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Untranslated leader region polymorphism of *Tvv1*, a retrotransposon family, is a novel marker useful for analyzing genetic diversity and relatedness in the genus *Vitis*

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Abstract Grapevine retrotransposons belonging to the *Tvv1* family share a single, highly conserved open reading frame but differ by their untranslated leader (UTL) region, which is highly variable in size. Amplification of the UTL region of *Tvv1* elements from 94 Vitaceae accessions reveals that each of them shows a unique pattern of UTL-derived bands, which is inherited in progenies but conserved between clones vegetatively propagated. The overall organization of genetic diversity of the Vitaceae at the inter and intraspecific level and relatedness among accessions described by UTL-derived bands was compared to those obtained using 15 microsatellite loci. Both fingerprinting methods show a similar grouping of *Vitis vinifera* accessions but UTL-based fingerprinting more accurately isolates the muscadine grapes from the American and Asian *Vitis*. Finally, sequence analysis of seven UTL regions determines that their size variation is essentially caused by large deletions/insertions within the internal region, whereas flanking regions are more conserved. UTL-based fingerprinting could be considered as a novel marker system specific of the genus *Vitis*; moreover, as this multiband genotype is stable between clones it is suitable to be used as a "DNA barcode" for *Vitis* identification.

Introduction

Retrotransposons are the most abundant and widespread class of transposable elements in plants, consisting of the long terminal repeats (LTR) and non-LTR retrotransposons (Kumar and Bennetzen 1999). They account for at least 50–80% of some grass genomes (Meyers et al. 2001). In the sequenced genomes of *Arabidopsis thaliana* and rice they constitute 4.8 and 22% of the current genome, respectively (Ma et al. 2004). LTR retrotransposons are divided in two classes, the *Ty1-copia* like (Flavell et al. 1992; Voytas et al. 1992) and the *Ty3-gypsy* like (Suoniemi et al. 1998) which present the same general structure with LTRs flanking an internal open reading frame (ORF) encoding the two major genes, *gag* and *pol*. Internal region starts by the primer binding site (PBS) and ends by the polypurine tract (PPT), two highly conserved sequence stretches that are involved in the replication cycle of the element. In addition, some elements display an untranslated leader region (UTL) located between the 5′ LTR and the ORF. Retrotransposons are closely related to retrovirus in their structure and transposition cycle. This copy-and-paste cycle comprises the synthesis of self-produced proteins that are required to synthesize an extrachromosomal DNA daughter copy, by reverse transcription of an intermediate RNA, prior to its reinsertion into the genome. The transposition mechanism is highly error-prone due to the lack of proofreading repair activity of reverse transcriptase and RNA polymerase. During reverse transcription misincorporation or insertion–deletions result in substitutions or frameshifts in the new DNA retrotransposed copies resulting in divergent sequences. Besides that at the chromosomal level recombination events can lead to genetic rearrangements (Preston 1996). Hence, episodes of retrotransposons activity occurring over millennia have formed classes of elements, each representing a progeny of active copies that has retrotransposed at some point in the past (Bennetzen et al. 1998). Variability within different families of retrotransposons has been extensively described. Several reports indicate that the

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LTR sequences, despite their functional importance, are one of the most rapidly evolving retrotransposon regions (Casacuberta and Grandbastien 1993; Casacuberta et al. 1995; Vicient et al. 2005). The UTL region can also display significant size variation to the point of being used to distinguish each member of *Stonor*, a maize retrotransposon family (Marillonnet and Wessler 1998). Although in most of the cases the difference in size is caused by difference in the number of repeats motifs (Fablet et al. 2006; Mugnier et al. 2005), the structural variation of *Stonor* seems to result from deletions generated during retrotransposition (Marillonnet and Wessler 1998).

The Vitaceae are woody climbers characterized by unique shoot architecture of leaf-opposed tendrils or inflorescences (Gerrath et al. 1998). The lack of clearly marked floral characteristics has resulted in a plethora of species differentiated mainly by highly variable vegetative characters, leading to a family of about 900 species classified in 14 genera such as *Parthenocissus*, *Ampelopsis*, *Cissus*, *Rhoicissus* and *Vitis*. However, relationships between genera have been proved difficult to establish (Gerrath et al. 1998). There is a taxonomic controversy concerning the genus *Vitis*, generally separated in two groups based on chromosome number (Goldy and Onokpise 2001). Following Bailey's classification (Bailey 1934) *Vitis* having 38 chromosomes are placed into subgenus *Euvitis* and those having 40 chromosomes into subgenus *Muscadinia*. *Euvitis* consists of approximately 40–60 species originated from Asia, 25 from North America and the single European species *Vitis vinifera*. The later is in turn subdivided in *V. vinifera* ssp *sativa*, the cultivated grapevine and *V. vinifera* ssp *silvestris*, the wild vine.

Nuclear and chloroplast molecular markers provide new tools to investigate the evolution of the Vitaceae. Based on plastid *rbcL* DNA sequences, a phylogenic analysis for 20 species of Vitaceae established an evolutionary trend from *Ampelopsis* ancestral to *Parthenocissus* intermediate and finally *Vitis* most derived, a result in agreement with floral and vegetative ontogenic traits (Ingrouille et al. 2002). A phylogenic analysis based on the sequencing of three chloroplast markers demonstrated complex multiple intercontinental relationships within Vitaceae of the northern hemisphere and between northern and southern hemispheres as well (Soejima and Wen 2006). In *Vitis*, microsatellites are the most widely used molecular markers (Sefc et al. 2001), and the sequence of a considerable number of *Vitis* microsatellite has been recently released (Di Gaspero et al. 2005; Merdinoglu et al. 2005). Genetic relationship among 14 *Vitis* species was established based on the nucleotide variation of eleven microsatellite loci. The absence of a clear cut difference between American and Asian species suggested a revision of the actual taxonomy reflecting the geographical distribution of species (Di Gaspero et al. 2000). Microsatellite markers are also very efficient to analyze genetic structure and differentiation in cultivated grape (Aradhya et al. 2003), in addition to cultivar identification (This et al. 2004), pedigree reconstitution (Bowers et al. 1999a), or genome mapping (Adam-Blondon et al. 2004; Doligez et al. 2006).

Tvv1 is a family of *Ty1-copia* like grapevine retrotransposons, whose consensus sequence was reconstituted by chromosome walking from the wine grape variety riesling (Pelsy and Merdinoglu 2002). In this previous study, we showed that *Tvv1* elements present in the studied genome possess a single, highly conserved ORF but differ by their UTL region highly variable in size. This internal region is flanked by two LTRs, which are at least 70% identical but contain a well conserved 47 pb-long stretch. Using primers designed in this conserved part of the 5′ LTR and at the beginning of the ORF, we amplified 28 UTL-derived major bands that allowed to differentiate *Tvv1* elements. In the present study, we have examined the set of UTL-derived bands produced from the *Tvv1* copies of 94 Vitaceae accessions. We showed that this set of bands is specific to each accession but conserved between clones of a same variety. After verifying that these bands were inherited in progenies, we studied the genetic relatedness between the 94 accessions based on UTL size similarity and compared it to the one obtained with 15 microsatellite loci. Finally, in order to elucidate the origin of this variable region, seven UTL-derived bands were sequenced and compared. Different types of retrotransposons-based molecular markers were previously developed, that exploit their insertional polymorphism such as S-SAP (Waugh et al. 1997) or RBIP (Flavell et al. 1998). This study demonstrates that internal polymorphism of the UTL region of *Tvv1* is a valuable tool to describe genetic diversity of *Vitis* at the inter and intraspecific level.

Materials and methods

Plant material

The plant material consisted of 94 Vitaceae accessions divided in two groups. The Vitaceae group included *Ampelopsis* (5 accessions), *Parthenocissus* (1 accession), and belonging to the *Vitis* genus *Muscadinia rotundifolia* (4 accessions), Asian species (5 accessions), North American species (17 accessions) and inter-specific hybrids (4 accessions; Table 1). A second group included all *V. vinifera* represented by cultivated grapevine varieties (52 accessions) and wild vines (6 accessions; Table 2). Grapevines were originated from France or cultivated in France while wild vines were originated from three locations, Ste Croix en Plaine (53) and Mandeure (C25) in France and Martigny (50) in Switzerland. Because of their dioicity, one male and

Table 1 Vitaceae accessions included in the study

Accession full name	Abbreviation	N°	Genus	Geographic origin
Couderc 3309	Couderc_3309	22	*Vitis*	Hybrid
Kober 5BB	Kober_5BB	31	*Vitis*	Hybrid
Vitis aestivalis	V_aestivalis	64	*Vitis*	North America
Vitis amurensis	V_amurensis	65	*Vitis*	Asia
Vitis arizonica	V_arizonica	66	*Vitis*	North America
Vitis berlandieri × colombard	V_berl_colomb	67	*Vitis*	Hybrid
Vitis berlandieri Planchon	V_berl_Planchon	68	*Vitis*	North America
Vitis candicans	V_candidans	69	*Vitis*	North America
Vitis cinerea	V_cinerea	70	*Vitis*	North America
Vitis cordifolia 9 Couderc	V_crdfCoud	71	*Vitis*	Hybrid
Vitis Davidii	V_Davidii	72	*Vitis*	Asia
Vitis doaniana	V_doaniana	73	*Vitis*	North America
Vitis ishikari	V_ishikari	74	*Vitis*	Asia
Vitis labrusca Concorde	V_labrusca_Conc	75	*Vitis*	North America
Vitis labrusca Isabelle	V_labrusca_Is	76	*Vitis*	North America
Vitis linsecumii	V_linsecumii	77	*Vitis*	North America
Vitis monticola Large Bell	V_monticola_LB	78	*Vitis*	North America
Vitis palmata	V_palmata	79	*Vitis*	North America
Vitis reticulata	V_reticulata	80	*Vitis*	Asia
Vitis riparia Gloire de Montpellier	V_riparia_Gloire	81	*Vitis*	North America
Vitis riparia Millardet	V_riparia_Mill	82	*Vitis*	North America
Vitis riparia Muller	V_riparia_Muller	83	*Vitis*	North America
Vitis rubra	V_rubra	84	*Vitis*	North America
Vitis rupestris du Lot	V_rupestris_Lot	85	*Vitis*	North America
Vitis titania	V_titania	92	*Vitis*	Asia
Vitis vulpina	V_vulpina	93	*Vitis*	North America
Muscadinia rotundifolia Carlos	M_rotund_Carlos	32	*Vitis*	North America
Muscadinia rotundifolia Dulcet	M_rotund_Dulcet	33	*Vitis*	North America
Muscadinia rotundifolia Régale	M_rotund_Regale	34	*Vitis*	North America
Muscadinia rotundifolia YxC	M_rotund_YxC	35	*Vitis*	North America
Ampelopsis aconitifolia		1	*Ampelopsis*	Asia
Ampelopsis cordata		2	*Ampelopsis*	North America
Ampelopsis heterophylla		3	*Ampelopsis*	Asia
Ampelopsis japonica		4	*Ampelopsis*	Asia
Ampelopsis pedunculata		5	*Ampelopsis*	Asia
Parthenocissus quinquefolia	P_quinquefolia	49	*Parthenocissus*	North America

Numbers are used in the correspondence analysis and abbreviation in the trees

one female stock were chosen for each location. All of them are stored in the ampelographic collection of INRA-Colmar (France).

The 42 riesling clones and 46 savagnins clones assessed to verify the stability of the UTL patterns at the intravarietal level are stored in the INRA-Colmar clonal collections.

UTL profile determination

Total DNA was purified from young expanded leaves from individual plants using Dneasy™ Plant Mini-Kit (Qiagen, Hilden, Germany) as described by the supplier. The pair of primers used to amplify the UTL regions of *Tvv1* elements was designed from riesling *Tvv1* sequences. The forward primer, Pltr1 (CCTAATTCAGGACTCTCAAT), was complementary to a consensus domain of the 5′ LTR and the reverse primer, P17 ((CT)AGAATTCTTACTCTCTTCC), to the beginning of the *gag* region. PCR amplification was carried out according to Pelsy and Merdinoglu (2002). Pltr1 was IRD 800 5′ labeled (MWG Biotech AG, Ebersberg, Germany) to resolve PCR fragments by electrophoresis on a 60 cm long acrylamide gel in a LiCor 4000L automated DNA sequencer (Lincoln, NB) using IRD41-labeled M13 fragments (50–1,206 bp) as size standard.

Table 2 Cultivated grapevine varieties and wild vine included in the study

Accession full name	Abbreviation	No.
Cultivated grapevine		
aligoté B	aligote	6
aubin vert N	aubin_vert	7
auxerrois B (AB57)	auxerrois	8
bachet noir N	bachet_noir	9
beaunoir N	beaunoir	10
cabernet franc N	cab_frc	11
cabernet sauvignon N	cab_sauv	12
carignan N	carignan	13
chardonnay B	chardonnay	14
chasselas blanc B (CB60)	chasselas	15
chenin B	chenin	16
cinsaut N	cinsaut	17
clairette B	clairette	18
colombard B	colombard	19
corbeau N	corbeau	20
côt N	cot	21
folle blanche B	folle	23
franc noir de la Haute Saone N	franc_noir_HS	24
gamay blanc Gloriod B	gamay_gloriod	25
gamay noir N	gamay	26
gewurztraminer B (GW48)	gewurztraminer	27
gouais B	gouais	28
grenache N	grenache	29
knipperlé B	knipperle	30
marsanne B	marsanne	36
mauzac B	mauzac	37
melon B	melon	38
merlot N	merlot	39
mourvèdre N	mourvedre	40
muscat cendré B	muscat_cendre	41
muscat d'Alexandrie B	muscat_Alex	42
muscat d'Alsace rouge N	muscat_Als_rouge	43
muscat de Hambourg N	muscat_Hambourg	44
muscat Ottonel B	muscat_Ottonel	45
muscat à petits grains B	muscat_PtGrains	46
muscat reine des vignes B	muscat_reinevig	47
muscat de Saumur B	muscat_Saumur	48
persan N	persan	50
peurion N	peurion	51
pinot noir N (PN162)	pinot_noir	52
riesling B (R49)	riesling	53
romorantin N	romorantin	54
roublot N	roublot	55
roussanne B	roussanne	56
sacy B	sacy	57
sauvignon blanc B	sauvignon	58
sémillon B	semillon	59
sylvaner B (SY50)	sylvaner	60
syrah N	syrah	61
tannat N	tannat	62
ugni blanc B	ugni_blc	63
viogner B	viognier	94
Wild vine		
Vitis silvestris 50 K (M)	V_silv_50 K	86
Vitis silvestris 50L (F)	V_silv_50L	87
Vitis silvestris 53I (F)	V_silv_53I	88
Vitis silvestris 53J (M)	V_silv_53J	89
Vitis silvestris C25S2B (M)	V_silv_C25S2B	90
Vitis silvestris C25S6 (F)	V_silv_C25S6	91

For each accession, UTL profile was performed at least twice and loaded on independent gels. Detection of UTL bands in each individual lane was performed using the RFLPscan (version 2.1) software (Scanalytics, Fairfax, VA). The software assigned a size to each band according to the size standard. The DNA sample of the variety pinot noir was systematically amplified and loaded. Reproducible UTL bands of pinot noir yielded a set of reference used to standardize UTL patterns of other accessions. Only reproducibly amplified bands were scored. According to the gel precision at different size range, bands were considered as identical with a 3 bp-difference of the estimated band molecular weight for bands between 1,200 and 1,000 bp, 2 bp-difference between 1,000 and 800 bp and 1 bp-difference for bands smaller than 800 bp. By assuming that DNA bands within the same molecular weight range share at least one *Tvv*1copy with the same UTL region, all the individual profiles were scored as a binary trait, where 0 and 1 correspond to the absence and presence of a given band, respectively.

Microsatellite analysis

Fourteen nuclear microsatellite markers were used in this study, 12 of them have been previously mapped and are scattered on nine of the 19 grapevine reference linkage groups (LG) (Doligez et al. 2006): ssrVrZAG25 (LG10), ssrVrZAG62 (LG7), ssrVrZAG79 (LG5), ssrVrZAG93 (LG2) (Sefc et al. 1999), VVS2 (LG11), VVS29 (LG1) (Thomas and Scott 1994), VVMD30 (LG15) (Bowers et al. 1999b), VvUCH12, VvUCH29 (Lefort et al. 2003), VMC1e11 (LG16), VMC2a12 (LG11), VMC3a9 (LG17), VMC5g7 (LG2), VMC8a7 (LG1) (Laboratories interested in these unpublished markers should contact Vitis Microsatllite Consortium, Agrogene SA, Moissy Cramayel, France). Microsatellite loci were amplified using one 6-FAM ,

HEX or NED fluorophore-labelled primer (PE Applied Biosystems, Warrington, UK). PCR amplifications were carried out according to Hocquigny et al. 2004. PCR fragments were resolved on an automated 310C ABI PRISM DNA sequencer (PE Applied Biosystems, Foster City, CA), and sized with an ROX labeled-internal standard (50–654 bp; PE Applied Biosystems). Microsatellite alleles were scored using GenScan (version 3.1) and Genotyper (version 2.5.2) softwares (PE Applied Biosystems).

At each locus, a genotype displaying one allele was considered as homozygous, whereas a genotype displaying two alleles was considered as heterozygous. Expected heterozygosity was calculated from the sum of squares of allele frequencies, as $He = 1 - \sum p_i^2$ where p_i is the frequency of allele i in the two sets of the accessions (Nei 1973). Observed heterozygosity (Ho) was calculated directly from the accession genotypes. Weir's fixation index was calculated as $F = 1 - Ho / He$ and the frequency of null alleles (r, Brookfiled 1996) was estimated as $r = (He - Ho)/(1 + He)$. At the end, all alleles were scored as 0/1 in a binary table, where 0 and 1 correspond to the absence and presence of a given allele, respectively.

Phylogenetic analysis

Genetic distances between all possible pairs of individuals and correlation between UTL-based and microsatellite genetic distance matrices investigated at three levels by the Mantel test of matrix correspondence were performed within GenAlEx V6 (Peakall and Smouse 2006). Outcomes of Mantel tests for correlation (r) between UTL-derived and microsatellite data were calculated for two set of accessions with a probability of statistical significance (P) based on 999 permutations. The correspondence analysis from binary data from the set of 94 accessions was performed within NTSYSpc software (version 2.02i). The most-parcimonious tree searches were performed with Phylo-win (Galtier 1996). Internal support was assessed using 500 bootstrap replicates. Bootstrap support is indicated as percentage of trees containing that group of accessions. Percentages of less than 50% were not reported.

Sequence analysis

The UTL bands amplified from riesling were cloned into the pCR® 4-TOPO cloning vector (InVitrogen, Groningen, NL), according to supplier instructions. Sequencing reactions were performed forward and reverse by PCR using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Samples were processed on the ABI3100 Genetic analyzer, a 16-capillary electrophoresis sequencing instrument. Computer-assisted analysis of the sequence data was performed using program DNAsis 2.1 (Hitachi Software Engineering Co. Ltd) and Vector NTI 7 (InforMax Inc.). The nucleotide data of the seven reported fragments appear in GenBank database under accession numbers: AF478386 and EU119272 to EU119277.

Results

Diversity of the UTL bands

The pair of primers, Pltr1 and P17, designed to amplify the UTL region of *Tvv1* elements produced specific profiles of PCR fragments from each of the 94 Vitaceae accessions (Fig. 1). An average number of 23 bands was scored per accession; however, it may depend on the considered group: 25.5 bands were found for cultivated grapevine (ranged from 17 to 34), 16.7 for *M. rotundifolia* (ranged from 16 to 22), 3.5 bands for *Ampelopsis* (ranged from 2 to 8) and 2 for the *Parthenocissus* accession (Table 3). Two hundred and forty two different UTL bands were scored in the set of 94 Vitaceae accessions, amongst which 153 bands (63%) were present in the *V. vinifera* group (cultivated grapevine and wild vine). The band sizes varied from 242 to 1,295 bp. However, considering the total number of 242 different bands 74% ranged between 1,200 and 801 bp (Fig. 2). All UTL-derived

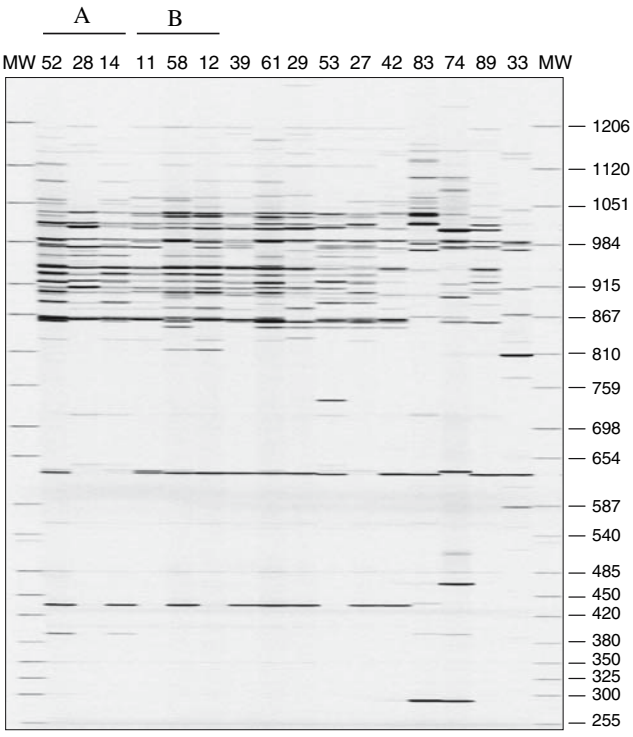

Fig. 1 UTL profile of 16 accessions. DNA sample are numbered in Table 1. **a** Pinot noir (52) and gouais (28) are the two parents of chardonnay (14); **b** cabernet franc (11) and sauvignon blanc (58) are the two parents of cabernet sauvignon (12)

Table 3 Genetic variability scores of the UTL fingerprinting

Genus	Subgenus	Species	Subspecies	No. of accessions	Total no. of scored bands	No. of distinct bands per group	No. of bands per accession	Mean no. of bands per accession	Band size range	No. of unique bands
Ampelopsis				5	17	12	2–8	3.4	242–1076	5
Parthenocissus				1	2	2	2	2	810–1043	0
Vitis	*Euvitis*	*V. vinifera*	*sativa*	52	1,323	146	17–34	25.5	292–1289	11
			silvestris	6	132	58	17–26	22	392–1200	0
		Hybrids		4	79	55	13–32	19.75	292–1154	4
		American *Vitis*		17	420	146	14–35	24.7	292–1295	26
		Asian *Vitis*		5	123	86	16–34	24.6	292–1278	6
	Muscadinia	*V. rotundifolia*		4	67	26	16–22	16.7	435–1150	3
Total				94	2,163	242	2–35	23	242–1295	55

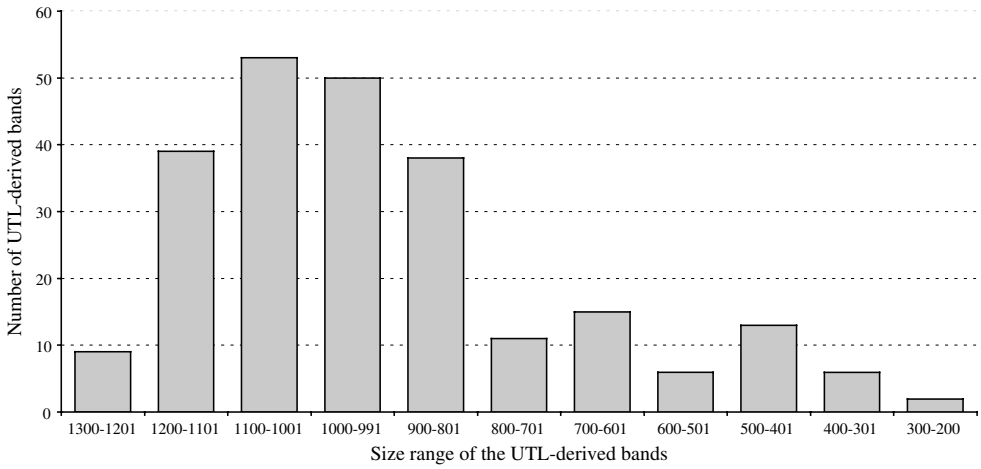

Fig. 2 Distribution of the 2163 UTL scored bands according to their length by range of 100 bp

bands were polymorphic but most of them were shared by at least two accessions. The most common band (989 bp) was present in 58 accessions including 49 *V.vinifera*, all four *M. rotundifolia* and five Asian and American *Vitis*. Most of the bands displayed by *Ampelopsis* or *Parthenocissus* accessions were absent in *Vitis*. Fifty-five unique bands (22.7%) were only scored in one accession, among which 26 in the American *Vitis* group and 11 in the cultivar group (Table 3). A maximum of four unique bands was scored for *V. arizonica* and *V. berlandieri* X colombard and a maximum of two for the cultivars grenache, mauzac and roussanne.

Inheritance of the UTL bands

Relatedness between different cultivated grapevines have been clearly established using microsatellite markers : for instance, pinot and gouais were determined to be the single pair of parents of 16 wine grapes including chardonnay (Bowers et al. 1999a). In the same way cabernet sauvignon is a progeny of cabernet franc and sauvignon blanc (Bowers and Meredith 1997). The pattern of UTL-derived bands displayed by cabernet sauvignon was in accordance with its genealogy: from the 24 reproducible scored bands, 14 bands were previously shared by both parents, three were inherited from cabernet franc and seven from sauvignon blanc (Fig. 1b). A comparable result was obtained comparing the 22 UTL-derived bands of chardonnay to its parents pinot and gouais patterns (Fig. 1a), hence confirming the inheritance of the UTL-derived bands.

Stability of the UTL-derived patterns

Since grapevines are propagated vegetatively, clones of a same variety are perpetuated virtually unchanged over time. UTL-derived patterns of 42 clones of riesling and 46 clones of savagnins were compared. These clones were confirmed to belong to their expected variety with a set of 30 microsatellite markers (data not shown). No difference in size neither in number of UTL-derived bands was observed for clones of the same variety (Fig. 3). This result showed the stability of the UTL-derived patterns at the clonal level.

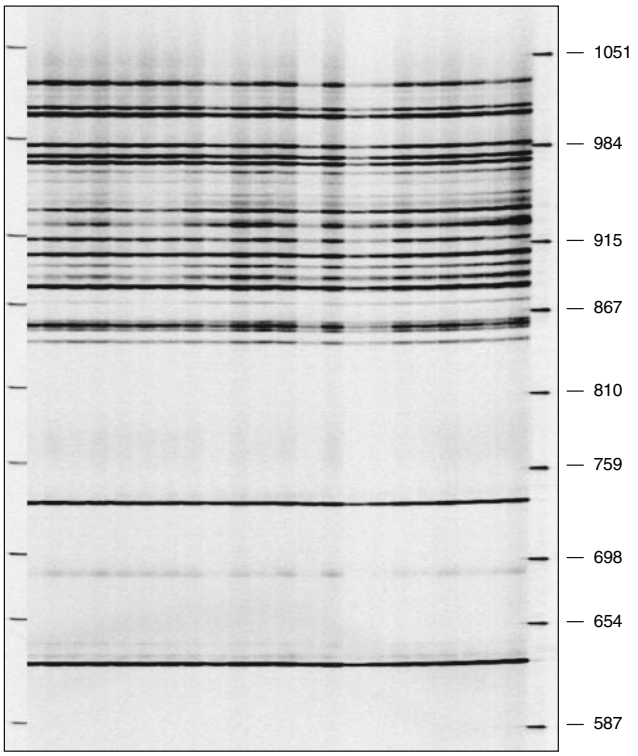

Fig. 3 UTL profile of 22 riesling clones

Conservation of microsatellite loci within Vitaceae

In parallel to UTL amplification study, the 94 accessions were analyzed with a set of 14 microsatellite markers that amplified 15 loci. A total number of 322 different alleles was scored among the whole set of 94 accessions, 129 alleles (40%) being present in the subset of *V. vinifera* accessions (Table 4). As a result, all accessions displayed a unique genotype. The number of alleles detected per locus ranged from 3 for VMC1e11a to 34 for VrZAG93 in the whole set, with an average of 21.5, and from 1 for VMC1e11a to 14 for VVS2 in the *V. vinifera* subset, with an average of 8.6. Expected (He) and observed (Ho) heterozygosity deviated substantially considering the whole set (mean value 0.811 vs. 0.640) and less significantly among *V. vinifera* accessions (mean value 0.705 vs. 0.661). The observed heterozygosity deficiency was clearly indicated when computing the Weir's fixation index that measures the deviation of the proportion of the observed heterozygotes from Hardy–Weinberg expectation. Negative values indicate an excess of heterozygotes and values above zero an excess of homozygotes. However, the probability of null allele was higher taking in consideration the whole set compared to the *V. vinifera* subset. It indicates that many apparent homozygotes are likely to be heterozygotes with one amplified and one null allele. It was very significant for locus VMC3a9 and to a lesser extent for VrZAG25 and VrZAG93. Added in proof, 12 accessions among 94 did not amplify locus VMC3a9, including 10 *V. vinifera*, one *M. rotundifolia*, and one *Vitis* accessions. In addition to the higher probability of null allele, the higher number of alleles per locus as well as the broader allele sizes confirmed the larger microsatellite diversity in the whole set of accessions compared to the subset of *V. vinifera*. All alleles were polymorphic and VMC1e11a-180, the most common allele, was shared by 61 accessions, including 45 *V. vinifera*, 9 *Vitis*, 4 *M. rotundifolia* and 3 *Ampelopsis* accessions. A total of 108 alleles (33.4%) were considered as unique in the set of 94 accessions based on their presence in only one genotype; however, the number of unique allele per genotype was very variable. The *Parthenocissus* accession that amplified 14 loci showed nine unique alleles for nine loci. All *Vitis* species and hybrids displayed at least one unique allele except *V. vulpina*. A maximum number of seven unique alleles for six markers was exhibited by the two species *V. aestivalis* and *V. monticola* Large Bell. Six alleles were only present in *M. rotundifolia* accessions, among which two were unique and one was shared by the four muscadine accessions. All *V. silvestris* alleles were present in the cultivated grapevine compartment. Only the two cultivars carignan and grenache amplified two unique alleles. Nineteen accessions reproducibly amplified three alleles for one to four of the markers except VVS29, VvUCH12 and UCH29, when two are expected for diploid species. Such tri-allelic combinations are typical of chimeric plants (Franks et al. 2002; Hocquigny et al. 2004).

UTL-derived and microsatellite patterns describe a phylogeny of Vitaceae

Computing correspondence analysis was used to compare the genetic structuring of the 94 Vitaceae accessions described by both fingerprinting methods. The UTL-derived data revealed three well-isolated clusters for the *M. rotundifolia*, the American and Asian *Vitis* and all *V. vinifera* accessions, respectively (Fig. 4a), excluding *Ampelopsis* and *Parthenocissus* accessions. The microsatellite data clustered *Ampelopsis*, *M. rotundifolia* and American and Asian *Vitis* accessions in overlapping groups (Fig. 4b). Next, genetic relatedness described by both methods was compared in two groups of accessions. The first group included the 39 *Vitis* accessions (Table 1) among them nine *V. vinifera* accessions, three cultivated grapevine (cabernet sauvignon, muscat d'Alexandrie and pinot noir) and six wild vines in addition the accession of *P. quinquefolia* were used as outgroup. The second group included the 58 *V. vinifera* accessions (Table 2) in addition with *M. rotundifolia* Carlos used as outgroup. In both groups, positive correlations ($r = 0.216$, $P < 0.001$ and $r = 0.328$, $P < 0.001$, respectively) were found between UTL and microsatellite

Table 4 Genetic variability scores of the microsatellite fingerprinting for a group including the 94 accessions and a subgroup 58 *V. vinifera* accessions (cultivated grapevine varieties and wild vines)

Locus	94 Accessions						58 *Vitis vinifera*					
	No. of alleles per locus	Allele size range	He	Ho	*F*	*r*	No. of alleles per locus	Allele size range	He	Ho	*F*	*r*
VMC1e11a	3	177–180	0.117	0.011	0.909	0.095	1	180	0	0		0
VMC1e11b	11	186–215	0.853	0.830	0.027	0.013	8	186–204	0.794	0.879	−0.108	−0.048
VMC2a12	23	89–128	0.840	0.628	0.253	0.116	9	89–125	0.719	0.776	−0.079	−0.033
VMC3a9	23	70–124	0.887	0.468	0.472	0.222	9	70–106	0.840	0.328	0.610	0.279
VMC5g7	27	157–217	0.875	0.691	0.209	0.098	13	177–216	0.780	0.845	−0.083	−0.036
VMC8a7	26	145–181	0.838	0.745	0.112	0.051	7	151–168	0.731	0.793	−0.085	−0.036
VrZAG25	20	184–258	0.845	0.489	0.421	0.193	8	184–245	0.697	0.552	0.208	0.086
VrZAG62	24	172–224	0.886	0.787	0.112	0.053	8	184–203	0.797	0.862	−0.081	−0.036
VrZAG79	17	233–278	0.902	0.702	0.221	0.105	12	233–257	0.859	0.793	0.077	0.035
VrZAG93	34	159–242	0.851	0.564	0.337	0.155	9	184–226	0.609	0.431	0.293	0.111
VVMD30	19	95–131	0.885	0.745	0.159	0.074	9	98–124	0.817	0.862	−0.055	−0.025
VVS2	18	120–156	0.863	0.670	0.224	0.104	14	126–152	0.806	0.741	0.08	0.036
VVS29	13	162–191	0.771	0.596	0.227	0.099	5	167–178	0.615	0.672	−0.093	−0.036
VvUCH12	32	123–239	0.845	0.702	0.169	0.078	8	128–209	0.680	0.621	0.0875	0.035
VvUCH29	32	205–308	0.911	0.649	0.288	0.137	9	205–307	0.831	0.759	0.0873	0.040
Mean values	21.5		0.811	0.618	0.276	0.106	8.6		0.705	0.661	0.057	0.025

Number of alleles par locus, expected heterozygosity (He) and observed heterozygosity (Ho) were calculated pooling all accessions into each group. *F* is the Weirs' fixation index; *r* is the frequency of null alleles

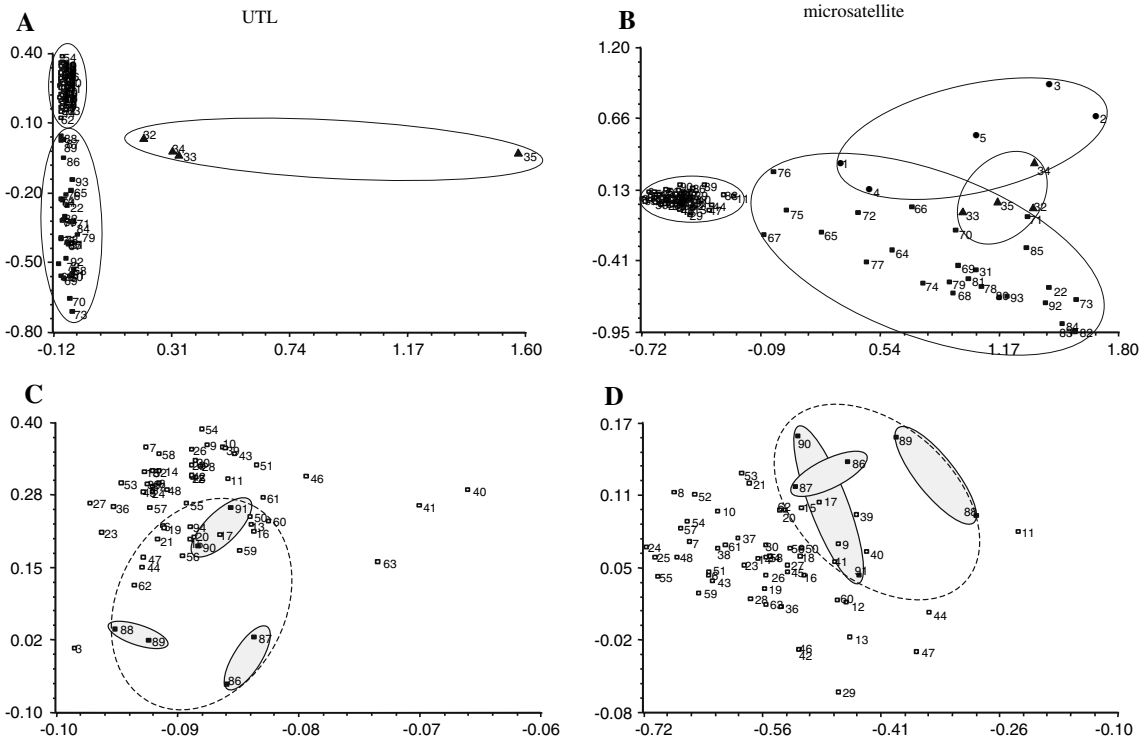

Fig. 4 Correspondence analysis of UTL and microsatellite data. In **a** and **b,** *Ampelopsis* and *Parthenocissus* (*black circles*); muscadine grapes (*black triangles*); *Vitis* (*black squares*); *V. vinifera* (*white squares*) are represented. **c** and **d** are focused on *V. vinifera* wild vines (*black squares*) and cultivated grapes (*white squares*). *Ellipses* are centered on the gravity center of each scatterplot

individual pairwise genetic distance matrices. Parcimonious analysis produced two single trees for each group of accessions, each tree revealing that each accession displayed a specific genotype. The topology of each tree was unique and showed major differences.

In the *Vitis* group, the position of the four *M. rotundifolia* accessions in a separate cluster was strongly bootstrap supported using both fingerprinting methods (Fig. 5a, b). The *V. vinifera* (cultivated and wild) accessions were separated from other *Vitis* species. Neither the UTL-derived nor the microsatellite trees showed a clear-cut difference between American and Asian species. In both *Vitis* trees, the general structuring of the *Vitis* was not supported by strong bootstrap values, except for the clustering of the two *V. labrusca* and the *V. riparia* accessions Millardet and Muller.

In the UTL-derived and microsatellite trees of the *V. vinifera* accessions only few relatedness received strong support: *V. vinifera* ssp *silvestris* accessions isolated from *V. vinifera* ssp *sativa* accessions, the cultivated grapevine varieties. But while UTL-derived data clustered wild vine closer from the camenère group to which belong cabernet franc, cabernet sauvignon and merlot (Fig. 5c), microsatellite clearly excluded them from the cultivated cluster (Fig. 5d). Relatedness between many accessions described in both trees was rather different, but not strongly bootstrap supported. However, both trees grouped muscat varieties in a main cluster and cabernet franc to cabernet sauvignon, parent and progeny, respectively (Bowers and Meredith 1997).

UTL sequence analysis

To elucidate the possible origin of the length variation at the UTL region, seven different UTL- derived bands were sequenced from the wine grape variety riesling (Pelsy and Merdinoglu 2002). Precise sizes of the bands ranged from 1,165 to 624 pb while the scored UTL-derived bands ranged from 1,200 to 630 bp. As expected for UTL-derived bands, all sequences displayed on one end a stretch of 90 to 105 pb from the 3′end of the 5′ LTR and on the other end the 22 first base pairs of the ORF. Between these conserved sequences, the precise UTL sequence varied from 512 to 1,038 bp. All UTL regions started by the nearly identical sequence TGGTATC(A/G) (G/T) AGCC, which corresponds to the PBS. A detailed analysis of three UTL regions named UTL 34, 31 and 23, which are 1,038, 851 and 512 bp-long, respectively, indicated that variations in size have essentially resulted from large deletions/insertions within the internal region. However, the highly variable region is flanked by two stretches approximately 100 bp-long, rather conserved between five of the seven UTL regions sequenced (Fig. 6). The two remaining UTL sequences started by the same PBS motif and ended by 28 pb conserved in the seven sequences. No repeated motifs were detected in any of the seven *Tvv1* UTLs.

Discussion

Polymorphism of the UTL region of *Tvv1* amongst the Vitaceae

Previous work on the *Tvv1* grapevine retrotranposon family revealed that its UTL region, upstream of the ORF, was highly variable in size and allowed the differentiation of some *Tvv*1 variants (Pelsy and Merdinoglu 2002). This study adds to our understanding that amongst the Vitaceae, copies belonging to the *Tvv1* family display UTL regions of different sizes. Patterns of UTL-derived amplifications were polymorphic at the genera, subspecies, species and varieties levels. The primers amplified the largest number of bands for *Vitis* species that decreased slightly for *M. rotundifolia* but dropped for *Ampelopsis* and *Parthenocissus* accessions. However, we have to take into account that primers were designed from *V. vinifera Tvv1* sequences, therefore, we think that *Tvv1* elements are present amongst all Vitaceae, but the very few bands amplified for *Ampelopsis* and *Parthenocissus* accessions could suggest a divergence in the *Tvv1* sequences in the genomes of these genera leading to a loss of specificity of the designed primers. Hence, this fingerprinting method could be considered as specific to the *Vitis* genus.

Considering only the 17 American *Vitis* accessions, they showed the highest score for distinct UTL-derived bands as well as for unique bands compared to the *V. vinifera* group of 52 varieties. Such a wider diversity at the interspecific level than at the intraspecific level is confirmed by the microsatellite data as the set of 94 studied accessions shows a larger number of alleles as well as a broader size range than the *V. vinifera* subset. Moreover, comparing American *Vitis* to cultivated grapevines the number of unique bands is much lower for cultivated grapevines despite a rather similar number of distinct UTL-derived bands. This most probably results from hybridization that led to varieties today cultivated that are more or less related (Aradhya et al. 2003; Meredith 2001). The particularity of the wine grape variety grenache that displays two unique UTL-derived bands is confirmed by the fact that it also displays two unique microsatellite alleles, so that this variety seems to be rather distant from other wine grape varieties, possibly because of its Spanish origin. Indeed, based on chloroplast polymorphisms, the genotypes of 70% of the Iberian Peninsula varieties are compatible with their being derived from western *silvestris* populations, while Central Europe varieties, among them French varieties, are more related to Middle

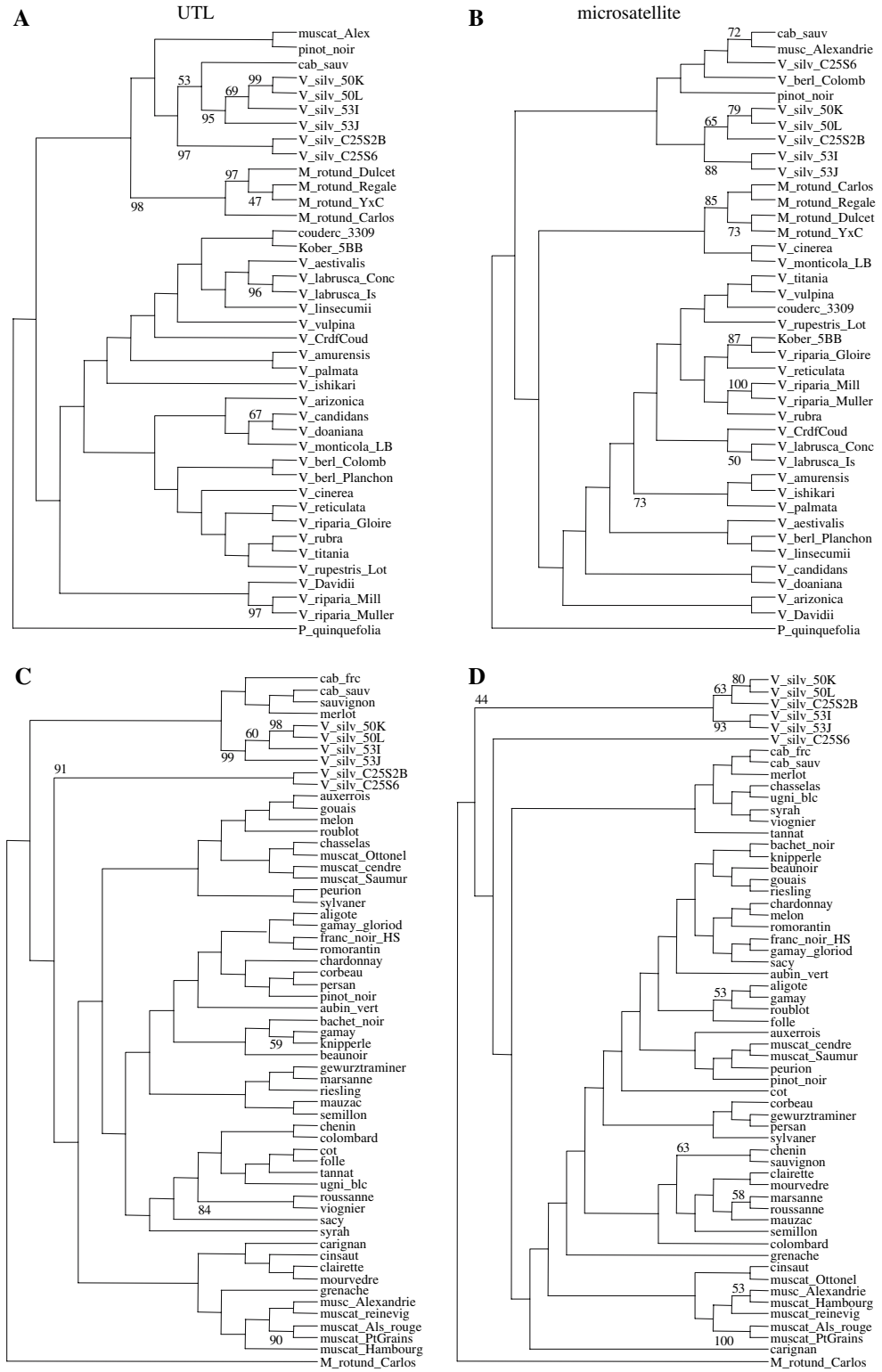

Fig. 5 Most parcimonious trees estimating the phylogenetic relatedness among the Vitaceae. Trees **a** and **b** group 39 *Vitis* accessions including three cultivated varieties and six wild vines. Trees **c** and **d** include the 58 *V. vinifera* accessions. Trees **a** and **c** re based on UTL data and trees **b** and **d** on microsatellite data. *P. quinquefolia* in the *Vitis* trees and *M. rotundifolia* Carlos in the *V. vinifera* trees are used as outgroup

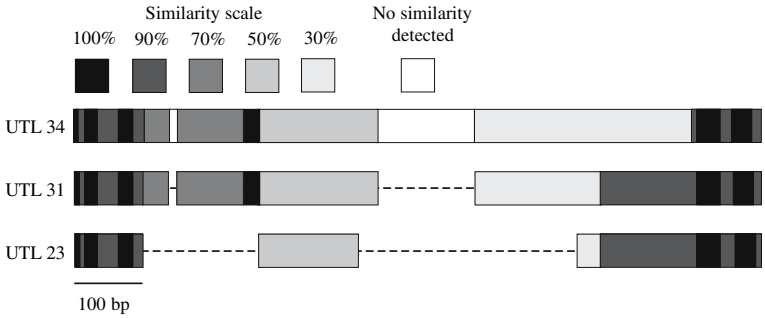

Fig. 6 Structure of three cloned UTL sequences. Similarity level decreases from 100% (*black boxes*) to no similarity (*white boxes*). Deletions are indicated by *dashed line*

East wild populations (Arroyo-Garcia et al. 2006). In addition, neither unique UTL band nor unique microsatellite allele were scored in the *V. vinifera* ssp *silvestris* accessions.

Comparison of phylogenic data

The relevance of the UTL fingerprinting was evaluated by a test Mantel testing that confirmed congruence between UTL-derived and microsatellite genetic distance within the *V. vinifera* group and in a lesser extend among *Vitis*. Then, we compared the relatedness among the *Vitis* described by these two types of polymorphic markers by constructing two most-parcimonious trees based on UTL-derived and microsatellite data, respectively. Although the topology of each tree is unique, this comparative study yields a number of similarities but also significant differences. Both trees show the clustering of *V. vinifera* accessions, of the four muscadine grapes and of few *Vitis* species. They also confirm the absence of clear-cut difference between American and Asian species (Di Gaspero et al. 2000), a result is in accordance with complex multiple intercontinental relationships within Vitaceae of the northern hemisphere (Soejima and Wen 2006). Focusing on the sibling wine grape cultivars of pinot and gouais (Bowers et al., 1999a), they all cluster together as well as cabernet sauvignon do with cabernet franc and sauvignon blanc. Such a result is in agreement with the Mendelian inheritance of the UTL-derived bands. Moreover, close genetic relatedness between muscat cultivars is also depicted in accordance with previous results (Aradhya et al. 2003).

The main difference comparing the two trees as well as the correspondence analysis visualizations is the clustering of the four muscadine grapes. The UTL data more closely cluster *V. vinifera* and *M. rotundifolia* while the microsatellite isolate the *M. rotundifolia* group with two American *Vitis* accessions. Despite the difference in chromosome number between *M. rotundifolia* and other *Vitis* species, 40 and 38, respectively, the clustering of muscadine grapes close to the *V. vinifera* group in the UTL tree is in agreement with a previous phylogenetic analysis based on plastid *rbc*L DNA sequences (Ingrouille et al. 2002). Comparing the trees as well as the correspondence analysis visualizations, the genetic structuring of wild vines appears lightly different between UTL-derived and microsatellite data but with both fingerprinting methods they were clustered out of the main cultivated group. Despite the lack of robustness of the clustering analysis, it seems that the internal UTL polymorphism of *Tvv1* elements describes an overall organization of genetic diversity of *Vitis* at the inter and intraspecific level that is different from the diversity described by microsatellite fingerprinting. It could be explain by a larger panel of stable UTL bands taken in consideration, less prone to homoplasy than microsatellite markers.

Origin of the UTL size variation

The UTL patterns of cultivars linked by known relatedness showed that most of the inherited UTL-derived bands were previously shared by parents and more generally by a large number of accessions. For these common UTL-derived bands, it is not possible to determine if they represent only one copy or more. They could represent ancient fixed copies that spread over time through natural intermixing. They could also be amplified from a number of daughter copies displaying UTLs of the same length that derived from some master copies of *Tvv1* shared by Vitaceae genomes. However, it is not possible to exclude that some UTL-derived bands scored as common between several accessions could display different sequences and then be homoplasic. Contrary to largely shared bands, UTL-derived bands that segregate in the progeny should represent one particular copy of *Tvv1*. As all bands scored in the progeny cultivars were present in their parents, the appearance of a new *Tvv1* copy with an UTL differing in size from the others seems to be a very unlikely.

Sequence analysis of seven UTL regions from riesling revealed that their size variation is essentially caused by large deletions/insertions within the internal region, whereas flanking regions are more conserved. On the contrary to *Drosophila 412* or *tirant* retrotransposons, no repeated motifs were detected in *Tvv1* UTLs (Fablet et al. 2006; Mugnier et al. 2005). Comparing the three cloned sequences named UTL 34, 31 and 23, successive deletions

could have taken place from UTL 34, the largest one, to UTL 23 the smallest (Fig. 5). These deletions could have occurred during retrotransposition as proposed for *Stonor*, a maize retrotransposon family (Marillonnet and Wessler 1998). However, in *Stonor*, the resulting structural variation seems to be largely restricted to the region just downstream of the 5′ LTR, which is not the case for *Tvv1* UTLs. With our present knowledge of the *Tvv1* family, the observed size variation between the three UTLs could also result from successive insertions from the smallest to the largest regions.

Retrotransposon UTL polymorphism as a new molecular marker

By a unique PCR-amplification, *Tvv1* UTL polymorphism describe a genotype specific of each of the *Vitis* accessions analyzed in this study, when a minimum of six standard microsatellite markers chosen for their high degree of allelic polymorphism are necessary to identify a variety (This et al. 2004). The congruence between UTL-derived and microsatellite data sets suggests that this method is applicable to genetic studies of *Vitis*. As clones belonging to a same variety display the same UTL-derived pattern, we assume that this multiband genotype is stable during the vegetative propagation cycles and can be used as a "DNA barcode" for *Vitis* identification. To our knowledge, this is the first description of a multilocus molecular marker based on the length variability of the internal UTL region, which we refer to as retrotransposon UTL polymorphism fingerprinting (RUP). Furthermore, RUP fingerprinting could be evaluated to describe genetic diversity of different plant species that display retrotransposons with a large UTL region such as *Tnt1* of tobacco (461 pb; Pouteau et al. 1991), *BARE 1* of barley (2,057 bp; Manninen and Schulman 1993) or *Stonor* of maize (2.0–2.2 Kb; Marillonnet and Wessler 1998).

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