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G. Di Gaspero · E. Peterlunger · R. Testolin
K.J. Edwards · G. Cipriani

Conservation of microsatellite loci within the genus *Vitis*

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Abstract Eleven microsatellites isolated from grapevine (*Vitis vinifera*) were used to study the degree of conservation of these sequences across different *Vitis* species. Nine microsatellites were newly isolated, the remaining two (VVS2 and VVS5) came from the literature. A preliminary assay on the conservation of priming sites was carried out on 14 non-*V. vinifera* species, including relevant taxa for breeding. *Parthenocissus quinquefolia* was added as representative of a related genus. Cross-species amplification was obtained in 94% of the 176 genotype×locus tested combinations. Three microsatellite loci were then cloned and sequenced in ten species. The microsatellite repeat was found present in all cases. The repeat region was often longer in *V. vinifera* than in the other species. Furthermore the non-source species showed interruptions in the repeat. In spite of these constraints, which could reduce the polymorphism of microsatellites in non-source species, the results demonstrate the possibility of extending the use of microsatellite markers to wild germplasm and interspecific hybrids. Point mutations have been found in microsatellite flanking regions and these variations have been used to investigate the genetic relationship among taxa. The Neighbor-joining tree that was obtained on the basis of ten nucleotide variations, showed that there is not a clear cut difference between American, Asian and European species and that the actual taxonomy which reflects the geographical distribution of species must most likely be revised. Moreover, in general, nucleotide variations which occur in microsatellite flanking regions provide new molecular tools for investigating the evolution of species.

Key words Simple sequence repeats · Inter-specific priming · Molecular markers · Grapevine

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G. Di Gaspero · E. Peterlunger · R. Testolin · G. Cipriani (✉)
Dipartimento di Produzione Vegetale e Tecnologie Agrarie,
University of Udine, via delle Scienze, 208-33100 Udine, Italy
e-mail: guido.cipriani@dpvta.uniud.it
Tel.: +39-0432-558635, Fax: +39-0432-558603

K.J. Edwards
IACR-Long Ashton Research Station,
Department of Agricultural Sciences, University of Bristol,
Long Ashton, Bristol BS18 9AF, UK

Introduction

Simple sequence repeats (SSRs) or microsatellites have become an important source of highly polymorphic genetic markers. These are widely used for the construction of linkage maps, the analysis of the genetic structure of populations and parentage testing (Rafalski et al. 1996). Lastly, they have been proposed for studies on the evolution of species (Goldstein and Pollock 1997).

The amplification of microsatellite loci by means of the PCR assay requires a knowledge of their DNA sequence, which is expensive and time consuming to obtain. One way to increase the utility of microsatellites, once they have been isolated and sequenced in a source species, is to transfer these markers to related species. There is a growing number of studies reporting cross-species conservation of repeat-flanking regions and, in some cases, even among very divergent taxa (for a review see Peakall et al. 1998). In fruit crops, heterologous amplification of SSRs has been tested in grapevine (Thomas and Scott 1993), *Prunus* (Cipriani et al. 1999), and *Actinidia* (Weising et al. 1996; Huang et al. 1998).

Grapevine is one of the oldest agricultural crops that mankind has used to produce table fruit, raisins, juice and wine (Olmo 1976). *Vitis vinifera* L. is cultivated world-wide and many important cultivars have been selected through the centuries. The inventory of grape cultivars described in the literature revealed the existence of more than 14000 putative cultivars (Alleweldt et al. 1990). This great genetic diversity originated from the old practice of growing seedlings noticed by chance and, to a less extent, by the breeding activity. The dispersion of grape cultivars or populations of different origin by means of migration and trade led to a high number of

synonymies, omonymies and ambiguities in cultivar identification that require a reliable method to be solved. Microsatellite DNA has been successfully used in grape for genomic studies, providing considerable resolving power for accurate variety labelling (Thomas and Scott 1993; Cipriani et al. 1994; Sefc et al. 1998a), pedigree reconstruction (Bowers and Meredith 1997; Sefc et al. 1998b) and genetic resources analysis (Lamboy and Alpha 1998).

Non-*V. vinifera* species and inter-specific hybrids are widely used as rootstocks. They are also a source of genes for breeding both table and wine cultivars (Alleweldt et al. 1990). Genetic diversity in grape is most likely considerable and numerous collections of germplasm have been established world-wide, but a detailed molecular analysis of these genetic resources has not yet been performed. Cross-species transportability of microsatellite markers seems to be a good tool for evaluating *Vitis* genetic resources. Although almost all the previous studies have demonstrated a wide cross-species amplification of microsatellite loci, no direct evidence for the presence of a repeat unit within the amplified regions has been provided.

With the aim of providing new insights on microsatellite locus conservation across *Vitis* species, we analysed 11 microsatellites developed for *V. vinifera* in 14 related species of the genus *Vitis*, including both sections *Vitis* and *Muscadinia*, as well as *Parthenocissus quinquefolia* (L.) Planch., a species belonging to a related genus of the same family.

Materials and methods

Plant material and DNA extraction

Three cultivars of *V. vinifera* L. ('Cabernet Sauvignon', 'Sultanina' and 'Syrah'), 14 species of both *Euvitis* and *Muscadinia* sections, and a species of the related genus *Parthenocissus* (*P. quinquefolia* (L.) Planch.) were used in the present study. The list of species is reported in Table 1. The plants came from the germplasm collection held at the Experimental Farm of the University of Udine, Italy.

DNA was extracted from 200 mg of young leaves using the method of Doyle and Doyle (1990) with the following modification: 1% PVP 40 (polyvinylpyrrolidone) was added to the extraction buffer.

Cross-species amplification of microsatellite loci

Nine microsatellite-containing DNA sequences were selected from several obtained by sequencing clones supplied by Agrogene (Moissy-Cramayel, France). Clones had been produced from a genomic library of *V. vinifera* cv 'Syrah' enriched for ten different microsatellite repeats. Primer pairs were designed in microsatellite flanking regions. Two further primer pairs amplifying the VVS2 and VVS5 microsatellite loci and published by Thomas and Scott (1993) were added to the set (see Table 2).

Polymerase chain reactions were carried out in a total volume of 25 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer, 1 unit of AmpliTaq Gold (PE Biosystems) and 50 ng of template DNA, using the following thermal profile: (1) 94°C for 10 min; (2) 94°C for 1 min, annealing temperature as reported in Table 2 for 1 min, 73°C for 1 min per 35 cycles; (3) 73°C for 7 min. PCR products were separated by electrophoresis using 2.5% MetaPhor agarose gels (FMC Bioproducts) in 0.5× TBE buffer at 4 V/cm. PCR was repeated at least twice when bad or no amplification occurred at the first attempt.

Microsatellite cloning and sequencing in *Vitis* species

Six microsatellites, including three AG/CT (VMC4D2, VMC4A5 and VVS2), two GCT/AGC (VMC4D4 and VMC4C6) and one AC/GT (VVS5), were amplified, cloned and sequenced in six *Vitis* species (*Vitis amurensis* Ruprecht, *Vitis berlandieri* Planch., *Vitis labrusca* L., *Vitis riparia* Mchx., *Vitis rupestris* Scheele and *Vitis vinifera* cv 'Sultanina') in a preliminary experiment.

Three loci (VMC4D4, VMC4C6, VVS5) were selected for their polymorphism in the repeat flanking regions and sequenced in an extended panel of ten species (Table 1), chosen to represent most of the taxonomic groups into which the genus *Vitis* is actually split (Galet 1988).

PCR-amplifications were performed as described above but with two exceptions: (1) the total volume was increased to 50 µl; and (2) the final elongation step was increased to 10 min. PCR products were separated in a 2% agarose gel (1% NuSieve, 1%

Table 1 List of taxa of the family Vitaceae used in cross-species amplification of 11 microsatellite loci

Species	Section/series[a]	Geographic origin[b]	
V. vinifera cv Cabernet Sauvignon	*Vitis/Viniferae*	Euro-Asia	*
V. vinifera cv Sultanina	*Vitis/Viniferae*	Euro-Asia	*
V. vinifera cv Syrah	*Vitis/Viniferae*	Euro-Asia	*
V. amurensis	*Vitis/Flexuosae*	East Asia	*
V. armata	*Vitis/Spinosae*	East Asia	*
V. aestivalis	*Vitis/Aestivalae*	North America	
V. arizonica	*Vitis/Arizonae*	North America	*
V. berlandieri	*Vitis/Cinereae*	North America	*
V. cinerea	*Vitis/Cinereae*	North America	
V. champinii	*Vitis/Candicansae*	North America	
V. doaniana	*Vitis/Candicansae*	North America	
V. longii	*Vitis/Candicansae*	North America	
V. labrusca	*Vitis/Labruscae*	North America	*
V. riparia var. Gloire	*Vitis/Ripariae*	North America	*
V. rupestris var. du Lot	*Vitis/Ripariae*	North America	*
V. rupestris var. Metallica	*Vitis/Ripariae*	North America	
V. slavinii	*Vitis/Ripariae*	North America	
V. rotundifolia	*Muscadinia*	North America	*
Parthenocissus quinquefolia		North America	*

[a] As reported by Galet (1988)
[b] The asterisks mark the species in which the three microsatellite loci were sequenced. (See Materials and methods for details)

Table 2 List of microsatellite loci analysed

Locus name	Primer sequence	Microsatellite motif[a]	Annealing temp. (°C)	Reference
VMC4A1	5′ATGCGACCTTAATAAATTGGGAA 5′AAGCTAGGCTTGTATGAGGGAGA	$(AG)_{20}$	61	Present study
VMC4D2	5′TGCAGATACCACATACCCACCT 5′AACAGCAAACATCCCAACTCAG	$(AG)_{16}$	61	Present study
VMC4F3	5′AAAGCACTATGGTGGGTGTAAA 5′TAACCAATACATGCATCAAGGA	$(AG)_{20}AA(AG)_9$	61	Present study
VMC4D4	5′GTCTTGTAATGGAACCAACTGC 5′AGATTGACCTGGACCTGAAACT	$(GCT)_9$	61	Present study
VMC4A5	5′ATTTTCCACAGGCAAACCACAT 5′TGTGGTTGTTGTAGCCTATCGG	$(AGAC)_5N_{31}(AG)_{14}$	61	Present study
VMC4H5	5′GATTTGTGACACTTGTGTAGCG 5′CAAGTGGAAAGCAATCTAGGAA	$(AG)_5TG(AG)_{33}$	61	Present study
VMC4C6	5′CTCCATCCCTATCTCATCAG 5′CTCTAACACCCAATCTCACA	$(GCT)_{11}$	51	Present study
VMC4G6	5′CCTTGAAGAGATGAGTTTGCTA 5′TATTTAACTTTGTGCCTCTGCT	$(AG)_{17}$	51	Present study
VMC4H6	5′GTATAGAACCACGCATCCAACA 5′CCCTTAGTTTCCTCGTGCTTTT	$(AG)_{23}$	61	Present study
VVS2	5′CAGCCCGTAAATGTATCCATC 5′AAATTCAAAATTCTTATTCAACTGG	$(AG)_9$	50	Thomas and Scott 1993
VVS5	5′ATTGATTTATCAAACACCTTCTACAT 5′TAGAAAGATGGAAGGAATGGTGAT	$(AC)_{21}$	50	Thomas and Scott 1993

[a] Microsatellite motif present in the clone sequenced from DNA library of *V. vinifera* cv Syrah

SeaKem, FMC Bioproducts) and recovered on a DEAE cellulose membrane (Schleicher and Schuell, NA-45). Bound DNA was eluted following the method of Sambrook et al. (1989), precipitated with 2.5 vol of absolute ethanol and 1/9 vol of 3 M Na-acetate and re-suspended in 10 µl of H_2O.

Cloning reactions were carried out with the TOPO TA Cloning kit (Invitrogen) using 1 µl of the purified DNA and following the instruction manual. Two to three single colonies for each DNA insert were picked up and cultured for plasmid DNA extraction. The clones were sequenced using an ABI 373A automated sequencer.

Data analysis

Multiple sequence alignments and tree construction with the Neighbor-joining method were realised by the CLUSTALX 1.64b package (Thompson et al. 1997). The N-J tree was drawn by TREEVIEW (Page 1996).

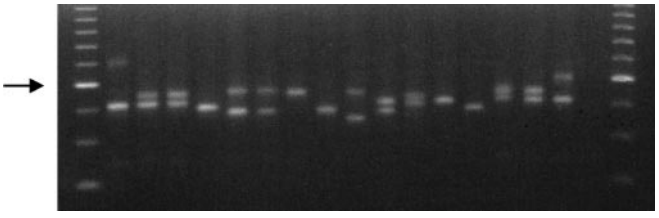

Fig. 1 PCR amplification and electrophoresis, into a 2.5% MetaPhor agarose gel, of the microsatellite locus VVS5 in 17 species and genotypes of the family Vitaceae. Lanes 1 and 19: 25 bp marker, the arrow points to the 125 bp band; lanes 2–18: *V. aestivalis*, *V. amurensis*, *V. arizonica*, *V. armata*, *V. berlandieri*, *V. champinii*, *V. cinerea*, *V. doaniana*, *V. labrusca*, *V. longii*, *V. rotundifolia*, *V. riparia*, *V. rupestris* du Lot, *V. rupestris* Metallica, *V. slavinii*, *V. vinifera* Cabernet Sauvignon, *Parthenocissus quinquefolia*

Results

Microsatellite characterisation in *V.vinifera*

The most frequent microsatellite repeat found in grapevine was the $(AG)_n$ repeat (data not reported). This was also the most frequent repeat in the panel of microsatellites selected for the assay on cross-species transportability (Table 2). Besides $(AG)_n$, which often occurred as a compound or imperfect motif, rare tri-nucleotide repeats were found, two of which were included in the assay (Table 2). The high frequency of the $(AG)_n$ repeat is in agreement with the preliminary results reported for grape by Thomas and Scott (1993).

Cross-species amplification

Eleven primer pairs which produced prominent and strong bands on an agarose gel in *V. vinifera* were also used for cross-species amplification in 15 non-*V. vinifera* accessions and *P. quinquefolia*.

A total of 176 PCRs was carried out (Table 3). One or two scorable bands of the expected size were obtained in 165 assays (94% of the examined cases). PCR was repeated at least twice when bad or no amplification occurred at the first attempt. In most cases (78%) bands were of good quality (Table 3 and Fig. 1). Mis-amplifications were mainly from the microsatellite VMC4A1 whose primers failed to amplify in 50% of the tested species. The VVS5 primers amplified a further faint band in

Table 3 Quality of cross-species amplification of 11 *V.vinifera* microsatellite loci amplified in 15 non-*V. vinifera* accessions and in *P. quinquefolia*. The numbers indicate: 1, strong amplification with one or two bands of expected size; 2, one or two prominent bands of expected size in a weak smear; 3, weak amplification of one or two bands of expected size; 4, no amplification; 5, amplified products of unexpected size

Species	VMC microsatellite loci										
	4A1	4D2	4F3	4D4	4A5	4H5	4C6	4G6	4H6	VVS2	VVS5
V. aestivalis	2	1	2	1	2	1	1	1	1	1	1
V. amurensis	5	1	1	3	2	1	1	1	1	1	1
V. arizonica	1	1	2	3	1	3	1	2	1	1	1
V. armata	1	1	1	3	1	1	1	1	1	1	1
V. berlandieri	4	1	1	3	1	1	1	1	1	1	1
V. champinii Texas	4	1	1	1	1	1	1	1	1	1	1
V. cinerea	1	1	1	3	1	1	1	1	1	1	1
V. doaniana	3	1	1	3	1	3	1	1	1	1	1
V. labrusca	1	1	2	3	1	1	1	1	1	1	1
V. longii	4	2	1	1	2	1	1	1	1	1	1
V. rotundifolia	1	3	1	1	2	1	1	1	1	1	1
V. riparia Gloire	4	1	1	1	1	3	1	1	1	1	1
V. rupestris du Lot	4	1	1	1	2	3	1	1	1	1	1
V. rupestris Metallica	4	1	1	1	2	3	1	1	1	1	1
V. slavinii	1	1	2	1	2	1	1	1	1	1	1
P. quinquefolia	4	1	4	1	1	4	1	1+5	1	1	4

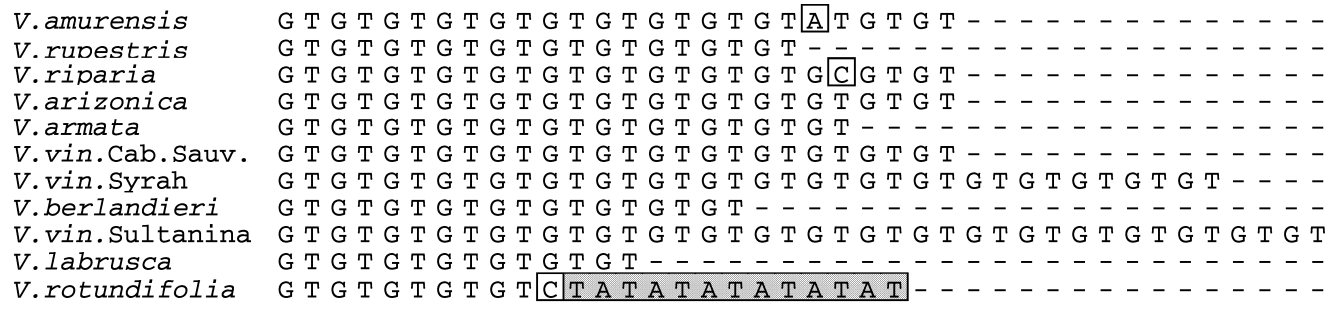

Fig. 2 ClustalX 1.64b multiple sequence alignment: structural evolution of VVS5 microsatellite repeats in 11 *Vitis* genotypes. Gaps (–) indicate length difference, *boxes* indicate interruptions and the *shaded box* indicates changes in the repeat type

all accessions, which was recognised as a second monomorphic 60 bp-long locus. Seven primers (64%) also gave successful amplifications in *P. quinquefolia*.

Sequence comparison in *Vitis* species

The preliminary sequencing assay carried out in a few species (data not shown) resulted in the presence of the repeat motifs in all the analysed sequences with the exception of the second locus amplified by the VVS5 primers, where the repeat was absent in all four checked species (*Vitis arizonica* Engelm., *V. berlandieri*, *Vitis rotundifolia* Mchx., *V. vinifera*). The sequences of the two loci amplified by VVS5 primers provided evidence that the short monomorphic fragment did not contain the microsatellite whereas the polymorphism of the longer fragments was due to the length variation of the microsatellite repeat.

The microsatellites showed a tendency to be shorter in non-*V. vinifera* species than in *V. vinifera* from which the sequences were first isolated. This has already been reported in the literature (Ellegren et al. 1995) and explained by the protocols followed in isolating microsatellite repeats, which sample the longest repeats in the source genome.

Beside variations in repeat length, the microsatellites exhibited further modifications within the repetitive structure, consisting of interruptions or changes in the repeat type.

Most of these variations found in the microsatellites examined could be summarized in terms of the example of the microsatellite VVS5, whose aligned sequences are reported in Fig. 2.

In that locus the long $(GT)_n$ repeat of the source species (*V. vinifera*) is shorter in most of the other species. Several single-base substitutions can also be noted. For instance, a G→A substitution occurs in *V. amurensis* and a T→C substitution in *V. riparia*. A more complex pattern can be seen in *V. rotundifolia* where a G→C substitution is followed by a new $(TA)_n$ repeat.

Fig. 3 ClustalX 1.64b multiple sequence alignment of locus VMC4D4 (EMBL accessions from AJ252873 to AJ252884). Gaps (–) have been placed to maximize the homology, *asterisks* (*) correspond to sequence positions with identical nucleotides in all the species. *Numbers* above the boxes indicate mutation sites. The *shaded box* indicates a microsatellite repeat ▶

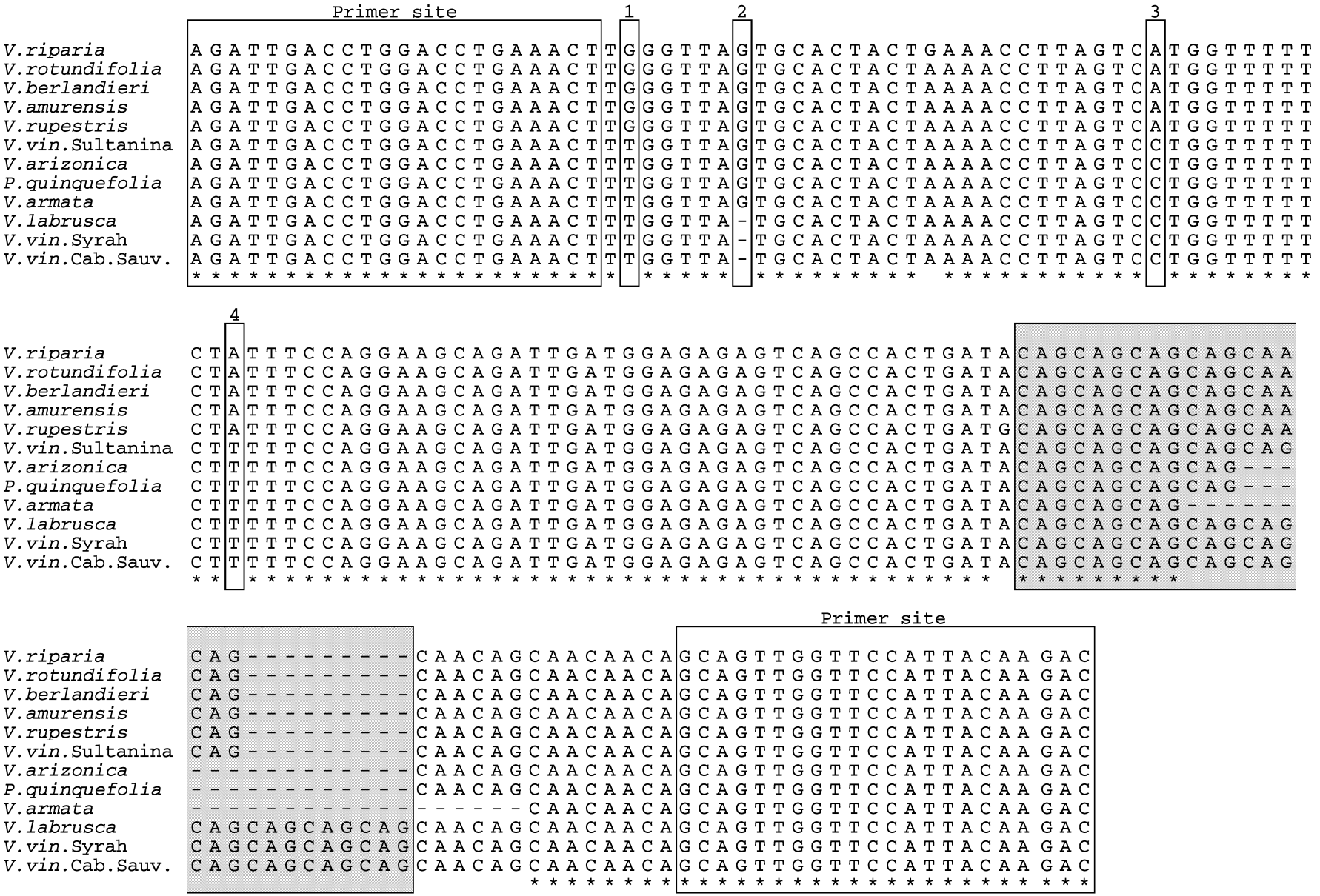
Primer site
1
2
3
4
Primer site
V.riparia
V.rotundifolia
V.berlandieri
V.amurensis
V.rupestris
V.vin.Sultanina
V.arizonica
P.quinquefolia
V.armata
V.labrusca
V.vin.Syrah
V.vin.Cab.Sauv.

Fig. 4 Mutation sites in three microsatellite loci across ten Vitaceae species

	Mutation sites									
	1	2	3	4	5	6	7	8	9	10
V. amurensis	G	G	A	A	C	C	T	T	G	C
V. arizonica	T	G	C	T	T	A	T	T	G	C
V. armata	T	G	C	T	T	A	T	T	G	G
V. berlandieri	G	G	A	A	T	A	A	T	G	G
V. labrusca	T	-	C	T	T	A	T	T	G	G
V. riparia Gloire	G	G	A	A	C	C	T	T	G	C
V. rotundifolia	G	G	A	A	T	A	A	T	G	G
V. rupestris du Lot	G	G	A	A	C	C	T	T	G	C
V. vinifera cv Cab.Sauvignon	T	-	C	T	T	A	T	T	G	G
V. vinifera cv Sultanina	T	G	C	T	T	A	T	A	A	G
V. vinifera cv Syrah	T	-	C	T	T	A	T	T	G	G
Parthenocissus quinquefolia	T	G	C	T	C	C	T	T	G	*

* No amplified product

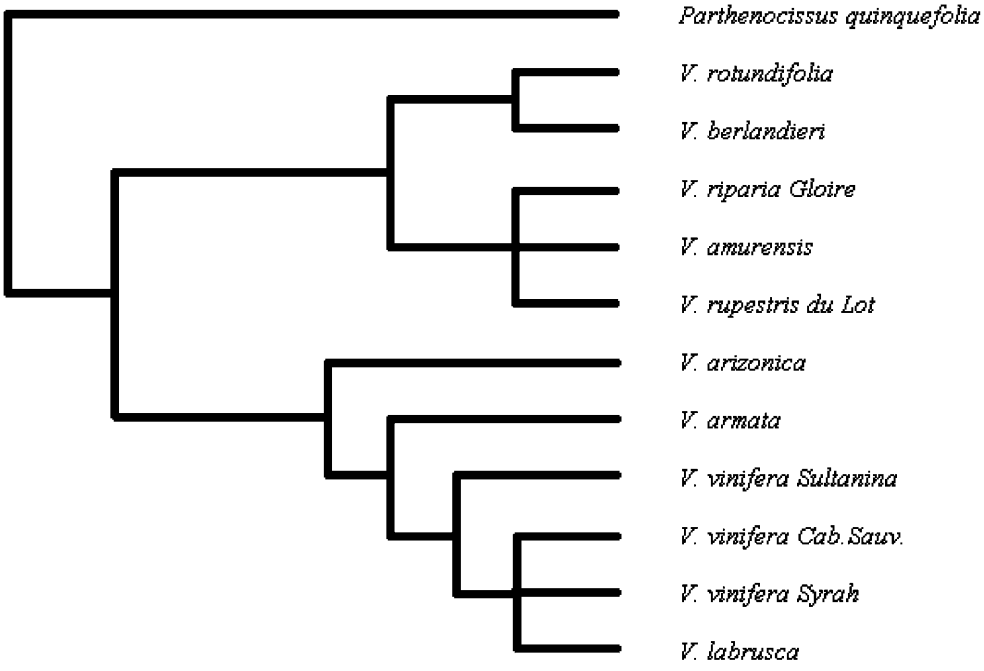

Fig. 5 Neighbor-joining dendrogram of genetic distance between 11 *Vitis* genotypes according to the mutation sites of Fig. 4. *P. quinquefolia* was forced as outgroup

Point mutations have also been observed along the microsatellite flanking regions. These variations have been considered in more detail in three loci (VMC4D4, VMC4C6, VVS5) that were sequenced in ten species (Fig. 3). Those three loci were also sequenced in three cultivars of *V. vinifera* to verify whether point mutations occurred within the species. Several nucleotide substitutions in microsatellite flanking regions were found in some species whereas no variability was found among the three genotypes of *V. vinifera*, with the exception of the cv 'Sultanina', that showed a double consecutive substitution (TG →AA, mutation sites 8 and 9) in one allele of the locus VMC4C6 and an insertion in the locus VMC4D4 (mutation site 2 in Fig. 4).

A total of ten 'true' mutation sites in microsatellite flanking regions was found (Fig. 4). These mutations were used to group the species according to their sequence homology (Fig. 5). Point mutations, when present in only one species were disregarded because in those cases a mis-sequencing could not be excluded on principle. The genetic distance was evaluated with the Neighbor-joining method with *P. quinquefolia* forced as an outgroup. The tree showed two main branches, each one including species of different geographic origin. In detail, the Asian species *V. amurensis* and *Vitis armata* Diels & Gilg were separated in the two main branches of the N-J tree. Additionally, the American species *V. arizonica* and *V. labrusca* were placed far from the geographically contiguous species *V. berlandieri*, *V. riparia* and *V. rupestris*. In contrast, the two American species *V. riparia*, *V. rupestris* and the Asian species *V. amurensis* had identical nucleotides at all polymorphic sites. Another group including the American species *V. labrusca* and the two European *V. vinifera* cultivars ('Cabernet Sauvignon' and 'Syrah') showed a similar genetic homology.

Discussion

The panel of species chosen to test the cross-species transportability of microsatellites isolated from *V. vinifera* included a wide range of species of the genus *Vitis*. The degree of transportability was very high. Ten loci (91%) were correctly amplified in all 15 examined *Vitis* species. The last locus, named VMC4A1, which exhibited a multi-banding pattern in *V. vinifera*, failed to be amplified in nearly half of the non-*V. vinifera* species. If that locus is disregarded, the efficiency of cross-species amplification would rise to 100%. Data suggest a high degree of conservation of microsatellite flanking regions in *Vitis*, much higher than that published for other fruit crops such as *Actinidia*, for which the cross-species transportability of microsatellites reached 75% (Huang et al. 1998), and *Prunus*, for which the transportability was much lower (59%) (Cipriani et al. 1999).

Besides the genus *Vitis* we tested another taxon belonging to the family Vitaceae and we obtained amplification in *P. quinquefolia* at 64% of the loci, a value far higher than that found by Peakall et al. (1998) in legume cross-genera amplification. All this evidence suggests that speciation in *Vitis*, and possibly in the whole family Vitaceae, took place rather recently.

Examples of conservation of non-coding DNA sequences across genera have been reported for a number of animals, such as cetaceans (Schlöetterer et al. 1991), rodents (Kondo et al. 1993), birds (Primmer et al. 1996), and fishes (Rico et al. 1996), as well as for crops like barley (Saghai-Maroof et al. 1994), *Citrus* (Kijas et al. 1995), *Brassica* (Szewc-McFadden et al. 1996), the Cucurbitaceae (Katzir et al. 1996), the legumes (Peakall et al. 1998) and also for grape (Thomas and Scott 1993; Lamboy and Alpha 1998).

While successful amplification across species indicates that flanking regions are highly conserved it does not guarantee the presence of the repeat within the sequence, which is a pre-requisite for using SSRs as molecular markers in non-source species. We have found the microsatellite repeat always to be present, although the source species exhibited a tendency to have longer repetitive regions than non-source species, as noted in legumes (Peakall et al. 1998). Moreover, differences between species in the repeat sequence were more complex than simple changes in the number of core motif repeats, as interruptions were frequently found in non-*V. vinifera* species, suggesting an ascertainment bias versus the species in which the microsatellites were first isolated (Goldstein and Pollock 1997).

The wide efficiency of cross-species amplification and the presence of microsatellite repeats in all species mean that polymorphic markers identified in one species could be used for genetic analysis in related species. This is an interesting finding for plants where microsatellites tend to be less frequent than in animals (Lagercrantz et al. 1993). The cross-species transportability of microsatellite markers is even more important in *Vitis*, where non-*V. vinifera* species and inter-specific hybrids are commonly used as rootstocks and as row materials in breeding programs.

Microsatellites are sometimes used for studies on the evolution of species (Goldstein et al. 1995). Both the variation in repeat length and mutations/insertions/deletions in flanking regions are matters of study, but such efforts have yet to yield results. Until the mechanism leading to the variation in the repeat length is better understood, the hyper-variable DNA regions have low reliability in evolutionary studies. On the other hand, the analysis of shared point mutations in regions flanking the repeat motif is, at present, more promising as an approach to the reconstruction of relationships among related taxa (Pépin et al. 1995; Ortì et al. 1997).

The similarity tree reported in Fig. 5 is based on such nucleotide substitutions or on insertions/deletions occurring in microsatellite flanking regions. Surprisingly, those data led us to group together some *Vitis* species representative of separated gene centers whereas other species actually growing in contiguous geographic regions spread into two less-genetically related groups.

In particular, the two American species *V. riparia* and *V. rupestris* were grouped together with *V. amurensis*, an Asian species which showed identical nucleotides at all mutation sites. Another group encompassed the American species *V. labrusca* and two cultivars of *V. vinifera* (‘Cabernet Sauvignon’ and ‘Syrah’).

Although this grouping seems to diverge from the traditional taxonomy based on geographical distribution and morphological similarities, our results do provide some support. Morphological affinities among the American species *V. labrusca* and the Asian species *Vitis coignetiae* Pulliat have already been reported in the past (De Lattin 1939), and this author suggested that a geographical separation could not always correspond to a genetic diversity. Furthermore, some species considered native to a given area show resistance to pathogens which are not endemic in that area, which indicates that those areas could not correspond to the center of origin of those species (He and Wang 1986). All this evidence is consistent with the theory that the genetic affinities among species which actually grow in different continents are due to a pre-glacial connection of gene centers (Lazic et al. 1968). Parfitt and Badenes (1997) reported that in the genus *Pistacia* two American species and one Asian species are closely related on the base of parsimony and cluster analysis, suggesting the presence of a common ancestor native to Asia. Mapping the geographical distribution of genetic variations in other plant genera would provide support for the hypothesis that the actual geographical location of a species does not always reflect its relationships and evolution history.

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