Scientia Horticulturae 244 (2019) 263–269

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Clonal propagation of cv. Italy grapes and the generation of genetic divergence among vineyards

Danuza Kelly Strioto[a], Afonso Carrasco Pepineli[a], Tauana Gibim Eisele[a], Giovana Carniatto Marinelli[a], Claudete A. Mangolin[b], Liriana Belizário Cantagali[b], Sandra A. de Oliveira-Collet[b], Maria de Fátima P.S. Machado[b,*]

[a] State University of Maringá, Maringá, PR, Brazil
[b] Department of Biotechnology, Genetics and Cell Biology, State University of Maringá, Maringá, PR, Brazil

ARTICLE INFO

Keywords:
Table grapes
Clonal polymorphism
Genetic divergence
Genetic identity

ABSTRACT

The genetic structure of vineyards with cv. Italy grapes, multiplied and maintained by vegetative propagation for more than fifty years in the region of Marialva, Brazil, was analyzed. Polymorphisms in 13 microsatellite loci and 14 ISSR markers were analyzed to estimate the genetic diversity indices of 57 plants obtained from four vineyards (V1 – V4). Higher genetic uniformity was detected by microsatellite and ISSR markers in V1 than in V2, V3, and V4 vineyards. Polymorphisms in microsatellite loci revealed only one somatic mutation at the molecular level. However, beyond the casual selection of samples for the establishment of new vineyards, propagation of this somatic mutation caused genetic divergence between the vineyards. Two molecular markers indicated moderate genetic divergence among the four vineyards with cv. Italy grapes. Polymorphisms in SSR loci and ISSR markers showed that clonal propagation of cv. Italy grapes may generate genetic divergence among vineyards.

1. Introduction

Somatic mutations during plant growth, deletions, retrotransposon activity, periclinal chimera formation, somatic recombination, epigenetic variations, and variable numbers of replications in microsatellite sequences are events caused by genetic diversity during the clonal propagation of grapes (Forneck, 2005). Since grape vines are perennial plants that may undergo stress during a long productive period, one or more of the above events are likely to occur. Different environmental stress conditions, such as pruning, pathogen attack, harsh climatic conditions or ultraviolet irradiation, enhance the occurrence of spontaneous somatic mutations, induce retrotransposon action and generate clonal polymorphisms and genetic diversity (review in Pelsy, 2010).

Somatic mutations in 17% of the microsatellite loci were detected after screening large numbers of loci and grapevine clones (Riaz et al., 2002). High polymorphism (22.4%) was detected in clones of the Pinot cultivar (Hocquigny et al., 2004) and Cabernet Sauvignon (21.4%; Moncada et al., 2006), while a low rate of polymorphism in microsatellite loci (10%) has been described by Moncada and Hinrichsen (2007) in their analysis of 20 Loci of 25 clones of Carmenére from Chile, France and Italy. Clonal polymorphisms in grapes have been a source of genetic variation used by breeders to improve the phenotypic features of classical cultivars (This et al., 2006). Investigating clonal polymorphisms and determining how grapevines are genetically structured are important in guiding cultural practices and in assessing the potential of vineyards to generate new cultivars.

Microsatellites are adequate molecular markers to evaluate polymorphisms and genetic diversity in plants because they are the loci of simple sequence repeats (SSRs) occurring in tandem and are abundantly distributed in coding or noncoding regions of plant genomes (Kalia et al., 2011). In addition to their high abundance in organisms, SSR loci have codominant inheritance (Litt and Luty, 1989); thus, SSR expansion or contraction may trigger the appearance of new alleles. In fact, new alleles may be the result of events caused by genetic diversity during clonal propagation. In addition, when SSR loci are in the coding regions of the plant genome, the alterations may ultimately lead to phenotypic changes (Li et al., 2004).

Primers for intersimple sequence repeats of DNA (ISSR markers) are also adequate molecular markers to evaluate genetic diversity in plants. ISSRs are DNA sequences of 100–3000 bp that are amplified by PCR using a single primer (16–20 bp) constructed from microsatellite sequences. Although ISSRs are dominant molecular markers, several

* Corresponding author.
E-mail address: mfpsmachado@uem.br (M.d.F.P.S. Machado).

https://doi.org/10.1016/j.scienta.2018.09.049
Received 17 July 2018; Received in revised form 12 September 2018; Accepted 18 September 2018

informative sequences are generated using larger and specific ISSR primers (more than 10 bp), with larger anchorage regions for SSR loci; highrepeatability in the pattern of amplified DNA segments is obtained with specific ISSR primers (Tsumura et al., 1996). ISSR primers have been used to discriminate different grape cultivars (Herrera et al., 2002; Argade et al., 2009).

The current study focuses on the polymorphisms in microsatellite loci and in ISSRs in vineyards with cv. Italy (*Vitis vinifera* L.) from different producers. The cultivar Italy (VIVC 5582) was originally a hybrid from the cross between Bicane × Muscat Hamburg introduced in Brazil in 1927. Culture started in the rural region of Marialva, a town in the northeastern region of the state of Paraná, Brazil, in 1962 (Camargo, 1998). With the cultivation of cv. Italy and the clonal propagation of its colored varieties, the town of Marialva became renowned as "the Brazilian capital of fine table grapes". During more than fifty years of vegetative propagation (1962–2013), somatic mutations or other events caused by clonal polymorphisms in cv. Italy vineyards generated phenotypic variants that were used as the sources of four new cultivars [cv. Rubi (Kishino and Mashima, 1980), cv. Benitaka (Sousa, 1996), cv. Redmeire (Pires et al., 2003) and cv. Haruna (Assis et al., 2013)]. The number of phenotypic variants (four) identified during 51 years of clonal propagation led to that hypothesis of high genetic stability in vineyards with cv. Italy. The objective of the present study was to evaluate clonal polymorphisms in vineyards of cv. Italy and the genetic divergence among clones employing microsatellites and ISSR markers. The assay reveals the genetic structure of vineyards with cv. Italy, multiplied and maintained by vegetative propagation for more than fifty years in the region of Marialva, Brazil.

2. Materials and methods

2.1. Samples of cv. Italy

In 2013, DNA was extracted from 57 plants obtained from four vineyards (V1 – V4) in the rural region of Marialva, PR Brazil (Table 1). The four vineyards were installed in the late 1990s, and the distance between them ranged between 2 km (V1 and V2) and 11 km(V1 and V3). The distance between V2 and V3 is greater than 10 km, and the distances between V1 and V4, V2 and V4, and V3 and V4 are greater than 6 and smaller than 7 km. Partially expanded leaves that were free of contaminants were collected from 12 to 16 plants (three leaves per each plant) from each vineyard (Table 1). Samples were individually stored in labeled plastic screen bags to avoid mixing of vineyards, maintained in ice (4 °C), transferred to the laboratory, frozen in liquid nitrogen and stored at −80 °C until DNA extraction.

2.2. DNA extraction

DNA was extracted from leaf tissues following the method described by Thomas et al. (1994), with minor modifications, which included the use of 100 mg of leaves from individual plants instead of 2.0 g of leaves. Samples were centrifuged for 10 min at 3000 rpm at room temperature (≈22 °C). After DNA extraction, DNA quantification was performed using a Picodrop Spectrophotometer (Pico 100 – Version 4.0/21/03/11). DNA concentrations averaged between 33 ng μL^{-1} and 450 ng μL^{-1} per sample. After quantification, DNA samples were diluted to a concentration of 10 ng μL^{-1}.

Table 1
Location of the vineyards (geographic coordinates) and number of plants from cv. Italy analysed.

Vineyards	Number of plants	Geographic coordinates
V1	16	23º 30'56" S / 51º47'57" W
V2	12	23º30'32" S / 51º49'01" W
V3	14	23º24'49" S / 51º48'54" W
V4	15	23º27'37" S / 51º46'28" W

2.3. Amplification reactions using microsatellite primers

Thirteen SSR primers that were previously developed for *Vitis vinifera* and defined as polymorphic in cv. Italy were used with the DNA samples. The primers VVS (Thomas and Scott, 1993), VVMD (Bowers et al., 1996; 1999), SCU (Scott et al., 2000), and UDV (Di Gaspero et al., 2005) were defined as polymorphic primers (UDV01, UDV11, UDV32, UDV34, UDV85, UDV96, VMC4A1, VMC4D4, VMC4C6, VVMD5, SCU08VV, SCU10VV, and SCU15VV).

Polymerase chain reaction (PCR) was performed with a Techne TC-512 thermal cycler. Amplifications were performed using the Touchdown PCR program (Don et al., 1991) with a total volume of 20 µL containing 25 ng of genomic DNA; reaction buffer (10 mM Tris − HCl, pH 8.8); 2.0 mM $MgCl_2$; 0.1 mM each of dATP, dGTP, dCTP, and dTTP; 0.3 µM each primer (F and R primers); 1 unit of Taq Polymerase Platinum (Invitrogen); and Milli-Q water to bring the reaction to the final volume. DNA amplification by primers UDV85, UDV96 and VMC4C6 occurred with initial denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, with annealing at 54 °C for 30 s and extension at 72 °C for 60 s. After these steps, a final extension was carried out at 72 °C for 10 min. Electrophoresis was performed with a 4% MS-8 agar gel and 0.5x TBE buffer (44.5 mM Tris-borate and 1 mM EDTA) at 60 V for 4 h. After electrophoresis, the gels were stained with ethidium bromide at 0.5 µg mL^{-1}, and images were taken with a Molecular Image LOCCUS L-PIX - HE by Picasa 3. The size of the PCR alleles was determined with a 100 bp DNA Ladder (Invitrogen).

Polymorphisms from SSR loci were analyzed with POPGENE 1.32 (Yeh et al., 1999) to estimate the average number of alleles per locus, the allele frequency in each locus and the genetic divergence between cv. Italy vineyards. Analysis of molecular variance (AMOVA, GenAlEx 6.2; Peakall and Smouse, 2006) explored the hierarchical partitioning of genetic variation within and between the four vineyards of cv. Italy.

2.4. Amplification reactions using intersimple sequence repeat primers

Four ISSR primers (ISSR-2, ISSR-5, ISSR-6, and ISSR-8) previously selected and defined as polymorphic in cv. Italy (Pepineli et al., 2014) and 10 other ISSR primers (ISSR-18, ISSR-19, ISSR-20, ISSR-21, ISSR-23, ISSR-807, ISSR-811, ISSR-813, ISSR-822, and ISSR-825) selected in the current study were used with the DNA samples. DNA amplification using the fourteen primers occurred with initial denaturation at 94 °C for 5 min, followed by 34 cycles at 94 °C for 1 min, with annealing at 49.5–51.5 °C for 1 min and extension at 72 °C for 1 min and 30 s. After these steps, a final extension was carried out at 72 °C for 7 min. Polymerase chain reaction (PCR) was performed with a Techne TC-512 thermal cycler. Amplifications were performed using volumes of 20 µL containing 15 ng of genomic DNA; reaction buffer (10 mM Tris − HCl, pH 8.8); 2.4 mM $MgCl_2$; 0.1 mM each of dATP, dGTP, dCTP, and dTTP; 0.25 µM primer; 1 unit of Taq Polymerase Platinum (Invitrogen); and Milli-Q water to bring the reaction to the final volume. Electrophoresis was performed with a 1.5% agar gel and 0.5x TBE buffer (44.5 mM Tris-borate and 1 mM EDTA) at 60 V for 4 h. After electrophoresis, the gels were stained with ethidium bromide at 0.5 µg mL^{-1}, and images were taken with a Molecular Image LOCCUS L-PIX - HE by Picasa 3. The size of the PCR fragments was determined with a 1 kb DNA Ladder (Invitrogen).

Polymorphisms from ISSR loci were analyzed as dominant markers [(1) presence and (0) absence of amplified DNA segments]. FreeTree software (Pavlícek et al., 1999) was used to perform bootstrap analyses to compare specimens between the four vineyards. The distance similarity matrix was computed with UPGMA (Sneath and Sokal, 1973), followed by Jaccard´s clustering method, with resampling analysis using 1000 replications. A dendrogram was constructed based on a