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Preliminary Clonal Characterization of Malvasia volcanica and Listan prieto by Simple Sequence Repeat (SSR) Markers in Free-Phylloxera Volcanic Vineyards (Lanzarote and Fuerteventura (Canary Islands, Spain))

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CONTENTS

1 ABSTRACT

Climate change is usually recognized as the most significant challenge facing viticulture in the 21st century. As a result, experts are increasingly emphasizing the need to explore the biodiversity within the species *Vitis vinifera* L. In this context, the present study investigated the intra-varietal biodiversity of two widely cultivated grapevine varieties on the Canary Islands of Lanzarote and Fuerteventura (Spain). These islands, characterized by desert-like climates, strong winds, volcanic soils, and phylloxera-free conditions, have presented uninterrupted grapevine cultivation for the past three to five centuries. Intra-varietal variability was detected in 93.46% of the 107 accessions analyzed. The most divergent samples were a Malvasia Dubrovacka (LNZ-87) and a Listan prieto (FTV-8), each exhibiting five distinct variations. Another Listan prieto accession (FTV-13) showed four variations. A group of seven individuals displayed three variations, including two Malvasia volcanica accessions (LNZ-12, LNZ-72) and five Listan prieto accessions (FTV-1, FTV-2, FTV-7, FTV-9, FTV-12). A set of 100 SSR markers was used to analyze this grapevine collection, of which 17 revealed variability. The most informative markers were VChr15b, VVIp34, VVMD32, VChr9b, VVMD5, VVMD28, and VMC4F3, while the least informative was VVNTM1, which detected no variation. The parentage of Malvasia volcanica (Malvasia Dubrovacka × Bermejuela) was supported by all SSR markers, assuming that three of them may involve a mutated parent.

Keywords: *Vitis vinifera* L.; microsatellite; clones; intra-varietal variability; Malvasia Dubrovacka (Malvasia aromatica); Bermejuela (Marmajuelo); pedigree.

2 INTRODUCTION

The grapevine (*Vitis vinifera* L.) is one of the world's most economically and historically important crops [1,2]. In 2022, the global vineyard surface was approximately 7.3 million hectares, and wine production exceeded 258 million hectoliters. That same year, international wine trade generated an estimated value of approximately €37.6 billion [3].

There are about 6,000 to 10,000 different varieties (unique genotypes) of *Vitis vinifera* L., of which around 1,100 are commercial cultivars used exclusively for winemaking. Remarkably, only 12 of these account for nearly 80% of the total cultivated vineyard area (these 12 varieties which represent the 1% of total genetic variability are: Cabernet

Sauvignon, Chardonnay, Merlot, Pinot noir, Syrah, Sauvignon blanc, Riesling, Muscat à petits grains blanc, Gewürztraminer, Viognier, Pinot blanc, and Pinot gris). This genetic variability is unlikely to increase in the near future; in fact, it may continue to decline due to several factors. This reduction of varietal diversity (genetic erosion) is driven by several factors: market globalization, the standardization of commercial wines, legal frameworks and *Appellation d'Origine Contrôlée /Protégée* (AOC/AOP) and climate change [4]. Nevertheless, contemporary viticulture places increasing emphasis on clonal selection, that is, the propagation of specific genotypes within a variety that exhibit desirable characters such as higher yields, greater disease resistance, unique aromatic profiles, or improved tolerance to water stress [5]. This intra-varietal diversity arises primarily from somatic mutations that have accumulated over time and been vegetatively propagated from the original cultivar. Some of these mutations are clearly expressed, for example in pigment biosynthesis pathways, while others are more discreet and phenotypically indistinguishable [6].

2.1 MOLECULAR TECHNIQUES FOR CLONE DISTINCTION

To detect, differentiate and characterize clones within a single grapevine variety, a wide array of molecular techniques has been developed. Among these, microsatellite or Simple Sequence Repeat (SSR) markers continue being the primary tool for varietal identification in grapevine due to their high polymorphic information content, multiallelic, and the availability of standardized protocols [7,8]. However, the relatively low mutation rate and genetic uniformity observed among most clones limit their ability to detect intra-varietal differences, as demonstrated in diverse studies [7,9,10]. Subsequently, more sensitive approaches were introduced to detect polymorphisms potentially undetectable by SSR, such as Amplified Fragment Length Polymorphism (AFLP) and its variants [10–12]. The development of retrotransposon-based marker systems including Sequence Specific Amplified Polymorphism (S-SAP), Retrotransposon-Microsatellite Amplified Polymorphism (REMAP), and *Inter*-Retrotransposon Amplified Polymorphism (IRAP), has further enhanced the resolution of clonal differentiation by enabling the detection of polymorphisms associated with transposable DNA elements [11,13]. Nevertheless, these techniques (AFLP, S-SAP, REMAP, IRAP) rely on dominant markers, which exclude the distinction between homozygous and heterozygous individuals. Additionally, they require meticulous standardization to ensure reproducibility across laboratories. Consequently, in

comparison to SSR, these methods are generally considered less suitable for genetic analysis in grapevine [11,14,15]. It is also worth noting that other techniques, such as Methylation-Sensitive Amplified Polymorphism (MSAP), have revealed that some clones exhibit distinct DNA methylation patterns despite displaying identical SSR or AFLP profiles [7]. In the past decade, next-generation sequencing (NGS)-based techniques have emerged as the most effective approaches for clonal discrimination in grapevine: 1) Whole-genome resequencing (WGR) and whole-genome sequencing (WGS) techniques enable the detection of point mutations, insertions, deletions, and structural variants that are specific to each clone, although their high cost currently limits their widespread application [14–16]. For instance, Calderón et al. used WGR to identify 941 Single Nucleotide Polymorphisms (SNPs) across four Malbec lines, which enabled the development of a customized genotyping panel capable of classifying 214 accessions into different clonal lineages [14]. Similarly, Gambino et al. employed WGS to establish a set of unique single nucleotide variations (SNVs) that distinguished clones based on morphological and geographical characteristics [15]. Additionally, Araya-Ortega et al. applied WGS to detect variants among clones of Carménère and Merlot, identifying between 5,000 and 6,000 clonal variants and validating functionally relevant polymorphisms through bioinformatics tools [16]. 2) Genome reduction techniques such as double digest restriction-site associated DNA sequencing (ddRADseq) [7] and genotyping-by-sequencing (GBS) have offered a more cost-effective and scalable solution, enabling the identification of thousands of informative SNPs [17–19]. For example, Villano et al. applied GBS to analyze 17 clones of ‘Greco Bianco’, identifying 14 unique genotypes and demonstrating how this technique can reveal variability even within varieties traditionally considered genetically homogeneous [17]. Similarly, Procino et al. analyzed 35 clones of Primitivo using GBS and detected over 38,000 SNPs, which allowed for the differentiation of three genetic groups associated with their geographical origin and with morphological and agronomic traits [18]. These SNPs can later be selected to design customized genotyping panels that enable high-throughput and precise clonal identification [7]. Likewise, Esteras et al. applied GBS to clones of Muscat of Alexandria’, detecting intra-varietal genetic variability that had not been previously identified with traditional SSR markers, thereby demonstrating the utility of this technique in characterizing clones within widely cultivated varieties [19]. 3) High-throughput amplicon sequencing (AmpSeq) has also proven to be a reliable tool for validating clonal SNPs across multiple accessions, increasing both genetic resolution and

clonal traceability. In a recent study, Urra et al. used AmpSeq to genotype clones from four commercial varieties, identifying up to 34 stable clonal polymorphisms and proposing their use as functional markers in traceability and certification schemes [20]. 4) However, commercial SNP chips, which are designed for varietal-level differentiation, often fail to detect rare mutations among closely related clones, as illustrated by the case of an 18K SNP array that was unable to distinguish among intra-varietal biotypes [13]. To address this limitation, SNP panels adapted to specific variety are being developed through the resequencing of representative clones. For example, Calderón et al. identified 941 reliable SNPs in four historical Malbec lines and used these markers to develop a customized panel that effectively classified 214 Malbec accessions into distinct clonal lineages [14].

Although, as previously mentioned, standard SSR markers typically fail to detect intra-varietal variation in most clones due to their relatively low mutation rate [7,9], the study by Migliaro et al. demonstrated that when locus selection was refined "*using an upgraded core set"* of SSR markers, it was possible to detect genetic differences even in subtle intra-varietal mutations, such as those affecting berry color [9]. Similarly, other studies have highlighted the utility of SSR for identifying clonal diversity under specific conditions: Jahnke et al. successfully distinguished closely related Pinot Noire clones using a carefully selected SSR set [21], and Meneghetti et al. confirmed that combining SSR with other molecular markers can effectively capture relevant intra-varietal variation [12].

2.2 *VITIS VINIFERA* L. AND CLIMATE CHANGE

Viticulture across large regions of the globe is facing significant challenges as a result of climate change, including rising temperatures, prolonged droughts, increased ultraviolet radiation, and a growing frequency of extreme weather events. One of the primary concerns is whether these changing climatic conditions will negatively impact grapevine yield and fruit quality. Among the key strategies to address these challenges, though still relatively underexplored, is varietal selection, which harnesses the existing genetic diversity within *Vitis vinifera* L. Across the broad range of grapevine cultivars, we find substantial variation in agronomic traits, including tolerance to cold, heat, and water stress, as well as differences in phenological development. To achieve high-quality wine production, the plant's phenology must align with the climatic conditions of the growing

region, in addition to demonstrating resilience to both abiotic and biotic stresses. As climate patterns shift, the phenological requirements of grapevines must also adapt, which can be addressed through the selection of optimal varietal and clonal combinations suited to local environmental conditions. Indeed, the concept of *terroir* fundamentally encompasses this alignment between grapevine genotype and regional climate [4,22].

2.3 THE CANARY ISLANDS AS A BIODIVERSITY HOTSPOT

The Canary Islands, with their unique geographical features and long-standing viticultural tradition, represent a remarkable case of biodiversity preservation and generation. In arrears to their isolation and phylloxera-free status, the archipelago has accumulated a high number of somatic mutations over more than five centuries, primarily due to the continuous vegetative propagation of grapevine material. The region can thus be considered a true hotspot for the generation of *Vitis vinifera* L. biodiversity, offering a wide repertoire of both varietal and clonal diversity that contributes to avoiding the homogenization of wine profiles. This is particularly relevant in light of the fact that, as previously mentioned, just 12 grapevine varieties account for approximately 80% of the global vineyard surface area, a trend that, when combined with the pressures of climate change, exacerbates the genetic erosion of the species [4,23,24].

Accordingly, the islands of Lanzarote and Fuerteventura (Canary Islands; Figure 1) represent a unique natural laboratory for evaluating clonal diversity developed under extreme environmental conditions.

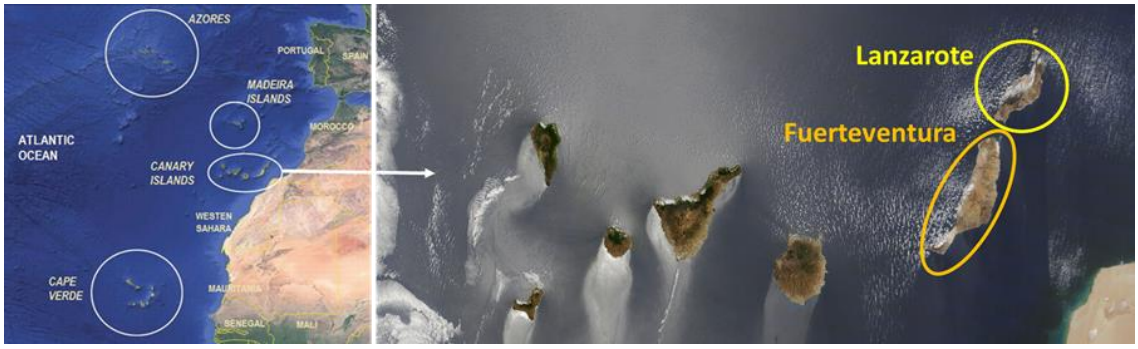

Figure 1. Geographical location of the Macaronesian region (left). The Canary Archipelago and detailed view of the islands of Lanzarote and Fuerteventura (right) [23,27,28].

Lanzarote, commonly known as the "Island of Volcanoes," features soils covered by volcanic *lapilli* (locally known as *rofe or picón*), originating from the Timanfaya

eruptions (1730–1736) [25]. The area blanketed by these *lapilli* constitutes what is now known as La Geria (20 km²) (Figure 2). This unique soil exhibits specific properties, including enhanced water retention and thermal insulation, which facilitate grapevine cultivation on the island [26,27]. Viticulture in Lanzarote is primarily characterized by singular agricultural practices, such as the planting of vines in "planting pits" the use of both natural and artificial sand mulching, and cultivation in trench-like structures called chabocos, all of which are adapted to optimize the limited water availability resulting from the island's low annual precipitation of just 90–120 mm [26].

Figure 2. Vineyard in La Geria (Lanzarote Island). Soil composed of *picón* or *rofe* (small black volcanic lapilli). Detail of the traditional "hole" planting strategy [26].

On the other hand, Fuerteventura, often referred to as the "Island of Wind", offers an even more arid environment, with annual rainfall dropping below 100 mm in some areas, high solar radiation, and intense wind erosion. In this situation, grapevines persist only in limited areas through a traditional agricultural system known as *gavias* (Figure 3), which efficiently captures and retains scarce rainwater from occasional precipitation events, enabling cultivation under such extreme climatic conditions [28]. Consequently, the inter and intra-varietal biodiversity of the Canary Islands stands out as a valuable resource for the identification and selection of varieties and clones with traits related to resilience

under global warming scenarios, such as drought tolerance, thus representing a promising long-term strategy for the sustainability of viticulture in Mediterranean and low-rainfall regions [23].

Figure 3. *Gavias* landscape on Fuerteventura Island [28].

3 HYPOTHESIS AND OBJECTIVES

Based on the above, the **primary objective** of this preliminary study was to detect samples exhibiting variation in their genetic profiles within the varieties Malvasia volcanica and Listan prieto. The **second objective** was to confirm the suitability of SSR markers for revealing differences resulting from the vegetative propagation of grapevine clones in geographically isolated and phylloxera-free areas (Lanzarote and Fuerteventura). Finally, the **third objective** was to confirm the pedigree of the Malvasia volcanica variety.

Hypothesis: There is intra-varietal variability in geographically isolated phylloxera-free areas, and the SSR technique is capable of detecting it.

4 MATERIALS AND METHODS

4.1 PLANT MATERIAL

4.1.1 Lanzarote Island

The population of Malvasia volcanica analyzed in this intra-varietal variability study comprised 86 accessions collected from across the island of Lanzarote. In addition, two individuals of Malvasia (Main Name [MN]: Malvasia Dubrovacka), identified by codes MAR1454 and MARMA467, and one accession of Bermejuela (MARMA467), were included for lineage analysis (Table S1). As shown, the accessions originated from various points across the island (29°02′06″N 13°37′59″W), representing a diversity of cultivation systems, abiotic conditions, and planting densities between vines, ranging from 1.5 to 2 meters in the northern zone, 1.5 meters in Tinajo, and up to 4 meters in La Geria. From each vine selected by technicians from the Island Council of Lanzarote and the Protected Designation of Origin (PDO) "Vinos de Lanzarote," 4 to 6 vine cuttings of approximately 15 cm were collected, separately bagged, and kept individually throughout the entire study. The plant material was sent to Rovira i Virgili University, where it was examined and labeled with laboratory identification codes, then stored at -20°C until further use.

4.1.2 Fuerteventura Island

The Listan prieto population analyzed in this intra-varietal variability study consisted of 18 accessions collected from various locations across Fuerteventura Island (Table S1). The majority of samples were obtained from the Betancuria area, along with two accessions from La Oliva (two samples), one from Puerto del Rosario, and one from Antigua. As in the previous case, 4 to 6 vine cuttings of approximately 15 cm were collected from each vine selected by technicians and winegrowers of the Majuelo Association. The cuttings were separately bagged and maintained individually throughout the study. They were also sent to Rovira i Virgili University, where they were reviewed, assigned laboratory identification codes, and preserved at -20°C until further analysis.

4.2 DNA EXTRACTION AND PURIFICATION

A proprietary method developed by this research group was used for DNA extraction, applicable to both leaf tissue [29] and recalcitrant tissues [30], as well as RNA extraction

from grape berries [31]. All procedures involved three main steps: preparation of the plant material, execution of the extraction protocol, and assessment of purity index (quality control). The wood DNA extraction protocol [30] employed in this study was optimized through the inclusion of two chloroform washing steps, aiming to more effectively eliminate proteins. Once DNA was extracted, its purity and concentration were assessed spectrophotometrically using the Thermo Fisher® Scientific NanoDrop™ 1000 Spectrophotometer (Waltham, MA, USA).

4.3 Simple Sequence Repeat (SSR) Markers

After verifying the quality of the DNA, the microsatellite regions of each sample were amplified using 100 SSR (simple sequence repeat) markers. These markers were selected based on their discriminatory capacity and polymorphism, according to previous studies (Table S2): VVS2, VVS3, VVS29 [32]; VVMD5, VVMD6, VVMD7, VVMD21, VVMD24, VVMD25, VVMD32 [33]; VVMD14, VVMD17, VVMD26, VVMD27, VVMD28, VVMD31, VVMD36 [34]; VrZAG7, VrZAG21, VrZAG25, VrZAG47, VrZAG62, VrZAG64, VrZAG67, VrZAG79, VrZAG83, VrZAG112 [35]; SCU06vv [36]; VMC1b11, VMC4f3 [37]; VvUCH11, VvUCH12, VvUCH19 [38]; VMC3D8, ISV2 (VMC6e1) [39]; VMC6e10 [40]; VVIb01, VVIq52, VVIh54, VVIp60, VVIn16, VVIn61, VVIb66, VVIv37, VVIv67, VVIn73, VVIp31, VVIv33, VVIb09, VVIb32, VVIp22, VVIp34, VVIp37, VVIn57, VVIp77, VVIt60, VVIv04, VVIv17, VVIv51, VVIv70 [41]; VMC4D9.2, VMC4G6 [42]; VRG1, VRG2, VRG3, VRG4, VRG7, VRG9, VRG10, VRG11, VRG13, VRG15, VRG16 [43]; VChr1b, VChr3a, VChr4a, VChr5b, VChr5c, VChr7b, VChr8a, VChr8b, VChr9a, VChr9b, VChr10a, VChr10b, VChr11b, VChr12a, VChr13a, VChr13b, VChr13c, VChr14b, VChr15a, VChr15b, VChr16a, VChr18a, VChr18b, VChr19a, VChr19b [44]; VVNTM1, VVNTM5 [45] (Figure S1). Nine of these markers are widely used as international reference loci by the scientific community [46] and have been selected by the OIV as standard descriptors for grapevine varieties and *Vitis* species (OIV 801–806).

4.4 DNA Amplification

SSR amplification was carried out using polymerase chain reaction (PCR) on various Applied Biosystems 2720 Thermal Cyclers (Foster City, CA, USA).

Each PCR reaction consisted of 4 ng of template DNA and 1 µM of each primer, with the forward primer labeled with a fluorescent dye (6- FAM: VVS3, VVMD7, VVMD24, VVMD25, VVMD28, VVMD32, VVMD36, VrZAG7, VrZAG47, VrZAG62, VrZAG83, VvUCH11, VvUCH19, VMC6e10, VVIb32, VVIp34, VVIp77,VVIv37, VVIq52, VVIn16, VVIp31, VVIb66, VVIv33, VMC4D9.2, VRG2, VRG3, VRG13, VChr1b, VChr5c, VChr8b, VChr9a, VChr9b, VChr10a, VChr10b, VChr12a, VChr13c, VChr15b y VVNTM1 ; HEX: VVS2, VVS29, VVMD6, VVMD21, VVMD26, VVMD27, VrZAG21, VrZAG25, VrZAG67, VrZAG79, VrZAG112, VMC4G6, VVIb09, VVIp22, VVIn57, VVIt60, VVIv04, VVIb01, VVIp60, VVIn61, VRG4, VRG9, VRG10, VRG15 VChr3a, VChr4a, VChr5b, VChr7b, VChr14b, VChr16a, VChr18a, VChr19a y VChr19b; NED: VVMD5, VVMD14, VVMD17, VVMD31, VrZAG64, SCU06vv, VvUCH12, VMC1b11, VMC3D8, ISV2 (VMC6e1), VMC4f3, VVNTM5, VVIp37, VVIv17, VVIv51, VVIv70, VVIn73, VVIh54, VVIv67, VRG1, VRG7, VRG11, VRG16 VChr8a, VChr11b, VChr13a, VChr13b, VChr15a y VChr18b) using the kit AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA). SSR amplification was carried out using the annealing temperatures and thermocycling regimes specified in Table S2. These temperatures and thermocycling regimes were selected based on the previously cited literature and further optimized in the laboratory.

4.5 AMPLIFIED FRAGMENTS ANALYSIS

Amplified products were mixed with 20 µL of deionized formamide and 0.5 µL of internal size standard (GeneScan 500 ROX, Applied Biosystems, Foster City, CA), and denatured at 95 °C for 3 minutes. Fragment separation was performed by capillary electrophoresis using an ABI PRISM 3730® Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The resulting electropherograms were analyzed using *Peak Scanner Software* (Applied Biosystems, NJ, USA) to size the amplified fragments. Each accession was analyzed at least twice using DNA from independent extractions to avoid possible errors (Figure S2).

4.6 SAMPLE IDENTIFICATION

The molecular profiles (MP-SSR) obtained for each SSR marker and accession were compared with published references and existing databases. The international SSR markers VVS2, VVMD5, VVMD7, VVMD27, VVMD28, ZAG62, and ZAG79 were cross-checked with data from the Vine Biology Database of the TECNENOL Research

Group [8,23,24,27,28,47,48]. In addition, markers VVMD25 and VVMD32 were compared with entries in the global "Vitis International Variety Catalogue" (VIVC) [49]. The TECNENOL database [8,23,24,27,28,47,48] also includes MP-SSR profiles for all varieties studied using SSR such as VVS3, VVS29, VVMD6, VVMD36, ZAG21, ZAG47, ZAG64, ZAG83, UCH11, UCH12, UCH19, SCU06, and VChr19a. The microsatellites VrZAG67 and VrZAG112 are documented in the 2018 book on cultivated grapevine varieties in the Canary Islands [50]. For Muscat of Alexandria, comparison was also possible with the following markers: VMC1b11, VMC4F3, VVIb01, VVIh54, VVIn16, VVIn73, VVIp31, VVIp60, VVIq52, VVIv37, VVIv67, VVMD21, and VVMD24 [51]. For the remaining SSR markers, MP-SSR profiles were not compared due to limited reference data in the literature, which typically focuses on the aforementioned markers—even when evaluating non-local cultivars such as Malvasia Fina, Malvasia Dubrovacka, or Listan prieto.

5 RESULTS

The results obtained are presented in three sections. The first provides an overview of the accessions that exhibited variations. Next, we describe the main characteristics of the SSR markers used in this study. Finally, we discuss the lineage analysis of Malvasia volcanica.

5.1 INTER-VARIETAL AND INTRA-VARIETAL VARIABILITY (CLONES IDENTIFIED: VARIATIONS RELATIVE TO THE MOST COMMON MOLECULAR PROFILE (MUTATIONS))

The study population consisted of 107 accessions collected from various locations across the islands of Lanzarote and Fuerteventura (Table S1). Specifically, 86 samples of Malvasia volcanica from Lanzarote were analyzed to assess intra-varietal variability, along with 2 accessions of Malvasia Dubrovacka [52] and 1 of the local Canarian variety known by its primary name (PN) as Bermejuela [23], in order to confirm the pedigree of Malvasia volcanica [53]. From Fuerteventura, 18 individuals of the Listan prieto variety were examined [28]. While no inter-varietal variability was detected among the Malvasia Dubrovacka and Listan prieto samples, four accessions within the Malvasia volcanica group were identified as belonging to other varieties*:* Muscat of Alexandria (LNZ-18 and

LNZ-52), a Greek white variety; Listan negro (LNZ-59), a local Canarian red variety; and the Portuguese white variety Malvasia fina (LNZ-69).

Intra-varietal variability was detected in 93.46% of the analyzed accessions (100 out of 107 individuals). Tables 1 and 2 present the samples showing differences relative to the most widespread or reference molecular profile (ARP) within the analyzed population, corresponding to 100 accessions. Thus, five individuals of the Malvasia volcanica variety (LNZ-, LNZ-28, LNZ-46, LNZ-57, LNZ-65), one sample of Listan prieto (FTV-15), and one accession of Muscat of Alexandria (LNZ-52) were identified as non-variable or matching the ARP (Assumed Reference Profile). The accessions exhibiting the greatest number of variations corresponded to a Malvasia Dubrovacka (LNZ-87) (Figure 4) and a Listan prieto (FTV-8) (Figure 5), each differing from the ARP at five SSR loci. Another Listan prieto accession (FTV-13) (Figure S3) showed four variations. The group presenting three variations comprised seven individuals: two belonging to the Malvasia volcanica variety (LNZ-12, LNZ-72) and five to the Listan prieto variety (FTV-1, FTV-2, FTV-7, FTV-9, FTV-12). A total of 36 individuals exhibited two variations: 30 of these corresponded to Malvasia volcanica, five to Listan prieto, and one to Malvasia Dubrovacka (LNZ-88). In 54 samples, only a single variation was detected. Of these, 45 were members of the Malvasia volcanica variety, five belonged to the Listan prieto cluster, and the remaining four were classified as Muscat of Alexandria (LNZ-18), Listan Negro (LNZ-59), Malvasia Fina (LNZ-69), and the local Canarian variety Bermejuela (LNZ-89).

As noted in the previous paragraph, the accessions exhibited the highest number of variations were LNZ-87 and FTV-8 (Tables 1 and 2, Figures 4 and 5). The Malvasia Dubrovacka from Lanzarote (LNZ-87) showed mutations at the SSR loci VVS3, VMC4F3, VVIv33, VVIn57, and VChr13b. The ARPs (Assumed Reference Profiles) for these five SSR were heterozygous, and the observed variations were as follows: 1) homozygosity at VVS3; 2) triallelism at VMC4F3, VVIv33, and VVIn57; and 3) retention of heterozygosity at VChr13b. The Listan prieto accession from Fuerteventura (FTV-8) exhibited variation at the SSR loci ZAG7, VChr9, VChr15b, VChr14b, and VChr13b. All corresponding ARPs were homozygous, and the observed variations in FTV-8 were as follows: 1) heterozygosity at VChr14b (Figures 5 and S2b) and VChr13b; 2) homozygosity with a change in allele size at VChr9; and (3) multi-allelic profiles at ZAG7 and VChr15b, with ZAG7 being triallelic (Figure S2d) and VChr15b tetra-allelic.

Table 1. Complete list of accessions showing variation in their molecular profile (MP-SSR) compared to the most widespread or reference profile (ARP). Variations typically involve the presence or absence of an allele (homozygosity or heterozygosity), triallelism, tetraallelism, or multiallelism. Some cases also present numerical variations affecting one or more peak families. List of SSR markers with unique profiles detected in some or all of the analyzed samples.

SSR No.	2	3	4	8	22	23	34	40	47	55	60	69	75	77	80	84	86	87	88	92	97	98	100
Peak size range / Fluorochrome colour	214-222	168-186	215-270	210-279	278-318	156-230	239-263	217-289	106–158	114-135	350-400	250-300	102-160	90-151	150-200	106-292	250-300	222-250	250-300	176-243	185-292	50-100	145-170
SAMPLE CODE / SSR	VVS3	VVS2	VVMD	VVMD28	VVIb0	VMC4F	VRG1	VVMD3	ZAG7	VChr13	VVIv3	VVIb0	VChr9	VChr15	VVIv5	VRG2	VVIn5	VVMD1	VVIt60	VChr14	VRG3	VVIp34	VChr13
LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1	LNZ-1
LNZ-2	LNZ-2*	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2	LNZ-2
LNZ-3	LNZ-3*	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3*	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3	LNZ-3
LNZ-4	LNZ-4*	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4	LNZ-4
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LNZ-71	LNZ-71*	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71	LNZ-71
LNZ-72	LNZ-72*	LNZ-72	LNZ-72	LNZ-72	LNZ-72	LNZ-72	LNZ-72	LNZ-72*	LNZ-72	LNZ-72*	LNZ-72	LNZ-72	LNZ-72	LNZ-72	LNZ-72	LNZ-72	LNZ-72	LNZ-72	LNZ-72	LNZ-72	LNZ-72	LNZ-72	LNZ-72
LNZ-73	LNZ-73*	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73*	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73	LNZ-73
LNZ-74	LNZ-74*	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74	LNZ-74
LNZ-75	LNZ-75*	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75	LNZ-75
LNZ-76	LNZ-76*	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76*	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76	LNZ-76
LNZ-77	LNZ-77*	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77	LNZ-77
LNZ-78	LNZ-78*	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78*	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78	LNZ-78
LNZ-79	LNZ-79*	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79	LNZ-79
LNZ-80	LNZ-80*	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80*	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80	LNZ-80
LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81*	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81	LNZ-81
LNZ-82	LNZ-82*	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82	LNZ-82
LNZ-83	LNZ-83*	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83*	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83	LNZ-83
LNZ-84	LNZ-84*	LNZ-82	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84*	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84	LNZ-84
LNZ-85	LNZ-85*	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85*	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85	LNZ-85
LNZ-86	LNZ-86*	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86	LNZ-86
FTV-1	FTV-1	FTV-1	FTV-1	FTV-1*	FTV-1	FTV-1	FTV-1	FTV-1	FTV-1	FTV-1	FTV-1	FTV-1	FTV-1*	FTV-1*	FTV-1	FTV-1	FTV-1	FTV-1	FTV-1	FTV-1	FTV-1	FTV-1	FTV-1
FTV-2	FTV-2*	FTV-2	FTV-2	FTV-2*	FTV-2	FTV-2	FTV-2	FTV-2	FTV-2	FTV-2	FTV-2	FTV-2	FTV-2	FTV-2*	FTV-2	FTV-2	FTV-2	FTV-2	FTV-2	FTV-2	FTV-2	FTV-2	FTV-2
FTV-3	FTV-3	FTV-3	FTV-3	FTV-3*	FTV-3	FTV-3	FTV-3	FTV-3	FTV-3	FTV-3	FTV-3	FTV-3	FTV-3*	FTV-3	FTV-3	FTV-3	FTV-3	FTV-3	FTV-3	FTV-3	FTV-3	FTV-3	FTV-3
FTV-4	FTV-4	FTV-4	FTV-4	FTV-4*	FTV-4	FTV-4	FTV-4	FTV-4	FTV-4	FTV-4	FTV-4	FTV-4	FTV-4	FTV-4*	FTV-4	FTV-4	FTV-4	FTV-4	FTV-4	FTV-4	FTV-4	FTV-4	FTV-4
FTV-5	FTV-5	FTV-5	FTV-5	FTV-5*	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5	FTV-5
FTV-7	FTV-7	FTV-7	FTV-7	FTV-7*	FTV-7	FTV-7	FTV-7	FTV-7	FTV-7	FTV-7	FTV-7	FTV-7	FTV-7	FTV-7*	FTV-7	FTV-7	FTV-7	FTV-7	FTV-7	FTV-7*	FTV-7	FTV-7	FTV-7
FTV-8	FTV-8	FTV-8	FTV-8	FTV-8	FTV-8	FTV-8	FTV-8	FTV-8	FTV-8*	FTV-8	FTV-8	FTV-8	FTV-8*	FTV-8*	FTV-8	FTV-8	FTV-8	FTV-8	FTV-8	FTV-8*	FTV-8	FTV-8	FTV-8*
FTV-9	FTV-9*	FTV-9	FTV-9	FTV-9*	FTV-9	FTV-9	FTV-9	FTV-9	FTV-9*	FTV-9	FTV-9	FTV-9	FTV-9	FTV-9	FTV-9	FTV-9	FTV-9	FTV-9	FTV-9	FTV-9	FTV-9	FTV-9	FTV-9
FTV-10	FTV-10	FTV-10	FTV-10	FTV-10*	FTV-10	FTV-10	FTV-10	FTV-10	FTV-10	FTV-10	FTV-10	FTV-10	FTV-10*	FTV-10	FTV-10	FTV-10	FTV-10	FTV-10	FTV-10	FTV-10	FTV-10	FTV-10	FTV-10
FTV-11	FTV-11	FTV-11	FTV-11	FTV-11*	FTV-11	FTV-11	FTV-11	FTV-11	FTV-11	FTV-11	FTV-11	FTV-11	FTV-11*	FTV-11	FTV-11	FTV-11	FTV-11	FTV-11	FTV-11	FTV-11	FTV-11	FTV-11	FTV-11
FTV-12	FTV-12	FTV-12	FTV-12	FTV-12*	FTV-12	FTV-12	FTV-12	FTV-12	FTV-12	FTV-12	FTV-12	FTV-12	FTV-12	FTV-12*	FTV-12	FTV-12	FTV-12	FTV-12	FTV-12	FTV-12*	FTV-12	FTV-12	FTV-12
FTV-13	FTV-13*	FTV-13	FTV-13	FTV-13*	FTV-13	FTV-13	FTV-13	FTV-13	FTV-13*	FTV-13	FTV-13	FTV-13	FTV-13	FTV-13*	FTV-13	FTV-13	FTV-13	FTV-13	FTV-13	FTV-13	FTV-13	FTV-13	FTV-13
FTV-14	FTV-14	FTV-14	FTV-14	FTV-14*	FTV-14	FTV-14	FTV-14	FTV-14	FTV-14*	FTV-14	FTV-14	FTV-14	FTV-14	FTV-14	FTV-14	FTV-14	FTV-14	FTV-14	FTV-14	FTV-14	FTV-14	FTV-14	FTV-14
FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15	FTV-15
FTV-16	FTV-16	FTV-16	FTV-16	FTV-16	FTV-16	FTV-16	FTV-16	FTV-16	FTV-16*	FTV-16	FTV-16	FTV-16	FTV-16	FTV-16	FTV-16	FTV-16	FTV-16	FTV-16	FTV-16	FTV-16*	FTV-16	FTV-16	FTV-16
FTV-18	FTV-18	FTV-18	FTV-18	FTV-18*	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18	FTV-18
FTV-20	FTV-20	FTV-20	FTV-20	FTV-20*	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20	FTV-20
FTV-32	FTV-32	FTV-32	FTV-32	FTV-32	FTV-32	FTV-32	FTV-32	FTV-32	FTV-32	FTV-32	FTV-32	FTV-32	FTV-32*	FTV-32	FTV-32	FTV-32	FTV-32	FTV-32	FTV-32	FTV-32	FTV-31	FTV-32	FTV-32
LNZ-18	LNZ-18	LNZ-18	LNZ-18*	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18	LNZ-18
LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52	LNZ-52
LNZ-87	LNZ-87*	LNZ-87	LNZ-87	LNZ-87	LNZ-87	LNZ-87*	LNZ-87	LNZ-87	LNZ-87	LNZ-87	LNZ-87*	LNZ-87	LNZ-87	LNZ-87	LNZ-87	LNZ-87	LNZ-87*	LNZ-87	LNZ-87	LNZ-87	LNZ-87	LNZ-87	LNZ-87*
LNZ-88	LNZ-88*	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88	LNZ-88*
LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89*	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89	LNZ-89
LNZ-59	LNZ-59*	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59	LNZ-59
LNZ-69	LNZ-69*	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69	LNZ-69
SAMPLE CODE / SSR	VVS3*	VVS29*	VVMD5*	VVMD28*	VVIb01	VMC4F3*	VRG16*	VVMD32*	ZAG7*	VChr13c*	VVIv33*	VVIb09*	VChr9b*	VChr15b*	VVIv51	VRG2	VVIn57*	VVMD14	VVIt60	VChr14b*	VRG3	VVIp34*	VChr13b*

LNZ-?	MALVASIA VOLCANICA
FTV-?	LISTAN PRIETO
LNZ-18 LNZ-52	MUSCAT OF ALEXANDRIA
LNZ-87 LNZ-88	MALVASIA AROMATICA
LNZ-89	BERMEJUELA
LNZ-59	LISTAN NEGRO
LNZ-69	MALVASIA FINA

	HETEROZYGOUS INDIVIDUAL (2 PEAK FAMILIES)
	HETEROZYGOUS INDIVIDUAL (2 PEAK FAMILIES). NUMERICAL VARIATION
	HOMOZYGOUS INDIVIDUAL (1 PEAK FAMILY)
	HOMOZYGOUS INDIVIDUAL (1 PEAK FAMILY). NUMERICAL VARIATION
	TRIALLELIC INDIVIDUAL (3 PEAK FAMILIES)
	TRIALLELIC INDIVIDUAL (3 PEAK FAMILIES). NUMERICAL VARIATION
	TETRAALLELIC INDIVIDUAL (4 PEAK FAMILIES)
	MULTIALLELIC INDIVIDUAL (MORE THAN 4 PEAK FAMILIES)
*	MUTATED INDIVIDUAL

Table 2. List of accessions showing variations in their molecular profile (MP-SSR), ordered from the highest to the lowest number of variations. Variations typically involve the presence or absence of an allele (homozygosity or heterozygosity), triallelism, or tetraallelism. Some cases also present numerical variations affecting one or more peak families.

LNZ-87	VVS3	VMC4F3	VVIv33	VVIn57	VChr13b
FTV-8	ZAG7	VChr9b	VChr15b	VChr14b	VChr13b
FTV-13	VVS3	VVMD28	ZAG7	VChr15b	
LNZ-17	VVS3	VVMD32	VChr9b		
LNZ-72	VVS3	VVMD32	VChr13c		
FTV-1	VVMD28	VChr9b	VChr15b		
FTV-2	VVS3	VVMD28	VChr15b		
FTV-7	VVMD28	VChr15b	VChr14b		
FTV-9	VVS3	VVMD28	ZAG7		
FTV-12	VVMD28	VChr15b	VChr14b		
LNZ-3	VVS3	VChr9b			
LNZ-21	VVS3	VChr9b			
LNZ-29	VVS3	VChr9b			
LNZ-32	VVS3	VChr9b			
LNZ-35	VVS3	VChr9b			
LNZ-45	VVS3	VChr9b			
LNZ-47	VVS3	VChr9b			
LNZ-48	VVS3	VChr9b			
LNZ-50	VVS3	VChr9b			
LNZ-54	VVS3	VChr9b			
LNZ-55	VVS3	VChr9b			
LNZ-56	VVS3	VChr9b			
LNZ-68	VVS3	VChr9b			
LNZ-78	VVS3	VChr9b			
LNZ-80	VVS3	VChr9b			
LNZ-83	VVS3	VChr9b			
LNZ-84	VVS3	VChr9b			
LNZ-85	VVS3	VChr9b			
LNZ-5	VVS3	VVS29			
LNZ-34	VVS3	VVS29			
LNZ-36	VVS3	VVS29			
LNZ-42	VVS3	VVS29			
LNZ-66	VVS3	VVS29			
LNZ-6	VVS3	VVMD32			
LNZ-8	VVS3	VVMD32			
LNZ-19	VVS3	VChr15b			
LNZ-24	VVS29	VChr9b			
LNZ-60	VVS3	VVIp34			
LNZ-73	VVS3	VVIb09			
LNZ-76	VVS3	VMC4F3			
FTV-4	VVMD28	VChr15b			
FTV-10	VVMD28	VChr9b			
FTV-11	VVMD28	VChr9b			
FTV-14	VVMD28	ZAG7			
FTV-16	ZAG7	VChr14b			
LNZ-88	VVS3	VChr13b			
LNZ-2	VVS3				
LNZ-4	VVS3				
LNZ-7	VVS3				
LNZ-9	VVS3				
LNZ-10	VVS3				
LNZ-11	VVS3				
LNZ-12	VVS3				
LNZ-13	VVS3				
LNZ-14	VVS3				
LNZ-15	VVS3				
LNZ-20	VVS3				
LNZ-22	VVS3				
LNZ-23	VVS3				
LNZ-25	VVS3				
LNZ-26	VVS3				
LNZ-27	VVS3				
LNZ-30	VVS3				
LNZ-33	VVS3				
LNZ-37	VVS3				
LNZ-38	VVS3				
LNZ-39	VVS3				
LNZ-43	VVS3				
LNZ-44	VVS3				
LNZ-49	VVS3				
LNZ-51	VVS3				
LNZ-53	VVS3				
LNZ-58	VVS3				
LNZ-61	VVS3				
LNZ-62	VVS3				
LNZ-63	VVS3				
LNZ-64	VVS3				
LNZ-67	VVS3				
LNZ-70	VVS3				
LNZ-71	VVS3				
LNZ-74	VVS3				
LNZ-75	VVS3				
LNZ-77	VVS3				
LNZ-79	VVS3				
LNZ-82	VVS3				
LNZ-86	VVS3				
LNZ-16	VVIp34				
LNZ-31	VChr9b				
LNZ-40	VChr9b				
LNZ-81	VChr9b				
LNZ-41	VVS29				
FTV-3	VVMD28				
FTV-5	VVMD28				
FTV-18	VVMD28				
FTV-20	VVMD28				
FTV-32	VChr9b				
LNZ-18	VVMD5				
LNZ-59	VVS3				
LNZ-69	VVS3				
LNZ-89	VRG16				
LNZ-1					
LNZ-28					
LNZ-46					
LNZ-57					
LNZ-65					
FTV-15					
LNZ-52					

LNZ-?	MALVASIA VOLCANICA
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	HETEROZYGOUS INDIVIDUAL (2 PEAK FAMILIES)
	HETEROZYGOUS INDIVIDUAL (2 PEAK FAMILIES). NUMERICAL VARIATION
	HOMOZYGOUS INDIVIDUAL (1 PEAK FAMILY)
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	TRIALLELIC INDIVIDUAL (3 PEAK FAMILIES)
	TRIALLELIC INDIVIDUAL (3 PEAK FAMILIES). NUMERICAL VARIATION
	TETRAALLELIC INDIVIDUAL (4 PEAK FAMILIES)

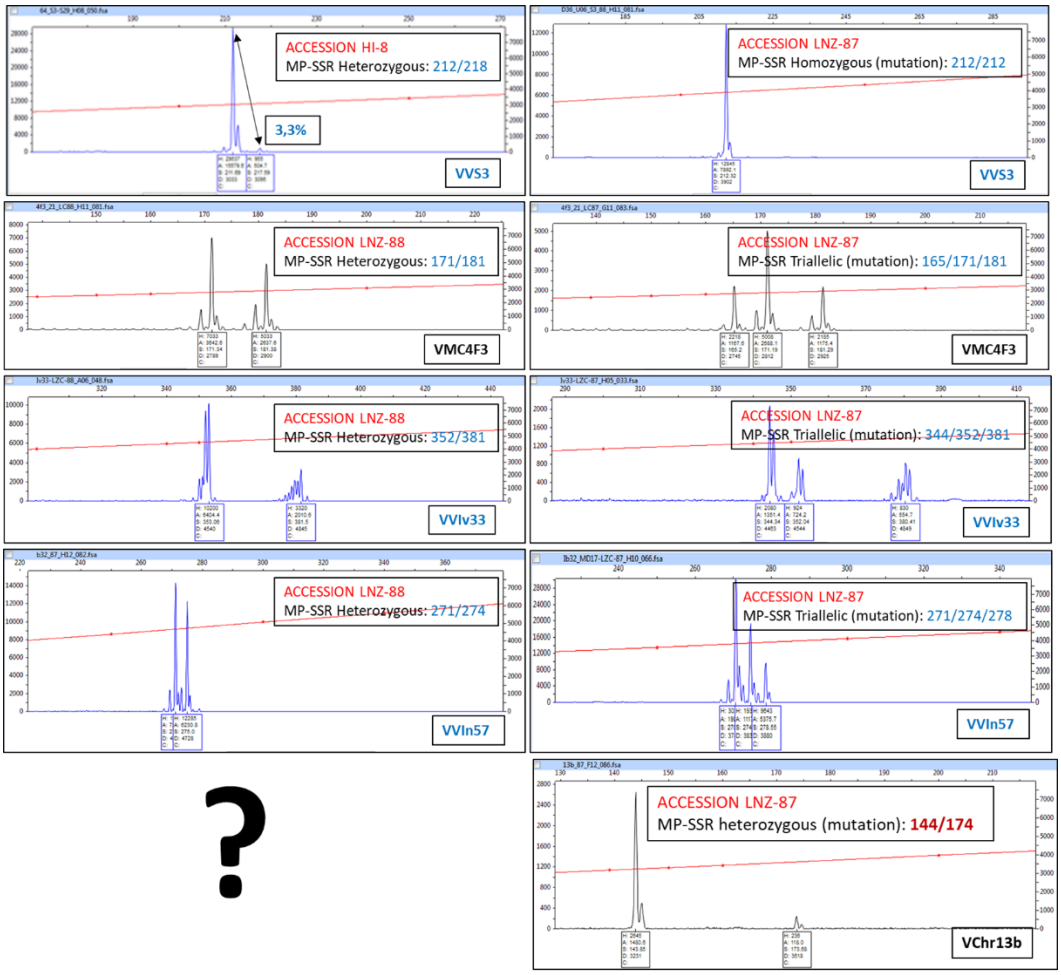

Figure 4. Malvasia Dubrovacka profiles (LNZ-87) showing variations compared to the most widespread or reference molecular profile (ARP). On the left, ARP electropherograms. HI: El Hierro Island; LNZ: Lanzarote Island; FTV: Fuerteventura Island. LNZ-87 is one of the two profiles with the highest number of variations. ?: The ARP profile of this SSR for Malvasia Dubrovacka is unknown (both samples of this variety, LNZ-87 and LNZ-88, showed the same result); based on their pedigree relationship, both are suspected to be mutated. Nº %: Examples of peak ratios (percentage indicating the ratio between the smallest and largest peaks), used to disregard extremely small peaks.

The accession FTV-13 exhibited four variations (VVS3, VVMD28, ZAG7, and VChr15b) (Tables 1 and 2, Figure S3). This sample of Listan prieto was heterozygous for VVS3 and VVMD28 in the ARPs, while the corresponding mutated profiles were homozygous. In contrast, ZAG7 and VChr15b had homozygous ARPs, with FTV-13 displaying heterozygosity at ZAG7 and triallelism at VChr15b.

The group of samples that showed three variations consisted of 7 accessions (Tables 1 and 2): 1) LNZ-17 varied at SSR loci VVS3 and VChr9b (homozygous variation) and

VVMD32 (heterozygous mutation with allelic shift); 2) LNZ-72 varied at SSR loci VVS3 and VChr13c (homozygous variation) and VVMD32 (triallelic); 3) FTV-1 showed mutations at SSR loci VVMD28 (homozygous variation), VChr9b (heterozygous variation), and VChr15b (triallelic); 4) FTV-2 varied at SSR loci VVS3 and VVMD28 (homozygous variation) and VChr15b (triallelic); 5) FTV-7 presented mutations at SSR loci VVMD28 (homozygous variation), VChr15b (triallelic), and VChr14b (heterozygous variation); 6) FTV-9 varying at SSR loci VVS3 and VVMD28 (homozygous variation) and ZAG7 (heterozygous variation); 7) FTV-12 mutated at SSR loci VVMD28 (homozygous variation), VChr15b (triallelic), and VChr14b (heterozygous variation).

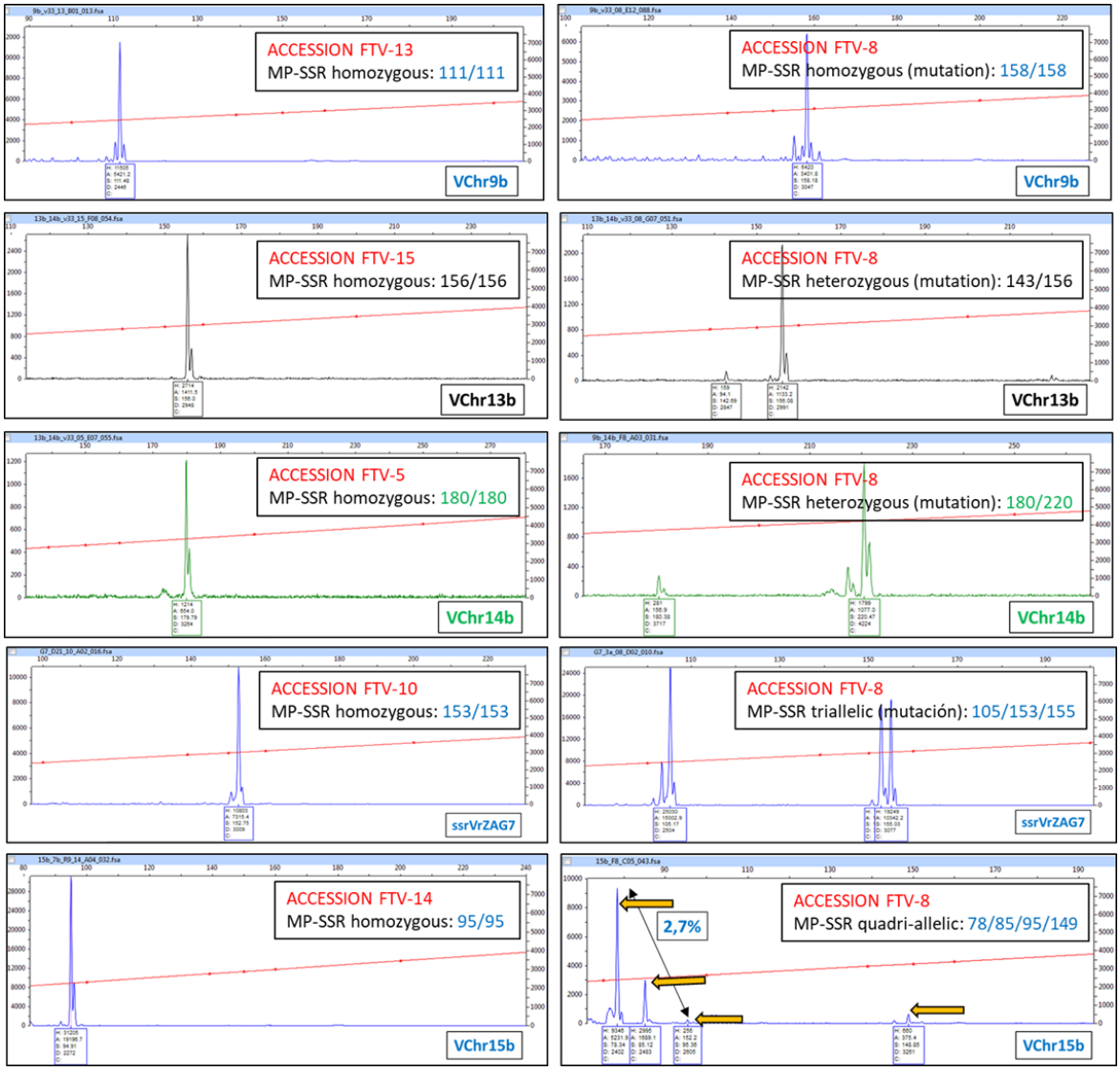

Figure 5. Listan prieto profiles (FTV-8) showing variations compared to the most widespread or reference molecular profile (ARP). On the left, ARP electropherograms. FTV-8 is one of the two profiles with the greatest number of variations.

The following group of accessions corresponded to those exhibited two variations (Tables 1 and 2). The samples of *Malvasia volcanica* (LNZ-) 3, 21, 29, 32, 35, 45, 47, 48, 50, 54, 55, 56, 68, 78, 80, 83, 84, and 85 showed variations at SSR loci VVS3 and VChr9b (homozygous variation), while accessions (LNZ-) 5, 34, 36, 42, and 66 presented mutations at SSR loci VVS3 (homozygous variation) and VVS29 (heterozygous variation). LNZ-6 and LNZ-8 varied at SSR loci VVS3 (homozygous variation) and VVMD32 (heterozygous variation). LNZ-19 changed at SSR loci VVS3 and VChr15b (Figure S2a) (homozygous variation). LNZ-24 showed variation at VVS29 (heterozygous variation) and VChr9b (homozygous variation). LNZ-60 exhibited mutation at SSR loci VVS3 (homozygous variation) and VVIp34 (triallelic with allelic shift). LNZ-73 varied at SSR loci VVS3 (homozygous variation) and VVIb09 (triallelic). LNZ-76 mutated at VVS3 (homozygous variation) and VMC4F3 (triallelic). FTV-4 showed variation at SSR loci VVMD28 (homozygous variation) and VChr15b (triallelic). Accessions FTV-10 and FTV-11 mutated at the same SSR loci, VVMD28 (homozygous variation) and VChr9b (homozygous variation with allelic shift). FTV-14 presented mutations at SSR loci VVMD28 (homozygous variation) and ZAG7 (heterozygous variation). FTV-16 varied at SSR loci ZAG7 (heterozygous variation) and VChr14b (heterozygous variation), while the Malvasia Dubrovacka variety (LNZ-88) was homozygous at SSR locus VVS3 and heterozygous at SSR locus VChr13

Finally, the following accessions exhibited a single variation: 1) varying at SSR locus VVS3 with homozygosity for the same, the samples of Malvasia volcanica (LNZ-) 2, 4, 7, 9, 10, 11, 12, 13, 14, 15, 20, 22, 23, 25, 26, 27, 30, 33, 37, 38, 39, 43, 44, 49, 51, 53, 58, 61, 62, 63, 64, 67, 70, 71, 74, 75, 77, 79, 82, and 86; 2) LNZ-16 exhibited a triallelic profile with allelic shift at SSR VVIp34; 3) accessions LNZ-31, LNZ-40, and LNZ-81 varied at SSR locus VChr9b (homozygous variation), while LNZ-41 showed heterozygous variation at SSR VVS29; 4) FTV-3, FTV-5, FTV-18, and FTV-20 presented homozygous variation at SSR VVMD28; 5) FTV-32 exhibited a heterozygous mutation at SSR VChr9b; 6) Muscat of Alexandria (LNZ-18) showed a heterozygous mutation with allelic shift at SSR VVMD5 compared to another sample of the same variety (LNZ-52) (Figure S2c); 7) The local Canary variety Bermejuela exhibited homozygous variation at SSR VRG16; 8) Finally, homozygous mutation at SSR VVS3 was observed in representatives of the Listan negro (LNZ-59) and Malvasia fina (LNZ-69) varieties.

5.2 SSR Markers Used: Effectiveness and Number of Alleles Computed

Out of the 100 SSR markers used in this study, 17 SSR (VVS3, VVS29, VVMD5, VVMD28, VMC4F3, VRG16, VVMD32, ZAG7, VChr13c, VVIv33, VVIb09, VChr9b, VChr15b, VVIn57, VChr14b, VVIp34, VChr13b) were able to detect intra-varietal variability in at least one of the four varieties (out of the seven analyzed in this study) where such variability is possible due to the presence of more than one clone (Table 1). The remaining six SSR (VVIb01, VVIv51, VRG2, VVMD14, VVIt60, VRG3) were include in Table 1 because they exhibited noteworthy specificity. For instance, SSR VVIb01 and VVIt60 showed triallelic profiles in both clones of the Malvasia Dubrovacka variety (LNZ-87 and LNZ-88). SSR VVIv51 displayed quadriallelism in the two clones of Muscat of Alexandria (LNZ-18 and LNZ-52) (Figure S2e), and triallelism in the local Canarian variety Bermejuela (LNZ-89). The only representatives of Listan negro and Malvasia fina (LNZ-59 and LNZ-69) exhibited a triallelic MP-SSR for SSR VVMD14. Finally, SSR VRG2 (Figure S2f) and VRG3 showed multiallelic profiles in Listan prieto; in contrast, VRG2 detected three alleles in Malvasia volcanica and the remaining varieties, while VRG3 detects four alleles in the same samples.

5.2.1 Effectiveness in Detecting Molecular Profiles

Table S3 presented a ranking of SSR markers classified according to their efficiency in detecting both inter-varietal and intra-varietal variability within the sampled population. Two versions of the table were provided: Table S3a highlighted the SSR that differentiated the highest number of MP-SSR profiles, whereas Table S3b focused on those with the greatest capacity to detect inter-varietal differences. As shown, although the specific markers differed between the two lists, the overall trends remained consistent.

Table S3a shows that the SSR marker distinguishing the highest number of MP-SSR profiles was VChr15b, which detected 11 distinct MP-SSR, although it did not differentiate between the Listan negro and Malvasia volcanica varieties. Nonetheless, it discriminated six different varietal groups out of the seven presents in the population, as well as one mutation in Malvasia volcanica and four variations in Listan prieto. The SSR VVIp34, VVMD32, and VChr9b followed, each of which distinguished nine MP-SSR profiles. SSR VVIp34 was able to differentiate seven varietal groups and two numerical variations within Malvasia volcanica. In contrast, VVMD32 differentiated six varietal

groups, failing to distinguish the MP-SSR of Malvasia Dubrovacka and Bermejuela, although it detected three variations among Malvasia volcanica samples. While VChr9b identified as many MP-SSR profiles as the others, it only differentiated five varietal groups, clustering Muscat of Alexandria with Listan prieto, and Listan negro with Bermejuela. Nevertheless, it detected one variation in Malvasia volcanica that coincided with a variation in Listan prieto, in addition to three other mutations within Listan prieto. In third place, a group of three SSR (VVMD5, VVMD28, VMC4F3) distinguished eight MP-SSR profiles, with all but VMC4F3 having differentiated the seven varieties in this collection of 107 accessions. As expected, based on the total number of MP-SSR detected, these SSR also identified intra-varietal variations. Sixteen SSR detected seven MP-SSR profiles (VVZAG21, VVIb01, VIn61, VRG10, VVMD24, VVIp60, VVIv67, ZAG7, VVIb32, ISV2, VChr8b, VChr3a, VMC6e10, VVMD14, VVIt60, and VChr13b). All of them, except for ZAG7 and VChr13b, were able to differentiate between the seven varieties. ZAG7, which distinguished only five varietal groups, could not differentiate between the MP-SSR of Malvasia fina, Malvasia volcanica, and Malvasia Dubrovacka, although it detected intra-varietal variability within Listan prieto. Similarly, SSR VChr13b identified five varietal groups, failing to distinguish between Listan negro, Malvasia fina, and Listan prieto, but it detected two variations in Malvasia Dubrovacka and Listan prieto. A slightly lower discrimination capacity was exhibited by the following 17 SSR, which did not distinguish between some varieties: VVS2, VVMD6, VVMD7, VVZAG79, VVIp31, ZAG112, VVIn16, VVIb66, VVIp37, VChr9a, VVIv33, VVIb09, VVIv17, VMC4D9.2, VVIp22, VRG9, and VVIn57, each of which detected six MP-SSR profiles. The subsequent group, capable of distinguishing five MP-SSR profiles, comprised 18 SSR (same as above, duplicated for clarity). Fifteen SSR detected four MP-SSR profiles (VVZAG83, VRG16, VVIq52, VVIv37, VVMD25, VVMD17, ZAG25, VChr13c, VChr10b, VVMD31, VChr11b, VChr7b, VChr13a, VVIv51, and VChr14b). Seventeen SSR detected three MP-SSR profiles (VVS3, VVUCH12, VVNTM5, VVIn73, VChr5b, VChr15a, VChr1b, VChr18b, VChr10a, VChr4a, VVMD26, VRG1, ZAG67, VRG7, VRG11, VRG15, and VChr19b). Finally, nine SSR distinguished only two MP-SSR profiles (VVS29, VChr19a, VRG4, VMC3D8, VChr12a, VChr16a, VRG2, VRG13, and VRG3), and SSR VVNTM1 did not discriminate any MP-SSR profiles.

When the classification criterion was changed to rank SSR according to their ability to detect inter-varietal variability (Table S3b) that is, whether they differentiated all seven

varieties defined in this study, the ranking shifted. Seventeen SSR exhibited maximum varietal discrimination, each distinguishing MP-SSR profiles for all seven varieties: VVIp34, VVMD5, VVMD28, VVZAG21, VVIb01, VIn61, VRG10, VVMD24, VVIp60, VVIv67, VVIb32, ISV2, VChr8b, VChr3a, VMC6e10, VVMD14, and VVIt60. Among these, VVIp34, VVMD5, and VVMD28 also exhibited a higher capacity to distinguish MP-SSR profiles, detected between eight and nine profiles, including intra-varietal variability. The following group of varieties, which fails to distinguish between two of the seven varieties present in our population, detecting only six varietal groups based on MP-SSR analysis, was compose of 18 SSR markers: VChr15b, VVMD32, VMC4F3, VVS2, VVMD6, VVMD7, VVZAG79, VVIp31, ZAG112, VVIn16, VVIb66, VVIp37, VChr9a, VVIv33, VVIv17, VMC4D9.2, VVIp22, and VRG9. This group included SSR VChr15b, which showed the highest overall discriminatory capacity within the collection, distinguishing 11 MP-SSR profiles as reported in Table S3a, as well as VVMD32 and VMC4F3, which distinguished nine and eight profiles, respectively. Twenty-three SSR distinguished five varietal groups (VChr9b, ZAG7, VChr13b, VVIb09, VVIn57, VVSD27, VVMD36, VVZAG47, VVZAG62, VVZAG64, UCH11, VVUCH19, SCU06, VMC1b11, VVMD21, VMC4G6, VVIh54, VChr8a, VChr5c, VChr18a, VVIp77, VVIv04, and VVIv70). Notably, the first five SSR in this group distinguished nine (VChr9b), seven (ZAG7 and VChr13b), and six (VVIb09 and VVIn57) MP-SSR profiles, respectively. Thirteen SSR distinguished four varietal groups (VVZAG83, VRG16, VVIq52, VVIv37, VVMD25, VVMD17, ZAG25, VChr10b, VVMD31, VChr11b, VChr7b, VChr13a, and VVIv51), all of which had an equivalent total capacity to differentiate MP-SSR profiles. A similar situation occurs with the following set of SSR markers, which is characterized by distinguishing only three varietal groups. This group consists of the following 18 SSR: VChr13c, VVS3, VVUCH12, VVNTM5, VVIn73, VChr5b, VChr15a, VChr1b, VChr18b, VChr10a, VChr4a, VVMD26, VRG1, ZAG67, VRG7, VRG11, VRG15, and VChr19b.An exception was SSR VChr13c, which also detected a mutation in Malvasia volcanica, thus identifying four MP-SSR profiles in total. Ten SSR distinguished two varietal groups (VChr14b, VVS29, VChr19a, VRG4, VMC3D8, VChr12a, VChr16a, VRG2, VRG13, and VRG3), with two exceptions: VChr14b, which distinguished four MP-SSR profiles, and VVS29, which distinguished three. Finally, SSR VVNTM1 showed no discriminatory power, as also noted in Table S3a.

5.2.2 Variations in Allele Number

The number of alleles detected for a given SSR varied depending on the variety being genotyped, as shown in Table 1, since MP-SSR were specific and unique to each variety and even to each individual (as illustrated in Figure 5 with the representative FTV-8 for VChr15b).

The variety Malvasia volcanica showed 9 SSR that detected 1, 2, 3, and even 4 alleles (Table S4). A total of 41% of the SSR were homozygous, 56% were heterozygous, 2% were triallelic, and the remaining 1% were tetraallelic. Among the homozygous SSR, two showed variations in their MP-SSR profiles. These were SSR VVS29, which exhibited a heterozygous variation in accessions LNZ-5 / LNZ-24 / LNZ-34 / LNZ-36 / LNZ-41 / LNZ-42 / LNZ-66, and SSR VVMD32, which showed: 1) heterozygous variation in samples LNZ-6 / LNZ-8; 2) heterozygous variation with numerical variation in sample LNZ-17; and 3) a triallelic case in sample LNZ-72. Six SSR showed variation within the group of heterozygous SSR. SSR VVS3 detected the highest number of variations, with 82 mutated (homozygous) individuals, followed by VChr9b with 23 homozygous individuals. SSR VChr13c and VChr15b each presented a single homozygous variation in samples LNZ-72 and LNZ-19 (Figure S2a), respectively, while SSR VMC4F3 (LNZ-76) and VVIb09 (LNZ-73) showed triallelic cases. SSR VRG2 and VVIp34 were triallelic in this variety, with the latter also presenting a triallelic profile with numerical variation in samples LNZ-16 / LNZ-60. Finally, SSR VRG3 was tetra-allelic in all Malvasia volcanica samples.

In the only variety from Fuerteventura Island (Listan prieto), this SSR kit showed the full spectrum of allele numbers previously described, as well as multiallelic individuals, although only 7 SSR detected variation among its 18 representatives (Table S5). This variety had 29 homozygous SSR, 68 heterozygous SSR, one tetra-allelic SSR (VVIp34), and two multiallelic SSR (VRG2 and VRG3). Among the homozygous SSR, five showed variation in their MP-SSR profiles: 1) ZAG7 was heterozygous in samples FTV-9 / FTV-13 / FTV-14 / FTV-16 and triallelic in sample FTV-8 (Figures 5 and S2d); 2) VChr9b showed two groups of homozygous accessions with numerical variation (FTV-8 (Figure 5) /// FTV-10 / FTV-11), two heterozygous samples (FTV-1 / FTV-32), and one triallelic individual (FTV-3); 3) VChr15b detected three groups of triallelic samples with different numerical compositions (FTV-1 / FTV-2 / FTV-13 /// FTV-4 / FTV-12 /// FTV-7) and one tetra-allelic accession (FTV-8, Figure 5); 4) VChr14b appeared as heterozygous with

three sample groups showing numerical variation (FTV-7 / FTV-16 /// FTV-12 /// FTV-8, (Figure 5); and 5) VChr13b showed heterozygous variation in sample FTV-8 (Figure 5). Among the 68 heterozygous SSR in this variety, only SSR VVS3 (FTV-2 / FTV-9 / FTV-13) and VVMD28 (FTV-1 / FTV-2 / FTV-3 / FTV-4 / FTV-5 / FTV-7 / FTV-9 / FTV-10 / FTV-11 / FTV-12 / FTV-13 / FTV-14 / FTV-18 / FTV-20) presented homozygous accessions.

The two accessions representing the variety Malvasia Dubrovacka (LNZ-87 and LNZ-88) also showed variation between them (Table S6). Twenty-three SSR expressed homozygosity in their MP-SSR profiles without any observed variation. Seventy-two SSR were heterozygous, with five SSR showing variations: homozygous (VVS3 in LNZ-87 / LNZ-88), heterozygous with numerical variation (VChr13b in LNZ-87 / LNZ-88), and triallelic (VMC4F3, VVIv33, and VVIn57 in LNZ-87). SSR VVIb01, VRG2, and VVIt60 showed triallelism in all samples, VRG3 was tetra-allelic, and VVIp34 displayed multiallelism. Table S7 shows the performance of this SSR kit for the variety Muscat of Alexandria (LNZ-18 and LNZ-52). Of the 100 SSR analyzed, 35 were homozygous, 62 were heterozygous, 1 was triallelic (VRG2), and 2 were tetra-allelic (VRG3 and VVIv51). Only one homozygous variation was detected for the heterozygous SSR VVMD5 in sample LNZ-18 (Figure S2c). The last three varieties, Listan negro (LNZ-59), Malvasia fina (LNZ-69), and Bermejuela (LNZ-89)—were each represented by a single accession. The variety Listan negro (Table S8) had 35% homozygous SSR, 61% with heterozygous profiles (with the exception of VVS3, which appeared homozygous), 3% triallelic SSR (VRG2, VVMD14, VVIp34), and one tetra-allelic SSR (VRG3). The variety Malvasia fina (Table S9) also had mostly heterozygous SSR (64 SSR´s) and presented one homozygous variation for VVS3. In addition, 33 SSR were homozygous, 2 were triallelic (VRG2 and VVMD14), and 2 were tetra-allelic (VRG3 and VVIp34). Finally, the Canarian variety Bermejuela (Tables S10 and S1) showed 28% homozygous SSR, 67% heterozygous SSR, 3% triallelic SSR (VVIb01, VRG2, VVIv51), and 2% tetra-allelic SSR (VRG2, VVIp34). One SSR likely showed variation in the sample VRG16, which was formally considered homozygous but was placed under the heterozygous column.

5.3 PEDIGREE OF THE MALVASIA VOLCANICA VARIETY

The pedigree relationship was satisfied for almost all of the 100 SSR. Three SSR were found to be discordant. Specifically, for SSR VVS3, VRG16, and VChr13b (Figure 6b),

the pedigree was not satisfied. As an example, Figure 6a shows the case of pedigree failure for SSR VVS3, as well as for SSR VChr13b (Figure 6b). In cases where one or both parents exhibited triallelism (VVIb01, VMC4F3 (Figure 6c), VVIv33, VVIv51, VVIn57, VVIt60), tetraallelism, or multiallelism (VVIp34) (Figure 6d), the pedigree was satisfied in all cases.

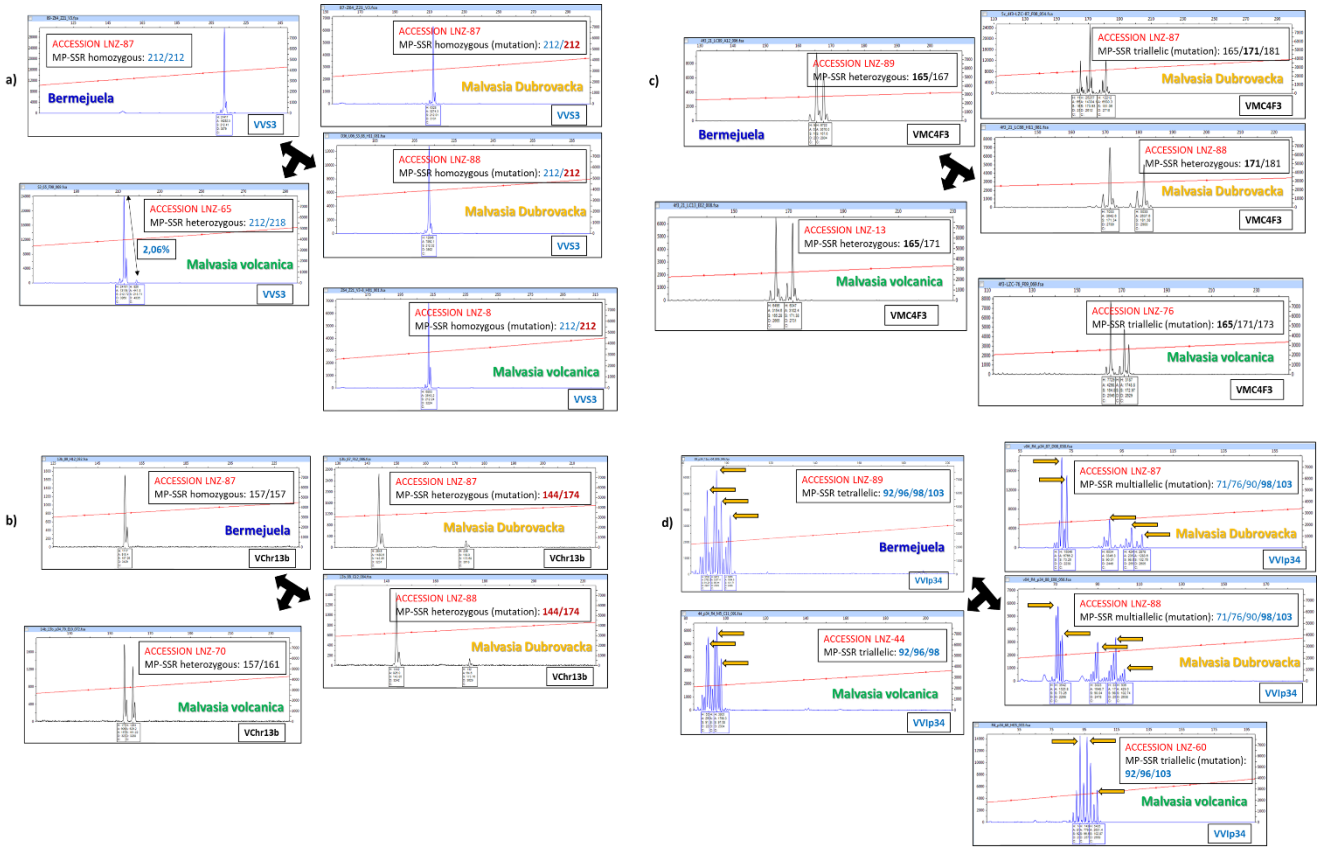

Figure 6. Pedigree of the Malvasia volcanica variety. Examples of four cases in which the parent varieties (Malvasia Dubrovacka and Bermejuela) are mutated. (a) The pedigree is not fulfilled. Malvasia volcanica is heterozygous, both parents being homozygous (although it may exhibit homozygous variation). (b) The pedigree is not fulfilled. Malvasia volcanica is heterozygous, but its alleles show a numerical change respect the Malvasia Dubrovacka. In triallelic (c) and, tetraallelic and multiallelic (c) cases, the pedigree is always fulfilled. Number in red: possible allele mutated.

6 DISCUSSION

The primary objective of this study was to investigate variations in MP-SSR markers (intra-varietal variability) within two grapevine varieties cultivated in the Canary Islands:

Malvasia volcanica (a local variety from Lanzarote Island) [8,23,24,27,28,47,48,50,52,53] and Listan prieto (a very ancient variety from mainland Spain, specifically Castilla) [54,55]. SSR markers were chosen as the genetic analysis tool in response to the historical and biogeographical uniqueness of Canary Islands vineyards [23,56], as well as the successful application of SSR in studies with samples collected across broad geographic regions [7,21]. In most European wine regions, the phylloxera crisis led to vineyard replanting with new and genetically limited materials, resulting in homogenization of plant material that often-constrained SSR' ability to detect clonal differences [57]. Conversely, Canary Islands vineyards, free from phylloxera, have preserved centuries-old plantings (over 500 years), propagated vegetatively, where the natural accumulation of somatic mutations over time has generated greater intra-varietal genetic diversity. As noted by Rade S. Jančić, vineyard aging correlates with an increase in clonal diversity [58]. The interest in Lanzarote Island was to initiate a study on its local and most extensively cultivated variety, Malvasia volcanica. For this purpose, 86 samples were genotyped, with the particularity that four accessions were identified as different varieties: Muscat of Alexandria (LNZ-18 and LNZ-52), Listan negro (LNZ-59), and Malvasia Fina (LNZ-69). This detection of MP-SSR profiles differing from the accession name (inter-varietal variability) and thus sampling errors can be logically explained. The old Canary Islands vineyards do not correspond to uniform plantations of a single variety; rather, they are mixed plantings with a high diversity of grapevine varieties. Additionally, the length of *Vitis vinifera* L. shoots [57] allows for intermixing among adjacent vines planted very closely, both in “hoyos” (planting pits) (Figure 2) and “chabocos” [27]. This explains the appearance of other varieties within this Malvasia volcanica population of 86 accessions. The study continued including these varieties to assess their behavior. On Fuerteventura Island, the focus was on the intra-varietal variability of the Listan prieto variety (18 individuals), as it is highly emblematic on this island. The low sample number reflected the historically residual viticulture on Fuerteventura due to nearly desert-like climatic conditions. Presently, it is the only island without a Protected Designation of Origin (PDO), although viticulture is resurging with the recent European legislation allowing vineyard irrigation [28].

For the reasons outlined above, the results were encouraging, with variability found in 93.46% of the accessions analyzed (100 out of 107 individuals) using 17 out of 100 SSR employed (Table 1). This overall result demonstrates: 1) SSR were effective in this

particular case, and 2) intra-varietal variability was present in the majority of samples. Specifically, for Malvasia volcanica, variability was 93.9% detected by 9 SSR, presenting heterozygous, numerically variable heterozygous, homozygous, triallelic, and numerically variable triallelic variants. SSR VRG3 was tetraallelic across all individuals, thus unable to detect variability, as was triallelic SSR VRG2 (Tables 1, 2, S4). For Listan prieto, 7 SSR detected 94.4% variability, with homozygous, numerically variable homozygous, heterozygous, triallelic, and tetra-allelic variants. SSR VVIp34 was fully tetra-allelic, and SSR VRG2 (Tables 1, 2, and S5, and Figure S2f) and VRG3 (Tables 1, 2, S5) were fully multi-allelic. For Malvasia Dubrovacka, variability reached 100%, with two samples showing heterozygous, numerically variable heterozygous, and triallelic variants across 5 SSR. These accessions exhibited cases of homozygous variations, heterozygous variations with numerical differences, and triallelism. The following SSR markers showed no capacity for differentiation: 1) VVIb01, VRG2, and VVIt60 as triallelic; 2) VRG3 as tetra-allelic; and 3) VVIp34 as multiallelic (Tables 1, 2, and S6). Among the two Muscat of Alexandria samples, only one SSR detected variability in accession LNZ-18, resulting in 50% variability. Here, SSR VRG3 was triallelic, while VVIv51 and VRG2 were tetra-allelic (Tables 1, 2 and S7). The other three varieties had only one representative each, making variation detection particularly challenging. For Listan Negro (LNZ-59), a single variation in SSR VVS3 was defined, which is included in the TECNENOL database. Notably, triallelism appeared in SSR VRG2, VVMD14, and VVIp34, and tetra-allelism in VRG3 (Tables 1, 2 and S8). Similarly, Malvasia Fina (LNZ-69) presented a single SSR VVS3 variation known from TECNENOL, with triallelism in VRG2 and VVMD14, and tetra-allelism in VRG3 and VVIp34 (Tables 1, 2 and S9). To conclude, Bermejuela showed a single presumed mutation in SSR VRG16, hypothesized based on its involvement in the pedigree of Malvasia volcanica as a progenitor (Section 3.3). SSR VVIv51 and VRG2 were triallelic, while VRG3 and VVIp34 were tetra-allelic for this variety (Tables 1, 2 and S10). Thus, the variety with the highest detected variability was Malvasia Dubrovacka, followed by Listan prieto, Malvasia volcanica, and Muscat of Alexandria showing the least variability. The other three varieties, represented by single individuals, lacked sufficient reference for conclusive analysis.

Accessions with the highest number of variations (Tables 1 and 2) were LNZ-87 (Malvasia Dubrovacka) (Figure 4) and FTV-8 (Listan prieto) (Figure 5), with five variations each. This was followed by FTV-13 with four variations (Figure S3). Three

variations were observed in two samples from Lanzarote and five from Fuerteventura. Thirty-six samples showed two variations (five from Fuerteventura), and 54 accessions had a single variation (with another five from Fuerteventura). Finally, seven samples showed no MP-SSR variation.

To interpret this classification, one may hypothesize considering that the evolution of grapevines on Lanzarote was shorter than on Fuerteventura. Between 1730 and 1736, the Timanfaya volcanic eruptions destroyed all crops [25], thus grapevine evolution on Lanzarote spans just over 300 years. In contrast, grapevines theoretically never disappeared on Fuerteventura, suggesting over 500 years of continuous evolution. Consequently, vines on Lanzarote had to be reintroduced from neighboring islands, which had uninterrupted evolution for over five centuries [25]. Moreover, natural selection shaped vines imported mainly from mainland Spain adapting to the harsh desert climate, especially on Fuerteventura, where evapotranspiration remains high due to lack of protective ground cover like *picón or rofe* [26,27].

This explains why Fuerteventura samples exhibited, both in absolute numbers and proportionally, higher variability than those from Lanzarote. Representative examples include FTV-8, FTV-13, and five additional samples from Fuerteventura with 5, 4, and 3 variations, respectively (Tables 1 and 2). Another accession with five variations was a Malvasia Dubrovacka sample from Lanzarote, possibly reflecting an evolution exceeding 500 years from another island before being introduced to Lanzarote. This sample appeared more evolved than any Malvasia volcanica sample, despite being one of its progenitors. Thus, if the crossing occurred on Lanzarote, all Malvasia volcanica samples would be less evolved, having fewer variations as they post-date the Malvasia Dubrovacka sample (LNZ-87). Regardless of the case(s), we assume that the process of incorporating variations into a given genome remains largely unknown in most instances. Although, in some cases, it may be possible to reliably estimate the timing of genome fragment introgression [59].

Regarding SSR efficiency for detecting intra-varietal variability in this grapevine collection, the best-performing SSR were those generating the highest absolute number of MP-SSR variants and, if possible, capable of distinguishing different varieties. In descending order of efficiency (Tables S2 and S3a): 1) VChr15b [44], which distinguished 6 variety groups and 5 variations, totaling 11 MP-SSR detections; 2) VVIp34 [41] with a total of 9 detections (2 variations), distinguishing 7 groups; alongside

the international SSR VVMD32, which distinguished 6 groups and 3 variations (9 total); 3) VChr9b [44], with 9 total MP-SSR detections (5 mutations), though distinguishing only 5 groups; 4) the international SSR VVMD5 and VVMD28 [34]; and 5) fifth in the ranking was the SSR marker designed by the Vitis Microsatellite Consortium, VMC4F3, which demonstrated a total efficiency that enabled the identification of 8 MP-SSR, including 2 variations and 6 varietal groups. The least efficient SSR were (Tables S2, S3a and b): 1) VVNTM1 [9,46], which failed to distinguish any MP-SSR; 2) a group of eight SSR that were only able to identify two MP-SSR (VChr19a, VRG4, VMC3D8, VChr12a, VChr16a, VRG2, VRG13, VRG3); and 3) a group of eighteen SSR with similarly low discriminatory power, each distinguishing only three MP-SSR (VVS3, VVUCH12, VVNTM5, VVIn73, VChr5b, VChr15a, VChr1b, VChr18b, VChr10a, VChr4a, VMD26, VRG1, ZAG67, VRG7, VRG11, VRG15, VChr19b, VVS29), among which only VVS29 was able to detect intra-varietal variation, but it was limited to discriminating just two varietal groups. Therefore, it can be concluded that 27% of the SSR used in this study showed little to no ability to discriminate MP-SSR. Conversely, if we consider the most efficient group to be those with the highest absolute potential to detect different MP-SSR in this collection, namely, those capable of distinguishing between 8 and 11 MP-SSR, this group represents 7% of the SSR used. If we also include those able to detect 7 distinct MP-SSR, the proportion of SSR with good discriminatory capacity rises to 23%.

Vitis vinifera L. is a diploid species characterized by a high level of heterozygosity, which confers a great capacity for inter-varietal variability [60]. This elevated heterozygosity is the result of its evolutionary history (hybridizations between local forms of the subspecies sylvestris and the domesticated subspecies) and its cross-pollinating sexual reproduction (driven by the dioecious nature of sylvestris) [61]. Nevertheless, some varieties exhibit long homozygous regions. Two main mechanisms explain this loss of heterozygosity: 1) cellular displacement in periclinal chimeras [62], and 2) chromosomal replacement or deletion events [63,64]. In our study, the variety that exhibited the highest heterozygosity was Malvasia Dubrovacka, with 72% of heterozygous loci (Table S6), followed by Listan prieto with 68% (Table S5), and Bermejuela with 67% (Table S10). Malvasia Fina presented 64% heterozygous *loci* (Table S9), while Muscat of Alexandria and Listan negro showed 62% and 61%, respectively (Tables S7 and S8). In contrast, the variety with the highest degree of homozygosity was Malvasia volcanica, with 41% of *loci* being homozygous (Table S4). The remaining percentages up to 100% are explained by the

presence of *loci* with more than two alleles. These are cases of triallelism, tetraallelism, and multiallelism (Table 1; Tables S4–S10). The emergence of such allelic configurations is primarily attributed to somatic mutations, mainly periclinal chimeras, accumulated over centuries of clonal propagation under anthropogenic selection pressure. However, as Gambino et al. [15] demonstrated, a chimeric plant does not necessarily exhibit multiallelism. Thus, in all the varieties studied within this population of 107 accessions, regions with three or more alleles have been identified (Tables 1, S4-S10). As an example, one of the most striking cases is observed in the SSR marker VVIp34, where heterozygous, triallelic, tetra-allelic, and multiallelic individuals appear, depending on the variety being genotyped (Figures 6d and 7). A frequent issue encountered is whether to consider or disregard a very small peak or family of peaks. This is an important decision, as it may determine whether an individual is classified as heterozygous or homozygous. When an electropherogram displays two families of peaks, and one of them is very small or extremely small, the smaller peak is considered valid if the peak ratio (between the smaller and the larger family) falls within or exceeds the range of 1.5 to 2 (Figures 4, 5, and S2). Otherwise, if the ratio is lower, the peak or peak family is disregarded and the MP-SSR is considered homozygous [65].

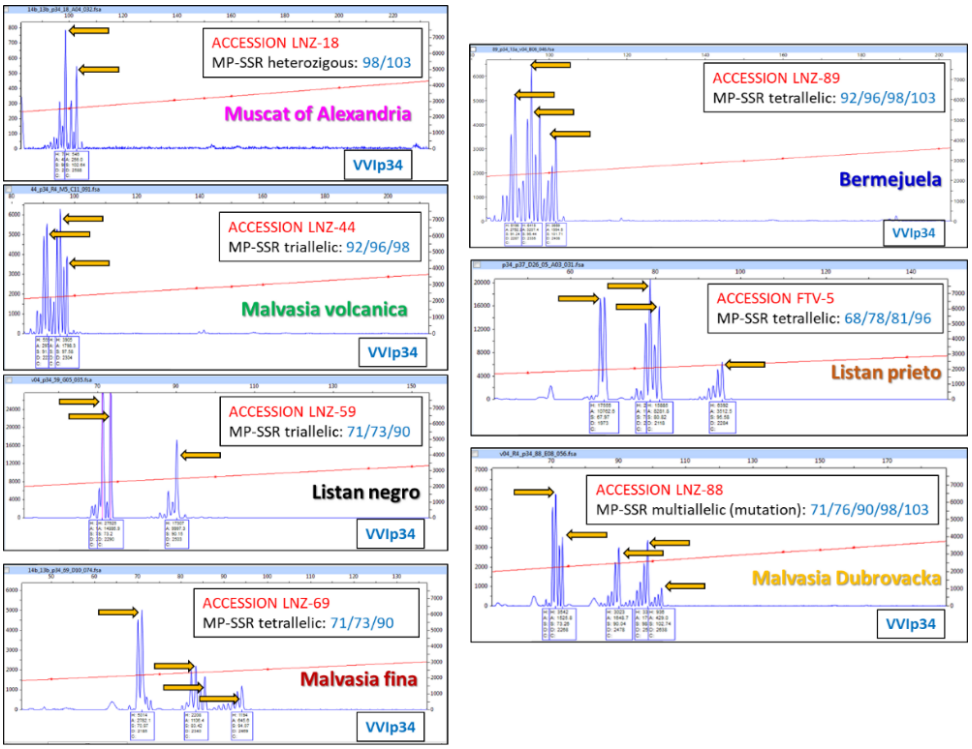

Figure 7. Detail of SSR VVIp34 showing the ability to generate heterozygous MP-SSR profiles in Muscat of Alexandria, triallelic profiles in Malvasia volcanica and Listan negro, tetraallelic profiles in Malvasia fina and Bermejuela, and multiallelic profiles in Malvasia Dubrovacka.

The possibility that Bermejuela and Malvasia Dubrovacka might be the progenitors of Malvasia volcanica was first proposed in 2006 in the book titled "*Variedades de Vid de Cultivo Tradicional en Canarias*", in which the authors hypothesized this relationship based on a study involving six SSR markers [53]. It was not until 2018 that Dr. Immaculada Rodríguez-Torres confirmed this possibility in her book "*Variedades de vid cultivadas en Canarias. Descriptores Morfológicos. Caracterización morfológica, molecular, agronómica y enológica*", using 11 SSR [50]. Subsequently, our research group published a study in the international journal OenoOne titled "Molecular characterization of Vitis vinifera L. local cultivars from volcanic areas (Canary Islands and Madeira) using SSR markers", in which this parental relationship was confirmed using 20 SSR markers [23]. As part of the present study on the intra-varietal variability of Malvasia volcanica, we considered it a suitable opportunity to confirm this pedigree using 100 SSR *loci*. However, not all SSR markers supported the proposed pedigree. Markers VVS3, VRG16, and VChr13b did not validate the parental relationships. Figures 6a and 6b provide two examples corresponding to markers VVS3 and VChr13b. In Figure 6a, the homozygous MP-SSR profiles of the two Malvasia Dubrovacka samples (variants LNZ-87 and LNZ-88) and the homozygous ARP of Bermejuela (LNZ-89) are shown, according to the TECNENOL database [8,23,24,27,28,47,48]. Also shown are the MP-SSR profiles of two Malvasia volcanica samples: one corresponding to a heterozygous ARP (LNZ-65), and the other to a mutated sample of Malvasia volcanica (LNZ-8), also recorded in the TECNENOL database [8,23,24,27,28,47,48]. The SSR VChr13b marker (Figure 6b) shows a Malvasia volcanica genotype that is heterozygous (157/161), while the putative parents are: 1) Bermejuela, which is homozygous (157/157), and 2) the two analyzed Malvasia Dubrovacka individuals, which are heterozygous (144/174) and possibly mutated (numerical variation). For this particular SSR, no information is available in the TECNENOL database [8,23,24,27,28,47,48], nor has any bibliographic reference been found for these varieties. For the SSR VRG16, the mutated parent is most likely Bermejuela, which is homozygous (250/250), while the two Malvasia Dubrovacka samples are heterozygous (240/250), and Malvasia volcanica is also homozygous (240/240). Assuming that the inconsistencies in these pedigrees are due to mutations in one of the parents, which is plausible given the low number of genotyped parental samples (only two Malvasia Dubrovacka accessions and one Bermejuela accession), it would be reasonable to affirm that the parents of Malvasia Volcánica are Malvasia Dubrovacka and the local Canarian variety Bermejuela. In cases where one or both progenitors exhibit

triallelism (e.g., VVIb01, VMC4F3, VVIv33, VVIv51, VVIn57, VVIt60), tetraallelism, or multiallelism (e.g., VVIp34), the pedigree is confirmed in all cases. Figures 6c and 6d illustrate two examples supporting these parental relationships through loci with more than two alleles.

7 CONCLUSIONS

Certainly, the most critical challenge viticulture faces in the 21st century is climate change. For this reason, experts emphasize the need to explore the biodiversity of the species *Vitis vinifera* L. at all levels. Obtaining plant material resilient to water stress, heatwaves (extreme temperatures), and excessive sunlight is essential as a key strategy to mitigate the effects of climate change. In this context, the present study explored the intra-varietal biodiversity of two grapevine varieties widely cultivated in the Canary Islands of Lanzarote and Fuerteventura (Spain). These islands are characterized by a desert climate and are strongly influenced by the trade winds and Saharan dust (Calima). In these volcanic, phylloxera-free areas, grapevines have evolved over three to five centuries, adapting to harsh abiotic conditions through both natural and anthropogenic (asexual reproduction) selection, incorporating relevant somatic mutations that have allowed them to persist to this day.

The aim of this study was to identify individuals exhibiting variation in their molecular profiles (clones) within the varieties Malvasia volcanica (a local variety from Lanzarote) and Listan prieto (a widely cultivated and extended variety in Fuerteventura). Among the 86 Malvasia volcanica accessions, inter-varietal variability was detected, identifying two Muscat of Alexandria accessions (LNZ-18 and LNZ-52), one accession corresponding to the local Canarian variety Listan negro (LNZ-59), and one accession identified as the Portuguese Malvasia Fina (LNZ-69). Additionally, 18 Listan prieto samples from Fuerteventura were analyzed. Overall, intra-varietal variability was found in 93.46% of the analyzed accessions (100 out of 107 individuals). The accessions with the highest number of variations were Malvasia Dubrovacka (LNZ-87) and Listan prieto (FTV-8), each exhibiting five variations. One Listan prieto accession (FTV-13) showed four variations. A group of seven individuals exhibited three variations, two belonging to Malvasia volcanica (LNZ-12, LNZ-72) and five to Listan prieto (FTV-1, FTV-2, FTV-7, FTV-9, FTV-12). A group of 36 samples presented two variations, while 54 accessions

showed only one variation. Finally, seven samples exhibited no variation in their MP-SSR profiles. The variety with the highest percentage of variation was Malvasia Dubrovacka (100%, based on only two individuals), followed by Listan prieto (94.4%, with 18 samples), Malvasia volcanica (93.9%, with 82 accessions), and Muscat of Alexandria (50%, with two individuals). For the remaining three varieties, no conclusive reference could be established due to each being represented by a single accession.

To study intra-varietal variability, 100 SSR markers were employed. Of these, 17 (VVS3, VVS29, VVMD5, VVMD28, VMC4F3, VRG16, VVMD32, ZAG7, VChr13c, VVIv33, VVIb09, VChr9b, VChr15b, VVIn57, VChr14b, VVIp34, and VChr13b) were informative in this population of 107 individuals. The most efficient markers were: 1) VChr15b; 2) VVIp34 and VVMD32; 3) VChr9b; 4) VVMD5 and VVMD28; and 5) VMC4F3, detecting 11, 9, and 8 MP-SSR, respectively. These correspond to only 7% of the SSR used. Including those that detected 7 distinct MP-SSR (a group of 16 SSR), the percentage of highly discriminating SSR rises to 23%. The least efficient SSR markers were: 1) VVNTM1; 2) VChr19a, VRG4, VMC3D8, VChr12a, VChr16a, VRG2, VRG13, and VRG3. Thus, 27% of the SSR used in this study (those detecting 0, 1, 2, or 3 MP-SSR) showed little to no capacity for MP-SSR discrimination.

The most homozygous variety was Malvasia volcanica, followed by Listan negro, Muscat of Alexandria, Malvasia Fina, Bermejuela, and Listan prieto. The most heterozygous variety was Malvasia Dubrovacka.

The pedigree of Malvasia volcanica is supported by the results from 100 SSR markers, under the assumption that for three SSR, one of the parents exhibits variation (mutation).

Therefore, under the studied conditions (phylloxera-free, volcanic, and isolated areas), it is possible to detect intra-varietal variability using SSR (microsatellite) markers.

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9 SUPPLEMENTARY MATERIALS

The following supporting information can be downloaded at: SUPPLEMENTARY MATERIAL.xlsx, Table S1. List of the 107 accessions collected on Lanzarote Island. General information on the resulting analyzed variety (VIVC) and entry registration details; Table S2. List of primers used for the amplification of the selected microsatellite regions. Characteristics; Figure S1. Approximation of the genomic SSR map used in this study. Consensus location of each of the regions selected for molecular characterization; Figure S2. Examples of samples showing variation (mutation) compared to the most widespread profile (ARP), including their replicates. Nº %: Examples of peak ratios (percentage indicating the ratio between the smallest and largest peaks), used to disregard extremely small peaks; Figure S3. Listan prieto profiles (FTV-13) showing variations compared to the most widespread or reference molecular profile (ARP). On the left, ARP electropherograms; Table S3a. List of SSR markers ranked from the most informative, detecting both intra-varietal and inter-varietal variability, to the least informative, which did not detect any variability, meaning it only identified a single MP-SSR. MofA: Muscat of Alexandria; LN: Listan negro; MF: Malvasia fina; MV: Malvasia volcanica; MD: Malvasia Dubrovacka; B: Bermejuela; LP: Listan prieto. Dark green cells correspond to SSR markers that did not detect any variation. Light green cells correspond to SSR markers that detected variation; Table S3b. List of SSR markers ranked from the most informative in distinguishing varieties to the least informative, which did not detect any differences, meaning it only identified a single MP-SSR. It is important to note that seven varieties were identified in this study. MofA: Muscat of Alexandria; LN: Listan negro; MF: Malvasia fina; MV: Malvasia volcanica; MD: Malvasia Dubrovacka; B: Bermejuela; LP: Listan prieto. Dark green cells correspond to SSR markers that did not detect any variation. Light green cells correspond to SSR markers that detected variation; Table S4. List of SSR markers showing predominant homozygosity, heterozygosity, and multiallelism in the Malvasia volcanica variety. *: Accessions showing variation; Table S5. List of SSR markers showing predominant homozygosity, heterozygosity, and multiallelism in the Listan prieto variety. *: Accessions showing variation; Table S6. List of SSR markers showing predominant homozygosity, heterozygosity, and multiallelism in the Malvasia Dubrovacka variety. *: Accessions showing variation; Table S7. List of SSR markers showing predominant homozygosity, heterozygosity, and multiallelism in the Muscat of Alexandria variety. *: Accessions showing variation; Table S8. List of SSR markers showing predominant homozygosity, heterozygosity, and multiallelism in the Listan prieto variety. *: Accessions showing variation; Table S9. List of SSR markers showing predominant homozygosity, heterozygosity, and multiallelism in the Malvasia fina variety. *: Accessions showing variation; Table S10. List of SSR markers showing predominant homozygosity, heterozygosity, and multiallelism in the Bermejuela variety. *: Accessions showing variation.