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Genomic analysis of *Grapevine Retrotransposon 1* (*Gret1*) in *Vitis vinifera*

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Abstract The complete sequence of the first retrotransposon isolated in *Vitis vinifera*, *Gret1*, was used to design primers that permitted its analysis in the genome of grapevine cultivars. This retroelement was found to be dispersed throughout the genome with sites of repeated insertions. Fluorescent in situ hybridization indicated multiple *Gret1 loci* distributed throughout euchromatic portions of chromosomes. REMAP and IRAP proved to be useful as molecular markers in grapevine. Both of these techniques showed polymorphisms between cultivars but not between clones of the same cultivar, indicating differences in *Gret1* distribution between cultivars. The combined cytological and molecular results suggest that *Gret1* may have a role in gene regulation and in explaining the enormous phenotypic variability that exists between cultivars.

Introduction

Retrotransposons, also known as retroelements, make up a large fraction of the nuclear genome in plants. These elements represent approximately 20% of the rice genome, whereas up to 90% of large genomes such as that of wheat are retroelement related (Jianxin et al. 2004; Flavell 1986). In their active state, retroelements transpose via an RNA intermediate that is reverse transcribed before integration into the host genome. Due to their dynamics and mobility, it is widely accepted that transposable elements increase genome size as well as generate molecular modifications, thus having an important role in genome evolution and speciation. There are three known categories of retrotransposons in plants, LINEs, SINEs, and long terminal repeat (LTR) containing elements. Elements containing the flanking LTRs can be further separated into two major families, defined by the order of the Reverse Transcriptase and Integrase domains in their *pol* genes. These are Ty3 or *gypsy*-like, and Ty1 or *copia*-like, with *gypsy*-like retrotransposons having the greatest similarity to retroviruses (Friesen et al. 2001).

Numerous studies on a number of plant species have been done involving the characterization and cytological localization of *gypsy*-like and *copia*-like retrotransposons. The distribution of these elements varies depending on the element type as well as the genome in question. Generally, LTR retroelements are disproportionably abundant in heterochromatin, such as in pericentric and terminal heterochromatic regions (Wong and Choo 2004; Belyayev et al. 2001). Nonetheless, there are a number of studies that show retroelements to be dispersed or clustered throughout the genome, including in gene rich regions (Sandhu and Kulvinder 2002; San-Miguel et al. 1996). Interestingly, diagnostic sequencing of a 280-kb genomic region containing two genes in maize showed this sequence to be composed primarily of nested retroelements inserted within each other (San-Miguel et al. 1996). Recent studies have confirmed that retrotransposons are not solely heterochromatic, but also insert in euchromatic regions, and often nearby genes (Vershinin et al. 2002). For example, *Tos17*, a rice *copia*-type element, was found to preferentially target gene rich regions in an experimental population (Miyao et al. 2003). Sequence analysis of the target sites for this element in the rice genome confirmed that 76% of its hotspots are located in coding sequences.

Due to their nature and distribution in plant genomes, retrotransposons have enormous potential as genetic tools. The conserved domains within the *gag-pol* coding regions allow for the study of specific retroelements for gene mapping and linkage analysis. Furthermore, LTR sequences permit experimental procedures

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such as retro-element-microsatellite-amplified-polymorphism (REMAP) and inter-retroelement-amplified-polymorphism (IRAP). These two experimental procedures have been extremely valuable for the development of molecular markers in plants (Kumar and Hirochika 2001; Schulman et al. 2004). REMAPs and IRAPs have been successfully used to map genes (Manninen et al. 2000), and to study genomic stability in allopolyploid species (Baumel et al. 2002). Furthermore, they were used to distinguish species across a genus (Kalendar et al. 1999), as well as to study genetic diversification in vegetatively propagated crop species (Bretó 2001).

Until recently, very little was known about retrotransposons in grapevine. *Vitis vinifera* has a diploid chromosomal number of $2n = 38$ and an approximate genome size of 500 Mb (Tomkins et al. 2001; Thomas et al. 2003). This is considered a medium sized genome, comparable to that of rice. Sequences isolated from a BAC library constructed from the cultivar Syrah analyzed against the SWISS-PROT database showed 41% to be retroelement related (Tomkins et al. 2001). Considering this high proportion of retroelements in the *Vitis* genome, it is surprising that the first complete retrotransposon sequence isolated in Grapevine, named *Gret1* (Grape retrotransposon 1), was only published in May 2004 (Kobayashi et al. 2004) *Gret1* is a *gypsy*-like LTR retrotransposon and was isolated as a retrotransposon induced mutation of *VvmybA1*, a gene affecting grape skin color. Previous to the characterization of *Gret1*, only a partial sequence for an LTR type retrotransposon, *Vine-1*, had been isolated from grapevine. *Vine-1* was found to be inserted into the fourth exon of *Adhr* gene from the *Adh* gene family in various grapevine cultivars (Verriès et al. 2000). Interestingly, although *Gret1* is a *gypsy*-type retrotransposon and *Vine-1* a *copia*-type, both were isolated from genomic regions directly upstream or inside coding sequences. Retrotransposons may therefore have a significant role in gene expression and phenotypic variation in grapevine.

Vitis vinifera is an agro-economically important fruit crop grown worldwide and particularly in Portugal, where a significant effort by the Portuguese Network for Vine Selection has resulted in the conservation and analysis of a number of experimental populations of clones from traditional Portuguese cultivars. These populations have been extremely valuable for the selection of superior phenotypes as well as the maintenance of the genetic variability that defines important Portuguese cultivars (Martins et al. 2002). Quantitative genetic studies have shown significant differences between clones in a number of wine associated phenotypes including fruit yield, acidity and anthocyanin concentration (Martins et al. 2002). There are approximately 300 distinct Portuguese cultivars traditionally used for winemaking, currently kept in the National Ampelographic Collection (J.E.J. Eiras-Dias, personal communication). Extensive studies with microsatellites have shown a large amount of genetic polymorphism between Portuguese cultivars (Sefc et al. 2000, Pinto-Carnide et al. 2003). In this work, we use four Portuguese cultivars, half of which are red wine producers. Included is Touriga Nacional, an important and well-studied Portuguese cultivar, for which we also observe clones. Combined with fluorescent in situ hybridization, we utilize molecular markers based on the LTRs of *Gret1* and analyze cultivars and clones by REMAP and IRAP. Our results show that *Gret1* is distributed throughout the *Vitis* genome and has multiple sites of repeated insertions. Specific REMAP and IRAP banding profiles indicate that *Gret1* insertion sites are cultivar specific. Taken together, our molecular and cytological results indicate that this retrotransposon may have a significant role in gene expression and phenotypic variability in grapevine.

Materials and methods

Plant material and DNA extraction

Vitis vinifera L. canes were collected from a number of experimental populations throughout Northern, Central and Southern Portugal, and kept at 4°C. One clone of cultivars Castelão, Cerceal Branco, Loureiro, and four Touriga Nacional clones (T0103, T1718, T3811 and T4236) were used in this study. For leaf and root material, canes were cut into approximately 20 cm shoots, carefully washed in 3% bleach, placed in water, and kept at a 16-h light (25°C)/8 h dark (20°C) cycle. For fluorescent in situ hybridization (FISH), root tips were collected, washed, fixed in ethanol/acetic acid (3:1 vol/vol) for 24 h, and stored at −20°C until use. To isolate genomic DNA, young leafs (<2 cm diameter) were collected, frozen in liquid nitrogen, and either used immediately or kept at −80°C until use. Frozen leaves (1 g) were ground to a fine powder in liquid nitrogen and DNA was extracted as previously described (Thomas et al. 1993). Quantification by 1% agarose gel electrophoresis showed sample yields to be between 50 μg/g and 100 μg/g FW.

Preparation of probe for the reverse transcriptase gene domain of *Gret1*

In order to construct the probe for Southern Blotting and FISH, the amino acid sequences of both *Gret1 gag-pol* precursor genes (accession AB111100) were compared to those of other published *gypsy*-type *gag-pol* precursors. The second ORF, at bases 8020–11103 of the entire retroelement was found to contain aa sequences most similar to those used to design degenerate primers for the RT gene domain in *gypsy*-like retrotransposons of rice (accession AB033235.1), Arabidopsis (accession AB033259) and maize (accession AF466202.2). Primers were therefore designed to amplify 417 nucleotides of the RT domain of the second *gag-pol* precursor (nucleotides 8236–8652 of *Gret1*, accession AB111100). The

sequences of the forward and reverse primers are shown in Table 1. Genomic DNA from Touriga Nacional clone T4236 was used and PCR was performed using primers *Gret1*-RT-Forward and *Gret1*-RT-Reverse as described by Friesen et al. (2001). The PCR products were separated by 1% gel electrophoresis, gel purified and sequenced in both directions with the above primers (Automatic Sequencer ABI3700, Applied Biosystems).

Southern blotting

Genomic DNA (5 μg) from each cultivar and clone was digested with *Bam*HI or *Bst*NI, according to the manufacturers' instructions (NewEngland Biolabs). Digested DNA was separated by 1% gel electrophoresis and alkaline transfered to Hybond N+ (Amersham). The Reverse Transcriptase gene domain of *Gret1* was prepared as described above and used at a concentration of 5 ng/cm^2 of blot. Southern Blotting was performed by the non-radioactive chemiluminescence method, ECL (Amersham) and the manufacturers' instructions were followed for probe labeling, hybridization and subsequent detection.

Fluorescence in situ hybridization (FISH)

Chromosome spreads and FISH were performed as previously described (Jones and Heslop-Harrison 2000), with the following modifications. Fixed root tips were digested with pectinase/cellulase in 1xEB for 5 h at 37°C, and chromosome squashes were performed in 60% glacial acetic acid. All four cultivars and clones were probed with the rDNA probe pTa71 as well as with the probe corresponding to the *Gret1* RT gene domain. The pTa71 probe is a 9 kb *Eco*RI fragment of the ribosomal DNA from wheat (*Triticum aestivum*). This probe was labeled with biotin-dUTP using a nick translation kit and conditions recommended by the supplier (Roche Applied Science). The probe for the RT gene domain of *Gret1* was prepared by incorporating digoxigenin-dUTP in the PCR reaction as instructed (Roche Applied Science). For FISH, 100–200 ng of each probe was used in the hybridization mixture. Digoxigenin-labeled probes were detected with anti-digoxygenin-fluorescein (Roche Applied Science) and biotin-labeled probes with Cy3-streptavidin (Sigma). Chromosomes were counterstained with 4′, 6-diamidino-2-phenylindole hydrochloride (DAPI) in Citifluor antifade mounting medium (AF1; Agar Scientific, Stansted, UK). Epifluorescence microscopy (Zeiss Axioskop 2) images were obtained by using a Zeiss AxioCam digital camera. Digital images were composed by using PHOTOSHOP (Adobe Systems, Mountain View, CA, USA).

REMAP and IRAP

The REMAP and IRAP were performed and compared between cultivars as well as Touriga Nacional clones. For this purpose, the 5′ and 3′ LTRs of *Gret1* (accession AB111100) were compared and primers designed to amplify in one direction of the insertion site of a complete *Gret1* retrotransposon or a solo *Gret1* LTR with primer *Gret1*LTR-reverse (inverse complementary of bases 4673–4700 of Gret1, or 2–29 of 5' LTR) and in the opposite direction for primer *Gret1*LTR-forward (bases 14912–14940 of *Gret1*, or 643–671 3 LTR). Two anchored primers were used for microsatellites based on the $(GA)_n$ and $(CT)_n$ dinucleotide repeats,as previously reported (Kalendar et al. 1999). Primer sequences are shown in Table 1. IRAP and REMAP PCR were performed in a 20 μl reaction mixture containing 20 μg of genomic DNA as previously described (Kalendar et al. 1999). All five primer combinations were used (*Gret1*LTR-reverse/Microsat-GA, *Gret1*LTR-reverse/Microsat-CT, *Gret1*LTR-forward/Microsat-GA, *Gret1*LTR-forward/Microsat-CT for REMAP, and *Gret1*LTR-reverse/ *Gret1*LTR-forward for IRAP). PCR products were separated by 1.5% agarose gel electrophoresis and detected by Ethidium Bromide staining.

Results

Gret1 RT gene domain

PCR with *Gret1*-RT-Forward and *Gret1*-RT-Reverse primers (Table 1) amplified a band from Touriga Nacional of approximately 400 bp as seen by gel electrophoresis. The purified product was sequenced using both forward and reverse primers, resulting in a 417 base pair fragment that showed complete sequence identity to

Table 1 Primer names and sequences

Primer	Sequence
Gret1 reverse transcriptase gene domain	
Gret1-RT-forward	5′-CGAGTTTGTGTAGATTACAC
Gret1-RT-reverse	5′-GCATTTAGAAGGATTTAGCTT
REMAP and IRAP	
*Gret1*LTR-reverse	5′-CGTGTTTCCCAGAGGGAGGGGTCCCTAC
*Gret1*LTR-forward	5′-CAACTAATTTATATTCC TCTCTCTAACCA
Microsat-GA	$(GA)_9C$
Microsat-CT	$(CT)_9G$

nucleotides 8236–8652 of the published *Gret1* sequence (accession AB111100). The amplified sequence therefore corresponded to the *Gret1* RT gene domain.

Genomic distribution of *Gret1* in *V. vinifera* cultivars and clones

Blots with restriction digested genomic DNA from the four cultivars and two T. Nacional clones probed with the 417 bp DNA fragment corresponding to the *Gret1* RT gene domain showed that this retroelement is significantly abundant in *Vitis* genome. This is evident as a smear from high to low molecular weight (Fig. 1). Southern Blots were repeated a number of times, with various amounts of genomic DNA. At high concentrations, the signal became a dark smear, and the bands became indistinguishable. At low concentrations, the signal disappeared completely except for the observed bands in the blot shown. There are some distinguishable high molecular weight bands when *Vitis* DNA is digested with restriction enzyme *Bam*HI. Although this endonuclease does not cut within the published coding *Gret1* sequence, there is one recognitions site in the 5' LTR and another in the 3'LTR for in the complete 10,440 bp sequence, resulting in a 9,598 bp fragment. The strong probe signal at the top of each lane indicates that there is a significant amount of large genomic DNA fragments containing the Gret1 RT gene domain in all four cultivars examined. The *Bam*HI recognition sites in the LTRs of the published sequence may therefore not be conserved in these cultivars. However, it is more likely that one or both of these recognition sites are absent in a significant portion of the total number of *Gret1* copies in the genomes of these cultivars. Enzyme *Bst*NI is a methylation insensitive isoschizomer of *Eco*RII. There are eight recognition sites for this endonuclease in the published sequence of the *Gret1 gag-pol* precursor genes, two of which should generate a 965 bp fragment that encompasses the *Gret1* RT probe used in this study. Accordingly, our results show the presence of a band approximately 950 bp in length for all the cultivars and clones analyzed. Interestingly, there are no visible higher molecular weight bands in the *Bst*NI digested DNA. This indicates that there is selection against changes in the RT coding region of *Gret1*. There is also a slightly smaller less intense band in all the cultivars, indicating that there may be a less abundant variant of *Gret1*.

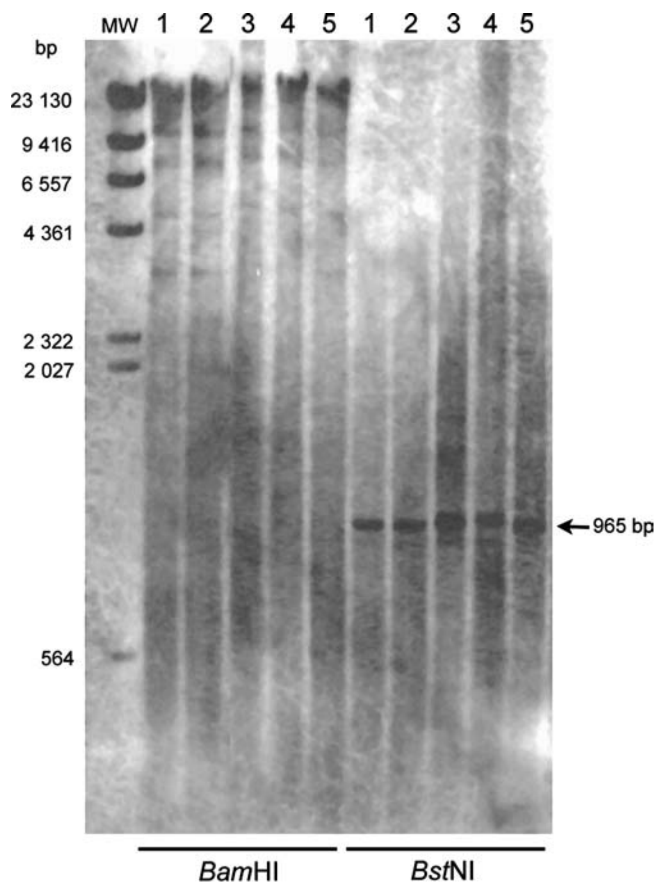

Fig. 1 Southern Blotting showing the genomic distribution of *Gret1* in *Vitis vinifera* cultivars and two Touriga Nacional clones. Molecular weight marker (MW, λ *Hin*dIII) with band sizes in base pairs (bp) is shown. Total genomic DNA (5 μg) of Touriga Nacional clones T0103 (*1*), T4236 (*2*), and cultivars Castelão (*3*), Cerceal Branco (*4*) and Loureiro (*5*) was digested with *Bam*HI or *Bst*NI and hybridized with a probe for the RT domain of *Gret1*. Southern with *Bam*HI digested DNA shows some high molecular weight bands as well as a continuous smear. Southern with *Bst*NI digested DNA shows the expected 965 bp fragment containing the *Gret1* RT domain, a less abundant slightly smaller band, and a smear. These results indicate a widespread *Gret1* distribution throughout the *Vitis* genome

Chromosome distribution of *Gret1 loci* in *V. vinifera*

In situ hybridization using the rDNA probe pTa71 showed 4 loci for the large rDNA repeat unit containing the 18S, 25S and 5.8S genes, as previously reported (Haas and Alleweldt 2000), (Fig. 2a, b). There were no measurable differences between cultivars or clones for *Gret1* labeling, evident as numerous signals detected throughout interphase (Fig. 2a) and metaphase (Fig. 2b) cells. At any focal point, 15–20 sites of *Gret1* hybridization were detected in all the cultivars and clones analyzed. *Gret1* was detected in at least half of the chromosomes. The observed *Gret1 loci* should correspond to insertion sites where this retroelement is present in multiple copies, since the hybridization of one single 417 bp probe would not emit sufficient fluorescence to be detected. Interestingly, all *Gret1* labeling sites are outside the heterochromatic chromosome domains, evident by the clear lack of overlap of Gret1 signals and the strong DAPI staining of heterochromatic domains.

Molecular analysis of *V. vinifera* cultivars and clones using REMAP and IRAP

The REMAP and IRAP profiles were compared between Touriga Nacional clones (Fig. 3) as well as between the four cultivars used in this study (Fig. 4).

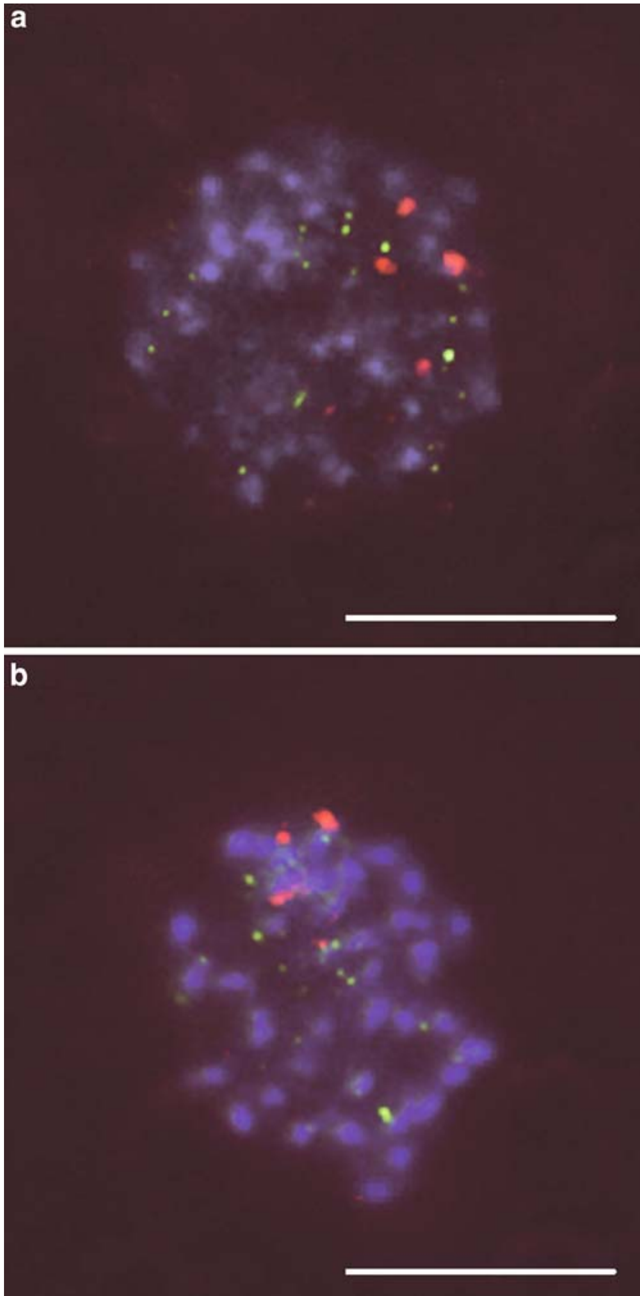

Fig. 2 Root-tip interphase (**a**) and metaphase (**b**) cells from Touriga Nacional clone T4236 after in situ hybridization with rDNA probe pTa71 (from wheat) and the *Gret1* RT domain. FISH and DAPI signals are superimposed. The four NORs (*red signals*) as well as a number of *Gret1 loci* (*green signals*) are visible in both cells. All *Gret1* labeling sites are outside the heterochromatic chromosome domains, distinguished by the characteristic strong DAPI staining. *Bar* 10 μm in (**a**) or 5 μm in (**b**)

REMAP amplified genomic regions where *Gret1* LTRs were flanked by microsatellites with proximities between 100 bp and approximately 2 kb. IRAP amplified genomic regions between two LTRs lying between 400 bp and 1.7 kb appart. Profile complexity ranged between one and 11 bands per reaction, with an overall average of 5 bands. All five IRAP and REMAP reactions amplified bands with various intensities in the four cultivars used in this study. There were no differences in REMAP and IRAP amplified bands between Touriga Nacional clones (Fig. 3). Although only three clones are shown here, these results were verified and repeated a number of times for a total of eight Touriga Nacional clones (not shown). On the other hand, both REMAP and IRAP showed distinct profiles for the four cultivars. All four cultivars were polymorphic for each the five primer combinations used (Fig. 4). These techniques did not allow for the disctimination between white (Cerceal Branco and Loureiro) and dark cultivars (Castelão and Touriga Nacional).

Discussion

The *gypsy*-type retrotransposon, *Gret1*, is the first complete retrotransposon sequence identified in *V. vinifera*. This element was isolated as a retrotransposon induced mutation of *VvmybA1*, a gene affecting grape skin color in most if not all grapevine cultivars in the world (Kobayashi et al. 2004). The authors hypothesize that *Gret1* inserted upstream of the *VvmybA1* coding sequence in a dark skinned ancestor and then a white skinned grape was produced. Some black-skinned *V. vinifera* cultivars still show a solo *Gret1* LTR upstream of the *VvmybA1* gene, suggesting that these cultivars were derived from white skinned progenitors by a reversion event involving recombination between LTRs. The two *Gret1* LTRs from the isolated clone differ at only four nucleotides, indicating a relatively recent insertion event (Kobayashi et al. 2004). Until the present study, there was no information regarding the genomic distribution of retrotransposons in grapevine. Here, we show that *Gret1* is abundant in grapevine, as seen by the large molecular weight bands in *Bam*HI digested DNA as well as the smears obtained by southern blotting. The smears also suggest that this retrotransposon is dispersed throughout the genome. Widely dispersed copies of *Gret1* will not be detected by in situ with the probe used in this study. Our in situ results therefore indicate numerous *loci* where *Gret1* is repeatedly inserted, as seen by multiple foci present in the euchromatic regions of at least half of the chromosomes. Due to the small size of grapevine chromosomes and the number of *loci* labeled by in situ, this retrotransposon is likely of limited use for linkage analysis in grapevine. The rDNA probe used as a control recognizes the 45S large repeat unit, in which the18S, 25S and 5.8S genes and intergenic sequences are arranged in tandem. In *V. vinifera*, this sequence has approximately 12 kb and has been estimated to be repeated approximately 2500 times in the diploid genome (Thomas et al. 1993). The dimension of the ribosomal probe is therefore comparable to that of the published *Gret1* sequence, which is 10.426 kb. Examining the size of the FISH signals generated by the Gret1 probe, we can conclude that this retrotransposon is present in a low to moderate number of copies at each *loci*.

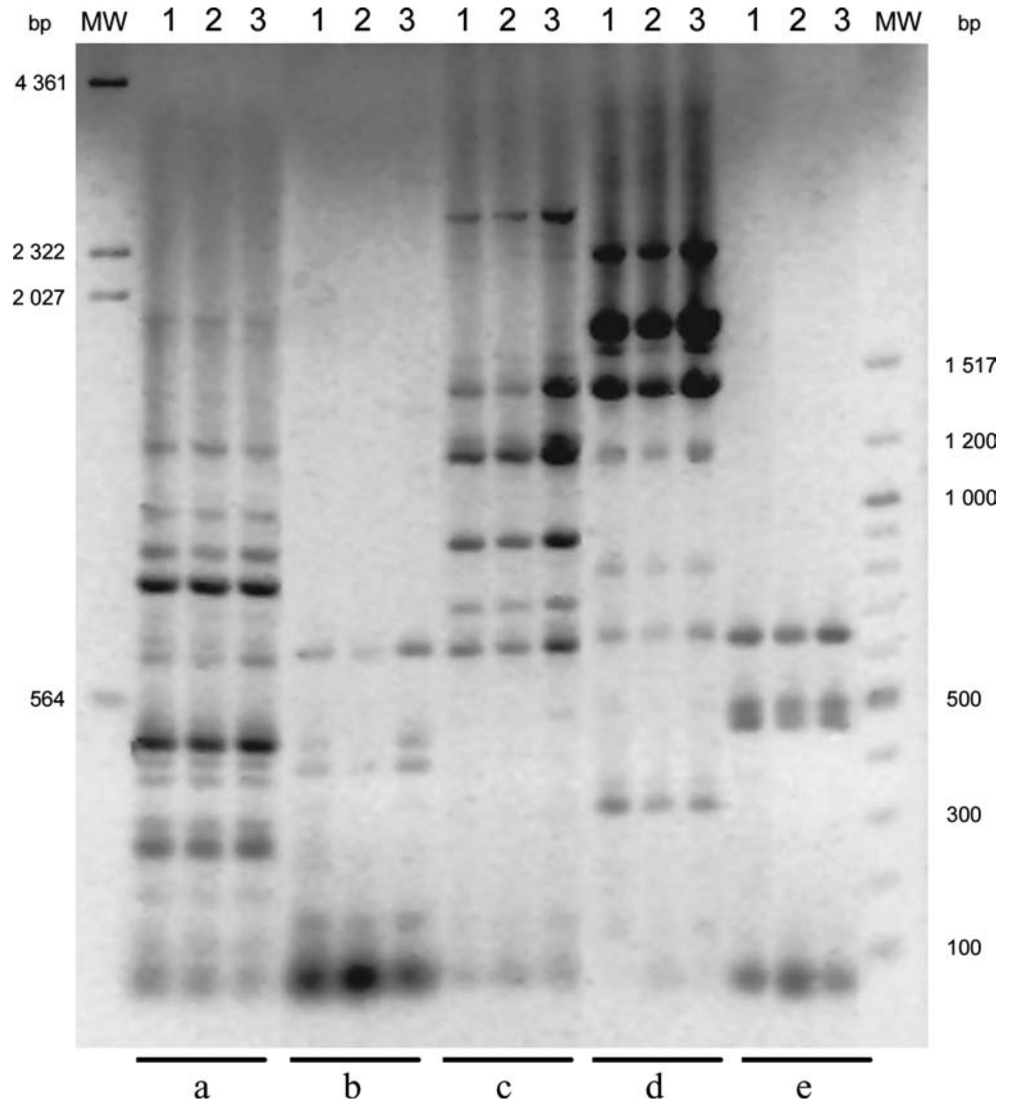

Fig. 3 Molecular characterization of Touriga Nacional clones using REMAP and IRAP. Molecular weight markers (MW, λ *Hin*dIII on the left and 100 bp DNA ladder on the *right*) with band sizes in base pairs are shown. Genomic DNA from Touriga Nacional clones T0103 (*1*), T1718 (*2*), T3811 (*3*) was used for REMAP and IRAP. The following primer combinations were analyzed: *Gret1*LTR-reverse/ Microsat-GA (**a**), *Gret1*LTR-reverse/ Microsat-CT (**b**), *Gret1*LTR-forward/Microsat-GA (**c**), *Gret1*LTR-forward/ Microsat-CT (**d**), and *Gret1*LTR-reverse/ *Gret1*LTR-forward (**e**). There were no differences in REMAP and IRAP profiles between T. Nacional clones

The only previous knowledge of the abundance of retrotransposons in the *Vitis* genome was that 45% of genomic sequences were retroelement related when compared to a protein database (Tomkins et al. 2001). This percentage may be overestimated due to sample size or underestimated due to lack of information of expressed or retroelement related *Vitis* sequences in the databases. Nevertheless, the results obtained here taken together with the above study indicate that retrotransposons are probably more abundant in *Vitis* than in rice, which has a similar sized genome. Mobile DNA elements such as retrotransposons have an important role in the genomes of plants and animals. From an evolutionary perspective, these elements are drivers of genomic modifications, a process essential for natural selection (Kazazian 2004). Additionally, retrotransposons are involved in important processes affecting gene regulation, and may have played a role in the evolution of RNA interference, an important gene silencing mechanism (Hodgetts 2004). Constraints imposed on mobile elements by the host genome are specific to the type of element, likely indicating specific evolutionary imposed functional constraints on each element type or family. Interestingly, all *Gret1 loci* observed in this study by in situ hybridization were found to lie in euchromatic, or gene rich, regions of chromosomes.

The in situ results indicating that *Gret1* is clustered in a number of *loci* would lead us to expect REMAP and IRAP to produce complex banding profiles. There are a number of possible explanations for the relatively few visible bands obtained by these techniques. One possibility is that the sites of repeated insertion represent genomic regions where *Gret1* is inserted in a tandem, evenly distributed fashion. This would not only explain the few bands obtained by IRAP but also the variation in their intensities. Tandem retrotransposon distribution has been previously reported for a number of *gypsy*-like elements (Fukui et al. 2001; Sanz-Alferez et al. 2003).. On the other hand, the in situ foci may represent sites where *Gret1* is clustered in a nested fashion as has previously been reported in maize (SanMiguel et al. 1996), thereby not detectable by IRAP or REMAP. Another

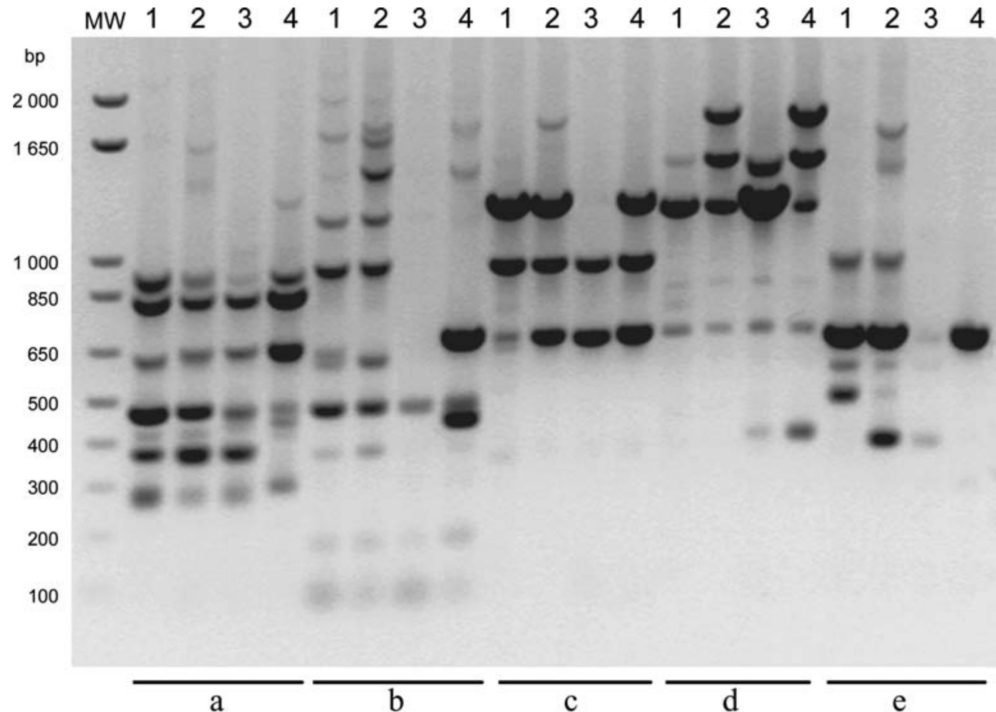

Fig. 4 Molecular characterization of *V. vinifera* cultivars using REMAP and IRAP. Molecular weight marker (MW, 1 kb^{+} DNA ladder) with band sizes in base pairs is shown. Genomic DNA from Castelão (*1*), Cerceal Branco (*2*), Loureiro (*3*), and Touriga Nacional (*4*) was used for REMAP and IRAP. The following primer combinations were analyzed: *Gret1*LTR-reverse/Microsat-GA (**a**), *Gret1*LTR-reverse/ Microsat-CT (**b**), *Gret1*LTR-forward/Microsat-GA (**c**), *Gret1*LTR-forward/ Microsat-CT (**d**), and *Gret1*LTR-reverse/ *Gret1*LTR-forward (**e**). All four cultivars showed different REMAP and IRAP profiles for each primer combination

explanation could be that the *Gret1 loci* detected by FISH represent incomplete retrotransposons, missing part or the whole LTR sequence, and therefore not detectable by IRAP or REMAP. Finally, the distance between *Gret1* copies in these clusters or between *Gret1* and the microsatellite loci used in this study may be too far apart to be amplified by conventional PCR.

There is an enormous amount of genetic variability between grapevine cultivars, measurable by numerous and complex ampelographic and organoleptic characteristics. Many important cultivars have been ''fingerprinted'' and there are a number of extensive databases with microsatellite profiles for cultivars from all over the world. In Portugal, there are over 300 cultivars that can be used for wine production, and a number of large populations of clones for some traditional cultivars. It has been shown that microsatellite profiles can distinguish between Portuguese cultivars but not clones of the same cultivar (Sefc et al. 2000; Pinto-Carnide et al. 2003). Although microsatellite markers have been extremely useful to identify cultivars, a number of *loci* need to be used to obtain sufficient discriminating power. Also, microsatellite data is not amenable to the calculation of genetic distances between cultivars. The evolutionary processes that gave rise to the huge variability between grapevine cultivars have therefore not been analyzed. Here, we show that the retrotransposon *Gret1* can be used to develop molecular markers that distinguish cultivars. Our REMAP and IRAP results show that, like with microsatellites, these techniques can identify cultivars but not clones. However, REMAPs and IRAPs amplify more than one *locus* simultaneously. Furthermore, these methodologies are straightforward to perform and cost efficient. Perhaps more importantly, the resulting patterns are sufficiently complex to calculate genetic distance between cultivars and therefore make evolutionary inferences. In this study we used two anchored primers for microsatellite *loci*, but this number can be greatly increased to provide more discriminating power. This will be imperative as sample size increases as well as to study closer genotypes. From a molecular perspective, our PCR results indicate that there are differences in *Gret1* genomic distribution between cultivars but not within cultivars. The mobility constraint on this element appears therefore to be cultivar specific. The molecular data showing widespread *Gret1* distribution and differences in IRAP and REMAP between cultivars, taken together with the in situ results showing sites of repeated insertions in gene rich regions, indicate that *Gret1* may have a role in defining genome structure and function in grapevine.

In conclusion, this work shows that *Gret1* is useful as a molecular marker, and that this retrotransposon may play an important role in the expression of phenotypes that characterize a cultivar. As more information on *Vitis* genomics becomes available, the abundance and importance of retrotransposons as well as other mobile DNA elements in grapevine will be further elucidated. Future studies involving the characterization of *Gret1* as well as other mobile elements in grapevine will be essential to understand the phenotypic and genotypic variability observable between cultivars. Of interest here is the recent revival of a 35-year-old model which proposed that expression of numerous genes of similar function to be integrated by members of a dispersed repeat family (Britten and Davidson 1969, Hodgetts

2004), such as *Gret1*. Due to its dispersed distribution throughout the genome and multiple sites of repeated insertions in gene rich chromosomal regions, *Gret1* may also prove useful as a tool to isolate and characterize coding sequences in grapevine. Finally, sequence analysis and expression profiles of genes flanking retrotransposons will aid in determining the functional implications of the differences in *Gret1* insertion events observed between cultivars.

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