Received: 17 August 2020 | Accepted: 11 May 2021 | Published: 25 June 2021
DOI:10.20870/oeno-one.2021.55.2.4473

OENO One
VINE AND WINE
OPEN ACCESS JOURNAL

A molecular marker set combining a retrotransposon insertion and SSR polymorphisms is useful for assessing diversity in *Vitis*

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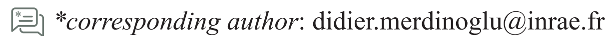
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Associate editor: Laurent Jean-Marie Torregrosa

ABSTRACT

Molecular markers, based on DNA polymorphisms, are useful tools for identifying individuals, establishing phylogenetic relationships, managing collections of genetic material or assisting breeding. In the present study, we developed a marker set to differentiate *Vitis* species, grapevine varieties or clones belonging to the same variety. This novel marker set combines, in four PCR amplifications, the presence/absence of a remarkable retrotransposon, *Tvv1*-Δ3460, inserted at its single locus and the SSR polymorphism present within its two LTRs. By studying a collection of *Vitaceae* accessions, we showed the prevalence of two allelic forms of *Tvv1*-Δ3460 - one of which was partially truncated - in *Vitis* species. Out of the twenty-five studied *Vitis* species, the insertion of a *Tvv1*-Δ3460 element was detected in twenty, including *Vitis vinifera.* The homozygous *vs* heterozygous state of the element insertion was determined by amplifying the empty site. Additionally, each *Tvv1*-Δ3460 LTRs included a microsatellite sequence useful for designing markers based on LTR length. The LTR-SSR markers distinguished most of the fifty-two cultivars and revealed polymorphism within five of the seven varieties studied.

KEYWORDS

molecular markers, grapevine, retrotransposon, SSR

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INTRODUCTION

Molecular markers are useful tools for identifying individuals, establishing phylogenetic relationships, managing collections of genetic material or assisting breeding. They are based on various types of DNA polymorphisms found in genomes (Gupta *et al.*, 1999; Schulman, 2006). Microsatellite markers, whose polymorphism takes advantage of the variable number of simple sequence repeats (SSR) at a given locus, is undoubtedly the most extended molecular marker system for grapevine. SSR markers are highly transferable, co-dominant, and very useful for identifying grapevine cultivars (Merdinoglu *et al*., 2005; This *et al*., 2004) and for studying *Vitis* phylogeny (Di Gaspero *et al.,* 2000). However, most available SSR markers fail to distinguish clones that derive from repeated vegetative propagation cycles from a unique single individual. Nevertheless, a standard set of five SSR markers (VMC3a9, VMC5g7, VVS2, VVMD30 and VVMD32) which can reveal clonal polymorphism has been proposed (Pelsy *et al.,* 2010). Somatic variations giving rise to clone diversity within grapevine varieties have also been investigated by genomic approaches; 15 distinguishable Chardonnay clones have been identified by 1620 SNPs and InDels, which can be exploited to define markers for clone-specific genotyping (Roach *et al.*, 2018).

Mobile elements are abundant, rapidly evolving and widespread in the genomes of plants. They actively contribute to molecular polymorphism and, therefore, form the basis of other molecular marker systems. They take advantage of the activity or the structural variations of transposable elements. Sequence-specific amplification polymorphisms (SSAP) markers, which reveal the pattern of insertion of elements belonging to the same family (Waugh *et al.,* 1997), have been developed to analyse genetic diversity and relatedness in the genus *Vitis* (Moisy *et al.*, 2008b). Retrotransposon polymorphism fingerprinting (RUP), which amplifies the highly variable untranslated leader (UTL) region of the *Tvv1,* has revealed a unique pattern in each of 94 *Vitaceae* accessions and is conserved between clones (Pelsy, 2007). Finally, inter retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP) have been shown to discriminate the white table grape cultivar Italia from its coloured variants (Rubi, Benitaka, Brasil and Black Star) derived from clonal propagation of somatic mutations (Strioto *et al.,* 2019).

In the grapevine genome, several retrotransposons have been found inside genes, such as *Vine1* in *Adhr* (Verriès *et al*., 2000), or close to genes, such as *Gret1 in* the promotor of *VVMybA1* (Kobayashi *et al.*, 2004). Others have been characterised by a computerized sequence similarity search and

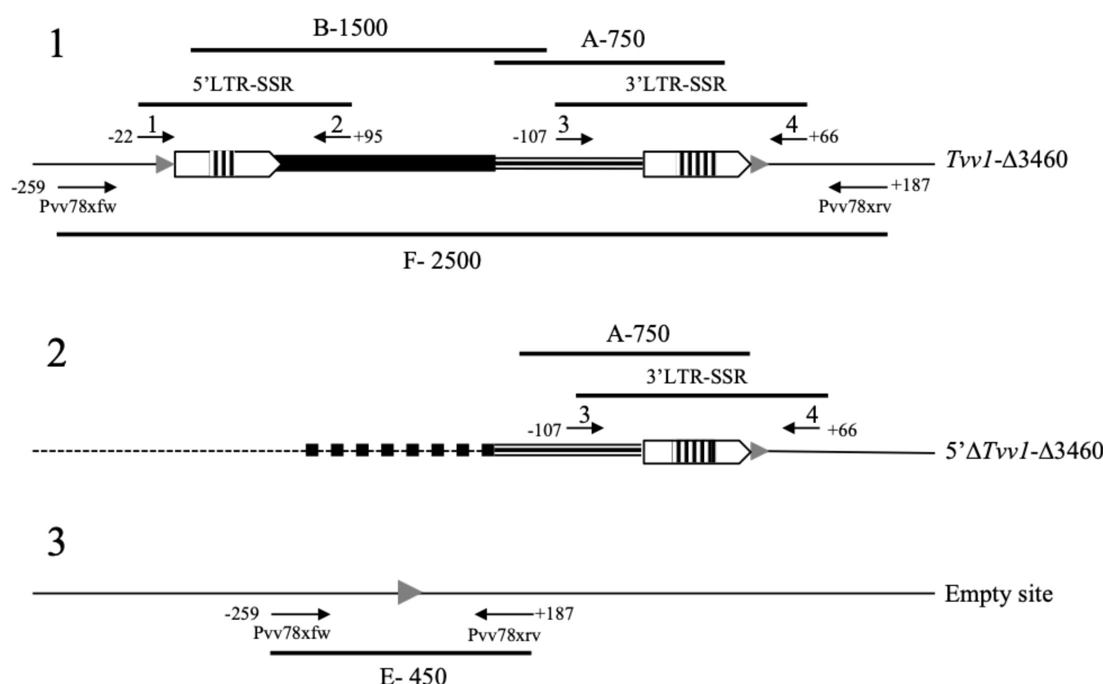

FIGURE 1. Schematic representation of the three alleles of the locus.

grouped into families sharing an amino acid identity of ≥ 90 % (Moisy *et al.,* 2008b), among which the *Tvv1* family, which is a *Ty1-copia* like LTR-retrotransposon, has been extensively studied (Moisy *et al.,* 2008a). *Tvv1* full-length copies of around 5 kb in size share an internal region with a single highly conserved open reading frame and, upstream, an untranslated leader (UTL) region which is highly variable in size. This internal region is flanked by LTRs between 149 and 157 bp long (Pelsy and Merdinoglu, 2002). The *Tvv1* family also comprises unique copies of remarkable *Tvv1* elements that have suffered large deletions and are fixed at single loci. Among them, *Tvv1*-Δ3460, which has undergone a major 3,460 bp long deletion in the coding sequence compared to the full-length copies, is located on chromosome 8 of the grapevine genome. In Pinot noir cv, *Tvv1*-Δ3460 is 2,074 pb long, but it slightly differs in size in the genomes of other varieties hosting this element, mainly because of the number of TA motifs in a microsatellite stretch located in both LTR sequences (Moisy *et al*., 2008a).

Molecular markers based on the insertion of retrotransposons can be used for identifying grapevine species and cultivars (D'Onofrio et al, 2010), but they generally fail to reveal clonal polymorphism (Pelsy, 2007). Nevertheless, transposable elements are responsible for the main proportion of somatic mutations affecting four Pinot Noir clones (Carrier *et al.,* 2012) and have been found to cause diversification of 15 Zinfandel clones (Vondras *et al*., 2019). In the present study, we developed a marker set based on the combination of the presence/absence of the remarkable retrotransposon *Tvv1*-Δ3460 at its insertion site and the SSR polymorphism within its LTRs. By studying a collection of *Vitaceae* accessions, we showed the relevance of *Tvv1*-Δ3460-based markers to distinguish in a simple way most of the *Vitis* accessions, and to reveal clonal polymorphism within different varieties.

MATERIALS AND METHODS

1. Plant material and DNA extraction

The plant material consisted in *Vitaceae* accessions divided into three groups. The *Vitaceae* group comprised *Ampelopsis* (5 accessions)*, Parthenocissus* (1 accession)*,* and *Vitis* genera (*Muscadinia rotundifolia* (4 accessions), Asian species (6 accessions)*,* North American species (16 accessions)) and inter-specific hybrids (2 accessions). The *V. vinifera* group contained cultivated grapevine varieties (52 accessions) and wild vines (6 accessions). Due to the dioicity of wild vines, a male and a female were chosen for each origin of sampling: Ste Croix en Plaine (53) and Mandeure (C25) in France, and Martigny (50) in Switzerland. All accessions are kept in the ampelographic collection of INRAE-Colmar (France). In addition, the segregation of the LTR-SSR alleles was assessed in a progeny of Riesling x Gewurztraminer comprising 11 randomly chosen individuals. The third group comprised seven wine grape varieties represented by a random collection of certified clones conserved in the French national repository (ENTAV, Le Grau du Roi, France), and by accessions recovered from field selections and kept in different germplasm repositories at INRAE, the French National Research Institute for Agriculture, Food and Environment : Cabernet franc (17 clones) , Cabernet-Sauvignon (37 clones), Chenin blanc (19 clones), Grolleau (48 clones), Pinot noir (23 clones), Riesling (27 clones), and Savagnin (45 clones including 22 Savagnin blanc, 19 Gewurztraminer, 4 Savagnin rose).

Total DNA was purified from young expanded leaves from individual plants using Dneasy ™ Plant Mini-Kit (Qiagen, Hilden, Germany) as described by the supplier.

2. PCR conditions and fragment analysis

According to Moisy *et al.* (2008a), the internal PCR products A-750 and B-1500 characterised the *Tvv1*-Δ3460 copy (Figure1). Due to the presence of full-length copies of *Tvv1* elsewhere in the genome, both amplifications also produce 4,3Kb and 4Kb long fragments respectively (Moisy *et al.*, 2008a). When present at its insertion site, *Tvv1*-Δ3460 was revealed by a long-range PCR product 2500 bp long, F-2500, using Pvv78x primers designed in the host flanking regions of the insertion. These primers also amplify the empty site, E-450, as well as a potential full-length *Tvv1* element that can be inserted at the site (Moisy *et al*., 2008a). To take advantage of the polymorphism of the microsatellite stretch located in both LTR sequences of *Tvv1*-Δ3460, 5' and 3' LTR-SSR markers primers were designed using Primer3 software (Rozen & Skaletsky, 1998) and synthesised by MWG Biotech AG (Ebersberg, Germany). One primer of each pair was HEX and 6-FAM fluorophore-labelled (PE Applied Biosystems, Warrington, UK) respectively to amplify the 5' and 3' LTR-SSR markers in multiplex (Table 1). All primer locations are given in Figure 1 and all new sequences in Table 1. PCR amplifications were carried out according

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to Hocquigny *et al*. (2004). The programme consisted of the following steps: 5 min at 94 °C, followed by 30 cycles of 30 s at 92 °C, 30 s at 52 °C, 30 s at 72 °C, and a final extension step of 7 min at 72 °C. 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, Foster City, CA). Microsatellite alleles were scored using GenScan (version 3.1) and Genotyper (version 2.5.2) software (PE Applied Biosystems, Foster City, CA). All polymorphisms were confirmed by at least two analysis.

1. *Tvv1*-Δ3460 insertion; 2: 5'Δ*Tvv1*-Δ3460 insertion and 3: the empty site

Arrows boxes represent LTRs including the SSR stretch, the black box the UTL and the triple line ORF regions. The 5 bp-sequence duplicated to flank the retrotransposon is represented by a triangle. Dotted lines represent the 5' deletion of 5'Δ*Tvv1*-Δ3460 and of its flanking region. Black arrows represent primers used in the study, whose numbers are shown in Table 1. Primers 1 and 2 amplify 5'LTR-SSR and primers 3 and 4 amplify 3'LTR-SSR. Pvv78x primers, located in the host region flanking the insertion, amplify either *Tvv1*-Δ3460 (F-2500) or the empty site (E-450). Primer positions are given according to the LTRs. Primers that generate A-750 an B-1500 fragments are given in Moisy *et al.* (2008a).

RESULTS

The presence/absence of *Tvv1*-Δ3460 at its expected insertion site was revealed by three PCR amplifications. The A-750 and B-1500 fragments, which overlap the specific internal region of *Tvv1*-Δ3460, characterised this element (Moisy *et al*., 2008a). The Pvv78x primers designed in the host flanking regions of the insertion amplified a 2500 bp-long fragment, F-2500, when *Tvv1*-Δ3460 was present. Conversely, this pair of primers produced a 450 bp-long fragment, E-450, when the site was empty. In some accessions, A-750 was amplified but not B-1500 or E-450. This combination of PCR fragments revealed a new element whose 5'LTR was truncated, which was named *5'*Δ*Tvv1*-Δ3460. No amplification of this new element was possible using the Pvv78x primers leading to the conclusion that its 5'host flanking region must have been deleted as well. Finally, the insertion of a full-length *Tvv1* copy, with an average size of 5 kb, was investigated at the given site by long-range PCR using Pvv78x primers. Among the accessions in the study, none revealed the expected 5,5 Kb-long fragment.

In addition, as can be seen in Table 1, two markers, 5' and 3' LTR-SSR, were developed to characterise *Tvv1*-Δ3460 LTRs. 5'LTR-SSR was amplified using primer 5_3460_fw, annealing upstream of *Tvv1*-Δ3460, and paired with primer 5_3460_rv designed in the UTL sequence of the element. Similarly, 3'LTR-SSR was amplified using primer 3_3460_fw, designed in the RNAse sequence of *Tvv1*-Δ3460, and paired with primer 3_3460_rv located downstream of the element.

1. *Tvv1*-Δ3460 in *Vitaceae* species

A collection of 34 *Vitaceae* species (Table 2) was investigated for the presence/absence of *Tvv1*-Δ3460. *Ampelopsis* species did not amplify A-750, B-1500 or E-450, which is most likely due to a lack of homology of the primers designed from the sequence of *V. vinifera* with the target sequence of *Ampelopsis*. The *Parthenocissus*, the 4 *Muscadinia* and 5 of 24 *Vitis* accessions amplified E-450 and not A-750 or B-1500, leading to the conclusion that *Tvv1*-Δ3460 is absent in these accessions, while the Pvv78x primers are effective at amplifying the empty site.

Of the remaining 19 *Vitis*, 14 accessions that amplified both A-750 and B-1500, but not E-450, kept *Tvv1*-Δ3460 in a homozygous state. Conversely, four accessions displayed A-750, B-1500 and E-450 indicating the presence of *Tvv1*-Δ3460 combined with its empty site. Finally, *Vitis rupestris du Lot* amplified A-750, but not B-1500 or E-450. This new pattern of amplification revealed the new element 5'Δ*Tvv1*-Δ3460. In this collection, 9 of the 18 North American *Vitis* or hybrids of North American accessions amplified

TABLE 1. 5'LTR-SSR and 3'LTR-SSR primers.

marker	primer name	nb	location	label	sequence
5'LTR-SSR	5_3460_fw	1	5' *Tvv1*-D3460 host region		CAGAGTCAAT TTCCTTCCCC AT
	5_3460_rv	2	UTL *Tvv1*-3460	HEX	CGTGACCCAA GAAGAAAAAG AA
3'LTR-SSR	3_3460_fw	3	Rnase *Tvv1*-3469	FAM	AGAGCAACTT GGGGATATTT TT
	3_3460_rv	4	3' *Tvv1*-D3460 host region		AGTCATTTGG AACCAGTGGA TC

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TABLE 2. Distribution of *Tvv1*-Δ3460, 5’Δ*Tvv1*-Δ3460 and the empty site within the *Vitaceae* panel and genotypes at 5’LTR-SSR and 3’LTR-SSR.

Accession name	Geographic origin	B-1500	A-750	E-450	F-2500	5’LTR-SSR		3’LTR-SSR	
		1500 bp	750 bp	450 bp	2500 bp	allele 1	allele 2	allele 1	allele 2
Ampelopsis aconitifolia	Asia	-	-	-		-		-	
Ampelopsis cordata	North America	-	-	-		-		-	
Ampelopsis heterophylla	Asia	-	-	-		-		-	
Ampelopsis japonica	Asia	-	-	-		-		-	
Ampelopsis pedonculata	Asia	-	-	-		-		-	
Parthenocissus quinquefolia	North America	-	-	+	-	-		-	
Muscadinia rotundifolia Carlos	North America	-	-	+	-	-		-	
Muscadinia rotundifolia Dulcet	North America	-	-	+	-	-		-	
Muscadinia rotundifolia Régale	North America	-	-	+	-	-		-	
Muscadinia rotundifolia YxC	North America	-	-	+	-	-		-	
Vitis aestivalis	North America	-	-	+	-	-		-	
Vitis amurensis	Asia	+	+	-		276	285	322	324
Vitis arizonica	North America	+	+	-		271	283	343	355
Vitis armata	Asia	+	+	-		271	278	323	
Vitis berlandieri Colombard	hybrid	+	+	+		240		324	
Vitis berlandieri Planchon	North America	+	+	+	+	277		322	
Vitis candicans	North America	-	-	+	-	-		-	
Vitis cinerea	North America	-	-	+	-	-		-	
Vitis cordifolia 9 couderc	hybrid	-	-	+	-	-		-	
Vitis Davidii	Asia	+	+	-		279	283	322	324
Vitis doaniana	North America	+	+	+		290		327	
Vitis ishikari	Asia	+	+	-		268	282	322	
Vitis labrusca Concorde	North America	+	+	-		277		332	349
Vitis labrusca Isabelle	North America	+	+	-		276		330	334
Vitis linsecumii	North America	-	-	+	-	-		-	
Vitis monticola Large Bell	North America	+	+	+		269		328	
Vitis reticulata	Asia	+	+	-	+	269		322	361
Vitis riparia Gloire de M	North America	+	+	-		252		331	
Vitis riparia Millardet	North America	+	+	-		273		331	355
Vitis riparia Muller	North America	+	+	-		273		331	355
Vitis rubra	North America	+	+	-	+	264	274	333	337
Vitis rupestris du Lot	North America	-	+	-	-	-		327	371
Vitis titania	Asia	+	+	-		252	280	328	333
Vitis vulpina	North America	+	+	-	+	275		331	

The sign ‘+’ indicates amplification of the fragment and ‘-‘ no amplification. *Tvv1*-Δ3460 is characterised by the amplification of A-750, B-1500 and F-2500, but not of E-450. 5’Δ*Tvv1*-Δ3460 is characterised by the amplification of A-750, but not of B-1500, E-450 or F-2500. The empty site is characterised by the amplification of E-450, but not of A-750, B-1500 or F-2500.

the empty site of insertion of *Tvv1*-Δ3460, but did not amplify any of the 6 Asian *Vitis*.

The polymorphism of the LTRs of the inserted elements were investigated using 5’ and 3’ LTR-SSR markers (Table 1). In the collection of the 19 *Vitaceae* species hosting at least one copy of *Tvv1*-Δ3460, a total of 18 alleles of 240 to 290 bp in size and 16 alleles of 322 to 371 bp in size were scored for 5’and 3’LTR-SSR markers respectively.

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TABLE 3. Distribution of *Tvv1*-Δ3460 and 5'Δ*Tvv1*-Δ3460 within the *Vitis vinifera* panel.

Amplified region	B-1500	A-750	E-450	5'LTR-SSR			3'LTR-SSR		
	1500 bp	750 bp	450 bp	Allele 1	Allele 2	Allele 3	Allele 1	Allele 2	Allele 3
Aligoté B	+	+	-	275	284		332	351	
Aubin vert B	+	+	-	284			348	351	
Auxerrois	+	+	-	284			348	351	
Bachet Noir N	+	+	-	278			330	348	
Beaunoir	+	+	-	275	278		330	332	
Cabernet franc N	+	+	-	277			330	342	
Cabernet-Sauvignon N	+	+	-	277			330	348	
Carignan N	+	+	-	276	284		346	351	
Chardonnay B	+	+	-	275	284		332	351	
Chenin B	+	+	-	276			348	352	
Cinsaut N	+	+	-	276			330	348	
Clairette B	+	+	-	276	278		330	348	
Colombard B606	+	+	-	276	277	279	330	353	
Corbeau N	+	+	-	277			330	348	
Côt N596	+	+	-	275			332	348	
Folle Blanche B	+	+	-	279			330	340	
Franc noir de la Haute Saone	+	+	-	284			348	351	
Gamay Blanc Gloriod B	+	+	-	284			332	351	
Gamay N	+	+	-	275	284		332	351	
Gewurztraminer R 643	+	+	-	277			330	348	
Gouais B	+	+	-	278	284		330	351	
Grenache N	+	+	-	275			330	340	
Grolleau B	+	+	-	285			349	351	
Knipperlé 61D	+	+	-	275	284		332	351	
Marsanne B	+	+	-	277			348		
Mauzac B	+	+	-	277			342	348	
Melon B	+	+	-	284			348	351	
Merlot N	+	+	-	277			330	348	
Mourvèdre	-	+	-	-			340	342	
Muscat d'Alexandrie B	+	+	-	239	240	276	324	330	
Muscat d'Alsace R	+	+	-	240	296		324	340	
Muscat cendré 336 B	+	+	-	275			332	348	
Muscat de Hambourg N	+	+	-	276			330	336	
Muscat de Saumur B	+	+	-	240			324	348	
Muscat Ottonel B	+	+	-	240			324	348	
Muscat Reine des Vignes B	+	+	-	276			330	348	

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Muscat petits grains	+	+	-	240	296		324	340	
Persan	+	+	-	275			332	348	
Peurion N	+	+	-	240	297		324	340	
Pinot N162	+	+	-	275			332	348	
Riesling B 49	+	+	-	285			351		
Romorantin B929	+	+	-	275	278		330	332	
Roublot	+	+	-	275	278		330	332	
Roussanne B	-	+	-	-			340	342	
Sacy B	+	+	-	278			330	348	
Sauvignon B	+	+	-	274			339	348	
Sémillon B	-	+	-	-			342	332	
Sylvaner 50	+	+	-	276	284		330	351	
Syrah n	+	+	-	274			332	348	
Tannat	+	+	-	274	278		330	332	
Ugni Blanc B	+	+	-	240	276		324	330	
Viogner B	+	+	-	278			330	342	
VSil50K	+	+	-	275			330	332	
VSil50l	+	+	-	275	277		328	330	348
VSil.53I	+	+	-	269	275	277	330	348	
VSil.53J	+	+	-	275	277		330	342	
VSil.C25S2B	+	+	-	275			332		
VSil.C1S6	+	+	-	274	284		332	351	

The sign '+' indicates amplification of the fragment and '-' no amplification. *Tvv1*-Δ3460 is characterized by A-750: +, B-1500: +, E-450: - and 5'Δ*Tvv1*-Δ3460 by A-750: +, B-1500: -, E-450 :-.

The genotypes at 5'LTR-SSR and 3'LTR-SSR are given.

The two LTR-SSR markers defined a specific genotype for all of these *Vitis* species, except for *V. riparia* Millardet and *V. riparia* Muller, which shared the same genotype. The LTR-SSR genotype of *V. rupestris du Lot* is consistent with the association of two copies of 5'Δ*Tvv1*-Δ3460, whose 3'LTR were different in size.

2. *Tvv1*-Δ3460 in *Vitis vinifera*

The presence/absence of *Tvv1*-Δ3460 was also evaluated in a collection of *V. vinifera* accessions comprising 52 cultivated varieties and 6 wild vines. On the one hand, all the accessions amplified A-750, while none amplified E-450, indicating the prevalence of *Tvv1*-Δ3460 insertion in the *V. vinifera* species. On the other hand, 55 of the 58 accessions amplified B-1500. The three varieties Mourvèdre, Roussanne and Semillon that did not amplify B-1500 presumably hosted only 5'Δ*Tvv1*-Δ3460 (Table 3).

In the *V. vinifera* collection, the amplification of 5' and 3' LTR-SSR markers each provided 13 alleles of 239 to 297 pb and 324 to 353 bp in size respectively. These two markers made it possible to characterise 38 genotypes which differed from those previously characterised in the collection of 34 *Vitaceae* species. Twenty-nine genotypes were displayed by one variety only, 5 genotypes by 2 varieties, 1 genotype by 3 varieties and 4 genotypes by 4 varieties. Varieties sharing the same genotype were known to be related such as the progeny of Pinot noir × Gouais blanc cross. Out of the 58 *Vitis vinifera,*

22 showed two alleles at each locus, indicating the presence of two full copies of *Tvv1*-Δ3460 with polymorphic LTRs. Thirty-one accessions, among them Pinot noir and Gewurztraminer, displayed one allele for 5'LTR-SSR and 2 alleles for 3'LTR-SSR. These varieties associated either two copies of *Tvv1*-Δ3460 with 5'LTRs of the same length

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and polymorphic 3'LTRs, or one copy each of *Tvv1*-Δ3460 and 5'Δ*Tvv1*-Δ3460. To clarify the 5' LTR-SSR genotype of Pinot noir (allele 275), 13 varieties known to be Pinot noir × Gouais blanc progeny (Bowers *et al.*, 1999) were considered. All of these varieties displayed one allele of Gouais blanc [278:284], either alone or associated with allele 275 of Pinot noir. This result suggests the segregation of a null allele of Pinot noir resulting from the lack of 5'LTR of *5'ΔTvv1*-Δ3460. Thus, Pinot noir is heterozygous [275:-], because of the association of *Tvv1*-Δ3460 and 5'Δ*Tvv1*-Δ3460, as well as Auxerrois or Melon [284:-].

The 5' LTR-SSR marker null allele resulting from the 5'Δ*Tvv1*-Δ3460 insertion is quite common and carries out segregation within many *V. vinifera* varieties. For example, the genotype of Cabernet-Sauvignon [277], whose parents are Cabernet franc [277] and Sauvignon B [274], must be heterozygous for the null allele [274: -] inherited from Sauvignon B [274:-].

Finally, two varieties, Marsanne and Riesling, and a *V. vinifera ssp. silvestris* accession, VSil. C25S2B, amplified only one allele at each locus. As none of these accessions amplified the empty site of insertion, their genotypes may result from the association of either two copies of *Tvv1*-Δ3460 with LTRs of the same length, or of *Tvv1*-Δ3460 and 5'Δ- *Tvv1*-Δ3460, which have 3'LTR of the same length. To clarify the genotypes of Riesling (alleles 285 at 5'LTR-SSR and 351 at 3'LTR-SSR) and Gewurztraminer (alleles 277 at 5'LTR-SSR and 330:348 at 3'LTR-SSR), the segregation of these alleles was analysed in 11 individuals of the progeny of a Riesling x Gewurztraminer cross. At 5'LTR-SSR locus, one descendant amplified both 277 and 285, three only 277, four only 285 and 3 did not amplify the marker. This result indicated the hemizygous genotypes of Riesling [285:] and Gewurztraminer [277:-] at 5'LTR-SSR. Conversely, all progeny displayed allele 351 of Riesling at 3'LTR-SSR, in association with either allele 330 (7 progeny) or allele 348 (7 progeny) of Gewurztraminer. The genotype of Riesling for the 3'LTR-SSR marker is therefore homozygous [351:351], while that of Gewurztraminer is heterozygote [330:348]. This pattern of amplification leads to the conclusion that the two varieties hosted *Tvv1*-Δ3460 and 5'Δ*Tvv1*-Δ3460, but Riesling has elements which display 3'LTR of identical size, while those of the Gewurztraminer elements are different.

3. LTR-SSR polymorphism within seven French wine grape variety collections

The capacity of the two *Tvv1*-Δ3460 LTR-SSR markers to reveal intra-varietal polymorphism was evaluated in seven clone collections of wine grape varieties: Cabernet franc, Cabernet-Sauvignon, Chenin blanc, Grolleau, Pinot noir, Riesling, Savagnin. These collections comprised

TABLE 4. 5'LTR-SSR and 3'LTR-SSR genotypes of clones belonging to seven wine grape varieties.

Variety	total clone nb	certified clone nb	5'LTR-SSR			3'LTR-SSR		
			Reference genotypes	Variant genotypes	Variant clone nb	Reference genotypes	Variant genotypes	Variant clone nb
Cabernet franc	17	2	277	277-**285**	1	330-342	**328**-342	1
							330-**332**-342	1
							330-**350**	1
Cabernet-Sauvignon	37	22	277	-		330-348	-	
Chenin	19	0	276	-		348-352	348-**350**-352	3
Grolleau	48		285	-		349-351	-	
Pinot noir	23	23	275	-		332-348	332-348-**350**	1
							332-**334**-348	1
Riesling	27	5	285	-		351	**339**-351	1
							351-**353**	2
Savagnins	45	7	277	**275**	1	330-348	330-**344**-348	1
							330-348-**358**	5
							330-**350**	1
							330-348-**350**	1
							330-348-**356**	1

New alleles are indicated in bold.

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a total of 216 accessions of certified clones and introductions which had been preserved in French repositories.

These collections of clones were chosen for comparison since they had previously been evaluated with 12 SSR markers for the collections of Grolleau, Cabernet franc, Chenin blanc, and with 30 markers for those of Cabernet-Sauvignon, Pinot noir, Riesling and Savagnin (Pelsy *et al.*, 2010).

In this study, the reference genotype was defined as that the genotype shared by the majority of the accessions of a collection (Table 4). All varieties amplified one allele at locus 5'LTR-SSR and six amplified two alleles at locus 3'LTR-SSR, except Riesling. Two variants were detected at locus 5'LTR-SSR: one Savagnin that displayed a 275 bp-long allele, instead of the 277 bp-long reference allele, and one Cabernet franc that showed the heterozygous genotype [277:285], instead of the reference genotype, which was most probably homozygous [277:277]. With the appearance of 10 new alleles, the 3'LTR-SSR locus was far more susceptible to polymorphism than 5'LTR-SSR (2 new alleles). One to 5 variant genotypes were observed in 5 of the varieties, mainly as a result of the addition of a new allele to the reference genotype, leading to triple-allele genotypes characterising periclinal chimeras.

Thus, clonal polymorphism was revealed in 5 of the 7 clone collections. Four variants were characterised out of the 17 clones of Cabernet franc, 3 out of the 19 clones of Chenin, 2 out of the 23 clones of Pinot noir, 3 out of the 27 clones of Riesling and 10 out of the 45 clones of Savagnin, most of the latter being Savagnin blanc. One clone of Cabernet franc was polymorphic at both loci. Altogether, it was possible to unambiguously distinguish 11 clones out of the 216 studied by a unique genotype, while 2 to 5 variants shared the same genotype.

Conversely, no variants were detected among the 37 and 48 clones of Cabernet-Sauvignon and Grolleau respectively.

DISCUSSION

Having effective and easy-to-use markers capable of quickly identifying *Vitis* species, grapevine varieties and clones belonging to the same variety is still a challenge today. In this study, we describe a novel set of molecular markers based on a combination of the variation in the presence of the remarkable retrotransposon *Tvv1*-Δ3460 at its insertion locus and the SSR polymorphism of its LTRs to assess grapevine diversity. We used it to explore species, varietal and intra-varietal diversity. In 4 PCR amplifications (B-1500, E-450 and 5' and 3' LTR-SSR in multiplex), it was possible to characterise all the species and varieties. In addition, the 3'LTR-SSR marker was shown to be highly relevant in revealing clonal polymorphism.

1. Presence vs absence of *Tvv1*-Δ3460 is informative

The *Tvv1*-Δ3460 locus showed different alleles in the *Vitaceae* species revealed by the long-range PCR using Pvv78x primers. *Ampelopsis* accessions did not amplify *Tvv1*-Δ3460 or its empty site, which is likely because the primers designed from a *V. vinifera* sequence were not homologous enough for their target sites in *Ampelopsis*, due to the phylogenetic distance between both genera. The *Parthenocissus*, the four *Muscadinia* and five of the American *Vitis* or hybrid accessions (*V. aestivalis*, *V. candidans*, *V. cinerea*, *V. cordifolia* Couderc and *V. linsecumii*) only amplified the empty site of the locus (Figure 2). This result agrees with previous studies which showed the divergence between the genera *Ampelopsis* and *Vitis*, but brought *Parthenocissus quinquefolia* as close as *Muscadinia* to *Vitis* (Pelsy, 2007).

Four American *Vitis* or hybrid accessions (*V. berlandieri* Colombard, *V. berlandieri* Planchon, *V. doaniana, V. monticola* Large Bell) amplified both the full and the empty site of *Tvv1*-Δ3460 (Figure 2). All the remaining accessions, including the Asian accessions and the wild or cultivated *V. vinifera,* displayed a *Tvv1*-Δ3460 element, but never the empty site. These results indicate that the *Tvv1*-Δ3460 insertion is specific to the *Vitis* species. Either the allele with the full site or the one with the empty site of *Tvv1*-Δ3460 were present homozygous or heterozygous in the American *Vitis* or hybrids accessions. Conversely, all the Asian accessions and *V. vinifera* varieties hosted a *Tvv1*-Δ3460 insertion at the locus. This insertion was most probably dispersed through natural intermixing, due to the close proximity of Asian *Vitis* with the European species. However, the empty site which is remained in half of American *Vitis* confirms the disjunction between the Old and New World (Zecca *et al.*, 2012).

The formation of deleted elements may occur either during the retrotransposition process prior to integration, or by illegitimate recombination

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within an integrated full-length element. Since no full copy of *Tvv1* was amplified via long-range PCR amplification using the Pvv78x pair, the large internal deletion characteristic of *Tvv1*-Δ3460 most likely occurred during the retrotransposition process before integration.

Finally, 5'Δ*Tvv1*-Δ3460, a new element that was subject to a deletion of the 5' region of *Tvv1*-Δ3460 and of its 5'host region, was identified in *V. rupestris* du Lot and in many *V. vinifera* varieties. Nevertheless, the 4 PCR does not allow us to conclude that the *V. rupestris* du Lot and *V. vinifera* varieties share the very same 5'Δ*Tvv1*-Δ3460 element, or that independent deletions lead to different 5'Δ*Tvv1*-Δ3460 elements.

In some varieties, two copies of *Tvv1*-Δ3460 or of 5'Δ*Tvv1*-Δ3460 can be combined, such as in Sylvaner or Semillon respectively, but with 3'LTRs of different lengths. Other varieties, such as Pinot noir, Riesling and Gewurztraminer, combine *Tvv1*-Δ3460 and 5'Δ*Tvv1*-Δ3460. The elements of Pinot noir and Gewurztraminer have 3'LTRs of different lengths, while those of Riesling have 3'LTR of the same length (Figure 2).

2. 5' and 3' LTR-SSR: highly informative markers which can be used to identify *Vitis* accessions

In the *Vitis* species which hosted at least one copy of *Tvv1*-Δ3460, the 5' and 3' LTR-SSR markers are well-conserved, defining a specific genotype for all of these *Vitis* species. Moreover, when considering the 58 *V. vinifera* accessions (cultivated grapevine varieties and wild vines), the same group studied with 14 SSR markers showed that the number of alleles detected per locus ranged from 1 for VMC1e11a to 14 for VVS2, with an average of 8.6 (Pelsy, 2007). 5' and 3' LTR-SSR each amplified 13 alleles; they are therefore among the most informative markers for identifying *V. vinifera* accessions.

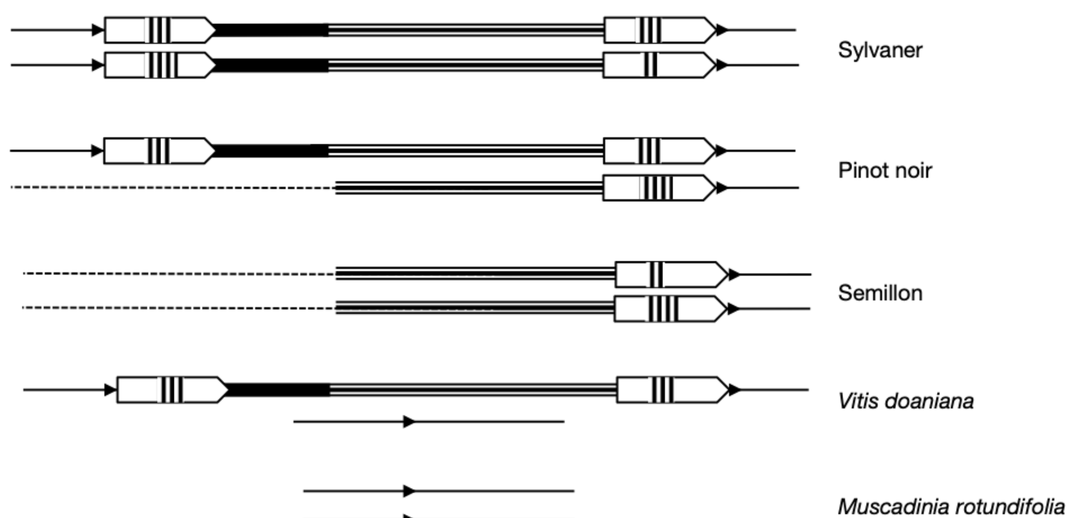
3. LTR-SSR polymorphism within seven French wine grape variety collections

Not all microsatellite markers can reveal clonal polymorphism within clone collections, and therefore a standard set of five microsatellite markers (VMC3a9,VMC5g7, VVS2, VVMD30, and VVMD32) has been proposed (Pelsy *et al.*, 2010).

It was possible to develop 5' and 3' LTR-SSR markers due to the systematic presence of an insertion - either of *Tvv1*-Δ3460 or 5'Δ*Tvv1*-Δ3460 - at the locus of different grape varieties, and to a TA microsatellite stretch in the LTR sequences of these *Tvv1* elements. Using these markers, polymorphism was assessed in the clone collections of seven varieties each comprising 17 to 48 clones.

The 3' LTR-SSR marker revealed heterozygous genotypes for all varieties, except Marsanne and Riesling. The analysis of a Riesling progeny showed that this variety is homozygous for this marker [351-351]. This assertion is confirmed

FIGURE 2. Combinations of the three alleles observed at the *Tvv1*-Δ3460 locus and example of accessions containing them.



Tvv1-Δ3460 is flanked by LTRs, the 5' region of 5'Δ*Tvv1*-Δ3460 is truncated and the empty site is symbolised by a triangle.

TABLE 5. number of distinguishable clones using one of the SSR sets and adding LTR-SSR markers.

Variety	total clone nb	distinguishable clones nb				
		SSR set 1	SSR set 2	SSR set 3	LTR-SSR	total
Cabernet franc	17		3		2	5
Chenin	19		3		0	3
Pinot noir	23	5			2	7
Riesling	27			1	2	3
Savagnins	45			5	4	9

SSR set 1 comprises VMC3a9, VMC5g7, VVS2, VVMD30, and VVMD32; set 2 comprises VMC3a9, VMC5g7, VVS2 and VVMD30 and set 3 comprises VMC3a9, VMC5g7, VVS2 and VVMD32.

by the identification of two variant clones of Riesling, which showed two new genotypes at this locus, [339:351] and [351:353], and two new alleles, 339 and 353, which derived from the reference allele 351. However, it was not possible to determine from these data whether Marsanne hosts *Tvv1*-Δ3460 with identical 5' and 3'LTRs or *Tvv1*-Δ3460 in association with 5'Δ*Tvv1*-Δ3460, both with an identical 3'LTR.

Variant genotypes were revealed by 3'LTR-SSR in five of the seven clone collections. Thus, this marker is more effective than all the previously identified SSR markers: indeed, VMC3a9, VMC5g7 and VVS2 revealed variants in four of these collections of clones, while VVMD30 and VVMD32 in three and two collections respectively.

The 5 'LTR-SSR marker, meanwhile, often only amplified a fragment in the studied varieties, as was the case for Pinot noir, Riesling and Gewurztraminer, which are all hemizygous due to the combination of *Tvv1*-Δ3460 and 5'Δ*Tvv1*-Δ3460, and 5'Δ*Tvv1*-Δ3460 resulting in a null allele with 5' LTR-SSR. Therefore, the 5' LTR-SSR marker is less effective at revealing clonal polymorphism than the 3' LTR-SSR marker.

In a previous study, the same clones were studied with SSRs to identify a standard set of five markers (VMC3a9, VMC5g7, VVS2, VVMD30 and VVMD32) which revealed clonal polymorphism within different varieties (Pelsy *et al.,* 2010). The addition of the LTR-SSR markers improved the identification of the clones in 4 of the 7 varieties by distinguishing new clones. Therefore, while the standard set markers made it possible to assign a unique genotype to 1 to 5 clones in the collections, the additional LTR-SSR markers allowed 2 to 4 new clones with a unique genotype to be identified, thus increasing the number of distinguishable clones; for example, for Savagnins, the number of clones displaying a unique genotype increased from 5 to 9 (Table 5).

Genomic analysis provides access to a large number of SNPs and InDels that can be exploited to define markers capable of characterising interclonal diversity in different grapevine varieties. Nevertheless, these approaches are limited by sequencing technology or the lack of a reference genome for the studied varieties (Roach *et al.*, 2018). Furthermore, SSR markers, which are highly transferable between different varieties and *Vitis* species, are simple tools which can be used to identify variant clones in a wide range of varieties. Thus, to increase the capacity for revealing clonal polymorphism with SSR markers, we recommend that 3 ′ LTR-SSR be added to the standard set of previously characterised VMC3a9, VMC5g7, VVS2, VVMD30 and VVMD32 (Pelsy *et al.*, 2010)

CONCLUSION

Deleted retroelements are unique, very stable and can be considered as Mendelian loci. They can be identified within different families of retrotransposon by PCR amplification with primers derived from the LTRs or the conserved PBS or PPT sequences. Using available grapevine genome sequences, their flanking sequences can then be identified to design primers that will yield easy-to-use co-dominant markers. Their specific dispersion in the *Vitaceae* make these markers valuable tools for studying their diversity.

Acknowledgements: We would like to thank Lionel Ley and the members of the experimental unit of INRAE-Colmar for their very valuable technical support in the production of plants both in the greenhouse and in the vineyard. We would also like to thank Cyndy Ponama, Gisèle

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Butterlin, Roxanne Hardy and Angélique Ardiller for the laboratory experiments.

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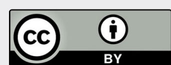

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