Molecular characterization of *Vitis vinifera* L. local cultivars from volcanic areas (Canary Islands and Madeira) using SSR markers

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ABSTRACT

Aim: This study characterises and identifies 79 grapevine accessions from the Canary Islands and 14 from Madeira using simple sequence repeat (SSR) analysis.

Methods and Results: A kit of 20 microsatellites or simple sequence repeats (SSRs) were used to obtain the molecular profiles of the 93 accessions in this study. The results allowed us to identify four new cultivars (Bienmesabe tinto, Burra volcanica, Vallera, Verijadiego negro), two new colour mutations (Listan rosa, Mollar cano rosado) and two unknown molecular profiles from Madeira. Furthermore, we propose that eight names of varieties be included in the *Vitis* International Variety Catalogue (*V*IVC) as prime names, and 38 accession names as synonyms, 19 of which are regarded as new synonyms of the 12 varieties. Finally, we also reported eight cases of mislabelling. The study of genetic structure shows that the cultivars from the Canary Islands and Madeira are strongly influenced by the Iberian Peninsula. We propose that 14 varieties and three sports (mutations) at are local to the Canary Islands: Albillo criollo, Bermejuela, Bienmesabe tinto, Burra volcanica, Albillo forastero, Huevo de gallo, Listan negro, Listan rosa, Malvasia di Sardegna rosada, Malvasia volcanica, Mollar cano rosado, Torrontes volcanico, Sabro, Uva de año, Vallera, Verijadiego, and Verijadiego negro.

Conclusions: It has been reliably shown that these cultivars have a characteristic genome: phylloxera never reached the Canary Islands so mutations, hybridations and human selection have been able to accumulate over 500 years. It is of great importance that this local plant material be conserved, given that it is part of our vine heritage. In the case of Madeira, it has not been possible to propose any local varieties because phylloxera did reach the island; this devastated the vineyards and there was a drastic reduction in local varieties. However, one unknown cultivar was the most characteristic genotype from this region.

Significance and impact of the study: This study shows the existence of non well-known varieties of *Vitis vinifera* L. that may be used to elaborate original wines, offering therefore new organoleptic sensations for the consumers. Furthermore, these results suggest that this volcanic area could be considered as one centre of origin of new cultivars of *Vitis vinifera* L. (Biodiversity Centre).

KEYWORDS

grapevine, microsatellite, identification, volcanic islands, DNA, molecular profile

INTRODUCTION

Vitis vinifera L., the grapevine, is one of the most important fruit species in the modern world. It is indigenous to southern Europe and western Asia, and is today cultivated worldwide. Wine and table grape sales contribute significantly to the economy of the major wine-producing countries. *Vitis vinifera* L. has a diploid genome with 38 haploid chromosomes and an estimated genome size of ~500 Mbp. Grape genotypes are highly heterozygous and nearly all modern cultivated varieties (cultivars) are hermaphroditic, self-fertile and out-cross easily (This *et al.*, 2006).

Macaronesia is a group of four North Atlantic archipelagos that extends outwards from the southwest (SW) of Europe to the southwest (NW) of Africa. This group consists of the Azores, Madeira, the Canary Islands and Cape Verde (Santamarta and Naranjo Borges, 2015). The present study focuses on the volcanic archipelagos of the Canary Islands and, to a lesser extent, on Madeira.

The Madeira archipelago is an autonomous region of the Republic of Portugal, which is 520 km from the African coast and 1,000 km from the European continent. It includes the islands of Madeira, Porto Santo, and the Desertas Islands, which are administered together with the separate archipelago of the Savage Islands. The general climate of the Madeira archipelago is mild, oceanic and humid, with moderate rainfall. It is greatly influenced by the subtropical anticyclone of the Azores and is mainly governed by the trade winds from the north and northeast (GEVIC (Gran Enciclopedia Virtual Islas Canarias), 2007).

The Canary archipelago is one of the autonomous communities of Spain. The islands sit just off the northwest coast of mainland Africa, 100 km west of the border between Morocco and Western Sahara. The main islands, from largest to smallest, are Tenerife, Fuerteventura, Gran Canaria, Lanzarote, La Palma, La Gomera, El Hierro and La Graciosa. The archipelago also has other islands and islets: Alegranza, Isla de Lobos, Montaña Clara, Roque del Oeste and Roque del Este. The climate is tropical and desert, moderated by the sea and in the summer by the trade winds. However, that can vary considerably as a function of altitude, orientation and orography, and different areas (coasts, mid-lying areas and mountain areas) have quite different precipitation, humidity, winds, etc. Generally, the precipitation patterns show little and highly varied rainfall. The variety of ecosystems and microclimates is favourable to the existence and development of many plant species, such as *Vitis vinifera* L. (GEVIC (Gran Enciclopedia Virtual Islas Canarias), 2007).

Several hypotheses have been put forward to explain the colonization of *Vitis vinifera* L. in the Canary and Madeira Islands. One hypothesis dates back to a half century before Christ, when Quinto Horacio Flaco claimed that "unpruned vine flowered continuously in the fortunate" (Moralejo Alvarez, 2011). However, for some time, it has been widely accepted that the vine was not part of the original flora of the Canary Islands and Madeira. Nevertheless, since seeds of the *Vitaceae* family have been found in several archaeological sites in the Canary Islands (Arco *et al.*, 2000), the theory that the vine is not part of the autochthonous flora has been reconsidered. The vine seeds found also seem to be wild and very similar to the North African biotypes of *Vitis vinifera* L. Thus, populations of *Vitaceae* may have existed in the Islands before the arrival of the first human group, a thesis that may seem obvious because, like all the native flora of the Archipelago, these plants have a tertiary origin; however, also like other populations of this flora, wild *Vitaceae* disappeared for reasons that are still unknown (Macías, 2005). Consequently, the first cultivated vine varieties were introduced by European colonization. Europeans first visited the Fortunate Islands (Macaronesia) until the fourteenth century, but it was not until the fifteenth century that monks, explorers, conquerors and traders introduced the first varieties of cultivated vine (domesticated).

The 19th century was marked by the entry of two major pests: powdery mildew (1852) and mildew (1878), but surprisingly the Macaronesia archipelagos (except Madeira) were not attacked by the phylloxera plague that devastated European vineyards and caused a drastic reduction in local varieties. It is for this reason that the Canary vineyards are considered to be the last stronghold of some of these varieties (Hidalgo, 2011). Today many viticulturist and growers in these islands can see new phenotypic features appearing in their vines. These new phenotypes may be due to hybridizations and/or genetic mutations accumulated over five

centuries. Therefore, selection (natural or anthropological) together with vegetative propagation (natural or anthropological) could have made significant changes to the first vine phenotypes over time, and have given rise to new varieties or different clones within the same variety (López *et al.*, 1993).

SSRs have been the most widely used markers for genotyping plants over the past 20 years because they are highly informative, codominant and multi-allele genetic markers (Ibáñez *et al.*, 2003). Moreover, SSRs are experimentally reproducible and transferable among related species. They are enormously useful in studies of population structure, genetic mapping and evolutionary processes and also for characterizing and identifying individuals (Emanuelli *et al.*, 2013).

The aim of this study was the molecular characterization of 79 accessions from the Canary Islands (El Hierro, La Gomera, La Palma, Lanzarote and Tenerife) and 14 accessions from Madeira (Madeira archipelago) using the simple sequence repeats (SSR) technique to plan germplasm conservation and to find genetic sources for breeding. More specifically, we carried out the detection of different errors (synonyms, mislabelling, etc.), evaluated genetic relationships among different varieties (possible pedigrees), and studied the genetic structure of this gene pool. The phylogeny and genetic relationships among grapevine cultivars is of great importance in genetic improvement, the preservation of biodiversity and the exploitation of traditional wines (Emanuelli *et al.*, 2013). Furthermore, this study may help to detect new genotypes and minimize the homogenization of wines from the Canary Islands and Madeira, and it would provide new material for breeding. Other equally important aspects to consider are the updating of the *Vitis* International Variety Catalogue Database (*V*IVC) and the possibility of discovering and demonstrating the uniqueness of the varieties of the Canary archipelago.

MATERIAL AND METHODS

1. Plant material

Ninety-three mature grapevine leaves (*Vitis vinifera* L.) were collected from the Canary Islands and Madeira (79 and 14 accessions, respectively), and conserved at -20°C until they were processed. Four well-characterized cultivars were also included as control samples (Marsal *et al.*, 2011): Chardonnay blanc, Garnacha blanca, Tempranillo tinto and Cabernet sauvignon cultivar plants from the Rovira i Virgili University experimental vineyard in Constantí (AOC Tarragona, Spain; 41°9′16.04″ (N) and 1°11′1.28″ (E)). Supporting Information 1 provides detailed information on all the accessions and all other necessary information.

2. Microsatellite genotyping

DNA was extracted using the method described by Marsal *et al.* (2013) (based on Fort *et al.* (2008) and Marsal *et al.* (2011) protocols). The grapevine genepool was genotyped at 20 SSR markers, which were selected for their capacity for discrimination and polymorphism in agreement with previous studies: VVS2, VVS3, VVS29 (Thomas and Scott, 1993); VVMD5, VVMD6, VVMD7 (Bowers *et al.*, 1996); VVMD27, VVMD28, VVMD36 (Bowers *et al.*, 1999b); VrZAG21, VrZAG47, VrZAG62, VrZAG64, VrZAG79, VrZAG83 (Sefc *et al.*, 1999); scu06vv (Scott *et al.*, 2000); VvUCH11, VvUCH12, VvUCH19 (Lefort *et al.*, 2002); VChr19a (Cipriani *et al.*, 2010). The international scientific community (This *et al.*, 2004; Maul and Röckel, 2015) uses seven of these as reference genetic markers.

Microsatellite amplifications were performed using polymerase chain reaction (PCR) analysis and a MyCycler thermocycler (BioRad Laboratories, Hercules, CA, USA). PCR was carried out with 50 ng of DNA and 1 µM of each primer, with an attached fluorescent dye in the upper primer (6-FAM: VVS3, VVMD7, VVMD28, VVMD36, VrZAG47, VrZAG62, VrZAG83, VvUCH11 and VvUCH19; HEX: VVS2, VVS29, VVMD6, VVMD27, VrZAG21, VrZAG79 and VChr19a; NED: VVMD5, VrZAG64, scu06vv, VvUCH12) using the AmpliTaq DNA Polymerase kit (Applied Biosystems, Foster City, CA). The SSRs were divided into three groups according to Marsal *et al.* (2011). The amplification products were mixed with 20 µL of deionized formamide and 0.5 µL of DNA size standard (GeneScan 500-ROX, Applied Biosystems), and denatured at 95 ˚C for 5 min. The fragments were separated by capillary electrophoresis with an ABI PRISM 3730® Genetic Analyzer (Applied Biosystems). Peak Scanner Software (Applied Biosystems) was used to size the amplified fragments. Each

cultivar was analysed twice to prevent possible errors.

3. Data analysis

GenAlEx 6.5 software (Peakall and Smouse, 2012) was used to estimate the four genetic parameters: the number of different alleles (Na), the number of effective alleles (Ne), observed heterozygosity (Ho), and expected heterozygosity (He). The probability of identity (PI) and the estimated frequency of null alleles (r) were calculated using th software Identity 1.0 (Wagner and Sefc, 1999). To distinguish homozygotes and heterozygotes for each locus, the data were considered codominant for the purposes of data analysis.

Population structure and identification of admixed individuals was performed using the model-based software program Structure 2.3 (Pritchard *et al.*, 2000; Falush *et al.*, 2003), which is a model-based Bayesian clustering method. In this model, a number of populations (K) are assumed to be present, and they are each characterized by a set of allele frequencies at every locus. Individuals in the sample are assigned to populations (clusters), or jointly to more populations if their genotypes indicate that they are admixed. All loci are assumed independent, and each K population is assumed to follow the Hardy–Weinberg equilibrium. The subsequent probabilities were estimated using the Markov Chain Monte Carlo (MCMC) method. The MCMC chains were run with a 100,000 burn-in period, followed by 1,000,000 iterations using a model allowing for admixture and correlated allele frequencies. The structure was run at least ten times by setting K from 1 to 15, and an average likelihood value, L (K), was calculated across all runs for each K. The mean log probability of the data for each K was calculated to determine the most appropriate number of clusters, and the value of K for which this probability was highest was selected. The ΔK was then calculated using the method described by Evanno *et al.* (2005). ΔK is a quantity based on the rate of change in the log probability of the data between successive K values.

In addition, principal coordinate analysis (PCoA) in GenAlEx 6.5 was used to further examine the genetic relationships between subpopulations on the basis of the same SSR data. PCoA was based on the standardized covariance of the genetic distances calculated for codominant markers.

The frequency-based assignment test (Paetkau *et al.*, 1995; Paetkau *et al.*, 2004), also available in GenAlEx 6.5, was first used to assign the accessions to each subpopulation generated by Structure. For each accession, a log likelihood value was calculated for each subpopulation using the allele frequencies of the respective subpopulations. An individual was assigned to the subpopulation with the highest log likelihood value.

Identity 1.0 software was also used to identify putative parentage relationships (Wagner and Sefc, 1999). This software prepares a list of the probable parent-progeny relationships on the basis of codominant inheritance (i.e. when the progeny receives one allele from one parent and the other allele from the other).

RESULTS

1. SSR polymorphism

The characterization of the efficiency of the 20 SSR markers studied is shown in Supporting Information 2 for a population of 44 varieties, which correspond to 41 molecular profile-SSR (MP-SSR) that are unique (without sports). In total, 49 out of the 93 initial accessions were not included because the results obtained indicated that they were synonyms of other accessions. Our population displayed between 5 and 29 alleles per locus, with a total of 257 alleles over the 20 loci, an unbiased expected heterozygosity (He) of 78.7 %, and 14 markers had an "r" value (the value of null alleles) lower than or equal to 0.01. The probability of obtaining identical genotypes using all 20 markers is 2.4×10^{-26} (cumulative PI*).

Seven SSRs for all 44 cultivars identified are shown in Supporting Information 3, together with the four control samples. In this case, the 14 varieties proposed as local next to the three colour mutations from Canary Islands were highlighted (14 varieties in blue and three cases of sports in green).

2. Cultivar analysis

2.1. Confirmation of accession name

The objective of this analysis was, first, to carry out an exhaustive bibliographic study to find out whether the name of the accession is

internationally known or not, and, second, to compare the MP-SSR obtained through microsatellite analysis with the MP-SSR found in the bibliographic sources (VIVC, Maul and Röckel, 2015; Rodríguez-Torres, 2018; *Vitis* Canarias, 2015).

For this reason, the 93 accession names were reviewed in the ampelographic section of the VIVC (Maul and Röckel, 2015), and in other bibliographical resources specialized in varieties cultivated in the Canary Islands (Zerolo *et al.*, 2006; Rodríguez-Torres, 2018; *Vitis* Canarias, 2015). All the information obtained from this bibliographic study is summarised in Supporting Information 4.

A total of 41 grapevine varieties and three colour mutations from the 93 accessions analysed were identified by the bibliographic study and the microsatellite analysis (Supporting Information 1, 3, and 4). Moreover, 49 accessions were synonyms or repetitions of other accessions. Of the 93 accessions, 91 were identified, of which there were 41 different varieties and three sports. Four of these genetic profiles coincide with others that do not have a described name. For that reason, we considered it necessary to give them a specific name after discussing with the scientist who identified them (I. Rodríguez-Torres). The selection of these new names took into account their history, their morphology or their organoleptic characteristics and the following prime names are proposed: Bienmesabe tinto, Burra volcanica, Torrontes volcanico and Vallera. Two other accessions were from two different cultivars that did not match any known genotype in the databases consulted (VIVC, Maul and Röckel, 2015; Rodríguez-Torres, 2018; *Vitis* Canarias, 2015). These genotypes were referred to as unknowns 2 and 3. Curiously, they were from Madeira. In Supporting Information 1 and 4 there is one prime name with number 1 not registered by VIVC (Maul and Röckel, 2015). This prime name (Uva de año) was used by other authors (Rodríguez-Torres, 2018). There are also seven names with number 2 (Bienmesabe tinto, Burra volcanica, Listan rosa, Mollar cano rosado, Torrontes volcanico, Vallera and Verijadiego negro) not registered by VIVC and the authors of this article and other authors specialized in Canarian cultivars (Rodríguez-Torres, 2018) propose should be included in VIVC. The prime names with number 3 are six varieties (Bienmesabe tinto, Burra volcanica, Torrontes

volcanico, Uva de año, Vallera and Verijadiego negro) with their MP-SSR not included in the VIVC (Maul and Röckel, 2015). It should be pointed out that there are 19 new accession names (highlighted in bold) and 19 existing accession names (highlighted in purple) that are not recorded as a synonyms of their corresponding cultivars nor have any bibliographic support in the VIVC (Maul and Röckel, 2015). Finally, eight accessions were cases of mislabelling (highlighted in red and by the symbol #).

It should be emphasized that the names proposed as "new prime name" or "new synonym" are only the names of the samples analysed, which were provided by the viticulturists (original information in Supporting Information 1). Therefore, other names used in the Canary Islands, and which may be valid candidates, have not been considered. In addition, unfortunately, the Madeira viticulturists did not provided us with any information about the samples.

2.2 Proposal of local varieties: bibliography study

After all cultivars had been identified and characterized, the next aim was to find which of them might be considered to be local varieties from the Canary Islands and Madeira. The two unknown varieties (from Madeira) were not included in the study because there was no available information about them. Consequently, our exhaustive bibliographical study (based on historical and lexical terms) led us to select 14 of the 41 identified varieties and three sports, given that most of them are mentioned in the specialised bibliography in cultivars grown in the Canary Islands and Madeira (Zerolo *et al.*, 2006; Rodríguez-Torres, 2018; *Vitis* Canarias, 2015). Specifically the following genetic profiles are proposed as local: Albillo criollo, Bermejuela, Bienmesabe tinto, Burra volcanica, Albillo forastero, Huevo de gallo, Listan negro, Listan rosa, Malvasia di Sardegna rosada, Malvasia volcanica, Mollar cano rosado, Sabro, Torrontes volcanico, Uva de año, Vallera, Verijadiego and Verijadiego negro. All are highlighted in Supporting Information 1 and 4 (14 varieties in blue, and the three sports in green). Curiously, none of the varieties proposed as local were from Madeira. The remaining 27 varieties were regarded as foreign.

3. Putative parentage relationships

Parentage was assessed using cultivars from the Canary-Madeira population used in this study. A total of three complete pedigrees and one incomplete pedigree were found in this population: 1) Malvasia di Sardegna x Bermejuela: Malvasia volcanica (Zerolo *et al.*, 2006; Rodríguez-Torres, 2018); 2) Palomino fino x Mollar cano: Listan negro (Zerolo *et al.*, 2006; Rodríguez-Torres, 2018); 3) Palomino fino x Verdelho branco: Albillo Forastero and Albillo criollo (Rodríguez-Torres, 2018); and 4) Alfrocheiro x Heben: Malvasia fina (Rodríguez-Torres, 2018). This last lineage was only obtained with data from Alfrocheiro as Heben was not present in this collection.

4. Genetic structure

4.1 Genetic structure of the Canary-Madeira population

This section focuses on the genetic structure of the Canary-Madeira population, and in particular, on the 14 cultivars and three sports proposed as local varieties from the Canary Islands. Although the Canary-Madeira population consisted of 44 varieties, three of them were not taken into account because they were closely related material; specifically, one sport included in the local population (Listan rosa) and two cultivars not included in the local population (Malvasia di Sardegna and Mollar cano). Three other cultivars were also excluded because they are interspecific crossing (Isabella and Flot rouge) or an author crossing (Malvasia branca de Sao Jorge). These genetic profiles were therefore regarded as redundant (Cabezas *et al.*, 2011; Marsal *et al.*, 2016; Marsan *et al.*, 2017) or as an artefact to *Vitis vinifera* L. population, or as an artificial crossing. These resting 38 genetic profiles were chosen for genetic structure analysis, 16 of which were proposed as local from the Canary Islands. The analysis was firstly made using the Structure software, which provided several genetic distributions for the population. Supporting Information 5 shows the distributions given by the Structure program, with K calculated using the method described by Evanno *et al.* (2005). In accordance with Supporting Information 5 (the graphical representation of ∆K), the best distribution was K=4 (4K). Therefore, the Canary-Madeira population was studied in detail when it was divided into four groups. The

distribution of the varieties made by the Structure program is showed in Figure 1. In this case, the varieties in each group have been ordered from highest to lowest according to their q value (the percentage of their inferred genome belonging to the cluster (Bacilieri *et al.*, 2013; Marsal *et al.*, 2017)). Therefore, when the population of 38 genotypes is divided into four subpopulations, nine cultivars are assigned to subPOP1 (six members with q ≥ 85 %), six to subPOP2 (four with q ≥ 85 %), 13 to subPOP3 (seven with q ≥ 85 %), and the remaining 10 to subPOP4 (seven with q ≥ 85 %). A total of 56 % of the varieties of the subPOP1 are from Portugal, 22 % are from Spain, 11 % are from France and the origin of the rest is uncertain. This subpopulation includes both white and red grape varieties (55 % white cultivars and 33 % red cultivars), and they are mainly used for winemaking (67 %). It is interesting to note that two (Albillo criollo and Vallera) of the 16 proposed local varieties placed in this subpopulation were considered as pure specimens. All members of subPOP2 are from Spain. This subpopulation also includes both red (33 %) and white grape varieties (67 %), wine cultivars (67 %) and "double-use" cultivars (table and wine) (34 %). In this subpopulation, there are three pure local variety from the Canary Islands (Burra volcanica, Verijadiego negro and Huevo de gallo), and one admixed cultivar (Sabro). SubPOP3, the third cluster, consists of 13 cultivars, and 77 % of these are from Spain. In this cluster, 69 % are white grape varieties and 54 % are used for winemaking. The rest are table or "double-use" cultivars. Nine of them were local varieties (Malvasia di Sardegna rosada, Malvasia volcanica, Mollar cano rosado, Bermejuela, Torrontes volcanico, Uva de año, Listan negro, Verijadiego y Albillo forastero). However, the Listan negro, Verijadiego y Albillo forastero varieties were considered to be an admixed genotype (q < 85 %). Finally, subPOP4 is more heterogeneous in terms of the origin of the varieties, given that 40 % are from Spain, 20 % from France, 20 % from Portugal, 10 % from Switzerland, and the remaining 10 % are of unknown origin. In this group, the red grape varieties are predominant, but 40 % are wine cultivars and the rest are table or "double-use" cultivars. In this group, there is one pure local cultivar (Bienmesabe tinto). PCoA (Figure 2) was carried out only on the 24 varieties for which at least 85 % of their inferred genome belonged to the cluster (q ≥ 85 %). Subsequently,
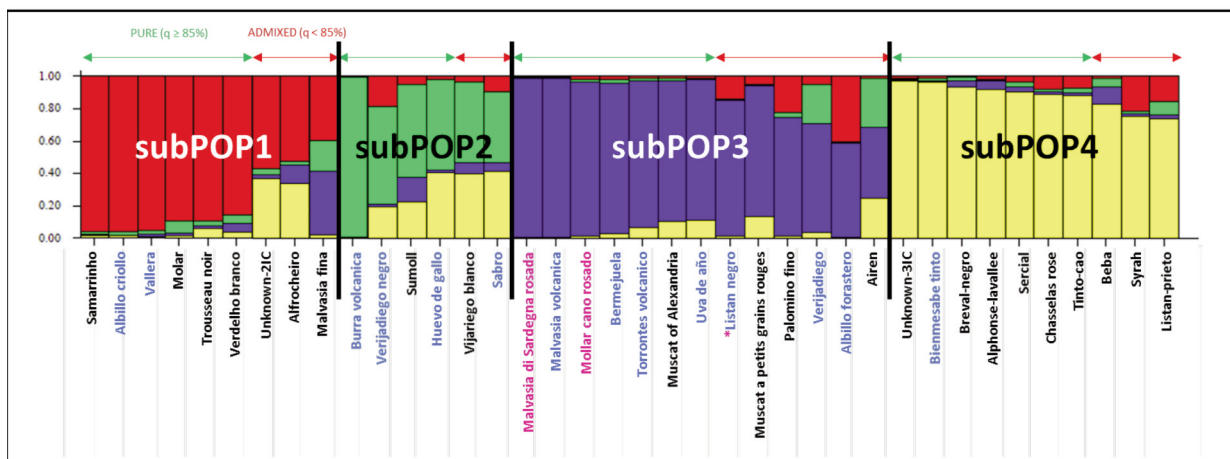
FIGURE 1. Representation of 38 individuals from Canary-Madeira Collection (IC-M) by Structure software, when the population is divided in 4 groups (4K). The green arrows include the cultivars that have a q ≥ 85 % and that are therefore selected for the study of the population structure. In this way, the population of 38 individuals will have 24 non admixed individuals (pure).

the GenAlEx 6.5 program carried out the assignment test, and assignment was deemed good in 96 % of cases (data not shown). Figure 2 shows the distribution of the 24 individuals when the population is divided into four groups (4K). Coordinate 1 explained 12.9 % of the overall variance, and it separated most of the individuals in the subPOP1, subPOP2 and subPOP4 (mainly located in the right-hand quadrants) from the subPOP3 subpopulation (located in the left-hand quadrants). Coordinate 2 explained 9.3 % of the total variance, and it separated the local varieties (highlighted in blue or purple, and mainly situated in the lower quadrants) from the foreign varieties.

4.2 Genetic structure of the local Canary varieties in a world population

The first step of this section was analysing the behaviour of a group of 17 varieties in comparison with a world population consisting of 290 varieties from 21 countries. We used a world population characterized by Marsal *et al.* (2017), without mutations (and sports), author crossing and interspecific crossing. Specifically, this group of 17 varieties includes the 14 local cultivars without sports (Albillo criollo, Bermejuela, Bienmesabe tinto, Burra volcanica, Albillo forastero, Huevo de gallo, Listan negro, Malvasia volcanica, Sabro, Torrontes volcanico, Uva de año, Vallera, Verijadiego and Verijadiego negro), two unknown cultivars (Unknown-2 and Unkown-3) and another Canarian local cultivar

(Albillo monte Lentiscal) that was not present in the samples provide for this study but was analysed for our group in a previous study. Both the world population and the Canary-Madeira population were identified using the same 20 SSRs. As in the previous section, the Structure program was used to obtain the most probable subpopulations (no. K). Supporting Information 6 shows the distribution of the 290 varieties given by the Structure program, with K being calculated by the method described in Evanno *et al.* (2005). Concerning the graphical representation of ΔK, the best distribution for this world population was for "K=2" (2K). Supporting Information 7 shows the detailed distribution of all the individuals when the population is divided into two groups alongside other information. Within each group, the varieties were ordered according to their q value. The cultivars are highlighted in different colour tones according to their q value. The admixed genotypes (q < 85 %) were not included in the genetic structure analysis. Therefore, the world population decreased from 290 to 251 genomes after the varieties with q ≥ 85 % had been selected. Then, assignments were made by the GenAlEx 6.5 program, and were successful in all cases (data not show). In this case, the subPOP1 consisted of 181 cultivars and the subPOP2 consisted of 109 varieties. Of the cultivars located in subPOP1, none is considered to be local to the Canary Islands: 29.8 % are from Italy, 27.6 % originate from France and Central Europe, 26 % are from the

Iberian Peninsula and 13.8 % are from Balkan Peninsula. The resting varieties are from the Near East of the Mediterranean Basin, the Caucasus or North Africa. Another characteristic of this group is that 54.7 % of its varieties are red grapes, 43 % are white, and the rest are rosé or rouge. In this subpopulation, 77 % of the varieties are used for winemaking, 21 % are "double-use" (table grapes and winemaking), and only the remaining 2 % are table varieties or triple use (raisin, table or wine). The subPOP2 is characterized by having 66 % of its members from Spain (14.6 % from Canary-Madeira group), and 17.4 % are from Italy, and the rest are from the Balkan Peninsula, the Near East, France and Central Europe. Another characteristic of the cultivars belonging to the subPOP2 is that 49.5 % are red grape varieties, 45.8 % are white, and the remaining 4.7 % are rosé or rouge grape varieties. Most are used for winemaking (66.7 %). Curiously, the 16 varieties proposed as local from the Canary and Madeira Islands can only be found here (although Albillo criollo and Unknown-2 are regarded as admixed). In the second step, we treated the more characteristic varieties of the Canary-Madeira Collection (13 local varieties and Unknown-3IC) as a single subpopulation (subPOP IC-M) to determine how it was related to the subPOP1 and subPOP2. In this case, the assignment test had a success rate of 95 % (data not shown). Figure 3-a shows the distribution of the subPOP1, subPOP2 and subPOP IC-M. Coordinate 1 explained 75.85 % of the overall variance, whereas Coordinate 2 explained 24.15 %. This representation clearly shows a split between the subPOP IC-M and the subPOP2, given that they are in opposite quadrants (at the ends). Although subPOP1 is in the lower quadrants with subPOP IC-M, the two subpopulations are very distant from each other, with subPOP1 in the right quadrant and subPOP IC-M in the left quadrant. In general, it can be observed that the subPOP IC-M is very distant from the other subpopulations, which is confirmed by the Fst values (data not show), as the subPOP IC-M has higher Fst values than the other subpopulations. The parameter of Fst is the correlation of randomly chosen alleles within the same subpopulation relative to the entire population; equivalently, the proportion of genetic diversity due to allele frequency differs among populations. The fixation index can range from 0 to 1, where 0 means complete sharing of
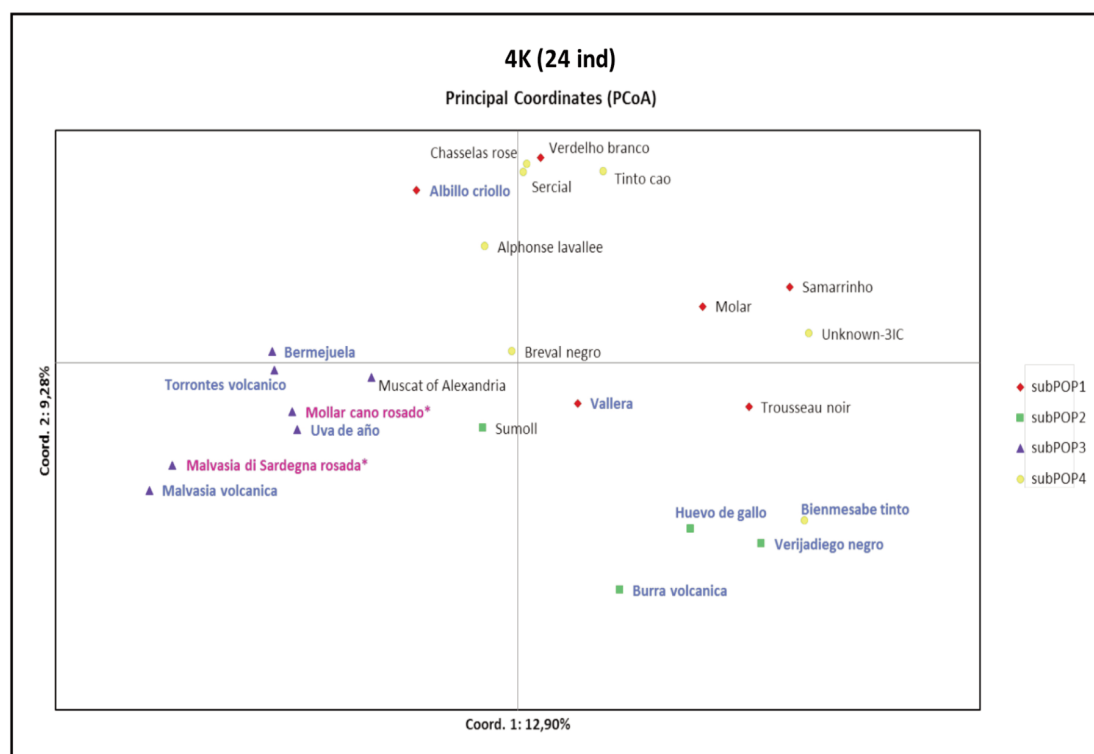
FIGURE 2. Representation of 24 non admixed individuals (q ≥ 85 %) from Canary-Madeira Collection by Principal Coordinates Analysis when the population is divided in 4 groups (4K).

genetic material and 1 means no sharing (Holsinger and Weir, 2009). However, the subPOP IC-M is much closer to that of subPOP2 (as expected, given that the varieties of subPOP IC-M initially belonged to the subPOP2). The main characteristic of the subPOP2 is that most of its cultivars are from the Iberian Peninsula and Italy. The subPOP1 is mainly made up of cultivars from Italy, France and Central Europe, and the Iberian Peninsula.

We also studied the relationship between the subPOP IC-M and six other subpopulations created from the geographical origin of the cultivars. The varieties were grouped in areas (clusters of nearby countries) because some of the countries have few varieties (Supporting Information 7). According to the literature (Imazio *et al.*, 2006; Arroyo-García *et al.*, 2006; Bacilieri *et al.*, 2013; Marsal *et al.*, 2017), the five subpopulations are: subPOP EASTMED-CAU (Algeria, Cyprus, Georgia, Israel, Lebanon, Tunisia and Turkey); subPOP BP (Bosnia and Herzegovina, Bulgaria, Croatia, Greece, Serbia, Slovenia and Montenegro); subPOP ITA (Italy); subPOP FRA-CEU (Austria, France, Germany, Hungary and Switzerland); and subPOP IP

(Spain and Portugal). Each variety was assigned to the country specified in VIVC (Maul and Röckel, 2015). Figure 3-b shows the distribution of these six areas described above (the geographical origin of the cultivar). In this case, coordinate 1 explains 44.27 % of the overall variance, and clearly shows a split between subPOP IC-M and subPOP IP (only located in the right-hand quadrant) and the other subpopulations. Coordinate 2 explains 19.30 % of the overall variance, and divides the subPOP ITA, subPOP FRA-CEU and subPOP IC-M (upper quadrant) from the rest of subpopulations, which are principally located in the lower left quadrant (except subPOP IP, which is in the lower right quadrant). This distribution shows that the subPOP IC-M is at some distance from all the subpopulations, but it is much closer to subPOP IP than to the others.

DISCUSSION

1. SSR polymorphism

The average expected heterozygosity was 0.787 (Supporting Information 2), which is considered relatively high, and is very similar to that
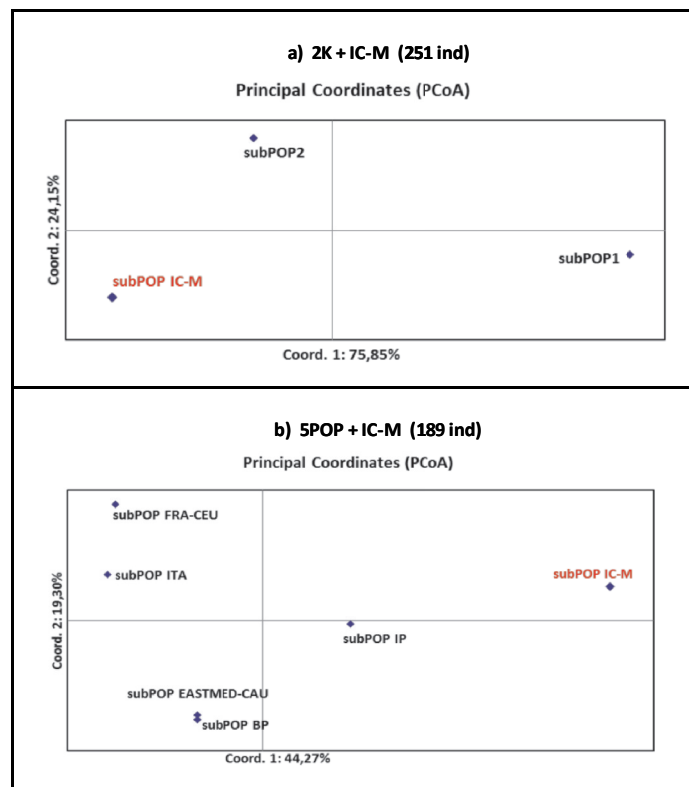
FIGURE 3. Representation of the subpopulations from world population by Principal Coordinates Analysis: a) 2K + IC-M subpopulation; b) 5 area-countries + IC subpopulation.
To do a better characterization of each group, only it was considered the varieties with q ≥ 85 % (non admixed individuals).

described by other authors (Costantini *et al.*, 2005 (0.79); Martinez *et al.*, 2006 (0.807); Stajner *et al.*, 2014 (0.79)). Consequently, the Canary-Madeira population shows considerable genetic diversity. The cumulative PI* was very low (2.4E^{-26}) so the 20 SSRs used were efficient at distinguishing between close varieties. This value was small enough to ensure that two plants with the same molecular profile in all loci were the same cultivar, with sports being the sole exception. In fact, in nine cases our PI* values were lower than the threshold (0.05) at which a microsatellite is considered hyperpolymorphic in grapes (Costantini *et al.*, 2005), and only VVS3, VVS29, and VVMD6 had values higher than 0.1. In addition, 13 markers had a probability of null alleles (r) lower than or equal to 0.01, meaning that there was a very small proportion of excess heterozygosity. This confirms that the homozygote profiles are true, so they would not have lost any alleles. All this data confirms that the 20 SSRs are appropriate for this study.

2. Cultivar analysis: Confirmation of accession name and proposal of local varieties

Eight of the prime names in this collection are not recorded in the VIVC: Bienmesabe tinto, Burra volcanica, Listan rosa, Mollar cano rosado, Torrontes volcanico, Vallera, Verijadiego negro and Uva de año (Supporting Information 1). All are the prime names of six cultivars and two sports that we propose should be included in the VIVC database. Uva de Año is a name that has been used by other authors (Rodríguez-Torres, 2018) but it does not appear in this database. Moreover, the MP-SSR of these eight cultivars are not described in VIVC. This suggests, therefore, that these names and theirs MP-SSR could be included in the VIVC database. The MP-SSR of Torrontes volcanico corresponds to MP-SSR of the cultivar named Pedro Ximenez/Torrontes by Rodríguez-Torres (2018). Interestingly, this molecular profile corresponds neither to MP-SSR Pedro Ximenez nor to the MP-SSR Torrontes, which indicates that it is a different cultivar.

We consider that 38 names from this collection are synonyms (either new synonyms or synonymous names for a particular cultivar). Of these, there are 19 accessions names that are not recorded or have no bibliographic support in the VIVC. These accession names are proposed as new synonyms given that they are very common in the Canary Islands. Furthermore,

19 accessions names are recommended as synonyms of 16 varieties. These names are already accepted synonyms for other varieties according to VIVC, but in the Canary Islands they are also commonly used to refer to the cultivars of this collection. Therefore, this information should also be included in the ampelographic section of VIVC (Maul and Röckel 2015). Finally, eight accessions have been identified as cases of mislabelling inasmuch as they all coincide with the prime name of other cultivars.

The studied Canary-Madeira population is made up of 41 varieties and three sports. However, according to several bibliographic resources specialized in the grapevines of the Canary Islands (Zerolo *et al.*, 2006; Rodríguez-Torres, 2018; *Vitis* Canarias, 2015), only 26 have been traditionally cultivated in these islands: Albillo criollo, Baboso blanco/Bastardo blanco, Baboso negro, Bastardo negro, Breval negro, Burra blanca, Castellana negra (Tintilla castellana), Forastera blanca (Albillo forastero), Gual, Huevo de gallo, Listan blanco de Canarias, Listan negro/Almuñeco, Listan prieto, Malvasia aromatica, Malvasia rosada, Malvasia volcanica, Marmajuelo, Moscatel de Alejandría, Negramoll, Torrontes volcanico (Pedro Ximenez/Torrontes by Rodríguez-Torres (2018)), Sabro, Tintilla, Verdello, Verijadiego, Vijariego blanco and Vijariego negro. For this reason, these 26 varieties have been selected and studied in detail to identify which can be considered as local varieties of the Canary Islands (Supporting Information 4). A particular case to highlight is that of three unknown varieties, which coincided with other unknown ones identified by other authors specializing in Canarian varieties (Rodríguez-Torres, 2018). In this case and by consensus of all the scientists who analysed their genetic profiles, the names of Bienmesabe tinto, Burra volcanica and Vallera were chosen, taking into account different aspects such as historical information (when available), phenological behaviour and plant morphology.

After an exhaustive bibliographic revision and molecular analyses, we proposed that 14 varieties and three sports are local to the Canary Islands. We found that their names and their MP-SSR are unique (except sports) in the world and to our knowledge they are only grown in the Canary Islands. These cultivars are Albillo criollo, Bermejuela, Bienmesabe tinto, Burra volcanica, Albillo forastero, Huevo de gallo,

Listan negro, Listan rosa, Malvasia di Sardegna rosada, Malvasia volcanica, Mollar cano rosado, Sabro, Torrontes volcanico, Uva de año, Vallera, Verijadiego and Verijadiego negro. Curiously, none of these come from Madeira. This suggests that, nowadays, the Canary Islands (unlike Madeira) keep genuine cultivars, which have been brought from different regions of the world over the years, because phylloxera never reached there. Unfortunately, Madeira suffered the disaster of phylloxera and probably lost many of its original varieties. However, there are two unknown varieties from Madeira (Supporting Information 1 and 4), but we cannot put forward a hypothesis about these since we have found no information.

3. Putative parentage relationships

In the Canarian and Madeiran population, we found four pedigrees. Two pedigrees (lineages 1 and 2) were reported in a study with nine SSRs (Zerolo *et al.*, 2006) and four pedigrees (lineages 1, 2, 3 and 4) were described in another study with 48 SNPs (Rodríguez-Torres, 2018). Cabezas *et al.* (2011) reported that 48 SNPs had a discrimination power similar to a set of 15 SSR markers. Therefore, the present study confirms these lineages through molecular characterization using 20 SSRs. It should be pointed out that lineage number 3 is not included or has no bibliography to support its inclusion in the VIVC.

4. Genetic structure

The Canary-Madeira population (Figures 1 and 2), can be divided into two large groups. One contains mainly foreign varieties (located in subPOP1 and subPOP4), and the other contains mainly local varieties (located in subPOP2 and subPOP3, these local varieties are highlighted in blue whereas their sports in purple). Curiously, it can be seen (Figure 2) that coordinate 2 separates these populations by placing the population of foreigners (almost totally) in the upper right quadrant and most of the local Canary-Madeira population in the lower quadrants. Conversely, coordinate 1 separates subPOP3 (isolating it in the lower left quadrant) from the rest of the subpopulations that disperse, occupying the quadrants on the right. SubPOP3 is characterized by having the highest proportion of local varieties at 69 %, which increases to 86 % if only pure local varieties are considered. Most of these cultivars come from Lanzarote Island. This

subpopulation groups some cultivars that have a strong influence of varieties from the east of the Mediterranean Basin (Malvasia di Sardegna or Muscat of Alexandria). The other group that includes a significant amount of local varieties (66.6 %) is subPOP2, which increases to 75 % when we consider only the pure individuals that come entirely from the Island of El Hierro (Burra volcanica, Huevo de gallo and Verijadiego negro). This subpopulation is characterized because three of its members have the ancestral Spanish cultivar Heben as the parent (Sumoll, Vijariego blanco (admixed) and Sabro (admixed)). The three local pure individuals together with the variety Bienmesabe tinto, from the island of La Palma and belonging to subPOP4, are the furthest local group. subPOP4 is the most disparate subpopulation: apparently its members are disconnected, although there is a certain Central European influence. Quite the opposite occurs in subPOP1 in which all its members, except the Vallera and Unknown-2IC varieties, are directly or indirectly related to the Central European ancestral variety known as Savagnin blanc. This last cultivar is the one of the parents of the varieties Samarrinho (PRT), Molar (PRT), Trousseau noir (a French cultivar widely implanted in Portugal and known by the name synonym of Bastardo negro), Verdelho branco (PRT) and Alfrocheiro (PRT), and the grandfather of the cultivars Albillo criollo (local cultivar from La Palma island) and Malvasia fina (PRT). Many authors support the theory that from the Middle Ages there was a large influx of Central European varieties through the Route of Santiago (Casanova *et al.*, 2011). This is possibly why the Savaging blanc cultivar was introduced in this area, since it is one of the varieties with more crossings in the northern Iberian Peninsula. Therefore, the Central European influence means that subPOP1 and subPOP4 are much related, between them occupying the upper right quadrant of the PCoA representation in Figure 2. These subpopulations only contain three local varieties: Albillo criollo from La Palma and Vallera from Tenerife (subPOP1) and Bienmesabe tinto from La Palma (subPOP4).

Consequently, the study of the genetic structure of the local Canary varieties in a world population must be based on the subPOP IC-M. This subpopulation is assumed to consist of 15 Canary cultivars (14 from this work and the Albillo monte Lentiscal from the world

population) and two varieties from Madeira (Unknown-2IC and Unknown-3IC), all of which belong to the area of subPOP IC-M (Supporting Information 7). The subPOP IC-M cultivars were removed from subPOP2, where most of the peninsular cultivars are found. The results show that these varieties have a direct relationship with the Iberian Peninsula. The assignment tests also show that the varieties in the subPOP IC-M are highly characteristic and constitute their own group (data not shown). The distribution of the subpopulations (Figure 3) shows that the subPOP IC-M is very distant from all other subpopulations. This proves the singularity of the molecular profiles of the subPOP IC-M. However, it is always nearer to the subPOP2 (Figure 3-a) and to the subPOP IP (Figure 3-b) than the others (Myles *et al.*, 2011). This may be because the main colonizers of these islands came from Spain and Portugal, so most of the first vines in the Canary Islands and Madeira come from these countries.

These results reinforce the idea that subPOP IC-M has characteristics that differentiates its varieties from the rest, although is clearly influenced by the peninsular population.

The Canary Islands never experienced the phylloxera plague. For 500 years the cultivars introduced by the first colonizers and the successive migrations have adapted to the new edaphoclimatic conditions (mutations) and hybridized spontaneously. In addition, grapegrowers have selected the most interesting specimens. All these actions have led to cultivars that are quite different from the initial ones, with characteristic and unique genetic profiles. Consequently, the Canary Islands and Madeira seem to be a biodiversity centre or a centre of origin of new cultivars of *Vitis vinifera* L.

CONCLUSIONS

This study proposes two new mutations, Listan rosa and Mollar cano rosado, which are sports of two well-known varieties (Listan negro and Mollar cano, respectively). The study has also detected four new cultivars for which there are no previous references (Bienmesabe tinto, Burra volcanica, Vallera and Verijadiego negro). Eight names of varieties have also been proposed for inclusion in the VIVC as new prime names (Bienmesabe tinto, Burra volcanica, Listan rosa, Mollar cano rosado, Torrontes volcanico, Uva de año, Vallera and Verijadiego negro).

Furthermore, the MP-SSR of five varieties is given (Bienmesabe tinto, Burra volcanica, Torrontes volcanico, Uva de año, Vallera and Verijadiego negro). We also suggest that 38accession names commonly used in the Canary Islands be added to the list of synonyms in the VIVC. Nineteen of these must be considered new synonyms of the 12 varieties (PN): Abillo grano chico (PN: Albillo criollo); Uvallón (PN: Breval negro), Huevo gallo (PN: Huevo de gallo); Uva olor (PN: Isabella); Muñeco negro, Negra gruesa, and Negra mulata (PN: Listan negro); Marmajuelo rosada (PN: Listan rosa); Bermejuelo, Vermejuela, and Marmajuelo blanco (PN: Bermejuela); Negramoll negra (PN: Mollar cano); Negramoll mulato, and Negramoll rosada (PN: Mollar cano rosado); Albillo grano pintado (PN: Muscat a petits grains rouges); Blanca peluda, and Viña Javier (PN: Palomino fino); Tintilla castellana (PN: Tinto cao). The rest, 19 synonymous names of 38, corresponding to 16 cultivars, are existing synonyms for other varieties. In this case, we suggest that they be incorporated as synonyms for the varieties of this collection. Two varieties are labelled as unknown and their MP-SSRs are provided. Eight cases of mislabelling were detected and identified.

The study of genetic structure reveals that the cultivars from the Canary and Madeira Islands have been strongly influenced by the Iberian Peninsula. Furthermore, 14 local varieties and three local sports from the Canary Islands have been proposed: Albillo criollo, Bermejuela, Bienmesabe tinto, Burra volcanica, Albillo forastero, Huevo de gallo, Listan negro, Listan rosa, Malvasia di Sardegna rosada, Malvasia volcanica, Mollar cano rosado, Torrontes volcanico, Sabro, Uva de año, Vallera, Verijadiego, and Verijadiego negro. They are all part of a single group with a genome characteristic of the Canary Islands. In Madeira, however, we were unable to find a local variety: the molecular profile of Unknown 3IC seems to be the most characteristic, but did not manage to identify this variety.

It can be concluded, therefore, that this volcanic area could be considered as a biodiversity centre or centre of origin of new cultivars of *Vitis vinifera* L.

support. We would also like to thank Rosa Pastor and Santiago Moreno for their support in the laboratory.

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