et al. 2007). Marzemina Nera was found to be heterozygous for the red allele (data not shown). As already seen for Cabernet Sauvignon and its bronze berry mutant phenotype 'Malian' (Walker et al. 2006), 'Marzemina Cenerenta' is supposed to harbor a mutation of the red allele of Marzemina Nera and the investigation of the possible origin of this mutation is ongoing. Marzemina Nera and 'Marzemina Cenerenta' are therefore a single variety and 'Marzemina Cenerenta' is a berry colour mutant.

Considering the positive probability of null alleles at 6 loci, Marzemina Bianca and Marzemina Nera differed at 23 alleles out of 60. Nevertheless they always shared one allele at each locus, thus suggesting a very close relationship compatible with a parent-offspring relationship. Marzemino is a famous red wine produced from the variety Marzemina Nera. This cultivar is an ancient one, known since the 16th century (Caló et al. 2001). The presence of rare alleles (Table S2) of Marzemina Nera with respect to Marzemina Bianca leads us to suppose that the former is the older variety. Although grapevines are mainly self-pollinated owing to cleistogamic flower morphology, self-pollination is not compatible with our data. Cleistogamy makes artificial cross-pollination difficult (Vouillamoz et al. 2003), but wind and insects are occasionally responsible for cross-pollination (Mullins et al. 1992). Furthermore, the first recorded deliberate cross between plants was made in *Dianthus* spp. in 1718 (North 1979). Because Marzemina Bianca was already cultivated before its first mention in 1679 (Agostinetti 1679), it is inferred that Marzemina Bianca resulted from a spontaneous cross in the 16th century between Marzemina Nera and a black berry variety heterozygous for the red allele or a white one. Suppositions about the second parent cannot be made but it should be looked for among other varieties cultivated in northeastern Italy during the same period. However, the chance of identifying the second parent of a cross that occurred long ago depends on the availability of ancient cultivars, and while many old varieties are still grown today, others were neglected and became extinct. It will therefore not be easy to complete the cross combination.

The genetic differentiation between Cabernet Franc and

'Cabernet Colli Euganei' is interesting. These samples did not match for 18 of the 60 scored SSR alleles. 'Cabernet Colli Euganei' has been grown in the area of Lissida (Euganean Hills) since 1870, when it was imported as Cabernet Franc from France. However, the comparison of our data with those in public databases shows that this cultivar matches the red wine cultivar Carmenère except for one allele at locus VVMD36 showing a difference of one repeat unit with respect to Carmenère. According to Moncada et al. (2005), who observed 2 polymorphic SSRs out of 21 tested while analyzing 65 clones of Carmenère, this difference can be explained as intraclonal diversity. When differences of this kind are found, the dominant allele should be taken as a reference and the putative divergent one as a mutant form (Crespan 2004). Confusion between Cabernet Franc and Carmenère is not rare in Italy, especially in the northeast (Fregoni 2000); many cultivars, although originally imported as Cabernet Franc, are Carmenère but are still called Cabernet Franc, as happens for 'Cabernet Colli Euganei'.

Precise detection and quantification of genetic variation is a prerequisite for the successful conservation and exploitation of plant genetic resources (Powell et al. 1996). The potential of molecular markers to yield different genotypes for as many grapevine cultivars as possible is of great interest and the selection of the most informative markers can reduce the number of loci that need to be investigated for reliable cultivar distinction (Sefc et al. 2000). To study genetic diversity, the genome of grapevine varieties was fingerprinted using SSR markers. Tri- and tetra-allelic profiles instead of the usual mono- or bi-allelic ones were detected in two varieties by analyzing 30 nuclear SSR loci. The sequencing of these alleles in 9 of the 18 observed cases confirmed their simultaneous presence in the leaf tissue. When the analysis was repeated using the DNA from berry skin of the same varieties, only bi-allelic profiles were observed. Most plants have three identifiable cell layers (L1, L2, and L3) which contribute to different parts of the plant. If *Vitis* plants do have three cell layers, as suggested by leaf variegation observations (Derman 1947), the most likely interpretation is that one of the two original alleles has mutated and, through vegetative propagation, this mutation has been maintained in one of the distinct cell layers of the apical meristem, while the original genotype is maintained in the other cell layers of the plant. So it is assumed that these tri-

allelic genotypes could identify chimeras in which the cell layers are genetically different. The effective genotype will therefore be derived from different cell layers. Chimerism has been seen to occur at an appreciable rate in many grapevine cultivars (Crespan 2004; Franks et al. 2002; Hocquigny et al. 2004; Moncada et al. 2006; Riaz et al. 2002). In this study 26 varieties were sampled from a total of 34 individuals, 30 microsatellite loci were analysed, and 19 mutations were found (18 tri-allelic genotypes and 1 tetra-allelic), resulting in a mutation frequency of 9×10^{-3} . This frequency is higher than the value previously found, 8.24×10^{-5} (Crespan 2004). This could be due to the small size of our population but might also be due to the high mutation rate detected in the variety 'Schiavetta Doretta', which presents a tri-allelic profile at 37% of the examined microsatellite loci. A possible explanation is that 'Schiavetta Doretta', being an ancient variety, could be defective in mismatch repair, thus undergoing a higher incidence of mutations. Indeed, the likelihood is quite high that more mutations are accumulated and fixed in an old variety. This chimeric situation can be the starting point for the origin of new alleles. This variety might thus represent an interesting source of clonal variability from the different cell layers. The recovery of these mutants might generate new agronomically useful phenotypes. New approaches to intra-cultivar genetic improvement may indeed be possible with the separation of genetically distinct plants derived from cell layers and regenerated from embryogenic cultures of clones (Franks et al. 2002). Because it is not possible to know a priori which was the mutant allele, only genotypes with a mono- or bi-allelic profile were included in the characterization of the local germplasm.

The cumulative probability of obtaining identical genotypes from different varieties at each of the 30 nuclear microsatellite loci was in the order of 1×10^{-17} , which means that the marker set chosen possesses a high discriminative power in all the studied varieties. Observed heterozygosity in our samples varied between 15.4% (VVMD36) and 92.3% (VMC2C7 and VrZAG112) with a mean of 66%, lower than the level of 81% found for Italian cultivars by Sefc et al. (2000). Nevertheless, this mean value is higher than expected under Hardy-Weinberg conditions, which suggests a high genetic variability of the studied germplasm. Grapevine is known to be very sensitive to inbreeding depression. Highly heterozygous plants were likely selected in the course of the domestication and cultivation of grapevines, when the genotypes were evaluated according to their agronomic performance. The excess of heterozygosity observed in this study might thus be due to both natural and human selection against homozygosity in grape plants. To investigate the genetic similarities among cultivars, a phenogram of the cultivars according to their genetic distance was constructed with all 26 denominations corresponding to 22 different genotypes (Fig. 1). The average phenetic distance among the 13 native varieties (without 'Corbinella', 'Cabernet Colli Euganei', and 'Tintoria') was 0.54, lower than the 0.6 reported for mid-European cultivars (Sefc et al. 1998). Moreover, the indigenous varieties were more similar to one another than to other *Vitis vinifera* cultivars and presented a mean expected heterozygosity (0.628) lower than that found in Italian cultivars (0.790) by Sefc et al.

(2000). The lower variation could be attributed to a limited number of founders of our varieties and to ascertainment bias. This suggestion is supported by some putatively first-degree-related cultivars (Marzemina Bianca, Raboso Veronese, and Raboso Piave; Marzemina Nera and Marzemina Bianca; 'Negrara Veronese' and 'Gruaja'), indicating a narrow genetic basis for this germplasm. However, rare alleles were abundant, suggesting that a large number of introgression events contributed to the founding of the germplasm: a higher proportion of unique alleles (34) was observed among the 13 native cultivars than among the international cultivars (12) (Table S2). Unique alleles can be derived from mutation events, since SSR loci are known to have a mutation rate per locus and per generation of 2.5×10^{-5} to 1×10^{-2} (Weber and Wong 1993), but may also represent wild germplasm or wild derivatives (Barkley et al. 2006). Despite the limited amount of genetic variation that could be ascribed to "rare allele variants" in the gene pool, the noticeable number of different rare alleles detected indicates that a large number of plants contributed to the genetic base of the gene pool. These alleles are an important source of biodiversity, which refers either to the number of alleles in a given population or to the number of rare alleles. 'Marzemina Nera Bastarda', Pignola, and 'Tintoria' showed the highest numbers of accession-specific alleles. 'Marzemina Nera Bastarda', in particular, is also very different from the others at the chloroplast level, presenting a unique haplotype (F). 'Marzemina Nera Bastarda' also stands apart from the native group in the phylogenetic tree: the average similarity between this variety and other local varieties is 40%, representing, with 'Schiavetta Doretta', the lowest similarity or greatest distance among the local varieties. Genetic diversity has an impact on the higher levels of biodiversity: for a species, maintaining the capacity to evolve requires the existence of genetic diversity (Templeton 1994). We therefore think that a conservation programme is necessary to safeguard these endangered varieties.

'Tintoria' is another variety showing many accession-specific alleles. In this case, however, the accession-specific alleles are thought to derive from a putative hybrid with a non-*vinifera* *Vitis* species. The interspecific origin of 'Tintoria' is supported by the chloroplast SSR markers. The chloroplast haplotype B, present only in this variety, is very common among American species and rare in *Vitis vinifera* and is considered the most ancestral allele (Arroyo-Garcia et al. 2002). The phylogenetic tree also supports this hypothesis: 'Tintoria' is different from all other varieties, reflecting its putative hybrid origin.

In Traminer and Pinot Nero, one allele was shared at each of the 30 nuclear SSR loci, thus confirming the findings of Regner et al. (2000), who proposed Riesling Renano and Traminer as the progenitors of Pinot Nero. Chloroplast microsatellite markers, when polymorphic, may further contribute to a more precise determination of pedigree relationships among varieties by clarifying the cross direction, which is impossible with nuclear markers. In most angiosperm species they are maternally inherited (Corriveau and Coleman 1988). For the genus *Vitis*, there is experimental evidence demonstrating the maternal inheritance plastids (Strefeler et al. 1992). In our study Traminer and Pinot Nero presented two different plastotypes, haplotypes I and

II, respectively (Table 5), thus suggesting Traminer as the male parent of Pinot Nero.

A further putative parent–offspring relationship was identified between Cabernet Franc and Merlot. Cabernet Franc shared at least half of its alleles with Merlot, which is the basic condition for a parent–offspring relationship. Furthermore, both cultivars originate from the same region of France and present a similar phenotype for some traits. For these reasons we favour the hypothesis of a parent–offspring relationship between them. The two different plastotypes observed in Merlot and Cabernet Franc, haplotypes III and I, respectively, indicate a male parent–offspring relationship between them but it has not yet been established which is the male parent and which is the offspring of the cross. To ascertain the origin of the two cultivars, the identification of the other parent is under investigation. ‘Gruaja’ and ‘Negrara Veronese’ also shared one allele per locus except at VrZAG64 and VMC2A3, indicating a possible parent–offspring relationship. This parentage is further supported by historical data. ‘Gruaja’ has been cultivated in the northern Vicenza area since the 18th century (Acanti 1754) but nowadays is grown on very few farms in a small area of this province (Breganze). ‘Negrara Veronese’ is a variety no longer grown to produce wine, but it was widespread in the past in the same area as ‘Gruaja’ (Caló et al. 2004). The two varieties present a similar phenotype for some traits (Caló et al. 2004) and were both sometimes confused with Raboso cultivars. ‘Gruaja’ and ‘Negrara Veronese’ instead belong to a different cluster in the phylogenetic tree (Fig. 1). We suppose that before the 18th century a natural cross involving one of the two varieties as parent produced the second one. Self-pollination is not compatible with our data. Suppositions of the second parent cannot be made but it should be looked for among other varieties cultivated in the same area during the same period.

The suggestion that Raboso Veronese is the offspring of Raboso Piave $\times$ Marzemina Bianca is supported by the high likelihood ratio values (Table 3): it is 1×10^{17} times more likely that Raboso Veronese is the progeny of Raboso Piave and Marzemina Bianca than of any two random cultivars. Because the allele frequencies of the population were calculated at 30 microsatellite markers for 22 cultivars and this is a relatively small number of individuals, the likelihood ratios were also calculated with 95% upper confidence limits for the allele frequencies; the proposed parentage was 1×10^6 times more probable than parentage with any two random cultivars. The three chloroplast SSR loci were monomorphic in the three cultivars so it cannot be distinguished between the maternal and paternal parents. Nevertheless, Crespan et al. (2006) indicated Raboso Piave as the putative maternal parent of the cross. Marzemina Bianca was widespread in northern Italy in the past but during the post-phylloxera era this variety was gradually substituted by new cultivars (Crespan et al. 2006). Raboso Veronese is a black berry variety similar, on an ampelographic basis, to Raboso Piave. The two cultivars were also sometimes considered synonymous (Cosmo 1936–1937). The first citation of Raboso Piave is in Agostinetti (1679), whereas mention of Raboso Veronese appears for the first time in the 19th century (Vianello and Carpené 2002). Raboso Veronese is

thought to result from a spontaneous cross between Marzemina Bianca and Raboso Piave in the 19th century.

The three chloroplast SSR markers showed polymorphisms in the germplasm and alleles differing from each other by 1 bp, thus confirming the stepwise mutation model, which can be explained by replication slippage at microsatellite loci (Freimer and Slatkin 1996). The three chloroplast SSR loci displayed a total of 8 alleles, and a species-specific allele at locus ccmp3 was determined in ‘Pattaresca’ (Table 5). This is a new allele never previously found in other studies. ‘Pattaresca’, along with ‘Gatta’, ‘Corbinella’, and Raboso Piave, was the most widely cultivated variety in the province of Padova before the Second World War (Marzotto 1925). It was later substituted by other more productive cultivars and is now no longer cultivated (Caló et al. 2004). The presence of this unique allele supports the need for a conservation strategy to preserve grapevine genetic diversity: a population cannot evolve nor adapt to environmental change without genetic diversity (Templeton 1994).

Intraspecific polymorphisms at loci ccmp3 and ccmp10 were also observed in *Actinidia*, *Lycopersicon*, and *Nicotiana* (Weising and Gardner 1999). As in other studies of different species (Arroyo-Garcia et al. 2002; Weising and Gardner 1999), ccmp10 showed the highest genetic diversity value (0.461) (Table 4). The preservation of polymorphisms at these loci suggests different evolutionary rates for chloroplast sequences that are conserved in different botanical families.

The average number of alleles at the chloroplast loci was lower in the international cultivars than in the local varieties. The average H_e values calculated for the chloroplast SSR loci were lower than those estimated for the nuclear SSR loci. This reflects the lower mutation rates and relative stability of the chloroplast genome with respect to the nuclear genome (Clegg et al. 1994). Eight plastotypes were recognized among all the cultivars. According to Arroyo-Garcia et al. (2002, 2006), most grapevine varieties possess plastotype I (54%) or II (15%). The third major plastotype was present only in the French cultivars Chardonnay and Merlot. Five accession-specific haplotypes were detected in the local varieties. In particular, plastotype VIII, present in many non-*vinifera* *Vitis* varieties (Arroyo-Garcia et al. 2002), was found only in ‘Tintoria’. Because this plastotype and other phenotypic traits belonging to American species are present in this variety (Tocchetti 1978), ‘Tintoria’ should be considered an interspecific hybrid. The other four plastotypes, IV, V, VI, and VII, were specific to ‘Agostana Nera’, ‘Marzemina Nera Bastarda’, ‘Pattaresca’, and ‘Schiavetta Doretta’, respectively. If locus ccmp5 in plastotype V is not considered, the resulting haplotype is found to be the same as a rare genotype spread among Iberian, Italian, Caucasian, and Iranian wild grapevine populations (Doulaty Baneh et al. 2007; Grassi et al. 2002, 2006). This rare wild plastotype typical of ‘Marzemina Nera Bastarda’, together with 5 specific nuclear alleles, should be ascribed to an ancient genotype. On this basis, ‘Marzemina Nera Bastarda’ is hypothesized to be one of the first domesticated grapevine varieties in northeastern Italy. Again, this variety should be considered a high-priority target for conservation of grapevine biodiversity to prevent genetic erosion. Moreover, we

think that 'Marzemina Nera Bastarda' will also be useful in future breeding programmes for its high resistance to disease (Caló et al. 2004). Plastotypes IV and VII of 'Agostana Nera' and 'Schiavetta Doretta' are infrequent haplotypes found only in Near Eastern *V. vinifera* subsp. *sylvestris* populations (Arroyo-Garcia et al. 2006). Accession-specific haplotype VI of 'Pattaresca', revealed for the first time in this work, should be considered typical of northeastern Italy. The presence of many specific haplotypes for the local varieties supports the hypothesis of a multi-geographical contribution of wild grapevine to the regional cultivated gene pool during different periods in the past. According to Grassi et al. (2003), some varieties could be derived from secondary domestication events or local breeding programmes from wild and cultivated accessions in the Mediterranean basin.

In conclusion, this work has shown that the characterization of a germplasm collection using microsatellites is very useful for its management. The observed parent-offspring relationships can be summarized as follows:

1. Merlot–Cabernet Franc, direct parentage line, different plastotypes;
2. Marzemina Bianca = Marzemina Nera × unknown variety;
3. Pinot Nero = Riesling Renano as the female parent × Traminer as the male parent;
4. Chardonnay = Gouais Blanc as the female parent × Pinot Nero as the male parent;
5. 'Gruaja'–'Negrara Veronese', direct parentage line, same plastotype; and
6. Raboso Veronese = Raboso Piave × Marzemina Bianca, same plastotype; it was not possible to determine the direction of the cross.

The majority of cultivars were demonstrated to constitute an independent source of genetic variation, and therefore a valuable source of variability for grapevine breeding programmes. The integration of our results with ampelographic data would be of great importance to unambiguously identify the native cultivars and could also be used for legal protection of cultivars. This germplasm therefore represents a unique, invaluable genetic resource for cultivated grapevines. Varieties such as 'Agostana Nera', 'Corbinella', 'Corbinona', 'Gatta', 'Marzemina Cenerenta', Marzemina Nera, 'Marzemina Nera Bastarda', 'Negrara Veronese', Pignola, 'Pattaresca', and 'Schiavetta Doretta' are major sources of new nuclear and (or) chloroplast alleles. Their disappearance from their natural habitat would be an irreversible loss for breeding programmes and the environment.

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